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Reversible coupling of individual phycobiliprotein isoforms during state transitions in the cyanobacterium *Trichodesmium* analysed by single-cell fluorescence kinetic measurements

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ABSTRACT

In the non-heterocyst, marine cyanobacterium *Trichodesmium* nitrogen fixation is confined to the photoperiod and occurs coevally with oxygenic photosynthesis although nitrogenase is irreversibly inactivated by oxygen. In previous studies it was found that regulation of photosynthesis for nitrogen fixation involves Mehler reaction and various activity states with reversible coupling of photosynthetic components. We now investigated these activity states in more detail. Spectrally resolved fluorescence kinetic measurements of single cells revealed that they were related to alternate uncoupling and coupling of phycobilisomes from and to the photosystems, changing the effective cross-section of PSII. Therefore, we isolated and purified the phycobiliproteins of *Trichodesmium* via ion exchange chromatography and recorded their UV/VIS absorption, fluorescence excitation and fluorescence emission spectra. After describing these spectra by mathematical equations via the Gauss-Peak-Spectra method, we used them to deconvolute the *in vivo* fluorescence spectra of *Trichodesmium* cells. This revealed that the contribution of different parts of the phycobilisome antenna to fluorescence quenching changed during the daily activity cycle, and that individual phycobiliproteins can be reversibly coupled to the photosystems, while the expression levels of these proteins did not change much during the daily activity cycle. Thus we propose that variable phycobilisome coupling plays a key role in the regulation of photosynthesis for nitrogen fixation in *Trichodesmium*.

1. Introduction

Trichodesmium is a genus of marine, filamentous cyanobacteria that fixes atmospheric nitrogen when other forms of nitrogen (nitrate or ammonia) are not available. It is the genus that contributes the largest single proportion to the total nitrogen fixation of the oceans [1]. Because of its diazotrophic (N_2 fixing) lifestyle, *Trichodesmium* is able to inhabit oligotrophic tropical and subtropical seas.

The enzyme nitrogenase, which mediates the fixation of molecular nitrogen, is directly and irreversibly inactivated by oxygen (reviewed by [2–5]). Heterocystous oxygenic cyanobacteria (e.g. *Anabaena*, *Nostoc*) solve this problem by spatial separation of nitrogen fixation to heterocysts and oxygen evolving photosynthesis to vegetative cells [6]. Others, e.g. *Cyanotheca* and *Lyngbya*, perform a temporal separation of both processes in the same cells (nitrogen fixation in the night), possibly mediated by changes in the redox state of the cell via thioredoxin [7].

Trichodesmium does neither possess heterocysts nor is performing nitrogen fixation during the night. In this organism nitrogen fixation is maximal in the middle of the light period, while oxygen release by photosynthesis shows one peak in the morning and another in the afternoon [8,9]. Although *Trichodesmium* does not have heterocysts or another form of permanent cell differentiation for nitrogen fixation, the enzyme nitrogenase is never expressed in all cells of a trichome simultaneously, but is temporarily expressed in a subset (10–20%) of cells, termed “diazocytes” [10]. Damage of the nitrogenase in these cyanobacteria is prevented mainly by two processes. During nitrogen fixation, dark respiration is elevated [8,11], but the main process of oxygen consumption seems to be the PSII-dependent Mehler reaction [11–13], which also supplies energy to nitrogen fixation. In this way, in *Trichodesmium* nitrogen fixation is inhibited by PSII inhibitors such as DCMU [12]. Via fluorescence kinetic microscopy [14] it was shown before [12] that PS II activity in *Trichodesmium* is homogenously high in all cells of the trichomes during most of the day, except for the period of nitrogen fixation during which a reversible partial

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differentiation occurs. This process leads to an enhancement of the Mehler reaction and therefore a decline of oxygen production in the nitrogen fixing cells. It involves the appearance of cells with a much higher basic Chl fluorescence yield (F_0) — compared to the non-diazotrophic period. These cells have been termed "bright cells" [12,15]. Two types of "bright cells" were found, only one of which is connected to nitrogen fixation (type I) while the other (type II, "very bright cells") can also occur without the presence of nitrogenase activity [15] but is related to stress [15,16]. Additionally, cells with unusually low fluorescence were found during and directly after the diazotrophic period [15].

Reversible switches between different fluorescence levels can occur within a few minutes, excluding that pigment synthesis/degradation could be their cause [15]. Küpper et al. [15] found that the higher F_0 results from a reversible uncoupling of PS II antenna proteins from the PS II reaction centre. But it remained unknown which parts of the antenna actually changed their coupling. In Chlorophyta the antenna proteins are chlorophyll containing proteins, while in cyanobacteria and red algae the phycobilisomes form the main antenna besides the chlorophyll-containing core antenna. The differential coupling of antenna proteins to the two reaction centres (PS I+PS II) is a process called "state transition" occurring in all photosynthetic organisms. This procedure regulates the amount of excitation energy directed to each reaction centre of both photosystems. It is due to reversible phosphorylation of LHC II in Chlorophyta [17] or still not completely understood mechanisms in the case of phycobilisomes. Phycobilisomes are known to diffuse very rapidly between both photosystems [18,19]. Recently, data of Liu et al. [20] using phycobilisomes isolated from red algae suggested that energetic decoupling of phycoerythrin can contribute to dissipation of excess energy.

The method of Chl fluorescence kinetic measurements allows for a detailed analysis of the photosynthetic apparatus of cyanobacteria and its regulation *in vivo* [19]. On the microscopic level, the Fluorescence Kinetik Microscope (FKM) allows for such measurements via the pulse amplitude modulation (PAM) principle with subcellular resolution [14,21]. In the current study, we used a recently introduced variant of this technique, spectrally resolved single-cell measurements of fluorescence kinetics in the FKM [21]. With this technique, we analysed the state transitions occurring during the daily cycle of photosynthetic activity in *Trichodesmium* and analysed them quantitatively for contributions of individual components of the antenna in order to find out more about the intricate regulation of photosynthesis for nitrogen fixation in this organism.

2. Material and methods

2.1. Cultivation

Trichodesmium strain IMS101 was grown in a continuous chemostat culture with a growth medium (called YBCHK) containing the following components: 420 mM NaCl, 10 mM KCl, 20 mM $MgCl_2$, 10 mM $CaCl_2$, 25 mM $MgSO_4$, 2.5 mM $NaHCO_3$, 464 μM H_3BO_3, 780 μM KBr, 50 μM KH_2PO_4, 68 μM NaF, 25 μM LiCl, 2 μM RbCl, 1 μM Fe-EDTA, 450 nM $NaIO_3$, 80 nM Na_2MoO_4, 20 nM $MnCl_2$, 7 nM $NiSO_4$, 2.5 nM $CoCl_2$, and 1 nM $CuSO_4$, dissolved in re-distilled water. The pH was adjusted to 8.2 with NaOH and the medium was sterilised via autoclaving. The flow rate of the medium was chosen to achieve an OD_{750nm} of the cultures of 0.5, resulting in about 0.7 l/d^{-1} flow in 2 l chemostats. The chemostats were aerated with air and the cultures maintained in a 12:12 h Light/Dark cycle (light on 8:00 am to 8:00 pm local time). The photon flux density during the light period followed a sinusoidal cycle simulating natural conditions, with a peak intensity of about 600 $\mu mol/m^{-2}/s^{-1}$. The light source was a panel of *OSRAM*® Dulux L 55W/12-950 (Osram, München, Germany, www.osram.com) fluorescent tubes.

2.2. Sampling times for in vivo measurements

Samples for measurements of nitrogenase activity assays and FKM analyses were taken five times a day: 7:00 am in the dark before onset of the light period, 10:00 am before pigment and enzyme synthesis, 1:30 pm in the middle of the light period, 5:00 pm time of nitrogenase decline, and 8:30 pm after the end of the light period.

2.3. Harvesting of the cells for pigment and protein extraction

For phycobiliprotein purification and pigment analysis, the cells were harvested by vacuum filtration with 50 mm GF 6 glass fibre filters (Whatman, UK), frozen in liquid nitrogen and stored at −80 °C. For preparative purification of phycobiliproteins, the outflow of all chemostats of the whole day was pooled. For analysing the diurnal cycle of pigment composition, 80 ml aliquots of sample were withdrawn from the cultures at the sampling times described above. For quantification of pigments and proteins in the measurements of daily cycles, the filters were frozen completely (i.e. the cells on the filter). For preparation of pure phycobiliproteins and of thylakoids, the cells were removed from the filter: at high densities on the filter, *Trichodesmium* filaments form a paper-like film on the filter that can be lifted off with forceps. This film was frozen in liquid nitrogen and stored at −80 °C.

2.4. Phycobiliprotein isolation

About 2.5 g of frozen cells were homogenised with the wheat mill Jupiter 872 with Messerschmidt stainless steel grinding engine (Messerschmidt Hausgeräte GmbH, Germany). The mill was pre-cooled with dry ice and cooled during grinding with liquid nitrogen. 20 ml of isolation buffer (0.1 M NaH_2PO_4, pH 6.8) was frozen as beads in liquid nitrogen and was added while grinding. The ground powder was molten and centrifuged at 16,000×*g* and 4 °C for 30 min. The pellet with thylakoids and membrane residues was treated as described in the next paragraph. The supernatant with phycobiliproteins was precipitated by addition of 40% ammonium sulphate and centrifuged at 16,000×*g* and 4 °C for 30 min. The pellet was resuspended in 50 ml dialysis buffer (NaH_2PO_4, 0.1 M, pH 6.8) and dialysed over night in the same buffer at 4 °C. During dialysis the buffer was exchanged 3 times. After that the sample was centrifuged at 25,000×*g* and 4 °C for 30 min.

2.5. Solubilisation of thylakoids and purification of Chl–protein complexes

The pellet with thylakoids that was obtained after ammonium sulphate precipitation (see above) was solubilised with 0.9% dodecyl-β-D-maltosid (DDM) in 0.3 M Tris/HCl pH 8.8 for 20 min on ice at a concentration of 0.2 mg Chl per ml of buffer. The solution obtained in this way was centrifuged 20 min at 20,000×*g* (4 °C) to pellet the membrane residues. The free pigment and remaining phycobiliproteins were separated from the Chl protein complexes by saccharose density gradient centrifugation for 17 h at 4 °C and 27,000×*g*. The UV/VIS absorption, fluorescence excitation and emission spectra of the Chl–protein complexes were measured and analysed in the same way as those of the purified phycobiliproteins (described below).

2.6. Purification of phycobiliproteins

The supernatant of the dialysed sample was loaded onto a DE52 FPLC column with a flow rate of 0.02–0.05 bed volumes per min. The elution was performed with wash buffer (NaH_2PO_4, 0.01 M, pH 6.8) and an exponential NaCl gradient from 0 to 400 mM with a flow rate of about 1 bed volume per minute. Each peak or shoulder on the recorder trace (measurement at 275 nm) represented one sample of which

absorption spectra were measured (see below). Then the interesting elution samples were concentrated in 15 ml concentration tubes with 10 kDa exclusion size (Amicon Ultra-15, Millipore Corporation, USA) at 4 °C and 4000×*g*. Further purification was achieved by re-running individual samples on a hydroxyapatite column (Calbiochem division of Merck KGaA, Darmstadt, Germany) with a 0–500 mM phosphate gradient (pH 6.8) and finally on a MonoQ column (GE Healthcare) with various NaCl gradients between 0 and 1000 mM in wash buffer (pH 6.8). Samples corresponding to peaks of absorption were collected and analysed by UV/VIS absorption spectroscopy for identification. Samples that seemed to be already pure according to their elution and the initial absorption spectrum were concentrated and then further analysed by UV/VIS absorption, fluorescence excitation and emission spectroscopy, SDS gel electrophoresis and Western blotting. The samples were stored on ice.

2.7. SDS gel and Western blot analysis of purified phycobiliproteins

For SDS gels and Western blotting, the OD_{275} (protein peak) of the samples was adjusted to 0.04 before loading them onto 12.5% Laemmli gels [22] that were run at 20 mA.

For silver staining, the gel was fixed over night with 100 ml fixative containing 50 ml methanol, 10 ml acetic acid, 40 ml H_2O and 50 µl formaldehyde. After 2× washing with water, the gel was incubated in 50% ethanol for 1 h, then treated with 0.04% sodium thiosulphate for 1 min and washed 3× with water. Afterwards it was incubated in 0.25% silver nitrate for 20 min and washed again 3× with water. For development, 4.5% (w/v) sodium carbonate in ddH_2O containing 0.05% (v/v) formaldehyde was used. Staining was stopped with a stop bath containing 50% methanol and 10% acetic acid in ddH_2O. For scanning the gel was washed with water. For Western blotting, an unstained gel was transferred to a nitrocellulose membrane (Protran BA 85 nitrocellulose membrane, Whatman, UK) using a wet blotting system (Hoefer Scientific Instruments, Holliston, Massachusetts, USA) at 200 mA for 1 h. Primary antibodies against B-phycoerythrin and phycocyanin were purchased from Rockland Immunochemicals (Gilbertsville, USA) and used in 1:1000 dilution. Blots were developed using alkaline phosphatase-coupled IgG (Sigma-Aldrich, Hamburg, Germany) followed by NBT/BCIP colorimetric reaction (Roche Diagnostics GmbH, F. Hoffmann-La Roche AG, Basel, Switzerland).

2.8. Measurements of macroscopic fluorescence excitation and emission spectra

2.8.1. Absorption analysis

The measurements were done using a Lambda 750 UV/VIS/NIR spectrometer (Perkin Elmer, Waltham, Massachusetts, USA) at wavelengths between 250 and 800 nm with 0.5 nm optical bandwidth (resolution), 0.2 nm point distance (sampling interval) and 0.12 s integration time per point. The samples were recorded in wash buffer (see above). For evaluation of the spectra, Microcal Origin software (versions 7G, and 8G, OriginLab, Northampton, Massachusetts, USA) was used.

2.8.2. Fluorescence analysis

The measurements were done in a LS50B fluorescence spectrometer (Perkin Elmer, Waltham, Massachusetts, USA). Fluorescence excitation spectra were recorded between 250 nm and 5 nm before the fluorescence emission peak, with detection set to the emission peak. Fluorescence emission spectra were recorded from 400 to 800 nm, with fluorescence excitation set to 365 nm since it turned out that all phycobiliproteins can be efficiently excited at this wavelength.

2.8.3. Gauss-Peak-Spectra

Both absorption and fluorescence spectra of purified phycobiliproteins were described by a series of Gaussian peaks to obtain equations of Gauss-Peak-Spectra for determining their contribution to absorption and fluorescence spectra in mixtures. This method was recently described (for chlorophylls and carotenoids) by Küpper et al. [23]; all details of the method and its application were described in that article. The equations and graphs of all Gauss-Peak-Spectra determined for the phycobiliproteins in the current work can be found in the supplementary material of the current article.

2.9. Protein quantification, determination of extinction coefficients

For determination of protein concentrations in the pure phycobiliprotein standards that were also used for the final UV/VIS absorption, fluorescence excitation and emission spectra, the "DC protein estimation kit" from BioRad Laboratories (Hercules, California, USA) was used. The correlation between UV/VIS absorption and protein quantification data was used for determining extinction coefficients of the phycobiliproteins. Approximate molar extinction coefficients were obtained by normalising the specific extinction coefficients to the molecular weights determined by calibrated SDS gel electrophoresis.

2.10. Analysis of Chl, carotenoids and phycobiliproteins in extracts

Chlorophyll and carotenoids were extracted with 100% acetone after filtrating 80 ml of culture on 25 mm GF/F glass fibre filters (Whatman), freezing the filters in liquid nitrogen and then lyophilising them. After 1 day of extraction at 4 °C in the dark, absorbance spectra from 350 nm to 750 nm were recorded in 0.2 nm intervals at an optical bandwidth (slit) of 1 nm. From these spectra, carotenoids and Chl were analysed using the Gauss-Peak-Spectra method according to Küpper et al. [23]. Phycobiliproteins were extracted after completion of the acetone extraction described above, which along with the pigments removed the membranes from the cells. This pre-treatment made the phycobilisomes easily accessible for extraction by incubation with phosphate buffered saline (PBS = 40 g/l^{-1} NaCl; 1 g/l^{-1} KCl; 7.2 g/l^{-1} Na_2HPO_4; 1.2 g/l^{-1} KH_2PO_4) for 1 day at 4 °C in the dark. Spectra of these extracts were recorded in the same way as those of the acetone extracts. Contents of the different phycobiliproteins were determined in the same way as chlorophylls and carotenoids, using the Gauss-Peak-Spectra obtained from the purified phycobiliproteins (complete equations and fitting library for SigmaPlot in the supplementary material).

2.11. Preparation of samples for FKM measurements

2 ml of cell suspension were taken from the chemostat cultures and filtered through a membrane filter to remove the medium. The cells were resuspended by washing the filter in 50 µl of YBCHK placed on the window of the measuring chamber (details in [15,16]). 50 µl of agarose (1.5% SeaKem Gold agarose in YBCHK) were mixed with the medium drop containing the cyanobacteria and covered with gas-permeable cellophane. The chamber was temperature controlled and pumped through with 30 ml/min^{-1} medium (27 °C) as described previously [14,15].

2.12. Fluorescence kinetic microscope (FKM) measurements

2.12.1. Intact cells

The spectrally resolved fluorescence kinetics on a single-cell level were measured as described in Küpper et al. [13,15,21], with excitation between 400–505 nm and fluorescence detection from 515–800 nm adjusted by the exciting LEDs and filter sets [21]. All measurements were done following an optimised protocol, as described in detail before [21]. An F_m measurement was followed by 90 s of darkness, and after F_0 was measured, 100 s of actinic light followed and finally 100 s dark relaxation without actinic light. The kinetics were analysed as described in [21]. In contrast to higher plants [21], in the current work

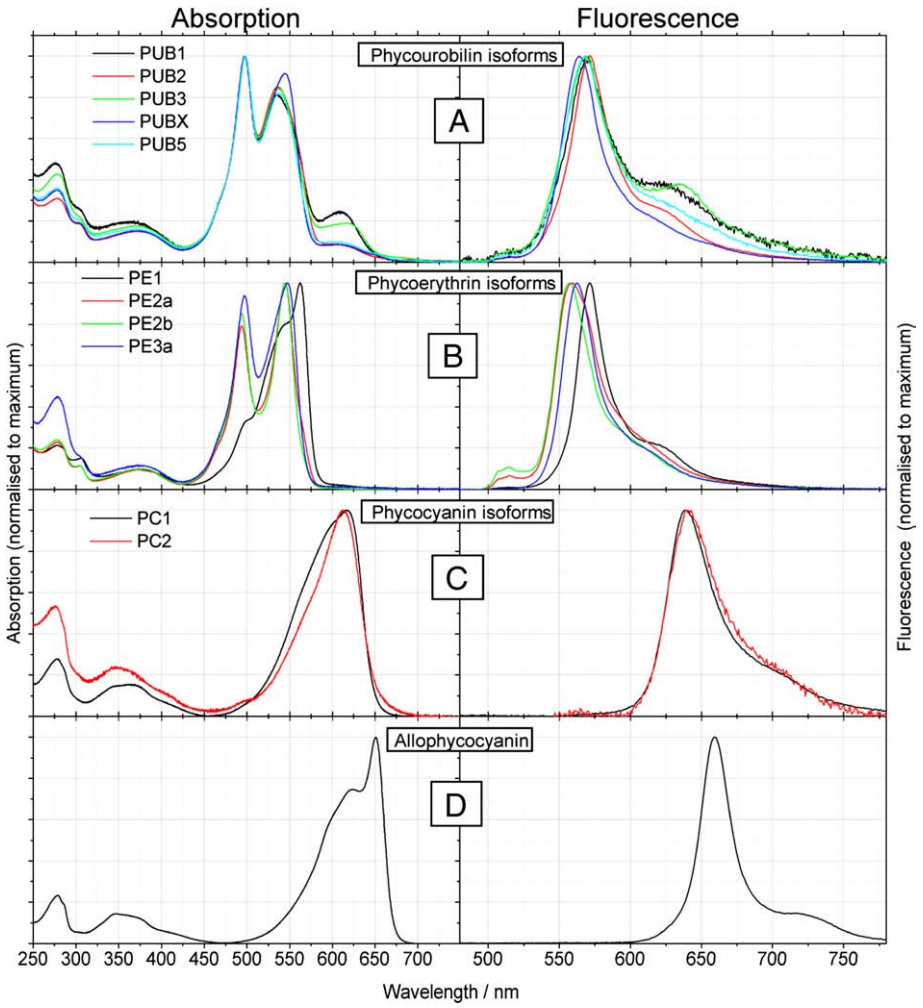


Fig. 1. Absorption and fluorescence emission spectra of purified phycobiliproteins from *Trichodesmium*. All maxima are normalised to 1 for easier comparison of the shape of the spectra. The numbers of the isoforms were given by their sequence of elution from the MonoQ column, the letters behind numbers denote isoforms that strongly overlapped in the elutions of the first run of the column and could only be separated by re-running a very shallow gradient around the salinity where the peak occurred in the first run (not all of these isoforms are shown here).

we could not include the spectrally resolved parameters of photochemical quenching. Photochemical quenching was originally also measured now, but it turned out that the heating of the LED in the FKM light source during the supersaturating flashes slightly red-shifted its spectrum, which increased the quantum yield of PE and PUB excitation but not of Chl excitation. Since so far we could not find out how much of an artefact this causes in spectra of photochemical quenching parameters, we do not present them in the current publication. This problem did not affect the measurements of non-photochemical quenching $q_{np}=F_m-F_m'$, because both F_m and F_m' were measured with the same saturating light, i.e. with the same excitation spectrum.

2.12.2. Phycobiliprotein standards

Solutions of isolated purified phycobiliproteins were analysed for the fluorescence emission spectra under the same optical conditions as the intact cells. This was done in order to have any instrument-specific bias (such as absorption in the optical system) influencing their spectra in the same way as those of the intact cells. For these calibration measurements, 10 µl of phycobiliprotein solution were placed in a counting chamber with 10 µm depth, i.e. similar to the diameter of a *Trichodesmium* cell. Then fluorescence was excited with a 365 nm peak emitting LED through the filter set that was also used for the *in vivo* measurements. The different excitation LED was chosen because only in the UVA range all phycobiliproteins can be excited efficiently with the same wavelength, while for *in vivo* measurements the inner part of the phycobilisomes (some of the phycoerythrins, phycocyanin and allophycocyanin) was intentionally only excited via energy transfer from the outer phycobilisome (mainly phycourobilin isoforms, plus some phycoerythrins). The emission spectra of the purified phycobiliproteins were used to set up Gauss-Peak-Spectra for deconvolution of *in vivo* fluorescence spectra as described for the macroscopic spectra above.

2.13. Measurement of nitrogenase activity

Nitrogenase activity was measured by the method of the acetylene reduction assay [24]. 20 ml samples were taken five times a day (see above) and incubated in 70 ml serum bottles. 10 ml of air were exchanged with 10 mL of acetylene. The samples were incubated for 1 h in an incubator with the same temperature and light/dark cycle as the main cultures. After the incubation time, 2 ml of the gas was taken out and analysed by gas chromatography (Hewlett Packard, USA, 5890 Series II equipped with flame ionization detector (FID) and 1.8 m packed steel column with Porapak N). The same was done with a control; containing only 20 ml of YBCHK medium to check the possible acetylene turnover not due to *Trichodesmium*.

3. Results

3.1. Purified phycobiliproteins

It turned out that *Trichodesmium* contains not only one protein in each of the known phycobiliprotein classes, i.e. phycourobilin (PUB), phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC), but many isoforms of PUBs and related PEs, as well as two isoforms of PCs (Fig. 1). The isoforms were confirmed (by SDS gels with Western blotting and spectroscopically) to occur also in the initial protein extract from *Trichodesmium*, i.e. they were not generated by artefactual modification during purification.

We found and purified one isoform of allophycocyanin (Figs. 1D, supplement 3) and purified it to run as a single peak on the MonoQ column. However, still a second band appeared on the SDS gels. This might be a residual contamination by a PC isoform as indicated by the absorption spectrum. It may not be visible in the fluorescence emission spectrum due to fluorescence resonance energy transfer (FRET), which would indicate that it is actually bound to APC, probably so strongly that it could not be separated by chromatography.

So far, we were able to purify two PC isoforms, only one of which is recognised by the commercial antibody while both are clearly phycocyanins according to their UV/VIS spectra (Fig. 1C). These isoforms differ from each other in the shape of their absorption spectra (Fig. 1C) and also in their size as judged by SDS gels (supplement 3). Interestingly, the smaller isoform 2 (PC2) has a lower chromophore/protein ratio than PC1, as judged by the ratio of the absorption peak of the protein backbone (275 nm) to that of the chromophores (about 610 nm). No further PC isoforms were present in *Trichodesmium* in amounts larger than 1% of the total PC amount (at least under the growth conditions used for the current study) as judged by SDS gels, UV/VIS spectra and FPLC profiles.

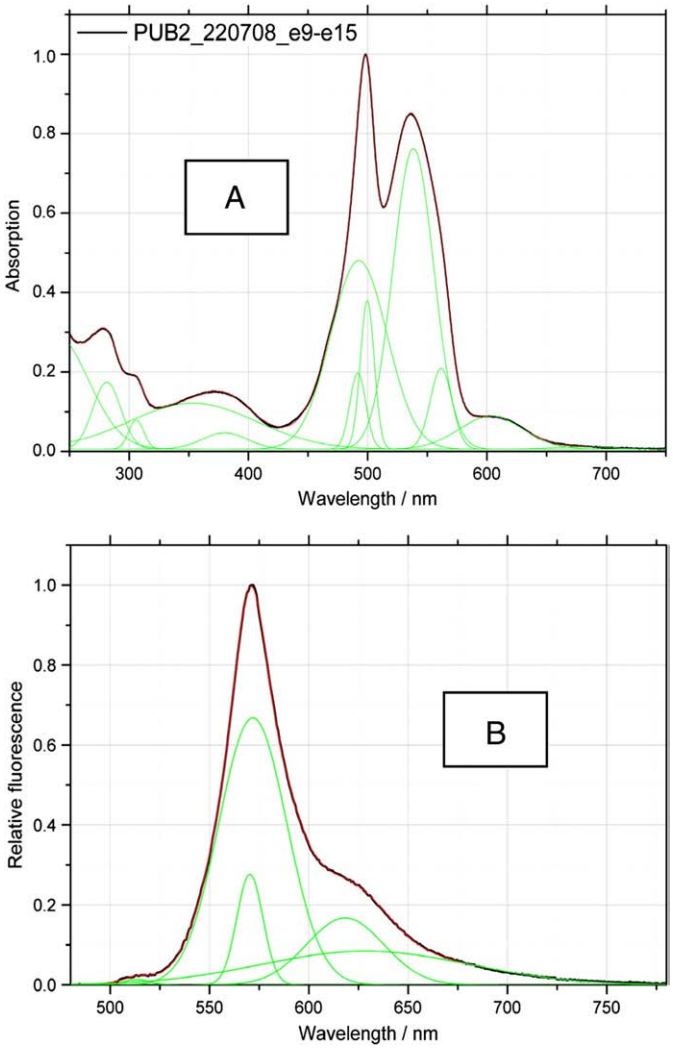


Fig. 2. Gauss-Peak-Spectra of phycourobilin isoform 2 in 10 mM sodium phosphate buffer pH 6.8. (A) Absorption, (B) Fluorescence emission. The Gauss-Peak-Spectra (equations) of all purified phycobiliproteins are in the supplementary material in the form of fitting libraries for SigmaPlot (SPSS Science, Chicago, Illinois, USA).

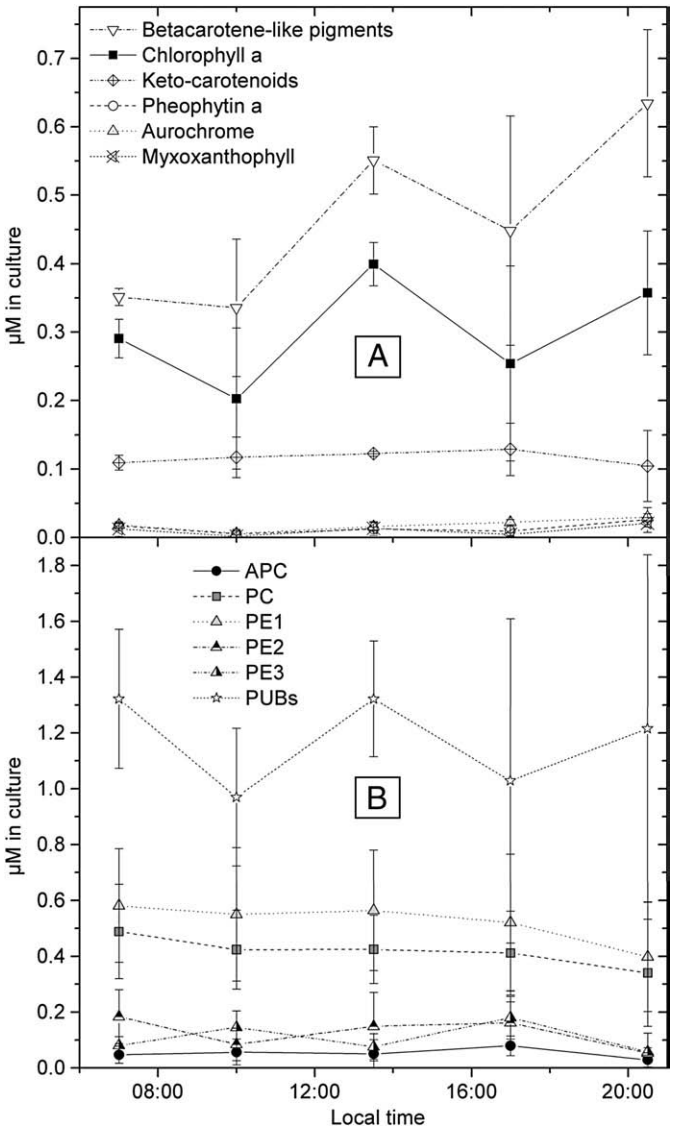


Fig. 3. Distribution of pigments and phycobiliproteins in the daily cycle. The concentration is indicated in µM. The sampling times were 7:00 am, 10:00 am, 1:30 pm, 5:00 pm and 8:30 pm. The data points shown represent the average and standard error of four measurements of four independent chemostats. (A) Pigments, (B) Phycobiliproteins.

Four isoforms of phycoerythrin were purified so far, each of which had a unique absorption and fluorescence spectrum although PE2a and PE2b were more similar to each other than the others (Fig. 1B). Besides the main PE peak at 550–570 nm, PE2a, PE2b and PE3 had a secondary absorption peak similar to the main absorption peak of PUBs, i.e. around 495 nm. In these three isoforms, additionally the main absorption peak was blue-shifted by about 15 nm compared to PE1, the same applied to the fluorescence emission spectra. Interestingly, PE2a and PE2b had an additional fluorescence peak at about 511 nm before the main peak at about 555 nm. It is possible that there are further PE isoforms present in *Trichodesmium*, since the peaks of PE2 and PE3

further subdivided on the MonoQ FPLC column, but these isoforms would have identical UV/VIS spectra and identical sizes according to SDS gels, so that we did not make further attempts of separation.

Five isoforms of PUBs were purified almost to homogeneity. They differed in their elution on the MonoQ column. Four of them (PUBs 1–3, PUB 5) had slightly different UV/VIS absorption spectra. PUBx had a much more different absorption spectrum because its secondary absorption peak was about 10% higher and about 10 nm red-shifted compared to the other PUBs. This isoform also exhibited the most distinct fluorescence emission spectrum, with the emission peak at the shortest wavelength and almost no shoulder behind the peak towards longer wavelengths (Fig. 1A). The PUBs were not recognised by the commercial PE antibody; to make them appear as bands on a Western blot with this antibody, the development had to be continued long enough so that other already very weak bands that were not visible on the silver gel became visible (not shown). It is likely that there are further PUB isoforms in *Trichodesmium*, since some PUB peaks (identified by their UV/VIS absorption and fluorescence spectra) coeluted with other proteins on all columns. But according to the size of those peaks and according to our deconvolution of *in vivo* spectra (see below) they should not contribute more than a few percent to the total amount of PUBs present in *Trichodesmium*.

The absorption and fluorescence spectra of all purified phycobiliproteins could be accurately described by Gauss-Peak-Spectra as shown in one example in Fig. 2. Generally, the deconvolution of the *in vivo* fluorescence spectra and of the absorption spectra of phycobiliprotein extracts with the GPS of the phycobiliproteins shown in Fig. 1 and contained in the GPS database in the supplementary material yields very small residuals. Therefore, we conclude that we were able to isolate and purify all major phycobiliprotein isoforms in *Trichodesmium* that are expressed under the current culture conditions.

3.2. Measurements of the daily activity cycle

3.2.1. General pattern of growth and nitrogenase activity

As intended, the measured chemostat cultures kept a constant OD_{750} of around 0.05±0.01, i.e. an about constant cell number, when comparing them at the same time of the day. This OD was achieved at a flow rate of about 0.7–0.8 l/d^{-1} at a volume of 2 l, corresponding to a growth rate of up to 0.4 d^{-1}, which is close to the maximum known for very healthy *Trichodesmium* cultures. During the day, the OD slightly increased from the morning until the evening (data not shown), as it had to have always the same OD in the morning despite the lack of growth during the night. The nitrogenase activity showed the typical pattern known for *Trichodesmium* (Supplement 4), making the current set of data comparable to the data of the control in our previous study [16]. There was no activity before the start of illumination (7:00 am), but early in the light period (10:00 am) the activity was already induced. In the middle of the light period (1:30 pm) the activity was highest and decreased afterwards (5:00 pm). At the beginning of the dark period (8:30 pm), there was still a slight acetylene turnover.

3.2.2. Expression levels of phycobiliproteins and pigment amounts

We quantified the amounts of phycobiliproteins in the PBS extracts taken at each of the five sampling timepoints during the daily activity cycle. This revealed some fluctuations of phycourobilin levels, but most of these were not significant (P=0.05) due to variations between individual cultures and measuring days. No pronounced changes in phycobiliprotein composition were found during the daily activity cycle (Fig. 3, lower panel). Furthermore, we analysed the pigment (Chl and carotenoid) distribution in daily activity cycle (Fig. 3, upper panel). The concentration of Chl increased slowly during the day like the phycobiliproteins, i.e. just reflecting the fact that during the day the growth of the culture was faster than the chemostat flow rate (see above). β-carotene-like pigments (β-carotene, β-cryptoxanthin and zeaxanthin, all having exactly the same chromophore) increased more strongly during the day, most likely as a defence against light stress. The concentration of keto-carotenoids remained nearly the same during the day, and was very low, as in the iron-replete culture in our previous study [16].

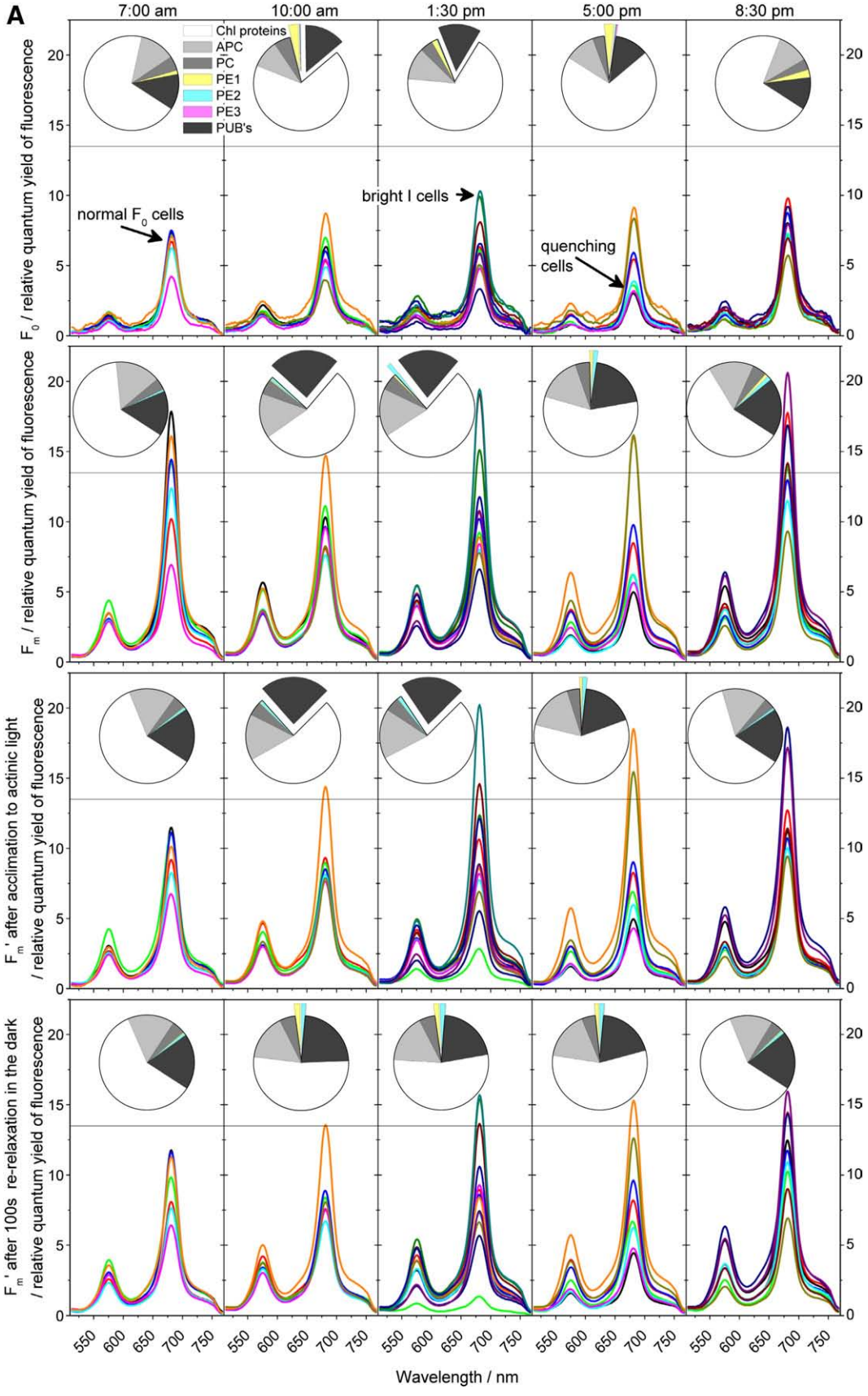
3.2.3. Spectrally resolved single-cell fluorescence kinetics

The spectral kinetics of *Trichodesmium* were the key elements of analysing the reversible coupling of antennae in *Trichodesmium*. Fig. 4 shows the daily changes of the spectral characteristics of fluorescence yields at various excitations (F_0, F_m, F_m') and of fluorescence parameters calculated from such values ($F_m - F_m'$). For each sampling hour during the day, spectra of several cells are shown to represent the variability in the population. In contrast, Fig. 5 presents the quantitative characterisation of selected cells, irrespective of the time of their appearance, which represent the different activity states according to our classification. The following fluorescence values and parameters were used for interpretation: (1) The basic fluorescence quantum yield (F_0) measured in dark adapted state, (2) the maximal dark-adapted fluorescence yield during a supersaturating flash in dark-acclimated cells (F_m) and (3) in light-acclimated cells (F_m'_i4), and (4) in cells after 100 s relaxation in the dark following the actinic light (F_m'_r3), (5) changes in the maximal fluorescence yield as they occur due to non-photochemical quenching and state transitions in response to actinic light ($F_m - F_m'$_i4) and (6) the same changes in the maximal fluorescence yield after the dark relaxation period ($F_m - F_m'$_r3). Chl proteins contributed by far the largest part to the total fluorescence spectrum, which was to be expected in view of the excitation at about 410–505 nm, which directly excited only Chl proteins, PUBs, PE2s and PE3. All other phycobiliproteins could only receive excitation energy via resonance energy transfer (FRET) from the directly excited phycobiliproteins and Chl proteins.

3.2.3.1. Basic fluorescence quantum yield F_0 (Figs. 4A and 5, top panels). In the early morning (7:00) the F_0 values for each single cell showed an almost homogenous distribution with a maximum value around 7. This distribution became more heterogeneous during the light period, as studied in a not spectrally resolved way earlier [15,16]. The strongest heterogeneity of F_0 values was again found at 1:30 pm, the main time of nitrogen fixation where the relative fluorescence yield of single cells fluctuated between 3 and 10 (Fig. 4A). The latter was the typical value of the "bright I" cells that were previously identified as most likely being the nitrogen fixing cells in *Trichodesmium* [12,15]. At 5:00 pm more cells with reduced fluorescence quantum yield were found, although still a few cells with high values were observed. The deconvolution of the F_0 spectra revealed differences in the ratio of the

Fig. 4. Spectral characterisation of the daily activity cycle — fluorescence emission spectra of fluorescence kinetic parameters. Each spectrum represents a single selected cell, which is labelled with the same colour in all graphs of the fluorescence parameters (e.g. F_0, F_m, q_{np}) at the same time. In this way it is possible to compare the spectrum of different parameters in the same cell. Fresh *Trichodesmium* samples were used for each cell at each time point. The pie charts and bar graphs illustrate the averaged contributions of individual phycobiliprotein types to these spectra, as determined by fitting with the Gauss-Peak-Spectra of the purified phycobiliproteins. (A) Basic fluorescence parameters F_0, F_m, F_m' in light acclimated state and F_m' after 100 s re-relaxation in the dark after the actinic light. The colour legend in the upper left panel applies to all pie charts. To make differences better visible at first glance, the parts of the pies representing the contributions of those phycobiliproteins where the most important changes occurred are slightly moved out of the circle. (B) Differences between the above-mentioned F_m and F_m' values. They show a combination of classical non-photochemical quenching and effects caused by differential coupling of antenna complexes. The colour legend in the upper left panel applies to all bar charts.

contributions of Chl–protein complexes compared to the various phycobiliproteins, and different contributions of individual phycobiliproteins. While the ratio between fluorescence contributions from Chl proteins to phycobiliproteins did not change much, it turned out that the fluorescence contribution of PE1 was 2–3× higher in the times before and after the nitrogen fixation peak compared to the other



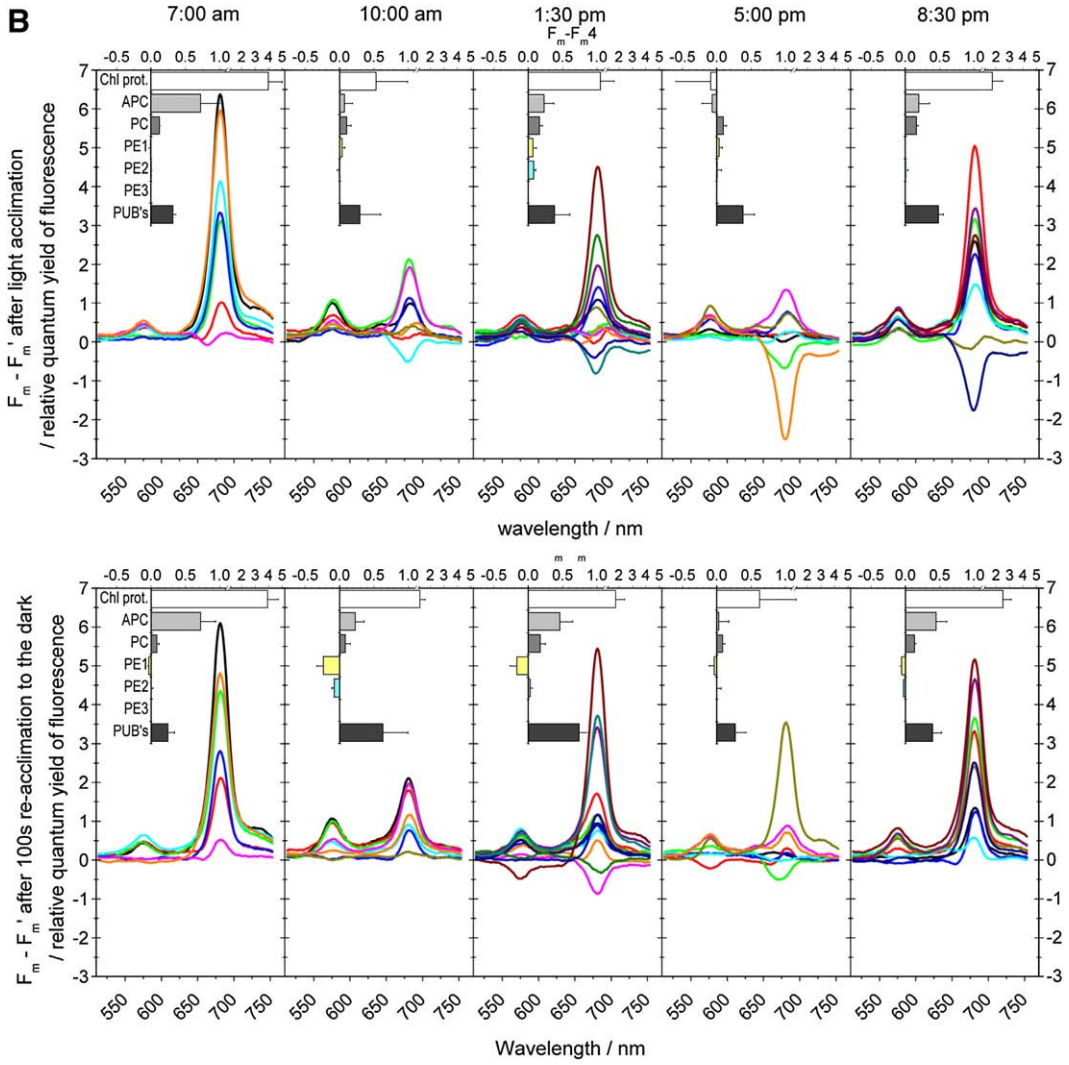


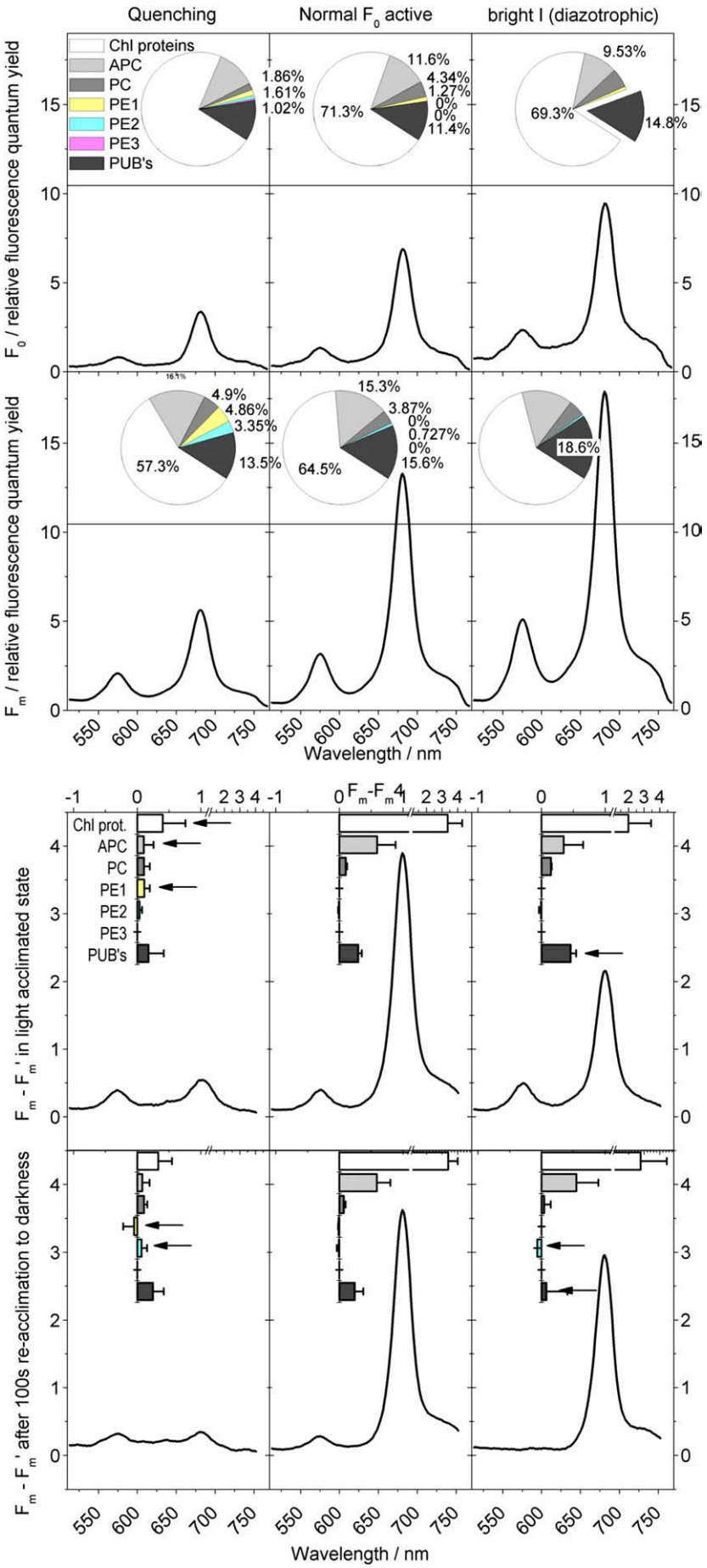
Fig. 4 (*continued*).

sampling time points. The contribution of PUBs, in contrast, was slightly increased during the peak of nitrogen fixation (13:30).

3.2.3.2. Maximal fluorescence quantum yield F_m (Fig. 4A, three lower panels; Fig. 5, second panel). In all F_m values the contribution of phycobiliproteins to the total fluorescence was higher than in F_0 most likely not only for physiological reasons but also (mainly?) due to the artefactual shift in the excitation spectra that prevented a reliable calculation of photochemical quenching parameters (see methods). But more interestingly, the contribution of phycobiliproteins to the F_m parameters varied in the daily activity cycle of *Trichodesmium*. It was generally higher at times of nitrogen fixation (i.e. during the day) compared to non-diazotrophic times, in particular 7:00 (at 20:30 there was still a bit of nitrogenase activity, see above). Further, the contribution of PE1 to dark-acclimated F_m increased from the morning to the evening, and the contribution of PE2 increased from morning until noon to remain constant until the evening. The changes in PE1 and PE2 contributions were hardly visible in the light-acclimated F_m', but became stronger again during re-acclimation to darkness. In the F_m' value measured after 100 s of dark relaxation, the contributions of both PE1 and PE2 were strongest in the diazotrophic period, and much weaker (PE2) or even absent (PE1) before and after the daily light period (i.e. at 7:00 and 20:30). These changes of PE1 contributions in the average values taken at the different timepoints during the daily cycle are closely linked to the appearance of cells with low fluorescence yield, termed "quenching cells" in our earlier non-spectral characterisation of *Trichodesmium* activity states [15]. These cells were now found to have a generally strong fluorescence contribution from PE1 and PE2, while in the "normal F_0 active" and "bright I" states the contribution of these phycobiliproteins was minimal (Fig. 5). "Bright I" cells had the highest proportion of PUB fluorescence among these three activity states; "normal F_0 active" cells had the highest contribution of Chl protein fluorescence.

3.2.3.3. Non-photochemical quenching/state transitions ($F_m - F_m'$) (Figs. 4B and 5 lower 2 panels). There were much higher fluctuations during the daily cycle in the values for non-photochemical quenching $F_m - F_m'$ than in the other parameters — almost each individual cell displayed a

Fig. 5. Spectral characterisation of the main activity states of *Trichodesmium* that were found and characterised by non-spectral fluorescence kinetic parameters in our earlier works [11,14,15]. Each spectrum represents an average of three to four cells of this activity state. (A) Basic fluorescence parameters F_0 and F_m. In the "normal F_0 active" state, all percentages of fluorescence contributions are shown, while in the other activity state only those percentages that markedly changed compared to the "normal F_0 active" state are labelled. (B) Differences between F_m and the F_m' values in light acclimated state and after 100 s re-relaxation in the dark after the actinic light. They show a combination of classical non-photochemical quenching and effects caused by differential coupling of antenna complexes.


Quenching
Normal F0 active
bright I (diazotrophic)
Chl proteins
APC
PC
PE1
PE2
PE3
PUB's
1.86%
1.61%
1.02%
71.3%
11.6%
4.34%
1.27%
0%
0%
11.4%
69.3%
9.53%
14.8%
4.9%
4.86%
3.35%
57.3%
13.5%
15.3%
3.87%
0%
0.727%
0%
64.5%
15.6%
18.6%
F0 / relative fluorescence quantum yield
Fm / relative fluorescence quantum yield
Wavelength / nm
Fm - Fm' in light acclimated state
Fm - Fm' after 100s re-acclimation to darkness
Chl prot.
Fm-Fm'
Wavelength / nm

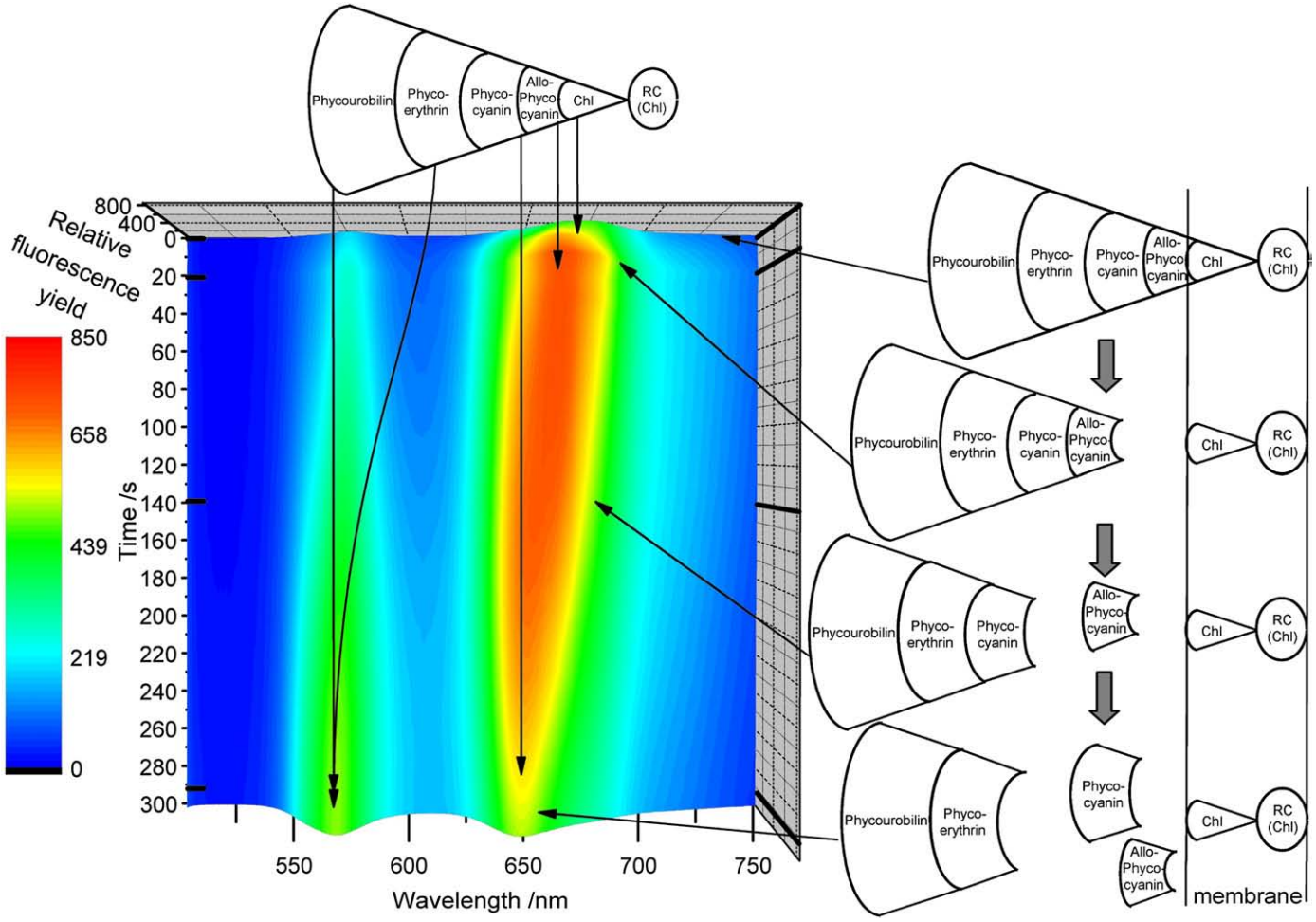


Fig. 6. Long-term F_t kinetics in actinic light of a selected *Trichodesmium* cell changing from the "normal F_0" state to the "bright II" state and then to the "normal F_0 inactive" state (compare scheme of all states in our earlier publication [14]). *Centre:* Measured fluorescence kinetics. *Top:* The scheme shows the normal arrangement of phycobiliproteins in *Trichodesmium*; the arrows point at the wavelengths of the emission peaks of individual phycobiliproteins. For comparison, the complete spectra of individual phycobiliproteins can be seen in Fig. 1. *Left:* The colour scale shows the colour encoding of the fluorescence yield. *Right:* The scheme illustrates the sequential uncoupling of the phycobilisome. Each arrow points at the time when this state occurred, so that the spectrum at this time can be compared with the proposed (un)coupling. The time slices are additionally marked by thick black lines on the time scales.

different spectrum for these parameters. Least heterogeneity in $F_m - F_m'$ was found, like for the basic fluorescence parameters, before the onset of the light period. At this time, almost all cells showed a strong peak in the $F_m - F_m'$ spectra in the position of Chl protein emission and a weaker peak in the phycobiliprotein emission. For $F_m - F_m'$ after 100 s re-relaxation, comparatively little heterogeneity was found not only before the onset, but also after the end of the daily light period (20:30). This is in line with the spectral characterisation of the main activity states (Fig. 5), since at 7:00 mainly cells of the "normal F_0 active" type were found. The non-photochemical quenching of the Chl proteins was clearly stronger at this time of the day and in this activity state compared to all other times of the day and activity states; this was found for both $F_m - F_m'$ parameters. The "quenching" = "low F_0" activity state was characterised by very low q_{np} values mediated by Chl proteins, but higher q_{np} by phycobiliproteins. This was obvious already from the size of the peaks in the spectra, but the deconvolution by the fitting of Gauss-Peak-Spectra additionally showed that in this activity state in particular PE1 and PE2 play a decisive role. The most likely diazotrophic "bright I" state was characterised by a strongly decreased contribution of PUBs to the difference $F_m - F_m'$ after re-acclimation to the dark. They further had a negative value of $F_m - F_m'$ for PE2, as it was observed for the low fluorescence cells for PE1 (Fig. 5). This means that fluorescence emission in this part of the spectrum increased during the measuring protocol, either as a response to the actinic light exposure or after switching the actinic light off.

3.2.3.4. F_t-record of the transition of a cell between three activity states (Fig. 6). A further important result of spectrally resolved fluorescence kinetic measurements was yielded by the incidental direct observation of a complete transition from the normal F_0 active state via the bright II state to the normal F_0 inactive state (as defined by Küpper et al. [15]). This transition involved dramatic changes in the amplitudes and wavelengths of the main fluorescence peaks (Fig. 6). In the first 20 s of this transition, a dramatic increase in the fluorescence both at 680 nm (Chl and APC) and 575 nm (PE's and PUBs) was observed. This was followed, in the next 80 to 100 s, by a shift of the main emission maximum from 680 nm to 650 nm (PC emission peak, see Fig. 1) that was accompanied by a further increase in PE + PUB fluorescence. Finally, in the remaining recorded kinetics, the main emission peak (now 650 nm = PC) strongly decreased, so that fluorescence emission in the detection range of the filters used for the camera in our earlier studies (650 to 710 nm; [12,15]) reached again the levels before these state transitions. In contrast, PE + PUB fluorescence further increased during the decline of the main peak, and levelled off when the main peak reached the "normal F_0" level.

4. Discussion

4.1. Purified phycobiliproteins

Many scientists have characterised phycobiliproteins of several cyanobacteria, e. g. *Nostoc* [25,26], *Gloeobacter violaceus* [27], *Mastigocladus*

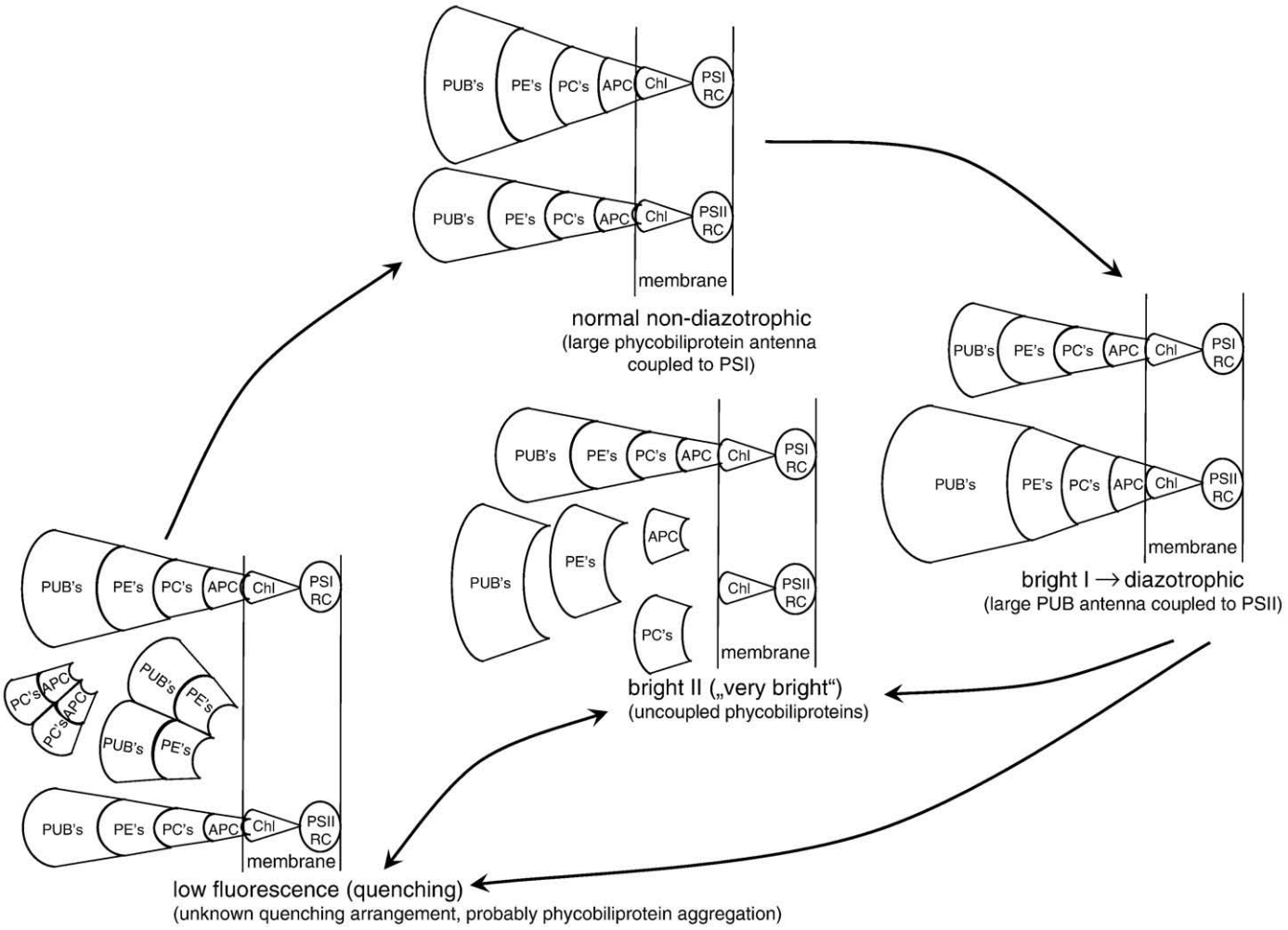


Fig. 7. Cartoon figure of the proposed model of reversible phycobiliprotein coupling in the different activity states of *Trichodesmium* cells (compare scheme of all states in our earlier publication [14]). The enhanced coupling of PUB to PSII in the bright I cells will lead to an enhancement of linear electron transport by PSII as required by the Mehler reaction that energizes nitrogen fixation while protecting nitrogenase from oxygen.

laminosus Cohn [28], and many other. Furthermore, Subranamiam et al. [29] characterised *Trichodesmium* via absorption and fluorescence spectra, and Küpper et al. [16] analysed *in vivo* single-cell and *in vitro* absorption spectra of *Trichodesmium* under normal and iron limited conditions. But the composition of phycobiliproteins in *Trichodesmium* was not characterised yet, and none of the phycobiliproteins from this or a related genus was isolated, purified and characterised.

We found that *Trichodesmium* contains an unusually large number of phycobiliprotein isoforms, in particular of phycourobilins (PUBs) and related forms of phycoerythrins (PEs) but also two phycocyanin (PC) isoforms. In contrast e.g. to *Nostoc* [26], however, only one isoform of allophycocyanin was found. Some of the phycobiliprotein isoforms in *Trichodesmium*, including the main PC isoform in *Trichodesmium*, were not recognised by commercial antibodies against normally conserved regions of phycobiliproteins, indicating that these isoforms should have a significantly different sequence compared to isoforms found in other cyanobacteria. Therefore, a sequence analysis of the purified *Trichodesmium* phycobiliproteins and a detailed search of the *Trichodesmium* genome for phycobiliproteins isoforms will be a part of a future study. Phycourobilins were known to constitute the main proportion of the total phycobiliprotein amount in *Trichodesmium* since the work of Subramaniam et al. [29], but the existence of isoforms was not analysed before. The relevance of this large number of isoforms is currently not clear. However, a hint to a possible answer was provided by our previous study on iron limitation effects in *Trichodesmium* [16], where in response to iron limitation an alternative larger PE isoform completely replaced the other isoform(s) that were detected on Western blots of the iron-replete cultures. This may indicate that *Trichodesmium* has different phycobiliprotein isoforms to have a better chance of acclimation to adverse environmental conditions. So far, only environment-responsive changes in the ratio between the main types of phycobiliproteins (PC vs. PE vs. APC) were investigated in other cyanobacteria (e.g. [30,31]), but except for our iron limitation study this has not been done for isoforms of PEs (incl. PUBs) and PCs. Therefore, we will investigate this possibility in more detail in future studies. Another possible benefit of having so many isoforms may be an optimal fine-tuning of light harvesting, and of energy transfer within the phycobilisomes. This might be concluded from the secondary peaks in the absorption, as well as secondary peaks and shifts in the fluorescence emission spectra observed for PE and PUB isoforms.

4.2. New insights into the regulation of photosynthesis for nitrogen fixation

The pattern of how *Trichodesmium* fixes nitrogen was already studied earlier as described in the introduction, and the current pattern is well in agreement with data obtained in the ocean (e.g. [12]) and in our previous study [16]. The residual nitrogenase activity half an hour after the end of the light period may indicate that the cells would still have had the capacity to fix nitrogen at this time, but did not do it efficiently because of the missing light that is required in

Trichodesmium to energize nitrogen fixation via the Mehler reaction. This aspect is interesting in view of the appearance of the presumably diazotrophic "bright I" cells also after the regular nitrogen fixation period, which was already observed in our previous study [16].

With help of spectrally resolved fluorescence kinetic microscopy the daily cycle of photosynthetic activity in *Trichodesmium* was analysed and compared to the nitrogen fixation activity. The different contributions of various parts of the antenna to the F_0, F_m and F_m' spectra, and in particular their changes during the day and even during 5 min of the measuring protocol, gave new insights into the mechanism of activity state changes in *Trichodesmium*, as schematically illustrated in Fig. 7.

Already the spectra of basic fluorescence parameters (F_0, F_m, and F_m') revealed that very strong differences in fluorescence properties exist between individual cells, and that systematic changes occur during the daily activity cycle. The strongest contribution of PC and particularly PUB was observed in the "bright I" cells. This indicates that in the bright I state a very large proportion of the available phycourobilin is coupled to PSII, as illustrated in Fig. 7. This coupling would increase, compared to a coupling to PS I, both the fluorescence of phycourobilin itself and also of the inner parts of the antenna, simply due to the larger number of excitons travelling through this "excitation funnel". If PUBs would be uncoupled, in contrast, the fluorescence yield of the other parts of the antenna would not be increased, i.e. the spectral composition of bright I cells compared to other activity state would be much more different than it is actually the case.

One surprise in this part of the study was that the "classical" types of PEs (PE1 and PE2), which are known from other cyanobacteria, usually contribute only little to the room temperature fluorescence emission of *Trichodesmium* cells, although they are abundant proteins. Part of this low fluorescence yield is certainly due to the fact that PE1 was not directly excited but could receive its energy only by FRET (see above), so that the changes in its fluorescence quantum yield show changes in its coupling to other components of the phycobilisomes. Nevertheless, in cells that enter the "fluorescence quenching state" [15], and thus at times of the day when such cells are abundant, a larger contribution of PE1 and PE2 fluorescence was observed. From the pattern of appearance of these cells [15,16], and from a few state transitions observed by chance [15], it seems that they are a recovery state after nitrogen fixation. The enhanced fluorescence quantum yield of PE1 and PE2 in the quenching cells could indicate that in this state, due to uncoupling, they do less efficiently transfer their excitons to the inner part of the light harvesting antenna. It is also possible that they receive excitation energy more efficiently from PUBs, which have an unusually low fluorescence contribution in this state (Fig. 7). In any case, in the low fluorescence state most of the antenna must be arranged in a way that most of the captured excitons are relaxed thermally. This could be caused by conformational changes of phycobiliproteins as it occurs when such proteins aggregate during precipitation. Additionally or alternatively, the quenching could be caused by the orange carotenoid protein characterised by Kirilovsky [32].

Non-photochemical quenching (measured here as $q_{np}=F_m-F_m'$) usually is a response to actinic light that lowers per definition the fluorescence yield in a non-photochemical way, as heat dissipation. In *Trichodesmium*, in contrast, q_{np} is most likely the result of antenna coupling/uncoupling in their role as light harvesting and energy transferring complexes respectively. This coupling is obviously reversible, as shown by our current data and schematically illustrated by Fig. 7. Uncoupling of phycobiliproteins from the cores of their photosystems, or re-coupling from PS I to PS II will increase their fluorescence quantum yield, leading to a seemingly negative q_{np}. On the other hand, if the opposite change of coupling (from PSII to PSI) or and aggregation of the phycobiliproteins occurs, the quantum yield decreases, causing a seemingly normal (positive) q_{np}. The much higher fluctuations during the daily cycle in the values for non-photochemical quenching F_m-F_m' than in the other parameters (almost each individual cell displayed a different spectrum for these parameters) are in line with our earlier observation that *Trichodesmium* cells can switch between activity states within the few minutes of the measuring protocol, causing a large heterogeneity in the cell population. A particularly impressive case of such a state transition was now spectrally resolved. The fact that both positive and negative peaks in the F_m-F_m' spectra appeared in both the Chl and the PE/PUB emission region, sometimes in the same and sometimes in the opposite direction, shows that these components of the light harvesting antenna can be coupled and uncoupled to/from the photosystems individually. And while PUB's are responsible for a particularly large proportion of the fluorescence of "bright I" cells, their fluorescence yield seems to change more slowly than that e.g. of APC, as revealed both by the q_{np} spectra indicative of state transitions and by the complete record of an activity state transition. Thus it can be concluded that even individual phycobiliproteins can reversibly (un)couple from/to the phycobilisomes. The biophysical mechanism of this will be an interesting topic for future investigations. This reversible coupling makes the antenna system of *Trichodesmium* extremely flexible, which seems to be a key element in the quick changes between the different (incl. diazotrophic vs. non-diazotrophic) activity states as illustrated in Fig. 7.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2009.01.001.

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