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LIGHT ACTION SPECTRA OF N_2 FIXATION BY HETEROCYSTOUS CYANOBACTERIA FROM THE BALTIC SEA[1]

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An on-line, laser photo-acoustic, trace gas detection system in combination with a stepper motor-controlled monochromator was used to record semicontinuous light action spectra of nitrogenase activity in heterocystous cyanobacteria. Action spectra were made of cultures of *Nodularia spumigena* Mertens ex Bornet & Flahault, *Aphanizomenon flos-aquae* Ralfs ex Bornet & Flahault, and *Anabaena* sp. and from field samples of a cyanobacterial bloom in the Baltic Sea. Nitrogenase activity was stimulated by monochromatic light coinciding the red and blue peaks of chl *a*, the phycobiliproteins phycocyanin (allophycocyanin) and phycoerythrin, and several carotenoids. Because nitrogenase is confined to the heterocyst, it was concluded that all photopigments must have been present in these cells, were involved in light harvesting and photosynthesis, and supplied the energy for N_2 fixation. The species investigated showed marked differences in their nitrogenase action spectra, which might be related to their specific niches and to their success in cyanobacterial blooms. Moreover, light action spectra of nitrogenase activity shifted during the day, probably as the result of changes in the phycobiliprotein content of the heterocyst relative to chl *a*. Action spectra of nitrogenase and changes in pigment composition are essential for the understanding of the competitive abilities of species and for the estimation of N_2 fixation by a bloom of heterocystous cyanobacteria.

Key index words: **Baltic Sea; cyanobacterial blooms; heterocystous cyanobacteria; light action spectra of nitrogenase activity; nitrogen fixation; on-line nitrogenase assay**

Cyanobacteria are oxygenic photoautotrophic prokaryotes comprising many species that are capable of N_2 fixation. Heterocystous cyanobacteria are particularly well adapted for diazotrophic growth, because they have confined the O_2-sensitive nitrogenase to special cells in which N_2 fixation is supported by PSI-mediated ATP generation and ferredoxin reduction (Wolk et al. 1994). However, the reducing equivalents necessary for N_2 fixation need to be imported as carbohydrate from vegetative cells (Böhme 1998, Curatti et al. 2002) because PSII is absent. Carbohydrates also fuel respiration, which is necessary to maintain virtually anoxic conditions in the heterocyst, which is necessary to protect nitrogenase. In addition, respiration also generates ATP and therefore partly covers the energy demand of nitrogenase in the light, whereas it is the only source of energy in the dark (Ernst et al. 1984, Staal et al. 2002). In the dark and at nonsaturating light, N_2 fixation is limited by ATP, whereas at saturating light the activity of nitrogenase is determined by the import of reducing equivalents or the potential activity of the enzyme (Staal et al. 2002).

Cyanobacteria are exposed to diurnal fluctuations of light and experience differences in irradiance when mixed in the water column. Light is not simply attenuated with the depth of water columns; its spectral quality is also subject to changes. Changes in spectral quality depend on the absorption spectra and scattering of the suspended particles within the water column and on absorption of water itself and the angle at which light impinges the water surface. Therefore, the remaining light available to the populations at greater depth is of a different spectral quality compared with the surface. Because N_2 fixation partly depends on photosynthetic activity, it is crucial to know which pigments play a part in the light stimulation of nitrogenase activity in heterocysts. This determines the organism's ecological success and affects the estimate of N_2 fixed by natural populations of heterocystous cyanobacteria. However, very little is known of the pigment composition of heterocysts and of the light action spectra for nitrogenase activity in cyanobacteria.

In summer, blooms of N_2-fixing cyanobacteria develop in the Baltic Sea. These blooms are composed of filamentous heterocystous species: *Nodularia spumigena* Mertens ex Bornet & Flahault, *Aphanizomenon flos-aquae* Ralfs ex Bornet & Flahault, and to a lesser extent *Anabaena* sp. These species all contain gas vesicles that provide them with buoyancy and usually form colonies or aggregates (Walsby et al. 1997), although *Anabaena* sp. is mostly encountered as single trichomes. The cyanobacteria in the Baltic Sea live in a dynamic environment and are subject to large changes in irradiation. This obviously affects N_2 fixation, but until now little attention has been paid to this problem.

The aim of this investigation was to measure light action spectra of nitrogenase activity in three cultured heterocystous cyanobacteria that are dominant in the

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Baltic Sea during cyanobacterial blooms and in a natural sample. This is the first time that high-resolution light action spectra were recorded for nitrogenase activity in cyanobacteria. This was possible because of the application of a monochromator in combination with an on-line near-real-time method for assaying nitrogenase activity by the acetylene reduction technique. The results allowed conclusions about the presence of various photopigments in the heterocysts and their involvement in light harvesting to cover the energy demand of N_2 fixation. The action spectra of nitrogenase revealed species-specific differences and were also variable during a diurnal cycle.

MATERIAL AND METHODS

Field samples. The measurements on field samples were done on samples taken from the Gotland Deep in the central Baltic Proper during a cruise on the Baltic Sea at the end of June 1999 on board of RV *Valdivia.* The daylight period lasted for approximately 20 h during that cruise. During sampling, the weather was calm and the water column was stratified with the thermocline at a depth of 16 m. Samples were taken from the upper mixed water column by vertical hauls of a plankton net (100 μm mesh size). Heterocystous cyanobacteria dominated the phytoplankton in the size fraction >100 μm. Microscopic observations revealed that *N. spumigena* and *A. flos-aquae* accounted for approximately 60% and 40%, respectively. *Anabaena* sp. was present in minor numbers (<2%). The ship followed a drifting buoy connected to a water anchor at the sampling station, ensuring sampling in the same water body.

Within 15 min after collection, 2–5 mL of sample was filtered on GF/F glass fiber filters (47 mm), rendering 2–5 μg of chl *a.* Filters were placed in the sample cell of the on-line acetylene reduction incubator (Staal et al. 2001), and measurements were started less than 30 min after sampling. Incubations were done at the *in situ* seawater temperature (14° C).

Laboratory cultures. Unialgal strains of *A. flos-aquae* (strain BS15), *N. spumigena* (strain BS19), and *Anabaena* sp. (strain BS18) were isolated from a bloom in the Gotland Deep, Baltic Sea (Culture Collection Department of Marine Microbiology, NIOO-KNAW). (The culture of *A. flos-aquae* has since been lost.) Isolation was done in artificial seawater made from one part ASN3 and two parts BG11 medium devoid of a source of combined nitrogen (Rippka et al. 1979). The salinity of this medium was approximately 9, corresponding to the location of sampling. The cultured organisms were grown in 250-mL Erlenmeyer flasks, containing 100 mL of growth medium, which were placed in an orbital, shaking (120 rpm), illuminated incubator (Gallenkamp, Loughborough, UK) at 20°C with an alternating light:dark cycle of 14:10 h. Fluorescent tubes (standard F30W/129, warm white, Sylvania, Munich, Germany) provided light at an incident irradiance of 60 μmol photons·m^{-2}·s^{-1}. Cultures were grown under this regime for 5 days before experiments were carried out. The cultures of *N. spumigena* that were used for monochromatic light-response curves were grown under continuous light. Only exponentially growing cultures were used.

Acetylene reduction. Nitrogenase activity was assayed using the acetylene reduction method (Hardy et al. 1968). For this purpose, we used the on-line, flow-through, acetylene reduction technique as described by Staal et al. (2001) with a gas mixture composed of 20% O_2, 70% N_2, 10% C_2H_2, and 0.04% CO_2. Ethylene was detected at high sensitivity by using a laser-based trace gas detector (Laser Photo-Acoustics [LPA], Life Science Trace Gas Exchange Facility: http://www.sci.kun.nl/tracegasfac) (te Lintel Hekkert et al. 1998). The on-line set-up combined with LPA detection of ethylene allowed continuous near-real-time measurements of nitrogenase activity. For use on board the research vessel, a mobile version of the LPA was constructed. The detection limit of ethylene was 0.2–0.5 ppb during calm weather and increased to 0.5–2 ppb in rough weather (>5 Beaufort). The sensitivity of the laboratory-based LPA was 0.01–0.25 ppb, depending on the amount of interfering gasses.

Light-response curves. Light-response curves of nitrogenase activity for different wavelengths of monochromatic light were recorded in the laboratory using a monochromator (grating 675 grooves mm^{-1}, Bausch and Lomb, Rochester, NY, USA) placed between a xenon lamp (Oriel, Stratford, CT, USA) and the incubation cell. Different light intensities were obtained by placing neutral density filters (Balzers, Liechtenstein) on top of the glass window of the cell. The monochromator had a gaussian-shaped transmission curve with a 12-nm bandwidth. Depending on the wavelength applied, the maximum obtainable monochromatic photon irradiances varied from 20 to 25 μmol photons·m^{-2}·s^{-1}.

Action spectra. Light action spectra of nitrogenase activity were obtained by running the monochromator using a stepper motor. The light source on board the ship was a slide projector, equipped with a 250-W halogen lamp, and in the laboratory the xenon lamp was used. The action spectra always started at 400 nm and ended at 720 nm. The step size was 0.2 nm and a semi-continuous spectrum scan was run at a speed of 8 nm·min^{-1}. The resolution achieved with this step size and wavelength width was sufficient to distinguish between the three major pigment classes that were present in the heterocysts.

Light action spectra were recorded at nonsaturating irradiances with a maximum of 12 μmol photons·m^{-2}·s^{-1} of monochromatic light, which varied with the wavelength. These low levels allowed linear normalization for differences in irradiance, that is, by dividing nitrogenase activity by the photon irradiance at each wavelength.

The relative contribution of the individual pigment classes to nitrogenase activity was estimated with a curve-fitting routine using the absorption spectra of the pigments found in the three species of cyanobacteria. The resolution of these spectra was 3–3.5 nm and equaled the resolution of the nitrogenase action spectra, which was the result of the 24-s sampling interval by LPA and the stepper motor speed (8 nm·min^{-1}). The absorption spectra of the acetone-extractable pigments were retrieved from the photodiode array detector of the HPLC. Attempts to derive pure phycobiliproteins from the cultures of *Anabaena* sp., *A. flos-aquae,* and *N. spumigena* failed. Therefore, solutions of almost pure phycoerythrin and phycocyanin were obtained from cultures of, respectively, *Pseudoanabaena* sp. (strain BS11) and *Synechococcus* sp. (strain BS12) (isolated from the Baltic Sea, Culture Collection Department of Marine Microbiology, NIOO-KNAW). Absorption spectra of these pigments were run on a spectrophotometer (ultraspec 4000, Pharmacia Biotech, Cambridge, UK) and corrected for the solvent. The pigment spectra were fed into a spreadsheet and multiplied by a variable parameter in the wavelength ranges 450–700 nm. The multiplied spectra were summed and compared with the measured N_2 fixation action spectrum. To find the smallest difference between the sum of the absorbances at each wavelength and the measured nitrogenase activity at that wavelength, parameters were varied automatically, using the solver function of Microsoft Excel. The sum of the spectra matched the nitrogenase action spectrum very well. The parameters found by this method were assumed to represent the relative contribution of the different pigments to the light-dependent nitrogenase activity and were used for monitoring changes in the pigment composition in the heterocyst during the light period.

Light measurements. Light was measured with a PAR light sensor (Licor 250, Licor, Lincoln, NE, USA) placed in the light spot generated by the monochromator. The PAR sensor is linear and measures photon irradiance in the spectral range of 400–690 nm. Calibration of the monochromator and the sensitivity of the PAR sensor for monochromatic light were carried out using a spectroradiometer (SR 9910-PC, Macam Photometrics Ltd., Livingston, Scotland).

Pigments. In vivo absorption spectra were measured with a double-beam spectrophotometer (UVIKON 940, Rotkreutz, Switzerland) with filter paper placed behind the quartz cuvettes to correct for back scattering (Shibata et al. 1954). The filters used

for the assay of nitrogenase activity were also used for the determination of chl *a*. Filters were lyophilized and frozen at –80°C until analysis. During the cruise filters were stored at –20°C; in the laboratory filters were lyophilized and stored at –80°C.

The lyophilized filters were extracted for 1 h by 90% acetone on ice in an ultrasonic water bath. The extracts were analyzed by HPLC (Waters, 2690, Waters, Milford, MA, USA) equipped with a photodiode detector (Waters, 996). Pigments were separated using a reverse-phase C18 column (Nova Pak C18, 150 × 3.9 mm, Waters) and a guard column packed with Bondapak C18. The mobile phase was 85:15 acetone:milliQ water (solvent A) and 100% acetone (solvent B), running at a flow rate of 1.1 mL·min^{-1}. Pigments were separated running a gradient of 75:25 A:B at t = 0–5 min and 35:65 A:B at t = 5.5–7 min. Samples were injected automatically using a cooled autosampler. Calibration runs for chl *a* were done after every 20 injections. Analysis of the chromatogram was carried out using the software package Millennium 3.2 (Milford, MA, USA).

RESULTS

Light action spectra of nitrogenase. Light action spectra of nitrogenase activity were recorded from cultures of the heterocystous cyanobacteria *A. flos-aquae*, *N. spumigena*, and *Anabaena* sp. that were isolated from a cyanobacterial bloom in the Baltic Sea and from a natural sample taken from this bloom (Fig. 1). These spectra revealed maxima at 665–680 and 430–445 nm, matching, respectively, the red and blue absorption peaks of chl *a*. In addition, the absorption peaks of the phycobiliproteins, phycocyanin and phycoerythrin, coincided with enhanced nitrogenase activities at 600–620 and 565 nm, respectively. A small stimulation of nitrogenase activity was seen at the wavelength range of 490–520 nm, which was attributed to carotenoids. Carotenoids were probably also responsible for the shoulder sometimes observed at 450–455 nm.

The action spectra revealed considerable differences among the three species investigated. In *A. flos-aquae*, light absorbed by phycobiliproteins stimulated nitrogenase activity more per photon than light absorbed by chl *a*, whereas the opposite was the case in *N. spu-*

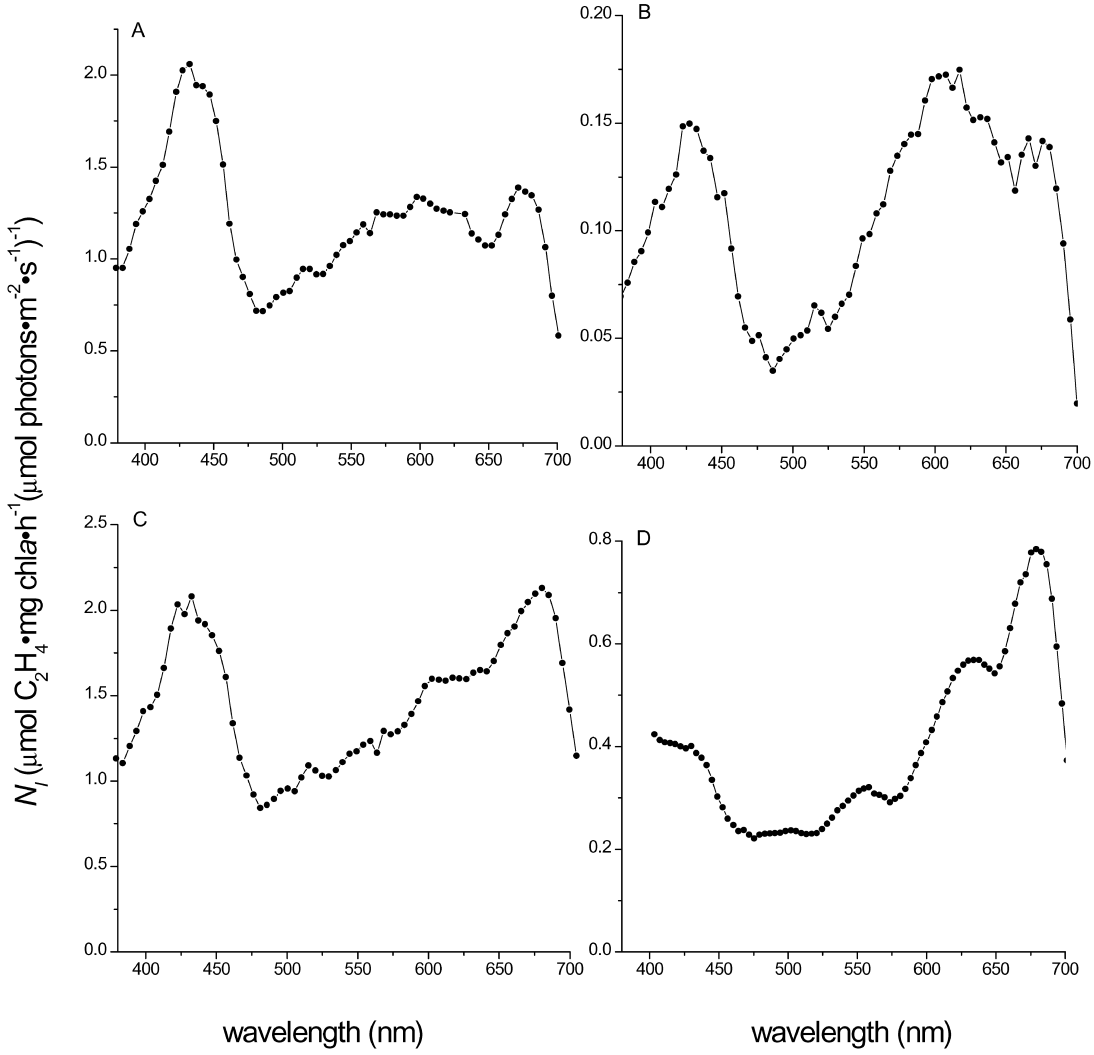

FIG. 1. Light action spectra of nitrogenase from the heterocystous cyanobacteria (A) *Anabaena* sp., (B) *Aphanizomenon flos-aquae*, and (C) *Nodularia spumigena* isolated from the Baltic Sea and from (D) a field sample from the Baltic Sea.

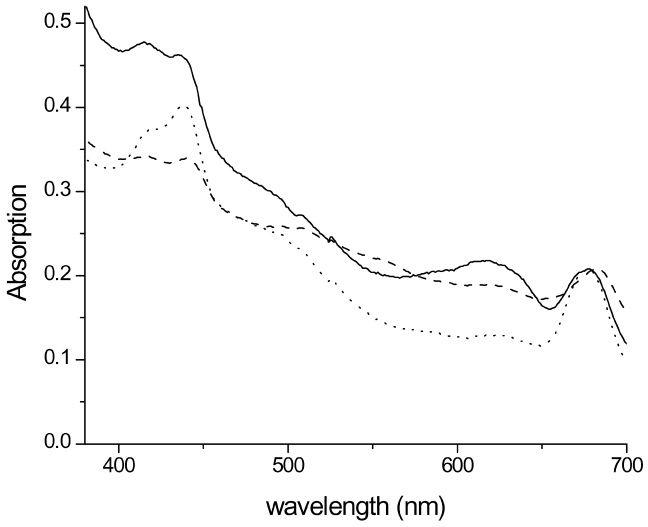

FIG. 2. *In vivo* absorption spectra of *Nodularia spumigena* (dotted line), *Aphanizomenon flos-aquae* (solid line), and *Anabaena* sp. (dashed line).

migena. An intermediate position was taken by *Anabaena* sp. (Fig. 1, A–C). To obtain a better insight into the relative contribution of the different pigments to nitrogenase activity, a curve-fitting routine was developed using the absorption spectra of the pigments. As was estimated from *in vivo* absorption spectra, the three cultures and the field samples contained, besides chl *a*, phycocyanin and phycoerythrin (Fig. 2). The shoulder around 490 nm in the *in vivo* spectra showed that carotenoids or xanthophylls were also present. There was also a strong absorption in the UV region, which was probably caused by mycosporine-like amino acids (Ehling-Schulz and Scherer 1999). The absorption ratios (A_{440}/A_{675}) were 1.55, 1.90, and 2.17 for, respectively, *Anabaena* sp., *N. spumigena*, and *A. flos-aquae*. To decide which carotenoids should be included in the fitting procedure, we analyzed the acetone extractable pigments (Table 1). *Anabaena* sp. and *N. spumigena* were identical in pigment signature, whereas *A. flos-aquae* distinguished itself by the presence of two additional pigments: astaxanthin and canthaxanthin. All carotenoids except astaxanthin were found in the field sample. *Aphanizomenon flos-aquae* was present in considerable numbers in the field sample (40% of the total number of diazotrophs), and therefore it may be that astaxanthin is only produced in laboratory-grown cultures or, alternatively, that our cultivated strain was different from the one that was dominant in the field (Barker et al. 2000).

Figure 3 depicts a representative example of the result from the curve-fitting routine. It shows the effect of different pigments involved in the light stimulation of nitrogenase in *N. spumigena*. The same was done for action spectra of *A. flos-aquae*, *Anabaena* sp., and the field samples (results not shown). The fitted light action spectrum gave a good indication of the relative contribution of the various pigments to nitrogenase activity (Fig. 3, solid line). When the carotenoids were omitted from the curve-fitting procedure, a good reconstruction was obtained of the nitrogenase action spectrum in the wavelength range above 520 nm. Yet, the fit was poor at the wavelengths at which the carotenoids absorb light (470–520 nm) (Fig. 3, dashed line) and at the blue absorption peak of chl *a*. This suggests that carotenoids were involved in the absorption of light energy and, ultimately, energization of nitrogenase in the heterocyst. Particularly, *Anabaena* sp. showed a relatively strong stimulation of nitrogenase activity by wavelengths at which the carotenoids have their absorption optimum (Fig. 1A). In the cultured *Anabaena* sp., the blue absorption peak of chl *a* was more important for nitrogenase activity relative to the red chl *a* peak. The differences in nitrogenase stimulation per photon between the blue and red peak ($N_{440}/N_{675} = 1.5$) in this species equaled the difference in light absorption of chl *a* ($A_{440}/A_{675} \approx 1.4$). Yet, this was not observed in the field samples ($N_{440}/N_{675} = 0.51$) and in samples of *N. spumigena* ($N_{440}/N_{675} = 0.98$) and *A. flos-aquae* ($N_{440}/N_{675} = 1.05$). The fitted action spectrum of *N. spumigena* was much higher in the blue region (420–450 nm) than the measured nitrogenase activity (Fig. 3). These differences were the reason why we omitted the blue region in the calculation of the relative contribution of pigments to nitrogenase activity.

According to the results of the curve-fitting procedures in all three strains, the only carotenoids that seem to be involved in photoenergy transfer were β-carotene and echinenone. However, the absorption spectra of carotenoids and xanthophylls overlap in the wavelength range 460–520 nm, and therefore the estimates of their contribution to nitrogenase activity might not be accurate.

TABLE 1. Presence or absence of pigments and their absorption maxima in acetone (Hirshberg and Chamovitz 1994) in cultures of *Anabaena* sp., *Aphanizomenon flos-aquae*, and *Nodularia spumigena* and in a field sample.

Pigment	Absorption maxima	*Anabaena* sp.	*A. flos-aquae*	*N. spumigena*	Field sample
Astaxanthin	480	−	+	−	−
Myxoxanthophyll	450, 478, 510	+	+	+	+
Canthaxanthin	471	−	+	−	+
Echinenone	460	+	+	+	+
β-Carotene	429, 452, 478	+	+	+	+
Chl *a*	431, 580, 618, 665	+	+	+	+

Pigments were measured by HPLC.

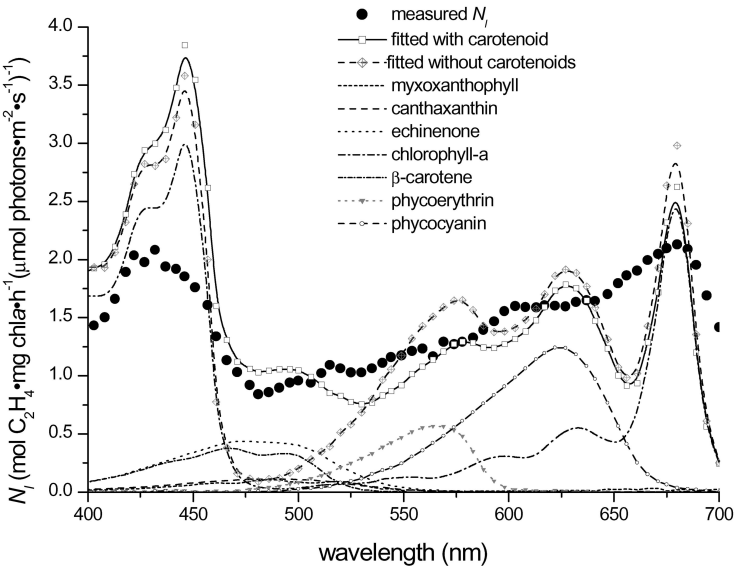

FIG. 3. A measured (solid circles) and a fitted (solid line with open squares) light action spectrum of nitrogenase from *Nodularia spumigena*. The contributions of the different pigments are shown that reconstructed the fitted action spectrum. Fitting was carried out with the solver function in Microsoft Excel varying the contributions of the individual pigments to the final fitted action spectrum (see Materials and Methods).

Daily changes of light action spectra of nitrogenase activity. Figure 4 depicts light action spectra of nitrogenase activity recorded from samples taken from a natural cyanobacterial bloom at three different times of the day: at 06:30 (2 h after sunrise), 12:30, and 18:30 (3 h before sunset). During the day, an increase in chl *a*-normalized nitrogenase activity was observed. In addition, the action spectrum changed during the day, which hinted to a change in pigment composition of the heterocyst. Using the curve-fitting routine we estimated the ratios of the three most important pigments: chl *a*, phycocyanin, and phycoerythrin. Figure 5D shows that the ratio of the integrated peaks of phycocyanin to chl *a* remained constant at approximately 0.9 throughout the day. In contrast, the ratio of phycoerythrin to chl *a* increased from 0.46 in the morning to 0.77 in the afternoon. Likewise, the ratio of phycoerythrin to phycocyanin increased during the day from approximately 0.49 to 0.84. Hence, it was concluded that the amount of phycoerythrin relative to phycocyanin increased in the heterocyst, thereby increasing its light-harvesting capacity in the 540- to 600-nm range (green-yellow).

Because this was unexpected, we also investigated whether the same phenomenon occurred in the cultures. For this purpose, the cultures were grown under an alternating light:dark regime and samples were taken during the light period at 4.5-h intervals. As was judged from nitrogenase activity measured at the wavelengths at which chl *a*, phycocyanin, and phycoerythrin absorb, the pigment composition of the heterocysts of all three species changed during the course of the light period. As was the case for the field samples, the ratio of phycoerythrin to chl *a* increased during the light period in all three species (0.15 to 0.65,

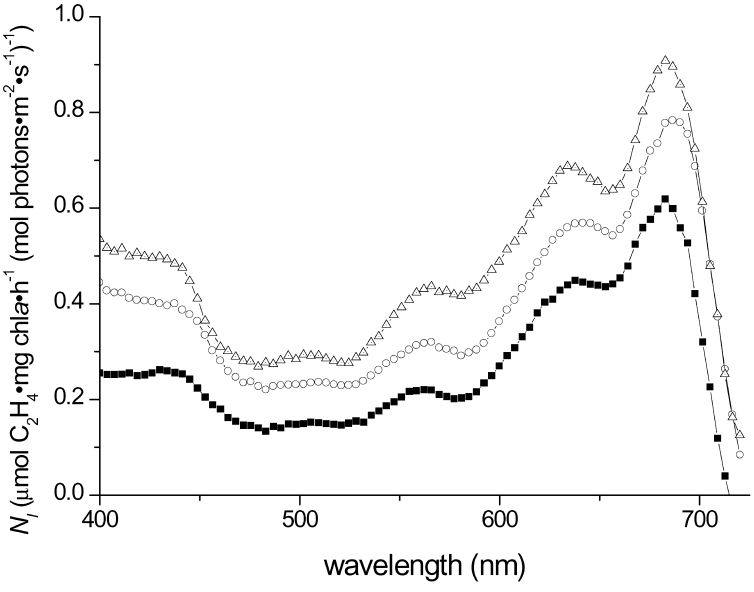

FIG. 4. Nitrogenase action spectra from natural samples (>20 µm fraction) taken from a cyanobacterial bloom in the Baltic Sea. Samples were taken three times during a light period: at 6:30 (solid squares), 12:30 (open circles), and 18:30 (open triangles). The action spectra were linearly corrected for differences in photon irradiance at the different wavelengths.

0.05 to 0.50 and 0.15 to 0.55 in *A. flos-aquae*, *Anabaena* sp., and *N. spumigena*, respectively) (Fig. 5, A–C). However, in contrast to the field observations, the ratio of phycocyanin to chl *a* increased as well. This was strongest in *A. flos-aquae*, where it changed from 0.50 to 1.00. In *N. spumigena* and *Anabaena* sp. the increases were much smaller (0.37 to 0.52 and 0.45 to 0.75, respectively) (Fig. 5, A–C). Consequently, the ratio of phycoerythrin to phycocyanin also increased (0.35 to 0.70, 0.15 to 0.7, and 0.40 to 1.10 in, respectively, *A. flos-aquae*, *Anabaena* sp., and *N. spumigena*). At the end of the following dark period, most pigment ratios returned to the starting value of the day before. Only the ratio of phycocyanin to chl *a* in *Anabaena* sp. did not change during the dark period. The ratio of phycoerythrin to phycocyanin in *N. spumigena* was lowered after the dark period but did not return to the starting value of the day before. In the field sample no extra measurements were taken at the beginning of the next light period, because the ship had moved to another station at that time.

Monochromatic light-response curves of nitrogenase activity. Light-response curves of nitrogenase activity were recorded for *N. spumigena* using white light and monochromatic light of 440, 560, 610, and 675 nm (Fig. 6). The rationale of the selected wavelengths was that ni-

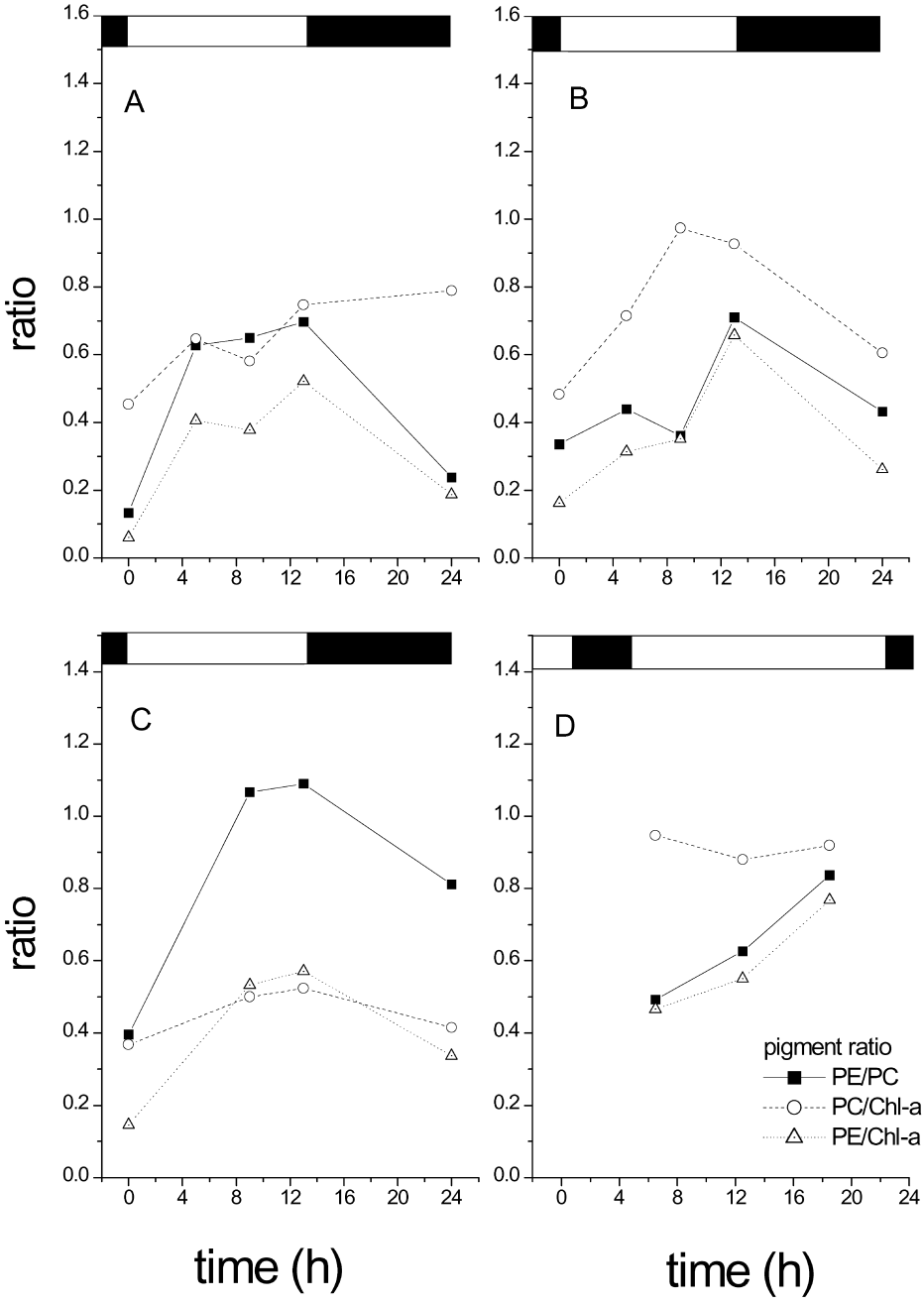

FIG. 5. Ratios of the contributions of the three major pigments, phycoerythrin, phycocyanin, and chl *a*, to the light stimulation of nitrogenase during a light period. The ratios were measured on cultures of (A) *Anabaena* sp., (B) *Aphanizomenon flos-aquae*, and (C) *Nodularia spumigena* and on (D) natural samples from the Baltic Sea. The bars at the top of the graphs indicate the light (white) and dark (black) periods.

trogenase activity peaks were observed due to absorption by chl *a* (440 and 675 nm) and the phycobiliproteins (phycoerythrin, 560–570 nm, and phycocyanin, 610–620 nm). The values were plotted as a percentage of the maximum nitrogenase activity at saturating white light minus the activity measured in the dark, according the equation

$$100\% = N_m^{white} = N_{tot}^{white} - N_d \quad (1)$$

N_{tot}^{white} and N_d were determined by assaying nitrogenase activity at saturating light (200 μmol photons·m^{-2}·s^{-1}) and in the dark. The difference between these values was set to 100%. We anticipated that at any given wavelength this value would be reached when supplied at a saturating photon irradiance. However, such irradiances of monochromatic light could not be obtained with our equipment. Light-response curves were fitted with the rectangular hyperbola model (Talling 1957):

$$N_I = N_m[\alpha I/(N_m + \alpha I)] + N_d \quad (2)$$

where N_I, N_m, and N_d denote the actual rate of nitrogenase activity at a given photon irradiance, the maximum light-dependent activity, and the dark nitrogenase activity, respectively. Note that the maximum N_I at saturating light equals the sum of N_m and N_d, which we refer to as N_{tot}. Because saturating monochromatic photon irradiances were not obtained, N_m was set to 100% in the fit procedure. At irradiances above 3–6 μmol photons·m^{-2}·s^{-1}, nitrogenase activity increased nonlinearly with irradiance. The light saturation coefficient, I_k, was calculated as N_m/α.

Of the four tested wavelengths, only 675 nm behaved more or less like white light. Both α and I_k approached the values obtained for white light (Table 2), and the data points followed those of white light (Fig. 6). The next most efficient wavelengths were 610 nm (phycocyanin) with an α and I_k of, respectively, 56% and 177% of the values of white light and 440 nm (the blue absorption peak of chl *a*) with α and I_k of, respectively, 45% and 222% of the values for white light. The least efficient wavelength was 560 nm (phycoerythrin), which revealed an α and I_k of 28% and 368%, respectively, of white light. At an irradiance of 10 μmol photons·m^{-2}·s^{-1}, nitrogenase activity at the wavelengths 675, 610, 440, and 560 nm were, respectively, 96%, 78%, 70%, and 51% of the activity at white light.

DISCUSSION

Light action spectra reveal information on the pigment composition of heterocysts. The action spectra of N_2 fixation in the three cultures of Baltic Sea heterocystous cyanobacteria, *N. spumigena*, *A. flos-aquae*, and *Anabaena* sp., and of natural samples of N_2-fixing cyanobacterial blooms in the Baltic Sea have clearly demonstrated the contribution of a variety of different pigments to support light stimulation of nitrogenase activity. This means that these pigments must have been involved in the harvesting of light and of transferring the energy to support N_2 fixation. Because N_2 fixation in heterocystous cyanobacteria is confined to the heterocyst and the ATP required for N_2 fixation is generated in these cells (Wolk et al. 1994), we conclude that the pigments were present in the heterocysts. Our conclusion is supported by the fact that the heterocysts of *N. spumigena* and *Anabaena* sp. show strong autofluorescence of phycobiliproteins (results not shown). This is not trivial because it has often been suggested that phycobiliproteins are mainly associated with the oxygenic PSII and therefore would be absent or not functional in heterocysts (Thomas 1970, Alberte et al. 1980). It is now known that in cyanobacteria, light harvested by phycobiliproteins may be transferred directly to PSI (Mullineaux 1994). Measurements of nitrogenase light-response curves of *N. spumigena* and *Anabaena* sp. in the presence of the inhibitor of PSII, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, showed no signifi-

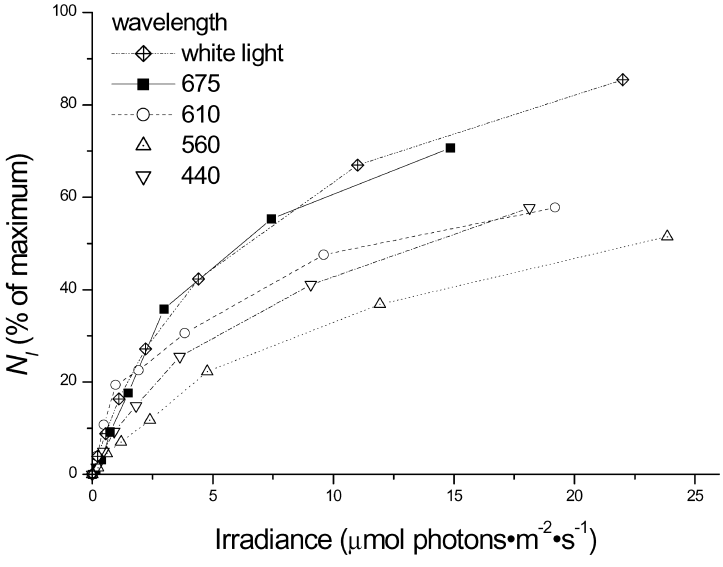

FIG. 6. Light saturation curves of nitrogenase at specific wavelengths in the heterocystous cyanobacterium *Nodularia spumigena*.

TABLE 2. Light affinity coefficient of the light-response curves illuminated with monochromatic light.

Wavelength	α (% of max N_m μmol photons·m^{-2}·s^{-1})	r^2	I_k
675	16.5 ± 0.7	0.99	6
610	10.3 ± 1.5	0.91	10
560	4.6 ± 0.2	0.99	20
440	8.2 ± 0.4	0.99	12
White light	18.2 ± 0.9	0.99	5

Light-response curves were fitted in Origin (Microcal Software Inc., Northampton, MA, USA using the equation $N_l = N_m[\alpha I/(Nm + \alpha I)] + N_d$, where N_l, N_m, and N_d denote nitrogenase activity in, respectively, the given photon irradiance, the maximum light-dependent activity at saturating irradiances, and in the dark. α is the light affinity coefficient, and I denotes irradiance. In this case only α was estimated by the model, because Nm and Nd were set to 100% to 0%, respectively (see text).

cant effect (data not shown). This indicates that the photostimulation of nitrogenase was mediated by PSI of the heterocyst and not due to the photosynthetic activity of the neighboring vegetative cells, that is, by increasing the amount of O_2 or carbohydrate. However, with respect to the presence of phycobiliproteins in heterocysts, contradictory reports have been published.

Although some studies concluded that the phycocyanin content of the heterocyst was virtually zero (Thomas 1970), others measured as much as 40% of the amount present in vegetative cells (Peterson et al. 1981). Likewise, phycoerythrin was not found in the heterocysts of various *Anabaena* species (Fay 1970, Thomas 1970, Peterson et al. 1981). Fay (1970) and Peterson et al. (1981) concluded that only light absorbed by chl *a* and phycocyanin was able to support nitrogenase activity in *A. variabilis* and *A. cylindrica*. However, Fay (1970) may have neglected a small hump at 575 nm in the action spectrum of nitrogenase activity in *A. cylindrica* that he published; this hump could have been ascribed to phycoerythrin. Yet, the concentration of phycoerythrin in heterocysts of *Aphanizomenon* and *Richelia* could equal or even exceed those of vegetative cells (Janson et al. 1994, 1995). The action spectra of nitrogenase showed unequivocally the contribution of phycoerythrin in our strain of *Anabaena*. Although we are aware that even closely related species may have different pigment compositions, the discrepancy could also be due to the very low phycoerythrin to chl *a* ratio in the beginning of the light period (0.05). If the strong variation of this ratio was a common property in heterocystous cyanobacteria, it would be important to take into account the point of time of sampling in a light: dark period.

The resolution of the action spectra was not sufficient to separate phycocyanin and allophycocyanin. Allophycocyanin shows a characteristic shoulder at 654 nm that was missing in the spectrum of phycocyanin we used for the fit procedure. This spectrum had an absorption ratio A_{620}/A_{654} of 3, twice the value of phycobiliproteins isolated from heterocysts of *Anabaena* PCC 7119 (Yamanaka and Glazer 1983). Hence, allophycocyanin was apparently absent in our extract, and this would explain the lower quality of the fit of the action spectra around 654 nm. However, nitrogenase activity was not lower at this wavelength, and therefore we are confident that allophycocyanin absorbs light and contributes to N_2 fixation in the heterocysts.

The action spectra of the three strains revealed the contribution of the carotenoids β-carotene and echinenone to nitrogenase activity, and hence we conclude these pigments were present in the heterocysts. The presence of carotenoids in heterocysts is not unusual (Fay 1969), and it is known that they may be very efficient in transferring light energy to PSI (Cogdell and Gardiner 1993).

Light response of N_2 fixation. The rationale for making light-response curves for monochromatic light was to determine whether nitrogenase activity increased linearly with photon irradiance within the range that was used for the action spectra. We assumed that light and nitrogenase activity correlated linearly up to irradiances of half of the I_k. Hence, at wavelengths close to 675 nm, irradiance should not exceed 3–4 μmol photons·m^{-2}·s^{-1}, whereas in the region 480–530 nm, nitrogenase activity increased linearly until 10 μmol photons·m^{-2}·s^{-1}. The irradiance of monochromatic light varied with the light source and differed between the wavelengths coming through the monochromator. We were careful not to exceed the maximum values determined for linear response of nitrogenase activity. It was therefore possible to compare the nitrogenase activity at each wavelength by dividing them by the photon density at which it was measured. Although monochromatic light-response curves were only measured for *N. spumigena*, it was anticipated that the other strains would behave similarly because there were only marginal differences in I_k when measured in white light.

Quantum efficiency of light harvesting. It was not possible to determine the quantum efficiencies of N_2 fixation for the pigments, because to do this the pigment concentrations in the heterocysts and the absorption cross-section must be known. *In vivo* absorption spectra reflect mainly the light absorption by the vegetative cells, which represent approximately 95% of the total number of cells. Even when the pigment concentrations in the heterocysts were known, α, which equals in this case $1/K_m$ in enzyme kinetics, would not be a good estimate of quantum efficiency. This parameter depends not only on pigment concentrations, but also on N_d and the concentration of nitrogenase (Staal et al. 2002).

To circumvent this problem, we followed another approach. Using the monochromatic light-response curves recorded in *N. spumigena*, we found that N_d and N_{tot} did not change during the day when grown under continuous light, and it is unlikely that pigment concentrations change during a measurement (40 min). Hence, it was possible to compare differences in quantum efficiency for different wavelengths within a pigment. When we compared quantum yield at 675 and 440 nm, we obtained unexpected results. The differ-

ences in quantum yield at these wavelengths were in contradiction to the higher absorption at the blue peak of extracted chl *a* ($A_{440}/A_{675} \approx 1.4$). The action spectra of *N. spumigena* and *A. flos-aquae* and the field samples showed a lower than expected stimulation of nitrogenase in the blue peak of chl *a*. A possible explanation for this paradoxical result is that several carotenoids and xanthophylls also absorb light at 440 nm without contributing to nitrogenase activity. Both myxoxanthophyll and canthaxanthin are known to have photoprotective properties in cyanobacteria, whereas they have no function as photosynthetic pigments (Lakatos and Büdel 2001). In addition, other nonphotosynthetic UV-absorbing pigments, such as mycosporine-like amino acids, absorb in the 440-nm region (Ehling-Schulz and Scherer 1999, Subramaniam et al. 1999). The *in vivo* spectra showed an absorption ratio A_{440}/A_{675} of 1.90 and 2.17 for, respectively, *N. spumigena* and *A. flos-aquae*, much higher than $A_{440}/A_{675} \approx 1.4$ that we would expect when only chl *a* absorbs blue light. The ratio A_{440}/A_{675} of 1.55, found in the *in vivo* absorption spectrum of *Anabaena* sp., indicates much lower absorption in the 440-nm region by other pigments than chl *a*. Moreover, *Anabaena* sp. was the only organism in which the N_2 fixation rate per photon was higher in the blue region than in the red region. Unfortunately, we did not measure *in vivo* spectra of field samples. However, the difference in relative quantum efficiency between the blue and red chl peak of the nitrogenase action spectra indicates that the A_{440}/A_{675} for the field sample is probably even higher than the one of *A. flos-aquae*, because in cyanobacteria a positive correlation between photoprotective pigments and irradiance is usually found (Lakatos and Büdel 2001). The irradiances in the Baltic Sea were much higher than those applied to the cultures, whereas in addition the latter were not exposed to UV light.

Chromatic acclimatization. Many phycoerythrin-containing cyanobacteria are capable of chromatic adaptation, that is, they adjust the relative amounts of phycocyanin and phycoerythrin to the prevailing spectral light quality and light quantity (Grossman et al. 1994, MacColl 1998). Our results have shown that phycoerythrin and, to a lesser extend, phycocyanin contribute to a varying extend to N_2 fixation during a day: night cycle. From this we conclude that the pigment composition of the heterocysts is variable during the period of 1 day. However, because we did not change the spectral quality of the light, the pigment changes in the heterocyst were not caused by chromatic acclimatization. The amount and composition of the phycobiliproteins may change as the result of the nutritional condition of cyanobacteria, particularly because of the nitrogen status of the cells (Wyman et al. 1985). It is tempting to speculate that the phycobiliproteins in the heterocyst can serve as a temporal nitrogen storage (Grossman et al. 1994). During the night when the rates of N_2 fixation are low, the phycobiliproteins and particularly phycoerythrin in the heterocysts may be degraded to allow an uninterrupted nitrogen supply to the vegetative cells. During the light, the higher rate of N_2 fixation would allow resynthesis of the phycobiliproteins. A similar theory was proposed to explain the observed changes in phycobiliprotein ratios within natural populations of the nonheterocystous N_2-fixing cyanobacterium *Trichodesmium* (Subramaniam et al. 1999).

Very little is known about the possibility of heterocysts to change their pigment composition, and it has been suggested that these cells are unable to synthesize phycobiliproteins (Yamanaka and Glazer 1983). Although indirectly through the measurement of nitrogenase activity, we provided for the first time strong evidence that heterocysts are able to vary the composition of their pigments. This can only be explained when heterocysts are capable of synthesizing and degrading phycobiliproteins actively.

The pronounced spectral differences of nitrogenase activity and their changes during a day:night cycle will have considerable consequences for the nitrogen budget of ecosystems in which heterocystous cyanobacteria are the dominant N_2 fixers. Simple N_2 fixation versus irradiance relationships that are used to calculate daily integrated amounts of nitrogen fixed may be biased when pigment changes and spectral differences are not taken into account (Stal and Walsby 2000).

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