ORIGINAL ARTICLE

Light dependency of nitrogen fixation in a coastal cyanobacterial mat

Ina Severin¹ and Lucas J Stal^{1,2}

¹Department of Marine Microbiology, Netherlands Institute of Ecology (NIOO-KNAW), Yerseke, The Netherlands and ²Department of Aquatic Microbiology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, The Netherlands

The fixation of nitrogen in cyanobacterial mats situated along the littoral gradient on a Dutch barrier island was investigated by using a high-resolution online, near-real-time acetylene reduction assay. Light-response curves of nitrogenase activity yielded a variety of physiological parameters that changed during a day–night cycle. The fitted parameters were used to calculate nitrogen fixation from the incident natural irradiance over several days in two different mat types. Mats occurring in the higher regions of the littoral were composed of a diverse community of cyanobacteria, consisting of both heterocystous and non-heterocystous filamentous species, whereas closer to the low water mark the mats contained mainly non-heterocystous filamentous cyanobacteria. Although the daily cycles of nitrogenase activity differed considerably between the two types of mats, the daily integrated rates of nitrogen fixation were the same. Moreover, the daily integrated nitrogen fixation seemed to be independent from the daily incident photon flux. The measurements further suggest that different types of diazotrophic cyanobacteria become active at different times of the day and that the composition of the mat community affects maximal and daily patterns of nitrogenase activity. Notwithstanding the apparent light independence of nitrogen fixation, the light-response curves as well as light action spectra unequivocally showed that cyanobacteria were the predominant nitrogen-fixing organisms in these mats. It is concluded that the diversity of nitrogen-fixing cyanobacteria leads to an optimization of this process.

The ISME Journal (2008) 2, 1077–1088; doi:10.1038/ismej.2008.63; published online 19 June 2008

Subject Category: geomicrobiology and microbial contributions to geochemical cycles

Keywords: action spectra; cyanobacteria; day–night cycles; light-response curves; microbial mat; nitrogen fixation

Introduction

The marine environment is often depleted of combined nitrogen and this nutrient then limits primary productivity (Paerl, 1990). This is particularly the case when the demand for nitrogen is high and microbial processes such as denitrification and anaerobic ammonium oxidation cause the continuous loss of bioavailable nitrogen. Under these circumstances, diazotrophic (N_2 fixing) microorganisms have an important ecological advantage. This is for instance the case in most microbial mats where N_2 fixation is often an important process (Bebout *et al.*, 1987). In coastal microbial mats, cyanobacteria have usually been regarded as the predominant diazotrophic organisms although other researchers

have also attributed N_2 fixation in cyanobacterial mats to associated heterotrophic bacteria (Zehr *et al.*, 1995; Olson *et al.*, 1999). In this model, N_2 fixation is the result of a joint venture of cyanobacteria and heterotrophic bacteria in which the former provide substrate and growth factors to the latter. The heterotrophic bacteria provide the cyanobacteria with fixed N_2 and CO_2 (Steppe *et al.*, 1996).

Nitrogenase, the enzyme responsible for the reduction of atmospheric N_2 to ammonium, is only found in prokaryotes (Zehr *et al.*, 2003). Among them cyanobacteria occupy a special place as many representatives are capable of fixing N_2 . Cyanobacteria can meet the high-energy demand of N_2 fixation (16 ATP for the reduction of 1 mol N_2), but the extremely oxygen-sensitive nitrogenase is incompatible with their oxygenic phototrophic mode of life (Fay, 1992). Therefore, cyanobacteria have evolved a variety of strategies to comply with this incompatibility. Among the most ingenious adaptations is the differentiation of a special cell type, the heterocyst, which is devoted to the fixation of N_2 . Heterocysts have lost photosystem II and hence the

Correspondence: LJ Stal, Department of Marine Microbiology, Netherlands Institute of Ecology, P.O. Box 140, 4400 AC Yerseke, The Netherlands.

E-mail: l.stal@nioo.knaw.nl

Received 13 March 2008; revised 20 May 2008; accepted 27 May 2008; published online 19 June 2008

capacity of oxygenic photosynthesis (Adams, 2000). Moreover, the cell envelope of heterocysts represents a gas diffusion barrier and any O_2 that enters the cell is scavenged by an efficient and high-affinity respiratory system (Walsby, 2007). Hence, heterocysts provide an anaerobic environment for nitrogenase and are a means to spatially separate oxygen-evolving photosynthesis from oxygen-sensitive N_2 fixation. Most non-heterocystous diazotrophic cyanobacteria fix N_2 exclusively under anaerobic conditions. However, a few types have evolved strategies that enable them to carry out aerobic N_2 fixation (Bergman *et al.*, 1997). Analogous to the heterocystous cyanobacteria, some species separate N_2 fixation temporally from photosynthesis by confining the former to the night. A few non-heterocystous species have evolved alternative strategies to allow N_2 fixation under aerobic conditions. The filamentous non-heterocystous cyanobacteria *Trichodesmium* spp. may combine spatial as well as temporal separation of the incompatible processes (Berman-Frank *et al.*, 2007).

Respiration is necessary to render the N_2 -fixing cyanobacterial cell anoxic, but it will also cover part of the energy demand of nitrogenase. Even so, light remains important as a source of energy for N_2 fixation in cyanobacteria. This is clearly demonstrated by the recordings of light-response curves of nitrogenase activity. These curves are similar to light-response recordings of photosynthesis (Staal *et al.*, 2002). Although the photosynthetic parameters are derived empirically from light-response curves using any model that fits the data, for nitrogenase it is anticipated that it should follow Michaelis–Menten enzyme kinetics. Light-response curves and the parameters of nitrogenase activity have therefore been derived by fitting using the rectangular hyperbola model (Staal *et al.*, 2002). The parameters derived from such light-response curves give important information on the physiological status of the N_2 -fixing cyanobacterium and their adaptation to different light regimes and during a daily cycle. Light is absorbed by a variety of photosynthetic pigments and thus the quality of light influences N_2 fixation in cyanobacteria. Light action spectra on nitrogenase activity are therefore useful to identify the pigments involved in N_2 fixation in cyanobacteria (Staal *et al.*, 2003a). Hitherto, neither light-response curves nor *in vivo* action spectra for nitrogenase activity have been published for microbial mat communities.

This paper focuses on microbial mats growing on sandy beaches of the barrier islands of the Wadden Sea (southern North Sea) (Stal *et al.*, 1985). These mats are locally known as ‘green beaches’ and may cover extensive areas. The colonization of the beach by cyanobacteria and their rapid growth has a profound influence on the local coastal morphodynamics. After cyanobacterial mats are established, a succession by higher plants takes place, changing the beach into a salt marsh-like community. Along

the littoral gradient, different types of microbial mats occur. Close to the low water mark, mats of the non-heterocystous filamentous cyanobacterium *Lyngbya aestuarii* develop. In the upper littoral, close to the dunes, microbial mats are more diverse and usually contain heterocystous cyanobacteria. The latter are less affected by seawater and receive freshwater from upwelling groundwater and from rain, while only irregularly inundated by the sea. The development of these mats and the subsequent succession by higher plants in this low-nutrient environment would be impossible without the fixation of N_2 .

The aim of this work was to measure the daily variations of nitrogenase activity in different types of coastal microbial mats of the barrier islands to gain a better understanding of the role of cyanobacteria in N_2 fixation in these microbial mat communities. Nitrogenase activity in these mats was measured for the first time by using the highly sensitive, online and near-real-time acetylene reduction assay (ARA). This approach allowed us to record light-response curves and light action spectra, providing insight in the contribution of cyanobacteria to nitrogenase activity. Although there is no doubt that cyanobacteria were the dominant-active diazotrophic organisms in these mats, it was found that the daily integrated N_2 fixation appeared independent of the daily incident photon flux. And although the two mat types differed considerably with respect to community composition and thus diurnal pattern of N_2 fixation, there was no difference in the daily integrated amount of nitrogen fixed. It appears that the mat community optimized this process.

Materials and methods

Sampling

The study site was located on the Dutch barrier island of Schiermonnikoog, which is situated in the Wadden Sea close to the mainland. The geographic coordinates of the site are $N53^{\circ}29'$ and $E6^{\circ}08'$. Microbial mats were found at the sandy beach covering the north bank of the island facing the North Sea (Figure 1). Areas of the beach are currently turning into a salt marsh resulting in mats partly overgrown by higher plants. Due to this succession and the gradually changing influence of the North Sea environment, different mat types develop along the littoral gradient.

Two sampling sites within this area were chosen based on microscopic observations of the cyanobacterial community composition as well as their location along the littoral gradient. Station I was located near the dunes and influenced by both seawater and freshwater. This area is only irregularly inundated, usually at spring tide and with northern winds. The mats were covered with a thin layer of sand deposited by wind. The mats found at

Figure 1 Geographical location of stations I and II on the island Schiermonnikoog.

this station revealed high cyanobacterial species diversity as judged by microscopic examinations and contained both heterocystous and non-heterocystous filamentous cyanobacteria as well as unicellular species. Diatoms represented another major group of phototrophic organisms. Station II was situated near the low water mark. Due to tidal inundation, seawater was far more important than the occasional rain showers and this distinguished it from station I. The cyanobacteria in these mats were mostly non-heterocystous forms, predominantly *L. aestuarii*. Diatoms were also an important component of these mats. Occasionally, heterocystous cyanobacteria have been observed, but these organisms did not seem to be an important structural part of this community.

For each 24 h measurement of nitrogenase activity, samples of the mats were collected using a corer with a diameter of 50 mm made of a PVC tube. The upper 2–3 mm of the mat was dissected using a sharp knife. After finishing the nitrogenase activity measurements, the sample was frozen in liquid nitrogen for later chlorophyll and other pigment analyses.

Acetylene reduction assay

Nitrogenase activity was measured using the ARA (Hardy *et al.*, 1968). We used the online method as

described by Staal *et al.* (2001). Ethylene concentration was measured using a laser-based photoacoustic (LPA) trace gas detection system (te Lintel Hekkert *et al.*, 1998) (Sensor Sense, Nijmegen, the Netherlands; <http://www.sensor-sense.nl/>). This instrument measures ethylene with an accuracy of <1% or 0.3 p.p.b.v., whichever is larger. The noise is 0.3 p.p.b.v. The ethylene background (as a contaminant in the acetylene) was ~10 p.p.b.v. Even at the lowest nitrogenase activities the concentration of ethylene was >100 p.p.b.v., allowing for highly accurate measurements with an error of less than 1%. The mat samples were incubated in a custom-made measuring cell under a continuous flow of 2 l h^{-1} of a gas mixture containing 20% O_2 , 70% N_2 (each containing 0.04% CO_2) and 10% C_2H_2 which was prepared using electronic mass flow controllers (Staal *et al.*, 2001). Gas mixtures and acetylene were purchased from Hoek-Loos and Messer (both in The Netherlands), respectively. The upper 2–3 mm of the mat was placed in a measuring cell with a diameter of 46 mm. The temperature of the cell was kept at 18°C using a Peltier element placed right beneath the bottom of the measuring cell. A halogen lamp (Model 460-F; Heinz Walz GmbH, Germany) served as the light source. Gas mixture and flow, temperature and light were controlled using a custom-made software program written in Testpoint.

Light-response curves

Light-response curves of nitrogenase activity were calculated from ethylene production rates at increasing as well as decreasing photon flux densities (PFDs) ranging from 0 to $300\mu\text{mol m}^{-2}\text{s}^{-1}$. The light-response curves obtained from increasing or decreasing PFDs were different and yielded different parameters from the fits. They were therefore both analyzed and considered. A complete light-response curve took 36 min, with nine different light intensity steps, each for 3 min, and a dark incubation for 9 min. Only values from the period after reaching steady-state conditions following the change of light intensity were taken into account (usually after ~1 min). Nitrogenase activity was normalized to the phototrophic biomass (mg chlorophyll a, chl a). Light-response curves were fitted using the rectangular hyperbola model (Staal *et al.*, 2002). Fitting the measured light-response curve to the rectangular hyperbola model was performed with the Solver function of Microsoft Excel (Microsoft Office 2003) by nonlinear least-squares fitting. All parameters (N_m , N_d and α) were derived from these fits and used to calculate I_k , N_{tot} and N_{tot}/N_d .

N_m is the maximum light-dependent nitrogenase activity under saturating PFDs, whereas N_d represents the light-independent activity. Any detection of N_m indicates the contribution of phototrophic organisms to community nitrogenase activity. N_d is the part of nitrogenase activity that is fueled by heterotrophic (respiration or fermentation)

metabolism only. $N_m + N_d = N_{\text{tot}}$ and the dimensionless ratio N_{tot}/N_d describes the degree of light dependency of N_2 fixation in the diazotrophic cell. When $N_{\text{tot}}/N_d = 1$, heterotrophic metabolism saturates the energy demand of nitrogenase and when $N_{\text{tot}}/N_d > 1$ light energy contributes to N_2 fixation. The larger this ratio, the more important light is compared to heterotrophic metabolism. Therefore, N_{tot}/N_d is an important measure of the physiology of nitrogenase activity in the mat, yielding information about the relative contribution of phototrophic and heterotrophic metabolism to N_2 fixation. I_k is the light saturation constant (identical to K_m in Michaelis–Menten kinetics) and is likewise biomass independent. It represents the light intensity at which nitrogenase would reach its maximum if the activity increased linearly with light intensity. Alpha (α) is the initial slope of the light-response curve, calculated as N_m/I_k , and represents the light affinity of nitrogenase activity.

Daily N_2 fixation

Natural PFDs ($\mu\text{mol m}^{-2} \text{s}^{-1}$) were recorded using a PAR (photosynthetic active radiation) light sensor (Licor 1000; Licor, Lincoln, NE, USA) connected to a data logger. The PFD was measured at intervals of 1 s and averages were stored every minute. The rate of nitrogenase activity at any time of the day and for any day was calculated from the fitted parameters obtained from the hourly measured light-response curves and the recorded natural PFDs according to equation (1).

$$N = N_m \left(\frac{\alpha I}{N_m + \alpha I} \right) + N_d \quad (1)$$

where N_m is the nitrogenase activity at saturating irradiances minus N_d , N_d , the nitrogenase activity measured in the dark, α , the light affinity coefficient for nitrogenase activity and I , the natural irradiance.

From these rates, the daily integral of acetylene reduction and the average amount of N_2 fixed per day were calculated. Daily fixed N_2 was calculated from acetylene reduction using a conversion factor of 4 (Jensen and Cox, 1983) and normalized to the surface area. Using this procedure, N_2 fixation was calculated for several days for both stations. Light-response curves measured with increasing as well as with decreasing PFDs were both used and compared.

Light action spectra of nitrogenase activity

In situ light action spectra of nitrogenase were obtained by using interference filters with a bandwidth of 10 nm (Knight Optical UK Ltd, UK) from 390 to 700 nm in 10 nm intervals. Nitrogenase activity was recorded under a constant incident light intensity before the interference filter. The measurement at each wavelength took 5 min; allowing steady-state conditions to establish after each

change of filter. Only values from the period after reaching steady-state conditions were taken into account. The transparency of the interference filters was measured for each filter. Maximum monochromatic PFDs were up to $50 \mu\text{mol m}^{-2} \text{s}^{-1}$, depending on the wavelength. Nitrogenase activity was normalized to the photon irradiance at each wavelength.

Pigments

Lyophilized and homogenized samples were extracted with 90% acetone. Pigments were determined by high-performance liquid chromatography (HPLC) (Dionex LC-02; Dionex Corporation, Sunnyvale, CA, USA). The HPLC was equipped with a photodiode array detector (PDA-100) and a fluorescence detector (RF-2000). Pigments were separated on a reverse-phase C18 column (QS Novapak 4 C18, 150×3.9 mm), running three mobile phases (A: 0.5 M ammonium acetate in 85% methanol, B: 90% acetonitrile and C: 100% ethyl acetate) at a flow rate of 0.8 ml min^{-1} (modified after Wright *et al.*, 1991). The gradient was changed stepwise from 60% solvent A and 40% solvent B to 100% solvent B, including steps with 20, 50 and 70% solvent C instead of solvent A. Identification of pigments was performed based on their retention times and absorption spectra. Concentrations were calculated from the peak areas using standards of known concentration.

Determination of bulk chlorophyll *a*

Chlorophyll *a* of the samples that were used for the nitrogenase measurements was extracted twice with 96% ethanol. The sediment was homogenized in the ethanol and left in the dark for 2 h at room temperature. The extract was centrifuged and the pellet was reextracted overnight, while the first extract was stored in the dark at 4°C . The two extracts were pooled and the absorption was measured at 665 nm in a spectrophotometer (Pharmacia Biotech—Novaspec II, Cambridge, UK). The amount of chl *a* in each sample was calculated using an absorption coefficient of $72.3 \text{ ml mg}^{-1} \text{ cm}^{-1}$ and related to the sample surface. For stations I and II, the chl *a* contents were 205.6 mg m^{-2} ($327 \mu\text{g}$ per mat sample) and 226.4 mg m^{-2} ($360 \mu\text{g}$ per mat sample), respectively.

Results

Light-response curves

Total nitrogenase activity (N_{tot}) was measured as ethylene (C_2H_4) production (ARA) and normalized to chl *a*. Light-response curves of the microbial mats were recorded during a period of 20 and 24 h for the stations I and II, respectively. For technical reasons we were unable to complete the full 24 h cycle for station I. Figure 2 illustrates representative light-response curves measured at increasing and

Figure 2 Nitrogenase activity versus irradiance curve for station II. Acetylene reduction assay (ARA). ●, Measured curve; —, fitted with rectangular hyperbola model. (a) Increasing photon flux densities and (b) decreasing photon flux densities.

decreasing PFDs (station II, 2125 hours). Nitrogenase activity was saturated at a PFD of ~ 270 and $\sim 135 \mu\text{mol m}^{-2} \text{s}^{-1}$ measured at increasing and decreasing PFDs, respectively.

The daily variation of the parameters derived from the light-response curves is depicted in Figure 3. Both stations showed considerable variation in nitrogenase activity during a diel cycle with most of it occurring during the night. The largest differences between parameters derived from measurements using increasing and decreasing PFDs were found for α and I_k . Much higher values for curves recorded using decreasing PFDs, especially at station I, were shown by α . In contrast, N_m showed higher values when recorded under increasing PFDs. The difference in N_d was less pronounced, showing similar values for station I and slightly higher values for increasing PFDs at station II. This resulted in a higher total nitrogenase activity recorded under increasing PFDs for station I and substantially higher values obtained for decreasing PFDs for station II (Figure 4). The daily patterns were similar for curves recorded using either increasing or decreasing PFDs. The daily pattern of the light saturation coefficient I_k that gives information about the affinity of nitrogenase toward light was similar for both stations but showed considerable differences when derived from curves recorded with increasing or decreasing PFDs. For increasing PFDs, I_k peaked at 2200 and 2030 hours at stations I and II, respectively, whereas there was a sharper and later peak for decreasing PFDs (0600 and 0300 hours for stations I and II, respectively).

N_{tot} and N_{tot}/N_d for both stations and for both light-response curves are depicted in Figure 4. In station II, N_{tot} was virtually 0 during the day and showed an increase during the first part of the night and a decrease during the second half, reaching a maximum just after midnight. This was the case for both light-response curves, although the highest rates were obtained when using the values from the light-response curve recorded with decreasing PFDs. In station I, nitrogenase activity was present throughout the day but the highest rates for N_{tot} were obtained at night and resembled that of station II. The N_{tot}/N_d ratio was calculated to reveal the degree

of light dependency of nitrogenase activity. This ratio is convenient because it is biomass independent and therefore specifically describes the activity in the N_2 -fixing cell. A ratio above 1 shows that respiration is not sufficient to meet the energy requirements of nitrogenase, making light-driven ATP generation necessary. Often the ratio is ~ 3 but deviations have been observed depending on the physiological state of the organism (Staal *et al.* 2002). Under decreasing PFDs, the N_{tot}/N_d ratio for station I showed a slightly decreasing trend during the course of the measurement, reaching a minimum value of 1.5. For station II, a stronger decrease in N_{tot}/N_d was observed at about 1800 hours, showing a minimum N_{tot}/N_d of 1.4 at 2030 hours, followed first by a slow and then by stronger increase at 0200 hours. The daily pattern for this ratio was totally different when obtained from the parameters of the light-response curve using increasing PFDs. For both stations, a peak (at 2200 and 2000 hours for stations I and II, respectively) in N_{tot}/N_d was observed followed by a decrease to minimum values of 2.1 and 1.6 for stations I and II, respectively (Figure 4).

Daily pattern of nitrogenase activity

Nitrogenase activity for natural PFD was obtained using the equations derived from the corresponding light-response curves. For both stations, the daily cycle of nitrogenase activity showed a consistent pattern almost independent from the differences in actual PFDs on 4 consecutive days. Therefore, only the pattern for 1 day (31 May 3101 June 2006) is shown (Figure 5).

For station I, chlorophyll a-normalized ethylene production ranged from 0.4 to 1.7 and 0.4 to $1.1 \mu\text{mol mg}^{-1}$ per hour for calculations based on increasing and decreasing PFDs, respectively. The trend was increasing and revealed three slight maxima at 2100, 0230 and 0600 hours. For the cycle calculated based on the measurements under increasing PFDs, the maxima at sunset and sunrise were much more pronounced. The daily cycle of nitrogenase activity at station II differed from that of station I. Nitrogenase activity increased from 1900 hours onward, resulting in a peak at about midnight, subsequently decreasing again until 0500 hours. Total nitrogenase activity (ARA) for both scenarios (increasing or decreasing PFDs) ranged from ~ 0.2 to $2.7 \mu\text{mol mg}^{-1}$ per hour. Under increasing PFDs, the calculation results in a broader peak with a slightly lower maximum.

Integrated nitrogenase activity also differed when based on increasing or decreasing PFDs, but these differences were similar for both stations. There was no relation between daily integrated nitrogenase activity and daily integrated photon flux. For both stations, the amount of ethylene produced by acetylene reduction per day was virtually the same (Table 1). Normalizing acetylene reduction to

Figure 3 Change in α , I_k , N_m and N_d . All parameters derived from measurements of nitrogenase activity versus irradiance curves (acetylene reduction assay, ARA) fitted with the rectangular hyperbola model. (a, c, e and g) Station I; (b, d, f and h) station II; (a, b) α ; (c, d) I_k ; (e, f) N_m ; (g, h) N_d . $\blacktriangle$, Increasing photon flux densities; $\bullet$, decreasing photon flux densities.

surface area, an average of 4.0 and 4.4 mmol m⁻² per day was obtained for stations I and II, respectively. This equals an average amount of 28 mg N m⁻² per day at station I and 31 mg N m⁻² per day at station II.

Light action spectra of nitrogenase

Light action spectra were recorded for microbial mats at stations I and II. These spectra showed maxima at 458, 490, 520, 610, 660 and 700 nm,

matching the *in vivo* absorption maxima of carotene or zeaxanthin (overlapping in the range of 460–520 nm), myxoxanthophyll, phycocyanin, allophycocyanin and chlorophyll a, respectively (Figure 6). The action spectra were similar for both stations as was the stimulation of nitrogenase activity at certain wavelengths.

The pigment composition, as revealed by HPLC analysis of both mats, confirmed the dominance of the pigments carotene and chlorophyll a (Table 2).

Figure 4 Change in N_{tot} (◆) and N_{tot}/N_d (■). All parameters calculated from measurements of activity versus irradiance curves (acetylene reduction assay, ARA) fitted with the rectangular hyperbola model. (A) Station I; (B) station II. (a1, b1) Increasing photon flux densities; (a2, b2) decreasing photon flux densities.

Figure 5 Representative 24 h cycle of nitrogenase activity as measured by acetylene reduction assay (ARA) and calculated on the basis of light-response curves and the natural incident photon flux densities (●). (A) Station I; (B) station II. (a1, b1) Increasing photon flux densities; (a2, b2) decreasing photon flux densities.

Table 1 Daily integrated nitrogenase activity ($\mu\text{mol C}_2\text{H}_4$ per mg chl a) and photon flux ($\mu\text{mol m}^{-2}$) for both stations and based on light response curves using increasing and decreasing photon flux densities

Date	Station I		Station II	
	Nitrogenase activity	Photon flux	Nitrogenase activity	Photon flux
28 May 2006				
Increasing	21.2	26 295	21.4	23 993
Decreasing	17.9		20.4	
29 May 2006				
Increasing	20.9	29 399	20.4	29 275
Decreasing	18.0		17.5	
30 May 2006				
Increasing	20.8	23 866	20.5	29 443
Decreasing	17.7		17.5	
31 May 2006				
Increasing	20.8	15 839	20.8	14 052
Decreasing	17.8		17.5	
Average	19.4		19.5	

Table 2 Pigment content ($\mu\text{g g}^{-1}$) for both stations and pigment absorption maxima (nm) in acetone^a

Pigments	Station I	Station II
α , β -Carotene	3.27	3.38
Chlorophyll a	36.53	43.73
Chlorophyll b	0.00	0.25
Chlorophyll c	0.84	0.85
Diadinoxanthin ^b	2.23	1.69
Pheophytin	1.88	1.48
Fucoxanthin	3.20	3.57

^aWright *et al.* (1991).^bIn eluant.

The pigment concentrations for both stations were similar. All pigments were present in the same order of magnitude. On the basis of the low amounts of chlorophyll species other than chlorophyll a, the pigments involved in light stimulation of nitrogenase activity can be attributed to cyanobacteria.

Discussion

Microbial mats have been studied extensively for more than 30 years. Due to the revolutionary developments in molecular ecology, much knowledge has been obtained about the composition and community structure of microbial mats. However, still very little is known about the physiology of microbial mats as well as the metabolic activities and their controlling factors. N_2 fixation is an important process in many microbial mats. It represents a large resource of nitrogen and can replenish any other bioavailable nitrogen that was lost from the system as the results of microbial activities such as denitrification and anaerobic ammonium oxidation. Notwithstanding the importance of N_2 fixation in microbial mats, measurements of this process have suffered from a lack of detail and resolution due to the unavailability of adequate methods. Online incubation of natural samples or cultures of diazotrophic cyanobacteria has shown its superiority compared to the traditional batch incubation in closed vials (Staal *et al.*, 2001). The online incubation is robust and keeps the sample largely unchanged due to a constant flow of a controlled gas mixture. The online incubation system can be connected to a gas chromatograph, which is traditionally used to measure the ethylene produced from acetylene by nitrogenase. However, the LPA trace gas detector allows a time resolution of ~ 5 s (compared to 5 min with the gas chromatograph) and is three orders of magnitude more sensitive, allowing measurements of very low ethylene concentrations (~ 10 p.p.b.). The combination of on-line incubation and LPA detection results in a near-real-time measurement of nitrogenase activity. This is the first time that this setup has

Figure 6 *In vivo* action spectra of nitrogenase. (a) Station I; (b) station II.

been applied to a microbial mat and we provide measurements with an unprecedented time resolution and sensitivity.

Because of the speed of the measurements we were able to record light-response curves during a 24 h cycle and analyzed two different mat types in this manner. Light-response curves provide much more information than a simple measurement at a set light intensity (usually assumed to be saturating) or at ambient light intensities. Ambient light may vary and only gives a snapshot of that particular point of time. Applying a set light intensity is equally unrealistic when one aims to determine the actual activity. Light-response curves of nitrogenase can be fitted using the rectangular hyperbola model (Staal *et al.*, 2002).

For both stations, changes in the parameters N_m , N_d , α and I_k were observed during the dark period. This hints to variations in the activity levels of the diazotrophic organisms due to an adaptation to changing conditions, to shifts in the active community during a 24 h day or to a combination of both. The parameters α and I_k showed the greatest difference between measurements using increasing or decreasing PFDs that was obvious from the dramatic difference in the initial slope of the curves (Figure 2). In both stations, a high affinity to light (α) was particularly observed at night and when decreasing PFDs were applied for the light-response measurements. The light-dependent part of nitrogenase activity was likewise highest at night, reaching a maximum at midnight. This strongly hints to the involvement of cyanobacteria for the nighttime nitrogenase activity. The combined effect of these two parameters was reflected in a high value for the light saturation constant, I_k , at sunset and sunrise when using increasing and decreasing PFDs, respectively. The increase in nitrogenase activity at sunset was expected in mats dominated by non-heterocystous cyanobacteria. For station I, N_m was slightly higher than N_d , whereas the opposite was the case in the mat containing almost exclusively non-heterocystous cyanobacteria. Heterocystous cyanobacteria were present at station I but virtually absent at station II. Heterocystous cyanobacteria are adapted to fix N_2 in the light concomitantly with oxygenic photosynthesis and therefore nitrogenase activity in the light was expected to be more important in station I compared to station II. The light-response curves recorded with increasing PFD in general revealed lower N_d and higher N_m than those recorded with decreasing PFDs (Figure 2).

Light-response curves were first recorded with increasing and subsequently with decreasing PFDs. The light-response curves obtained in this way were different as a result of hysteresis. This effect has been observed before (Staal M and Stal LJ, unpublished observations) and was attributed to a gradual adaptation of the organism to the increasing PFDs resulting in a slightly different behavior when

measuring the second light-response curve using decreasing PFDs. A similar hysteresis has been observed when recording O_2 response curves of nitrogenase activity (Staal *et al.*, 2003b). In the case of microbial mats, hysteresis may be caused by the dynamics of oxygen. During the measurement at increasing PFDs, the mat turns from a virtually anoxic situation (after 9 min of dark incubation) to a situation with oxygen supersaturation. During the dark period, oxygen is quickly respired, resulting in anoxic conditions. Energy supply for nitrogenase is exclusively covered by anaerobic heterotrophic processes (for example, fermentation of storage carbohydrate by the cyanobacteria). It has been shown that oxygen concentrations in microbial mats may drop rapidly upon transfer into the dark (Villbrandt *et al.*, 1990), ruling out respiration as a possible energy source for dark nitrogenase activity. The measurements under increasing PFDs may therefore reflect the situation in the mat at night. As soon as the mat is illuminated during the recording of the light-response curve, nitrogenase is fueled by energy derived from light as well as from aerobic respiration, made possible through oxygen produced by photosynthesis. Light-independent nitrogenase activity measured under these conditions is lower than when measuring light-response curves under decreasing PFDs. In the latter case, the system is supersaturated with oxygen, which fuels nitrogenase activity in addition to light. Hence, the measurement using decreasing PFDs might represent the daytime situation. Oxygen profiles recorded in microbial mats have demonstrated that oxygen is limited to the very top layer of the mat (up to 0.5 mm) under low light conditions, whereas it may penetrate deeper (up to 4 mm) during the day (Jørgensen *et al.*, 1979).

As argued above, increasing and decreasing PFDs for light-response curves are inappropriate for the situation in the mat at sunset and sunrise, respectively, and we would therefore interpret the peaks of I_k as possible measurement artifacts. Combining the results of the two light-response curves using the line of arguments above would result in a curve without clear peaks at sunset and sunrise. Nevertheless, I_k values would remain higher at night, a tendency that is more pronounced at station I and might also hint to changes in the active diazotrophic community. The peaks in N_{tot}/N_d observed at sunset and sunrise are also considered possible measurement artifacts for the same reason. As is the case with I_k , these peaks disappear when combining the results according to the conditions most likely present in the mat at different times of the day. In that case no clear trend would be observed for the daily pattern of N_{tot}/N_d .

The hysteresis effect observed when recording the light-response curves under increasing and decreasing PFDs may be explained in terms of the prevailing oxygen profiles in the mat during incubations. In accordance with the presumptions mentioned

Figure 7 Representative 24 h cycle of nitrogenase activity as measured by acetylene reduction assay (ARA) and calculated on the basis of natural incident photon flux densities and the fitted parameters of the light-response curves using increasing and decreasing photon flux densities for night- and daytime situation, respectively (●). (a) Station I; (b) station II.

above, we calculated the daily nitrogenase activity pattern under natural light conditions by using the parameters derived from the light-response curve under increasing and decreasing PFDs for the night and daytimes, respectively. This pattern is depicted in Figure 7 and is in fact a combination of the patterns shown in Figure 5.

The diel pattern of nitrogenase activity as observed in station II is typical for a non-heterocystous filamentous cyanobacterium such as *L. aestuarii* and has been reported by others for mats dominated with this organism (Villbrandt *et al.*, 1990). For the mats in station I the situation is more complex. Mats of heterocystous cyanobacteria are rare but in those with reported diel cycles of nitrogenase activity it was confined to the daytime (Stal, 1995). The daily pattern of nitrogenase activity for station I, containing heterocystous as well as non-heterocystous cyanobacteria, was characterized by another nitrogenase activity maximum in the dark period in between the sunset and sunrise peaks. This could be taken as an indication of a shift in the actively N₂-fixing microbial community. This possibility is currently addressed by investigating the transcription of the *nifH* gene. In station II, nitrogenase

activity under ambient light conditions was similar to that of a young microbial mat described previously. An increase was recorded at ~2000 hours, reaching its maximum at midnight and subsequently decreasing to values similar to the ones before the dark period at ~1800 hours. This pattern is characteristic for N₂ fixation by non-heterocystous cyanobacteria and agrees with the observed dominant cyanobacterial component of this mat (Bebout *et al.*, 1987).

Bebout *et al.* (1987) considered competition for energy and inhibition by photosynthetically evolved oxygen as the most important determinants for the daily variations in N₂ fixation. Decreased photosynthetic activity at daytime resulted in a significantly lower nitrogenase activity during the night (Bebout *et al.*, 1993). Hence, light was an important source of energy and responsible for the supply of reductant for N₂ fixation. In this study, daily integrated N₂ fixation under natural light was almost identical for the two stations and was also constant over several consecutive days. N₂ fixation was therefore independent from the daily integrated incident light. The efficiency of light-independent nitrogenase activity is probably the most important factor that controls the total N₂ fixation of this ecosystem. Although this could be erroneously taken as a support for chemotrophic bacteria as the only diazotrophic agents in these microbial mats, the light-response curves as well as the light action spectra of nitrogenase activity provide a strong support for the dominant role of cyanobacteria. If heterotrophic diazotrophs would be the only active N₂-fixing organisms in the mat, no stimulation of nitrogenase activity under increasing PFD could be observed. Nevertheless, microbial mats are complex communities containing a plethora of different types of microorganisms. The potential for N₂ fixation is widely distributed among Bacteria and Archaea. Cyanobacterial mats contain a high diversity of *nifH* genes, many belonging to heterotrophic organisms (Zehr *et al.*, 1995; Olson *et al.*, 1999). However, these diazotrophic organisms may not all be abundant or expressing nitrogenase. When measuring *nifH* expression in microbial mats, cyanobacterial transcripts are found in addition to those belonging to heterotrophs (Steppe and Paerl, 2005), among which sulfate-reducing bacteria (Steppe and Paerl 2002). Mat-forming cyanobacteria excrete low-molecular carbon compounds during fermentation in the dark that serve as substrate for sulfate-reducing bacteria. If these bacteria fix N₂ they could provide the cyanobacteria with combined nitrogen (Steppe *et al.*, 1996). However, we observed an instantaneous light response, confirming the involvement of phototrophic diazotrophs.

The light action spectra showed the involvement of pigments typical for cyanobacteria such as chlorophyll *a* and phycocyanin. The peak at 510 nm could be assigned to myxoxanthophyll, a well-known carotenoid of cyanobacteria. Although

carotenoids are not known to serve as a photosynthetic light-harvesting pigment, it has been shown previously that light in that wavelength stimulates N_2 fixation in pure cultures and natural communities of cyanobacteria (Staal *et al.*, 2003a). The high value of nitrogenase activity in the UV region is probably an artifact. The interference filters in the UV region have a very low transparency and nitrogenase activity was normalized to the PFD that penetrates the filter. The same may have been true for the far-red region, although it has been shown that PS-I is a major driver of nitrogenase activity in cyanobacteria. However, most important is that the action spectra show that cyanobacteria are involved in N_2 fixation.

With the results obtained so far, characteristics of biological N_2 fixation as well as the daily nitrogenase activity pattern of a complex community were shown to be highly variable during the course of a day. Reasons for that may be found in the adaptation of active organisms to changing conditions, to shifts in the active community during a 24 h day or to a combination of both and need further investigation. Although we do not exclude the possibility that heterotrophic and anoxygenic phototrophic bacteria contribute to N_2 fixation in the microbial mats, we conclude that cyanobacteria are responsible for the bulk of it.

Acknowledgements

We thank Dr Eric Boschker for his valuable comments on an earlier version of the paper. This work was financially supported by the Netherlands Research Council (NWO) contract ALW 815.01.003 and by the Schure-Beijerinck-Popping funds (KNAW). This is publication 4264 of the Netherlands Institute of Ecology (NIOO-KNAW).

References

- Adams DG. (2000). Heterocyst formation in cyanobacteria. *Cur Opin Microbiol* **3**: 618–624.
- Bebout BM, Fitzpatrick MW, Paerl HW. (1993). Identification of the sources of energy for nitrogen-fixation and physiological characterization of nitrogen-fixing members of a marine microbial mat community. *Appl Environ Microbiol* **59**: 1495–1503.
- Bebout BM, Paerl HW, Crocker KM, Prufert LE. (1987). Diel interactions of oxygenic photosynthesis and N_2 fixation (acetylene-reduction) in a marine microbial mat community. *Appl Environ Microbiol* **53**: 2353–2362.
- Bergman B, Gallon JR, Rai AN, Stal LJ. (1997). N_2 fixation by non-heterocystous cyanobacteria. *FEMS Microbiol Rev* **19**: 139–185.
- Berman-Frank I, Lundgren P, Chen Y, Küpper H, Kolber Z, Bergman B *et al.* (2007). Segregation of nitrogen fixation and oxygenic photosynthesis in the marine cyanobacterium *Trichodesmium*. *Science* **294**: 1534–1537.
- Fay P. (1992). Oxygen relations of nitrogen fixation in cyanobacteria. *Microbiol Rev* **56**: 340–373.
- Hardy RWF, Holsten RD, Jackson EK, Burns RC. (1968). The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. *Plant Physiol* **43**: 1185–1207.
- Jensen BB, Cox RP. (1983). Direct measurements of steady-state kinetics of cyanobacterial N_2 uptake by membrane-leak mass spectrometry and comparisons between nitrogen fixation and acetylene reduction. *Appl Environ Microbiol* **45**: 1331–1337.
- Jørgensen BB, Revsbech NP, Blackburn TH, Cohen Y. (1979). Diurnal cycle of oxygen and sulfide microgradients and microbial photosynthesis in a cyanobacterial mat sediment. *Appl Environ Microbiol* **38**: 46–58.
- Olson JB, Litaker RW, Paerl HW. (1999). Ubiquity of heterotrophic diazotrophs in marine microbial mats. *Aquat Microb Ecol* **19**: 29–36.
- Paerl HW. (1990). Physiological ecology and regulation of N_2 fixation in natural waters. *Adv Microb Ecol* **11**: 305–344.
- Staal M, Stal LJ, te Lintel Hekkert S, Harren FJM. (2003a). Light action spectra of N_2 fixation by heterocystous cyanobacteria from the Baltic Sea. *J Phycol* **39**: 668–677.
- Staal M, te Lintel Hekkert S, Harren F, Stal LJ. (2001). Nitrogenase activity in cyanobacteria measured by the acetylene reduction assay: a comparison between batch incubation and on-line monitoring. *Environ Microbiol* **3**: 343–351.
- Staal M, te Lintel Hekkert S, Harren FJM, Stal LJ. (2003b). Effects of O_2 on N_2 fixation in heterocystous cyanobacteria from the Baltic Sea. *Aquat Microb Ecol* **33**: 261–270.
- Staal M, te Lintel Hekkert S, Herman P, Stal LJ. (2002). Comparison of models describing light dependence of N_2 fixation in heterocystous cyanobacteria. *Appl Environ Microbiol* **68**: 4679–4683.
- Stal LJ. (1995). Physiological ecology of cyanobacteria in microbial mats and other communities. *New Phytol* **131**: 1–32.
- Stal LJ, van Gemerden H, Krumbein WE. (1985). Structure and development of a benthic marine microbial mat. *FEMS Microbiol Ecol* **31**: 111–125.
- Steppe TF, Olson JB, Paerl HW, Belnap J. (1996). Consortial N_2 fixation: a strategy for meeting nitrogen requirements of marine and terrestrial cyanobacterial mats. *FEMS Microbiol Ecol* **21**: 149–156.
- Steppe TF, Paerl HW. (2002). Potential N_2 fixation by sulfate-reducing bacteria in a marine intertidal microbial mat. *Aquat Microb Ecol* **28**: 1–12.
- Steppe TF, Paerl HW. (2005). Nitrogenase activity and *nifH* expression in a marine intertidal microbial mat. *Microb Ecol* **49**: 315–323.
- te Lintel Hekkert ST, Staal M, R Nabben RHM, Zuckermann H, Persijn S, Stal LJ *et al.* (1998). Laser photoacoustic trace gas detection, an extremely sensitive technique applied in biological research. *Instrum Sci Tech* **26**: 157–175.
- Villbrandt M, Stal LJ, Krumbein WE. (1990). Interactions between nitrogen-fixation and oxygenic photosynthesis in a marine cyanobacterial mat. *FEMS Microbiol Ecol* **74**: 59–72.

- Walsby AE. (2007). Cyanobacterial heterocysts: terminal pores proposed as sites of gas exchange. *Trends Microbiol* **15**: 340–349.
- Wright SW, Jeffrey SW, Mantoura RFC, Llewellyn CA, Bjornland T, Repeta D *et al.* (1991). Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. *Mar Ecol Prog Ser* **77**: 183–196.
- Zehr JP, Braun S, Litaker W, Steppe T, Paerl HW. (1995). Diversity of heterotrophic nitrogen fixing genes in a marine cyanobacterial mat. *Appl Environ Microbiol* **61**: 2527–2532.
- Zehr JP, Jenkins BD, Short SM, Steward GF. (2003). Nitrogenase gene diversity and microbial community structure: a cross-system comparison. *Environ Microbiol* **5**: 539–554.