

ORIGINAL ARTICLE

Regulation of *nif* gene expression and the energetics of N₂ fixation over the diel cycle in a hot spring microbial mat

Anne-Soisig Steunou¹, Sheila I Jensen², Eric Brecht³, Eric D Becraft⁴, Mary M Bateson⁴, Oliver Kilian¹, Devaki Bhaya¹, David M Ward⁴, John W Peters⁴, Arthur R Grossman¹ and Michael Kühl²

¹Department of Plant Biology, The Carnegie Institution, Stanford University, Stanford, CA, USA; ²Marine Biological Laboratory, Department of Biology, University of Copenhagen, Copenhagen, Denmark;

³Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT, USA and ⁴Department of Land Resources and Environmental Science, Montana State University, Bozeman, MT, USA

Nitrogen fixation, a prokaryotic, O₂-inhibited process that reduces N₂ gas to biomass, is of paramount importance in biogeochemical cycling of nitrogen. We analyzed the levels of *nif* transcripts of *Synechococcus* ecotypes, NifH subunit and nitrogenase activity over the diel cycle in the microbial mat of an alkaline hot spring in Yellowstone National Park. The results showed a rise in *nif* transcripts in the evening, with a subsequent decline over the course of the night. In contrast, immunological data demonstrated that the level of the NifH polypeptide remained stable during the night, and only declined when the mat became oxic in the morning. Nitrogenase activity was low throughout the night; however, it exhibited two peaks, a small one in the evening and a large one in the early morning, when light began to stimulate cyanobacterial photosynthetic activity, but O₂ consumption by respiration still exceeded the rate of O₂ evolution. Once the irradiance increased to the point at which the mat became oxic, the nitrogenase activity was strongly inhibited. Transcripts for proteins associated with energy-producing metabolisms in the cell also followed diel patterns, with fermentation-related transcripts accumulating at night, photosynthesis- and respiration-related transcripts accumulating during the day and late afternoon, respectively. These results are discussed with respect to the energetics and regulation of N₂ fixation in hot spring mats and factors that can markedly influence the extent of N₂ fixation over the diel cycle.

The ISME Journal (2008) 2, 364–378; doi:10.1038/ismej.2007.117; published online 6 March 2008

Subject Category: microbial population and community ecology

Keywords: *Synechococcus*; microsensor; gene expression; nitrogen fixation; Yellowstone National Park; *in situ*

Introduction

The microbial mats in alkaline siliceous hot springs in Yellowstone National Park, especially Octopus and Mushroom Springs, are among the most intensively studied natural microbial communities (reviewed in Ward *et al.*, 1998, 2006). Studies of microbial mat model systems are yielding new insights into fundamental aspects of the biodiversity and functional ecology of microorganisms (Ward *et al.*, 2008), and their interactions with biogeochemical processes now and over evolutionary time scales (Hoehler *et al.*, 2001; Des Marais, 2003). These hot spring microbial mats represent relatively

stable microbial ecosystems composed of photoautotrophic, photoheterotrophic, chemoautotrophic and heterotrophic organisms (Pierson *et al.*, 1984, 1985; Ferris and Ward, 1997; Hanada *et al.*, 1997, 2002; Ward *et al.*, 1998; Pierson, 2001; Boomer *et al.*, 2002). Cyanobacterial populations are the dominant primary producers, providing fixed carbon to the heterotrophs of the community; cyanobacteria can also alleviate the demand for exogenous, reduced nitrogen as many are capable of fixing N₂ (Pearson *et al.*, 1979; Stal and Krumbein, 1985). Hot spring microbial mats maintain a diversity of cyanobacteria that varies horizontally with temperature, as the effluent waters flow away and cool off with increasing distance from the source. There is also a vertical organization of various microbes in the mat, which might reflect adaptations to the light microclimate and chemical gradients in the mats (Ramsing *et al.*, 2000; Ward *et al.*, 2006).

Correspondence: A-S Steunou, Department of Plant Biology, The Carnegie Institution, 260 Panama Street, Stanford, CA, USA. E-mail: steunou@cgm.cnrs-gif.fr

Received 8 November 2007; revised 4 December 2007; accepted 4 December 2007; published online 6 March 2008

In the lower temperature range (40–50 °C), hot spring mats are dominated by filamentous cyanobacteria such as *Phormidium* or *Plectonema* species (Walter *et al.*, 1976; Cady and Farmer, 1996; Farmer *et al.*, 1997; Ward *et al.*, 1998; Ward and Castenholz, 2000; Namsaraev *et al.*, 2003; Lau *et al.*, 2005). However, at higher temperatures (>55 °C), only one morphotype of unicellular, but ecologically diverse cyanobacteria, assigned to the genus *Synechococcus*, persists within the upper 1–2 mm of the mat (Ward *et al.*, 1998, 2006). Within and below this surface, cyanobacterial layer, filamentous anoxygenic phototrophic (FAP) bacteria related to *Roseiflexus castenholzii* and *Chloroflexus aurantiacus* form the bulk of the mat biota (Nübel *et al.*, 2002), with *Roseiflexus* spp assemblages dominating at 60 °C and *Chloroflexus* spp assemblages dominating at higher temperatures (for example, 70 °C) (Nübel *et al.*, 2002). *Chloroflexus* spp also appear to be dominant in sulfidic hot springs, while *Roseiflexus* spp dominate non-sulfidic springs (Ward *et al.*, 1997; van der Meer *et al.*, 2000; Nübel *et al.*, 2002).

Recently, the genomes of two thermophilic *Synechococcus* ecotypes, *Synechococcus* OS-A and OS-B', which have been identified as abundant components of the microbial mat of both Octopus Spring and the neighbouring Mushroom Spring, were sequenced (whole-genome accession nos. NC_007775 and NC_007776, respectively). *Synechococcus* OS-B' is most abundant at temperatures of 55–60 °C, while *Synechococcus* OS-A is most abundant at temperatures of 60–65 °C. The genomes of these organisms have similar gene content, but large-scale genome architecture is not maintained, with the longest syntenic gene clusters extending approximately 30 kbp (Bhaya *et al.*, 2008). Interestingly, *nif* genes were identified in this cluster, suggesting that both organisms were capable of N₂ fixation (Steunou *et al.*, 2006). The nitrogenase gene cluster of the hot spring *Synechococcus* spp spans a 23-kbp region of the genome and encodes proteins required for catalytic activity (NifHDK), for synthesizing the FeMo cofactor, and for allowing maturation and stability of the nitrogenase protein complex (NifWX₂NEBS₂UHDKVZT) (Jacobson *et al.*, 1989; Kim and Burgess, 1996; Lee *et al.*, 1998; Rangaraj and Ludden, 2002; Hu *et al.*, 2004; Rubio and Ludden, 2005).

The nitrogenase enzyme complex is extremely O₂ sensitive, and *nif* transcripts are generally absent in cells exposed to O₂ and/or sources of reduced nitrogen such as ammonia (Wang *et al.*, 1985; Fay, 1992). Several strategies have evolved in N₂-fixing microorganisms that help to protect the nitrogenase from inactivation by O₂, especially in cyanobacteria since they perform oxygenic photosynthesis. Filamentous, N₂-fixing cyanobacteria often differentiate to form specialized N₂-fixing cells, heterocysts, that exclusively contain the nitrogenase complex. Heterocysts typically have little or no O₂ evolution, and predominantly exhibit cyclic photosynthetic

electron flow (Tel-Or and Stewart, 1977), increased respiratory activity (Peterson and Burris, 1976; Golden and Yoon, 2003; Valladares *et al.*, 2003) and develop a thick cell wall with low gas permeability (Walsby, 1985; Murry and Wolk, 1989; Wolk *et al.*, 1994).

Non-heterocystous diazotrophic cyanobacteria may spatially (Carpenter and Price, 1976; Fay, 1992) or temporally separate oxygenic photosynthesis from N₂ fixation (Reddy *et al.*, 1993). Their photosynthetic activity is high during the day when there is high excitation energy, while N₂ fixation dominates in the evening and night under oxygen-depleted conditions. Furthermore, some cyanobacteria, such as the marine species *Cyanothece* sp (*Synechococcus*-RF1) and *Trichodesmium* sp, exhibit nitrogenase activity during the day, with spatiotemporal alterations in the appearance of photosynthesis, respiration and N₂ fixation that may be controlled by the circadian clock (Schneegurt *et al.*, 1994; Huang *et al.*, 1999; Berman-Frank *et al.*, 2001).

The mechanisms by which *nif* genes are regulated in unicellular cyanobacteria are largely unknown. In some diazotrophic bacteria, particularly members of the γ -proteobacteria, *nif* gene activity is regulated by the O₂-responsive regulatory system comprised of NifL and NifA (Martinez-Argudo *et al.*, 2004). In other diazotrophs such as the α -proteobacterium *Rhodobacter capsulatus*, *nif* genes can be regulated by the anaerobic sensory system that involves RegA and RegB (Joshi and Tabita, 1996; Elsen *et al.*, 2000, 2004). However, based on *Synechococcus* OS-B' and OS-A full genome information, the regulators NifL, NifA, RegA and RegB are not present in these hot spring cyanobacteria (Bhaya *et al.*, 2008).

Alternatively, expression of *nif* genes might be controlled by nitrogen availability or the energetic status of the diazotrophic cells (Rabouille *et al.*, 2006). NtcA, a transcriptional activator involved in global nitrogen control in cyanobacteria, binds promoters containing the consensus nucleotide sequence GTAN₈TAC. In the absence of ammonium, NtcA regulates the transcription of genes encoding polypeptides required for the uptake and assimilation of various nitrogen sources, including nitrate and nitrite (Luque *et al.*, 1994). Hence, the levels of *ntcA* transcripts can be used as indicators of the nitrogen status in the mat (Lindell and Post, 2001). NtcA is encoded on both the *Synechococcus* OS-A (CYA_1799) and OS-B' (CYB_2533) genomes, and we localized putative NtcA-binding sites upstream of several genes in the genomes of *Synechococcus* OS-A and OS-B'; for example, upstream of genes for nitrite reductase (*nirA*), the photosystem II (PSII) reaction center protein, the ammonium transporter (*amt*), glutamine synthetase (*glnA*) and NtcA itself (Steunou *et al.*, 2006). However, there are no convincing NtcA-binding sites in the promoter regions of the *nif* gene clusters.

The identification of nitrogenase gene clusters in two predominant *Synechococcus* ecotypes in microbial mats from Octopus Spring (Steunou *et al.*, 2006) led to studies of *in situ* gene expression that demonstrated accumulation of *nif* transcripts during the evening when the mat became anoxic, while these transcripts were barely detectable during the day when the mat was hyperoxic. Furthermore, nitrogenase activity was detected during the night, with little or no measurable activity during the day. In the present study, we investigated the kinetics of change over the diel cycle of *nif* transcript abundance, NifH polypeptide levels and nitrogenase activity in the microbial mat of Mushroom Spring, Yellowstone National Park, an alkaline hot spring with similar characteristics and mat composition to those of nearby Octopus Spring. Our study provides new insights into the biosynthesis of the nitrogenase complex and the dynamics and energetics of thermophilic cyanobacterial N₂ fixation activity in hot spring microbial mats.

Materials and methods

Sample collection

Samples were collected on 30 June–1 July 2005, 30 September–1 October 2005 and 17–20 September 2006 from a northwestern effluent channel of Mushroom Spring, an alkaline siliceous hot spring in the White Creek area of Yellowstone National Park (Ward *et al.*, 2006). The sampling site had an average temperature of 60 °C (±1 °C) over the entire sampling period. The mat was ~1 cm thick, with *Synechococcus*-like cells populating the top 1–1.5 mm; other cyanobacterial morphotypes were not observed by microscopic analysis and were not found in earlier studies (Ward *et al.*, 2006). Mat samples (~1.13 cm²) were collected using a cork borer, and the top 2 mm of each core containing the entire cyanobacterial layer was immediately excised, frozen in liquid N₂ and stored for nucleic acid and protein extraction, or was incubated with acetylene for measuring nitrogenase activity (see below).

The down welling quantum irradiance at each sampling time and throughout the diel experiments was measured using an LI-1400 Datalogger (LI-COR Biosciences, Lincoln, NE, USA) equipped with a quantum irradiance sensor. The temperature at the sampling site was monitored by an electronic thermometer equipped with a thermocouple (DualogR; DigiSense, Vernon Hill, IL, USA).

Microsensor measurements

Microprofiles of O₂ concentration in the microbial mat were measured *in situ* with an electrochemical Clark-type O₂ microsensor (Revsbech and Ward, 1984; Revsbech, 1989) as described earlier (Steunou *et al.*, 2006). The O₂ microsensor was calibrated in a

small semiclosed beaker filled with source water (~70 °C) that was continuously flushed with air by a hand-operated air pump connected to a ceramic diffuser in the beaker. The temperature of the water was monitored by an electronic thermometer (Omnitherm, Germany). As the water cooled down by the continuous purging with air, O₂ microsensor signals equivalent to 100% air saturation were recorded over the experimental temperature range. Zero O₂ readings were obtained at the measuring site when the O₂ microsensor penetrated into deeper anoxic layers of the mat. The O₂ concentration in 100% air-saturated spring water at *in situ* temperature (60 °C) and elevation (~2250 m above sea level), C_S, was estimated as:

$$C_S = \left(\frac{P_{2250\text{ m}} - P_w(60^\circ\text{C})}{P_{0\text{ m}}} \right) 0.2095 \alpha(60^\circ\text{C}) \frac{M(\text{O}_2)}{V_m}$$

where $P_{2250\text{ m}}$ and $P_{0\text{ m}}$ are the atmospheric pressures at 2250 m (=770 mbar) and 0 m (=1013 mbar), respectively, $P_w(60^\circ\text{C})$ =199 mbar is the water vapor pressure, $\alpha(60^\circ\text{C})$ =0.01923 is the Bunsen absorption coefficient, $M(\text{O}_2)$ =32 g mol⁻¹ is the molecular mass of O₂ and V_m =22.414 mol l⁻¹ is the molar volume. With these values, we obtain a C_S =3.24 mg O₂ per l=101 µmol O₂ per l.

Net O₂ production was calculated using Fick's first law: $J = -D_0(dC/dz)$, where (dC/dz) is the slope of the linear O₂ concentration profile in the diffusive boundary layer just above the mat surface, and D_0 is the molecular diffusion coefficient of O₂ in water. We used a value of $D_0(60^\circ\text{C})$ =4 × 10⁻⁵ cm² s⁻¹ (Han and Bartels, 1996).

Nitrogenase activity

In situ nitrogenase activity was assayed by the acetylene reduction technique as previously described (Steunou *et al.*, 2006). We note that our samples were incubated in argon-flushed vials. Consequently, our nitrogenase activity measurements in darkness could be affected to some extent by this removal of O₂, which would cause a somewhat diminished energy supply. However, during the incubation with air, O₂ would also be rapidly depleted during 1–2 h incubation since the mat samples would rapidly consume O₂ on all exposed surfaces. A more exact study of these effects would involve *in situ* incubations at a series of time intervals and under a range of defined O₂ and light levels, but such a detailed methodological investigation was not possible within the scope and sampling limit of our study.

Nitrogenase activity over the diel cycle. In June and September 2005, core mat samples (~1.13 cm²) were collected and the top 2 mm removed and placed in 10 ml serum vials that were sealed under a constant stream of argon. Two milliliters of argon-flushed Mushroom Spring water were added, and the vials were incubated for 15 min in the hot spring

at *in situ* temperature and irradiance. The nitrogenase assay was initiated with the injection of 1 ml of O₂-free acetylene. Vials were incubated in the spring for an additional 2 h before the reaction was stopped by injection of 0.2 ml of formaldehyde. Each assay was performed in triplicate. The nitrogenase activity reported represents the total activity over the 2 h incubation. The ethylene production in samples was quantified by gas chromatography (Shimadzu GC-8A with a flame ionization detector).

Dark shift experiment. On 20 September 2006 at 0500 hours, we followed the same protocol as described below. At ~0530 hours, 20 cores (~0.56 cm²) were collected and placed in 20 separate vials. One set of ten vials was maintained at ambient conditions, while a second set was covered with black tape to isolate the vials from light. All vials were incubated in the hot spring at 60 °C, and at the times 0600, 0700, 0730, 0800, 0900 and 1000 hours, two vials were injected with 1 ml of O₂-free acetylene. After 2 h of incubation, the reactions were stopped by injection of 0.2 ml of formaldehyde and ethylene production in samples quantified by gas chromatography.

Light shift experiment. Eight cores (~0.56 cm²) were collected in September 2006 and placed in eight vials that were sealed under a constant argon stream. Two milliliters of argon-sparged water were added. Duplicate vials were placed under different light intensities from a Halogen bulb (0, 25, 100 and 200 μmol photons m⁻² s⁻¹) and incubated 15 min in the hot spring at 60 °C. At 2200 hours, 1 ml of O₂-free acetylene was injected into each vial and at 2400 hours, the reactions were stopped by injection of 0.2 ml of formaldehyde. The nitrogenase activity reported represents the total activity over the 2 h incubation. The ethylene production in each of the samples was quantified by gas chromatography.

Temperature shift experiment. In September 2006 at 0745 hours, six cores (~0.56 cm²) were collected at 60 °C and placed in six vials that were sealed under a constant stream of argon. Two milliliters of argon-flushed water were added. The vials were incubated for 15 min in the hot spring at 60 °C. At 0800 hours, 1 ml of O₂-free acetylene was injected into each vial and placed at different sites in the spring corresponding to temperatures of 40, 50, 60, 65, 70 and 75 °C. The vials were incubated in the spring for 2 h and were then processed as described above.

RNA extraction

Mat samples, collected in September 2005, over the diel cycle, that had been frozen and stored at -80 °C were dispersed by vortexing in 1 ml of 10 mM NaAc (sodium acetate) (pH 4.5), with 0.5 g of glass beads (150–212 μm; Sigma-Aldrich, St Louis, MO, USA) in

a 2-ml screw-cap microfuge tube. Cells were harvested by centrifugation (21 460 g) for 1 min at 4 °C, the cell pellet resuspended in 250 μl of 10 mM NaAc (pH 4.5) and 37.5 μl of 500 mM Na₂-EDTA (pH 8.0), and RNA was extracted as previously described (Steunou *et al.*, 2006). Isolated RNA was treated twice with 7 U per reaction of RNase-free DNase (Qiagen Inc., Valencia, CA, USA) for 20 min, the reaction was stopped with 1 volume of phenol/chloroform (1:1), and the RNA in the aqueous phase was precipitated during a 20-min incubation at -20 °C after the addition of 0.1 volume of 10 M LiCl and 2.5 volume of 100% ethanol. The RNA concentration was determined by absorption at 260 nm. On the basis of the PCR amplification in the absence of reverse transcriptase, none of the RNA samples used exhibited detectable genomic DNA contamination (data not shown).

Real-time RT-PCR

Single-stranded cDNA synthesis was performed using reverse transcriptase (RT), DNase-treated RNA (as described above) and specific reverse primers designed for *Synechococcus* OS-B' *nifH*, *nifD*, *psbB*, *cpcE*, *coxA*, *cydA*, *adhE*, *acs*, *pdhB* and *ackA* genes. The accession number for all *Synechococcus* OS-B' genes is NC_007776. Because of the high homology of the nitrogenase, the photosynthesis, the respiration and the fermentation genes between *Synechococcus* OS-B' and *Synechococcus* OS-A (NC_007775) ecotypes, the primer pairs that were designed specifically against *Synechococcus* OS-B', are likely to anneal to transcripts from other related *Synechococcus* ecotypes within the mat (Steunou *et al.*, 2006). Primers were annealed to 100 ng of total RNA extracted from mat samples and extended for 45 min in the presence of 2.5 mM dNTPs at 55 °C using 200 U of RT Superscript III (Invitrogen Corporation, Carlsbad, CA, USA). The primer pairs used (Table 1) were designed to generate single-stranded cDNA of ~200 nucleotides. The single-stranded DNA synthesized in the RT reactions served as template for real-time RT-PCR (qPCR) amplifications, which were performed using the DyNAmo HS SYBRGreen qPCR Kit (Finnzymes, Espoo, Finland) and the Engine Opticon System (Bio-Rad, South San Francisco, CA, USA). The specific amplification protocol was 1 cycle at 95 °C for 10 min, and 44 cycles at 94 °C for 10 s, 56 °C for 15 s, 72 °C for 8 s and a final incubation of 72 °C for 10 min. We determined both the absolute (Whelan *et al.*, 2003) and relative (normalized to the T1 sample, collected at 1330 hours) levels of each specific RNA among all environmental samples.

Dark shift experiment

On 20 September 2006, at 1200 hours, eight cores (~1.13 cm²) were collected and each was placed into a sealed vial. Two milliliters of spring water was

Table 1 Primers used for qPCR analysis of *Synechococcus* OS-B' transcript levels

Gene	Function	Forward primer	Reverse primer
<i>nifH</i>	Nitrogenase iron protein	GGAAGAAAACGGAGCCTAC	CGCCAGAGTAGGCGTATTTTC
<i>nifD</i>	Nitrogenase molybdenum-iron protein α chain	GCAGATTGCGGAGTAAAATC	GCCGAAGGTGTTCAACCCCGG
<i>cpcE</i>	Phycocyanobilin lyase α subunit	CTGGGAAGCCCTTATCGAG	GTCGTAGGCTCGAGTTGGAG
<i>psbB</i>	PS II chlorophyll binding protein	CTGCCCAAGTCTCTGCTCTTC	CAGGATAACCGGGAAGGTCTC
<i>cydA</i>	Cytochrome <i>d</i> quinol oxydase subunit I	GTCGATGGGAAAATTTGTGGTG	GATCGCCAAGACCACTTTGAG
<i>coxA</i>	Cytochrome <i>c</i> oxidase subunit I	GTTGGCAGGAACCTCTTCC	CAACAGGTCAAAGCTGAGC
<i>pflB</i>	Pyruvate formate lyase	CTAGGTCCGCACAGCGGAG	ACGTGCGGTTAATGACATCC
<i>adhE</i>	coA-linked acetaldehyde dehydrogenase-alcohol dehydrogenase	CAAACCGGTGCGAGTCTTGATC	GGATCCGAAACCAGAGCATG
<i>acs</i>	Acetyl-CoA synthase	GCGGGACGAAGTCCTTAATC	CTCGTTTTTCGCAAAGAACCTC
<i>psaB</i>	PSI reaction center protein	GGGCATGGATGTGTTGTTGTC	GACCAAGATCAAGGTGGTG
<i>ntcA</i>	Nitrogen-responsive regulatory protein	CCGTCTGGTCAGCTTTTG	GGTCTGTACGGTGATCTTC
<i>glnA</i>	Glutamine synthetase, type I	CGAGCCCTTGGACAAAGATAT	GATTGATGGGGTTGATCTCG

Abbreviation: qPCR, real-time reverse transcriptase-PCR.

The transcripts analyzed encode proteins associated with nitrogen metabolism, photosynthesis, respiration and fermentation. The gene name, protein name/function and primer sequence are presented. The accession numbers for all *synechococcus* OS-B' genes are NC_007776.

added to each vial. Four vials were kept dark (covered by black tape), and all of the vials were incubated in the hot spring at the *in situ* temperature and irradiance. Two vials (one darkened and the other illuminated) were removed from the spring following 2, 5, 20 and 60 min incubations. The water from the vials was removed and the cores were frozen in liquid N₂ and stored at -80°C until analysis.

Protein preparation for SDS-PAGE

Mat samples were collected at thirteen time points over the diel cycle in September 2005, and the top 2 mm of each was excised, frozen in liquid N₂ and stored at -80°C . The cores ($\sim 0.56\text{ cm}^2$) and cells within the cores were disrupted using a bead beater (BioSpec Products, Bartlesville, OK, USA) at full power. Disruptions of each core were performed by four consecutive treatments, 30 s for each, at 4°C in 250 μl of ice-cold buffer (50 mM Tris-HCl, 50 mM EDTA, 5 mM NaCl) and 2 μl DTT (1 M), 5 μl of protease inhibitor cocktail (Calbiochem, La Jolla, CA, USA) and 100 mg of 150–215 μm glass beads (Sigma-Aldrich) in a 2-ml screw-cap tube. The lysates were separated into a membrane and soluble fraction by centrifugation (11 750 g) for 5 min at 4°C . The pellets were resuspended by sonication in 100 μl of Na₂CO₃ (0.1 M) and 100 μl of DTT (0.1 M) followed by the addition of 200 μl of the sample buffer (5% SDS, 10 mM EDTA, 20% sucrose). The samples were boiled for 1 min and insoluble debris removed from samples by centrifugation (11 750 g) for 5 min at room temperature. The supernatants were collected and frozen in liquid nitrogen until further analysis. The concentration of the protein was determined using the Bio-Rad protein assay based on the method of Bradford with bovine serum albumin as a reference standard as described by the manufacturer.

Immunoblot analyses

NifH antibody (kindly provided by Dr Paul Ludden) was raised against a mixture of purified NifH proteins from *Azotobacter vinelandii* and *Rhodospirillum rubrum* and further purified using affinity chromatography (http://info.med.yale.edu/mbb/koelle/protocols/protocol_Ab_affinity_purif.html). For western blot analyses, 60 μg of protein from the mat samples were resolved by Tris-HCl-SDS-PAGE (12% polyacrylamide) and transferred onto PVDF membranes (VWR, West Chester, PA, USA) by semidry electrotransfer in buffer (31 mM Tris, 48 mM glycine, 5% methanol). Blots were incubated with a 1000-fold dilution of anti-NifH antibody for 12 h (at 4°C) followed by incubation with a 10 000-fold dilution of a horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI, USA) for 6 h and detected by chemiluminescence (Pierce, Rockford, IL, USA).

Results and discussion

Oxygen dynamics and nitrogenase activity over the diel cycle

We monitored *in situ* O₂ dynamics and nitrogenase activity of the Mushroom Spring mat over the diel cycle during September 2005 (Figures 1a and b). The large fluctuation of irradiance during the initial daylight period was a consequence of varying cloud cover. Overall, the irradiance over the diel cycle was generally lower in September (maximum sustained values were generally $<1500\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$, and mostly below $1000\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$) than in a second diel cycle experiment conducted in June 2005 (maximum sustained values were $\sim 2000\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$) (Supplementary Figure 1); also, day length during the September collection was shorter (12.5 h of daylight in September, 14 h of daylight in June).

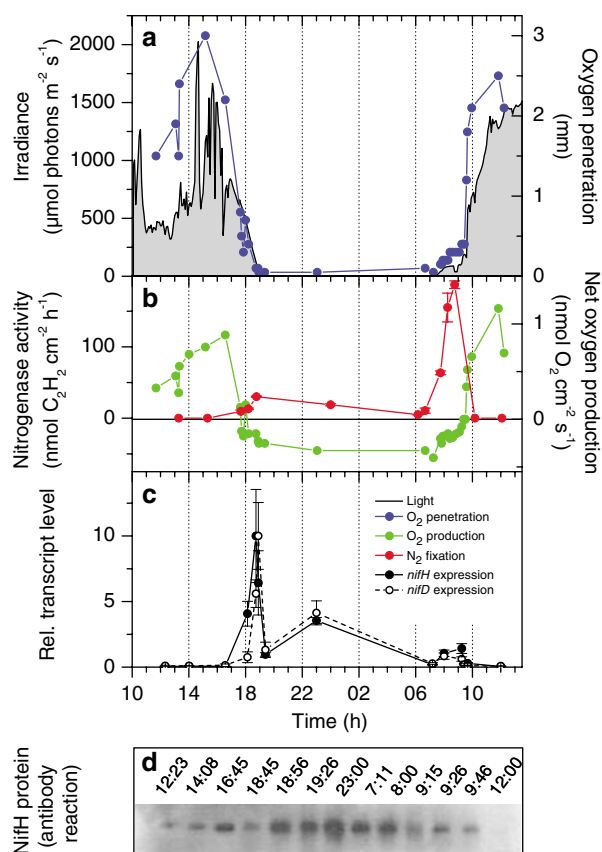


Figure 1 *In situ* nitrogenase activity, levels of NifH subunit and transcripts encoding NifH and NifD, and oxygen penetration and net production over the diel cycle in the microbial mat of Mushroom Spring in September 2005. (a) Incident down welling irradiance (μmol photons m⁻² s⁻¹) and O₂ penetration (mm) in the hot spring mat over the diel cycle. (b) *In situ* nitrogenase activity (nmol ethylene per cm² per h) and net O₂ production (nmol O₂ cm⁻² s⁻¹) over the diel cycle. (c) Relative abundance of transcripts encoding the nitrogenase subunits NifH and NifD over the diel cycle. (d) NifH protein accumulation over the diel cycle. Western blot analysis measuring NifH protein levels over the diel cycle were performed using immunoreactions with affinity purified NifH antibodies (see Materials and methods). Curves and colors are defined in the inset of panel c. Nitrogenase activity and gene expression data points represent means ± s.e. (N=3).

The O₂ conditions in the mat were strongly linked to irradiance (Figures 1a and b). At an irradiance of >250 μmol photons m⁻² s⁻¹, that is, the compensation irradiance, cyanobacterial photosynthesis surpassed O₂ consumption in the mat, causing net O₂ production and increased O₂ penetration. The maximal O₂ penetration depth was ~2.5–3 mm under the highest irradiance (Figure 1a, at ~1430 hours). Oxygenic photosynthesis was confined to the upper 1–1.5 mm layer of the cyanobacterial zone, which was supersaturated with O₂ at high irradiance (Supplementary Figure 2). At irradiances below 250–300 μmol photons m⁻² s⁻¹, the mat was strongly O₂ depleted, and a minimal O₂ penetration depth of <0.1–0.2 mm was observed throughout the night (Figure 1a). The switch between highly oxic and

almost complete anoxic conditions in the mat occurred rapidly, that is, whenever the irradiance declined below the compensation point for periods of time >15–30 min in the late afternoon and after 1800 hours (Figure 1 and Supplementary Figure 2).

In the morning, the irradiance increased more slowly due to a shadowing effect of the tree-lined hills surrounding Mushroom Spring. In this time window of dim morning light, O₂ penetration and photosynthetic O₂ production increased gradually over almost 3 h (from ~0630 to 0930 hours) (Figures 1a and b and Supplementary Figure 2). During this time interval, O₂ consumption was still higher than O₂ production in the mat, but the onset of photosynthesis provided a significant boost in available energy within the mat as compared to the situation during the night. This was reflected in the increasing O₂ penetration depth, gradually alleviating diffusive O₂ limitation within the upper mat layers. As irradiance further increased in the morning, O₂ production in the mat intensified dramatically, and over a 10- to 15-min interval the mat became supersaturated in the upper layers (Figures 1a and b and Supplementary Figure 2).

The detailed pattern of nitrogenase activity over the diel cycle, characterized in September 2005, showed two peaks (Figure 1b), with no activity observed during the day, when the upper several millimeters of the mat were oxic (Figure 1a and Supplementary Figure 2). A small peak of activity was measured in the evening at ~1830 hours as the mat became O₂ depleted. However, much higher activities were observed at ~0800 hours, when the light intensity was still relatively low (~200 μmol photons m⁻² s⁻¹) and O₂ had not yet accumulated in the mat. In September 2005, nitrogenase activity peaked in the evening at 40 nmol ethylene produced per cm² per h, decreased to some extent during the remainder of the evening, and as the light intensity increased during sunrise, peak activity reached 187 nmol ethylene produced per cm² per h (Figure 1b). We obtained similar results when this experiment was performed in June 2005, although we only had a single point showing the morning rise (Supplementary Figure 1). Interestingly, we noted a significant difference in the absolute level of nitrogenase activity in the June and September samples. In June, the activity was much higher in the evening (~100 nmol ethylene per cm² per h), throughout the night (~60 nmol ethylene per cm² per h) and in the morning (~538 nmol ethylene produced per cm² per h).

We also investigated the temperature dependence of the *in situ* nitrogenase activity in September 2006 by placing mat samples from the 60 °C sampling site at different sites in the spring corresponding to water temperatures of 40, 50, 60, 65, 70 and 75 °C. Although sampling restrictions prevented replication, these measurements showed highest nitrogenase activities at 60 (peak) and 65 °C (Supplementary Figure 3). Lowest activity was found at 40 °C, while

intermediate levels of nitrogenase activity were detected at 50, 70 and 75 °C. This temperature dependence roughly followed the previously measured temperature dependence of growth and photosynthesis for *Synechococcus* isolates from the hot spring mat (Allewalt *et al.*, 2006) and suggest thermal adaptation of N₂ fixation activity in the mat.

nif transcript and polypeptide accumulation over the diel cycle

In addition to measuring nitrogenase activity, we collected mat samples over the diel cycle for RNA and protein analyses. Total RNA from each sample was purified and evaluated for levels of *nif* transcripts from *Synechococcus* ecotypes using qPCR. Total protein from the mat was extracted and analyzed by western blots for the presence of the NifH subunit.

nif transcript abundance may be controlled by both O₂ levels and the energetic of the mat at any given time, and possibly also by the concentration of reduced nitrogen compounds in the environment. Almost no *nifH* or *nifD* transcripts were detected when ambient irradiance levels were >500 µmol photons m⁻² s⁻¹ (Figure 1c); at this irradiance, there was significant O₂ accumulation in the upper 2–3 mm of the mat (Supplementary Figure 2). However, *nif* transcripts were detected at lower irradiances and accumulated in the early evening, although they declined significantly over the course of the night. This decline may be a consequence of the rapid decrease in the energy supply caused by O₂ limitation during the night (see below), which could have a strong impact on overall anabolic processes in the cell. Furthermore, in contrast to the strong peak in nitrogenase activity in the early morning (between 0700 and 1000 hours), there was little increase in *nifH* or *nifD* transcript levels over this same period. Under increasing irradiance during the morning, the mat became oxic and the nitrogenase activity declined to near zero. The increase in nitrogenase activity observed during the early morning was thus not a consequence of increased transcript accumulation, but more likely a consequence of an alleviation of ATP limitation as a result of (i) increasing availability of O₂, which can be used for respiration, and/or (ii) elevated cyclic photosynthetic ATP production.

We used monospecific antibodies to track the level of the NifH subunit over the diel cycle. The NifH polypeptide was only detected in the membrane fraction and had an apparent molecular mass of ~32 kDa. At noon, very little NifH was observed in mat samples, but the level of this polypeptide increased during the evening, following the increase in *nifH* mRNA (Figures 1c and d). However, the level of the NifH polypeptide remained high throughout the night, with the first noticeable decrease in its level at 0915 hours, as the morning light became more intense and O₂ levels in the mat increased

significantly (Figures 1a and b and Supplementary Figure 2b). Furthermore, like the *nif* transcripts, the polypeptide level did not increase when nitrogenase exhibited peak activity in the morning (see Figures 1b–d). These findings strongly suggest that the peak level of the nitrogenase activity in the early morning is a consequence of an increase in enzyme activity resulting from elevated availability of ATP and reductant, a direct consequence of the initiation of photosynthetic electron transport, which would also increase the oxygen available for respiration.

Processes associated with energetics of the mat

We analyzed the abundance of transcripts associated with metabolic processes in the *Synechococcus* ecotypes required for the production of ATP and reductant; these processes include photosynthesis, respiration and fermentation. Both ATP and reductant are essential for the synthesis of nitrogenase transcripts and polypeptide subunits, as well as for the activity of the enzyme, which has a high energetic demand.

Photosynthesis. Transcripts encoding proteins associated with photosynthetic function, *cpcE* (phycocyanobilin lyase α subunit) and *psbB* (photosystem II chlorophyll binding protein), were relatively high during the afternoon and began to significantly decline by 1800 hours, when the irradiance was declining (Figures 2a and b). At night, the transcript levels were barely detectable, but they rose markedly during the low light conditions of the early morning. Moreover, the increase in transcript levels in the morning was congruent with the morning rise in nitrogenase activity. Over this time period, the mat is still a net consumer of O₂, but as light levels increase gradually, photosynthetic O₂ generation overtakes respiratory O₂ consumption. These results strongly suggest that the increase in transcripts encoding proteins associated with photosynthesis is a response to the light environment and probably not to oxic conditions. This conclusion is also supported by the finding that mat samples exposed to low levels of light during the night show marked increases in levels of transcripts encoding proteins involved in photosynthetic function (unpublished data). The responses of the photosynthetic genes could be a consequence of light perception by a specific photoreceptor (Lohr *et al.*, 2005; Kehoe and Gutu, 2006), changing the energetic or redox state of the cell associated with the initiation of photosynthetic electron transport in the morning (Elsen *et al.*, 2004; Wormuth *et al.*, 2006), and may also be associated with a circadian clock function to some extent.

Respiration. For genes encoding respiratory proteins, *coxA* (cytochrome *c* oxidase subunit I) and *cydA* (cytochrome *d* quinol oxidase subunit I), peak

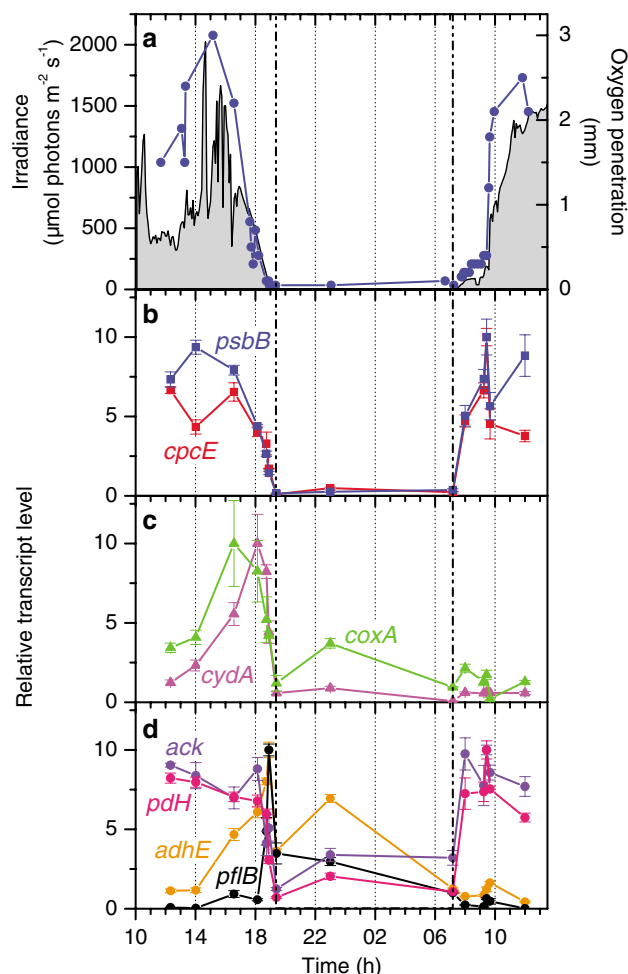


Figure 2 Real-time reverse transcriptase (RT)-PCR (qPCR) analysis of relative transcript levels from genes involved in energy metabolism in *Synechococcus* ecotypes. (a) Curves showing the light intensity and O₂ penetration in the mats over the diel cycle; this data, the same as in Figure 1, provide a reference for evaluating the transcript data presented in the remaining panels. The levels of transcript encoding proteins with (b) photosynthetic (*psbB*, *cpcE*), (c) respiratory (*cydA*, *coxA*) and (d) fermentation functions (*pf1B*, *pdH*, *adhE*, *acs*) measured over the diel cycle. Error bars on graphs indicate the mean \pm s.d.

expression occurred as evening approached (Figure 2c), although the light levels were still relatively high and the upper mat layers were oxic. The increase in the level of transcripts for respiratory proteins precedes the increase in *nif* mRNAs and raises the possibility of coordination of the two processes; high respiration rates would lower the O₂ tension in the mat when the light levels are still relatively high, which, in turn, would allow activation of *nif* genes and the production of nitrogenase prior to complete light extinction. The O₂ depletion in the mat during the night coincides with a decline in *cydA* and *coxA* transcript levels and interestingly, transcript levels do not increase significantly in the morning, when light levels rise and O₂ penetration in the mat increases. Thus, the absolute O₂ or light levels may not play a major role in regulating the

level of terminal oxidase transcripts; it is more likely that the respiratory genes are under circadian control.

Fermentation. An extended period of almost complete anoxia occurs during the night, where O₂ is rapidly consumed in the upper <0.1 mm of the mat and oxygenic respiration is thus limited to a very thin surficial zone (Supplementary Figure 2), where O₂ may also be used for reoxidation of H₂, of other fermentation and anaerobic respiration products such as sulfide (Dillon *et al.*, 2007) or methane (Anderson *et al.*, 1987). In this situation, the diffusive supply of O₂ through the boundary layer strongly limits its availability in the mat. Fermentation is thus the major energy-generating pathway for cyanobacteria in layers below 0.1 μ m during periods of anoxia in the mat (Nold and Ward, 1996). Genes encoding proteins associated with *Synechococcus* fermentative metabolism showed a complex behavior (Figure 2 and Supplementary Figure 4), with some transcripts like pyruvate dehydrogenase (*pdH*) and acetate kinase (*ack*) declining in the evening and others like CoA-linked acetaldehyde dehydrogenase-alcohol dehydrogenase (*adhE*) and pyruvate formate lyase (*pf1B*) increasing. Similar results were observed in studies of the Octopus Spring mat (Steunou *et al.*, 2006). Those transcripts that increase, encode proteins likely to facilitate the fermentative production of formate, acetyl-CoA and ethanol (Supplementary Figure 4), which sustains cellular energetic demands but can only support low rates of N₂ fixation. Transcripts encoding proteins associated with fermentation that increased in abundance as evening approached usually declined significantly over the course of the night and remained low until late afternoon. As for the *nif* genes, this decline may be a consequence of the rapid decrease in the energy supply, which could have a strong impact on overall anabolic processes. A more detailed investigation of the role of *Synechococcus* fermentation in mat energetics is clearly needed and would involve a quantification of pools and fluxes of fermentation products and the dynamics of H₂ and interspecies H₂ transfer, which may affect the redox state of fermentation products (Anderson *et al.*, 1987).

Regulation of transcription of *nif* genes

It is not resolved how the *nif* genes are controlled in the cyanobacteria of hot spring microbial mats. On the basis of the present study, the activity of the nitrogenase complex appears to be sensitive to O₂, as in other systems, while regulation of *nif* transcript levels appears to be more complex. The *nifH* and *nifD* transcripts increased in abundance in the early evening, when light levels declined to <250 μ mol photons m⁻² s⁻¹ and the mat became depleted of O₂. This suggests that it is the O₂ tension that controls transcript abundance. However, we also noted a

decline in *nifH* and *nifK* transcript levels over the course of the evening and night, when fermentation became dominant, lowering the energy yield of the cyanobacteria. Thus, the levels of *nif* transcripts may also be sensitive to the energetic conditions of the cell. However, we cannot exclude potential circadian control of *nif* gene expression; the *kai* genes, which are associated with circadian control in cyanobacteria (Bell-Pedersen *et al.*, 2005), are present in the genomes of *Synechococcus* OS-A and OS-B' (the accession numbers for *kaiABC* genes in *Synechococcus* OS-B' are CYB_0490, CYB_0489 and CYB_0488; in *Synechococcus* OS-A, CYA_1902, CYA_1901 and CYA_1900 [6]).

To address the question of whether or not circadian control is important for *nif* gene expression, we collected mat samples in September 2006 during midday, when the irradiance was 500–1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Half of the samples were incubated in the light and the other half in the dark; samples were maintained under these conditions for various times. Total RNA from each sample was purified and evaluated for levels of *nifH* and *cpcE* transcripts. As shown in Figure 3a, the level of *nifH* mRNA increased in the dark (relative to the light-incubated samples) to ~6- and ~13-fold after 20 min and 60 min, respectively. Furthermore, the levels of both the *psaB* and *cpcE* transcripts showed some decline in the dark over the same period. These results suggest that circadian control is unlikely to play a major role in regulating expression of the *nif* genes, unless circadian control can be overridden by a direct response of the system to microenvironmental conditions, such as O₂ levels.

The activity of the *nif* genes might be controlled by nitrogen availability and the regulatory element NtcA, a transcriptional activator associated with global nitrogen control in cyanobacteria. The *glnA* gene, which encodes glutamine synthetase, is subject to NtcA regulation. As shown in Figure 3b, both *ntcA* and *glnA* transcripts are readily detected during the day, but decrease sharply beginning at 1800 hours. The levels of these transcripts remain low during the night, but increase to near maximal values by 0700 hours. Elevated levels of *ntcA* transcript during the day may thus reflect a scarcity of fixed nitrogen in the hot spring environment and indicate the need for the cells to activate pathways for scavenging a range of different nitrogen compounds over the period in which CO₂ fixation is high. Furthermore, the decline in *glnA* and *ntcA* transcripts in the evening, in conjunction with the observed increase in *nif* transcript abundance (and the development of nitrogenase activity), suggests that the fixation of N₂ augments the supply of reduced nitrogen.

Regulation of nitrogenase activity

On the basis of the results presented above, we hypothesize that elevation of nitrogenase activity

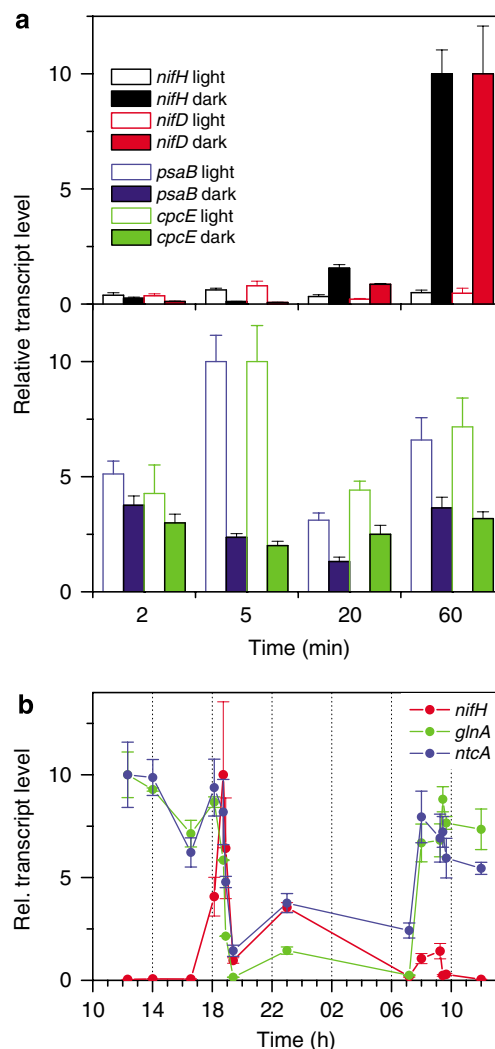


Figure 3 Regulation of *Synechococcus* ecotype genes associated with N₂ fixation and assimilation and photosynthesis. (a) Effect of a shift from light to dark on the abundance of *nifH* and *nifD*, and *psaB* and *cpcE* transcripts *in situ*. This experiment was performed in September 2006 during midday (1200 hours) and the levels of *nifH*, *nifD*, *cpcE* and *psaB* transcripts were monitored for the mat under ambient light condition, or maintained in the dark during 2, 5, 20 or 60 min. (b) Relative transcript level of *ntcA*, *glnA* and *nifH* over the diel cycle. Error bars on graphs indicate the mean \pm s.d.

(an energetically demanding process) in the morning, reflects an alleviation of energy limitation when increased ATP production by photosynthesis and respiration is initiated (Rabouille *et al.*, 2006; Staal *et al.*, 2007). To test this hypothesis, we incubated mat cores *in situ* in the dark, while other cores remained in ambient light during the morning, as the sun was rising. As shown in Figure 4a, high nitrogenase activity was observed in the sample that was left in the dim morning light, while very low-level nitrogenase activity (similar to levels measured during the night) was observed in the sample that was kept in the dark. Conversely, when mat cores were exposed to light in the middle of the night, we

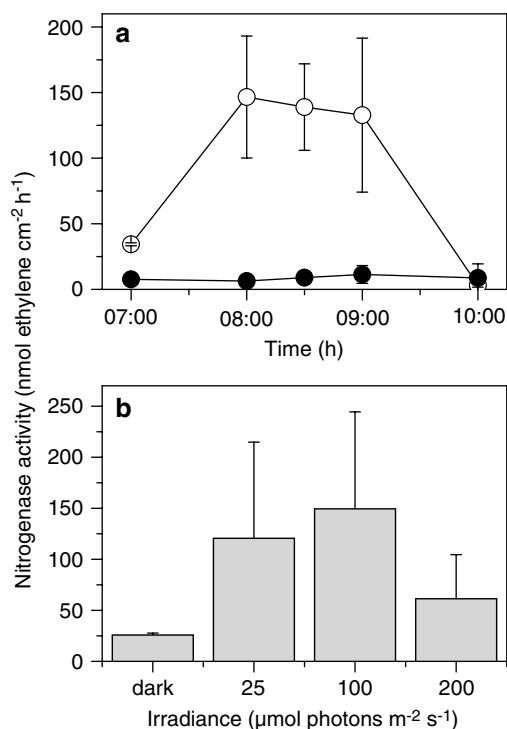


Figure 4 Regulation of the nitrogenase activity in mat samples: (a) Nitrogenase activity during the morning (0700–1000 hours) in mat samples under ambient light condition (open symbols), or maintained in the dark (solid symbols). (b) Nitrogenase activity during the night following exposure of a part of the mat to 25, 100 or 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Symbols indicate means \pm s.e. ($N=2$).

noted an increase in nitrogenase activity that was dependent upon light intensity (Figure 4b). We interpret this result to indicate that light (at 25 and 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) allows for photosynthesis, causing elevated levels of ATP and reductant, which in turn would stimulate nitrogenase activity. However, under conditions used for this experiment, at 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, cyanobacterial photosynthesis produced more O₂ than could be consumed by respiration, and the accumulation of O₂ in the mat caused some inhibition of the nitrogenase activity.

Even during the evening and at low irradiance when the mat experiences strong O₂ limitation, O₂ from the air–mat interfaces can still diffuse into the mat where it is consumed in the upper 0.1–0.2 mm (Supplementary Figure 2). This O₂ consumption may contribute energy for cyanobacterial N₂ fixation within this zone, during the night. However, as in other mat systems, this strong O₂ consumption may also be due to respiration of other bacteria in the mat, as well as reoxidation of reduced products from fermentation and anoxic respiration processes such as sulfate respiration or methanogenesis. In hypersaline mats and cyanobacterial biofilms, reoxidation of sulfide has been shown to consume most of the O₂ at nighttime (Kühl and Jørgensen, 1992; Canfield and Des Marais, 1993). It has previously been shown that

both H₂ and sulfide are generated at significant rates in the Mushroom Spring mat during nighttime and that these reduced compounds become depleted at the mat–water interface (van der Meer *et al.*, 2005; Dillon *et al.*, 2007; M Kühl *et al.*, unpublished results). Therefore, it is not possible at this point to make an exact estimate of the amount of O₂ consumption that is channeled into cyanobacterial respiration, the ATP production of which could supply N₂ fixation at the very surface of the mat during nighttime. To understand this in more detail, combined microsensor measurements of O₂, pH, H₂S and H₂ in concert with sampling and slicing of mat samples into different zones for subsequent gene expression and nitrogenase activity analysis would be necessary.

In summary, our results strongly suggest that energy generation from early morning photosynthetic activity is critical for much of the morning peak in nitrogenase activity that occurs in the Mushroom Spring microbial mat. Simple gravimetric integration of the activity curve shows that ~59% of the total diel nitrogenase activity occurs in the 2- to 3-h-long morning peak period in September (Figure 1). This early morning period, characterized by dim light and low O₂ levels but increased O₂ turnover, seems to be of major importance for the nitrogen budget of the hot spring mat cyanobacteria. To allow for the elevated nitrogenase activity observed during this period, the cyanobacteria need to make efficient use of the slowly increasing light availability. This may partly be reflected by the rapid, early morning increase in transcripts encoding proteins involved in photosynthesis. Furthermore, transcripts encoding respiratory proteins did not significantly increase during this early morning period. Since photosynthetic and respiratory electron transport pathways in cyanobacteria intersect and compete for electrons at the level of the cytochrome *b₆f* complex, it is critical that these processes be coordinated, and some studies suggest that respiration may be inhibited in the light (Scherer *et al.*, 1988). From these data, we hypothesize that the early morning peak of N₂ fixation may reflect low rates of respiration and efficient photosynthetic electron transport that stimulates a rise in both ATP and nicotinamide adenine dinucleotide phosphate levels.

Nitrogenase activity, hydrogen and the microbial community

Non-heterocystous, diazotrophic cyanobacteria in marine or hypersaline microbial mats also exhibit a diel pattern of N₂ fixation. Similar to the hot spring mats, N₂ fixation in some mats is suppressed during the day and increases after sunset, but becomes maximal either during the night or in the early morning (Stal *et al.*, 1984; Villbrandt and Krumbein, 1990; Paerl *et al.*, 1996; Omoregie *et al.*, 2004). In the microbial mat community of a tropical hypersaline

lagoon (Pinckney and Paerl, 1997), an important role for N₂ fixation has been attributed to diazotrophic anoxygenic phototrophs (purple phototrophic bacteria).

In our study, the measured nitrogenase activity might be the sum of cyanobacterial activity plus activities associated with other diazotrophic bacteria. The 'draft' genome sequence of *Roseiflexus* RS1, a FAP isolated from the Octopus Spring microbial mat, contains the *nifHBDK* genes (CG Klatt *et al.*, unpublished data) but genes required for the synthesis and maturation of nitrogenase and the *nifLA* genes could not be identified. Preliminary analysis of *Roseiflexus* RS1 gene expression *in situ* did not detect *nif* transcripts (CG Klatt *et al.*, personal communication). Thus, it is still unclear whether this organism can synthesize a functional nitrogenase. None of the *nif* genes appear to be present on the *Chloroflexus* sp genomes (http://genome.jgi-psf.org/mic_home.html).

Even if *Roseiflexus* and *Chloroflexus* cannot fix N₂, their metabolisms may still be closely linked to cyanobacterial N₂ fixation. Indeed, the genomes of neither *Synechococcus* OS-B' nor OS-A encode hydrogenases. Most cyanobacteria contain two different Ni-Fe hydrogenases called the uptake and bidirectional hydrogenases (Ghirardi *et al.*, 2007). The uptake hydrogenase, encoded by *hupSL* genes, is coupled to N₂ fixation and is needed for recycling of H₂ that is produced by the nitrogenase. All cyanobacterial *hupSL* genes sequenced to date are highly conserved, with 83.8–95.1% nucleotide identity (Tamagnini *et al.*, 2002). The bidirectional hydrogenase, encoded by *hox* genes, may function as an electron valve that controls the accumulation of electrons in the photosynthetic electron transport chain and may also facilitate elimination of excess reductant generated during fermentation metabolism (Ludwig *et al.*, 2006). As far as we know, all of the N₂ fixing cyanobacteria that have been characterized possess an uptake hydrogenase, except for *Synechococcus* sp BG 043511, which only has the bidirectional enzyme (Ludwig *et al.*, 2006). In the genomes of *Synechococcus* OS-B' and OS-A, neither *hup* nor *hox* genes have been identified (based on homology). This suggests that at least some of the *Synechococcus* strains in the hot spring mats are unable to recycle the H₂ produced by the nitrogenase. The H₂ might be released and used by other bacteria such as *Chloroflexus* or *Roseiflexus* RS1, both of which have hydrogenases (Klatt *et al.*, 2007). Thus, these bacteria or others, may be able to use the H₂ generated by the cyanobacteria as a source of both energy and reductant, especially in the early morning, when hydrogen stimulates incorporation of CO₂ into FAP lipids (van der Meer *et al.*, 2005; Klatt *et al.*, 2007). Similar crossfeeding of hydrogen could fuel sulfate-respiring populations in the mat (Dillon *et al.*, 2007). By-products of cyanobacterial nitrogen fixation in the mats may thus provide an important functional link between the mat microbes.

Conclusion

This study provides a detailed account of *in situ* N₂ fixation and its regulation in hot spring microbial mats. Our *in situ* studies are summarized in a conceptual model showing the factors that influence nitrogenase activity over the diel cycle in the hot spring mats (Figure 5). During the day, because of cyanobacterial oxygenic photosynthesis, the upper few millimeters of the mat are supersaturated with O₂. Under these conditions, the *nif* genes are not expressed. With declining irradiance toward the end of the day, the O₂ concentration in the mat drops because of (i) a decline in photosynthetic O₂ evolution and (ii) sustained, or increased, respiratory consumption of O₂ by cyanobacteria and other microbes of the community. As the mat becomes progressively O₂ depleted, both *nif* and specific fermentation transcripts increase, polypeptides are synthesized and assembled into active complexes, and N₂ fixation is initiated. However, by the time the level of the nitrogenase becomes maximal and the activity is fully established, photosynthetic energy production has decreased substantially. Oxygen is depleted strongly in the upper 1–2 mm of the mat due to respiration and reoxidation of reduced compounds and a limited oxygen supply from overlaying spring water by mass transfer across the diffusive boundary layer. The only source of energy for cyanobacterial N₂ fixation in the anoxic part of the mat would be derived from the fermentation of organic carbon that had been fixed during the previous day. The high energetic cost of N₂ fixation (16 ATP per N₂ fixed) and the low energy yield of fermentation (2–4 ATPs per glucose metabolized) are likely to preclude high-level N₂ fixation during the night, and also make it dependent on the amount of fixed carbon stored in the cells during the

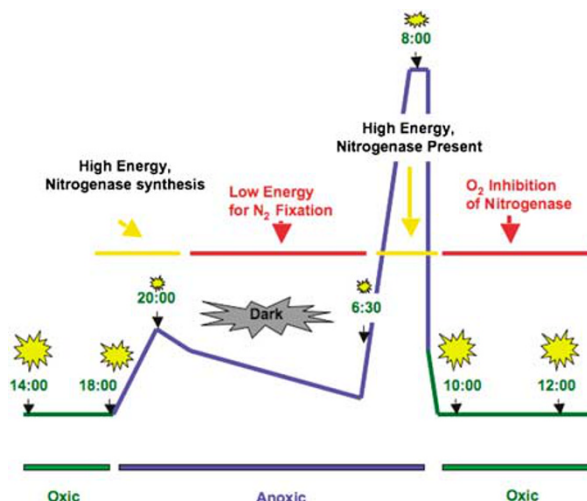


Figure 5 Conceptual model depicting changes in nitrogenase activity over the diel cycle. The figure depicts the relative nitrogenase activity, light conditions, the O₂ status and the energetics of the mat over the diel cycle.

previous day, until the next light period. A similar scenario has been proposed for hypersaline mats (Bebout *et al.*, 1993).

In the morning, with increasing irradiance, nitrogenase activity increases in congruence with photosynthetic activity. This increased nitrogenase activity is not a consequence of increased nitrogenase transcript or protein levels, but reflects elevated production of ATP and reductant. The mat remains largely anoxic with net O₂ consumption for some hours, until increasing irradiance drives the rate of photosynthetic O₂ evolution above the rate of respiratory O₂ consumption. This conclusion is supported by our findings that (i) maintaining the mat in the dark in the morning inhibits the increase in nitrogenase activity, (ii) exposure of mat samples to low light levels in the middle of the night promotes N₂ fixation and (iii) high light levels inhibit dark fixation. When the irradiance increases to the point where O₂ begins to accumulate in the mat, the nitrogenase activity becomes strongly inhibited, the levels of Nif subunits decline and the *nif* genes are not expressed.

Nitrogenase activity dependence on photosynthetic electron transport may reflect both total photosynthetic activity over the diel cycle as well as the temporally limited window of activity associated with sunrise. We noticed that there is a difference in the extent of nitrogenase activity measured in June and September. The overall pattern of nitrogenase activity for both times of year were the same, but the cumulative activity in the evening and the peak activity associated with the initiation of photosynthetic electron transport in the morning were both higher in the June experiment. The higher nighttime and peak nitrogenase activities observed in June may reflect climatic or seasonal factors. In June, there is likely to be significantly more overall photosynthesis and accumulation of fixed carbon since the day length is longer, and the light intensities throughout the day, on average, were higher in comparison to the September experiment. Therefore, we speculate that more energy was available in the evening transition allowing for synthesis of higher amounts of nitrogenase before the onset of energy limitation during the night. Elevated accumulation of polysaccharide reserves in June relative to September could also stimulate fermentation metabolism during the night, leading to a greater availability of energy and carbon backbones for nocturnal N₂ fixation.

In summary, we combined ecophysiological methods to quantify the mat microenvironment and nitrogenase activity *in situ* together with biochemical and molecular techniques to explore the level of the NifH protein and the expression patterns associated with numerous genes involved in energy metabolism. This integrated approach has yielded new insights into the complex dynamics of N₂ fixation in hot spring cyanobacterial mats and shows that N₂ fixation in thermophilic *Synechococcus* sp

is closely linked to their energy metabolism, which shows pronounced shifts during a diel cycle, and may also involve functional interactions with other microbes in the mat community. In the future, we hope the approach of integrating *in situ* data with *ex situ* experiments with ecologically relevant isolates will generate testable hypotheses and will reveal more insights into the complex and evolved interactions between mat microbes.

Acknowledgements

We thank Anni Glud for construction of microsensors used in this study; and Chris G Klatt, William Herpson and Melanie Melendrez for their excellent help in the field. The NifH antibody used in this study was kindly provided by Dr Paul Ludden. Dr Marc Staal is gratefully acknowledged for discussions and valuable suggestions on improving the manuscript. We thank Don Bryant for providing draft *Roseiflexus* and *Chloroflexus* genome sequences, which were obtained as part of a Joint Genome Institute Department of Energy project to determine genome sequences of FAPs. We thank the US National Park Service and personnel from Yellowstone National Park for their permission to conduct this work and their helpful assistance. The research was funded by the Frontiers in Integrative Biology Program of the National Science Foundation Grant EF-0328698 and by the National Institutes of Health GM069938 (JWP). MK and SIJ acknowledge additional support from the Danish Natural Science Research Council.

References

- Allewalt JP, Bateson MM, Revsbech NP, Slack K, Ward DM. (2006). Effect of temperature and light on growth of and photosynthesis by *Synechococcus* isolates typical of those predominating in the octopus spring microbial mat community of Yellowstone National Park. *Appl Environ Microbiol* **72**: 544–550.
- Anderson KL, Tayne TA, Ward DM. (1987). Formation and fate of fermentation products in hot spring cyanobacterial mats. *Appl Environ Microbiol* **53**: 2343–2352.
- 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.
- Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL *et al.* (2005). Circadian rhythms from multiple oscillators: lessons from diverse organisms. *Nat Rev Genet* **6**: 544–556.
- Berman-Frank I, Lundgren P, Chen YB, Kupper H, Kolber Z, Bergman B *et al.* (2001). Segregation of nitrogen fixation and oxygenic photosynthesis in the marine cyanobacterium *Trichodesmium*. *Science* **294**: 1534–1537.
- Bhaya D, Grossman AR, Steunou AS, Khuri N, Cohan FM, Hamamura N *et al.* (2008). Population level functional diversity in a microbial community revealed by comparative genomic and metagenomic analyses. *ISMEJ* **100**: 207–219.

- Boomer SM, Lodge DP, Dutton BE, Pierson B. (2002). Molecular characterization of novel red green non-sulfur bacteria from five distinct hot spring communities in Yellowstone National Park. *Appl Environ Microbiol* **68**: 346–355.
- Cady SL, Farmer JD. (1996). Fossilization processes in siliceous thermal springs: trends in preservation along the thermal gradient. *Ciba Found Symp* **202**: 150–170.
- Canfield DE, Des Marais DJ. (1993). Biogeochemical cycles of carbon, sulfur, and free oxygen in a microbial mat. *Geochim Cosmochim Acta* **57**: 3971–3984.
- Carpenter EJ, Price CC. (1976). Marine *Oscillatoria* (*Trichodesmium*): explanation for aerobic nitrogen fixation without heterocysts. *Science* **191**: 1278–1280.
- Des Marais DJ. (2003). Biogeochemistry of hypersaline microbial mats illustrates the dynamics of modern microbial ecosystems and the early evolution of the biosphere. *Biol Bull* **204**: 160–167.
- Dillon JG, Fishbain S, Miller SR, Bebout BM, Habicht KS, Webb SM et al. (2007). High rates of sulfate reduction in a low-sulfate hot spring microbial mat are driven by a low level of diversity of sulfate-respiring microorganisms. *Appl Environ Microbiol* **73**: 5218–5226.
- Elsen S, Dischert W, Colbeau A, Bauer CE. (2000). Expression of uptake hydrogenase and molybdenum nitrogenase in *Rhodobacter capsulatus* is coregulated by the RegB-RegA two-component regulatory system. *J Bacteriol* **182**: 2831–2837.
- Elsen S, Swem LR, Swem DL, Bauer CE. (2004). RegB/RegA, a highly conserved redox-responding global two-component regulatory system. *Microbiol Mol Biol Rev* **68**: 263–279.
- Farmer JD, Bebout B, Jahnke LL. (1997). Fossilization of coniform (*Phormidium*) stromatolites in siliceous thermal springs, Yellowstone National Park. *GSA (abs)* **29**: 295.
- Fay P. (1992). Oxygen relations of nitrogen fixation in cyanobacteria. *Microbiol Rev* **56**: 340–373.
- Ferris MJ, Ward DM. (1997). Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. *Appl Environ Microbiol* **63**: 1375–1381.
- Ghirardi ML, Posewitz MC, Maness PC, Dubini A, Yu J, Seibert M. (2007). Hydrogenases and hydrogen photoproduction in oxygenic photosynthetic organisms. *Annu Rev Plant Biol* **58**: 71–91.
- Golden JW, Yoon HS. (2003). Heterocyst development in *Anabaena*. *Curr Opin Microbiol* **6**: 557–563.
- Han P, Bartels DM. (1996). Temperature dependence of oxygen diffusion in H₂O and D₂O. *J Phys Chem* **100**: 5597–5602.
- Hanada S, Kawase Y, Hiraishi A, Takaichi S, Matsuura K, Shimada K et al. (1997). *Porphyrobacter tepidarius* sp. nov., a moderately thermophilic aerobic photosynthetic bacterium isolated from a hot spring. *Int J Syst Bacteriol* **47**: 408–413.
- Hanada S, Takaichi S, Matsuura K, Nakamura K. (2002). *Roseiflexus castenholzii* gen. nov., sp. nov., a thermophilic, filamentous, photosynthetic bacterium that lacks chlorosomes. *Int J Syst Evol Microbiol* **52**: 187–193.
- Hoehler TM, Bebout BM, Des Marais DJ. (2001). The role of microbial mats in the production of reduced gases on the early Earth. *Nature* **412**: 324–327.
- Hu Y, Fay AW, Dos Santos PC, Naderi F, Ribbe MW. (2004). Characterization of *Azotobacter vinelandii* *nifZ* deletion strains. Indication of stepwise MoFe protein assembly. *J Biol Chem* **279**: 54963–54971.
- Huang TC, Lin RF, Chu MK, Chen HM. (1999). Organization and expression of nitrogen-fixation genes in the aerobic nitrogen-fixing unicellular cyanobacterium *Synechococcus* sp. strain RF-1. *Microbiology* **145** (Part 3): 743–753.
- Jacobson MR, Cash VL, Weiss MC, Laird NF, Newton WE, Dean DR. (1989). Biochemical and genetic analysis of the *nifUSVWZM* cluster from *Azotobacter vinelandii*. *Mol Gen Genet* **219**: 49–57.
- Joshi HM, Tabita FR. (1996). A global two component signal transduction system that integrates the control of photosynthesis, carbon dioxide assimilation, and nitrogen fixation. *Proc Natl Acad Sci USA* **93**: 14515–14520.
- Kehoe DM, Gutu A. (2006). Responding to color: the regulation of complementary chromatic adaptation. *Annu Rev Plant Biol* **57**: 127–150.
- Kim S, Burgess BK. (1996). Evidence for the direct interaction of the *nifW* gene product with the MoFe protein. *J Biol Chem* **271**: 9764–9770.
- Klatt CG, Bryant DA, Ward DM. (2007). Comparative genomics provides evidence for the 3-hydroxypropionate autotrophic pathway in filamentous anoxygenic phototrophic bacteria and in hot spring microbial mats. *Environ Microbiol* **9**: 2067–2078.
- Kühl M, Jørgensen BB. (1992). Microsensor measurements of sulfate reduction and sulfide oxidation in compact microbial communities of aerobic biofilms. *Appl Environ Microbiol* **58**: 1164–1174.
- Lau E, Nash CZ, Vogler DR, Cullings KW. (2005). Molecular diversity of cyanobacteria inhabiting coniform structures and surrounding mat in a Yellowstone hot spring. *Astrobiology* **5**: 83–92.
- Lee SH, Pulakat L, Parker KC, Gavini N. (1998). Genetic analysis on the NifW by utilizing the yeast two-hybrid system revealed that the NifW of *Azotobacter vinelandii* interacts with the NifZ to form higher-order complexes. *Biochem Biophys Res Commun* **244**: 498–504.
- Lindell D, Post AF. (2001). Ecological aspects of *ntcA* gene expression and its use as an indicator of the nitrogen status of marine *Synechococcus* spp. *Appl Environ Microbiol* **67**: 3340–3349.
- Lohr M, Im CS, Grossman AR. (2005). Genome-based examination of chlorophyll and carotenoid biosynthesis in *Chlamydomonas reinhardtii*. *Plant Physiol* **138**: 490–515.
- Ludwig M, Schulz-Friedrich R, Appel J. (2006). Occurrence of hydrogenases in cyanobacteria and anoxygenic photosynthetic bacteria: implications for the phylogenetic origin of cyanobacterial and algal hydrogenases. *J Mol Evol* **63**: 758–768.
- Luque I, Flores E, Herrero A. (1994). Molecular mechanism for the operation of nitrogen control in cyanobacteria. *EMBO J* **13**: 2862–2869.
- Martinez-Argudo I, Little R, Shearer N, Johnson P, Dixon R. (2004). The NifL-NifA System: a multidomain transcriptional regulatory complex that integrates environmental signals. *J Bacteriol* **186**: 601–610.
- Murry MA, Wolk CP. (1989). Evidence that the barrier to the penetration of oxygen into heterocysts depends upon two layers of the cell envelope. *Arch Microbiol* **151**: 469–474.
- Namsaraev ZB, Gorlenko VM, Namsaraev BB, Buriukhaev SP, Iurkov VV. (2003). The structure and biogeochemical

- activity of the phototrophic communities from the Bol'sherechenskii alkaline hot spring. *Mikrobiologiya* **72**: 228–238.
- Nold SC, Ward DM. (1996). Photosynthate partitioning and fermentation in hot spring microbial mat communities. *Appl Environ Microbiol* **62**: 4598–4607.
- Nübel U, Bateson MM, Vandieken V, Wieland A, Kühl M, Ward DM. (2002). Microscopic examination of distribution and phenotypic properties of phylogenetically diverse chloroflexaceae-related bacteria in hot spring microbial mats. *Appl Environ Microbiol* **68**: 4593–4603.
- Omoregie EO, Crumbliss LL, Bebout BM, Zehr JP. (2004). Determination of nitrogen-fixing phylotypes in *Lyngbya* sp. and *Microcoleus chthonoplastes* cyanobacterial mats from Guerrero Negro, Baja California, Mexico. *Appl Environ Microbiol* **70**: 2119–2128.
- Paerl HW, Fitzpatrick M, Bebout BM. (1996). Seasonal nitrogen fixation dynamics in a marine microbial mat: potential roles of cyanobacteria and microheterotrophs. *Limnol Oceanogr* **41**: 419–427.
- Pearson HW, Rowsley R, Kjeldsen CK, Walsby AE. (1979). Aerobic nitrogenase activity associated with a non-heterocystous filamentous cyanobacterium. *FEMS Microbiol Lett* **5**: 163–167.
- Peterson RB, Burris RH. (1976). Conversion of acetylene reduction rates to nitrogen fixation rates in natural populations of blue-green algae. *Anal Biochem* **73**: 404–410.
- Pierson BK. (2001). O phototroph, o chemotroph, where art thou? *Trends Microbiol* **9**: 259–260.
- Pierson BK, Giovannoni SJ, Castenholz RW. (1984). Physiological ecology of a gliding bacterium containing bacteriochlorophyll a. *Appl Environ Microbiol* **47**: 576–584.
- Pierson BK, Giovannoni SJ, Stahl DA, Castenholz RW. (1985). *Heliothrix oregonensis*, gen. nov., sp. nov., a phototrophic filamentous gliding bacterium containing bacteriochlorophyll a. *Arch Microbiol* **142**: 164–167.
- Pinckney JL, Paerl HW. (1997). Anoxygenic photosynthesis and nitrogen fixation by a microbial mat community in a Bahamian hypersaline lagoon. *Appl Environ Microbiol* **63**: 420–426.
- Rabouille S, Staal M, Stal LJ, Soetaert K. (2006). Modeling the dynamic regulation of nitrogen fixation in the cyanobacterium *Trichodesmium* sp. *Appl Environ Microbiol* **72**: 3217–3227.
- Ramsing NB, Ferris MJ, Ward DM. (2000). Highly ordered vertical structure of *Synechococcus* populations within the one-millimeter-thick photic zone of a hot spring cyanobacterial mat. *Appl Environ Microbiol* **66**: 1038–1049.
- Rangaraj P, Ludden PW. (2002). Accumulation of 99Mo-containing iron-molybdenum cofactor precursors of nitrogenase on NifNE, NifH, and NifX of *Azotobacter vinelandii*. *J Biol Chem* **277**: 40106–40111.
- Reddy KJ, Haskell JB, Sherman DM, Sherman LA. (1993). Unicellular, aerobic nitrogen-fixing cyanobacteria of the genus *Cyanothece*. *J Bacteriol* **175**: 1284–1292.
- Revsbech NP. (1989). An oxygen microelectrode with a guard cathode. *Limnol Oceanogr* **34**: 474–478.
- Revsbech NP, Ward DM. (1984). Microelectrode studies of interstitial water chemistry and photosynthetic activity in a hot spring microbial mat. *Appl Environ Microbiol* **48**: 270–275.
- Rubio LM, Ludden PW. (2005). Maturation of nitrogenase: a biochemical puzzle. *J Bacteriol* **187**: 405–414.
- Scherer S, Almon H, Boger P. (1988). Interaction of photosynthesis, respiration and nitrogen fixation in cyanobacteria. *Photosynth Res* **15**: 95–114.
- Schneegurt MA, Sherman DM, Nayar S, Sherman LA. (1994). Oscillating behavior of carbohydrate granule formation and dinitrogen fixation in the cyanobacterium *Cyanothece* sp. strain ATCC 51142. *J Bacteriol* **176**: 1586–1597.
- Staal M, Rabouille S, Stal LJ. (2007). On the role of oxygen for nitrogen fixation in the marine cyanobacterium *Trichodesmium* sp. *Environ Microbiol* **9**: 727–736.
- Stal LJ, Grossberger S, Krumbein WF. (1984). Nitrogen fixation associated with the cyanobacterial mat of a marine laminated microbial ecosystem. *Mar Biol* **82**: 217–224.
- Stal LJ, Krumbein WE. (1985). Nitrogenase activity in the non-heterocystous cyanobacterium *Oscillatoria* sp. grown under alternating light–dark cycles. *Archs Microbiol* **143**: 67–71.
- Steunou AS, Bhaya D, Bateson MM, Melendrez MC, Ward DM, Brecht E et al. (2006). *In situ* analysis of nitrogen fixation and metabolic switching in unicellular thermophilic cyanobacteria inhabiting hot spring microbial mats. *Proc Natl Acad Sci USA* **103**: 2398–2403.
- Tamagnini P, Axelsson R, Lindberg P, Oxelfelt F, Wunschiers R, Lindblad P. (2002). Hydrogenases and hydrogen metabolism of cyanobacteria. *Microbiol Mol Biol Rev* **66**: 1–20.
- Tel-Or E, Stewart WDP. (1977). Photosynthetic components and activities of nitrogen-fixing isolated heterocysts of *Anabaena cylindrica*. *Proc R Soc London Ser B* **198**: 61–86.
- Valladares A, Herrero A, Pils D, Schmetterer G, Flores E. (2003). Cytochrome c oxidase genes required for nitrogenase activity and diazotrophic growth in *Anabaena* sp. PCC 7120. *Mol Microbiol* **47**: 1239–1249.
- van der Meer MT, Schouten S, Bateson MM, Nübel U, Wieland A, Kühl M et al. (2005). Diel variations in carbon metabolism by green nonsulfur-like bacteria in alkaline siliceous hot spring microbial mats from Yellowstone National Park. *Appl Environ Microbiol* **71**: 3978–3986.
- van der Meer MT, Schouten S, De Leeuw JW, Ward DM. (2000). Autotrophy of green non-sulphur bacteria in hot spring microbial mats: biological explanations for isotopically heavy organic carbon in the geological record. *Environ Microbiol* **2**: 428–435.
- Villbrandt M, Krumbein WF. (1990). Interactions between nitrogen fixation and oxygenic photosynthesis in a marine cyanobacterial mat. *FEMS Microbiol Ecol* **74**: 59–72.
- Walsby AE. (1985). The permeability of heterocysts to the gases nitrogen and oxygen. *Proc R Soc Lond B* **226**: 345–366.
- Walter MR, Bauld J, Brock TD. (1976). Microbiology and morphogenesis of columnar stromatolites (*Conophyton*, *Vacerrilla*) from hot springs in Yellowstone National Park. In: Walter MR (ed). *Stromatolites*. Elsevier Scientific: New York. pp 273–310.
- Wang ZC, Burns A, Watt GD. (1985). Complex formation and O₂ sensitivity of *Azotobacter vinelandii* nitrogenase and its component proteins. *Biochemistry* **24**: 214–221.
- Ward DM, Bateson MM, Ferris MJ, Kühl M, Wieland A, Koepfel A et al. (2006). Cyanobacterial ecotypes in the

- microbial mat community of Mushroom Spring (Yellowstone National Park, Wyoming) as species-like units linking microbial community composition, structure and function. *Philos Trans R Soc Lond B Biol Sci* **361**: 1997–2008.
- Ward DM, Castenholz RW. (2000). Cyanobacteria in geothermal habitats. In: Potts M, Whitton B (eds). *Ecology of Cyanobacteria*. Kluwer Academic Publishers: The Netherlands. pp 37–59.
- Ward DM, Cohan FM, Bhaya D, Heidelberg JF, K hl M, Grossman A. (2008). Genomics, environmental genomics and the issue of microbial species. *Heredity* **100**: 207–219.
- Ward DM, Ferris MJ, Nold SC, Bateson MM. (1998). A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. *Microbiol Mol Biol Rev* **62**: 1353–1370.
- Ward DM, Santegoeds CM, Nold SC, Ramsing NB, Ferris MJ, Bateson MM. (1997). Biodiversity within hot spring microbial mat communities: molecular monitoring of enrichment cultures. *Antonie van Leeuwenhoek* **71**: 143–150.
- Whelan JA, Russell NB, Whelan MA. (2003). A method for the absolute quantification of cDNA using real-time PCR. *J Immunol Methods* **278**: 261–269.
- Wolk CP, Ernst A, Elhai J. (1994). Heterocyst metabolism and development. In: Bryant DA (ed). *The Molecular Biology of Cyanobacteria*. Kluwer: Dordrecht. pp 76–82.
- Wormuth D, Baier M, Kandlbinder A, Scheibe R, Hartung W, Dietz KJ. (2006). Regulation of gene expression by photosynthetic signals triggered through modified CO₂ availability. *BMC Plant Biol* **6**: 15.

Supplementary Information accompanies the paper on The ISME Journal website (<http://www.nature.com/ismej>)