The ISME Journal (2013) 7, 2105–2115

www.nature.com/ismej

ORIGINAL ARTICLE

Low temperature delays timing and enhances the cost of nitrogen fixation in the unicellular cyanobacterium *Cyanothece*

Verena S Brauer[1,2,3], Maayke Stomp[1], Camillo Rosso[1], Sebastiaan AM van Beusekom[1], Barbara Emmerich[1], Lucas J Stal[1,4] and Jef Huisman[1]

[1]*Department of Aquatic Microbiology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, The Netherlands;* [2]*Department of Theoretical Biology, Center for Ecological and Evolutionary Studies, University of Groningen, Groningen, The Netherlands;* [3]*Laboratoire Ecologie des Systèmes Marins Côtiers ECOSYM, UMR 5119, CNRS, IRD, Ifremer, Université Montpellier 2, Montpellier, France and* [4]*Department of Marine Microbiology, Royal Netherlands Institute for Sea Research (NIOZ), Yerseke, The Netherlands*

Marine nitrogen-fixing cyanobacteria are largely confined to the tropical and subtropical ocean. It has been argued that their global biogeographical distribution reflects the physiologically feasible temperature range at which they can perform nitrogen fixation. In this study we refine this line of argumentation for the globally important group of unicellular diazotrophic cyanobacteria, and pose the following two hypotheses: (i) nitrogen fixation is limited by nitrogenase activity at low temperature and by oxygen diffusion at high temperature, which is manifested by a shift from strong to weak temperature dependence of nitrogenase activity, and (ii) high respiration rates are required to maintain very low levels of oxygen for nitrogenase, which results in enhanced respiratory cost per molecule of fixed nitrogen at low temperature. We tested these hypotheses in laboratory experiments with the unicellular cyanobacterium *Cyanothece* sp. BG043511. In line with the first hypothesis, the specific growth rate increased strongly with temperature from 18 to 30 °C, but leveled off at higher temperature under nitrogen-fixing conditions. As predicted by the second hypothesis, the respiratory cost of nitrogen fixation and also the cellular C:N ratio rose sharply at temperatures below 21 °C. In addition, we found that low temperature caused a strong delay in the onset of the nocturnal nitrogenase activity, which shortened the remaining nighttime available for nitrogen fixation. Together, these results point at a lower temperature limit for unicellular nitrogen-fixing cyanobacteria, which offers an explanation for their (sub)tropical distribution and suggests expansion of their biogeographical range by global warming.
The ISME Journal (2013) **7,** 2105–2115; doi:10.1038/ismej.2013.103; published online 4 July 2013
Subject Category: Microbial population and community ecology
Keywords: climate change; day–night cycle; nitrogenase; nitrogen fixation; respiration; unicellular cyanobacteria

Introduction

The global distribution of dinitrogen (N_2)-fixing cyanobacteria across the world's oceans exhibits a striking relationship with temperature. Marine N_2-fixing cyanobacteria occur in high numbers only in the tropics and subtropics, at water temperatures above 20 °C, whereas they are virtually absent from temperate and polar regions (Staal *et al.*, 2003; Stal, 2009). This temperature-related geographic pattern seems to hold for different phylogenetic groups of oceanic N_2-fixing cyanobacteria, such as the filamentous *Trichodesmium* (Capone *et al.*, 1997; Lugomela *et al.*, 2002; Chen *et al.*, 2003; Langlois *et al.*, 2005, 2008), unicellular cyanobacteria (UCYN) of the groups B and C (Mazard *et al.*, 2004; Staal *et al.*, 2007; Church *et al.*, 2008; Langlois *et al.*, 2008; Moisander *et al.*, 2010) and the heterocystous symbionts of diatoms (Foster *et al.*, 2007; Dore *et al.*, 2008; Fong *et al.*, 2008). Representatives of the uncultured symbiotic UCYN-A show peak abundances in subtropical waters, but it appears that their temperature range is somewhat broader (Needoba *et al.*, 2007; Church *et al.*, 2008; Langlois *et al.*, 2008; Moisander *et al.*, 2010).

The effect of temperature on the global distribution pattern of N_2-fixing cyanobacteria might be direct or indirect. Indirect temperature effects may operate for instance through negative correlations of

Correspondence: VS Brauer, Department of Aquatic Microbiology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, P.O. Box 94248, 1090 GE Amsterdam, The Netherlands.
E-mail: v.s.brauer@uva.nl
Received 21 December 2012; revised 19 May 2013; accepted 24 May 2013; published online 4 July 2013

temperature with the availability of nitrate and ammonium, which may favor the dominance of N_2 fixers in warm waters, because nitrogen fixation provides an important additional source of nitrogen (Monteiro *et al.*, 2011). Furthermore, high surface temperature strengthens water column stratification, which may favor the dominance of floating cyanobacteria such as the diazotroph *Trichodesmium* (Sonntag and Hense, 2011). Temperature can also be directly responsible for the ecological success of N_2 fixers in the tropical ocean, for example, if their nitrogenase activity is favored by high temperature. For instance, Breitbarth *et al.* (2007) showed that the temperature range for growth and N_2 fixation of *Trichodesmium* IMS-101 in laboratory experiments matched the biogeographical distribution of *Trichodesmium* in the ocean. Similar experiments revealed that N_2-fixing UCYN also have their temperature niche above 20 °C (Falcón *et al.*, 2005; Webb *et al.*, 2009).

A possible physiological mechanism explaining the thermal properties of N_2-fixing cyanobacteria was proposed by Staal *et al.* (2003) and Stal (2009). These authors hypothesized that N_2-fixing cyanobacteria are restricted to warm waters, because at low temperature the organism is unable to maintain sufficiently low levels of oxygen in the N_2-fixing cell to avoid the inactivation of nitrogenase. Here we refine this hypothesis for the UCYN groups B and C, which contribute substantially to the global oceanic nitrogen budget (Zehr, 2011; Großkopf *et al.*, 2012). Representatives of UCYN-B and -C exhibit a strong diurnal pattern in N_2 fixation, respiration and photosynthesis (Peschek *et al.*, 1991; Schneegurt *et al.*, 1994b; Colón-López *et al.*, 1997; Červený and Nedbal, 2009). In order to protect nitrogenase from high oxygen concentrations produced during photosynthesis, these organisms fix nitrogen typically during the night. Their nocturnal N_2 fixation is fueled by respiration of the glycogen pool that has been built up by photosynthesis during the daytime (Schneegurt *et al.*, 1994a; Dron *et al.*, 2012).

As pointed out by Staal *et al.* (2003) and Stal (2009), respiration of glycogen requires oxygen, and UCYN therefore face an important trade-off. Oxygen levels in the cell should be sufficiently low to prevent inactivation of nitrogenase, yet the influx of oxygen should be sufficiently high to enable respiration for N_2 fixation. Oxygen diffusion into cells shows only a weak temperature dependence (Figure 1a). In contrast, respiration and N_2 fixation are enzymatic processes that, in potential, will increase faster with temperature than oxygen diffusion. Hence, at high temperature the actual N_2-fixation activity may become limited by the oxygen influx (Figure 1a). Conversely, at low temperature nitrogenase activity will be the rate-limiting step for N_2 fixation. However, the respiration rate needs to be high enough to protect nitrogenase against the diffusive influx of oxygen. A low nitrogenase activity in combination with sustained high levels of respiration results in high respiration costs per molecule of N_2 fixed at low temperature (Figure 1b). We thus derived two testable hypotheses: (1) N_2-fixation rates of UCYN will increase more strongly with temperature at low than at high temperature (Figure 1a) and (2) low temperature enhances the respiratory cost of N_2 fixation (Figure 1b).

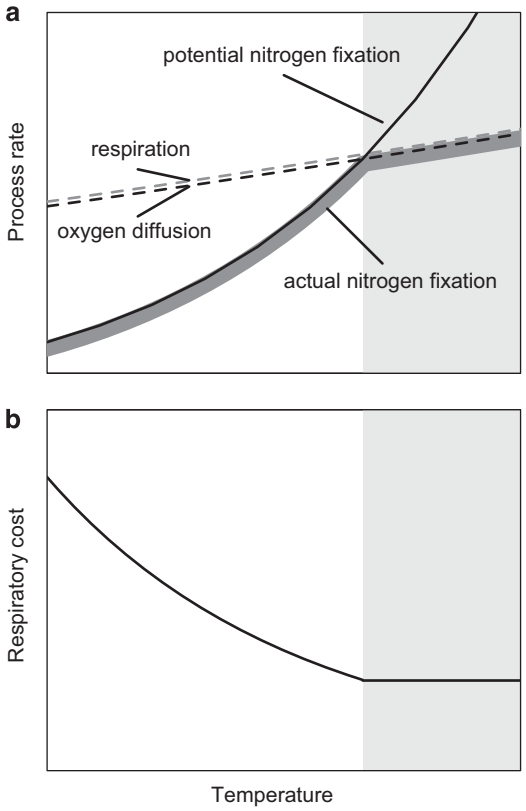

Figure 1 Conceptual visualization of the two hypotheses explaining temperature dependence of N_2 fixation in unicellular cyanobacteria. (**a**) Rates of oxygen diffusion (black dashed line) and potential N_2 fixation (black solid line) as function of temperature. The actual N_2-fixation rate (thick grey line) equals the potential N_2-fixation rate at low temperature, but is limited by the oxygen diffusion rate at high temperature. The respiration rate (grey dashed line) matches the rate of oxygen diffusion into the cells in order to maintain a low-oxygen environment for nitrogenase. (**b**) Comparison of the respiration rate with the actual N_2-fixation rate, in panel **a**, shows that the respiratory cost of N_2 fixation (that is, the oxygen respired per molecule of N_2 fixed) will increase strongly at low temperature. Background shading indicates temperature regions where nitrogen fixation is controlled by the reaction rate of nitrogenase (white background) or the rate of oxygen diffusion (dark background).

In this study we test these hypotheses by investigating N_2 fixation and respiration rates of the unicellular cyanobacterium *Cyanothece* BG 043511 at different temperatures. This strain assimilates nitrate if sufficient nitrate is available, but switches to N_2 fixation if nitrate has been depleted (Agawin *et al.*, 2007). Hence, by manipulating the nitrogen source, we can compare the respiration rates of *Cyanothece* under diazotrophic versus non-diazotrophic growth conditions to infer the respiratory cost of N_2 fixation. In addition, we measured

the light response of the N_2 fixation and growth rates of *Cyanothece* at different temperatures. Our results show strong effects of temperature on the respiratory cost as well as the timing of N_2 fixation in *Cyanothece*, which may offer a mechanistic explanation for the temperature dependence of the global biogeographical distribution of many other N_2-fixing UCYN as well.

Materials and methods

Experimental design

The strain *Cyanothece* sp. Miami BG043511, which belongs to the group of UCYN-C, was grown in batch cultures with a volume of 50 ml in 250 ml Erlenmeyer flasks on artificial sea water medium enriched with nutrients (Mitsui and Cao, 1988). The mineral medium was provided either with 1.5 mM nitrate (non-diazotrophic condition) or without combined nitrogen (diazotrophic condition). The cultures were unialgal but not axenic. However, regular microscopic inspection confirmed that abundances of other bacteria remained low (<1% of the total biomass) for the entire duration of the experiments. The cultures were grown under an alternating 12:12 h light:dark cycle. A shaking cryostat bath controlled the temperature and guaranteed continuous mixing of the cultures. We investigated the growth rate and N_2-fixation rate of *Cyanothece* at seven different temperatures from 14 to 38 °C, nine light intensities from 3 to 133 μmol photons $m^{-2} s^{-1}$ and with or without nitrate in the mineral medium using a full factorial design. This resulted in $7 \times 9 \times 2 = 126$ different experimental treatments. Before the experiments, the *Cyanothece* cultures were acclimated to the applied temperature and nitrogen source for at least 1 week, using an intermediate light intensity of ~30 μmol photons $m^{-2} s^{-1}$.

Growth rates

The specific growth rate of each population was determined during the exponential growth phase. We used one batch culture per experimental treatment. Cell numbers in this batch culture were counted in triplicate on 5 consecutive days using an automated cell counter (Casy Cell Coulter, Schaerfe System GmbH, Reutlingen, Germany). Specific growth rates were calculated as the slope of the regression line of the natural logarithm of population density versus time.

At each experimental temperature, the light response of the specific growth rate, $\mu(I)$, was described by the Monod equation (Monod, 1950; Huisman, 1999):

$$\mu(I) = \frac{\mu_{\max} I}{(\mu_{\max}/\alpha) + I} \quad (1)$$

where I is light intensity, $\mu_{\max}$ is the maximum specific growth rate and α is the initial slope of the $\mu(I)$ function. Equation (1) was fitted to the measured growth rates using non-linear regression with minimization of the residual sum of squares (R version 2.13.1).

Subsequently, the temperature dependence of the maximum specific growth rate was described by the Arrhenius equation (Gillooly *et al.*, 2001):

$$\mu_{\max}(T) = c\, e^{\frac{-E}{kT}} \quad (2)$$

where c is a normalization constant, E is the activation energy, k is Boltzmann's constant (8.62×10^{-5} eV K^{-1}) and T is the absolute temperature in Kelvin. The activation energy quantifies the temperature dependence, and was calculated as the slope of the linear regression of $\ln(\mu_{max})$ plotted against the inverse of temperature, $1/kT$. Activation energy was converted to Q_{10}-values according to Vasseur and McCann (2005).

Nitrogen fixation rates

N_2-fixation rate was determined during the exponential growth phase using the acetylene reduction assay (Hardy *et al.*, 1968). For each experimental treatment, whole-night N_2 fixation was measured in duplicate incubations over a 12-h dark period. In addition, we used six consecutive incubations of 2 h each to follow the temporal pattern of nocturnal N_2 fixation. Whole-night incubations were done in 5-ml crimp-top vials (Chrompack, Middelburg, The Netherlands) using 1 ml of *Cyanothece* culture and a headspace acetylene concentration of 20%. For the 2-h incubations, 3 ml of culture and 11% acetylene was used. After incubation, gas samples of 300 μl were withdrawn from the headspace with a disposable syringe and injected into a gas chromatograph (GC14A, Shimadzu, Kyoto, Japan) to measure the concentrations of ethylene and acetylene. The gas chromatograph was equipped with a flame ionization detector and a 25-m long wide-bore silica-fused Porapak U column (0.53 mm inner diameter; Chrompack). The temperatures of oven, injector and detector were set to 60, 100 and 150 °C, respectively. N_2 fixation was expressed as acetylene reduction, and was calculated according to Stal (1988) using acetylene as internal standard.

Analogous to the specific growth rates, the light response of N_2 fixation was fitted to the Monod equation (Equation 1) by expressing the whole-night N_2 fixation as a function of the light intensity provided during the preceding daytime. Subsequently, the maximum whole-night N_2 fixation obtained from the Monod equation ($n_{\max}$) was fitted to the Arrhenius equation (Equation 2) to quantify its temperature dependence.

Respiration rates

Respiration was monitored during the 12-h dark period using automated recording of oxygen consumption. *Cyanothece* was grown in Erlenmeyer

flasks under high-light conditions (130 μmol photons $m^{-2} s^{-1}$) and a 12:12 h light:dark cycle, at five different temperatures (from 18 to 30 °C) under both N_2-fixing and non-N_2-fixing conditions. The cultures were continuously mixed with magnetic stirrers. Each Erlenmeyer flask was connected to a temperature-controlled flow-through cuvette with a chamber volume of ~5 ml. Every 30 min a peristaltic pump (Watson Marlow 101U, Falmouth, UK) connected to a computer-controlled USB-time relay (H-TRONIC, Hirschau, Germany) flushed the flow-through cuvette during 7 min with a new air-saturated sample taken from the Erlenmeyer flask. Subsequently, the pump was stopped and the decline in oxygen concentration was measured online during 10 min by using optical oxygen sensors connected to a fiber optic oxygen transmitter (Oxy-4-mini, PreSens, Regensburg, Germany). The respiration rate was calculated as the slope of the regression line of oxygen concentration versus time.

Cellular carbon and nitrogen

At the beginning and end of the 12-h dark period, 5 to 10 ml of the high-light cultures were filtrated on pre-combusted glass fiber filters (GF/F, Watson). The filters were rinsed with demineralized water and stored at −20 °C. Amounts of organic carbon and nitrogen on each filter were determined with an element analyzer (NA-2500, Thermo Scientific, Whaltham, MA, USA) to determine cellular carbon and nitrogen contents.

Cost of nitrogen fixation

We used two measures to quantify the respiratory cost of N_2 fixation. First, we calculated the gross cost of N_2 fixation (C_{gross}), which we defined as the whole-night respiration of N_2-fixing cultures divided by the whole-night N_2 fixation:

$$C_{gross} = \frac{\int_0^{12h} r_{N2}(t)dt}{\int_0^{12h} Nf(t)dt} \quad (3)$$

where $r_{N2}(t)$ is the respiration rate of the N_2-fixing culture as function of time, and $Nf(t)$ is its N_2-fixation rate. Accordingly, the gross cost of N_2 fixation includes respiration associated directly with N_2 fixation as well as respiration required for the maintenance of other cellular processes.

Second, we calculated the net cost of N_2 fixation (C_{net}), defined as the difference between the whole-night respiration of N_2-fixing and non-N_2-fixing cultures divided by the whole-night N_2 fixation:

$$C_{net} = \frac{\int_0^{12h} [r_{N2}(t) - r_{NO_3}(t)]dt}{\int_0^{12h} Nf(t)dt} \quad (4)$$

where $r_{NO3}(t)$ is the respiration rate of the non-N_2-fixing culture as function of time. Hence, the net cost of N_2 fixation excludes the maintenance cost associated with other metabolic processes. For both measures, a conversion factor of 4 was used to calculate N_2 fixation from acetylene reduction (for example, Stal, 1988).

Results

Growth and nitrogen fixation

The specific growth rate of *Cyanothece* was an increasing decelerating function of light intensity (Figure 2). The exact response to light, however, depended on temperature and nitrogen source. At 14 °C, *Cyanothece* showed only marginal growth under non-N_2-fixing conditions, while we were unable to grow *Cyanothece* diazotrophically at this temperature (Figure 2a). At 18 and 22 °C, *Cyanothece* grew well under both N_2-fixing and non-N_2-fixing conditions, and the specific growth rate already leveled off at a light intensity of 40–50 μmol photons $m^{-2} s^{-1}$ (Figures 2b and c). At 34 and 38 °C, the specific growth rate had not yet leveled off at a light intensity of >150 μmol photons $m^{-2} s^{-1}$ (Figures 2f and g).

Whole-night N_2 fixation in cultures without nitrate was also an increasing decelerating function of the light intensity received during the preceding daytime, and varied with temperature (Figure 3). N_2 fixation (acetylene reduction) was not detectable in the cultures with nitrate (results not shown), which confirmed that *Cyanothece* grows non-diazotrophically when provided with sufficient nitrate. For the diazotrophic cultures, we compared the amount of nitrogen fixed per cell calculated from acetylene reduction (N_{fix}) with the accumulation of nitrogen in cells calculated from the cellular nitrogen contents at the beginning and end of the dark period (N_{acc}). Both values generally agreed quite well, with an average ratio of $N_{fix}/N_{acc} = 0.86$ (s.e. = 0.22; $N = 63$), which illustrates the consistency of the data.

The light responses were fitted to the Monod equation (solid lines in Figures 2 and 3), and the parameter values obtained from these Monod fits are plotted as function of temperature in Figure 4. Up to 30 °C, the maximum specific growth rate of the N_2-fixing and non-N_2-fixing cultures increased with temperature in a similar manner (Figure 4a), with activation energies of 0.81 eV (95% confidence interval: 0.76–0.85 eV, $df = 3$, $P < 0.001$; $Q_{10} = 2.89$) and 0.90 eV (95% confidence interval: 0.70–1.11 eV, $df = 3$, $P = 0.0134$; $Q_{10} = 3.28$) under N_2-fixing and non-N_2-fixing conditions, respectively. The maximum specific growth rate continued to increase with temperature up to 34 °C under non-N_2-fixing conditions, whereas it leveled off above 30 °C under N_2-fixing conditions. Hence, at high temperature, *Cyanothece* reached much higher specific growth rates under non-N_2-fixing than under N_2-fixing conditions.

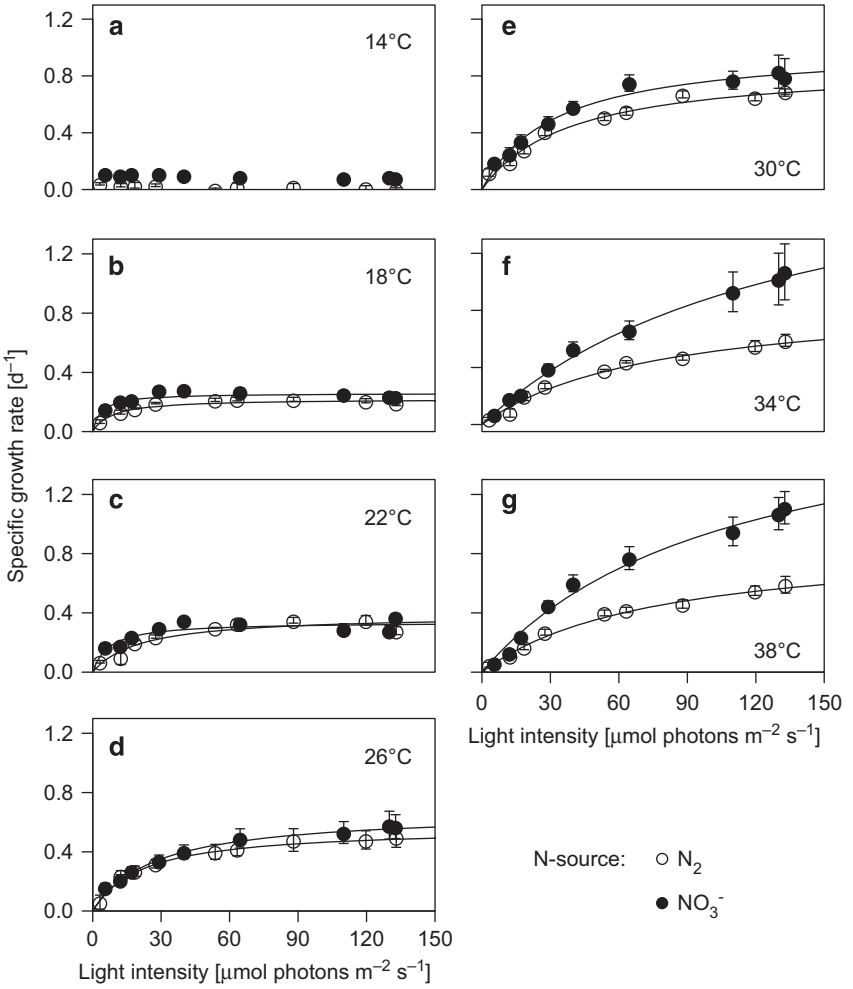

Figure 2 Light-response curves of the specific growth rate at different temperatures. (**a**) 14 °C, (**b**) 18 °C, (**c**) 22 °C, (**d**) 26 °C, (**e**) 30 °C, (**f**) 34 °C, (**g**) 38 °C. The panels compare the light response of N_2-fixing cultures (open circles) versus cultures grown on nitrate (closed circles). Each data point is based on a time series of five measurements. The specific growth rate was calculated as the slope of the linear regression of ln[population density] versus time; error bars represent the s.e. of the slope ($N=5$). Solid lines show light-response curves fitted by the Monod equation.

Maximum whole-night N_2 fixation showed a unimodal response to temperature (Figure 4b). At 14 and 18 °C, whole-night N_2 fixation did not exceed the detection limit in most cases (see Figure 3b). Maximum whole-night N_2 fixation increased strongly with temperature from 22 to 30 °C, with an activation energy of 1.27 eV (95% confidence interval: 0.05–2.5 eV, $df=2$, $P=0.29$; $Q_{10}=5.2$). Above 30 °C, however, maximum N_2 fixation decreased with temperature (Figure 4b).

The initial slope of the light-dependent growth rate (α_μ) at 18 and 22 °C was higher for non-N_2-fixing cultures than for N_2-fixing cultures (Figure 4c). Above 22 °C, the initial slope of the light-dependent growth rate seemed largely temperature independent, and was essentially similar under both N_2-fixing and non-N_2-fixing conditions. The initial slope of the light-dependent whole-night N_2 fixation (α_n) showed considerable variation, but seemed also largely independent of temperature (Figure 4d). The low value of α_n at 18 °C might be an outlier due to the technical difficulty to obtain an accurate light-response curve from the low N_2-fixation rates measured at this temperature (cf. Figure 3b).

Nocturnal patterns of nitrogen fixation and respiration

As a next step, we investigated how temperature affects temporal patterns of N_2 fixation (acetylene reduction) and respiration (oxygen consumption) during the 12-h dark period. This revealed that N_2 fixation and respiration exhibited very similar nocturnal patterns (Figure 5). In most cases, N_2 fixation started after the onset of darkness and ceased before daybreak. However, the precise timing of the N_2-fixation activity was strongly determined by temperature. The N_2-fixation peak shifted from the end of the dark period at 18 °C to the beginning of the dark period at 30 °C (Figures 5a–e). N_2 fixation reached the highest rate at intermediate temperatures from 21 to 27 °C (Figures 5b–d). At each temperature, N_2-fixation rates in the night increased

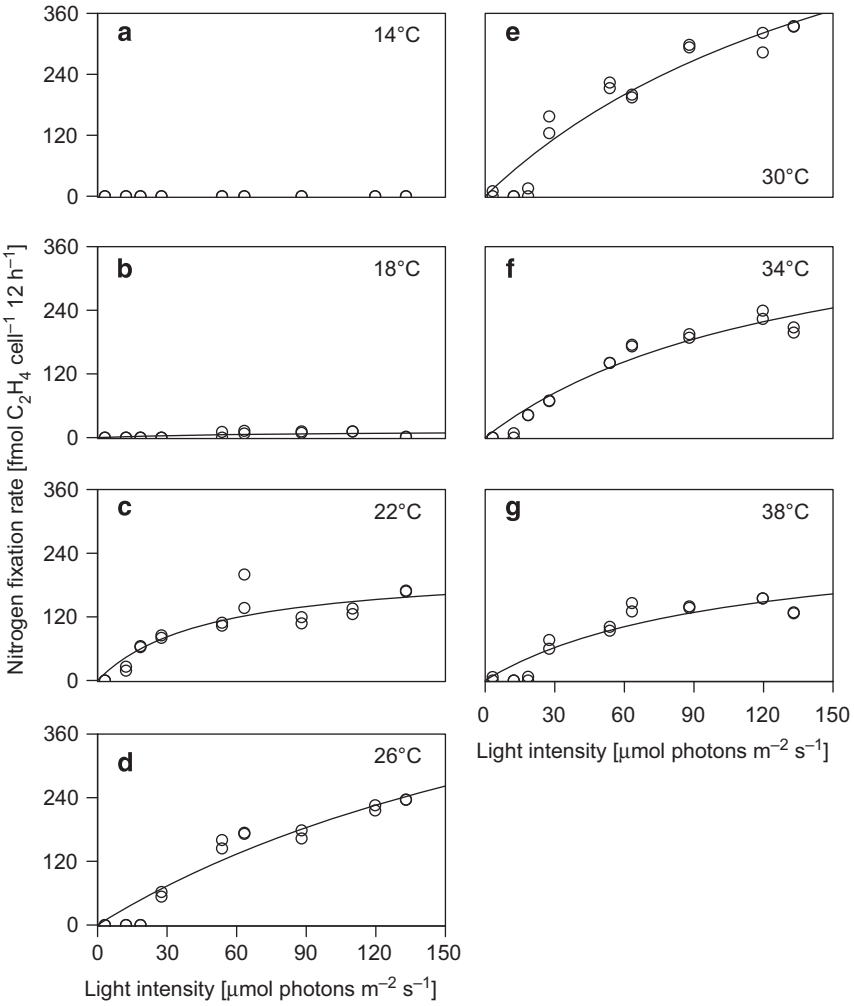

Figure 3 Light-response curves of whole-night N_2 fixation at different temperatures. (**a**) 14 °C, (**b**) 18 °C, (**c**) 22 °C, (**d**) 26 °C, (**e**) 30 °C, (**f**) 34 °C, (**g**) 38 °C. Each data point represents an independent incubation. N_2 fixation is expressed as acetylene reduction, integrated over the entire 12-h night. Light intensities were measured during the preceding daytime. Solid lines show light-response curves fitted by the Monod equation.

with the light intensity provided during the preceding daytime, but light intensity did not affect the temporal pattern of N_2 fixation (Figures 5a–e).

The temporal pattern of respiration reflected that of N_2 fixation (Figures 5f–j). Respiration rates of N_2-fixing cultures reached a maximum during the night, and the timing of this maximum shifted from the end of the dark period at low temperature to the beginning of the dark period at high temperature (open circles in Figures 5f–j). The highest respiration rates were found at intermediate temperatures (Figures 5g–i). In contrast, respiration rates of non-N_2-fixing cultures remained low and were basically constant during the dark period (closed circles in Figures 5f–j).

Cost of nitrogen fixation

We introduced two measures of the respiratory cost per molecule of N_2 fixed. The gross cost of N_2 fixation considers the whole-night respiration of the N_2-fixing culture (that is, the area underneath the open circles in Figures 5f–j integrated over the 12 h-dark period), whereas the net cost considers the difference in whole-night respiration between the N_2-fixing and non-N_2-fixing culture (that is, the area between the open and closed circles in Figures 5f–j integrated over the 12-h dark period). Both measures strongly increased with decreasing temperature (Figure 6a). At 18 °C, the gross cost of N_2 fixation was about five times higher than at 30 °C, whereas the net cost of N_2 fixation was about four times higher. Likewise, the C:N ratio of the cells determined at the end of the 12-h dark period increased at low temperature, particularly for the N_2-fixing cultures at 14 and 18 °C (Figure 6b).

Discussion

Mechanisms controlling temperature dependence

The results show that temperature has major effects on growth, N_2 fixation and respiration of the unicellular N_2-fixing cyanobacterium *Cyanothece*.

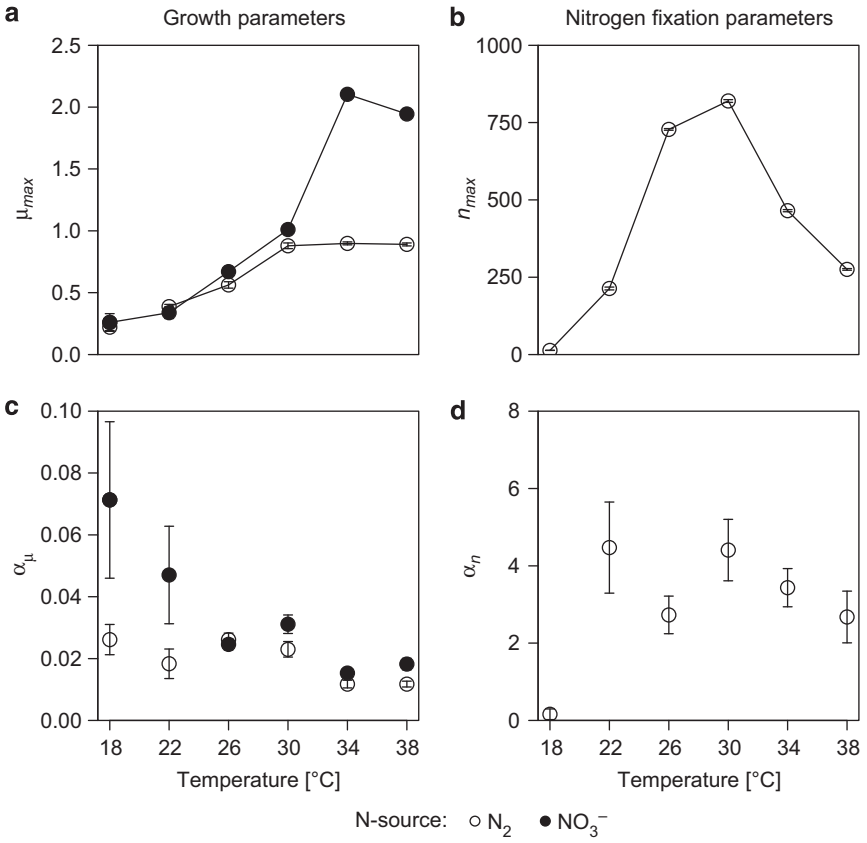

Figure 4 Monod parameters for the light response of the specific growth rate and of the whole-night N_2 fixation, plotted as function of temperature. (**a**) Maximum specific growth rate, μ_{max}; (**b**) maximum whole-night N_2-fixation, n_{max}; (**c**) initial slope, α_μ, of the light-response curves of the specific growth rates, (**d**) initial slope, α_n, of the light-response curves of whole-night N_2 fixation. Open circles represent N_2-fixing cultures; closed circles represent cultures grown on nitrate. The parameter estimates are based on the Monod fits shown in Figure 2 ($N=9$) and Figure 3 ($N=18$). Error bars represent s.e. of the parameter estimates.

In agreement with the first hypothesis, displayed in Figure 1a, the maximum specific growth rate of *Cyanothece* grown under N_2-fixing conditions showed a strong temperature dependence at low temperature but a weak temperature dependence at high temperature (Figure 4a). The switch from strong to weak temperature dependence occurred at 30 °C, suggesting that the diffusive influx of oxygen (and possibly N_2) limited a further increase of the diazotrophic growth rate at higher temperatures. In contrast, the specific growth rate under non-N_2-fixing conditions still increased steeply with temperature at this temperature range, demonstrating that cells were capable of a much higher growth rate if they were not limited by the N_2-fixation process (Figure 4a).

Although the specific growth rate leveled off above 30 °C, the maximum whole-night N_2 fixation declined above this temperature. Gallon *et al.* (1993) observed a similar decline of the N_2-fixation rate in the cyanobacteria *Anabaena cylindrica* and *Gloeothece*. Their results indicate that the decline is probably not caused by thermal inactivation of nitrogenase, because *in vitro* studies showed that thermal inactivation of nitrogenase required much higher temperatures. Instead, Gallon *et al.* (1993) attributed the decreased N_2-fixation rate to an enhanced sensitivity of nitrogenase to oxygen inhibition at elevated temperature. Another possible explanation is that the observed decrease of the acetylene reduction rate above 30 °C does not represent a decrease of the actual N_2-fixation rate. This latter explanation would be compatible with the observation that the growth rate did not decline (Figure 4a) and cellular C:N ratio remained low (Figure 6b) above 30 °C.

Respiratory cost of nitrogen fixation

Our findings support the hypothesis, in Figure 1b, that the respiratory cost of N_2 fixation increases at low temperature (Figure 6a). One might argue that our *Cyanothece* cultures were not axenic, and that other bacteria may have confounded the respiration measurements. However, our cultures were provided with a mineral medium designed exclusively for photoautotrophic organisms, without suitable substrates to respire by heterotrophic bacteria. Microscopic inspection confirmed only minor contamination by other bacteria, suggesting that their contribution to the total respiration rate was probably small compared with that of *Cyanothece*.

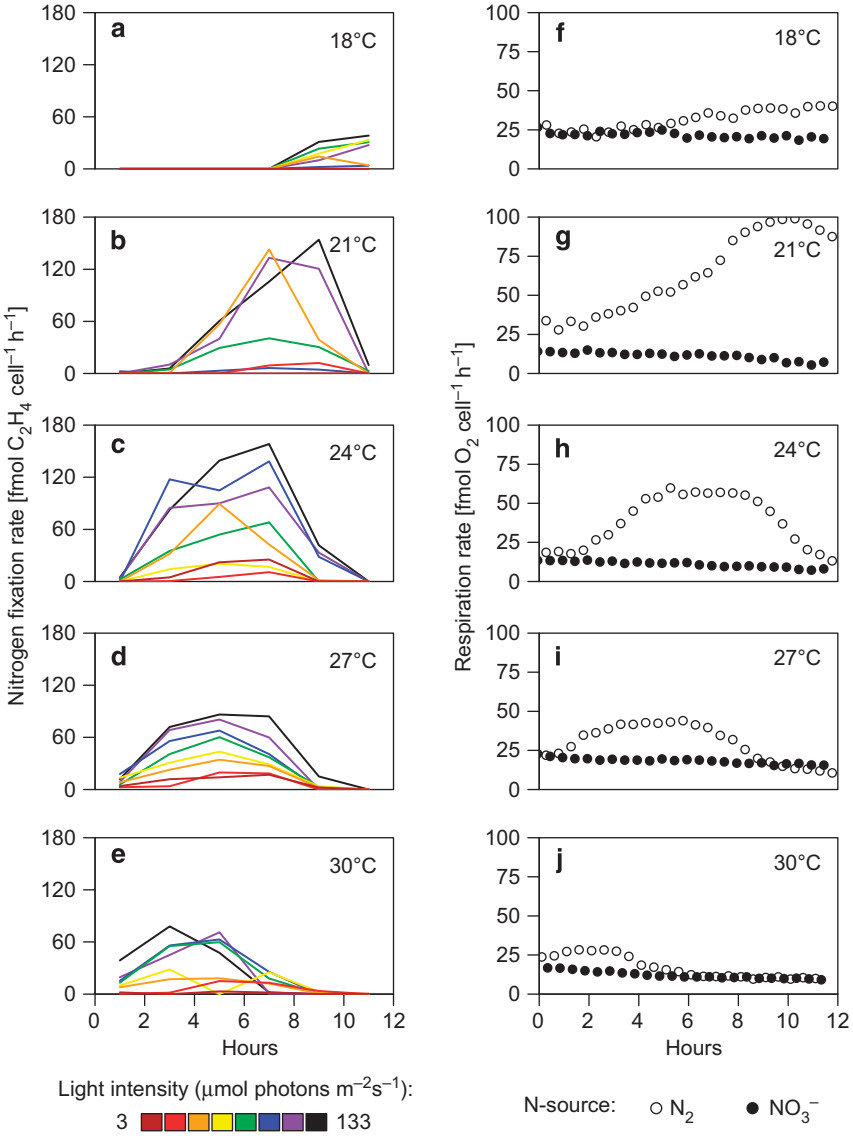

Figure 5 Nocturnal patterns of N_2 fixation (acetylene reduction) and respiration (oxygen consumption) at different temperatures. (**a–e**) The N_2-fixation rate was measured during six consecutive intervals of two hours each, covering the entire 12-h dark period. The different light intensities provided during daytime are indicated by different colors. (**f–j**) Respiration rate of N_2-fixing cultures (open circles) and non-N_2-fixing cultures (closed circles) during the 12-h dark period, after exposure to a daytime light intensity of 130 µmol photons $m^{-2} s^{-1}$. N_2 fixation and respiration rates were measured in separate cultures.

Furthermore, respiration rates under N_2-fixing conditions were much higher than in the nitrate-grown cultures, and showed the same temporal pattern as the nitrogenase activity. This clearly indicates a predominant contribution of *Cyanothece* to the respiration rate, because one would not expect such patterns when the respiration rate would be dominated by contaminating heterotrophic bacteria. Finally, the net respiratory cost of N_2 fixation calculated with Equation (4) is based on the difference in respiration rate between N_2-fixing and nitrate-grown cultures. Hence, this calculation removes the background respiration not associated with N_2 fixation, such as the respiratory contributions of contaminating bacteria. Our results therefore support unequivocally the hypothesis that the respiratory cost of N_2 fixation increases with decreasing temperature, as predicted by theory.

It is well known that N_2-fixing cyanobacteria often have elevated respiration rates compared with non-N_2-fixing organisms (for example, Wastyn *et al.*, 1988; Peschek *et al.*, 1991). This is typically ascribed to the high energy demand of N_2 fixation (Bergman *et al.*, 1997). However, the observed increase in respiratory cost at low temperature suggests that part of the respiratory oxygen consumption is independent of the energy demand of N_2 fixation, and is used to maintain low intracellular oxygen levels. Großkopf and LaRoche (2012) estimated that the N_2-fixing UCYN *Crocosphaera watsonii* invested only 40% of the energy obtained from respiration directly into N_2 fixation and maintenance

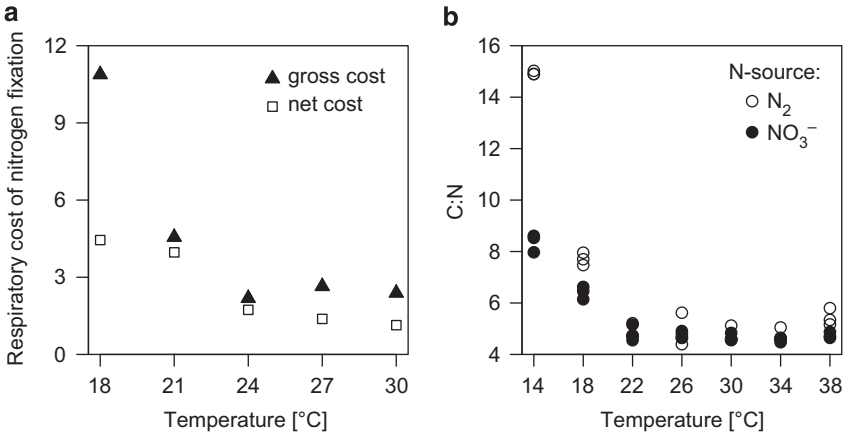

Figure 6 Respiratory cost of N_2 fixation. (**a**) Gross respiratory cost (closed triangles) and net respiratory cost (open squares) of N_2 fixation as function of temperature. Gross respiratory cost represents the ratio of whole-night respiration over whole-night N_2 fixation, net respiratory cost represents the same ratio but corrected for respiration not associated with N_2 fixation. (**b**) Cellular C:N ratio of N_2-fixing (open circles) and non-N_2-fixing cultures (closed circles). The C:N ratios were measured at the end of the 12-h dark period in cultures exposed to 80–130 μmol photons $m^{-2} s^{-1}$ during the preceding daytime.

metabolism, while up to 60% of the respiratory cost represented an indirect cost for removal of intracellular oxygen or reversal of oxidative damage, for example, by *de novo* nitrogenase synthesis. Our results show that this indirect cost varies with temperature. More specifically, the fourfold rise in net respiratory cost above the baseline value (Figure 6a) suggests that at low temperature, the indirect cost associated with oxygen removal may constitute up to 80% of the total cost of N_2 fixation.

We currently do not know which oxygen-scavenging mechanisms are present in *Cyanothece* sp. BG 043511. One possible strategy is 'respiratory protection,' where organisms possess a branched electron transport chain with multiple terminal oxidases, as has been shown for a number of bacteria and the unicellular N_2-fixing cyanobacterium *Gloeothece* (Maryan *et al.*, 1986; Gallon, 1992). An alternative strategy is 'autoprotection,' where the nitrogenase enzyme protects itself by reducing oxygen to H_2O and H_2O_2 (Gallon, 1992; Bergman *et al.*, 1997).

High respiratory cost of N_2 fixation at low temperature have also been found in diazotrophic symbionts of terrestrial plants. Using *Frankia*-infected root nodules of alder, Winship and Tjepkema (1985) showed that nitrogenase activity decreased faster with temperature than the respiration rate. As a consequence, the respiratory cost per molecule of fixed N_2 rose sharply at low temperature, similar to our findings. This indicates that the high respiratory cost of N_2 fixation at low temperature, required to maintain low intracellular oxygen concentrations, might be a common challenge for N_2-fixing organisms in both aquatic and terrestrial habitats.

Timing of dinitrogen fixation

In addition to the predicted results, we found that temperature has also a remarkable effect on the timing of N_2 fixation. The timing of N_2 fixation and many other processes involved in the nitrogen and carbon metabolism of the genus *Cyanothece* is under the tight control of a circadian clock. N_2 fixation and respiration show a distinct 24-h periodicity, and photosynthesis peaks about 12 h out of phase (Peschek *et al.*, 1991; Schneegurt *et al.*, 1994b; Colón-López *et al.*, 1997; Červený and Nedbal, 2009). The circadian oscillations of N_2-fixation and other processes are expressed independently of external triggers, under continuous light (Colón-López *et al.*, 1997; Toepel *et al.*, 2008; Červený and Nedbal, 2009), under continuous darkness (Schneegurt *et al.*, 1994b) and under various day–night cycles (Červený and Nedbal, 2009; Toepel *et al.*, 2009). Studies on the phosphorylation cycle of the cyanobacterial clock protein KaiC demonstrated that the period of oscillations is independent of nutrient status and temperature (Nakajima *et al.*, 2005; Tomita *et al.*, 2005). Likewise, our study shows that differences in light intensity during the daytime do not affect the timing of N_2 fixation during the night.

However, decreasing the temperature strongly delays the onset of N_2 fixation (Figure 5). A possible explanation might be that the synthesis of nitrogenase proceeds at a lower rate at low temperature. In N_2-fixing UCYN nitrogenase is inactivated by oxygen during the day and has to be resynthesized *de novo* every night (Mullineaux *et al.*, 1981; Huang *et al.*, 1988; Gallon, 1992; Bergman *et al.*, 1997). Thus, even if the circadian clock is temperature-compensated and always initiates the synthesis of nitrogenase at exactly the same time, there might be a temperature-dependent delay in the delivery of the functional enzyme. This may cause a decrease in N_2-fixation capacity if low temperature would delay the synthesis of active nitrogenase to such an extent that the remaining nighttime becomes insufficient to cover the nitrogen demands of the cell. The observed delay in the timing of N_2 fixation may thus contribute to the steep decline of the whole-night

N_2-fixation activity (Figure 4b) and to the high cellular C:N ratio (Figure 6b) at low temperature.

Biogeographical distribution

Although many environmental factors may influence the distribution of N_2-fixing cyanobacteria, such as the availability of dissolved nitrogen, iron and phosphorus (for example, Sañudo-Wilhelmy *et al.*, 2001; Mills *et al.*, 2004; Monteiro *et al.*, 2011), there is ample evidence that temperature has also an important role (Stal, 2009). According to our laboratory results, *Cyanothece* can grow over a temperature range from 18 to at least 38 °C. This is in good agreement with field observations, which indicate that N_2-fixing UCYN-B and -C occur only in natural waters above 20 °C (Mazard *et al.*, 2004; Langlois *et al.*, 2005; Staal *et al.*, 2007; Church *et al.*, 2008; Langlois *et al.*, 2008; Moisander *et al.*, 2010). Their global biogeographical distribution might thus reflect the direct effect of temperature on the physiology and growth of these organisms (see also Breitbarth *et al.*, 2007). In nature, competitive interactions with other species may further restrict the temperature range of N_2-fixing UCYN, particularly below 21 °C when the cost of N_2 fixation becomes high. Our experiments thus suggest that the lower temperature limit of N_2-fixing UCYN-B and -C is due to the enhanced respiratory cost and strongly delayed timing of N_2 fixation at low temperature.

Together, these results provide novel experimental evidence for the general hypothesis that the temperature sensitivity of N_2 fixation is a key determinant of the biogeographical distribution of N_2-fixing UCYN across the world's oceans. An important implication of these findings is that global warming is likely to facilitate the expansion of N_2-fixing UCYN to higher latitudes.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

We thank Joke Westerveld and Frans van der Wielen for technical assistance in gas chromatography, Hans Matthijs for scientific discussions and Franjo Weissing for facilitating the research. We are grateful to the two anonymous reviewers for their constructive comments on the manuscript. The research of VSB, MS and JH was supported by the Earth and Life Sciences Foundation (ALW), which is subsidized by the Netherlands Organization for Scientific Research (NWO).

References

Agawin NSR, Rabouille S, Veldhuis MJW, Servatius L, Hol S, van Overzee HMJ *et al.* (2007). Competition and facilitation between unicellular nitrogen-fixing cyanobacteria and non–nitrogen-fixing phytoplankton species. *Limnol Oceanogr* **52**: 2233–2248.

Bergman B, Gallon JR, Rai AN, Stal LJ. (1997). N_2 fixation by non-heterocystous cyanobacteria. *FEMS Microbiol Rev* **19**: 139–185.

Breitbarth E, Oschlies A, La Roche J. (2007). Physiological constraints on the global distribution of *Trichodesmium*: effects of temperature on diazotrophy. *Biogeosciences* **4**: 53–61.

Capone DG, Zehr JP, Paerl HW, Bergman B, Carpenter EJ. (1997). *Trichodesmium*, a globally significant marine cyanobacterium. *Science* **276**: 1221–1229.

Červený J, Nedbal L. (2009). Metabolic rhythms of the cyanobacterium *Cyanothece* sp. ATCC 51142 correlate with modeled dynamics of circadian clock. *J Biol Rhythms* **24**: 295–303.

Chen YLL, Chen HY, Lin YH. (2003). Distribution and downward flux of *Trichodesmium* in the South China Sea as influenced by the transport from the Kuroshio Current. *Mar Ecol Prog Ser* **259**: 47–57.

Church MJ, Björkman KM, Karl DM, Saito MA, Zehr JP. (2008). Regional distributions of nitrogen-fixing bacteria in the Pacific Ocean. *Limnol Oceanogr* **53**: 63–77.

Colón-López MS, Sherman DM, Sherman LA. (1997). Transcriptional and translational regulation of nitrogenase in light-dark- and continuous-light-grown cultures of the unicellular cyanobacterium *Cyanothece* sp. strain ATCC 51142. *J Bacteriol* **179**: 4319–4327.

Dore JE, Letelier RM, Church MJ, Lukas R, Karl DM. (2008). Summer phytoplankton blooms in the oligotrophic North Pacific Subtropical Gyre: historical perspective and recent observations. *Progr Oceanogr* **76**: 2–38.

Dron A, Rabouille S, Claquin P, Le Roy B, Talec A, Sciandra A. (2012). Light–dark (12:12) cycle of carbon and nitrogen metabolism in *Crocosphaera watsonii* WH8501: relation to the cell cycle. *Environ Microbiol* **14**: 967–981.

Falcón LI, Pluvinage S, Carpenter EJ. (2005). Growth kinetics of marine unicellular N_2-fixing cyanobacterial isolates in continuous culture in relation to phosphorus and temperature. *Mar Ecol Prog Ser* **285**: 3–9.

Fong AA, Karl DM, Lukas R, Letelier RM, Zehr JP, Church MJ. (2008). Nitrogen fixation in an anticyclonic eddy in the oligotrophic North Pacific Ocean. *ISME J* **2**: 663–676.

Foster RA, Subramaniam A, Mahaffey C, Carpenter EJ, Capone DG, Zehr JP. (2007). Influence of the Amazon River plume on distributions of free-living and symbiotic cyanobacteria in the western tropical North Atlantic Ocean. *Limnol Oceanogr* **52**: 517–532.

Gallon JR. (1992). Reconciling the incompatible: N_2 fixation and O_2. *New Phytol* **122**: 571–609.

Gallon JR, Pederson DM, Smith GD. (1993). The effect of temperature on the sensitivity of nitrogenase to oxygen in the cyanobacteria *Anabaena cylindrica* (Lemmermann) and *Gloeothece* (Nägeli). *New Phytol* **124**: 251–257.

Gillooly JF, Brown JH, West GB, Savage VM, Charnov EL. (2001). Effects of size and temperature on metabolic rate. *Science* **293**: 2248–2251.

Großkopf T, LaRoche J. (2012). Direct and indirect costs of dinitrogen fixation in *Crocosphaera watsonii* WH8501 and possible implications for the nitrogen cycle. *Front Microbiol* **3**: 236.

Großkopf T, Mohr W, Baustian T, Schunck H, Gill D, Kuypers MMM *et al.* (2012). Doubling of marine dinitrogen-fixation rates based on direct measurements. *Nature* **488**: 361–364.

Hardy RWF, Holsten RD, Jackson EK, Burns RC. (1968). Acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. *Plant Physiol* **43**: 1185–1207.

Huang TC, Chow TJ, Hwang IS. (1988). The cyclic synthesis of the nitrogenase of *Synechococcus* RF-1 and its control at the transcription level. *FEMS Microbiol Lett* **50**: 127–130.

Huisman J. (1999). Population dynamics of light-limited phytoplankton: microcosm experiments. *Ecology* **80**: 202–210.

Langlois RJ, Hümmer D, LaRoche J. (2008). Abundances and distributions of the dominant nifH phylotypes in the Northern Atlantic Ocean. *Appl Environ Microbiol* **74**: 1922–1931.

Langlois RJ, LaRoche J, Raab PA. (2005). Diazotrophic diversity and distribution in the tropical and subtropical Atlantic Ocean. *Appl Environ Microbiol* **71**: 7910–7919.

Lugomela C, Lyimo TJ, Bryceson I, Semesi AK, Bergman B. (2002). *Trichodesmium* in coastal waters of Tanzania: diversity, seasonality, nitrogen and carbon fixation. *Hydrobiologia* **477**: 1–13.

Maryan PS, Eady RR, Chaplin AE, Gallon JR. (1986). Nitrogen fixation by *Gloeothece* sp. PCC 6909: respiration and not photosynthesis supports nitrogenase activity in the light. *J Gen Microbiol* **132**: 789–796.

Mazard SL, Fuller NJ, Orcutt KM, Bridle O, Scanlan DJ. (2004). PCR analysis of the distribution of unicellular cyanobacterial diazotrophs in the Arabian Sea. *Appl Environ Microbiol* **70**: 7355–7364.

Mills MM, Ridame C, Davey M, LaRoche J, Geider RJ. (2004). Iron and phosphorus co-limit nitrogen fixation in the eastern tropical North Atlantic. *Nature* **429**: 292–294.

Mitsui A, Cao S. (1988). Isolation and culture of marine nitrogen-fixing unicellular cyanobacteria *Synechococcus*. In: Packer L, Glazer AN (eds) *Methods in Enzymology, Cyanobacteria* vol 167. Academic Press: San Diego, pp 105–113.

Moisander PH, Beinart RA, Hewson I, White AE, Johnson KS, Carlson CA *et al.* (2010). Unicellular cyanobacterial distributions broaden the oceanic N_2 fixation domain. *Science* **327**: 1512–1514.

Monod J. (1950). La technique de culture continue, théorie et applications. *Annales de l'Institut Pasteur (Paris)* **79**: 390–410.

Monteiro FM, Dutkiewicz S, Follows MJ. (2011). Biogeographical controls on the marine nitrogen fixers. *Global Biogeochem Cycles* **25**: GB2003.

Mullineaux PM, Gallon JR, Chaplin AE. (1981). Nitrogen fixation in cultures of the cyanobacterium *Gloeocapsa* (*Gloeothece*) sp. 1430/3 incubated in the dark. *J Gen Microbiol* **124**: 141–146.

Nakajima M, Imai K, Ito H, Nishiwaki T, Murayama Y, Iwasaki O *et al.* (2005). Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. *Science* **308**: 414–415.

Needoba JA, Foster RA, Sakamoto C, Zehr JP. (2007). Nitrogen fixation by unicellular diazotrophic cyanobacteria in the temperate oligotrophic North Pacific Ocean. *Limnol Oceanogr* **52**: 1317–1327.

Peschek GA, Villgrater K, Wastyn M. (1991). 'Respiratory protection' of the nitrogenase in dinitrogen-fixing cyanobacteria. *Plant Soil* **137**: 17–24.

Sañudo-Wilhelmy SA, Kustka AB, Gobler CJ, Hutchins DA, Yang M, Lwiza K *et al.* (2001). Phosphorus limitation of nitrogen fixation by *Trichodesmium* in the central Atlantic Ocean. *Nature* **411**: 66–69.

Schneegurt MA, Sherman DM, Nayar S, Sherman LA. (1994a). Oscillating behavior of carbohydrate granule formation and dinitrogen fixation in the cyanobacterium *Cyanothece* sp. strain ATCC 51142. *J Bacteriol* **176**: 1586–1597.

Schneegurt MA, Tucker DL, Ondr JK, Sherman DM, Sherman LA. (1994b). Metabolic rhythms of a diazotrophic cyanobacterium, *Cyanothece* sp. strain ATCC 51142, heterotrophically grown in continuous dark. *J Phycol* **36**: 107–117.

Sonntag S, Hense I. (2011). Phytoplankton behavior affects ocean mixed layer dynamics through biological-physical feedback mechanisms. *Geophys Res Lett* **38**: L15610.

Staal M, Meysman FJR, Stal LJ. (2003). Temperature excludes N_2-fixing heterocystous cyanobacteria in the tropical ocean. *Nature* **425**: 504–507.

Staal M, te Lintel Hekkert S, Brummer GJ, Veldhuis M, Sikkens C, Persijn S *et al.* (2007). Nitrogen fixation along a north-south transect in the eastern Atlantic Ocean. *Limnol Oceanogr* **52**: 1305–1316.

Stal LJ. (1988). Nitrogen fixation in cyanobacterial mats. In: Packer L, Glazer AN (eds) *Methods in Enzymology, Cyanobacteria* vol 167. Academic Press: San Diego, pp 474–484.

Stal LJ. (2009). Is the distribution of nitrogen-fixing cyanobacteria in the oceans related to temperature? *Environ Microbiol* **11**: 1632–1645.

Toepel J, McDermott JE, Summerfield TC, Sherman LA. (2009). Transcriptional analysis of the unicellular, diazotrophic cyanobacterium *Cyanothece* sp. ATCC 51142 grown under short day/night cycles. *J Phycol* **45**: 610–620.

Toepel J, Welsh E, Summerfield TC, Pakrasi HB, Sherman LA. (2008). Differential transcriptional analysis of the cyanobacterium *Cyanothece* sp. strain ATCC 51142 during light-dark and continuous-light growth. *J Bacteriol* **190**: 3904–3913.

Tomita J, Nakajima M, Kondo T, Iwasaki H. (2005). No transcription-translation feedback in circadian rhythm of KaiC phosphorylation. *Science* **307**: 251–254.

Vasseur DA, McCann KS. (2005). A mechanistic approach for modeling temperature-dependent consumer-resource dynamics. *Am Nat* **166**: 184–198.

Wastyn M, Achaty A, Molitor V, Peschek GA. (1988). Respiratory activities and aa_3-type cytochrome oxidase in plasma and thylakoid membranes from vegetative cells and heterocysts of the cyanobacterium *Anabaena* ATCC 29413. *Biochim Biophys Acta* **935**: 217–224.

Webb EA, Ehrenreich IM, Brown SL, Valois FW, Waterbury JB. (2009). Phenotypic and genotypic characterization of multiple strains of the diazotrophic cyanobacterium, *Crocosphaera watsonii*, isolated from the open ocean. *Environ Microbiol* **11**: 338–348.

Winship LJ, Tjepkema JD. (1985). Nitrogen fixation and respiration by root nodules of *Alnus rubra* Bong.: effects of temperature and oxygen concentration. *Plant Soil* **87**: 91–107.

Zehr JP. (2011). Nitrogen fixation by marine cyanobacteria. *Trends Microbiol* **19**: 162–173.