Diel nitrogen fixation pattern of *Trichodesmium*: the interactive control of light and Ni

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*Trichodesmium*, a nonheterocystous cyanobacterium widely abundant in the surface water of the tropical and subtropical ocean, fixes dinitrogen under high light conditions while concurrently undergoing photosynthesis. The new production considerably influences the cycling of nitrogen and carbon in the ocean. Here, we investigated how light intensity and nickel (Ni) availability interplay to control daily rates and diel patterns of N₂ fixation in *Trichodesmium*. We found that increasing Ni concentration increased N₂ fixation rates by up to 30-fold in the high light treatment. Cultures subjected to high Ni and light levels fixed nitrogen throughout most of the 24 H light:dark regime with the highest rate coinciding with the end of the 12 H light period. Our study demonstrates the importance of Ni on nitrogen fixation rates for *Trichodesmium* under high light conditions.

Nitrogen fixation is a critical process to provide new bioavailable nitrogen to phytoplankton in the ocean, where nitrogen is considered to be the most important limiting nutrient for phytoplankton growth in the ocean. *Trichodesmium*, a filamentous cyanobacterium, is a particularly important oceanic diazotroph due to its substantial contribution of fixed nitrogen and new production to the tropical and subtropical ocean. Previous laboratory and field studies indicate that the nitrogen fixing ability of *Trichodesmium* may be controlled by the supply of Fe and P in the surface waters. Our recent laboratory studies show that Ni availability also exhibits a crucial role in the nitrogen fixing process of *Trichodesmium*, most likely due to the presence of Ni in Ni-superoxide dismutase, which is needed to protect nitrogenase from reactive oxygen species (ROS) generated through photosynthesis. These two studies indicate that the sufficient supply of Ni is essential to sustain the growth of *Trichodesmium*, particularly under high light conditions. The light intensities in the surface water of the tropical and subtropical ocean, which *Trichodesmium* inhabits, can reach ∼2000 μEM⁻²S⁻¹ during high noon in summer. In addition, both laboratory and field studies have shown that the growth and nitrogen fixation rates of *Trichodesmium* are positively correlated with light intensity. *Trichodesmium*, a nonheterocystous cyanobacterium, carries out oxygen-producing photosynthesis and nitrogen fixation simultaneously during the light period. This photosynthetic O₂ production is problematic as nitrogenase is known to be irreversibly inactivated by oxygen and other ROS. The mechanisms that *Trichodesmium* possesses to simultaneously carry out nitrogen fixation and photosynthesis in a strong light environment remain unclear.

Results

We carried out two culture experiments to investigate the coupled effect of light intensity and Ni availability on nitrogen fixation rates and cellular growth rates (see details in Methods section). The effect of light intensity and the concentration of biologically available dissolved inorganic Ni species (referred to here as Ni⁺) on nitrogen fixation rates were determined over a 6H light period and a 24H light/dark cycle. We observed that the cellular growth rate in the high light and Ni-sufficient treatment was slightly higher than rates observed in the lower light treatments (Fig. 1). The growth rates obtained in cultures subjected to 250 μEM⁻²S⁻¹ were 0.33 and 0.35 day⁻¹ for the treatments with 13 and 67 pM Ni⁺, respectively. The growth rates in cultures subjected to a light intensity of 600 μEM⁻²S⁻¹ were higher, 0.40 and 0.48 day⁻¹ respectively, for the low and high-Ni treatments. These growth rates were determined prior to collection of cells for trace metal quota determination and while the cells were in the exponential growth phase. The influence of light intensity on growth rates was consistent with previous laboratory and field observations that high light enhances *Trichodesmium* growth. The 6H acetylene reduction assay shows that at the lower light intensity, 250 μEM⁻²S⁻¹, the nitrogen fixation rates were 2 to 6 times higher in cultures with relatively high Ni availability (Ni⁺ = 67 pM) than the cultures with relatively low Ni availability (Ni⁺...
At the relatively high light intensity, 600 μE m⁻² s⁻¹, the nitrogen fixation rates for the high Ni cultures were 6 to 10 times higher than in the low Ni cultures (Fig. 2). The observed increase in nitrogen fixation rates by *Trichodesmium* with increasing Ni concentration indicates that the capacity to fix nitrogen is limited by Ni availability, especially at high light intensities.

The first experiment covered only 6H during the middle of the photoperiod and was initiated 2 hours after the light period commenced. We then carried out a second experiment employing similar conditions as the 6H experiment to determine nitrogen fixation rates for an entire 24H light:dark diurnal cycle. In agreement with the results observed from the 6H experiment, we found that the Ni sufficient cultures (Ni⁺ = 67 pM) achieved much higher rates, as much as 30 times higher, than the low-Ni cultures (Ni⁺ = 13 pM) at the high light intensity (Fig. 3). The effect of Ni was less striking at the relatively low light intensity used (Fig. 3). The results of the 24H experiment further validate the hypothesis that Ni availability and light intensity interact to influence the nitrogen fixation in *Trichodesmium*.

More importantly, the 24H experiment revealed a new diel pattern of nitrogen fixation, exhibiting the maximum rate near or at the end of the light phase and with the nitrogen fixation process sustained during the dark phase for the high Ni-high light treatment (Fig. 3). This unique temporal profile is in contrast to what was observed for cultures at the lower light intensity (250 μE m⁻² s⁻¹), where the window of nitrogen fixation only occurred for a short period near or at the end of the light phase.
The cellular metal quotas of Fe and Ni were shown by normalizing their total intracellular concentrations to phosphorus as a biomass proxy (Fig. 4). The cellular Fe:P ratios increased by a factor of 1.5 with the increase in light intensity. These cellular iron quotas may be associated with the biochemical iron requirements for nitrogen fixation through the associated Fe containing enzymes, such as nitrogenase and various Fe enzymes of the photosynthetic apparatus. In addition, the higher Fe:P ratio is also likely attributed to the higher iron availability to the Trichodesmium under higher light intensity, which is linked to photo redox cycling of Fe(III)-EDTA chelates. Because the cycling rates are proportional to light intensity, the higher light intensity would result in higher cellular iron uptake rates by increasing steady-state concentrations of dissolved inorganic ferrous and ferric iron species. The cell Ni:P ratios in the high-Ni cultures were about 2-fold higher than those in the low-Ni cultures. However, the Ni quotas of the cultures at high light intensity (600 \(\mu\)E m\(^{-2}\) s\(^{-1}\)) were comparable or slightly lower than those in the low light cultures. We had expected that the increase in light intensities should lead to elevated levels of ROS, which should have entailed the use of even more Ni-SOD and elevated uptake for Ni. The cellular metal concentration is equal to the uptake rate divided by the specific growth rate, thus when the growth rates decline, the cellular metal levels will increase provided that the metal uptake rate remains constant. We found that the specific growth rates of the high Ni treatments were 0.40 and 0.48 d\(^{-1}\) for the growth period prior to the measurement of Ni quota under the low and high light conditions, respectively. If the Ni uptake rate remained constant, that difference in the growth rates would result in a 20% increase in the cellular Ni quota, explaining most of the observed increase in the quota.

Our study demonstrates the importance of Ni on nitrogen fixation rates for Trichodesmium under high light conditions. A 12-fold increase was observed for cultures with the high light-high Ni treatment compared to the cultures with low Ni treatment for the 24H period. Aside from the marked increase in total nitrogen fixed, the increase in both Ni and light may extend the nitrogen fixation process beyond the photoperiod. The effect of Ni is related to the role of Ni-SOD in reducing oxidative stress by removing superoxide radicals, and in Ni-Fe uptake hydrogenases, which increases the efficiency of \(N_2\) fixation by utilizing the \(H_2\) released as a by-product of \(N_2\) fixation. The extent to which Ni regulates \(N_2\)- and C-fixation in
Trichodesmium in the ocean needs to be investigated to further understand how Ni influences the occurrence and distribution of this diazotroph in the ocean. Further field enrichment experiments should be carried out to test whether Ni may be a limiting factor in the tropical and subtropical oceans, particularly under high light conditions. It would also be interesting to learn whether the 2 nM dissolved Ni generally observed in the surface water of the subtropical and tropical oceans are bioavailable to Trichodesmium, and whether the nitrogen fixation rates and the abundance of Trichodesmium are regulated by bioavailable Ni concentrations. The findings of these studies may be important for understanding the distributions and activities of Trichodesmium and the environmental controls on its nitrogen fixation in modern and ancient oceans.

**Methods**

**Trichodesmium cultures.** Materials used for culturing were carefully washed with Micro-90 solution, rinsed, soaked with 10% hydrochloric acid solution, and rinsed thoroughly with superpure Milli-Q water. All necessary procedures including the medium preparation, culturing, and harvesting of cells for trace metal quota determination were carried out in a class 100 trace-metal clean laboratory. The nonaxenic cultures of *Trichodesmium erythraeum* (obtained from the National Center for Marine Alga and Microbiota) were grown in 1 L trace metal clean polycarbonate bottles (Nalgene, USA) with a trace metal-defined medium modified from the original recipe. The medium was passed through Chelex-100 resin prior to the addition of trace metals. The dissolved total concentrations of the trace metals were at the following values: Fe, Mo, Mn, Zn, Co, Se, and Cu at 400, 100, 10, 10, 10, and 10 nM, respectively. The availability of the trace metals added was controlled by adding 20 μM of ethylenediaminetetraacetic acid (EDTA). The different Ni treatments were achieved by adding total Ni concentrations of 20 and 100 nM, which resulted to 13 pM and 67 pM respectively of inorganic Ni (Ni(I)) in the culture media. The total initial concentration of P was 50 μM and the B-vitamins were added at the suggested levels. These culture conditions were designed such that P and Fe levels are sufficient in the culture medium. The cultures were kept in a temperature-controlled growth chamber fixed at 26 °C with different light treatments at 250 and 600 μmol quanta m⁻² s⁻¹. Photon irradiances were achieved by placing the culture bottles in appropriate distances from the light source, and were verified by measuring the light penetration PAR into a seawater-filled polycarbonate bottle using a submersible radiometer (Biophotrical Instruments Inc. QSL 2100). The growth chamber was kept at a 12:12 H:light:dark cycle. All sets of treatments were carried out in triplicates.

**Growth rates and intracellular metal quota.** The growth of the cultures was monitored by measuring the total cellular volume using a Beckman Coulter Counter Multisizer 3 until decline in the biomass was observed for all culture bottles. As explained elsewhere, the use of the Coulter counter provides a precise and reliable way of monitoring growth rates. The growth rates were determined on different days, between days 5 to 13, during the log-linear phase of the growth curve. Determination of intracellular quotas was done by harvesting *Trichodesmium* cells while on the exponential phase of the growth curve (indicated in Fig. 1). The cells were filtered onto acid washed polycarbonate filters (25 mm with 5 μm pore size), washed with ultrapure Milli-Q water and subsequently decomposed before analysis. The elemental composition was determined using HR-ICPMS (Element XR, Thermo Scientific).

**Nitrogen fixation rates.** The nitrogen fixation rates were estimated using the acetylene reduction method following the steps outlined elsewhere. Two sets of experiments were conducted to elucidate the effect of Ni availability and light intensity on the N₂ fixation rates. The first experiment was for a short incubation period lasting for 6H and was intended to study the rates while the second was designed to cover a 24H period to study the diel pattern of N₂ fixation. In brief, 10 ml aliquots of the cultures (duplicate samples were prepared from each of the triplicate bottles per treatment for each time point) were transferred to 20 ml vials (Agilent). The vials were sealed using Teflon-coated caps and 2 ml air was drawn using a syringe. The air in the sealed vials was replaced by 2 ml of freshly-prepared acetylene to initiate the experiment. The vials were then incubated at the same growth conditions for 2 to 6 hours in the 6H experiment and 2 to 24 hours for the 24H experiment. The time-point experiment was designed so that nitrogen fixation was stopped after every 2 hours during the light phase (for both experiments) and after every 3 hours during the dark phase (for the 24H experiment). The nitrogen fixation experiment was started to coincide with the start of the light phase, which commenced at 9AM, of the light-dark cycle. After incubation for the desired period, 2 ml of headspace was drawn and the gaseous sample was subsequently analyzed for acetylene using an Agilent 7890A gas chromatograph equipped with a Poropak N column (Agilent) and a flame ionization detector. Estimation of the dinitrogen reduction was taken from the acetylene reduction using a conversion ratio of 4:1, and the assumption that the Bunsen coefficient for ethylene is 0.084. Nitrogen fixation experiments were conducted while cells were at the exponential growth stage. The rate at a specific time point was calculated by subtracting the accumulated C₂H₂ at the preceding point divided by the duration.

1. Capone, D. G., Zehr, J. P., Paerl, H. W., Bergman, B. & Carpenter, E. J. *Trichodesmium*, a globally significant marine cyanobacterium. *Science* 276, 1221–1229 (1997).
2. Karl, D. et al. The role of nitrogen fixation in biogeochemical cycling in the subtropical North Pacific Ocean. *Nature* 388, 533–538 (1997).
3. Zehr, J. P. et al. Unicellular cyanobacteria fix N₂ in the tropical North Pacific Ocean. *Nature* 412, 635–638 (2001).
4. Carpenter, E. J., Subramaniam, A. & Capone, D. G. Biomass and primary productivity of the cyanobacterium, *Trichodesmium*, in the southwestern tropical North Atlantic Ocean. *Deep-Sea Res. 51*, 173–203 (2004).
5. Capone, D. G. et al. Nitrogen fixation by *Trichodesmium* spp.: An important source of new nitrogen to the tropical and subtropical North Atlantic Ocean. *Global Biogeochem. Cycle.* 19, GB0204; DOI:10.1029/2004GB002331 (2005).
6. Berman-Frank, I., Cullen, J. T., Shaked, Y., Sherrell, R. M. & Falkowski, P. G. Iron availability, cellular iron quotas, and nitrogen fixation in *Trichodesmium*. *Limnol. Oceanogr.* 46, 1249–1260 (2001).
7. Sainudo-Wilhelmy, S. A. et al. Phosphorus limitation of nitrogen fixation by *Trichodesmium* in the central Atlantic Ocean. *Nature* 411, 68–69 (2001).
8. Mills, M. M., Ridame, C., Davey, M., La Roche, J. & Geider, R. J. Iron and phosphorus co-limit nitrogen fixation in the eastern tropical North Atlantic. *Nature* **429**, 292–294 (2004).

9. Ho, T.-Y. Nickel limitation of nitrogen fixation in *Trichodesmium*. *Limnol. Oceanogr.* **58**, 112–120 (2013).

10. Ho, T.-Y., Chu, T. H. & Hu, C. L. Interrelated influence of light and Ni on *Trichodesmium* growth. *Front. Microbiol.* **4**, 139; DOI:10.3389/fmicb.2013.00139 (2013).

11. Carpenter, E. J. et al. The tropical diatomophytic phytoplankter *Trichodesmium*: biological characteristics of two common species. *Mar. Ecol. Prog. Ser.* **95**, 295–304 (1993).

12. Breithbarth, E., Wohlers, J., Kläs, J., LaRoche, J. & Peeken, I. Nitrogen fixation and growth rates of *Trichodesmium* IMS-101 as a function of light intensity. *Mar. Ecol.-Prog. Ser.* **359**, 25–36 (2008).

13. Gallon, J. R. The oxygen sensitivity of nitrogenase: A problem for biochemists and microorganisms. *Trends Biochem. Sci.* **6**, 19–23; DOI:10.1016/0968-0004(81)90008-6 (1981).

14. Latifi, A., Ruiz, M. & Zhang, C. C. Oxidative stress in cyanobacteria. *FEMS Microbiol. Rev.* **33**, 258–278 (2009).

15. Berman-Frank, I. et al. Segregation of nitrogen fixation and oxygenic photosynthesis in the marine cyanobacterium *Trichodesmium*. *Science* **294**, 1534–1537 (2001).

16. Saino, T. & Hattori, A. Diel variation in nitrogen fixation by a marine blue-green alga, *Trichodesmium thiebautii*. *Deep-Sea Res.* **25**, 1259–1263 (1978).

17. Ōhki, K., Zehr, J. P. & Fujita, Y. Regulation of nitrogenase activity in relation to the light-dark regime in the filamentous non-heterocystous cyanobacterium *Trichodesmium* sp. NIBB 1067. *J. Gen. Microbiol.* **138**, 2679–2685 (1992).

18. Tuit, C., Waterbury, J. L. & Ravizza, G. Diel variation of molybdienum and iron in marine diatomophytic cyanobacteria. *Limnol. Oceanogr.* **49**, 978–990 (2004).

19. Chen, Y. R., Zehr, J. P. & Mellon, M. Growth and nitrogen fixation of the diatomophytic filamentous nonheterocystous cyanobacterium *Trichodesmium* sp. IMS-101 in defined media: evidence for a circadian rhythm. *J. Phycol.* **32**, 916–923 (1996).

20. Tamagnini, P. et al. Cyanobacterial hydrogenases: diversity, regulation and applications. *FEMS Microbiol. Rev.* **31**, 692–720 (2007).

21. Wilson, S. T., Foster, R. A., Zehr, J. P. & Karl, D. M. Hydrogen production by *Trichodesmium erythraeum*, *Cyanobaethus* sp. and *Crocosphaera watsonii*. *Aquat. Microb. Ecol.* **59**, 197–206 (2010).

22. Dupont, C. L., Barbeau, K. & Palenik, B. B. Ni uptake and limitation in marine Synechococcus strains. *Appl. Environ. Micro.* **74**, 23–31 (2008).

23. Whittaker, S., Bille, K. D., Kustka, A. B. & Falkowski, P. G. Quantification of nitrogenase in *Trichodesmium* IMS101: implications for iron limitation of nitrogen fixation in the ocean. *Environ. Microbiol. Rep.* **3**, 54–58 (2011).

24. Kustka, A. B., Sanudo-Wilhelmy, S., Carpenter, E. J., Capone, D. G. & Raven, J. A. A revised estimate of the iron use efficiency of nitrogen fixation, with special reference to the marine cyanobacterium *Trichodesmium spp.* (Cyanophyta) *J. Phycol.* **39**, 12–25 (2003).

25. Anderson, M. A. & Morel, F. M. M. The influence of aqueous iron chemistry on the uptake of iron by the coastal diatom *Thalassiosira weissflogii*. *Limnol. Oceanogr.* **27**, 789–813 (1982).

26. Sunda, W. G. & Huntsman, S. A. Effect of pH, light, and temperature on Fe-EDTA chelation and Fe hydrolysis in seawater. *Mar. Chem.* **84**, 35–47 (2003).

27. Sunda, W. G. & Huntsman, S. A. Interactive effects of light and temperature on iron limitation in a marine diatom: Implications for marine productivity and carbon cycling. *Limnol. Oceanogr.* **56**, 1475–1488 (2011).

28. Sunda, W. G. Feedback interaction between trace metal nutrients and phytoplankton in the ocean. *Front. Microbiol.* **3**, 204; DOI:10.3389/fmicb.2012.00204 (2012).

29. Goebel, N. L., Edwards, C. A., Carter, B. J., Achilles, K. M. & Zehr, J. P. Growth and carbon content of three different-sized diatomophytic cyanobacteria observed in the subtropical North Pacific. *J. Phycol.* **44**, 1212–1220 (2008).

30. Capone, D. G. & Montoya, J. P. [Nitrogen fixation and denitrification] *Method in Microbiology, Marine microbiology* [Paul, J. (ed.)] [501–515] (Kluwer Academic, Netherlands, 2001).

31. Breitbarth, E., Mills, M. M., Friedrichs, G. & LaRoche, J. The Bunsen gas solubility coefficient of ethylene as a function of temperature and salinity and its importance for nitrogen fixation assays. *Limnol. Oceanogr. Methods* **2**, 282–288 (2004).

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Author contributions

T.Y.H. conceptualized the study; I.B.R. and T.Y.H. planned and conducted the experiment; I.B.R. and T.Y.H. analyzed the data and wrote the paper.

Additional information

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