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Nickel limitation of nitrogen fixation in *Trichodesmium*

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Abstract

I show that the growth of *Trichodesmium*, the primary diazotrophic phytoplankton in tropical and subtropical oceans, can be limited by Ni availability in both trace metal–defined culture media and natural seawater when the supply of Fe and P is sufficient. I further show that the increase of Ni concentrations elevates cellular superoxide dismutase (SOD) activities and nitrogen fixation rates, suggesting that NiSOD may be involved in the protection of nitrogenase from superoxide inhibition during photosynthesis in this nonheterocystous diazotroph.

Trichodesmium accounts for half of new production in the tropical and subtropical oceans and may play a significant role in material cycling globally (Capone et al. 1997; Karl et al. 1997; Davis and McGillicuddy 2006). Although studies have shown that the growth of *Trichodesmium* might be limited by P and Fe (Berman-Frank et al. 2001*a*; Sañudo-Wilhelmy et al. 2001; Mills et al. 2004), the environmental factors that regulate the abundance and activity of *Trichodesmium* are still not fully understood. Genome studies revealed that *Trichodesmium* contains *sodN* and *sodA* (Palenik et al. 2003; Dupont et al. 2008), genes encoding Ni- and Mn-containing superoxide dismutases (SODs), respectively. However, Ni is generally not included as an essential trace metal in marine phytoplankton culture media (Price et al. 1989; Ho et al. 2003) unless urea is used as a nitrogen source (Price and Morel 1991). For *Trichodesmium*, Ni is not included in the culture medium recipe of the YBCII medium, as suggested by the US National Center for Marine Algae and Microbiota (NCMA), although Ni is used in YBCIII medium (Chen et al. 1996). Whether Ni is an essential trace metal for the growth of *Trichodesmium* remains unknown.

Trichodesmium, a nonheterocystous cyanobacterium, can carry out oxygen-producing photosynthesis and nitrogen fixation simultaneously. Nitrogenase is known to be irreversibly inactivated by reactive oxygen species (ROS) (Gallon 1981). It remains uncertain how nitrogenase survives during the photosynthetic period in *Trichodesmium*. The study of Berman-Frank et al. (2001*b*) suggested that temporal and spatial segregation of nitrogen fixation and photosynthesis within the photoperiod could be important for the protection. Additionally, the cellular antioxidant enzymes, SOD, might play an important role on protecting nitrogenase from ROS inhibition during photosynthetic periods (Gallon 1981; Puppo and Rigaud 1986). Oxygen produced through photosystem II in chloroplasts is photo-reduced to generate superoxide radicals, which is then disproportionated by SOD to peroxides and oxygen (Asada 1999; Latifi et al. 2009). Catalases, Fe metalloenzymes, then detoxify peroxide to water and oxygen (Asada 1999; Latifi et al. 2009). Previous studies showed that SOD levels in both heterocystous and nonheterocystous diazotrophs were relatively high when they carried out nitrogen fixation, and SOD activities were relatively low when ammonium was utilized as a nitrogen source, also suggesting that SOD was involved in protecting the overall process of dinitrogen fixation from the inhibition of ROS (Puppo and Rigaud 1986). An early study observed Ni-dependent growth for an *Oscillatoria* sp. isolated from coastal mud samples (Baalen and O'Donnell 1978). Whether NiSOD plays essential roles in nitrogen fixation of *Trichodesmium* deserves investigation. Here, I hypothesize that Ni can be essential for the growth of *Trichodesmium* by influencing NiSOD expression and nitrogen fixation.

In this study, I measured the background Ni concentration in the YBCII medium commonly used to cultivate *Trichodesmium* and then prepared a trace metal–defined culture medium to investigate the importance of Ni availability to *Trichodesmium* growth. *Trichodesmium* was grown in trace metal–defined artificial culture media and natural seawater, in which the concentrations of bioavailable nitrogen were below the detection limit, 20 nmol L^{-1}. Finally, the cellular SOD activities and nitrogen fixation rates in *Trichodesmium* were determined with the diazotrophs growing in the culture media mediated with various Ni concentrations.

Methods

Nonaxenic *Trichodesmium erythraeum* IMS101 was purchased from the NCMA. Cultures of *Trichodesmium* were grown in trace metal–clean polycarbonate bottles in a temperature-controlled growth chamber at 26°C with an illumination of 90 μmol quanta m^{-2} s^{-1} under a 12 : 12 h light : dark cycle. An artificial culture medium was prepared using the recipe for the YBCII culture medium as suggested by NCMA (Chen et al. 1996). The strain was first maintained in the unchelexed YBCII medium for about 30 generations and was then transferred to trace metal–defined YBCII media. To prepare a trace metal–defined culture medium, we remove trace metal impurities from the salt solution of the YBCII medium by passing the artificial seawater through chelating resins in a clean room. Medium sterilization, medium preparation, and trace metal control and manipulation for the trace metal–defined YBCII media

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Table 1. The concentrations (nmol L^{-1}) of dissolved trace metals in unchelated YBCII medium, chelated YBCII medium (before adding trace metals), and the surface seawater collected in the SCS. It should be noted that the background concentrations of trace metals in the unchelexed artificial culture medium can vary from time to time and from laboratory to laboratory because the culture medium prepared in different laboratories by different people with the use of different brands of chemicals would cause different levels of contamination through the preparation procedures.

	Concentration (n = 3)						
Culture medium	Ni	Fe	Mn	Zn	Cu	Co	Cd
Unchelated YBC II	130±4	8.9±0.3	7.0±0.6	13±1	3.1±0.2	0.11±0.004	0.80±0.04
Chelated YBC II	0.04±0.01	0.15±0.01	0.02±0.02	0.15±0.3	0.10±0.2	0.005±0.001	0.010±0.001
SCS surface water	2.0±0.01	0.14±0.06	3.0±0.05	0.74±0.3	1.3±0.06	0.027±0.005	0.10±0.005

generally follows the procedures for preparing trace metal–defined culture media (Price et al. 1989; Ho et al. 2003).

Previous studies showed that cell volume was closely correlated with carbon content in various marine phytoplankton (Thompson et al. 1991; Verity et al. 1992; Montagnes et al. 1994), indicating that cell volume is a reliable parameter for the estimate of biomass or growth rates in phytoplankton, including *Trichodesmium* (Tuit et al. 2004). Because the *Trichodesmium* strain used in this study existed in a single trichome state during the growth period, the total cell volume of *Trichodesmium* samples can be determined by a particle counter. I made an extra effort in this study to evaluate the accuracy of total cell volume obtained by Coulter Counter (Beckman Coulter, Multisizer 3). The total cellular volume of the same *Trichodesmium* culture samples were repeatedly determined simultaneously by Coulter Counter and a microscope (Carl Zeiss, Axio Imager A2) with hemocytometer (Marienfeld, Neubaueer). Using the microscope and hemocytometer, the lengths and widths of all trichomes per unit sample volume were measured, and the total cell volume was estimated by multiplying the total length of trichomes with the cross-sectional area. Overall, the precision of the replicate analysis of the total cell volume obtained by the Coulter Counter was much better than the microscope – 1–5% and 30–60%, respectively. The total cellular volume obtained by the particle counter was comparable or slightly lower than the value obtained by the microscope with hemocytometer, indicating that the particle counter can be a reliable method of estimating the total cell volume of *Trichodesmium*.

We observed by microscope that individual cell volume varied significantly from cell to cell in each trichome. However, the variations of the averaged cell volume of the *Trichodesmium* grown in various Ni treatments were analytically insignificant because the individual variations were mostly canceled out by dividing total cell volume by total cell numbers. Additionally, we observed consistent trichome size during growth periods in different Ni treatments, suggesting that average cell size was comparable during the growth period in this study. Thus, the increase in total cellular volume can be a good estimate of the increase in total cell numbers or the increase in growth rates. Total cell numbers can be estimated by using the conversion factor of average cell volume per cell, which mostly ranged from 200 to 250 μm^3 per cell in this study. We also observed that chlorophyll *a* (Chl *a*) concentrations by fluorometric analysis (Turner Designs) exhibited strong linear correlations with total cell volume measured, with correlation coefficients (r^2) up to 0.99 ($p < 0.01$) during the exponential phase. The conversion factor of cell volume to Chl *a* was about 0.10 ng chlorophyll per 10^6 μm^3 of cell volume.

To determine intracellular trace metal quotas accurately, we modified the concentrations of Fe and ethylenediaminetetraacetic acid (EDTA) in the original YBCII culture medium (Chen et al. 1996). It is likely that high levels of Fe added to the culture medium would be precipitated and adsorbed on the algal surface (Ho et al. 2003). We increased the EDTA concentration from 2 to 20 μmol L^{-1} and decreased the total Fe concentration from 400 to 100 nmol L^{-1} in YBCII medium so that the inorganic Fe concentration was about 500 pmol L^{-1} (Ho et al. 2003). Unless otherwise noted, *Trichodesmium* was grown under the modified trace metal–defined medium. Initial phosphate concentration in the medium was 50 μmol L^{-1}; bioavailable nitrogen was not added, and the background concentrations of nitrate and nitrite were below the detection limit in the culture medium. Other trace metal concentrations in the medium were 20, 4, 1, 2.5, and 11 nmol L^{-1} for Mn, Zn, Cu, Co, and Mo, respectively. Cells were grown twice for at least 10 generations in the trace metal–defined culture medium with a 50 nmol L^{-1} Ni concentration and then transferred to new media enriched with total dissolved Ni concentrations varying from 0 to 400 nmol L^{-1}.

Trace metal concentrations in natural seawater and culture media were measured by an automated pretreatment method with a high-resolution inductively coupled plasma spectrometer (HR-ICPMS) (Ho et al. 2010). The natural seawater used in this study was collected at an offshore basin site in the northern South China Sea (SCS) by using trace metal–clean techniques and using Teflon-coated Go Flo bottles (General Oceanics) mounted on a Teflon-coated rosette equipped with Kevlar wire. The total dissolved Fe and Ni concentrations in the natural water were 0.14 and 2.0 nmol L^{-1}, respectively (Table 1).

To determine intracellular trace metal quotas, cultured cells were harvested onto acid-washed 25 mm polycarbonate filters with a 10 μm pore size in a class 100 trace metal–clean laboratory during the light portion of the light–dark cycle. The filtered cells were rinsed with trace metal–clean seawater, the chelexed seawater, to remove the culture medium residue. The trace metal–clean seawater is the seawater passed through a column filled with chelex 100

resins, in which trace metal concentrations are extremely low (Table 1). Trace metal quotas in the phytoplankton and trace metal concentrations in the seawater were then determined using a HR-ICPMS (Thermo Scientific Element XR) fitted with a desolvation system (Elemental Scientific). The Fe quotas in the cells we have observed were around 10 mmol mol^{-1} P, which was slightly higher than the Fe quotas observed in other marine phytoplankton grown in a low-Fe culture medium (Ho et al. 2003), thus indicating that extracellular Fe adsorption on *Trichodesmium* was insignificant. The details of the analytical precision, accuracy, and detection limits of the ICPMS method for seawater and phytoplankton were described in our previous studies (Ho et al. 2003, 2007, 2010).

Two Ni enrichment experiments in natural seawater were carried out in this study. First, 10 mL of the cultured species *Trichodesmium* IMS 101, maintained in the modified YBCII medium enriched with 50 nmol L^{-1} Ni, was inoculated in 1 liter of natural seawater collected from the surface water of the SCS. The background concentrations of bioavailable nitrogen in the surface water were below detection limits, and the background-dissolved concentrations of phosphate, Ni, and Fe were 20, 2.0, and 0.14 nmol L^{-1}, respectively. The natural seawater was sterilized by filtering the water through 0.2 μm acid-washed polycarbonate filters and was amended with 50 μmol L^{-1} phosphate, 100 nmol L^{-1} Fe, and other trace metals—except Ni.

A preliminary Ni enrichment experiment was also carried out in the field by using natural surface seawater collected in the northern SCS during 02–12 June 2010. The surface seawater was collected at depths of 20–30 m, where nitrate and nitrite concentrations in the seawater were below detection limits. Seawater samples were then filtered through 200 μm acid-washed polycarbonate filters to remove large zooplankton in trace metal–clean laminar flow benches on board. Three 1 liter trace metal–cleaned polycarbonate bottles amended with different concentrations of Ni, Fe, and P were used. No nutrients or trace metals were added to the control sample bottle. The Ni-only bottle was enriched with 10 nmol L^{-1} Ni, and the third bottle was enriched with 10 nmol L^{-1} Ni, 100 nmol L^{-1} Fe, and 5 μmol L^{-1} phosphate. The total incubation time was 13 days, including 9 days on board and 4 days in laboratory. The culture was maintained on board with a light intensity of 30 μmol quanta m^{-2} s^{-1} under a 12 : 12 light : dark cycle at 27°C. After being brought back to the laboratory, the samples were maintained in growth chambers at 90 μmol quanta m^{-2} s^{-1} under a 12 : 12 h light : dark cycle at 26°C. We filtered 500 to 1000 mL of the samples and resuspended the filtered trichomes in 5 mL of original filtered seawater. The total numbers of trichomes in the suspended samples were then counted by using a fluorescence microscope with green excitation (Carl Zeiss, Axio Imager A2).

SOD activities were determined using a cytochrome *c* (Cyt *c*) with xanthine-xanthine oxidase method based on the ability of SOD to inhibit the reduction of Cyt *c* by scavenging superoxide anion radicals produced by the xanthine xanthine oxidase system (Flohe and Otting 1984). One unit of SOD activity is defined as the amount of enzyme that produced a 50% inhibition of Cyt *c* reduction. The calibration curve, with an r^2 of > 0.99 ($p < 0.01$), was first established between the reciprocal of the absorbance of ferrous Cyt *c* and the enzymatic activity by using seven SOD standards. For each bottle, a linear correlation was observed between biomass and SOD activities. The SOD activity of each sample was obtained using the SOD assay for four different sample volumes. I then collected *Trichodesmium* samples through filtration, resuspended the cells in 1 mL of seawater, and determined the cell volume. The cells were broken with sonication under an ice-water slurry. After observing pigment release, I centrifuged the samples and removed the particulate material. Then, 100 μL of cell sample was added into a 3 mL quartz cuvette prefilled with 2.89 mL of phosphate buffer solution, which contained xanthine and Cyt *c*. Then, 10 μL of xanthine oxidase was added to the cuvette for analysis (Flohe and Otting 1984).

To determine nitrogen fixation activity, 20 mL Agilent flat-bottom headspace vials fitted with Teflon-coated silicone septa were used. Both the vials and the septa were cleaned rigorously using Micro-90 (International Products Corporation) cleaning solution and 1 mol L^{-1} superpure HCl (Seastar) to remove trace metal impurities. The 20 mL vials were first soaked in 2% Micro-90 overnight and then cleaned with Milli-Q water. They were then filled with 10% superpure HCl overnight and rinsed thoroughly with Element-grade Milli-Q water immediately before use. We then determined nitrogen fixation rates with the acetylene reduction method (Capone and Montoya 2001). The *Trichodesmium* samples grown under treatments ranging from 10 to 100 nmol L^{-1} Ni were added into each vial with an upper gas volume of 10 mL. Right before the lights went on, 10 mL aliquots of *Trichodesmium* culture were added to the 20 mL vials with sterile, acid-washed tips, which were then sealed using Al caps and acid-washed septa with the Teflon end toward to the samples. The vials were then incubated in the original incubator for 6 to 12 h. Four-point time courses from 6 to 12 h were prepared for each individual treatment. Two milliliters of the 10 mL headspace in the vials was removed and replaced with 2 mL of acetylene generated from carbide (Capone and Montoya 2001). Then, gas-tight plastic needles were used to add 2 mL of 99.9% acetylene to the serum sample vials to reach a 20% final concentration in the upper gas phase. The vials were then gently shaken to mix the seawater containing *Trichodesmium* in the vials. The subsamples were incubated in the original growth chamber for 6–12 h under the same growth irradiance and temperature as the original cultures. We then sampled 1 mL of the headspace gas from the vials and injected it into clean, empty 10 mL serum vials for ethylene analysis. The ethylene production rate was measured using an Agilent 7890A gas chromatograph with a Poropak N column (Agilent, HP-PLOT Al_2O_3 S) and a flame ionization detector. The ethylene concentrations were quantified using the calibration curve obtained from the ethylene standard. Nitrogen fixation rates for *Trichodesmium* in the Ni treatments were estimated by using the acetylene reduction method with a ratio of 4 : 1 for the

conversion of acetylene reduction to dinitrogen reduction (Capone and Montoya 2001). The Bunsen coefficient for C_2H_2 under growth conditions is 0.086 and is negligible. The results were calculated and presented as N_2 pmol h^{-1} $cell^{-1}$ (Capone and Montoya 2001).

Results

Using the traditional unchelexed YBCII medium (Chen et al. 1996), I observed normal growth of *Trichodesmium* without adding Ni or bioavailable nitrogen. I then measured Ni concentrations in the unchelexed medium using the automated flow injection ion chromatography pretreatment method coupled with HR-ICPMS (Ho et al. 2010). The background concentrations of Ni in the unchelexed medium prepared in our laboratory generally ranged from 50 to 150 nmol L^{-1} (Table 1), showing that Ni was present as a contaminant in the medium and that removing trace metal impurities in the medium was required to study trace metal nutrition for *Trichodesmium*. We then prepared trace metal–defined culture medium by passing the YBCII seawater through chelating resins to remove trace metal impurities as described in the Methods section (Table 1). The background Ni concentrations in the chelated seawater was < 0.1 nmol L^{-1}. Using trace metal–defined YBCII culture medium, we observed that the maximum sustainable biomass of *Trichodesmium* was significantly higher in the presence than in the absence of added Ni (Fig. 1). Using the conversion factor of 250 μm^3 per cell, the total maximum sustainable biomass or estimated cell numbers of *Trichodesmium* in the Ni-replete medium was approximately 64,000 cells per mL of seawater (Fig. 1). This value is 10 times greater than for the treatment without Ni. The growth rates were 0.19, 0.30, and 0.31 d^{-1} for 0, 4, and 40 nmol L^{-1} Ni treatments, respectively (Fig. 1A). After adjusting the concentrations of EDTA and Fe in the medium from 2 μmol L^{-1} and 400 nmol L^{-1} to 20 μmol L^{-1} and 100 nmol L^{-1}, a similar Ni limitation result was observed. *Trichodesmium* barely grew in the medium without Ni (Fig. 1B). The growth rates were 0.05, 0.16, and 0.16 d^{-1} for 0, 40, and 400 nmol L^{-1} Ni treatments, respectively (Fig. 1B). We observed that the lowest dissolved Ni concentration in the culture medium required to maintain optimal growth rates and biomass was around 50 nmol L^{-1}. The intracellular Ni quotas ranged from 0.50 to 1.0 mmol mol^{-1} P in *Trichodesmium* growing in the chelated medium with sufficient Ni.

For Ni enrichment experiments in natural seawater, the growth of *T. erythraeum* IMS101 was significantly slower in the natural seawater without the addition of 10 nmol L^{-1} Ni (Fig. 2). Additionally, the results of the preliminary field addition experiment in the South China Sea also suggest that Ni addition could enhance *Trichodesmium* growth in the ocean. We observed 5, 64, and 66 trichomes L^{-1} in the control, Ni-only, and Ni-Fe-P–treated bottles, respectively, at a station (117°51′E; 20°03′N) and observed 22, 46, and 35 trichomes L^{-1} in the control, Ni-only, and Ni-Fe-P–treated bottles, respectively, at the other station (118°30′E; 21°28′N). However, because this field experiment was carried out with the use of a single bottle with long

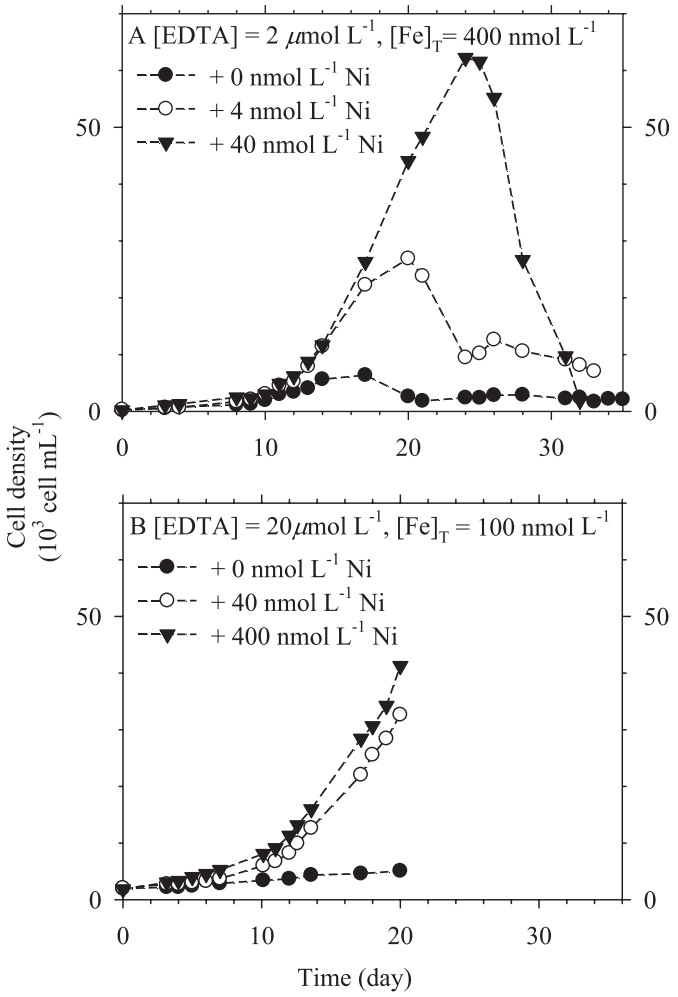

Fig. 1. Comparison of the sustainable cellular biomass or the growth curves of *Trichodesmium* grown in trace metal–defined YBCII media with different Ni concentrations. Cell numbers were estimated by the conversion of total cell volume measured. (A) The total Ni concentrations in the media were 0, 4, and 40 nmol L^{-1}. The concentrations of EDTA and Fe in the media were 2 μmol L^{-1} and 400 nmol L^{-1}, respectively. Initial phosphate concentration in the medium was 50 μmol L^{-1}; bioavailable nitrogen concentration was below detection limit. The growth rates were 0.19, 0.30, and 0.31 d^{-1} for the treatments with the addition of 0, 4, and 40 nmol L^{-1} Ni, respectively. The rates were calculated during the log-linear portion ($r^2 > 0.96$) of the curve between days 8 and 16 for the treatments with Ni added and between days 8 and 14 for the treatment without Ni added. (B) The experiment was carried out with six different Ni concentrations: 0, 40, 80, 100, 200, and 400 nmol L^{-1}. The growth rates of the five treatments with Ni added were all similar. Only the growth curves of the treatments with 0, 40, and 400 nmol L^{-1} Ni added are shown here. Cell counting ended on day 20. The EDTA and Fe concentrations in the media were 20 μmol L^{-1} and 100 nmol L^{-1}, respectively. The growth rates during the exponential growth period were 0.05, 0.17, and 0.16 d^{-1} for 0, 40, and 400 nmol L^{-1} Ni treatments, respectively. The growth rates were calculated during the log-linear portion ($r^2 > 0.99$) of the curve between days 6 and 20.

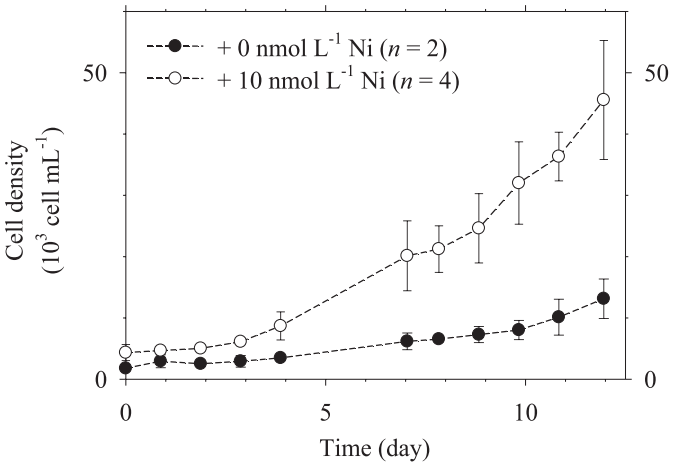

Fig. 2. Comparison of the growth curves and sustainable cellular biomass of *Trichodesmium* grown in natural surface seawater with and without Ni addition. Cell numbers were estimated by the conversion of total cell volume. The control treatments (solid circle, $n = 2$) were not spiked with extra Ni; the enriched treatments (open circle, $n = 4$) were spiked with 10 nmol L^{-1} Ni. The growth rates of *Trichodesmium* were 0.14 and 0.22 d^{-1} for the treatments in which Ni was not added and added, respectively. The errors were propagated with the errors of the growth rates in each culture. The seawater was the surface water of the SCS, in which the original Ni concentrations were 2 nmol L^{-1}. The bioavailable nitrogen concentrations in the seawater were below detection limit, and the seawater was enriched with 50 μmol L^{-1} phosphate and 100 nmol L^{-1} Fe.

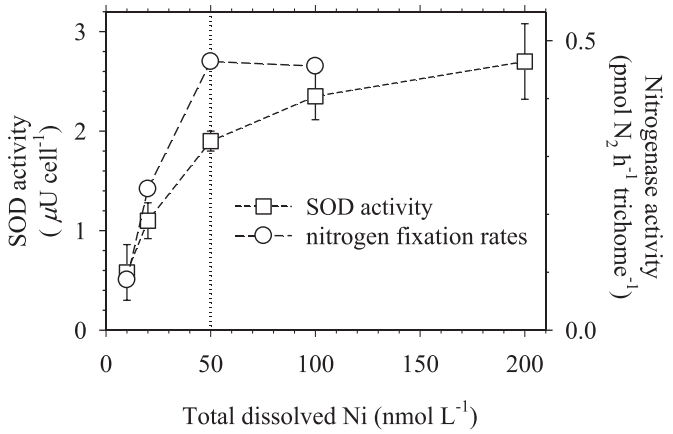

Fig. 3. The SOD activities and nitrogen fixation rates of *Trichodesmium* grown in media with various Ni concentrations. Total cell numbers were estimated by the conversion of total cell volume. The culture media were prepared by trace metal–defined medium with 20 μmol L^{-1} EDTA and 100 nmol L^{-1} total Fe. Total dissolved Ni concentrations were 10, 20, 50, 100, and 200 nmol L^{-1}, which were equivalent to 6.7, 13, 33, 67, and 133 pmol L^{-1} of inorganic Ni, respectively (Westall et al. 1976; Price and Morel 1991). The range of these Ni concentrations was chosen to correspond to the result observed in Fig. 1B. The growth rates did not increase once total dissolved Ni concentrations were above 40 nmol L^{-1} in the culture medium. The growth rates were 0.14 and 0.11 d^{-1} for the treatments with Ni concentrations of 10 and 20 $nmol^{-1}$, respectively. The growth rates for the treatments with Ni concentrations $>$ 50 nmol L^{-1} ranged from 0.18 to 0.22 d^{-1} with no correlation to Ni concentration. The SOD activities were obtained from the average of three to four replicate samples of a single-culture bottle (Table 2). The error bars show the standard deviation of the replicate analysis. Analyses of total dissolved Ni concentrations for nitrogen fixation rates were 10, 20, 50, and 100 nmol L^{-1}. The unit of the nitrogen fixation rates was shown by using the conversion factor of 50 cells per trichome for rate comparison with previous field studies.

incubation time, the results should be considered preliminary, and they were consistent with the laboratory findings reported in this study.

The results in Fig. 3 show that Ni availability can regulate both SOD activities and nitrogen fixation activities in *Trichodesmium*. The cell-normalized SOD activities of *Trichodesmium* were positively correlated with Ni concentration in the artificial medium at Ni concentrations $<$ 50 nmol L^{-1}, and a fivefold difference in activity existed between the lowest and highest Ni treatments (Fig. 3). Elevated Ni availability also significantly enhanced nitrogen fixation rates in *Trichodesmium*. Cell volume–normalized nitrogen fixation activities increased linearly with increasing Ni concentrations in *Trichodesmium* at Ni concentrations $<$ 50 nmol L^{-1} and saturate at Ni concentrations $>$ 50 nmol L^{-1} under the experimental conditions (Fig. 3). Overall, the nitrogen fixation rates ranged from 0.08 to 0.48 pmol N_2 production h^{-1} $trichome^{-1}$, with Ni concentrations ranging from 10 to 100 nmol L^{-1}. The nitrogen fixation rates were comparable to the value observed in the field studies, which mostly ranged from 0.10 to 2.0 pmol N h^{-1} $trichome^{-1}$ (Capone et al. 2005).

Discussion

Ni limitation in Trichodesmium—The results of Figs. 1, 2 indicate that *Trichodesmium* grows much more quickly in seawater enriched with Ni and that the 2 nmol L^{-1} Ni in the natural surface seawater might not be enough to support its maximum biochemical demand for optimal growth in seawater with otherwise sufficient nutrient concentrations. During the exponential growth period, the growth rates and the increase in total cell volume or biomass yield of the 4 and 40 nmol L^{-1} Ni treatments seem to be similar in the early growing stage, which may be due to Ni carry-over effect in the cells or a sufficient Ni uptake in the early stage (Fig. 1A). The growth rates observed in the modified low-Fe, high-EDTA culture medium were relatively lower than the rates observed in high-Fe, low-EDTA medium (Fig. 1B), possibly because of relatively low Fe availability. Overall, these results indicate that optimal growth of *Trichodesmium* requires an ample supply of Ni. A recent field study observed that Ni can be a limiting factor for nondiazotrophic cyanobacteria (Dupont et al. 2010). Dupont et al. (2010) found that cyanobacteria growth can be enhanced with Ni addition in the Gulf of California. Their radioactive tracer experiments also suggested that much of natural dissolved Ni might not be bioavailable to small phytoplankton in seawater. These findings and my study both suggest that more extensive Ni enrichment experiments in the tropical and subtropical

Table 2. The SOD activities, Mn quota, and proportion of Ni in NiSOD to total cellular Ni concentrations in *Trichodesmium* grown under different Ni concentrations. The dissolved Ni concentrations in culture media are shown in the first column. The Ni_{SOD} and Ni_{total} columns are the particulate or cellular Ni concentrations in SOD and total cellular material per unit volume of seawater. Because NiSOD activity per unit weight is similar to bovine CuZnSOD, the equivalent total cellular SOD concentrations (by mass) per cell can be estimated by dividing measured SOD activities per cell (the second column) by the activity-to-concentration ratio of the bovine CuZnSOD standard used (5030 units mg^{-1}). Because NiSOD is a hexamer with a subunit of molecular mass 13.4 kDa, the cellular NiSOD concentration per cell (third column) is then obtained by dividing the SOD mass by the molecular mass of a NiSOD subunit. The estimated intracellular Ni concentrations in SOD per cell (Ni_{SOD}) are obtained by multiplying the cellular NiSOD concentrations with cell concentrations in the medium.

Ni_{total} (nmol L^{-1})	Cellular SOD activity (μU $cell^{-1}$)	NiSOD concentration* (amol $cell^{-1}$)	Medium cell concentration (10^3 cell mL^{-1})	Mn quota† (mmol mol^{-1} P)	Ni quota† (mmol mol^{-1} P)	Ni_{SOD} (nmol L^{-1})	Ni_{total} (nmol L^{-1})	Ni_{SOD}:Ni_{total} (%)
n	3–4	3–4	2	2	2	3–4	2	
10	0.58±0.28	4.3±4.0	26±1.2	0.51	0.22	0.11±0.10	1.20±0.18	9±9
20	1.1±0.18	8.0±2.8	22±1.1	0.45	0.21	0.18±0.06	0.80±0.03	23±8
50	1.9±0.10	15±1.5	34±0.1	0.54	0.52	0.51±0.05	3.7±0.13	14±2
100	2.5±0.23	19±3.5	33±1.4	0.53	0.73	0.62±0.12	3.6±0.11	17±4
100	2.2±0.05	16±0.8	35±2.2	0.54	0.85	0.56±0.08	5.7±0.15	10±1
200	2.7±0.38	20±5.5	32±2.3	0.52	1.08	0.64±0.20	8.6±0.10	15±2

* Assuming that half of the SOD activities measured (second column) were attributed to NiSOD.
† The average deviations of the replicate analysis of the metal quotas were all $< 10\%$.

oceans are needed to further investigate whether Ni might be an independent or colimiting factor for *Trichodesmium* and other diazotrophs in the oceans.

The dissolved concentrations of Ni are usually around 2 nmol L^{-1} or higher in oceanic surface waters, although the vertical profiles of dissolved Ni in the global oceans generally exhibit strong linear correlation with major nutrients (Mackey et al. 2002). Compared with other essential trace metals like Fe and Zn, dissolved Ni concentrations in the surface waters of contemporary open oceans are relatively high. It is unclear why *Trichodesmium* do not use up the dissolved Ni in the oceanic surface waters. One possibility is that much of the 2 nmol L^{-1} dissolved Ni is not bioavailable to *Trichodesmium* and other prokaryotic phytoplankton that require Ni. The bioavailability of dissolved trace metals to phytoplankton are speciation specific. The speciation of organic ligands for Ni might be an important factor influencing its bioavailability to phytoplankton. For example, studies show that *Trichodesmium* or cyanobacteria can take up the Fe chelated by siderophores but not the Fe complexed by porphyrin (Achilles et al. 2003). However, diatoms can take up the Fe complexed by porphyrin (Hutchins et al. 1999). Without further understanding of the bioavailability of a dissolved trace metal to a specific phytoplankton phylum, the total dissolved concentrations might not be a useful parameter to evaluate whether a trace metal can be limiting to *Trichodesmium*. Additionally, the study of Hudson and Morel (1993) pointed out that Ni complexation kinetics is slow, which might require microalgae to devote a large amount of their membrane space to Ni acquisition. The other possible explanation for the relatively high surface concentrations is that phytoplankton might be unable to take up Ni efficiently to deplete concentrations because of slow kinetics.

The dissolved Ni concentrations in the tropical region in the Western Pacific, where *Trichodesmium* is relatively abundant, are around 2 nmol L^{-1} (Mackey et al. 2002), which is the lowest concentration that has been observed in the global ocean (Mackey et al. 2002; this study). Total dissolved Ni concentrations of surface waters might elevate in temperate and polar regions, where the abundance of *Trichodesmium* is much lower than the tropical and subtropical regions. Further studies can be carried out to evaluate the possible causal relationship between the abundance of *Trichodesmium* and the distribution of total dissolved Ni concentrations in the global ocean.

Ni supply vs. the activities of *SOD and nitrogen fixation*—The results shown in Fig. 3 indicate that the biochemical utilization of Ni could be used for the NiSOD cofactor in *Trichodesmium* and that Ni depletion would limit NiSOD synthesis and nitrogen fixation rates. Although *Trichodesmium* also possesses MnSOD (Palenik et al. 2003; Dupont et al. 2008), Mn quotas do not change with Ni concentrations when SOD activities increase by a factor of five with increasing Ni concentrations in the culture medium (Table 2). Cellular Mn quotas ranged from 0.45 to 0.54 mmol mol^{-1} P under Ni-replete and -deplete media and do not show any systematic relationship to Ni availability, suggesting that MnSOD does not replace

NiSOD in *Trichodesmium* when Ni is depleted. Further experiments by covarying Mn and Ni availability could shed light on the interaction between Mn and Ni in terms of SOD expression.

Table 2 also presents the estimated proportion of Ni in NiSOD to the total intracellular Ni taken up in *Trichodesmium*. Total cellular SOD activities of *Trichodesmium* are estimated by the calibration curves obtained from bovine CuZnSOD standards. It is reasonable to estimate the Ni quota of NiSOD through the specific activity of CuZnSOD because previous studies on NiSOD have already observed that NiSOD exhibits similar specific activity as CuZnSOD (Youn et al. 1996; Barondeau et al. 2004). The cellular Ni concentrations estimated from the total SOD activity would be the upper limit of Ni concentrations in NiSOD. The cellular concentrations may be close to the Ni concentrations in NiSOD because the increased activities were mostly derived from NiSOD (Table 2). By assuming that only half of the SOD measured was due to NiSOD, the percentages of Ni in NiSOD ranged from 9% to 23% of cellular Ni (Table 2). These results indicate that NiSOD is essential for nitrogen fixation in *Trichodesmium* and may be the sole SOD involved in the protection of the nitrogenase used in nitrogen fixation.

In addition to NiSOD, it should be noted that Ni is also used for hydrogenases, another Ni enzyme in cyanobacterial diazotrophs (Tamagnini et al. 2007; Bothe et al. 2010). Most of the studies regarding Ni requirements of hydrogenase in cyanobacteria have focused on the heterocystous cyanobacterium, *Anabaena cylindrica* (Daday et al. 1985). Ni availability does significantly influence H_2 uptake rates in *Anabaena,* but the activities of hydrogenase do not influence the growth rates and nitrogen fixation rates (Daday et al. 1985). Thus, no advantage was conferred by the ability to metabolize hydrogen gas in terms of growth rates or total nitrogen fixed. Further study in the diazotroph, *Azotobacter vinelandii*, similarly observed that hydrogen recycling was too low to benefit cellular energy metabolism or nitrogenase activities (Linkerhagner and Oelze 1995). These studies suggest that hydrogenase might not be a limiting factor for nitrogen fixation in cyanobacterial diazotrophs. There are still no systematic studies reporting the activities or Ni requirements of hydrogenase in *Trichodesmium*.

The essential requirement of Ni in *Trichodesmium* suggests that intracellular Ni in phytoplankton could be an important source of Ni preserved in the organic matter of sedimentary deposits when *Trichodesmium* or other diazotrophs requiring Ni were dominant primary producers in oceanic surface waters. Indeed, there is a strong linear correlation between organic matter and Ni concentrations in marine black shales (Piper and Calvert 2009). A recent field study also observed elevated V concentrations in *Trichodesmium* (Nuester et al. 2012). Vanadium nitrogenase could have replaced Mo nitrogenase in marine diazotrophs in ancient oceans when Mo concentrations were depleted (Raymond et al. 2004; Boyd et al. 2011). It is well known that the concentrations of porphyrin-bound Ni and V are abundant in organic-rich marine sediments and crude oils. The concentration ratios of Ni and V can be used to distinguish the origins of crude oils (Quirke 1987). Abiotic geochemical processes have been considered to be the major mechanism for the formation of organic Ni and V preserved in crude oils and organic-rich sediments. The coincidence of elevated Ni and V observed in *Trichodesmium* suggests that Ni and V in sedimentary organic matter could come at least in part from biogenic material when diazotrophs using Ni and V were dominant. The elemental concentrations of Ni and V can be compared with the isotopic composition of nitrogen in sedimentary organic material to investigate this possibility. The isotopic composition and fractionation effect of Ni in *Trichodesmium* might be a useful indicator to evaluate the likelihood of the biological origin for the Ni and V.

This study demonstrates that Ni is essential for nitrogen fixation in *Trichodesmium* and that Ni is an important factor regulating SOD activities and nitrogen fixation rates in this diazotroph. Preliminary field experiments in this study suggest that Ni could be a significant limiting factor for the growth of *Trichodesmium*. Future field studies conducted by applying Ni addition or the antibody of NiSOD could thoroughly explore whether Ni can be an independent limiting or colimiting factor for nitrogen fixation in *Trichodesmium* or other diazotrophs in the oceans. In terms of biochemical functions of Ni in *Trichodesmium*, the results shown in Fig. 3 indicate that Ni could be used in NiSOD to protect nitrogenase from ROS inhibition during photosynthesis in the nonheterocystous diazotroph. The findings of this study have potential implications for interpreting distributions and activities of *Trichodesmium* and for the existing models of mechanistic controls on nitrogen fixation in modern and ancient oceans.

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