Limnol. Oceanogr., 58(6), 2013, 2029–2036

doi:10.4319/lo.2013.58.6.2029

Ammonium uptake and dinitrogen fixation by the unicellular nanocyanobacterium *Crocosphaera watsonii* in nitrogen-limited continuous cultures

Takako Masuda, Ken Furuya,* Taketoshi Kodama,[a] Shigenobu Takeda,[b] and Paul J. Harrison[c]

Department of Aquatic Bioscience, The University of Tokyo, Yayoi, Bunkyo, Tokyo, Japan

Abstract

Ammonium uptake and nitrogen (N) fixation of the unicellular nanocyanobacterium *Crocosphaera watsonii* isolated from the western subtropical North Pacific were determined in N-limited continuous cultures. Six steady-state growth rates ranging from 0.10 to 0.35 d^{-1}, corresponding to 20–75% of the maximum growth rate, were established under saturating light. Unlike other larger diazotrophs, nitrogen fixation of *C. watsonii* was not inhibited by ambient ammonium ranging from < 3 to 59 nmol L^{-1}, and nitrogen fixation did not vary consistently with dilution rate and ranged from 4.4 to 12.9 fmol N $cell^{-1}$ d^{-1}, with the highest rates at intermediate dilution rates. In contrast, ammonium uptake increased significantly with increasing dilution rates over the range of 10 to 80 fmol N $cell^{-1}$ d^{-1} and contributed 65–95% to the daily cellular N requirement. The dissolved organic nitrogen (DON) excretion increased with increasing dilution rate; however, only a small portion of assimilated nitrogen was excreted as DON. In contrast, in ammonium-free medium, where N assimilation occurred only by dinitrogen (N_2) fixation, 60% of the fixed N was excreted. Interestingly, ammonium enrichment did not increase the growth rate of *C. watsonii*, but cellular contents of N, phosphorus, and chlorophyll *a* significantly increased for most dilution rates compared with cells grown in ammonium-free medium. *C. watsonii* was capable of fixing N_2 while taking up ammonium at environmentally relevant low concentrations of < 3 nmol L^{-1}, and N_2 fixation was independent of nanomolar NH_4^+ concentrations. Therefore, *C. watsonii* can compete with nondiazotrophic phytoplankton for ammonium in oligotrophic subtropical gyres.

Nitrogen (N) may limit primary production in the subtropical gyres, and sources of dissolved inorganic nitrogen (DIN) such as nitrate (NO_3^-) and ammonium (NH_4^+) play a critical role in biological production. Dinitrogen (N_2) fixation is one of the main processes that drives nitrogen cycling in the open ocean. Recent studies (Montoya et al. 2004; Shiozaki et al. 2009) have unequivocally demonstrated that nitrogen fixers contribute a significant portion to new production in nitrate-depleted subtropical waters.

While the filamentous cyanobacteria *Trichodesmium* has been considered to be the main nitrogen fixer, nano-sized unicellular cyanobacteria (nanocyanobacteria) are gaining increasing attention as important marine nitrogen fixers (Zehr et al. 2001; Montoya et al. 2004). The genes attributable to the diazotrophic nanocyanobacteria occur extensively in subtropical and tropical waters, including the Pacific Ocean.

Although they have access to a large pool of N_2, some diazotrophs are able to utilize fixed nitrogen (i.e., DIN; Mulholland and Capone 1999; Agawin et al. 2007). Therefore, they can compete for DIN with non-diazotrophic phytoplankton. Thus, DIN utilization by diazotrophs is a key in the regulation of biological productivity in nitrogen-limited subtropical waters. However, our knowledge on nitrogen uptake of diazotrophs is mainly for *Trichodesmium*, and relatively little is known about marine diazotrophic nanocyanobacteria.

The present study aims to clarify the relationship between N_2 fixation and NH_4^+ uptake of diazotrophic nanocyanobacteria using continuous cultures of the diazotrophic nanocyanobacteria *Crocosphaera watsonii* isolated from the western subtropical Pacific. Ammonium was supplied at six different dilution rates in the continuous cultures, and NH_4^+ uptake and N_2 fixation were measured along with various cell parameters. Since NO_3^- is usually exhausted down to the nanomolar level at the surface in the western subtropical Pacific (Hashihama et al. 2009; Kitajima et al. 2009; Kodama et al. 2011), we examined NH_4^+ utilization at environmentally relevant nanomolar NH_4^+ concentrations as a regenerated source of nitrogen for this diazotroph and how ammonium influences N_2 fixation under a range of N-limited cultures of *C. watsonii*.

Methods

Culture—A seawater sample collected from the surface of western subtropical Pacific (28°56′N, 131°11′E) on 11 September 2006 was spread on agar plates prepared from a nitrogen-free TMV medium (Prufert-Bebout et al. 1993) and incubated at 26°C under saturating light. Colonies that formed on the surface were picked up and transferred to a nitrogen-free TMV liquid medium. A clonal diazotroph strain was established, the *nifH* base sequence of which had two replacements, sharing 323 out of the total 325 bases with *C. watsonii* strain WH8501 (Zehr et al. 2001), corresponding to one amino acid difference among 108

* Corresponding author: furuya@fs.a.u-tokyo.ac.jp

Present addresses:
[a] National Research Institute of Fisheries Science, Fisheries Research Agency, Fukuura, Yokohama, Japan
[b] Graduate School of Fisheries Science and Environmental Studies, Nagasaki University, Bunkyo, Nagasaki, Japan
[c] Department of Earth and Ocean Sciences, University of British Columbia, Vancouver, British Columbia, Canada

amino acids of *nifH* from *C. watsonii* WH8501. Based on this 99.1% identity with *C. watsonii* strain WH8501, our clone was identified to be *C. watsonii*.

The maximum growth rate of *C. watsonii* was determined in triplicate batch cultures at 24°C on a 12 : 12 h light : dark cycle with 200 μmol photons m^{-2} s^{-1}. A medium was prepared from 1 month–aged seawater collected from the surface of the western North Pacific Ocean (34°20′N, 138°40′E), which was enriched with 50 μmol L^{-1} NH_4Cl, 20 μmol L^{-1} NaH_2PO_4, a vitamin mix, and trace metals. The composition and concentrations of the vitamin mix and trace metals were the same as those of the f/2 medium. Growth rates were also determined under the same conditions as above using the same medium without NH_4^+ addition in order to compare the growth rate derived from N_2 fixation vs. NH_4^+ uptake.

Continuous cultures were maintained under the same temperature, light, and nutrient conditions as were used for the batch cultures. A single chemostat was prepared for each dilution rate. A 1.8 liter culture was grown in a 2.5 liter, three-port polycarbonate magnetically stirred culture vessel fitted with a tetra-fluoroethylene stir bar with a polypropylene and polyvinylidene fluoride stirring assembly (Nalgen Nunc). Once the culture reached the exponential growth phase, the vessel was connected to a peristaltic pump, which supplied fresh medium continuously. All tubing was acid-cleaned and sterilized by autoclaving. Culture vessels were continuously mixed by a suspended magnetic stir bar at 60 revolutions min^{-1}. A weak flow of 0.2 μm filtered air maintained a slight positive pressure in the culture. All of the containers, including tubing and connectors, were replaced every 3 or 4 d. The cultures were not axenic, but aseptic techniques were used to minimize bacterial contamination. Bacterial cell numbers were consistently 4 orders of magnitude lower than that of *C. watsonii* and were considered insignificant. Steady state was determined by measuring cell density and in vivo chlorophyll (Chl) fluorescence for 10 consecutive days or longer. In vivo fluorescence was measured using a fluorometer (TD700, Turner Designs).

C. watsonii grew at the same rate of 0.46 ± 0.02 d^{-1} in the batch culture with and without NH_4^+ addition. Based on these rates, chemostats were run at dilution rates between 0.10 and 0.35 d^{-1}, corresponding to 20–75% of the maximum growth rate. Steady states were usually established by day 20 after the start of the culture. A culture in NH_4^+–free medium was also maintained at a dilution rate of 0.20 d^{-1} in order to assess cellular responses due only to N_2 fixation.

Analyses and calculations—At steady state, the growth rate (μ) of *C. watsonii* was equal to the dilution rate (D). The volume of the culture overflow was monitored daily to confirm the dilution rate. In *C. watsonii,* N_2 fixation occurs during the dark period, and carbon assimilation and cell division occur during the light period (Dron et al. 2012). Our strain of *C. watsonii* was confirmed to also fix N_2 only during the dark period. Based on this diel metabolic periodicity, subsampling from the overflow of chemostats was made every day for three consecutive days at the end of the light period. Therefore, biological and chemical determinations were made in triplicate. The cell number of *C. watsonii* in the overflow was counted using a flow cytometer (PASIII, Partec) equipped with 10 mW argon ion lasers. Bacteria were also counted by staining with 10 μmol L^{-1} of a fluorescence nucleic acid dye SYBR® Green (Molecular Probes).

The cell size of *C. watsonii* was measured under an epifluorescence microscope. Cells were stained with 50 μg mL^{-1} of 4′,6-diamidino-2-phenylindole, mixed with 0.025 mol L^{-1} Tris-HCl (pH 7.0), and then filtered onto a black membrane filter (0.2 μm pore size, Millipore). Cellular dimensions were measured for 50 cells of *C. watsonii* under a ×100 objective, and cell volume was calculated assuming that cells were elliptical in shape. Biovolume was calculated by multiplying cell volume by cell density.

NH_4^+ and PO_4^{3-} in the medium were determined manually by the indophenol and molybdenum blue methods, respectively (Strickland and Parsons 1972). Concentrations of NH_4^+ and PO_4^{3-} in the culture were measured using a highly sensitive colorimetry system that consisted of an AutoAnalyzer II (Technicon) and a Liquid Wave-guide Capillary Cell (World Precision Instruments; Hashihama et al. 2009; Kodama et al. 2011) after filtration through a 0.2 μm cellulose acetate in-line filter (DISMIC®, Advantec). Detection limits for both NH_4^+ and PO_4^{3-} were 3 nmol L^{-1}.

Total nitrogen (TN), total phosphorus (TP), dissolved organic nitrogen (DON), and phosphorus (DOP) were determined by the persulfate oxidation method (Hansen and Koroleff 1999). Samples for TN and TP were stored frozen until analyzed, and those for DON and DOP were immediately filtered through a 0.2 μm DISMIC filter and stored frozen until analysis. One milliliter of borate-buffered persulfate solution was added to test tubes containing 9 mL of sample, and the tubes were tightly capped. The contents were mixed and autoclaved at 121°C for 60 min. After cooling, the contents were mixed and the concentrations of nitrate and ammonium were measured by standard methods using a TRAACS2000 (Bran+Luebbe). PO_4^{3-} concentrations in the micromolar range were measured manually by the molybdenum blue method (Strickland and Parsons 1972). Detection limits of DON and DOP analysis were 0.12 μmol L^{-1}.

The nitrogen budget was estimated from the following equation:

$$\frac{dN}{dt} = N_{2fix} + D(NH_{4med} + DON_{med} - NH_{4ambient} - DON_{ambient}) - \mu X Q_N \quad (1)$$

where N_{2fix} is N_2 fixation rate (μmol N L^{-1} d^{-1}); D is dilution rate (d^{-1}); NH_{4med} and DON_{med} are NH_4^+ and DON concentrations in the medium (μmol N L^{-1}), respectively; $NH_{4ambient}$ and $DON_{ambient}$ are NH_4^+ and DON concentrations in the culture (μmol N L^{-1}), respectively; μ is growth rate (d^{-1}); X is cell density (cells L^{-1}); and Q_N is cellular N quota (fmol N $cell^{-1}$). DON excretion was estimated from Eq. 2 with the assumption

that ambient DON concentrations were constant once steady state was established.

$$\frac{d\mathrm{DON}}{dt} = D(\mathrm{DON_{med}} - \mathrm{DON_{ambient}}) - \mu X \mathrm{Q_{DON}} \quad (2)$$

where Q_{DON} is cellular DON quota (fmol N cell^{-1}), and excretion was calculated as a negative value of μQ_{DON} (fmol N cell^{-1} d^{-1}). Ammonium uptake was similarly calculated from Eq. 3:

$$\frac{d\,\mathrm{NH_4}}{dt} = D(\mathrm{NH_{4med}} - \mathrm{NH_{4ambient}}) - \mu X \mathrm{Q_{NH_4}} \quad (3)$$

where Q_{NH4} is cellular NH_4 quota (fmol N cell^{-1}), and uptake was calculated as μQ_{NH4} (fmol N cell^{-1} d^{-1}).

The P budget was estimated from Eq. 4:

$$\frac{d\mathrm{P}}{dt} = D(\mathrm{PO_{4med}} + \mathrm{DOP_{med}} - \mathrm{PO_{4ambient}} - \mathrm{DOP_{ambient}}) - \mu X \mathrm{Q_P} \quad (4)$$

where PO_{4med}, and DOP_{med} are PO_4^{3-} and DOP concentrations in the medium (μmol P L^{-1}), respectively; $PO_{4ambient}$ and $DOP_{ambient}$ are PO_4^{3-} and DOP concentrations in the culture (μmol P L^{-1}), respectively; and Q_P is cellular P quota (fmol P cell^{-1}).

N_2 fixation was determined by the uptake of $^{15}N_2$ (Montoya et al. 1996). The culture was introduced into acid-cleaned vials in triplicate and spiked with $^{15}N_2$ gas (99.8 atom% ^{15}N; SI Science). The samples were incubated for 24 h under the same light and temperature conditions as the continuous cultures. N_2 fixation activity was also measured by the acetylene reduction assay following the method of Kitajima et al. (2009). Six milliliters of culture was introduced into 12 mL HCl-rinsed glass vials with five replicates. After sealing with a butyl rubber stopper, 1.5 mL of acetylene (99.9999% [v : v], Kouatsu Gas Kogyo) was injected by replacing the same volume of headspace. After a 24 h incubation, ethylene was analyzed. The produced ethylene was converted to fixed nitrogen with a molar ratio of 4 : 1 (Montoya et al. 1996). Particulate N (PN) and particulate carbon were determined using a Flash EA elemental analyzer (Thermo Electron). An aliquot of overflow was filtered onto a pre-combusted 25 mm GF/F filter and maintained at −20°C until analyzed. The filters were dried at 50°C overnight, exposed to HCl fumes for 3 h to remove residual inorganic carbon, and packed in tin capsules after dehydration. Natural ^{15}N abundance in PN was determined on the same samples that were used for PN. Particulate P was calculated from TP minus PO_4^{3-}.

Alkaline phosphatase activity (APA) was assayed by emission of 3-O-methylfluoresein at 520 nm released from 3-O-methylfluorescein phosphate after the excitation at 435 nm (Healey and Hendzel 1979). The assay was made in triplicate in the dark at in situ temperatures. The assay time was 10 min for dilution rates of 0.10 and 0.15 d^{-1} and 1 h for 0.20–0.35 d^{-1}. The emission was calibrated against standard solutions of 3-O-methylfluorescein over a range from 0 to 0.66 μmol L^{-1}. Artificial seawater was used as a blank.

Methodological comparison of N_2 fixation rates—As a result of the slow dissolution of gaseous $^{15}N_2$ in water, the conventional bubbling method has been reported to underestimate N_2 fixation rates (compared with the $^{15}N_2$-enriched seawater method; Mohr et al. 2010). Since the work of Mohr et al. (2010) was published after our experiments were conducted, an additional experiment was performed to compare N_2 fixation rates determined by both methods. Cultures maintained in NH_4^+-added and NH_4^+-free medium at $D = 0.20$ h^{-1} were used. Each culture was introduced into eight acid-cleansed vials, $^{15}N_2$ as a gas bubble was added to four vials, and $^{15}N_2$-enriched medium was added to the other four vials. The samples were incubated for 24 h under the same light and temperature conditions as those described above.

Results

Cellular composition and NH_4^+ availability—The availability of NH_4^+ influenced the C and N metabolism of *C. watsonii* (Table 1). Cells grown in NH_4^+-free cultures were less abundant and contained significantly lower C, N, and Chl *a* content per biovolume, with a lower N : P ratio and a higher C : N ratio, than did those in the NH_4^+-added cultures at the same dilution rate of $D = 0.20$ d^{-1}. However, there was no difference in phosphate content.

In the NH_4^+-added cultures at different dilution rates, cell densities decreased linearly from 5.0×10^5 to 2.2×10^5 cells mL^{-1} with increasing dilution rates (Table 1). Cell volume ranged between 77 and 131 μm^3, and no obvious relationship was noted between cell volume and dilution rates. Volume-specific cellular C, N, and Chl *a* content showed no significant trend with dilution rates. Similarly, there was no trend with dilution rate for cellular C : N and N : P ratios, which ranged from 7.0 to 9.2 (mean of 8.5 ± 0.8) and from 1.4 to 2.6 (mean of 2.0 ± 0.2), respectively.

Nutrient concentrations in cultures—Surprisingly, NH_4^+ was detected in the NH_4^+-free culture, suggesting exudation of NH_4^+. Ambient NH_4^+ concentrations were highest at $D = 0.10$ d^{-1} and decreased with increasing dilution rates (Table 2). *C. watsonii* took up NH_4^+ down to very low concentrations of < 3 nmol L^{-1} at $D = 0.35$ d^{-1}, suggesting that the cells were the least N stressed at this dilution rate. In contrast, PO_4^{3-} was not exhausted at all dilution rates, but its concentration tended to be lower at lower dilution rates and the lowest at $D = 0.15$ d^{-1}. P per cell revealed no indication of P storage at high dilution rates. APA was detected at low dilution rates and was probably associated with the low ambient PO_4^{3-} concentrations, indicating P deficiency as well as probable N limitation at these low dilution rates.

DON remained constant regardless of dilution rates, ranging from 11.4 to 13.4 μmol L^{-1}, but DON in the NH_4^+-free culture was lower than that in the NH_4^+-added culture. DOP was consistently below detection limits at all dilution rates.

Cellular processes and NH_4^+ availability—Cells maintained in the NH_4^+-free culture at $D = 0.20$ d^{-1} had no NH_4^+ uptake, and, hence, the N assimilation rate of this culture was 75% lower compared to that of the NH_4^+-added culture.

Table 1. Summary of cell density; cell volume; cellular carbon, nitrogen, phosphorus, and Chl; and cellular N : P and N : P molar ratios of *C. watsonii* grown in continuous culture. Standard deviations for triplicate determination are in parentheses.

Dilution rate (d^{-1})	Cell density ($\times 10^8$ cells L^{-1})	Cell volume (μm^3)	C per biovolume (fmol μm^{-3})	N per biovolume (fmol μm^{-3})	P per biovolume (fmol μm^{-3})	Chl per biovolume (fg μm^{-3})	C : N ratio	N : P ratio
NH_4^+-added medium								
0.10	5.0(0.1)	77(6)	7.51(0.41)	0.82(0.11)	0.32(0.04)	2.5(0.0)	9.2	2.6
0.15	4.4(0.7)	131(8)	4.97(0.18)	0.58(0.04)	0.31(0.01)	2.1(0.0)	8.6	1.9
0.20	4.0(0.4)	106(5)	6.09(0.22)	0.72(0.08)	0.30(0.02)	5.0(0.0)	8.5	2.4
0.25	2.4(0.3)	121(5)	6.49(0.28)	0.77(0.04)	0.35(0.01)	4.9(0.0)	8.4	2.2
0.30	2.6(0.2)	100(6)	5.86(0.23)	0.84(0.06)	0.50(0.02)	3.7(0.0)	7.0	1.7
0.35	2.2(0.0)	94(6)	5.04(0.28)	0.56(0.02)	0.39(0.02)	3.0(0.0)	9.0	1.4
NH_4^+-free medium								
0.20	2.0(0.0)	165(7)	4.04(0.16)	0.37(0.04)	0.25(0.03)	1.5(0.0)	11.0	1.5

The NH_4^+-free and NH_4^+-added cultures both excreted ~ 3–4 fmol $cell^{-1}$ d^{-1} of DON (Table 3). Even though this value was similar in both cultures, it represented ~ 60% of daily assimilated N for the NH_4^+-free culture, indicating that *C. watsonii* released a considerable portion of its fixed N_2 under the NH_4^+-free condition.

In the NH_4^+-added cultures at different dilution rates, cellular NH_4^+ uptake rates increased eightfold with increasing dilution rates, from 10 to 80 fmol $cell^{-1}$ d^{-1} (Table 3). In contrast, cellular N_2 fixation rates determined by $^{15}N_2$ uptake and the acetylene reduction assay were similar and did not vary systematically compared to NH_4^+ uptake over the range of dilution rates; the highest N_2 fixation rate of 12.9 fmol $cell^{-1}$ d^{-1} was observed at D = 0.25 d^{-1}. The comparison of the two methods of measuring N_2 fixation rates revealed that the bubbling method underestimated N_2 fixation by 49 ± 1% in NH_4^+-added medium and by 44 ± 2% in NH_4^+-free medium compared with rates determined by the $^{15}N_2$-enriched seawater method, supporting previous results by Mohr et al. (2010). Since the rates determined by the two methods were of the same order of magnitude, and given that our whole data set was obtained simultaneously with the bubbling method, we used N_2 fixation rates determined by this method thereafter.

Cellular N assimilation (i.e., NH_4^+ uptake plus $^{15}N_2$ fixation) increased fivefold with dilution rate (15.6 fmol $cell^{-1}$ d^{-1} at D = 0.10 d^{-1} to 84.6 fmol $cell^{-1}$ d^{-1} at D = 0.35 d^{-1}), and N_2 fixation contributed only 5–35% to the total cellular N assimilation. Thus, when NH_4^+ was available, *C. watsonii* depended mainly on NH_4^+ uptake from the medium, and N_2 fixation contributed only 5–35% to their daily N requirement. The N_2 fixation rate in the NH_4^+-free cultures was within the range of those in the NH_4^+-added cultures, indicating that N_2 fixation was independent of NH_4^+ availability at a low concentration.

In the NH_4^+-added cultures, DON excretion rate increased sevenfold with increasing dilution rates, from 1.4 fmol N $cell^{-1}$ at D = 0.1 d^{-1} to 10.7 fmol N $cell^{-1}$ at D = 0.35 d^{-1} (Table 3). DON excretion represented 7.7–12.6% of the cellular N assimilation. Instantaneous N- and P-specific growth rates estimated from Eqs. 1 and 3 were significantly correlated (r^2 = 0.81, $p < 0.05$) with dilution rates (Fig. 1). While P-specific growth rate coincided well with dilution rate, N-specific growth rates were 2.8 times higher than the dilution rate (i.e., the slope of the regression [2.8] was significantly higher than 1 [$p < 0.05$]). When the datum point at D = 0.35 was excluded, the slope was calculated to be 2.2 and significantly higher than 1 ($p < 0.05$).

Discussion

Cellular composition and NH_4^+ availability—NH_4^+ availability (i.e., NH_4^+-free vs. NH_4^+-added cultures at D = 0.20 d^{-1}) altered cellular composition significantly, as observed in the elevated cellular C, N, and Chl *a* contents. This agrees with the findings of Dekaezemacker and Bonnet (2011), who showed that an NH_4^+ addition increased C and N content of *C. watsonii* WH0003. In

Table 2. NH_4^+, PO_4^{3-}, DON, and DOP concentrations and APA of *C. watsonii* grown in continuous cultures. Standard deviations ($n = 3$) are in parentheses. PO_4^{3-} concentrations at dilution rates of 0.10 and 0.15 were determined by high-sensitivity colorimetry. na, lower than detection limit.

Dilution rate (d^{-1})	NH_4^+ (nmol L^{-1})	PO_4^{3-} (nmol L^{-1})	DON (μmol L^{-1})	DOP (μmol L^{-1})	APA (fmol h^{-1} $cell^{-1}$)
NH_4^+-added medium					
0.10	59(40)	52(14)	13.4(0.0)	na	1.6(0.0)
0.15	25(5)	49(15)	13.1(0.2)	na	1.4(0.0)
0.20	15(7)	$5.7(0.2)\times10^3$	13.2(0.4)	na	0.0(0.0)
0.25	8(4)	$7.1(0.2)\times10^3$	11.4(0.7)	na	0.0(0.0)
0.30	9(3)	$8.8(0.0)\times10^3$	12.9(0.4)	na	0.0(0.0)
0.35	<3	$11.4(0.1)\times10^3$	13.3(0.5)	na	0.0(0.0)
NH_4^+-free medium					
0.20	10(7)	$10.9(0.1)\times10^3$	10.9(0.7)	na	0.0(0.0)

addition, the elevated cellular N under high-NH_4^+ supply is consistent with the findings of Shi et al. (2010), who reported that cyanophycin genes are present in the *Crocophaera* genome and are transcribed in a diel pattern reflecting N_2 fixation. Cyanobacteria are capable of storing nitrogen as proteins, and *Cyanothece* and *Trichodesmium* store nitrogen in cyanophycin granules (Reddy et al. 1993; Finzi-Hart et al. 2009). This observation is supported by the complete genome sequence of *Cyanothece,* which indicated its capability to store metabolic products in inclusion bodies (Welsh et al. 2008).

The C : N ratio of *C. watsonii* was relatively constant, with a mean of 8.5 ± 0.2 in NH_4^+-added medium over the range of dilution rates and 11.0 in N-free medium and within the range of reported values (Dron et al. 2012). However, N : P ratios for *C. watsonii* with a mean of 2.0 ± 0.2 were considerably lower compared with those reported by Knapp et al. (2012). In their batch cultures with varying NO_3^- and PO_4^{3-} concentrations, *C. watsonii* exhibited N : P ratios ranging from 31 to 40 during the exponential phase and from 38 to 62 during the stationary phase. The low N : P ratios in our study may be partially related to the N limitation in the longer term steady-state continuous cultures used in our study compared to the batch cultures used by Knapp et al. (2012). The stoichiometric flexibility of relatively large cells of *Trichodesmium* under nutrient limitation is well recognized (Holl and Montoya 2008), and the N : P ratio increased with increasing P limitation (Krauk et al. 2006).

Nutrient concentrations in cultures—C. watsonii was able to decrease NH_4^+ down to < 3 nmol L^{-1}. Previously, utilization of NH_4^+ and NO_3^- by *C. watsonii* has been examined only at 0.2 μmol L^{-1} or 200 nmol L^{-1} (Dekaezemacker and Bonnet 2011). Similar studies for the marine diazotrophs *Gloeothece* and *Cyanothece* have been conducted (Flynn and Gallon 1990; Agawin et al. 2007), but the analytical detection limit of NH_4^+ or NO_3^- in these previous studies was > 50 nmol L^{-1} (i.e., 10 times higher than our concentrations). Therefore, this study comprises the first report on the N uptake capacity of a subtropical diazotroph growing at very low nanomolar NH_4^+ concentrations. The surface water of the North Pacific subtropical gyre, in which *C. watsonii* is ubiquitous (Zehr et al. 2001; Moisander et al. 2010), is known to be an N-limited oligotrophic area (Hashihama et al. 2009; Kitajima et al. 2009) where NH_4^+ concentrations at the surface are < 100 nmol L^{-1}. In fact, in the western subtropical Pacific where our strain of *C. watsonii* was isolated, surface NH_4^+ concentrations varied from < 3 to 36 nmol L^{-1} (T. Kodama and K. Furuya unpubl.). These observations suggest that *C. watsonii* can compete with non-diazotrophic

Table 3. NH_4^+ uptake and N_2 fixation, N assimilation (i.e., NH_4^+ uptake plus N_2 fixation), and DON excretion of *C. watsonii* grown in continuous cultures. Percentages of cellular DON excretion rate per N assimilation rate are also given. N_2 fixation was determined by both $^{15}N_2$ uptake (^{15}N) and acetylene reduction assay (ARA). Standard deviations ($n = 3$) are in parentheses. nd, not detectable.

Dilution rate (d^{-1})	NH_4^+ uptake rate (fmol $cell^{-1}$ d^{-1})	N_2 fixation $rate_{15N}$ (fmol $cell^{-1}$ d^{-1})	N_2 fixation $rate_{ARA}$ (fmol $cell^{-1}$ d^{-1})	N assimilation rate (fmol $cell^{-1}$ d^{-1})	DON excretion rate (fmol $cell^{-1}$ d^{-1})	DON excretion: N assimilation (%)
NH_4^+-added medium						
0.10	10.1(0.1)	5.5(0.7)	3.5(0.1)	15.6	1.4	9
0.15	17.0(0.7)	4.4(0.8)	8.7(0.3)	21.3	2.2	10
0.20	24.9(0.4)	6.2(0.5)	10.9(0.2)	31.1	3.3	11
0.25	51.1(0.3)	12.9(0.5)	15.1(0.3)	64.0	4.9	8
0.30	56.7(0.2)	6.5(0.6)	8.8(0.4)	63.2	7.2	11
0.35	80.0(0.0)	4.6(0.6)	5.9(0.5)	84.6	10.7	13
NH_4^+-free medium						
0.20	nd	7.3(0.7)	7.4(0.6)	7.3	4.4	60

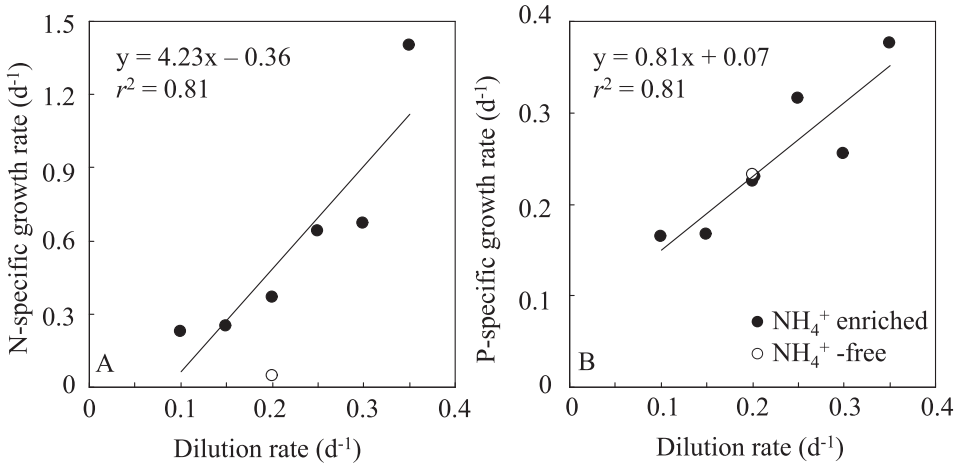

Fig. 1. (A) N- and (B) P-specific growth rates as a function of dilution rate. Filled and open circles denote cells grown in NH_4^+-enriched and NH_4^+-free medium, respectively. Least-squares regression lines for the cells grown with NH_4^+ are shown.

phytoplankton for NH_4^+ in oligotrophic subtropical waters.

Cellular processes and NH_4^+ availability—Surprisingly, cellular N_2 fixation did not differ with or without NH_4^+ in the medium. This is a remarkably unique feature of this nanocyanobacterium, because the presence of micromolar levels of NO_3^-, NH_4^+, and urea are known to inhibit N_2 fixation in the relatively large cells of *Trichodesmium* (Holl and Montoya 2005). The nitrogenase enzyme is inhibited by high DIN concentrations (Dixon and Kahn 2004). A similar inhibition of N_2 fixation by DIN has been reported for the unicellular cyanobacterium *Gloeothece* (Mullineaux et al. 1983) and the freshwater filamentous cyanobacteria *Plectonema boryanum* and *Anabeana* sp. (Rice et al. 1982; Rai et al. 1992). While direct comparison of the results of the present study with those of the previous studies is not warranted, the difference in DIN concentrations should be noted. The previous studies were conducted in the micromolar range of DIN. Dekaezemacker and Bonnet (2011) reported an inhibition of N_2 fixation of *C. watsonii* after the addition of 1–10 μmol L^{-1} of NH_4^+, but not for a lower addition of 0.2 μmol L^{-1} (i.e., 200 nmol L^{-1}) NH_4^+. A similar observation was made in *Trichodesmium* IMS101 by Holl and Montoya (2005), who reported that repression of N_2 fixation in the presence of ammonium and nitrate depends on their concentration and that nitrate of < 5 μmol L^{-1} does not inhibit N_2 fixation. This implies that the ambient NH_4^+ concentration is important when establishing the inhibitory effect of nitrogenous compounds on N_2 fixation for *C. watsonii* and in agreement with our study, in which nanomolar NH_4^+ did not inhibit N_2 fixation. Since *C. watsonii* is expected to encounter nanomolar-level nutrient patches at the surface in the oligotrophic ocean (T. Kodama and K. Furuya unpubl.), it is advantageous for *C. watsonii* to maintain its ability to assimilate N via both NH_4^+ uptake and N_2 fixation when NH_4^+ is available even at nanomolar levels.

The maximum growth rate was not enhanced by NH_4^+ amendment in the batch culture. On the other hand, NH_4^+ uptake rates increased by an eightfold measure (10–80 fmol N $cell^{-1}$ d^{-1}) with increasing dilution rates since more NH_4^+ was available at higher dilution rates. A similar observation was reported for *C. watsonii* at higher NH_4^+ concentrations (Dekaezemacker and Bonnet 2011) and for NO_3^- uptake of *Trichodesmium* at a growth rate of 0.3 d^{-1} (Holl and Montoya 2005). These observations suggest that NH_4^+ uptake is not essential for its growth but that NH_4^+ is preferred when it is available.

Enhanced DON excretion also influenced NH_4^+ availability, and DON excretion increased with increasing dilution rates. *Trichodesmium* released 50–90% of its recently fixed N_2 during growth in culture (Mulholland and Bernhardt 2005) as DON, amino acids, and NH_4^+ (Capone et al. 1994; Mulholland et al. 2004). NH_4^+ and/or DON have been reported to accumulate in seawater in and around the *Trichodesmium* blooms (Karl et al. 1997). Compared to *Trichodesmium*, information on nitrogen release by unicellular nanocyanobacteria is limited. A model analysis coupled with competition experiments in a NH_4^+-free medium suggests that *Cyanothece* excreted up to 89% of its recently fixed N (Agawin et al. 2007). Furthermore, Dron et al. (2012) analyzed the diel change in N_2 fixation and cellular N content and suggested that *C. watsonii* can excrete 26–66% of its recently fixed N during the light period. In the present study, excreted DON represented 60% of the fixed N in NH_4^+-free chemostats, and this rate falls within reported values for unicellular cyanobacteria. However, when NH_4^+ was available for uptake, DON release was low, varying from only 8% to 13% of the total assimilated N. Thus, compared with the high excretion of DON in the NH_4^+-free culture with only N_2 fixation, the NH_4^+ that was taken up in the NH_4^+-added culture was not actively released as DON.

We were surprised that the highest ambient NH_4^+ concentrations occurred at the lowest dilution rates. In continuous cultures of a diatom, concentrations of the

limiting nutrient, ammonium or silicate, were higher at the lowest dilution rates than at intermediate dilution rates, suggesting that cells at very low dilution rates were 'overstressed' and could not take the limiting nutrient down to low concentrations (Harrison et al. 1976). This suggests that the cells at the lower dilution rates were somewhat physiologically impaired (i.e., both N and P limited) and not able to take NH_4^+ down to very low nanomolar concentrations. Dekaezemacker and Bonnet (2011) reported an increase of up to 100 nmol L^{-1} of NH_4^+ at the end of the dark period for *C. watsonii* that grew in the NH_4^+-free medium, indicating that NH_4^+ is excreted by *C. watsonii.* This supports our observation of a detectable amount of NH_4^+ in the NH_4^+-free culture and higher concentrations of NH_4^+ at lower dilution rates.

Surprisingly, N-specific growth rates were considerably higher than dilution rates. It is possible that the co-occurrence of NH_4^+ uptake and N_2 fixation as N assimilation pathways and their interaction may explain these higher N-specific growth rates. Since P-specific growth rates were similar to dilution rates, the cellular P budget was adequately estimated.

Summary of N budget—With increasing N availability (i.e., increasing dilution rate) there were fewer cells, but surprisingly, N, P, and Chl per biovolume showed little change and, hence, no evidence of N limitation at low dilution rates based on cellular composition. However, the higher ambient NH_4^+ concentrations at the lowest dilution rates and the onset of P deficiency (i.e., high APA activity) suggest that these cells were more physiologically impaired. As dilution rate increased, ambient NH_4^+ decreased, while PO_4^{3-} increased as expected under N limitation. At the higher dilution rates, ammonium uptake increased, but N_2 fixation was surprisingly constant. Since NH_4^+ uptake increased eightfold, total N assimilation (NH_4^+ plus N_2 fixation) increased, as did DON excretion, but as a percentage of excretion with respect to total N assimilation, DON excretion was small and nearly constant over the range of dilution rates. Therefore, much of the NH_4^+ taken up was utilized for growth, and little was excreted at higher dilution rates. In contrast, with no NH_4^+ uptake (i.e., in N-free medium) and only N_2 fixation, cell numbers, cell volume, and cellular N, P, and Chl *a* were very low and excretion as a percentage of total N assimilated was very high, suggesting that these N-stressed cells retain little of the N_2 that is fixed when they are N stressed.

Effect of bacterial contamination—The cultures that we used were not axenic, and the possibility of heterotrophic bacterial uptake of ammonium in our analysis was not excluded. However, cell number of heterotrophic bacteria was stable at each dilution rate (data not shown), indicating that the growth rate of heterotrophic bacteria was constant and similar at each dilution rate. Furthermore, the cell number of heterotrophic bacteria was consistently 4 orders of magnitude lower than that of *C. watsonii* at each dilution rate, and the daily N requirement of heterotrophic bacteria estimated from cell density, cell size, and cellular C : N ratio and its growth rate ranged from 2.7 to 9.5 pmol N L^{-1} d^{-1}. These N requirements were 6 orders of magnitude lower than those of *C. watsonii* (3.4–7.2 μmol N L^{-1} d^{-1}), as determined from Table 1. Therefore, the possible influence of heterotrophic bacterial contamination on N uptake and N fixation in *C. watsonii* cells was considered to be negligible.

Ecological implications—The present study demonstrates a fundamental difference in N_2 fixation of this nanocyanobacteria compared to that of other larger marine diazotrophs (i.e., N_2 fixation is not inhibited by the presence of nanomolar concentrations of NH_4^+) and that N_2 fixation is independent of NH_4^+ availability and growth activity. This difference may be that other larger diazotrophs reduce their N_2 fixation activity in the presence of much higher (i.e., micromolar) DIN concentrations, while ambient nanomolar concentrations were present in our cultures. In addition, our results clearly showed a significant contribution of NH_4^+ uptake to the total N assimilation, even at nanomolar ambient NH_4^+ concentrations. These results suggest that *C. watsonii* assimilates nitrogen by both N_2 fixation and NH_4^+ uptake when it is available in the oligotrophic ocean and that it contributes to global biogeochemical nitrogen budgets.

Acknowledgments

We thank Satoshi Kitajima for providing an enrichment culture of *Crocosphaera watsonii* used in the present study, Takuhei Shiozaki for his assistance with the isotopic analysis, and Mitsuhide Sato for his analysis of alkaline phosphatase activity. We appreciate two anonymous reviewers for constructive comments on the manuscript. This study was financially supported by Ministry of Education, Culture, Sports, Science and Technology (MEXT) Grants-in-Aid for Scientific Research on Priority Areas (21014006) and on Innovative Areas (New Ocean Paradigm on Its Biogeochemistry, Ecosystem and Sustainable Use [NEOPS]: 24121001 and 24121005).

References

Agawin, N. S. R., S. Rabouille, M. J. W. Veldhuis, L. Servatius, S. Hol, H. M. J. van Overzee, and J. Huisman. 2007. Competition and facilitation between unicellular nitrogen-fixing cyanobacteria and non-nitrogen-fixing phytoplankton species. Limnol. Oceanogr. **52:** 2233–2248, doi:10.4319/lo.2007.52.5.2233

Capone, D. G., M. D. Ferrier, and E. J. Carpenter. 1994. Amino acid cycling in colonies of the planktonic marine cyanobacterium *Trichodesmium thiebautii*. Appl. Environ. Microbiol. **60:** 3989–3995.

Dekaezemacker, J., and S. Bonnet. 2011. Sensitivity of N_2 fixation to combined nitrogen forms (NO_3^- and NH_4^+) in two strains of the marine diazotroph *Crocosphaera watsonii* (Cyanobacteria). Mar. Ecol. Prog. Ser. **438:** 33–46, doi:10.3354/meps09297

Dixon, R., and D. Kahn. 2004. Genetic regulation of biological nitrogen fixation. Nat. Rev. Microbiol. **2:** 621–631, doi:10.1038/nrmicro954

Dron, A., S. Rabouille, P. Claquin, B. Le Roy, A. Talec, and A. Sciandra. 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, doi:10.1111/j.1462-2920.2011.02675.x

FINZI-HART, J. A., AND OTHERS. 2009. Fixation and fate of C and N in the cyanobacterium *Trichodesmium* using nanometer-scale secondary ion mass spectrometry. Proc. Natl. Acad. Sci. USA **106:** 6345–6350, doi:10.1073/pnas.0810547106

FLYNN, K. J., AND J. R. GALLON. 1990. Changes in intracellular and extracellular α-amino acids in *Gloeothece* during N_2-fixation and following addition of ammonium. Arch. Microbiol. **153:** 574–579, doi:10.1007/BF00245267

HANSEN, H. F., AND F. KOROLEFF. 1999. Determination of nutrients, p. 159–228. *In* K. Grasshoff, K. Kremling, and M. Ehrhardt [eds.], Methods of seawater analysis, 3rd ed. Wiley-VCH.

HARRISON, P. J., H. L. CONWAY, AND R. C. DUGDALE. 1976. Marine diatoms grown in chemostats under silicate or ammonium limitation. I. Cellular chemical composition and steady-state growth kinetics of *Skeletonema costatum*. Mar. Biol. **35:** 177–186.

HASHIHAMA, F., K. FURUYA, S. KITAJIMA, S. TAKEDA, T. TAKEMURA, AND J. KANDA. 2009. Macro-scale exhaustion of surface phosphate by dinitrogen fixation in the western North Pacific. Geophys. Res. Lett. **36:** L03610, doi:10.1029/2008GL036866

HEALEY, F. P., AND L. L. HENDZEL. 1979. Fluorometric measurement of alkaline phosphatase activity in algae. Freshw. Biol. **9:** 429–439, doi:10.1111/j.1365-2427.1979.tb01527.x

HOLL, C. M., AND J. P. MONTOYA. 2005. Interactions between nitrate uptake and nitrogen fixation in continuous cultures of the marine diazotroph *Trichodesmium* (Cyanobacteria). J. Phycol. **41:** 1178–1183, doi:10.1111/j.1529-8817.2005.00146.x

———, AND ———. 2008. Diazotrophic growth of the marine cyanobacterium *Trichodesmium* IMS101 in continuous culture: Effects of growth rate on N_2-fixation rate, biomass, and C:N:P stoichiometry. J. Phycol. **44:** 929–937, doi:10.1111/j.1529-8817.2008.00534.x

KARL, D., R. LETELIER, L. TUPAS, J. DORE, J. CHRISTIAN, AND D. HEBEL. 1997. The role of nitrogen fixation in biogeochemical cycling in the subtropical North Pacific Ocean. Nature **388:** 533–538, doi:10.1038/41474

KITAJIMA, S., K. FURUYA, F. HASHIHAMA, S. TAKEDA, AND J. KANDA. 2009. Latitudinal distribution of diazotrophs and their nitrogen fixation in the tropical and subtropical western North Pacific. Limnol. Oceanogr. **54:** 537–547, doi:10.4319/lo.2009.54.2.0537

KNAPP, A. N., J. DEKAEZEMACKER, S. BONNET, J. A. SOHM, AND D. G. CAPONE. 2012. Sensitivity of *Trichodesmium erythraeum* and *Crocosphaera watsonii* abundance and N_2 fixation rates to varying NO_3^- and PO_4^{3-} concentrations in batch cultures. Aquat. Microb. Ecol. **66:** 223–236, doi:10.3354/ame01577

KODAMA, T., K. FURUYA, F. HASHIHAMA, S. TAKEDA, AND J. KANDA. 2011. Occurrence of rain-origin nitrate patches at the nutrient-depleted surface in the East China Sea and the Philippine Sea during summer. J. Geophys. Res. **116:** C08003, doi:10.1029/2010JC006814

KRAUK, J. M., T. A. VILLAREAL, J. A. SOHM, J. P. MONTOYA, AND D. G. CAPONE. 2006. Plasticity of N:P ratios in laboratory and field populations of *Trichodesmium* spp. Aquat. Microb. Ecol. **42:** 243–253, doi:10.3354/ame042243

MOHR, W., T. GROSSKOPF, D. W. R. WALLACE, AND J. LAROCHE. 2010. Methodological underestimation of oceanic nitrogen fixation rates. Plos One **5:** e12583, doi:10.1371/journal.pone.0012583

MOISANDER, P. H., AND OTHERS. 2010. Unicellular cyanobacterial distributions broaden the oceanic N_2 fixation domain. Science **327:** 1512–1514, doi:10.1126/science.1185468

MONTOYA, J. P., C. M. HOLL, J. P. ZEHR, A. HANSEN, T. A. VILLAREAL, AND D. G. CAPONE. 2004. High rates of N_2 fixation by unicellular diazotrophs in the oligotrophic Pacific Ocean. Nature **430:** 1027–1031, doi:10.1038/nature02824

———, M. VOSS, P. KAHLER, AND D. G. CAPONE. 1996. A simple, high-precision, high-sensitivity tracer assay for N_2 fixation. Appl. Environ. Microbiol. **62:** 986–993.

MULHOLLAND, M. R., AND P. W. BERNHARDT. 2005. The effect of growth rate, phosphorus concentration, and temperature on N_2 fixation carbon fixation, and nitrogen release in continuous culture of *Trichodesmium* IMS101. Limnol. Oceanogr. **50:** 839–849, doi:10.4319/lo.2005.50.3.0839

———, D. A. BRONK, AND D. G. CAPONE. 2004. Dinitrogen fixation and release of ammonium and dissolved organic nitrogen by *Trichodesmium* IMS101. Aquat. Microb. Ecol. **37:** 85–94, doi:10.3354/ame037085

———, AND D. G. CAPONE. 1999. Nitrogen fixation, uptake and metabolism in natural and cultured populations of *Trichodesmium* spp. Mar. Ecol. Prog. Ser. **188:** 33–49, doi:10.3354/meps188033

MULLINEAUX, P. M., A. E. CHAPLIN, AND J. R. GALLON. 1983. Synthesis of nitrogenase in the cyanobacterium *Gloeothece* (*Gloeocapsa*) sp. CCAP1430/3. J. Gen. Microbiol. **129:** 1689–1696.

PRUFERT-BEBOUT, L., H. W. PAERL, AND C. LASSEN. 1993. Growth, nitrogen fixation, and spectral attenuation in cultivated *Trichodesmium* species. Appl. Environ. Microbiol. **59:** 1367–1375.

RAI, A. N., M. BORTHAKUR, AND B. BERGMAN. 1992. Nitrogenase derepression, its regulation and metabolic changes associated with diazotrophy in the nonheterocystous cyanobacterium *Plectonema boryanum* PCC-73110. J. Gen. Microbiol. **138:** 481–491, doi:10.1099/00221287-138-3-481

REDDY, K. J., J. B. HASKELL, D. M. SHERMAN, AND L. A. SHERMAN. 1993. Unicellular, aerobic nitrogen-fixing cyanobacteria of the genus *Cyanothece*. J. Bacteriol. **175:** 1284–1292.

RICE, D., B. J. MAZUR, AND R. HASELKORN. 1982. Isolation and physical mapping of nitrogen fixation genes from the cyanobacterium *Anabaena* 7120. J. Biol. Chem. **257:** 13157–13163.

SHI, T., I. ILIKCHYAN, S. RABOUILLE, AND J. P. ZEHR. 2010. Genome-wide analysis of diel gene expression in the unicellular N_2-fixing cyanobacterium *Crocosphaera watsonii* WH 8501. ISME J. **4:** 621–632, doi:10.1038/ismej.2009.148

SHIOZAKI, T., K. FURUYA, T. KODAMA, AND S. TAKEDA. 2009. Contribution of N_2 fixation to new production in the western North Pacific Ocean along 155°E. Mar. Ecol. Prog. Ser. **377:** 19–32, doi:10.3354/meps07837

STRICKLAND, J. D. H., AND T. R. PARSONS. 1972. A practical handbook of seawater analysis, 2nd ed. Fisheries Research Board of Canada.

WELSH, E. A., AND OTHERS. 2008. The genome of *Cyanothece* 51142, a unicellular diazotrophic cyanobacterium important in the marine nitrogen cycle. Proc. Natl. Acad. Sci. USA **105:** 15094–15099, doi:10.1073/pnas.0805418105

ZEHR, J. P., AND OTHERS. 2001. Unicellular cyanobacteria fix N_2 in the subtropical North Pacific Ocean. Nature **412:** 635–638, doi:10.1038/35088063

Associate editor: Heidi M. Sosik

Received: 03 March 2013
Accepted: 23 July 2013
Amended: 24 July 2013