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Sensitivity of N_2 fixation to combined nitrogen forms (NO_3^- and NH_4^+) in two strains of the marine diazotroph *Crocosphaera watsonii* (Cyanobacteria)

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ABSTRACT: Despite the increasing recognition of the biogeochemical importance of marine unicellular diazotrophic cyanobacteria, the environmental factors controlling their distribution and activity in the ocean are still very poorly understood. In particular, very few studies have considered the effect of combined nitrogen (N) on unicellular diazotroph activity, although their presence has been reported in surface waters exhibiting micromolar concentrations of dissolved inorganic nitrogen (DIN). Here, we studied the effect of nitrate (NO_3^-) and ammonium (NH_4^+) additions (0.2 to 10 µmol l^{-1}) on N_2 fixation and N uptake in 2 strains of *Crocosphaera watsonii* (WH8501 and WH0003) maintained in batch cultures in an N-free medium (YBC II) and a 12 h light:12 h dark cycle. Compared with the untreated control, increasing concentrations of NH_4^+ to 10 µmol l^{-1} rapidly inhibited night-integrated N_2 fixation by up to 36 and 83% for WH8501 and WH0003, respectively. In contrast, night-integrated N_2 fixation was not significantly inhibited at any NO_3^- concentration. These results contrast with data obtained on *Trichodesmium* spp. (Cyanobacteria) in which night-integrated N_2 fixation is inhibited up to 50% at the same NO_3^- concentrations. This study indicates that *C. watsonii* is able to take up combined nitrogen while keeping its ability to fix dinitrogen at high rates.

KEY WORDS: *Crocosphaera watsonii* · Cyanobacteria · Inhibition · N_2 fixation · Ammonium · Nitrate · Nitrogen uptake

INTRODUCTION

There has been considerable debate concerning the state of the oceanic nitrogen (N) budget over the last decades (Gruber & Sarmiento 1997, Codispoti et al. 2001, Gruber 2004). This debate comes from uncertainties on biological processes driving the main sink and source terms of fixed N in the ocean. Sink terms are dominated by biological processes converting fixed N to dinitrogen (N_2) (through denitrification and anammox [anaerobic ammonium oxidation] mainly occurring in N-rich Oxygen Minimum Zones, OMZs), and source terms are dominated by biological N_2 fixation (the reduction of N_2 into the bioavailable form of ammonium, NH_4^+). Estimates using field data suggest that marine N_2 fixation (~125–160 Tg N yr^{-1}) only compensates for ~25 to 50% of N-loss processes (~165–400 Tg N yr^{-1}) (Gruber & Sarmiento 1997, Brandes & Devol 2002, Codispoti 2007). However, new N_2-fixing organisms and ecological niches suitable for N_2 fixation are being discovered, which may lead to increased global N_2 fixation estimates; most research efforts dedicated to marine N_2 fixation before 2000 mainly focused on the filamentous cyanobacterium *Trichodesmium* (Carpenter 1983, Capone et al. 1997), and the conventional view was that N_2 fixation takes place in warm oligotrophic N-depleted surface waters (Karl et al. 1992, Mahaffey et al. 2005). Other planktonic organisms such as unicellular cyanobacteria, heterotrophic eubacteria and archaea

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were then found to express the *nifH* gene encoding the nitrogenase enzyme catalyzing N_2 fixation (Zehr et al. 1998, 2001, Braun et al. 1999), and N_2 fixation rates associated with these organisms have been recognized to be equal to or exceed those reported for *Trichodesmium* spp. (Montoya et al. 2004). Unicellular diazotrophs account for 75% of total activity in coastal and oceanic waters of the south west and equatorial Pacific (Biegala & Raimbault 2008, Bonnet et al. 2009). More recently, biological and geochemical studies have reported the presence of active diazotrophs in more diverse and previously unexpected environments, some containing micromolar levels of combined nitrogen (1.5 to 4 µmol NO_3^- l^{-1}) (Short & Zehr 2007, Bonnet et al. 2011, Webb et al. 2009) such as High Nutrient Low Chlorophyll (HNLC) waters or above OMZs in upwelling systems (Deutsch et al. 2007, Zehr & Kudela 2011, Fernandez et al. 2011) where sinks of N may be spatially coupled with N_2 fixation in waters exhibiting micromolar nitrate (NO_3^-) concentrations.

Despite the increasing recognition of the biogeochemical importance of unicellular diazotrophic organisms, their role is still not explicitly taken into account in most global biogeochemical budgets and models. Moreover, the environmental factors controlling their distribution and activity in the ocean are still very poorly known. In particular, few studies have considered the effect of combined nitrogen (NH_4^+ or NO_3^-) on unicellular diazotroph activity, although the presence of such organisms has been reported in N-rich waters and in oligotrophic gyres (e.g. Church et al. 2005) in which seasonal mixing, vertical diffusion and atmospheric inputs represent a substantial source of new N, potentially inhibiting the N_2 fixation activity. The objectives of this study were (1) to determine the threshold of N_2 fixation inhibition under environmentally relevant concentrations of dissolved inorganic nitrogen (DIN) such as NO_3^- and NH_4^+, and (2) to investigate the ability of unicellular diazotrophs to take up these combined nitrogen forms.

We performed this study on the unicellular diazotrophic cyanobacterium *Crocosphaera watsonii*. This nanoplanktonic strain was chosen as a good representative of marine unicellular diazotrophs. Furthermore *Crocosphaera watsonii* WH8501 has the genomic potential to assimilate NH_4^+, and reduce NO_3^- and NO_2^- (Hewson et al. 2009). It is widespread in the Pacific (Zehr et al. 2001, Campbell et al. 2005, Church et al. 2008) and in the North Atlantic (Langlois et al. 2008), and is one of the main models of marine unicellular diazotrophs to date available in culture.

MATERIALS AND METHODS

Culture conditions

Two unialgal cultures of *Crocosphaera watsonii* WH8501 (Waterbury & Rippka 1989, Zehr & Turner 2001) and WH0003 (Webb et al. 2009) were grown in batch cultures, with a 12 h light:12 h dark cycle, a daytime photon flux of 180 µmol photons m^{-2} s^{-1} and a temperature of 27°C, which has been reported as optimal for the growth of these 2 strains (Webb et al. 2009). Both strains were cultivated in triplicate in artificial seawater N-free YBC II medium (50 µM PO_4^{3-}, Chen et al. 1996). Each culture was grown in a 2 l Nalgene® polycarbonate magnetic culture vessel (Nalgene Nunc International) fitted with a tetrafluoroethylene stir bar gently mixing the culture. The initial culture volume was 1.5 l. The cultures were not axenic, but standard sterilization procedures and manipulation under laminar flow hoods were followed to minimize contamination. We checked that bacteria present in the medium did not show any diazotrophic activity by filtering culture through polycarbonate 2.0 µm porosity filters and measuring acetylene reduction activity in the filtrate. Bacteria did not show any N_2 fixation activity and thus did not interfere with the *C. watsonii* N_2 fixation. Moreover, most of the carbon biomass and biovolume in the cultures was associated with *C. watsonii* (99.5 and 99.8%, respectively, for the strain WH8501, and 99.9 and 99.9%, respectively, for the strain WH0003).

Experimental procedure

The growth rates of the 2 strains were monitored for 23 d using *in vivo* chlorophyll *a* fluorescence (model Trilogy, Turner Designs) and cell abundances (flow cytometry, MoFlo cell sorter, Dako). At the beginning of the exponential growth phase of each strain, 700 ml of each triplicate vessel were taken and mixed together. The final volume (2.1 l for each strain) was divided among 9 sterile culture flasks to which different amounts of inorganic nitrogen were added (as 2 mmol l^{-1} solution of either $NaNO_3$ [99.99 Suprapur, Merck] or NH_4Cl [99.995 Suprapur, Merck]), in order to reach final concentrations of 0.2, 1, 5 and 10 µmol l^{-1} and facilitate comparisons with experiments performed on *Trichodesmium* spp. (Holl & Montoya 2005). For each strain, vessels were left untreated as controls. These additions were made 90 min before the start of the dark period (i.e. before synthesis of the enzyme nitrogenase catalysing N_2 fixation, Shi et al. 2010). Immedi-

ately after nitrogen additions, samples were collected in duplicates from each vessel for acetylene reduction assays performed every 2 h in duplicates. Samples were also collected every 2 h for triplicate measurements of NH_4^+ and NO_3^- concentrations. Finally, samples were collected for cell abundance determination 3 times during the night.

N_2 fixation analyses

Nitrogenase activity was measured by the acetylene reduction technique (Capone 1993) in replicate flasks. Incubations were performed in 25 ml vials fitted with Teflon-lined septum caps. Vials were filled with 15 ml of culture and 1 ml of acetylene was added in the 10 ml head space (10% gaseous fraction). Two vials for each condition were incubated under the same light and temperature conditions as the original culture. Every 2 h, the ethylene concentration in the head space was measured in duplicate in each vial (4 per condition, n = 2) by gas chromatography using an Agilent µ-GC 200 equipped with a Flame Ionization Detector. Acetylene reduction rates were calculated for each 2 h interval using a reduction ratio (C_2H_2:N_2) of 4:1 to convert acetylene reduction to N_2 fixation rates (Capone & Montoya 2001). The rates were then normalized per cell using the flow cytometry data.

Nutrient analyses

Samples for NH_4^+ analysis were collected in 40 ml SHOTT flasks and analyzed by the fluorescent method as described in Holmes et al. (1999) using a Trilogy fluorometer (Turner Designs, CDOM module) (detection limit = 11 nmol l^{-1}).

Samples for NO_3^- concentrations were collected in acid-washed 20 ml polyethylene flasks and immediately poisoned with 200 µl $HgCl_2$ (i.e. final concentration of 20 µg ml^{-1}) (Kirkwood 1992) and stored at 4°C until analysis using an AutoAnalyzer 3 Digital Colorimeter (Bran Luebbe) according to standard automated colorimetric methods (Aminot & Kerouel 2007) (detection limit = 69 nmol l^{-1}).

Cell analyses

Samples for flow cytometry were taken at the beginning, middle and end of the dark period to check the stability of cell concentrations during the experimental period: 1.8 ml of culture was fixed with 0.2 ml of 30% glutaraldehyde and counted using a MoFlo.

For carbon (C) and N contents, 13 ml of culture were filtered 9 h after the beginning of the dark period through precombusted (6 h, 450°C) Whatman GF/F filters (diameter 25 mm, porosity ≈ 0.7 µm), using a low vacuum pressure (<100 mm Hg). Following filtrations, filters were placed into 2 ml precombusted glass vials, dried for 48 h at 55°C and stored until analysis. Analyses were performed on an Integra-CN mass spectrometer calibrated using glycine every 10 samples.

Cell specific uptake rates of NH_4^+ and NO_3^- were calculated from the rates of disappearance of NH_4^+ and NO_3^- from the medium and then normalized to cell abundances obtained by flow cytometry analysis.

Statistical analyses

For each control and nitrogen treatment, cell abundance means were compared between the 3 time points using a 1-way ANOVA and a Fisher's protected least significant difference test (n = 3, df = 8). For each nitrogen treatment, maximum N_2 fixation means and night-integrated N_2 fixation were compared to the control mean and the control night-integrated N_2 fixation using a paired Student's *t*-test (4 measurements, n = 2, df = 1).

RESULTS

N_2 fixation: effect of NH_4^+

In the control treatment of the WH8501 strain (no nitrogen added), nitrogenase activity started 4 h after the beginning of the dark period, and reached a maximum of 10×10^{-7} nmol N_2 $cell^{-1}$ h^{-1} 9.3 h after the onset of the dark period (Fig. 1A). Nitrogenase activity remained detectable at the beginning of the light period but decreased quickly to become undetectable 2.8 h after the beginning of the day. We observed a measurable inhibition of maximum N_2 fixation with NH_4^+ additions, except at the lower end of the range of NH_4^+ additions (0.2 µmol l^{-1}) (Fig. 1B); the maximum N_2 fixation rate decreased significantly (*t*-test, $p < 0.05$) by 11.5 ± 1.2% (8.9×10^{-7} nmol N_2 $cell^{-1}$ h^{-1}), 8.1 ± 0.5% (9.3×10^{-7} nmol N_2 $cell^{-1}$ h^{-1}) and 37.7 ± 3.9% (6.3×10^{-7} nmol N_2 $cell^{-1}$ h^{-1}) relative to the control after 1, 5 and 10 µmol l^{-1} of NH_4^+ were added, respectively (Table 1, Fig. 1C–E).

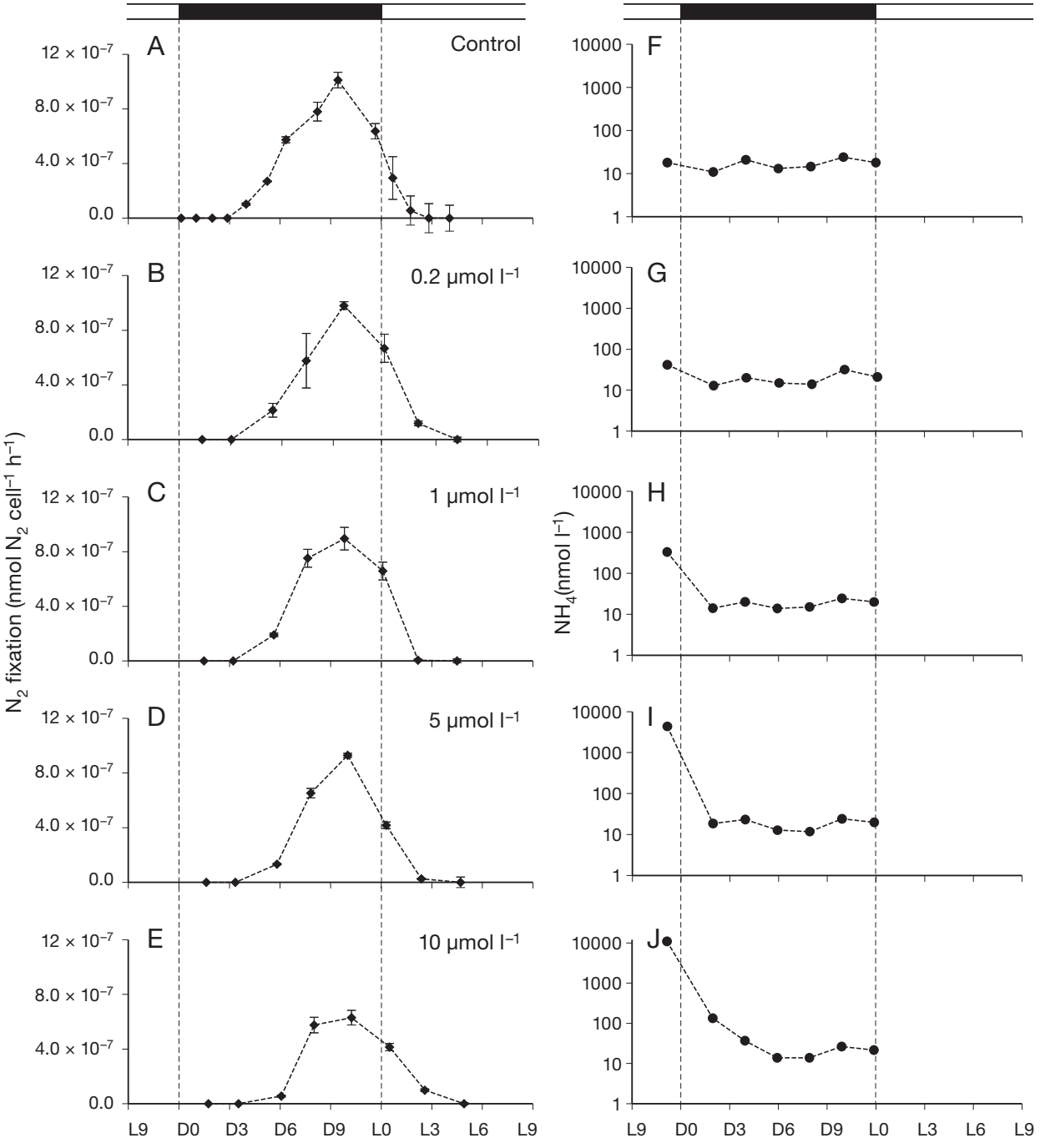

Fig. 1. *Crocosphaera watsonii*. Effect of NH_4^+ additions on N_2 fixation rates (left panels) through the night period in cultures of strain WH8501 (mean ± SD, n = 4) and on NH_4^+ concentrations (right panels) for (A,F) untreated condition, for addition of (B,G) 0.2 µmol l^{-1}, (C,H) 1 µmol l^{-1}, (D,I) 5 µmol l^{-1} and (E,J) 10 µmol l^{-1}. The *x*-axis represents the time: L = light, D = dark, followed by the corresponding hour after the beginning of the light or dark cycle . The *y*-axis of the right panels is a decimal logarithmic scale adjusted for the NH_4^+ concentration in the medium

In the control treatment of the strain WH0003, the N_2 fixation cycle exhibited a similar pattern compared to strain WH8501. N_2 fixation started 4.1 h after dark, reached a maximum of 10.5×10^{-7} nmol N_2 $cell^{-1}$ h^{-1} after 9.1 h of darkness, and decreased quickly with light but was still detectable 1.4 h after the onset of the light period (Fig. 2A). However, the effect of NH_4^+ additions on this strain was more

Table 1. *Crocosphaera watsonii*. Inhibition of N_2 fixation maximum and night-integrated N_2 fixation (mean ± SD) of strains WH8501 and WH0003 under different dissolved inorganic nitrogen (DIN) enrichments (paired Student's *t*-test, n = 2, df = 1). *Significantly different compared to control condition ($p < 0.05$)

Strain	DIN addition		Inhibition of N_2fixation maximum (%)	Inhibition of night-integrated N_2 fixation (%)
	Type	Final concentration (µmol l^{-1})		
WH8501	NH_4^+	0.2	3.0 ± 0.2	–
		1	11.5 ± 1.2*	5.6 ± 0.1*
		5	8.1 ± 0.5*	21.5 ± 0.6*
		10	37.7 ± 3.9*	36.9 ± 0.8*
	NO_3^-	0.2	5.1 ± 0.5	–
		1	5.1 ± 0.3	–
		5	23.8 ± 3.3*	–
		10	11.8 ± 0.9*	–
WH0003	NH_4^+	0.2	–	–
		1	20.8 ± 2.7*	1.2 ± 0.1*
		5	40.6 ± 12.3*	37.3 ± 2.6*
		10	80.0 ± 24.7*	83.3 ± 4.1*
	NO_3^-	0.2	14.2 ± 2.1	–
		1	10.8 ± 1.9	–
		5	3.6 ± 0.8	–
		10	–	5.0 ± 0.2

important in terms of intensity of inhibition and synchronization of nitrogenase activity than that observed for the strain WH8501 (Fig. 2B–E). The N_2 fixation maximum was significantly (*t*-test, $p < 0.05$) inhibited by 20.8 ± 2.7% (8.3×10^{-7} nmol N_2 $cell^{-1}$ h^{-1}), 40.6 ± 12.3% (6.2×10^{-7} nmol N_2 $cell^{-1}$ h^{-1}) and 80.0 ± 24.7% (2.1×10^{-7} nmol N_2 $cell^{-1}$ h^{-1}) relative to the control after 1, 5 and 10 µmol NH_4^+ l^{-1} additions, respectively (Table 1). Moreover, we noticed in the 10 µmol NH_4^+ l^{-1} treatment a clear delay in the onset of the nitrogenase activity, which started 5.8 h later than in the untreated control.

N_2 fixation: effect of NO_3^-

Experiments indicate that NO_3^- additions only affected N_2 fixation for the WH8501 strain in the 5 and 10 µmol NO_3^- l^{-1} treatments. In these cases, the maximum values of N_2 fixation decreased significantly (*t*-test, $p < 0,05$) by 23.8 ± 3.3% (7.7×10^{-7} nmol N_2 $cell^{-1}$ h^{-1}) and 11.8 ± 0.9% (8.9×10^{-7} nmol N_2 $cell^{-1}$ h^{-1}) relative to the control (Table 1, Fig. 3D–E). This maximum occurred 2 h after the control maximum (Fig. 3D–E).

For the WH0003 strain, the N_2 fixation maxima did not show any significant decrease, but a delay of 3.5 h after the control maximum in the 0.2 and 1 µmol NO_3^- l^{-1} treatments, and a 1.8 h delay in the 5 and 10 µmol NO_3^- l^{-1} treatments was observed (Fig. 4B–E).

When considering the total N_2 fixation integrated over the dark period, our results indicate that NO_3^- additions did not cause any significant inhibition for the 2 strains (Table 1).

N uptake

Experiments performed on strain WH8501 indicate that the initial concentration of NH_4^+ was 20 nmol l^{-1} at the beginning of the experiment (Fig. 1F). The added NH_4^+ began being consumed within 3 h after the additions of 0.2, 1 and 5 µmol NH_4^+ l^{-1} (Fig. 1G–I), and within 5 h for +10 µmol NH_4^+ l^{-1} (Fig. 1J), and decreased to the original background concentration of 20 nmol l^{-1} at the end of the experiment in all treatments (Fig. 1G–J).

Experiments performed on strain WH0003 show that the concentration of NH_4^+ was undetectable at the beginning of the experiment and increased throughout the dark period to reach 100 nmol l^{-1} at the beginning of the light period (Fig. 2F). The added NH_4^+ began being consumed within 3 h after the addition of 0.2 and 1 µmol NH_4^+ l^{-1} (Fig. 2G–H), and within 7 h for the 5 and 10 µmol NH_4^+ l^{-1} additions (Fig. 2I–J), reaching the same concentration (10 to 20 nmol l^{-1}) as was observed in the control during the middle of the dark period (Fig. 2G–J). In all treatments, concentrations returned to 100 nmol l^{-1} by the end of the night.

For both strains, NH_4^+ did not only affect the intensity and the timing of the maximum of N_2 fixation, but also affected the total integrated N_2 fixed during the dark cycle (Table 1, Fig. 5A). The inhibitory effect of NH_4^+ on night-integrated N_2 fixation was more important for the strain WH0003 compared to WH8501; for example, an addition of 10 µmol NH_4^+ l^{-1} significantly inhibited (*t*-test, $p < 0.05$) the night-integrated N_2 fixation of the strain WH8501 by 36.9 ± 0.8%, whereas the strain WH0003 was inhibited by 83.3 ± 4.1% (Table 1, Fig. 5A).

Cell specific uptake rates of NH_4^+ were similar for both strains for the 0.2 to 5 µmol NH_4^+ l^{-1} additions (Fig. 5B). However, the strain WH0003 exhibited higher uptake rates for the 10 µmol NH_4^+ l^{-1} addition, with a cell specific uptake rate of 2.8×10^{-3} nmol N $cell^{-1}$ h^{-1} compared to the strain WH8501, for which the uptake rate for the 10 µmol l^{-1} addition was 1.5×10^{-3} nmol N $cell^{-1}$ h^{-1} (Fig. 5B).

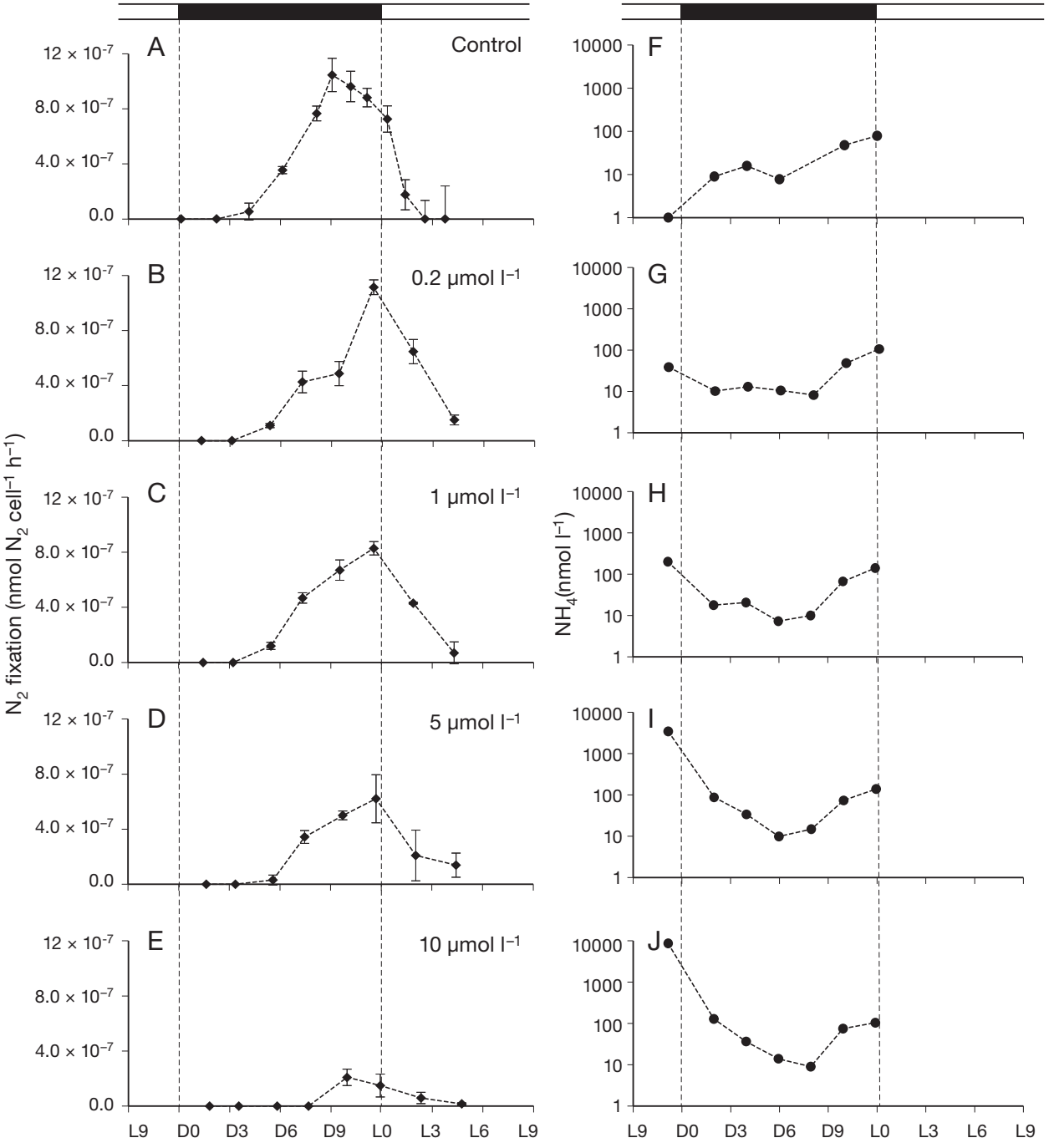

Fig. 2. *Crocosphaera watsonii*. Effect of NH_4^+ additions on N_2 fixation rates (left panels) through the night period in cultures of strain WH0003 (mean ± SD, n = 4) and on NH_4^+ concentrations (right panels) for (A,F) untreated condition, for addition of (B,G) 0.2 µmol l^{-1}, (C,H) 1 µmol l^{-1}, (D,I) 5 µmol l^{-1} and (E,J) 10 µmol l^{-1}. The *x*-axis represents the time: L = light, D = dark, followed by the corresponding hour after the beginning of the light or dark cycle. The *y*-axis of the right panels is a decimal logarithmic scale adjusted for the NH_4^+ concentration in the medium

Experiments performed with NO_3^- indicate slower or incomplete consumption of NO_3^- over the course of the experiment compared with NH_4^+. For the strain WH8501, NO_3^- was completely consumed after 0.5, 3.5, and 7.5 h for additions of 0.2, 1 and 5 µmol NO_3^- l^{-1}, respectively (Fig. 3G–I). In the 10 µmol l^{-1} treatment, NO_3^- was not exhausted and 4 µmol NO_3^- l^{-1} was still remaining in the medium 11.5 h after the initial addition (Fig. 3J).

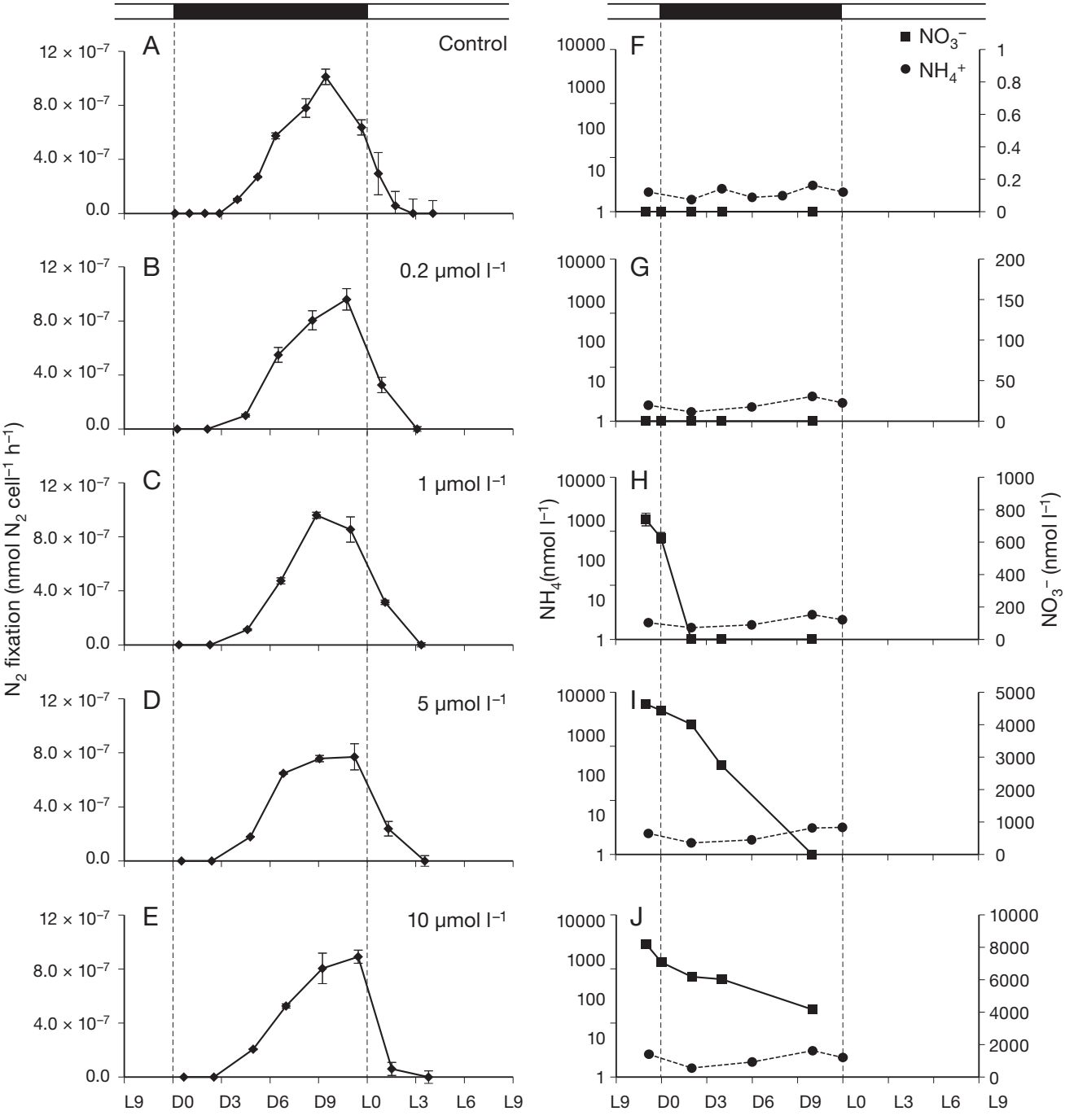

Fig. 3. *Crocosphaera watsonii*. Effect of NO_3^- additions on N_2 fixation rates (left panels) through the night period in cultures of strain WH8501 (mean ± SD, n = 4), and on NH_4^+ concentrations (dashed curves) and NO_3^- concentrations (solid lines) (right panels) for (A,F) untreated condition, for addition of (B,G) 0.2 µmol l^{-1}, (C,H) 1 µmol l^{-1}, (D,I) 5 µmol l^{-1} and (E, J) 10 µmol l^{-1}. The *x*-axis represents the time: L = light, D = dark, followed by the corresponding hour after the beginning of the light or dark cycle

For the strain WH0003, NO_3^- was completely consumed after 1.5, 3.5, 7.5 and 11.5 h, respectively for the 0.2, 1, 5 and 10 µmol NO_3^- l^{-1} treatments (Fig. 4G–J). Similar to NH_4^+ uptake, cell specific uptake rates of NO_3^- increased with increasing amount of NO_3^-, except for the strain WH8501 at 5 µmol NO_3^- l^{-1}, where uptake capacities reached a saturation at 5.1 × 10^{-4} nmol N $cell^{-1}$ h^{-1}. For the 10 µmol NO_3^- l^{-1} addition for strain WH0003, the uptake rate still increased to a maximum of 2.0×10^{-3} nmol N $cell^{-1}$ h^{-1} (Fig. 5C).

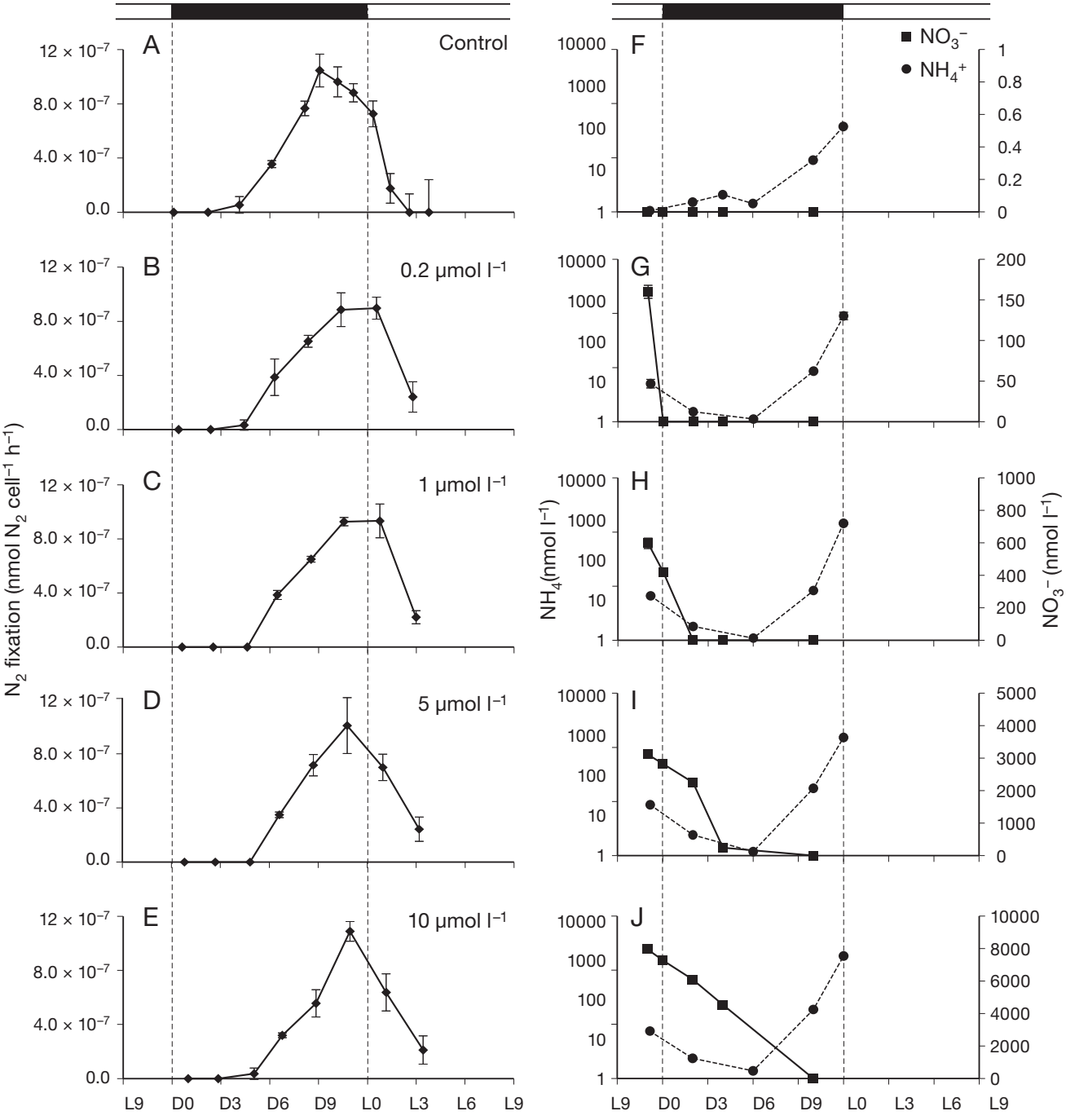

Fig. 4. *Crocosphaera watsonii*. Effect of NO_3^- additions on N_2 fixation rates (left panels) through the night period in cultures of strain WH0003 (mean ± SD, n = 4), and on NH_4^+ concentrations (dashed curves) and NO_3^- concentrations (solid lines) (right panels) for (A,F) untreated condition, for addition of (B,G) 0.2 µmol l^{-1}, (C,H) 1 µmol l^{-1}, (D,I) 5 µmol l^{-1} and (E, J) 10 µmol l^{-1}. The *x*-axis represents the time: L = light, D = dark, followed by the corresponding hour after the beginning of the light or dark cycle

It should be noted that the NO_3^- additions did not result in any NH_4^+ contamination or excretion for the experiments performed with the strain WH8501 (Fig. 3G–J). NH_4^+ concentrations remained constant (20 nmol l^{-1}) regardless of the amount of NO_3^- added. In experiments performed with the strain WH0003, NH_4^+ concentrations increased up to 40 nmol l^{-1} with NO_3^- additions, possibly due to a small contamination with NH_4^+ (Fig. 4G–J), although the NH_4^+ concentration decreased within 3 h after addition to return to

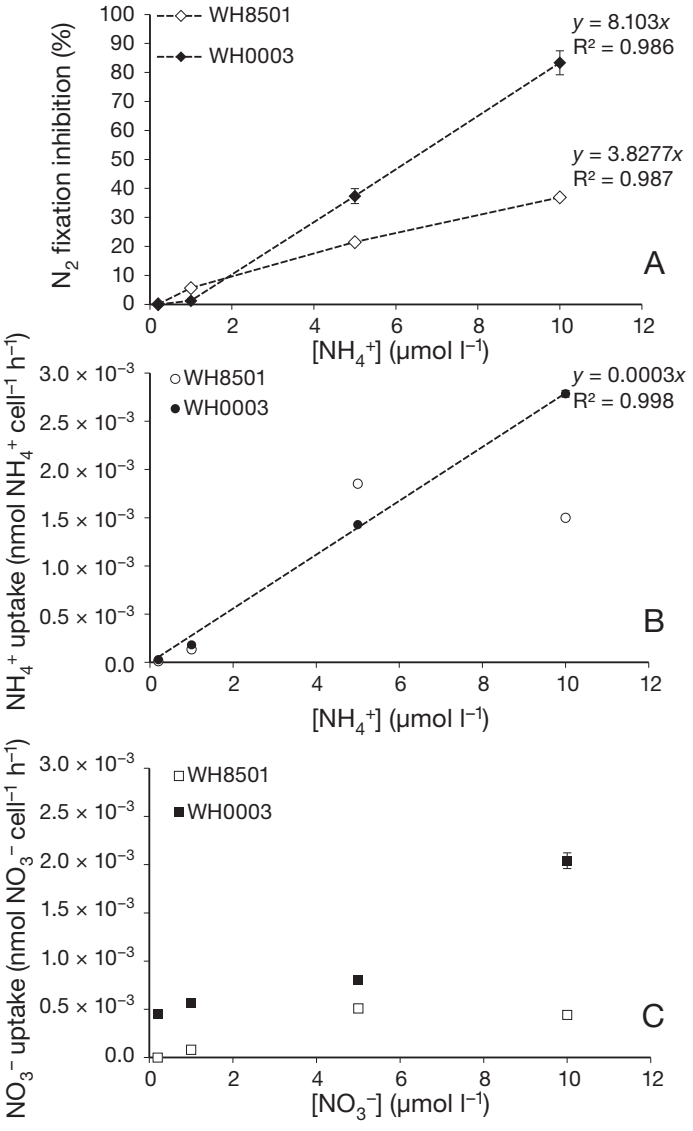

Fig. 5. *Crocosphaera watsonii*. N_2 fixation and dissolved inorganic nitrogen (DIN) uptake. (A) Relative inhibition of night-integrated N_2 fixation in each condition of NH_4^+ addition in comparison to the N_2 fixation in the control (no inhibitions were found in NO_3^- additions), (B) NH_4^+ uptake rate, and (C) NO_3^- uptake rate as a function of the DIN addition concentration for the 2 strains WH8501 (open symbols) and WH0003 (black symbols)

the concentration in the control (20 nmol l^{-1}). Regardless of the type or magnitude of N amendment, the strain WH0003 showed an increase in NH_4^+ concentration up to 100 nmol l^{-1} in the control as well as in all the treatments at the end of the dark period, indicating that this strain excretes NH_4^+ before the light period.

Cell abundances measured at 3 time points (at the end of the light period, during the middle of the dark period, and at the beginning of the new light period) in each condition did not change over the course of the experiments or between the treatments (1-way ANOVA, $p < 0.05$), validating the normalisation of N_2 fixation rates and uptake data by cell abundance.

Cellular C and N content

In control conditions, the cellular C and N content was 5.0×10^{-4} nmol C $cell^{-1}$ and 0.8×10^{-4} nmol N $cell^{-1}$ for strain WH8501 and 13×10^{-4} nmol C $cell^{-1}$ and 1.2×10^{-4} nmol N $cell^{-1}$ for the strain WH0003, resulting in C:N ratios of 5.2 and 9.4 for the strains WH8501 and WH0003, respectively (Table 2).

Crocosphaera watsonii showed variations in cellular C and N content and C:N ratios with the elevation of DIN in the medium (Table 2). Strain WH8501 showed variation in cellular C content with 4.6×10^{-4} nmol C $cell^{-1}$ for the 10 µmol NH_4^+ l^{-1} addition and 6.0×10^{-4} nmol C $cell^{-1}$ for 10 µmol NO_3^- l^{-1}. For this strain, the N content remained stable between 0.8 to 0.9×10^{-4} nmol N $cell^{-1}$ regardless of the type or magnitude of N enrichment, resulting in C:N ratios of 4.8 and 6.0 after 10 µmol l^{-1} additions of NH_4^+ and NO_3^-, respectively.

Table 2. *Crocosphaera watsonii*. Cellular carbon and nitrogen content and C:N ratio of strains WH8501 and WH0003 under different dissolved inorganic nitrogen (DIN) enrichments 9 h after the beginning of the dark period. na: not available

Strain	DIN addition		Cellular C (10^{-4} nmol C $cell^{-1}$)	Cellular N (10^{-4} nmol N $cell^{-1}$)	C:N
	Type	Final concentration (µmol l^{-1})			
WH8501	control		5.0	0.8	5.2
	NH_4^+	0.2	5.0	0.8	5.3
		1	4.8	0.8	5.3
		5	5.0	0.8	5.2
		10	4.6	0.8	4.8
	NO_3^-	0.2	4.8	0.8	5.1
		1	na	na	na
		5	na	na	na
		10	6.0	0.9	6.0
WH0003	control		12.9	1.2	9.4
	NH_4^+	0.2	13.5	1.3	9.1
		1	13.8	1.3	9.2
		5	12.3	1.4	7.8
		10	17.8	1.4	11.1
	NO_3^-	0.2	12.9	1.4	7.8
		1	12.6	1.3	8.1
		5	13.2	1.5	7.4
		10	14.2	1.6	7.7

The cellular C content of strain WH0003 was higher compared to the control for the 10 µmol l^{-1} DIN additions, with values of 17.8 × 10^{-4} nmol C $cell^{-1}$ for NH_4^+ and 14.2 × 10^{-4} nmol C $cell^{-1}$ for NO_3^-. The N content increased with increasing DIN in the medium to reach 1.4 × 10^{-4} nmol N $cell^{-1}$ with a 10 µmol l^{-1} addition of NH_4^+ and 1.6 × 10^{-4} nmol N $cell^{-1}$ with the addition of 10 µmol NO_3^- l^{-1}. C:N ratios thus varied from 7.7 to 8.1 with NO_3^- and from 7.8 to 11.1 with NH_4^+ additions.

DISCUSSION

Because some cyanobacteria have the ability to fix N_2, most of the studies dedicated to N_2 fixation have been performed in environments devoid of N. However, our understanding of marine nitrogen fixation is constantly evolving, and emerging satellite (Westberry et al. 2005), geochemical (Deutsch et al. 2001, 2007), and biological (Fernandez et al. 2011) evidence suggests that diazotrophy may not be limited to the nitrate-depleted surface waters of the oligotrophic ocean. This recognition encouraged us to investigate how 2 sources of combined nitrogen (NO_3^- and NH_4^+) will affect the diel N_2 fixation cycle of *Crocosphaera watsonii*.

Validation of the spike time

Nitrogenase activity results obtained in this study indicate that *Crocosphaera watsonii* fix dinitrogen during the dark period, with a maximum of activity at the end of the night, and a rapid decrease after the onset of the light period. This result is in accordance with recent molecular studies based on *nifH* gene expression (Mohr et al. 2010, Pennebaker et al. 2010, Shi et al. 2010), which confirm that *C. watsonii* has a maximum enrichment factor of *nifH* transcripts at the beginning of the dark period and a minimum between the beginning and the middle of the light period. There is a delay of several hours between the maximum *nifH* expression recorded by Shi et al. (2010) and the maximum nitrogenase activity measured in this study, which is probably due to the time necessary to activate the functional enzyme. In order to study the potential effect of NH_4^+ and NO_3^- on nitrogenase activity, we decided for this study to perform the spike before the synthesis of the new pool of nitrogenase for the night (i.e. 90 min before the start of the dark period).

NH_4^+ effect on N_2 fixation

Our results show that *Crocosphera watsonii* can simultaneously fix N_2 and utilize NH_4^+. These results are consistent with data obtained in exponentially growing batch cultures of *Trichodesmium* spp. (Mulholland & Capone 2001), which show a clear inhibition of nitrogenase activity by more than 50% after a 10 µmol NH_4^+ l^{-1} treatment. NH_4^+ is a metabolic end product of the N_2 fixation reaction and is able to directly decrease the activity of the enzyme (Thomas et al. 1982) and thus probably resulted in a decrease of nitrogen fixation fluxes in our experiment. It is thus not surprising that the magnitude of N_2 fixation inhibition was proportional to the magnitude of the NH_4^+ spike in our experiments.

NO_3^- effect on N_2 fixation

One of the most striking results in our experiments was the very small degree to which NO_3^- inhibited N_2 fixation, even at high concentrations (5 and 10 µmol l^{-1}), which are representative of ambient NO_3^- concentrations found in upwelling systems or HNLC waters where N_2 fixation occurs (Moutin et al. 2005, Bonnet et al. 2009, Fernandez et al. 2011). As the NO_3^- additions were performed before the maximum of *nif* gene transcript production (Shi et al. 2010), and because nitrogenase is synthesized de novo each night and degraded each day (Tuit et al. 2004, Saito et al. 2011), these results would indicate that high NO_3^- concentrations do not inhibit the synthesis of the enzyme nitrogenase in *Crocosphaera watsonii*.

These data contrast with those obtained in *Trichodesmium* spp. cultures, in which pulses of 10 µmol NO_3^- l^{-1} inhibited N_2 fixation by 35% in batch cultures (Mulholland et al. 2001), and up to 50% in continuous cultures (Holl & Montoya 2005). N_2 fixation is energy-consuming, and NO_3^- reduction needs one third less energy (Karl et al. 2002). If the high rate of NO_3^- uptake was only to economize on energy, N_2 fixation would be inhibited and NO_3^- assimilation would dominate consumption. This hypothesis could explain the inhibition of N_2 fixation by NO_3^- for *Trichodesmium* spp. due to the shift of some cellular reserves and machinery to synthesize nitrate reductase (Mulholland et al. 2001), but not the maintenance of nitrogenase activity by *Crocosphaera watsonii* found in this study. Furthermore, to be synthesized, nitrogenase and nitrate reductase require molybdenum (Mo) (Bagchi et al. 1985). The Mo metabolism of *Trichodesmium*

spp., which maintains an internal pool of Mo (Tuit et al. 2004), is different from the one of *C. watsonii*, which takes up a new pool of Mo every night (Tuit et al. 2004). Moreover, *C. watsonii* has 3 to 300 times lower intracellular Mo content than *Trichodesmium* spp. under obligate diazotrophy (Tuit et al. 2004), indicating potentially lower Mo requirements for nitrogenase synthesis and better ability to synthesize nitrate reductase. We did however notice a delay from 2 to 4 h in the occurrence of the N_2 fixation maximum in NO_3^- enriched treatments, reflecting the possible competition between N_2 fixation and NO_3^- uptake for energy, reserves and machinery in the cells.

These experiments indicate that micromolar NO_3^- additions induced immediate consumption of NO_3^-, proving that *Crocosphaera watsonii* is able to take up nitrate during the night and continues to fix N_2. These results indicate that N_2 fixation in the ocean would not be inhibited by pulses of DIN, such as Saharan dust deposition or mixing events. Other recent culture studies indicate that N_2 fixation by *C. watsonii* is ~60 to 80% reduced, although not entirely stopped, by 5 to 16 µM NO_3^-, after the cultures are acclimated for 10 generations to these NO_3^- concentrations and are grown with 0.5 to 1.0 µM PO_4^{3-} (A. N. Knapp unpubl. data). This result suggests that while maximum rates of N_2 fixation by *C. watsonii* are unlikely in waters with significant NO_3^- concentrations, lower rates of N_2 fixation are still possible in N-rich areas such as the Peru-Chile upwelling system (Fernandez et al. 2011).

Comparison of the two *Crocosphaera watsonii* strains

In the control treatments, the night-integrated N_2 fixation of the strain WH0003 was higher (13%) than in the WH8501 strain. This result is consistent with data obtained by Webb et al. (2009) who attributed higher rates of N_2 fixation in the WH0003 strain to imbalanced C:N ratios (N limitation due to high CO_2 fixation), compared to the strain WH8501, which would be relatively balanced or more C limited when grown in the same conditions. These hypotheses are confirmed here by the lower cellular C:N ratio of the WH8501 strain (5.2) compared to the strain WH0003 (9.4) in control conditions.

One night after DIN additions, regardless of DIN type and concentration, the cellular C:N ratio remained relatively low and stable (Table 2) for the strain WH8501, indicating that this strain is able to maintain stable cellular quotas. In contrast, the C:N ratio for the strain WH0003 varied between treatments, with an excess of C (Table 2), while the N content increased with the addition of DIN compared to the control, which is in accordance with its higher cell specific uptake rates of DIN (compared to WH8501). This imbalance is partially compensated for by the concomitant higher intracellular C compared to the control; the strain WH0003 is known to produce and excrete high quantities of extracellular polysaccharides (EPS, rich in C) compared to WH8501 (Webb et al. 2009) and it is possible that WH0003 decreased the excretion of EPS under high DIN conditions to balance the C:N ratio in the cells, but further studies would be required to precisely understand those mechanisms.

N-nutrition of diazotrophs

For 10 µmol l^{-1} DIN additions, WH8501 showed saturating uptake kinetics, while DIN uptake rates for strain WH0003 continued to increase over the range of DIN additions. This limitation of uptake for WH8501 could explain the lower inhibition of N_2 fixation relative to WH0003. The capacity of strain WH8501 to use NH_4^+ is limited because of the plateau in uptake rates. Furthermore it cannot modulate its cellular N content to respond to variations in environmental conditions; the maximum inhibition of N_2 fixation by NH_4^+ is only 32%, showing the limit of N metabolism in this strain and proving that it could not be much more inhibited in these culture conditions. For NH_4^+ additions, the N nutrition from N_2 fixation represents from 0.1 to 30.2% and from 0.5 to 47.3% of the total N uptake (NH_4^+ uptake + N_2 fixation), respectively, for 10 and 0.2 µmol NH_4^+ l^{-1} treatments of strains WH8501 and WH0003, respectively.

For NO_3^- additions, strain WH8501 shows saturating NO_3^- uptake kinetics while strain WH0003 is able to assimilate NO_3^- at concentrations of 10 µmol l^{-1} without any saturation in uptake rates. Furthermore, NO_3^- uptake does not reduce N_2 fixation rates in either strain. In these cases, N nutrition based on N_2 fixation represents from 2.1 to 12.3% and from 0.6 to 2.8% of the total N uptake (NO_3^- uptake + N_2 fixation), respectively, for 5 and 1 µmol NO_3^- l^{-1} treatments of strain WH8501 and for 10 and 0.2 µmol NO_3^- l^{-1} treatments of strain WH0003.

By comparison, the addition of 10 µmol NO_3^- l^{-1} inhibited N_2 fixation by *Trichodesmium* spp. under comparable culturing conditions by up to 50% com-

pared with control conditions, and N nutrition for these *Trichodesmium* cultures based on N_2 fixation still represented 8% (Holl & Montoya 2005). Proportionally, *Crocosphaera watsonii* is more efficient than *Trichodesmium* spp. at taking up DIN while keeping its ability to fix dinitrogen at high rates. However, after DIN pulses, N_2 fixation represents a small part of the total N uptake.

CONCLUSION

These culture experiments report the first data on N_2 fixation sensitivity to combined nitrogen forms for *Crocosphaera watsonii*, a representative of unicellular diazotrophs, and indicate that these organisms are efficient at assimilating NH_4^+ and NO_3^- while simultaneously fixing N_2. We show that N_2 fixation is clearly inhibited by NH_4^+, proportionally to the amount of NH_4^+ added. The most striking result is that unicellular diazotrophs continue to fix N_2 without inhibition, even after micromolar NO_3^- additions. This study implies that actively N_2-fixing populations may not be restricted to the nitrate depleted surface waters of the oligotrophic ocean as previously thought, but probably inhabit larger ecological niches and geographical distributions. This wide distribution is currently confirmed by the recent increasing number of field studies reporting the presence of active diazotrophs in N-rich ecosystems, including nutrient enriched estuarine and coastal waters (Short & Zehr 2007, Rees et al. 2009), eddies (Church et al. 2009), and OMZs (Fernandez et al. 2011). Contrary to *Trichodesmium* spp., unicellular diazotrophs have also been found deeper in the euphotic zone (Moisander et al. 2010), and to remain active throughout a larger timescale over the annual cycle, including the spring bloom (nitrogen repleted conditions) and the summer (phosphate depleted conditions), in Mediterranean temperate waters (Garcia et al. 2006, Bonnet et al. 2011).

However, *Crocosphaera watsonii* is the only marine unicellular diazotroph to date available in culture and it would be interesting to know if our results apply more broadly to other marine diazotrophs, such as heterotrophic bacteria or the uncultivated and widely distributed unicellular cyanobacterial Group A (Zehr et al. 2008, Moisander et al. 2010). This newly sequenced diazotroph lacks genes for assimilatory nitrate or nitrite reduction (Tripp et al. 2010), and is thus potentially even less sensitive to high nitrate concentrations compared to the Group B *C. watsonii* tested in this study.

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