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Hydrogen production by *Trichodesmium erythraeum* *Cyanothece* sp. and *Crocosphaera watsonii*

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ABSTRACT: Diazotrophic cyanobacteria are important components of marine ecosystems, where they contribute to primary production and provide a source of fixed nitrogen (N). During biological fixation of atmospheric nitrogen (N_2), hydrogen is produced as an obligate by-product. The present study investigated the potential contribution of 4 marine diazotrophs to the pool of dissolved H_2 in the oceans. N_2 fixation, as measured by acetylene reduction, and H_2 production rates were monitored throughout the diel period in cultures of the filamentous *Trichodesmium erythraeum* strain IMS101, and the unicellular organisms *Cyanothece* sp. strain ATCC 51142 and *Crocosphaera watsonii* strains WH8501 and WH0002. H_2 production coincided with diel variations in N_2 fixation for each strain regardless of whether N_2 fixation peaked during the day or night. Chlorophyll-normalized rates of H_2 production ranged 100-fold from a maximum of 3 nmol µg chl a^{-1} h^{-1} in *T. erythraeum* IMS101 cultures to 0.03 nmol µg chl a^{-1} h^{-1} in *Crocosphaera watsonii* WH0002. Overall, the ratio of net H_2 produced to N_2 fixed varied from 0.05 to 0.003 in the unicellular cyanobacteria, compared to 0.3 in the filamentous *T. erythraeum* IMS101, indicating that unicellular cyanobacteria produce less, or alternatively, re-assimilate more of the H_2 produced during N_2 fixation. *Crocosphaera watsonii* has recently been identified as a significant source of fixed N in the marine environment, and an efficient recycling of H_2 would provide a valuable source of energy to their respiratory electron transport chain. Furthermore, the magnitude of H_2 produced by *T. erythraeum* IMS101 strongly implicates this organism in the production of H_2 in the upper ocean.

KEY WORDS: N_2 fixation · Hydrogen · Cyanobacteria

INTRODUCTION

The ocean is a net source of hydrogen (H_2) to the atmosphere (Schmidt 1974, Hauglustaine & Ehhalt 2002), where it acts as an indirect greenhouse gas by influencing the concentrations of methane in the troposphere and water vapor in the stratosphere (Ehhalt & Prather 2001). Although the global ocean is typically classified as being 2 to 3 times supersaturated with respect to atmospheric H_2 concentrations (Seiler & Schmidt 1974), the net production of H_2 in surface seawater is restricted to tropical and subtropical biomes (Herr et al. 1981). Within the upper water column, vertical profiles of H_2 display the highest concentrations within the top 50 m, where values range from 1 to 3 nmol l^{-1}, followed by sharp decreases in concentration with depth, becoming under-saturated typically within 100 m of the surface (e.g. Conrad & Seiler 1988, Moore et al. 2009).

There is conflicting evidence of a diel cycle associated with H_2 concentrations in the upper water column of the open ocean. A clear increase during the daytime was reported in the oligotrophic South Atlantic (Herr et al. 1984), compared to a weak daytime peak in the equatorial Atlantic (Conrad & Seiler 1988), and no diel variation in the California Current System (Setser et al. 1982). The longer-term temporal variability of dissolved H_2 concentrations over several months has been reported for high-latitude coastal waters (Punshon et al. 2007).

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The most important environmental parameter influencing ambient H_2 concentrations was suggested by Conrad (1988) to be the biological fixation of nitrogen (N_2). H_2 evolution is an obligate by-product of the enzymatic N_2 fixation reaction, summarized in Eq. (1):

$$N_2 + 8\,H^+ + 8\,e^- + 16\,ATP \rightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\,Pi \quad (1)$$

where Pi is inorganic phosphate. H_2 is formed during the binding of a N_2 molecule to the molybdenum-iron (MoFe) subunit of the nitrogenase enzyme complex, prior to the reduction of N_2 to ammonia (Lowe & Thorneley 1984, Howard & Rees 2006). According to Eq. (1), which represents the least energetically costly stoichiometry for N_2 fixation, the theoretical production of H_2 under N_2-saturating conditions should be equimolar to the rate of N_2 fixation (Burns & Hardy 1975). However, diazotrophs contain uptake hydrogenase, which recycles the H_2 produced during N_2 fixation, and measured rates of net production of H_2 are typically much less than the theoretical maximum (Bothe et al. 1980).

A recent oceanographic cruise across the equatorial Pacific revealed a strong correlation between the rate of N_2 fixation and H_2 supersaturation (Moore et al. 2009). Further evidence for N_2 fixation as the mechanism responsible for dissolved H_2 comes from laboratory-based research on photobiological H_2 production (reviewed by Asada & Miyake 1999, Prince & Kheshgi 2005, Rupprecht et al. 2006). A large and diverse range of microbes have been analyzed for their H_2 production rates, and the more extensively examined N_2 fixing cyanobacteria include *Anabaena* sp. (Masukawa et al. 2002), *Nostoc punctiforme* (Schütz et al. 2004), and *Synechococcus* sp. (Mitsui & Suda 1995).

Despite the fact that N_2 fixing microorganisms have been studied for their ability to produce H_2, it remains unclear how different diazotrophs contribute to dissolved H_2 dynamics in the marine environment. The majority of simultaneous measurements of N_2 fixation and H_2 production in the marine environment have been conducted on *Trichodesmium* spp., a filamentous non-heterocystous cyanobacterium that fixes N_2 during the daytime while evolving oxygen via photosynthesis (Capone et al. 1997). However, results are conflicting, as laboratory-maintained cultures showed a positive correlation between H_2 production and N_2 fixation (Punshon & Moore 2008), yet field-collected colonies showed no correlation with N_2 fixation rates (Scranton 1983). Resolving this discrepancy is of particular importance to the marine H_2 cycle, given that *Trichodesmium* spp. is found throughout warm oligotrophic waters and is estimated to contribute 25 to 50% of the new production in the North Pacific Subtropical Gyre (Karl et al. 1997).

To better understand the role of N_2 fixing *Cyanobacteria* in H_2 cycling in the upper ocean, cultures of 3 model marine *Cyanobacteria* were analyzed for their N_2-fixing and H_2-production capabilities. The test organisms included *Trichodesmium erythraeum* strain IMS101, *Cyanothece* sp. strain ATCC 51142, and *Crocosphaera watsonii* strains WH8501 and WH0002.

MATERIALS AND METHODS

Culture conditions. The *Cyanobacteria* strains used in this work are listed in Table 1 and were grown using fixed N-free media: YBC II medium at pH 8.0 and salinity of 34 for *Trichodesmium erythraeum* IMS101 (Chen et al. 1996), and SO medium (pH 8.0, salinity 28) (Waterbury & Willey 1988) for *Cyanothece* sp. ATCC 51142 and both strains of *Crocosphaera watsonii*. All cultures were maintained at 26°C using a 12:12 h square-wave light:dark cycle with a light intensity of 44 µmol photons m^{-2} s^{-1}. This light intensity is approximately equivalent to a depth of 110 m at Stn ALOHA. Growth of all cultures was monitored by daily measurements of *in vivo* fluorescence with a TD-700 Turner Designs fluorometer. Chlorophyll *a* (chl *a*) measurements were made at the beginning of each experiment. Triplicate aliquots of cultures were filtered onto 25 mm Whatman GF/F filters and the chl *a* extracted in 5 ml of 90% acetone for 24 h at –20°C before being analyzed using a Turner Designs Model 10-AU fluorometer (Strickland & Parsons 1972).

The cultures were grown in 500 ml plastic culture flasks and transferred to borosilicate glass vials for experimental analysis. Typical volumes for sample analysis were 200 ml (for the unicellular diazotrophs)

Table 1. *Cyanobacteria* strains examined in the present study

Strain	Location isolated	Year isolated	Source
Trichodesmium erythraeum IMS101	North Carolina coast	1992	Prufert-Bebout et al. (1993)
Crocosphaera watsonii WH8501	Tropical South Atlantic	1984	Waterbury & Rippka (1989)
Crocosphaera watsonii WH0002	Hawaii	2000	Webb et al. (2009)
Cyanothece sp. ATC51142	Gulf of Mexico	1993	Reddy et al. (1993)

and 40 ml (for *Trichodesmium erythraeum* IMS101), which were analyzed in 240 ml and 76 ml glass vials, respectively. The smaller sample volume for *T. erythraeum* IMS101 was due to the magnitude of the H_2 produced. The glass vials were crimp-sealed with standard gray butyl rubber stoppers and aluminum seals to ensure the incubations were gas-tight. For each organism, the measurements of H_2 and ethylene (C_2H_4) were run on separate experimental flasks due to the residual H_2 in the cylinder of acetylene (C_2H_2) (Praxair) contaminating the samples and inundating the H_2 analyzer.

The analytical method was designed to measure continual rates of acetylene reduction and H_2 production in the *Cyanobacteria* cultures using on-line automated systems (Fig. 1). These 2 systems are described separately below ('Hydrogen measurements' and 'Nitrogenase activity').

Hydrogen measurements. The analytical protocol for H_2 analysis was designed to minimize contamination from the ambient atmosphere and sampling equipment. The gray stoppers for the glass vials were pierced with two 1/16 inch (1.6 mm) PEEK tubes (VICI Valco Instruments): an inlet which extended to the base of the flask, and an outlet which reached just inside the crimp-seal. The culture was continually purged with zero-H_2 (<10 ppt) air at a flow rate of 5.4 ml min^{-1}. The outlet tubing incorporated a 13 mm diameter hydrophobic filter to prevent any accidental injections of seawater and passed through a nafion drier (Perma Pure) to remove water vapor. The sample gas stream was connected directly from the nafion drier to the injection port of the reduced gas analyzer. Between each sample analysis, the tubing was disconnected, cleaned and purged with ultra-high-purity air (Airgas). One unavoidable feature of the analytical set-up is the inherent delay in purging the sample of H_2; i.e. if H_2 production were to cease, this would not be instantly recorded by the analyzer. Controlled measurements showed that it took 45 min to completely purge H_2 from the larger 240 ml glass vial used for the unicellular diazotrophs and 15 min to purge the 76 ml glass vial used for *Trichodesmium erythraeum* IMS101.

H_2 concentrations were measured using a reduced gas analyzer that incorporated a mercuric oxide bed coupled to a reducing compound photometer (Peak Laboratories). In brief, the reduction of heated mercuric oxide by H_2 gas liberated mercury vapor (HgO (solid) + H_2 → Hg (vapor) + H_2O) which was quantitatively detected using an ultraviolet absorption photometer located immediately downstream from the reaction site. The gas flow exiting the detector passed through an activated charcoal mercury scrubber before venting. Samples were injected via a 2 ml sample loop typically every 5 min onto 2 analytical columns maintained at 104°C. The first column was packed with Unibeads 1S (60/80 mesh, 1/8 inch [3.2 mm] diameter, and 16.5 inch [41.9 cm] length), and the second one with Molecular Sieve 13X (60/80 mesh, 1/8 inch [3.2 mm] diameter, and 81 inch [2.06 m] length). The retention time for H_2 was 51 s. The analytical precision based on the comparison of 4 samples was ±2% and the detection limit was 0.2 nmol l^{-1} h^{-1}, equating to a H_2 production rate in our experiments of 3 pmol (µg chl *a*)$^{-1}$ h^{-1}. Blank controls, consisting of filtered autoclaved seawater, never produced H_2 above the detection limit. The analyzer was calibrated using a 1 ppm H_2 standard (Scott Marrin) and serially diluted using zero-H_2 air. Over the experimental period, the

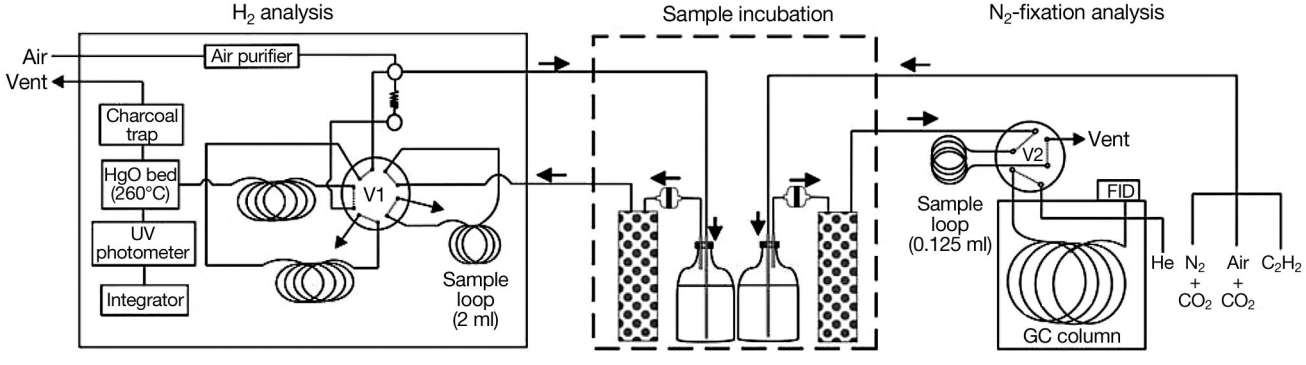

Fig. 1. Analytical design for H_2 production and acetylene reduction assays. During the experimental period, cultures were kept in the incubator (dashed line) and purged under continual gas flow (indicated by the direction of the arrows). The exit lines incorporated hydrophobic filters and nafion driers to remove water vapor from the sample gas stream. The gas lines led either to a 10-port switching valve (V1) which injected 2 ml of the sample stream for H_2 analysis using a HgO bed (Peak Laboratories), or a 6-port switching valve (V2) which injected 0.125 ml onto the gas chromatography (GC) column for ethylene analysis by gas chromatography-flame ionization detection (GC-FID) (Agilent Technologies). The diagram shows the valves in their 'purge' position

instrument drift was negligible (<0.1% difference in the calibration slope).

Nitrogenase activity. Nitrogenase activity was measured using an on-line monitoring C_2H_2 reduction assay similar to that described by Staal et al. (2001). The system was modified to keep the sample conditions identical to those used in the H_2 analysis (Fig. 1). The main modification was to replace the stainless steel sample cell with a 240 ml crimp-sealed borosilicate glass vial fitted with an inlet and outlet. One drawback of using a larger volume was that it increased the time required to purge the sample to 16 min at a gas flow rate of 17 ml min^{-1} (compared to 4 min using the sample cell described by Staal et al. 2001). The purge gas ran continually and was composed of 70% N_2 (containing 300 ppm CO_2), 20% O_2 (containing 300 ppm CO_2), and 10% C_2H_2. A consistent and reproducible signal was recorded for C_2H_4 production at a concentration of 10% C_2H_2 without the risk of starving the cells of nitrogen at a higher C_2H_2 concentration. A C_2H_4 background peak was quantified on blank samples and subsequently subtracted from the measurements. After exiting the sample culture, the gas flow was dried by passing through a nafion drier to a 6-port switching valve that injected 125 µl into an Agilent Technologies gas chromatograph (GC) (Model 6850 Series II) fitted with a fused silica Porapak U capillary column (25 m × 0.53 mm; Chrompack). The conditions of the GC were as follows: injector, detector, and oven temperatures were 90, 155, and 45°C, respectively. The carrier gas was helium at a flow rate of 3.6 ml min^{-1}, and the H_2 and air to the detector were 35 and 400 ml min^{-1}, respectively. The retention time for C_2H_4 was 1.94 min and 1 full run took 4 min. The detection limit for this particular set-up was approximately 0.8 nmol C_2H_4 l^{-1} h^{-1}, which equates to a C_2H_4 production rate of 0.03 nmol C_2H_4 (µg chl *a*)$^{-1}$ h^{-1}. The software used was Chemstation version A.09.03 (Agilent Technologies). C_2H_4 calibrations were performed using dilutions of 2 C_2H_4 standards (100 ppm and 9.9 ppm; Scotty Gases). Nitrogenase activity is reported as C_2H_4 production, except when the ratio of N_2 fixation to H_2 production is compared with the theoretical stoichiometry in Eq. (1). To convert C_2H_4 production rates to N_2 fixation, a ratio of 4 mol of C_2H_4 produced per mol N_2 reduced was used (Capone 1993, Mulholland et al. 2004).

Experimental manipulation. To test whether the H_2 evolved was due to nitrogenase activity, ammonium (NH_4^+) was added as a source of fixed nitrogen to all cultures (final concentration of 20 µmol l^{-1}). NH_4^+ has previously been used to inhibit nitrogenase synthesis in both natural populations (Capone et al. 1990) and cultures of *Trichodesmium erythraeum* IMS101 (Chen et al. 1996).

RESULTS

The production of H_2 was detected in all cultures of *Trichodesmium erythraeum* IMS101, *Cyanothece* sp. ATCC 51142, and both strains of *Crocosphaera watsonii.* H_2 production was correlated with rates of ethylene production in all instances and was suppressed in all cultures by the addition of NH_4^+ as a fixed source of nitrogen, indicating that nitrogenase was the likely source of the H_2.

Trichodesmium erythraeum IMS101

Nitrogenase activity followed a diel cycle, with activity increasing rapidly after the beginning of the light period (Fig. 2). C_2H_4 production reached a maximum of 32 nmol C_2H_4 (µg chl *a*)$^{-1}$ h^{-1} in the cultures. H_2 production in *Trichodesmium erythraeum* IMS101 cultures also occurred during the light period (08:00 to 20:00 h). Net production of H_2 began at the onset of the light period and increased rapidly for the first 3 h. The highest rates of H_2 production reached 3 nmol H_2 (µg chl *a*)$^{-1}$ h^{-1}. The peak of H_2 production in *T. erythraeum* IMS101 was broad compared to the unicellular cultures and extended for 3 to 4 h before concentrations decreased. On 1 sampling occasion H_2 was still being produced at the end of the light period (0.1 to 0.3 nmol H_2 [µg chl *a*]$^{-1}$ h^{-1}) and the onset of the dark period stimulated an immediate decrease in H_2 production (Fig. 2A).

Cyanothece sp. ATCC 51142

An increase in nitrogenase activity by *Cyanothece* sp. ATCC 51142 was detected approximately 1 h after the onset of the dark period and increased steadily to reach a maximum rate of 24 nmol C_2H_4 (µg chl *a*)$^{-1}$ h^{-1} in the middle of the night (Fig. 3). Maximum rates of nitrogenase activity were sustained for 1 to 2 h before decreasing again and were typically back to zero before the end of the dark period. Net H_2 production also increased during the night, although it was delayed by 4 h after the onset of the dark period, approximately 2 h after the onset of C_2H_4 production. Concentrations of H_2 increased for 2 h, reaching a maximum production rate of 0.3 to 0.5 nmol H_2 (µg chl *a*)$^{-1}$ h^{-1}. H_2 production subsequently declined and was not detected by the end of the dark period. In comparison to the 2 h time difference between the increase of H_2 and C_2H_4 production in the *Cyanothece* sp. ATCC 51142 cultures, both H_2 and C_2H_4 production decreased at approximately similar times.

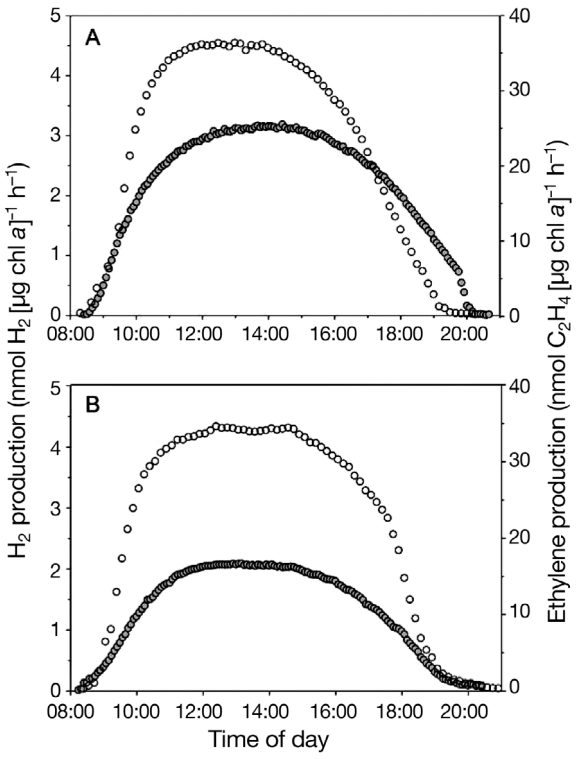

Fig. 2. *Trichodesmium erythraeum* strain IMS101. Chlorophyll-normalized rates of ethylene production (○) and net H_2 production (●). Measurements were repeated on (A) 26 May and (B) 2 June 2009. The light period in both experiments extended from 08:00 to 20:00 h

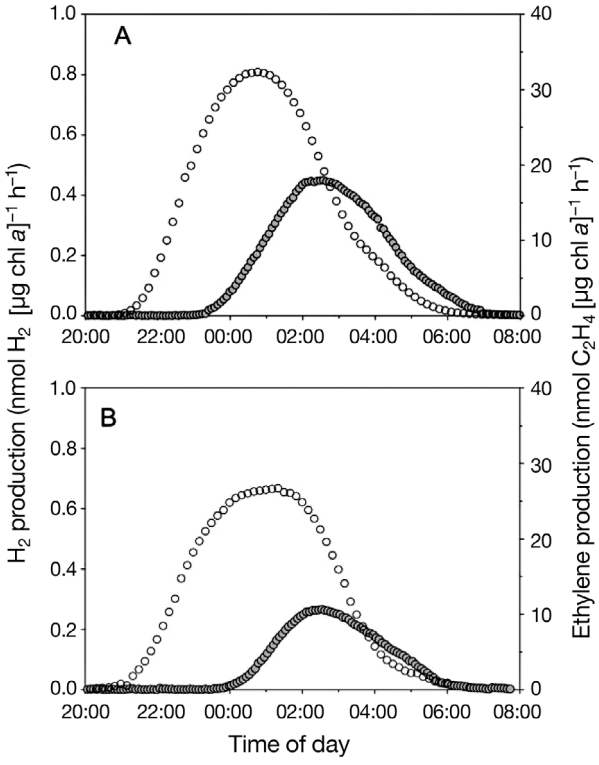

Fig. 3. *Cyanothece* sp. strain ATCC 51142. Chlorophyll-normalized rates of ethylene production (○) and net H_2 production (●). Measurements were repeated on (A) 28 May and (B) 4 June 2009. The dark period in both experiments extended from 20:00 to 08:00 h

Crocosphaera watsonii

Nitrogenase activity occurred during the dark period for both strains of *Crocosphaera watsonii*. The maximum rates of C_2H_4 production were 24 and 16 nmol C_2H_4 (µg chl *a*)$^{-1}$ h^{-1} for strains WH8501 and WH0002, respectively (Figs. 4 & 5). Maximum H_2 production rates were 0.028 and 0.018 nmol H_2 (µg chl *a*)$^{-1}$ h^{-1} for strains WH8501 and WH0002, respectively. These values represent the lowest concentrations of H_2 measured in the *Cyanobacteria* cultures during the present study. Net production rates of H_2 were an order of magnitude lower than with *Cyanothece* sp. ATCC 51142 and were close to the detection limit for the analytical system.

During the experimental incubations, both *Crocosphaera watsonii* strains WH8501 and WH0002 formed a mat on the bottom of the culture glass vial, even in the presence of a continual gas flow. This was minimized by gentle swirling of the flask throughout the sampling period. This is likely to have contributed to the higher variability of the measurements observed in the *C. watsonii* cultures (e.g. Fig. 4A), which likely also reflect the low H_2 concentration.

N_2 fixation

The stoichiometry of H_2 production as part of the N_2 fixation process can be considered by converting C_2H_4 production to N_2 fixation. *Trichodesmium erythraeum* IMS101 was not only the dominant producer of H_2, but also produced the highest concentration of H_2 relative to N_2 fixation. The H_2 production to N_2 fixation ratios for *T. erythraeum* IMS101 ranged from 0.25 to 0.34, compared to 0.04 to 0.055 for *Cyanothece* sp. ATCC 51142. The 2 strains of *Crocosphaera watsonii* produced the least amount of H_2 relative to nitrogenase activity, and the average H_2 production to N_2 fixation ratios were 0.004 and 0.003 for strains WH8501 and WH0002, respectively.

Addition of NH_4^+

The addition of NH_4^+ inhibited nitrogenase activity and H_2 production in the cultures (Fig. 6A). It was added in the light or dark period prior to the active nitrogenase period for each organism. The rates of C_2H_4 production decreased by 80 to 90% on all occa-

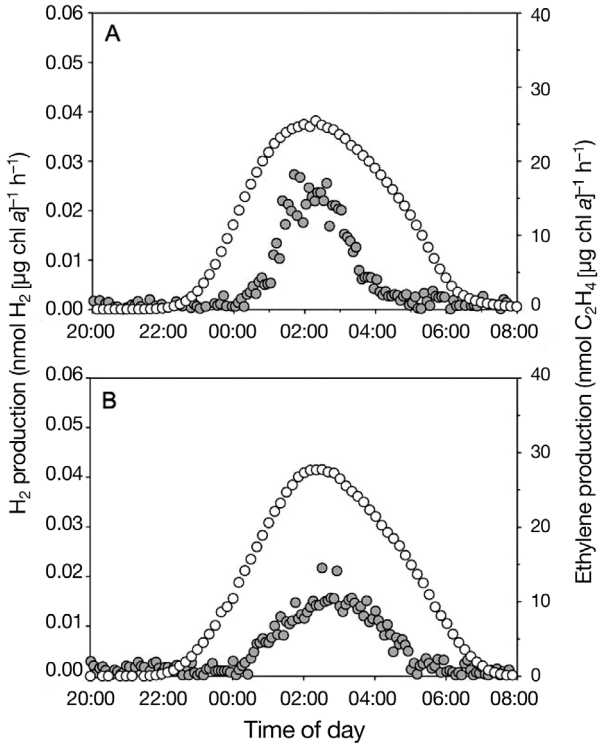

Fig. 4. *Crocosphaera watsonii* strain WH8501. Chlorophyll-normalized rates of ethylene production (○) and net H_2 production (●). Measurements were repeated on (A) 5 June and (B) 20 June 2009. The dark period in both experiments extended from 20:00 to 08:00 h

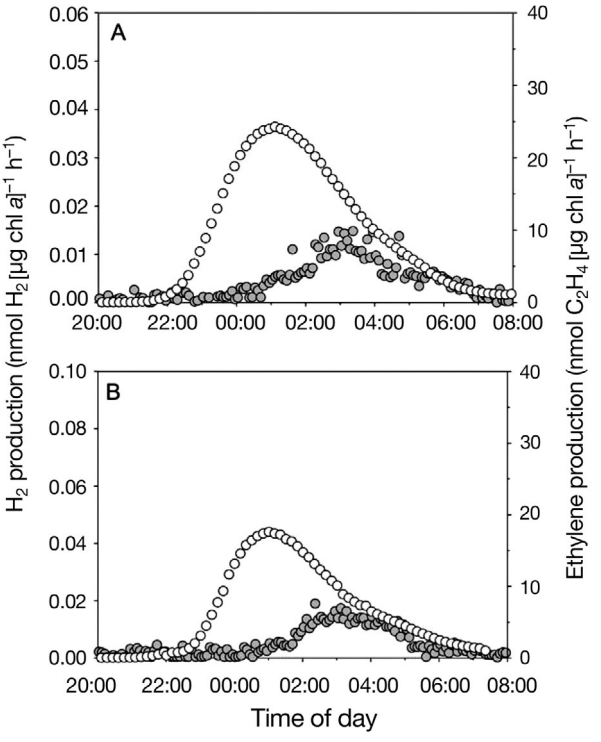

Fig. 5. *Crocosphaera watsonii* strain WH0002. Chlorophyll-normalized rates of ethylene production (○) and net H_2 production (●). Measurements were repeated on (A) 6 June and (B) 14 June 2009. The dark period in both experiments extended from 20:00 to 08:00 h

sions. Fig. 6A shows the effect of adding NH_4^+ during the middle of the light period to a culture of *Trichodesmium erythraeum* IMS101 that was actively fixing N_2 (20 nmol C_2H_4 [µg chl *a*]$^{-1}$ h^{-1}) and producing H_2 (~1 nmol H_2 [µg chl *a*]$^{-1}$ h^{-1}). There was an immediate decrease in the rate of C_2H_4 and H_2 production, and concentrations of H_2 subsequently decreased steadily over the next 2 to 3 h. Similarly, C_2H_4 and H_2 production were also inhibited in *T. erythraeum* IMS101 by removing the light source (Fig. 6B). In comparison to adding NH_4^+, C_2H_4 production decreased more rapidly to reach 10% of pre-treatment values within 40 min when the lights were turned off. H_2 production also decreased, yet there was little apparent difference between the 2 treatments.

DISCUSSION

Diazotrophic *Cyanobacteria* are widespread in the world's oceans where they are estimated to provide approximately 100 Tg yr^{-1} of fixed N to the marine ecosystem (Karl et al. 2002). As a by-product of the N_2 fixation process, the diazotrophic *Cyanobacteria* also produce H_2 in the surface waters of the world's oceans. The present study measured N_2 fixation and H_2 production in cultures of several N_2 fixing *Cyanobacteria* (*Trichodesmium erythraeum* IMS101, *Cyanothece* sp. ATCC 51142, and *Crocosphaera watsonii* WH8501 and WH0002) to determine their potential contribution to the dissolved H_2 present in the upper ocean.

All of the cultures of *Cyanobacteria* exhibited diel patterns of nitrogenase activity as previously reported, with fixation during the light period for *Trichodesmium erythraeum* IMS101 (Chen et al. 1996, Berman-Frank et al. 2001) and night-time nitrogenase activity for *Cyanothece* sp. (Sherman et al. 1998) and *Crocosphaera watsonii* (Tuit et al. 2004). Outside of these time periods, no nitrogenase activity was detected using the continual on-line acetylene reduction assay. C_2H_4 production rates in *T. erythraeum* IMS101 cultures reached a maximum of 32 nmol C_2H_4 (µg chl *a*)$^{-1}$ h^{-1}, which is comparable to previous measurements (Chen et al. 1996). Compared to the unicellular *Cyanobacteria*, nitrogenase activity by *T. erythraeum* IMS101 cultures extended over a longer time period. The rates of C_2H_4 production in *Crocosphaera watsonii* WH8501 and WH0002 (24 and 16 nmol C_2H_4 [µg chl *a*]$^{-1}$ h^{-1}, respectively) were higher than those recently reported for *Crocosphaera watsonii* WH8501

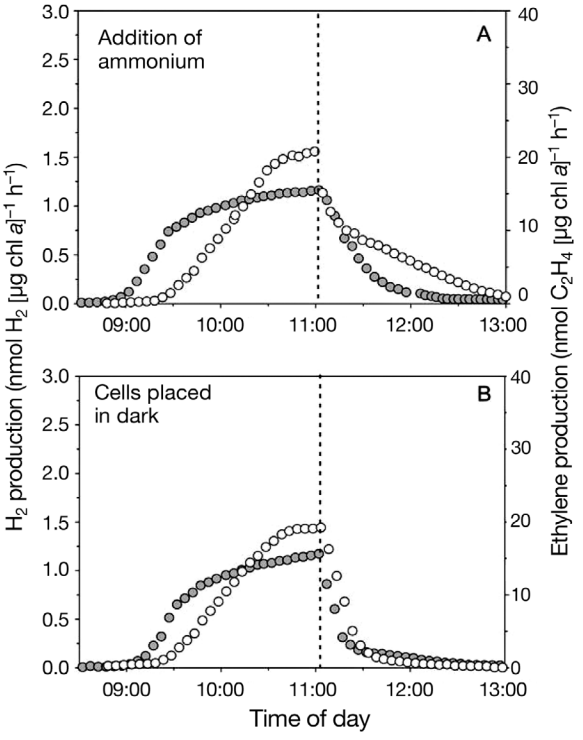

Fig. 6. *Trichodesmium erythraeum* strain IMS101. Effect of (A) ammonium and (B) darkness on ethylene production (○) and net H_2 production (●). The dashed line represents when (A) ammonium was added (final concentration: 20 µmol l^{-1}) or (B) the light source was removed

by Webb et al. (2009). One possible reason for the variation is the difference in the acetylene-reduction assay protocols. The on-line GC method used in the present study measured the flux of ethylene, compared to discrete batch incubations that can be incubated for over an hour. Comparisons between the discrete and continuous measurements have been documented for *Nodularia spumigena* (Staal et al. 2001), but should be carried out for other diazotrophic organisms, which may respond differently to the experimental conditions.

The highest rates of H_2 production were measured in cultures of *Trichodesmium erythraeum* IMS101, with production reaching 3 nmol H_2 (µg chl *a*)$^{-1}$ h^{-1}. These rates exceed those reported by Punshon & Moore (2008) for the same strain of *T. erythraeum* (0.061 to 0.71 nmol H_2 [µg chl *a*]$^{-1}$ h^{-1}), which most likely reflects the higher N_2 fixation rates measured in the present study (10 nmol N_2 [µg chl *a*]$^{-1}$ h^{-1}) compared to the cultures of Punshon & Moore (2008) (0.59 to 4.71 nmol N_2 [µg chl *a*]$^{-1}$ h^{-1}). The rate of H_2 production by *T. erythraeum* IMS101 is comparable to previously reported rates of 7.5 to 20 nmol H_2 (µg chl *a*)$^{-1}$ h^{-1} reported for other filamentous *Cyanobacteria* such as *Anabaena* sp. and *Nostoc* sp. (Bothe et al. 1980). In comparison to the filamentous *Cyanobacteria*, the rates of H_2 production measured in the unicellular *Cyanobacteria* were lower (0.3, 0.04, and 0.03 nmol H_2 [µg chl *a*]$^{-1}$ h^{-1} for *Cyanothece* sp. ATCC 51142 and *Crocosphaera watsonii* WH8501 and WH0002, respectively). These rates are lower than night-time H_2 production rates of 1.7 and 0.2 nmol H_2 (µg chl *a*)$^{-1}$ h^{-1} reported for the unicellular, N_2 fixing *Cyanothece* sp. strain 7822 and *Synechococcus* sp. strain 7425, respectively (Van der Oost et al. 1987). However, in the study by Van der Oost et al. (1987), the rates of H_2 production exceed the stoichiometric rate of N_2 fixation, indicating another source of H_2 in addition to nitrogenase. In contrast, the H_2 production rates of the unicellular *Cyanobacteria* analyzed in the present study compare favorably with those reported for other unicellular, albeit non-N_2 fixing, *Cyanobacteria*, which range from 0.02 to 0.48 nmol H_2 (µg chl *a*)$^{-1}$ h^{-1} for *Synechococcus* sp. and 0.06 H_2 (µg chl *a*)$^{-1}$ h^{-1} for *Synechocystis* sp. strain PCC 6308 (Howarth & Codd 1985).

The C_2H_4 production and H_2 production results of the present study complement the findings of previous work, that also showed that the filamentous *Cyanobacteria* fix more N_2 per cell volume (Mahaffey et al. 2005) and produce more H_2 (Schütz et al. 2004) than their unicellular counterparts. Ratios of H_2 production to N_2 fixation can be compared with the gross theoretical stoichiometric model of 1:1 (Eq. 1) by converting C_2H_4 production to N_2 fixation using a ratio of 4 mol C_2H_4 produced per mol N_2 reduced (Jensen & Cox 1983, Capone 1993, Mulholland et al. 2004, Tuit et al. 2004). Ratios of H_2 production to N_2 fixation for *Trichodesmium erythraeum* IMS101, *Cyanothece* sp. ATCC 51142, and *Crocosphaera watsonii* WH8501 and WH0002 were 0.28, 0.05, 0.004, and 0.003, respectively. The deficit in H_2 values from the 1:1 ratio is most likely due to H_2 measurements not accounting for the subsequent re-incorporation of H_2 by uptake hydrogenase (Bothe et al. 1980). Measurements of gross H_2 production were not undertaken during the present study, but are an integral component of future work to resolve the cycling of H_2 by diazotrophs. Possible methods to estimate gross H_2 production rates include $^3H_2$-exchange assays (e.g. Paerl 1983), the inhibition of uptake hydrogenase by carbon monoxide (Smith et al. 1976), or genetic manipulation of the hydrogenase gene (e.g. Lindberg et al. 2002, Masukawa et al. 2002). The H_2:N_2 ratios will also be affected by the conversion factor selected for converting C_2H_2 reduction rates to N_2 fixation rates, as variation in this ratio, either between species or at different stages of growth, would influence the calculated H_2 yield (Punshon & Moore 2008). Another potential factor affecting the H_2:N_2 ratio is the consumption of the H_2 by chemoheterotrophic microorganisms. Although sterile labo-

ratory techniques were used at all times, the cultures used in the present study were not axenic.

The ability of *Trichodesmium erythraeum*, *Cyanothece* sp., and *Crocosphaera watsonii* to re-incorporate the H_2 produced during N_2 fixation has important implications for cellular and ecological bioenergetics. The production of H_2 during N_2 fixation has previously been described as energetically wasteful (Prince & Kheshgi 2005) as the H_2 component of N_2 fixation requires 2 electrons, compared to the 6 electrons required to reduce N_2 to NH_3 and at least 2 molecules of ATP per electron are needed (Eady 1996). One strategy for a diazotroph to reduce this energy loss is to reincorporate the H_2 released via hydrogenase enzymes (Stephenson & Stickland 1931). Uptake hydrogenases, which re-assimilate the H_2 produced by nitrogenase, have been found in all N_2 fixing *Cyanobacteria* examined, including *Trichodesmium* spp. (Saino & Hattori 1982), *Cyanothece* sp. (Stöckel et al. 2008), and *Crocosphaera* sp. (Tamagnini et al. 2004).

The re-incorporation of H_2 has numerous potential benefits for *Cyanobacteria* and the functioning of nitrogenase. The uptake hydrogenase could provide electrons for the electron transport chain, via a Knallgas reaction, resulting in phosphorylation of ADP to ATP and the simultaneous consumption of oxygen (Bothe et al. 1974, Robson & Postgate 1980). Alternatively, hydrogenase could provide electrons to photosystem I, which would generate reductant, rather than ATP, from the H_2 produced by nitrogenase (Dixon 1972). In the present study, the lower net production rates by the unicellular *Cyanobacteria* may indicate that they are more efficient at recycling the H_2 produced by nitrogenase, as previously suggested by Schütz et al. (2004). The recycling of H_2 could also explain the offset observed during the diel cycle between H_2 production and N_2 fixation (e.g. Fig. 3). The lag time between the N_2 fixation and the H_2 production rate may reflect a higher rate of H_2 re-assimilation during the period of maximum N_2 fixation.

In addition to the importance of H_2 cycling at the cellular level, the results of the present work allow the diversity and activity of N_2 fixing *Cyanobacteria* in the marine environment to be linked with the concentration of dissolved H_2. A recent oceanographic cruise across the equatorial Pacific measured dissolved H_2 concentrations and also N_2 fixation using size-fractionated $^{15}N_2$ uptake (Moore et al. 2009). The <10 µm size class (assumed to be unicellular *Cyanobacteria*) contributed 36 to 100% to the whole community N_2 fixation rates, which ranged from 0.18 to 9.0 µmol N_2 m^{-3} d^{-1}. Estimated rates of net H_2 production across the equatorial Pacific ranged from 0 to 0.059 µmol H_2 m^{-3} d^{-1} with an average of 0.17 µmol H_2 m^{-3} d^{-1} (Moore et al. 2009). Applying the H_2:N_2 ratios from the present study (0.3 and 0.003 for *Trichodesmium erythraeum* IMS101 and *Crocosphaera watsonii*, respectively) to average values of H_2 production and N_2 fixation for the whole cruise indicate H_2 production rates for *T. erythraeum* and *C. watsonii* of 0.129 and 0.003 µmol H_2 m^{-3} d^{-1}, respectively. These estimates support previous suggestions that *Trichodesmium* spp. makes a substantial contribution to the pool of dissolved H_2 in the upper ocean (Scranton 1983, Punshon & Moore 2008). However, careful corroboration of the laboratory-based measurements with *in situ* net H_2 production rates in the natural environment is required. This is particularly pertinent because measurements of H_2 production by field-collected colonies of *T. thiebautii* revealed H_2 production to N_2 fixation ratios ranging from 0.01 to 0.06 (Scranton et al. 1987), an order of magnitude lower than cultures of *T. erythraeum* IMS101 analyzed in the present study. One possible explanation is the difference in light intensity between cultures and field-collected organisms. However, an increased light intensity increases N_2 fixation in *Trichodesmium* spp. (Berman-Frank et al. 2001) and would therefore be predicted to increase, rather than decrease, H_2 production. Efforts are currently being made to improve the experimental design for further physiological experiments to measure N_2 fixation and H_2 production.

It should be noted that in addition to a nitrogenase source of H_2, the fermentation of organic material by *Cyanobacteria* can also produce H_2 (Schütz et al. 2004). In the present study, the addition of NH_4^+ to the cultures inhibited both N_2 fixation and H_2 production, indicating that nitrogenase was the source of the H_2 measured. This is to be expected, as the cultures were constantly aerated, which constrains the anaerobic conditions required for H_2 production via fermentation by *Cyanobacteria* (Rupprecht et al. 2006), and furthermore the medium was devoid of fermentable organics. In the oxygenated upper ocean, fermentation metabolism by *Cyanobacteria* is likely to be minor in comparison to aerobic respiration (Stal & Moezelaar 1997) and H_2 is more likely to evolve from N_2 fixation.

CONCLUSIONS

The present work showed the production of H_2 by *Trichodesmium erythraeum* IMS101, *Cyanothece* sp. ATCC 51142, and *Crocosphaera watsonii* in conjunction with N_2 fixation. As demonstrated by previous work on filamentous and colonial *Cyanobacteria*, cultures of *T. erythraeum* IMS101 produced comparatively high concentrations of H_2 relative to the rates of N_2 fixation. In contrast, the unicellular diazotrophs pro-

duced less H_2, which is considered to reflect a higher efficiency of H_2 scavenging following its production by nitrogenase. An efficient recycling of H_2 would alleviate some of the rigorous metabolic requirements associated with nitrogenase activity by providing ATP, a supply of reductant and mitigating the effects of oxygen. Therefore, intracellular cycling of H_2 may prove to be essential for an efficient functioning of these diazotrophs, which have recently been identified as key players in the fixation of N_2 in the open ocean. Despite the important role of unicellular diazotrophs in global N_2 fixation, it appears that *Trichodesmium* spp. is producing much of the dissolved H_2 in the upper oceans. This suggests that changes in the distribution and physiology of *Trichodesmium* spp. could also indirectly affect the levels of dissolved H_2 in the ocean. This would not only have repercussions for the ocean as a source of H_2 to the atmosphere, but could also affect the availability of H_2 to marine microorganisms as a potential source of energy and reducing power. The role of H_2 in the energy budget of the upper ocean is not well known and the present study is an important reminder of its availability.

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