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 (N₂), hydrogen is produced as an obligate by-product. The present study investigated the potential contribution of 4 marine diazotrophs to the pool of dissolved H₂ in the oceans. N₂ fixation, as measured by acetylene reduction, and H₂ 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. H₂ production coincided with diel variations in N₂ fixation for each strain regardless of whether N₂ fixation peaked during the day or night. Chlorophyll-normalized rates of H₂ 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 H₂ produced to N₂ 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 H₂ produced during N₂ fixation. *Crocosphaera watsonii* has recently been identified as a significant source of fixed N in the marine environment, and an efficient recycling of H₂ would provide a valuable source of energy to their respiratory electron transport chain. Furthermore, the magnitude of H₂ produced by *T. erythraeum* IMS101 strongly implicates this organism in the production of H₂ in the upper ocean.

KEY WORDS: N₂ fixation • Hydrogen • Cyanobacteria

INTRODUCTION

The ocean is a net source of hydrogen (H₂) 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 H₂ concentrations (Seiler & Schmidt 1974), the net production of H₂ in surface seawater is restricted to tropical and subtropical biomes (Herr et al. 1981). Within the upper water column, vertical profiles of H₂ 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 H₂ 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 H₂ concentrations over several months has been reported for high-latitude coastal waters (Punshon et al. 2007).
The most important environmental parameter influencing ambient H₂ concentrations was suggested by Conrad (1988) to be the biological fixation of nitrogen (N₂). H₂ evolution is an obligate by-product of the enzymatic N₂ fixation reaction, summarized in Eq. (1):

\[
\text{N}_2 + 8 \text{H}^+ + 8 \text{e}^- + 16 \text{ATP} \rightarrow 2 \text{NH}_3 + \text{H}_2 + 16 \text{ADP} + 16 \text{Pi}
\]

where Pi is inorganic phosphate. H₂ is formed during the binding of a N₂ molecule to the molybdenum-iron (MoFe) subunit of the nitrogenase enzyme complex, prior to the reduction of N₂ to ammonia (Lowe & Thorneley 1984, Howard & Rees 2006). According to Eq. (1), which represents the least energetically costly stoichiometry for N₂ fixation, the theoretical production of H₂ under N₂-saturating conditions should be equimolar to the rate of N₂ fixation (Burns & Hardy 1975). However, diazotrophs contain uptake hydrogenase, which recycles the H₂ produced during N₂ fixation, and measured rates of net production of H₂ 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 N₂ fixation and H₂ supersaturation (Moore et al. 2009). Further evidence for N₂ fixation as the mechanism responsible for dissolved H₂ comes from laboratory-based research on photobiological H₂ 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 H₂ production rates, and the more extensively examined N₂-fixing and H₂-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>
<thead>
<tr>
<th>Strain</th>
<th>Location isolated</th>
<th>Year isolated</th>
<th>Source</th>
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<tbody>
<tr>
<td><em>Crocosphaera watsonii</em> WH0002</td>
<td>Hawaii</td>
<td>2000</td>
<td>Webb et al. (2009)</td>
</tr>
<tr>
<td><em>Cyanothece</em> sp. ATCC51142</td>
<td>Gulf of Mexico</td>
<td>1993</td>
<td>Reddy et al. (1993)</td>
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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 H₂ 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 H₂ and ethylene (C₂H₄) were run on separate experimental flasks due to the residual H₂ in the cylinder of acetylene (C₂H₂) (Praxair) contaminating the samples and inundating the H₂ analyzer.

The analytical method was designed to measure continual rates of acetylene reduction and H₂ 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 H₂ 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-H₂ (<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 setup is the inherent delay in purging the sample of H₂; i.e. if H₂ production were to cease, this would not be instantly recorded by the analyzer. Controlled measurements showed that it took 45 min to completely purge H₂ 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.

H₂ 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 H₂ gas liberated mercury vapor (HgO (solid) + H₂ (vapor) + H₂O) 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 H₂ 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 H₂ production rate in our experiments of 3 pmol (µg chl a)⁻¹ h⁻¹. Blank controls, consisting of filtered autoclaved seawater, never produced H₂ above the detection limit. The analyzer was calibrated using a 1 ppm H₂ standard (Scott Marrin) and serially diluted using zero-H₂ air. Over the experimental period, the

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**Fig. 1.** Analytical design for H₂ 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 H₂ 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$_2$H$_2$ 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$_2$H$_2$. A consistent and reproducible signal was recorded for C$_2$H$_4$ production at a concentration of 10% C$_2$H$_2$ without the risk of starving the cells of nitrogen at a higher C$_2$H$_2$ concentration. A C$_2$H$_4$ 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$_2$H$_4$ 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$_2$H$_4$ h$^{-1}$, which equates to a C$_2$H$_4$ production rate of 0.03 nmol C$_2$H$_4$ (µg chl a)$^{-1}$ h$^{-1}$. The software used was Chemstation version A.09.03 (Agilent Technologies). C$_2$H$_4$ calibrations were performed using dilutions of 2 C$_2$H$_4$ standards (100 ppm and 9.9 ppm; Scotty Gases). Nitrogenase activity is reported as C$_2$H$_4$ 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$_2$H$_4$ production rates to N$_2$ fixation, a ratio of 4 mol of C$_2$H$_4$ 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 nmol 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$_2$H$_4$ production reached a maximum of 32 nmol C$_2$H$_4$ (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$_2$H$_4$ (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$_2$H$_4$ 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$_2$H$_4$ production in the *Cyanothece* sp. ATCC 51142 cultures, both H$_2$ and C$_2$H$_4$ production decreased at approximately similar times.
Nitrogenase activity occurred during the dark period for both strains of *Crocosphaera watsonii*. The maximum rates of C$_2$H$_4$ production were 24 and 16 nmol C$_2$H$_4$ (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$_2$H$_4$ 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$_2$H$_4$ production decreased by 80 to 90% on all occa-
Fig. 4. Crocosphaera watsonii strain WH8501. Chlorophyll-normalized rates of ethylene production (O) and net H₂ production (D). 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 (O) and net H₂ production (D). 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 NH₄⁺ during the middle of the light period to a culture of Trichodesmium erythraeum IMS101 that was actively fixing N₂ (20 nmol C₂H₄ [µg chl a]⁻¹ h⁻¹) and producing H₂ (~1 nmol H₂ [µg chl a]⁻¹ h⁻¹). There was an immediate decrease in the rate of C₂H₄ and H₂ production, and concentrations of H₂ subsequently decreased steadily over the next 2 to 3 h. Similarly, C₂H₄ and H₂ production were also inhibited in T. erythraeum IMS101 by removing the light source (Fig. 6B). In comparison to adding NH₄⁺, C₂H₄ production decreased more rapidly to reach 10% of pre-treatment values within 40 min when the lights were turned off. H₂ 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 N₂ fixation process, the diazotrophic Cyanobacteria also produce H₂ in the surface waters of the world’s oceans.

The present study measured N₂ fixation and H₂ production in cultures of several N₂ fixing Cyanobacteria (Trichodesmium erythraeum IMS101, Cyanothecce sp. ATCC 51142, and Crocosphaera watsonii WH8501 and WH0002) to determine their potential contribution to the dissolved H₂ 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 Cyanothecce 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. C₂H₄ production rates in T. erythraeum IMS101 cultures reached a maximum of 32 nmol C₂H₄ (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 C₂H₄ production in Crocosphaera watsonii WH8501 and WH0002 (24 and 16 nmol C₂H₄ [µg chl a]⁻¹ h⁻¹, respectively) were higher than those recently reported for Crocosphaera watsonii WH8501.
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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 H₂ production were measured in cultures of *Trichodesmium erythraeum* IMS101, with production reaching 3 nmol H₂ (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 H₂ (µg chl a⁻¹ h⁻¹)), which most likely reflects the higher N₂ fixation rates measured in the present study (10 nmol N₂ (µg chl a⁻¹ h⁻¹)) compared to the cultures of Punshon & Moore (2008) (0.59 to 4.71 nmol N₂ (µg chl a⁻¹ h⁻¹)). The rate of H₂ production by *T. erythraeum* IMS101 is comparable to previously reported rates of 7.5 to 20 nmol H₂ (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 H₂ production measured in the unicellular Cyanobacteria were lower (0.3, 0.04, and 0.03 nmol H₂ (µg chl a⁻¹ h⁻¹) for *Cyanobethe* sp. ATCC 51142 and *Crocosphaera watsonii* WH8501 and WH0002, respectively). These rates are lower than night-time H₂ production rates of 1.7 and 0.2 nmol H₂ (µg chl a⁻¹ h⁻¹) reported for the unicellular, N₂ fixing *Cyanobethe* 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 H₂ production exceed the stoichiometric rate of N₂ fixation, indicating another source of H₂ in addition to nitrogenase. In contrast, the H₂ production rates of the unicellular *Cyanobethe* analyzed in the present study compare favorably with those reported for other unicellular, albeit non-N₂ fixing, *Cyanobethe*, which range from 0.02 to 0.48 nmol H₂ (µg chl a⁻¹ h⁻¹) for *Synechococcus* sp. and 0.06 H₂ (µg chl a⁻¹ h⁻¹) for *Synechocystis* sp. strain PCC 6308 (Howarth & Codd 1985).

The C₂H₄ production and H₂ production results of the present study complement the findings of previous work, that also showed that the filamentous *Cyanobethe* fix more N₂ per cell volume (Mahaffey et al. 2005) and produce more H₂ (Schütz et al. 2004) than their unicellular counterparts. Ratios of H₂ production to N₂ fixation can be compared with the gross theoretical stoichiometric model of 1:1 (Eq. 1) by converting C₂H₄ production to N₂ fixation using a ratio of 4 mol C₂H₄ produced per mol N₂ reduced (Jensen & Cox 1983, Capone 1993, Mulholland et al. 2004, Tuit et al. 2004). Ratios of H₂ production to N₂ fixation for *Trichodesmium erythraeum* IMS101, *Cyanobethe* sp. ATCC 51142, and *Crocosphaera watsonii* WH8501 and WH0002 were 0.28, 0.05, 0.004, and 0.003, respectively. The deficit in H₂ values from the 1:1 ratio is most likely due to H₂ measurements not accounting for the subsequent re-incorporation of H₂ by uptake hydrogenase (Bothe et al. 1980). Measurements of gross H₂ production were not undertaken during the present study, but are an integral component of future work to resolve the cycling of H₂ by diazotrophs. Possible methods to estimate gross H₂ production rates include ³H₂-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 H₂:N₂ ratios will also be affected by the conversion factor selected for converting C₂H₄ reduction rates to N₂ fixation rates, as variation in this ratio, either between species or at different stages of growth, would influence the calculated H₂ yield (Punshon & Moore 2008). Another potential factor affecting the H₂:N₂ ratio is the consumption of the H₂ by chemo-heterotrophic microorganisms. Although sterile labo-
The ability of *Trichodesmium erythraeum*, *Cyanobacteria* sp., and *Crocophaga 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 & Keshgi 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), *Cyanothecaceae* sp. (Stöckel et al. 2008), and *Crocophaga 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 to 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 *Crocophaga 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, *Cyanothecaceae* sp., ATCC 51142, and *Crocophaga 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 H₂, which is considered to reflect a higher efficiency of H₂ scavenging following its production by nitrogenase. An efficient recycling of H₂ 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 H₂ may prove to be essential for an efficient functioning of these diazotrophs, which have recently been identified as key players in the fixation of N₂ in the open ocean. Despite the important role of unicellular diazotrophs in global N₂ fixation, it appears that Trichodesmium spp. is producing much of the dissolved H₂ in the upper oceans. This suggests that changes in the distribution and physiology of Trichodesmium spp. could also indirectly affect the levels of dissolved H₂ in the ocean. This would not only have repercussions for the ocean as a source of H₂ to the atmosphere, but could also affect the availability of H₂ to marine microorganisms as a potential source of energy and reducing power. The role of H₂ 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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LITERATURE CITED

- Burns RC, Hardy RWF (1975) Nitrogen fixation in bacteria and higher plants. Springer Verlag, Heidelberg


Strickland JDH, Parsons TR (1972) A practical handbook of seawater analysis. Fisheries Research Board of Canada, Ottawa, ON


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