

Hydrogen Cycling by the Unicellular Marine Diazotroph *Crocospaera watsonii* Strain WH8501[∇]

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The hydrogen (H₂) cycle associated with the dinitrogen (N₂) fixation process was studied in laboratory cultures of the marine cyanobacterium *Crocospaera watsonii*. The rates of H₂ production and acetylene (C₂H₂) reduction were continuously measured over the diel cycle with simultaneous measurements of fast repetition rate fluorometry and dissolved oxygen. The maximum rate of H₂ production was coincident with the maximum rates of C₂H₂ reduction. Theoretical stoichiometry for N₂ fixation predicts an equimolar ratio of H₂ produced to N₂ fixed. However, the maximum rate of net H₂ production observed was 0.09 nmol H₂ μg chlorophyll *a* (chl *a*)⁻¹ h⁻¹ compared to the N₂ fixation rate of 5.5 nmol N₂ μg chl *a*⁻¹ h⁻¹, with an H₂ production/N₂ fixation ratio of 0.02. The 50-fold discrepancy between expected and observed rates of H₂ production was hypothesized to be a result of H₂ reassimilation by uptake hydrogenase. This was confirmed by the addition of carbon monoxide (CO), a potent inhibitor of hydrogenase, which increased net H₂ production rates ~40-fold to a maximum rate of 3.5 nmol H₂ μg chl *a*⁻¹ h⁻¹. We conclude that the reassimilation of H₂ by *C. watsonii* is highly efficient (>98%) and hypothesize that the tight coupling between H₂ production and consumption is a consequence of fixing N₂ at nighttime using a finite pool of respiratory carbon and electrons acquired from daytime solar energy capture. The H₂ cycle provides unique insight into N₂ fixation and associated metabolic processes in *C. watsonii*.

The biological production of hydrogen (H₂) can occur as a by-product of photosynthesis, fermentation, and N₂ fixation (22). Of these three metabolic pathways, N₂ fixation remains a particularly enigmatic process, and to date there is no clear explanation for why H₂ evolves during the reduction of N₂ (11). The unfavorable energy cost of N₂ fixation can be mitigated by reassimilating the released H₂ via uptake hydrogenase enzyme activity (30). The coupled production and consumption of H₂ during cellular nitrogenase activity creates a H₂ cycle that can be hidden from measurements of ambient environmental H₂ concentrations and fluxes, depending upon the overall efficiency of H₂ assimilation (Fig. 1).

For most cultures of phototrophic marine diazotrophs grown under optimal conditions, complete reassimilation of H₂ is not achieved, and the excess H₂ is lost to the surrounding environment. This excess H₂ equates to the net production of H₂ and is expressed as the ratio of H₂ formed to N₂ fixed or the H₂/N₂ ratio. To date, H₂/N₂ ratios have mainly been measured on filamentous, colony-forming diazotrophs such as *Anabaena* spp. and *Trichodesmium* spp. with H₂ production rates of up to 20 nmol H₂ μg chlorophyll *a* (chl *a*)⁻¹ h⁻¹ and H₂/N₂ ratios ranging from 0.01 to 0.48 (3, 20, 24). H₂ production has also been quantified in unicellular diazotrophs (12, 16, 17, 32),

although the H₂ measurements have rarely been performed in conjunction with rates of N₂ fixation. However, recent H₂ measurements of two N₂-fixing unicellular cyanobacteria species reached a maximum of 1.38 nmol H₂ μg chl *a*⁻¹ h⁻¹, with H₂/N₂ ratios ranging from 0.003 to 0.05, indicating an effective reassimilation of H₂ can occur under certain conditions (34).

H₂ cycling in marine diazotrophs has important ecological implications both for the cell and for the marine H₂ cycle. Surface waters of low-latitude oceans are typically 200 to 300% supersaturated in dissolved H₂ with respect to atmospheric concentrations (25), implying a sustained localized production of H₂. The source of the dissolved H₂ is thought to be biological N₂ fixation (7); however, the relative contributions of diverse diazotrophic communities and *in situ* controls on H₂/N₂ ratios are not well constrained. N₂ fixation is performed by a suite of diazotrophs typically identified by their nitrogenase gene (*nifH*) sequences amplified directly from oceanic water samples (35). The importance of unicellular diazotrophs, including *Crocospaera* spp., in marine N₂ fixation has recently become widely recognized (36). Size-fractionated rates of N₂ fixation indicate that in the oligotrophic ocean, <10-μm microorganisms, which include the unicellular cyanobacteria, make a substantial contribution to the daily N₂ fixation (9, 18). Correlating the species-specific production of H₂ with the activity and biomass of diazotrophs will help elucidate dissolved H₂ cycling in the upper ocean.

We examined the cycling of H₂ in cultures of *Crocospaera watsonii* strain WH8501, a marine unicellular diazotroph, and correlated it with other metabolic parameters, including N₂ fixation measured via acetylene (C₂H₂) reduction, O₂ produc-

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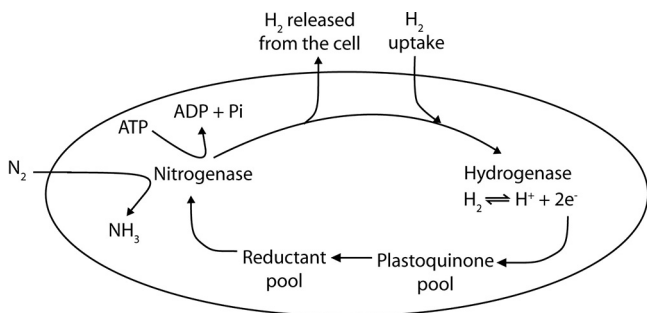


FIG. 1. H_2 is formed during N_2 fixation by the binding of a N_2 molecule to the molybdenum-iron protein of the nitrogenase enzyme complex, prior to the reduction of N_2 to ammonia (11, 15). The most energetically favorable theoretical *in vivo* stoichiometry predicts that one mole of H_2 is produced for every mole of N_2 reduced: $N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$. The production of H_2 consumes 25% of the electron flux through nitrogenase and diazotrophs mitigate this loss of potential energy by reassimilating the H_2 via uptake hydrogenase (21, 30). The electrons produced by uptake hydrogenase either generate reductant or ATP with simultaneous consumption of O_2 (3). (Adapted from reference 32a.)

tion and consumption, and photosynthetic efficiency. Carbon monoxide (CO) was used as an inhibitor of intracellular H_2 reassimilation to reveal the H_2 cycling that can occur in conjunction with nitrogenase activity. H_2 reassimilation by *C. watsonii* was shown to be very efficient in our laboratory experiments, which is considered to be a consequence of the temporal separation between daytime photosynthetic activity and nighttime N_2 fixation. Therefore, the present study not

only reveals the cell's H_2 cycle but also provides insight into the metabolism of nitrogenase in *C. watsonii*.

MATERIALS AND METHODS

C. watsonii strain WH8501 cultures were grown in 1.8-liter batch volumes and maintained in custom-built 2-liter glass incubator vessels. The experimental design permitted both discrete (chl *a*, cell counts) and continuous (O_2 , photosynthetic efficiency, C_2H_2 reduction, and net H_2 production) measurements. The growth and maintenance of the cultures, the incubator vessels, and the measurements are described below.

Stock cultures of *C. watsonii* were maintained in plastic culture flasks in SO medium (pH 8.0, salinity 28) (33) at 26°C using a 12-h square-wave light-dark (LD) cycle with a light intensity of $45 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$. When the cells were in exponential growth phase, 400 ml of culture with typical cell density of 10^5 cells ml^{-1} was used to inoculate 1.4 liters of SO medium in 2-liter incubation vessels known as photosynthetic response incubation and manipulation system (PRIMaS). The incubation chamber was 300 mm high and had a 100-mm diameter, with 3-mm glass thickness. The lid of the vessel was made of polytetrafluoroethylene (PTFE) and incorporated ports for the oxygen and pH probes (described below), an air inlet and outlet, and a discrete sampling port (Fig. 2). The inoculation and assembly of the incubator vessels were conducted under sterile conditions, and the cultures were monitored for bacterial contamination by flow cytometry analysis (described below).

The PRIMaS allowed control and manipulation of the light level, temperature, and the air composition and flow rate entering the incubator. The light source consisted of light-emitting diodes (8 warm white LEDs; Lumileds LXHL-NWG8) located at the bottom of the unit. Reflection of the light from the white PTFE lid helped to distribute the light evenly throughout the culture vessel. The temperature was maintained at 26°C. A series of mass flow controllers (Aalborg Instruments & Controls, Inc.) maintained the flow rate (50 ml min^{-1}) of air supplied to the culture. However, when H_2 measurements were conducted, the cultures were purged with ultrahigh purity air (Airgas) containing zero- H_2 (<10 ppt). To prevent clumping of cells at the bottom of the vessel, a stir bar (Nalgene, Rochester, NY) designed for stirring at low speeds was used to keep the cells suspended. In addition to the standard 12-h LD light regime, during one exper-

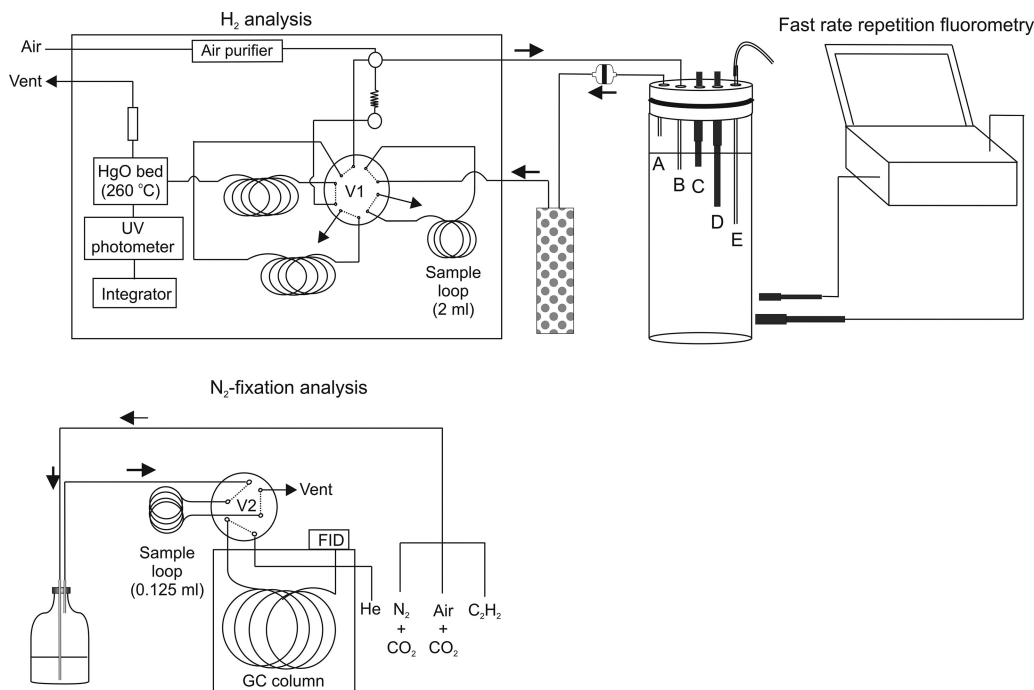


FIG. 2. Schematic of the experimental design for measuring H_2 production, fast repetition rate fluorometry and dissolved O_2 in a culture of *C. watsonii* maintained at 26°C on a 12-h LD cycle in a 2-liter glass vessel. The vessel lid incorporated an outlet (A) and inlet (B) for gas transfer, an O_2 probe (C) and a pH probe (D), and a port for discrete sampling (E). Measurements of C_2H_2 reduction were made on aliquots of *C. watsonii* subsampled from the incubator and analyzed using the on-line GC as previously described (34).

imental manipulation, *C. watsonii* cultures that had been maintained under five cycles of 12-h LD were exposed to constant light (24-h light) and monitored for 48 h.

To assess cell biomass and growth rates, discrete samples for chl *a* and cell counts were taken from the incubators using the sampling port every 24 h during the middle of the light period. For chl *a*, triplicate aliquots (3 ml) of culture were filtered onto 25-mm Whatman GF/F filters and extracted in 5 ml of 90% acetone for 24 h at -20°C before being analyzed by using a Turner Designs Model 10-AU fluorometer (28). For cell counts, 900 μl of culture were fixed with ultrapure-TEM grade Tousimis glutaraldehyde (final concentration, 0.25%) at room temperature for 20 min and then flash frozen in liquid nitrogen. The samples were stored at -80°C until processed using an InFlux flow cytometer (Becton Dickinson, San Jose, CA). The data were analyzed by using FlowJo software (Tree Star, Oregon) to obtain a volumetric estimate of cell density (cells ml^{-1}).

Measurements of C_2H_2 reduction were made using a modified online gas chromatography (GC) system, as previously described (27). Subsamples (30 ml) of the culture were taken from the incubator vessel immediately after the onset of the dark period, transferred to 76-ml borosilicate glass vials, and crimp sealed. The borosilicate vials were continually flushed with a gas stream (17 ml min^{-1}) composed of 70% N_2 (containing 300 ppm CO_2), 20% O_2 (containing 300 ppm CO_2), and 10% C_2H_2 . After exiting the sample culture, the gas flow was dried by passage through a Nafion drier (Permapure) to a 6-port switching valve, which injected 125 μl into an Agilent Technologies GC (model 6850, series II) fitted with a fused silica Porapak U capillary column (25 m by 0.53 mm; Chrompack). The operating conditions of the GC were the same as previously reported (34), and the detection limit with the analytical configuration was 0.03 nmol $\text{C}_2\text{H}_4\ \mu\text{g chl } a^{-1}\ \text{h}^{-1}$. Nitrogenase activity is expressed in terms of C_2H_4 production, except when the rates of N_2 fixation are compared to the net H_2 production. To convert C_2H_4 production rates to N_2 fixation, a ratio of 4 mol of C_2H_4 produced per mol of N_2 reduced was used (4).

Net H_2 production was quantified using a reduced gas analyzer (RGA; Peak Laboratories, Mountain View, CA) as previously described (34), with a few minor modifications. In contrast to C_2H_2 reduction measurements, which were conducted on subsamples, the RGA was directly connected to the incubator vessel (Fig. 2). The reason for having a higher biomass for H_2 measurements compared to C_2H_4 production was based on previous measurements of net H_2 production by *C. watsonii* WH8501, which revealed very low quantities of H_2 (34). The disadvantage of this design is that the analyzer was sensitive to trace quantities of H_2 emitted by the O_2 probe, and therefore a duplicate incubation was run in parallel to the incubator vessel containing the O_2 probe. In contrast to measurements of net H_2 production, gross H_2 production was measured by incubating discrete 30-ml subsamples of *C. watsonii* in the presence of an inhibitor of hydrogenase, described in full below.

In addition to net H_2 and C_2H_4 production, continual measurements were made for O_2 , pH, and variable fluorescence. Dissolved O_2 was measured by using a commercially available O_2 sensor (DO 1200; Sesonex, California). The sensor uses a Galvanic cell situated behind a high-density polyethylene membrane (16-mm diameter). The pH was measured by using mini-electrodes (Cole-Parmer, C-29044-00) and housed in a black Delrin cover. Variable fluorescence was measured by using a custom built benchtop fast repetition rate fluorometer (FRRF), which measures fluorescence transients induced by a series of subsaturating excitation pulses from a blue (470-nm) light emitting diode (350 mA 220 Lumens) to derive photosynthetic parameters (14). The FRRF was used to determine the photochemical quantum yield (F_v/F_m), which is the ratio of the maximum change in variable fluorescence (F_v) to the maximum fluorescence yield (F_m). This was determined from the initial dark-adapted fluorescence (F_0), and F_m , when all PSII reaction centers are photochemically reduced [$F_v/F_m = (F_m - F_0)/F_m$].

Alongside the measurements described above, a series of inhibitor experiments were carried out to further investigate H_2 cycling by *C. watsonii*. To derive an estimation of gross H_2 production, CO was added to a culture of *C. watsonii*. CO is an inhibitor of hydrogenase and all reactions catalyzed by nitrogenase except for the reduction of H^+ to H_2 (26). The CO inhibitor experiments were carried out on 30-ml aliquots of *C. watsonii* cultures subsampled from the incubator unit at selected times during the dark period. The 30-ml aliquots were added to 76-ml glass vials and crimp sealed. The aliquots were flushed with CO-free air for 10 min at 20 ml min^{-1} , injected with CO (final concentration, 20 mM), and incubated in the dark for 30 min. Separate experiments indicated that the production of H_2 increased linearly within an incubation time of 60 min. Headspace samples from the crimp-sealed glass vials were then injected into the analyzer. For the CO experiments, a longer Unibead column (2.44 m by 3.18 mm) was installed in the RGA as the primary column (Fig. 2). The longer column allowed the carrier flow to be diverted to waste after H_2 had eluted and

been quantified, avoiding the deleterious effects of CO (20 mM) reaching the mercuric oxide bed of the analyzer.

Two separate inhibitor experiments investigated the supply and demand for electrons by nitrogenase in relation to H_2 production and variable fluorescence measurements. For these inhibitor experiments, two additional cultures of *C. watsonii* were grown in the incubator units and monitored over several days. The inhibitors were added via the discrete sampling port of the incubator unit and the culture was subsequently subsampled for N_2 fixation and net H_2 production as described above. The supply of electrons to nitrogenase was inhibited by the addition of 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) (20 μM final concentration) at the end of the light period. DBMIB blocks the transfer of electrons from the plastoquinone (PQ) pool (Fig. 1) to cytochrome *b₆f* (8, 10). The demand for electrons by nitrogenase was independently inhibited by the addition of ammonium chloride (NH_4Cl) as previously reported (5). The NH_4Cl (final concentration, 20 μM) was added 9 h after the beginning of the light period.

RESULTS

Net H_2 production was measured in *C. watsonii* batch cultures grown in 1.8-liter volumes at $45\ \mu\text{mol quanta m}^{-2}\ \text{s}^{-1}$ and 26°C . In conjunction with C_2H_4 production, photosynthesis, and respiration measurements, net H_2 production also displayed strong diel cycle (Fig. 3).

The onset of net H_2 production was observed 4 to 5 h into the dark period (Fig. 3a), reaching a maximum rate of 0.09 nmol $\text{H}_2\ \mu\text{g chl } a^{-1}\ \text{h}^{-1}$ after 8 to 9 h in the dark period. Maximum rates of net H_2 production were never sustained for more than 1 h and subsequently decreased to low levels before the onset of the light period. The net production of H_2 coincided with C_2H_4 production with nearly identical temporal patterns (Fig. 3b). C_2H_4 production began ~ 3 h into the dark period, and a maximum C_2H_4 production rate of 22.5 nmol $\text{C}_2\text{H}_4\ \mu\text{g chl } a^{-1}\ \text{h}^{-1}$ occurred 7 h after the onset of the dark period.

The daily pattern of dissolved O_2 concentrations displayed the expected accumulation of O_2 during the light period due to photosynthesis and the respiratory O_2 consumption in the dark period (Fig. 3c). It should be noted that as the cultures were maintained under constant airflow (50 ml min^{-1}), dissolved O_2 concentrations were continually pushed toward equilibrium with the air supply. Therefore, the increase in dissolved O_2 concentration observed in the final 5 h of the dark period most likely reflects deceleration of the respiratory process with the O_2 equilibrating toward the ambient level. Despite this, three distinct phases are evident in the respiratory O_2 consumption (Fig. 3c). Prior to the onset of N_2 fixation at ~ 3 h into the dark period (Fig. 3b), the dissolved O_2 decreased at a rate of about $3.2\ \mu\text{mol h}^{-1}$, reflecting the respiratory utilization of organic carbon. Since the respiratory and photosynthetic electron utilization pathways are shared in cyanobacteria, this respiratory electron flow reduces the PQ pool, resulting in almost total collapse of the variable fluorescence within the first 10 min after switching the light off (Fig. 3d). The respiratory O_2 utilization increased by a factor of two following the onset of N_2 fixation based on the change in the dissolved O_2 slope. The midpoint of this phase corresponds to the highest gradient in the N_2 fixation curve, possibly indicating the highest demand for energy/electrons. Incidentally, this point also corresponds to the transient increase in the F_v/F_m ratio at ~ 5 h into the dark period (Fig. 3d), most likely reflecting a brief reoxidation of the PQ pool due to the strongest demand for the respiratory electrons that transiently outpaces their supply. The dissolved

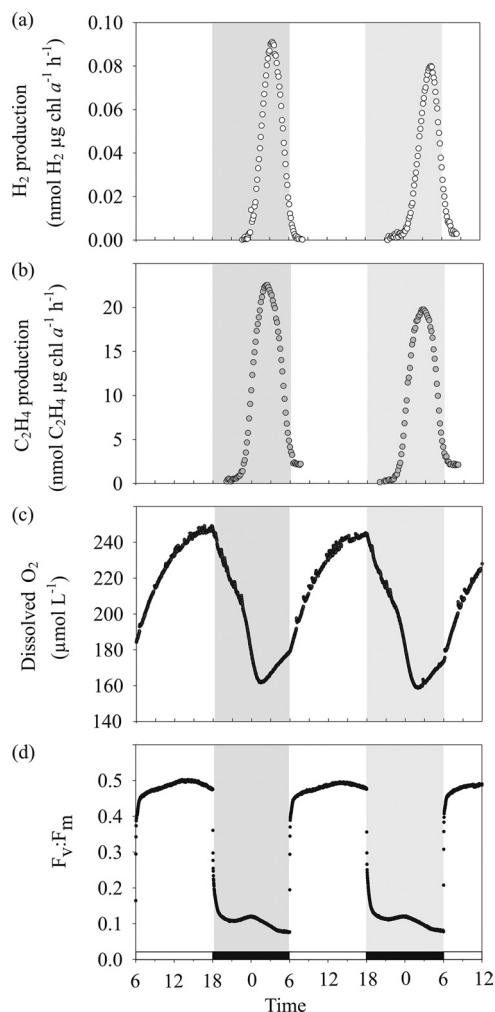


FIG. 3. Metabolism of *C. watsonii* under 12-h LD conditions. (a) H_2 production; (b) N_2 fixation, shown as C_2H_4 production; (c) O_2 concentration as measured by an O_2 electrode; (d) photosynthetic quantum yield measured using fast repetition rate fluorometry. Dark periods during each diel cycle are indicated as solid blocks on the lower section of Fig. 3d.

O_2 minimum coincides with the maximum of N_2 fixation and is followed by the third phase, with increasing concentration of dissolved O_2 , as previously described.

After the onset of illumination, the photosynthetic quantum yield of PS II (F_v/F_m) increased rapidly within 10 min, reaching a level ranging from 0.5 to 0.55 during the light phase, with the maximum yield occurring 6 h into the light period (Fig. 3d). This pattern was observed in all *C. watsonii* cultures from the second day after inoculation of the incubator vessels and onward. At the onset of the dark phase, the F_v/F_m ratio rapidly decreased and then displayed a more gradual decline throughout the night to reach a minimum yield (0.1) just prior to the onset of the light period. The night-time F_v/F_m profile was characterized by a transient increase, which consistently occurred 3 h into the dark period. At this time, the F_v/F_m ratio increased to a maximum of 0.15 before declining again.

The diel cycling in H_2 and C_2H_4 production observed during 12-h LD cycles continued to occur when the *C. watsonii* cul-

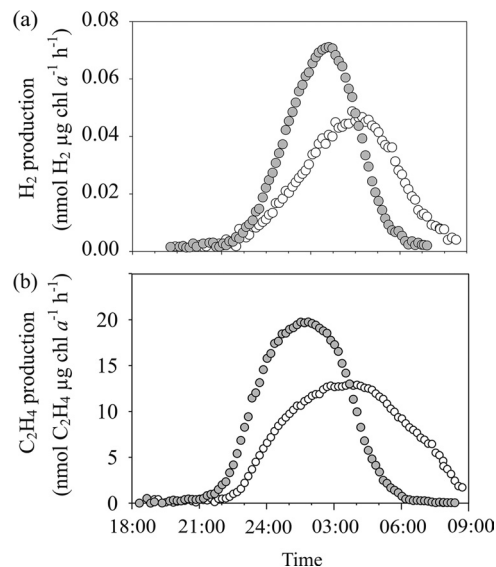


FIG. 4. H_2 production (a) and C_2H_4 production (b) in *C. watsonii* cultures exposed to a constant light regime (open circles) compared to a 12-h LD regime (shaded circles). The cultures were maintained in the incubators for five cycles of 12-h LD before switching to constant light. The dark period would have extended from 18:00 to 06:00.

tures were maintained under constant illumination (Fig. 4). Maximum rates of C_2H_4 production and net H_2 production under constant light regime were 65 and 68%, respectively, of the 12-h LD values. In both instances, the peak of maximum rates was delayed by 1 to 2 h. Furthermore, C_2H_4 and net H_2 production extended for a longer time period. Therefore, although the maximum rates of production are lower in 24 h light, the C_2H_4 and net H_2 production rates integrated over a 24 h period were 93 and 88%, respectively, of the 12-h LD values.

The addition of CO to aliquots of *C. watsonii* cultures subsampled from the incubator vessels had a dramatic effect on H_2 concentrations when it was added during the period of nitrogenase activity. Outside of this time period, e.g., at the onset of the dark period, the addition of CO had no effect on H_2 production (data not shown). The effect of H_2 production in the presence of CO (Fig. 5b) was evident compared to the unamended culture (Fig. 5a). The addition of CO increased the maximum rate of H_2 production 40-fold from 0.09 to 3.5 $nmol H_2 \mu g chl a^{-1} h^{-1}$.

To further elucidate the pathways of respiratory electron transport in N_2 fixation, cultures of *C. watsonii* were amended with NH_4Cl and the electron transport inhibitor DBMIB in separate experiments. Immediately after the addition of NH_4Cl to the incubator vessel, there was a slight decrease in the F_v/F_m ratio by 0.05 (Fig. 6a). At the onset of the dark period, 2 h later, the typical decrease in the F_v/F_m ratio was observed, indicating active utilization of the respiratory carbon and PQ pool reduction. In contrast to the unamended cultures, the transient increase in the F_v/F_m ratio after 3 h was absent, indicating the loss of the nitrogenase-based sink for the respiratory electrons. This loss is consistent with the 95% inhibition of C_2H_4 production by NH_4Cl (Fig. 6b). The addition of the DBMIB produced a response similar to that of NH_4Cl . There

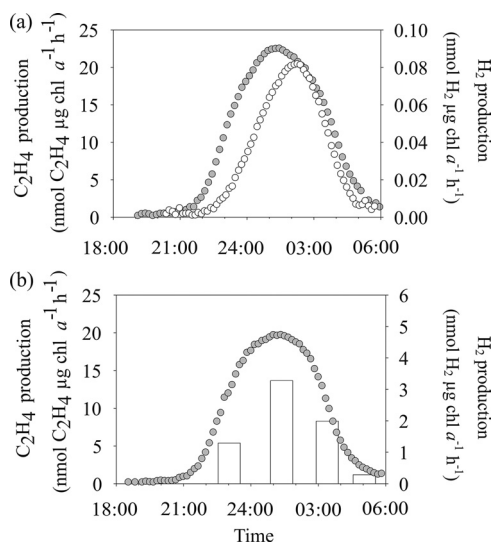


FIG. 5. Effect of CO on H_2 production by *C. watsonii*. (a) Continual measurements of H_2 production (open circles) and C_2H_4 production rates (shaded circles) with no CO added; (b) H_2 production (open bars) measured in the presence of CO (final concentration, 20 mM) on discrete subsamples of *C. watsonii* during the dark period and compared to C_2H_4 production rates (shaded circles), which have no CO added. Note the difference in the y axis scale for H_2 production between panels a and b.

was no transient F_v/F_m change observed after 3 h (Fig. 6c), and the rate of C_2H_4 production decreased by 82% of the control measurements. In this instance, however, these effects are due to inhibition of electron flow from the PQ pool by DBMIB.

DISCUSSION

The H_2 cycle was investigated in the unicellular diazotroph *C. watsonii*, which was maintained in batch culture. During the experimental period, the growth rate was 0.3 divisions day^{-1} with a quantum yield of photosynthesis (F_v/F_m ratio) of 0.52. This is at the higher end of reported F_v/F_m values for cyanobacteria, which typically range from 0.1 to 0.6 (29), indicating that the cells were physiologically healthy (14). The production of H_2 and C_2H_4 under standard 12-h LD light regimes and constant illumination indicates that these metabolic processes operate under circadian control, as previously shown for nitrogenase gene expression in *C. watsonii* (19).

The net H_2 production rates by *C. watsonii* reached a maximum of 0.09 $nmol H_2 \mu g chl a^{-1} h^{-1}$ compared to the N_2 fixation rates of 5.5 $nmol N_2 \mu g chl a^{-1} h^{-1}$. Therefore, *in vivo* net H_2 evolution rates are <2% of the theoretical stoichiometry (see the stoichiometry equation in the legend to Fig. 1). The production of H_2 is integral to the N_2 fixation process (11), and we hypothesized that the low H_2/N_2 ratio was a result of efficient H_2 re-assimilation by *C. watsonii*. The highly effective assimilation of H_2 by *C. watsonii* is particularly apparent compared to other diazotrophs, such as *Trichodesmium erythraeum*, where H_2/N_2 ratios are equal to 0.3 (34). Furthermore, the H_2/N_2 ratio increased dramatically when CO was added to subsamples of *C. watsonii* cultures. The rates of H_2 production in the presence of CO were 40 times greater than unamended measurements (Fig. 5) and represented 70% of the equimolar

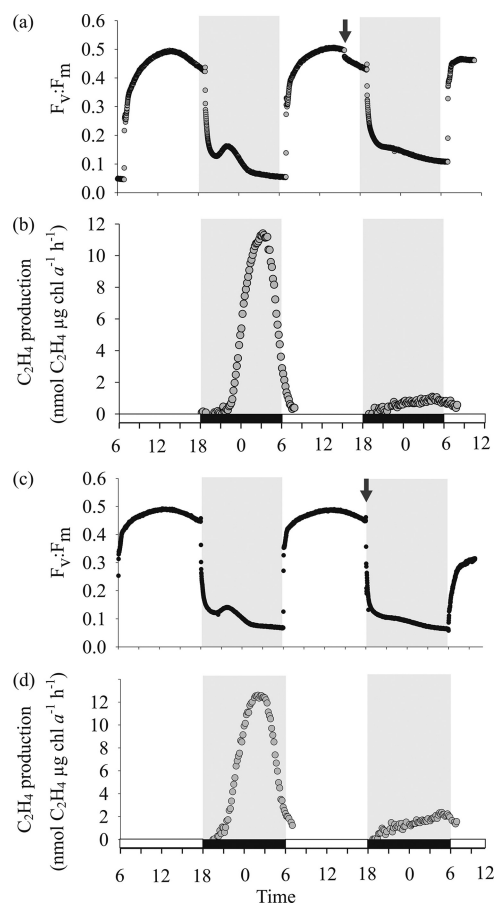


FIG. 6. Effect of NH_4Cl (final concentration, 20 μmol) on the F_v/F_m ratio (a) and C_2H_4 production (b) and effect of DBMIB (final concentration, 20 μmol) on the F_v/F_m ratio (c) and C_2H_4 production (d). The arrows in panels a and c indicate when the inhibitors were added to the separate experiments. Both treatments inhibited N_2 fixation while eliminating the transient peak in the nighttime F_v/F_m ratio.

theoretical stoichiometry (see equation [legend to Fig. 1]). The use of CO to reveal the hidden cycling of H_2 has been demonstrated for cultures of other diazotrophs, including *Trichodesmium* spp. (23), *Azotobacter chroococcum* (26), and *Anabaena cylindrica* (2). These studies revealed that the maximum H_2 production rates were elicited when CO was added together with C_2H_2 , or DCMU in the case of *Trichodesmium*. C_2H_2 was not used as an inhibitor in the present study because commercially available gas cylinders of C_2H_2 (e.g., Praxair) contain H_2 in excess of the concentrations produced by *C. watsonii* (13).

The efficient recycling of H_2 by *C. watsonii* can be explained by nitrogenase activity within unicellular diazotrophs. *Crocospaera* sp. protects its nitrogenase from inactivation by temporally separating N_2 fixation and photosynthetic (O_2 -producing) activities (6). Therefore, *Crocospaera* fixes N_2 at night using the energy and reductant provided via respiration and use of photosynthetically fixed carbon (1). This places a greater demand on the cell to re-assimilate the H_2 produced by nitrogenase as the photosynthetic pool of fixed carbon cannot be replenished until the following daytime (31). Uptake hydrogenase activity feeds the electrons from the assimilated H_2 back

into the electron transport chain to form either reductant (e.g., ferredoxin) or to produce ATP with the simultaneous consumption of O₂ (3).

Additional evidence of efficiency in the metabolic machinery of *C. watsonii* is revealed in the F_v/F_m measurements. The F_v/F_m profile for *C. watsonii* decreases at the beginning of the dark period due to a reduction of the PQ pool (e.g., Fig. 3d). This rapid decrease is followed by a smaller transient increase in F_v/F_m ratio 3 to 5 h later, indicating the partial reoxidation of the PQ pool. The addition of NH₄Cl and DBMIB revealed the relationship between PQ oxidation and the transiently high demands for the respiratory electrons due to nitrogenase activity. The addition of NH₄Cl and DBMIB eliminated this transient by either reducing this demand by inhibiting nitrogenase activity or by blocking the electron pathway toward nitrogenase (see equation [legend to Fig. 1]). Furthermore, at the same time the rate of O₂ drawdown increases due to accelerated respiratory activity required to support both the energy and the electron requirements of nitrogenase (Fig. 3c). The F_v/F_m profiles therefore highlight the central role of PQ in the cellular electron transport system. It acts as an intermediary electron carrier between photosystem II and photosystem I in photosynthesis but also mediates the flow of the respiratory electrons. Interestingly, the dual function of PQ in both respiratory and photosynthetic electron transport systems in *A. cylindrica* was revealed by the analysis of H₂ metabolism (8). The addition of DBMIB inhibited the H₂ uptake in either the respiratory or photosynthetic electron transport chains, indicating the PQ is also the primary electron acceptor for uptake hydrogenase (8). Future experiments investigating H₂ oxidation by cyanobacteria and the response of the intracellular respiratory electron transport system should consider complementary measurements of the F_v/F_m ratio.

Conclusion. The daily patterns of photosynthesis, respiration, and N₂ fixation of *C. watsonii* cultures were measured alongside the theoretical, the net, and the gross fluxes of H₂ associated with these processes. Our measurements revealed an active cycling of H₂ by *Crocospaera* that is remarkably efficient under the culture conditions investigated in the present study. This efficiency appears to be driven by the temporal separation of photosynthesis and N₂ fixation, resulting in a more stringent limitation of exclusively respiratory energy and electron pools available to nitrogenase. The unraveling of a hidden H₂ cycle in *Crocospaera* is an important component in understanding how these cyanobacteria combine metabolic processes, e.g., photosynthesis, respiration, and N₂ fixation in a single unicellular compartment. Furthermore, as we understand more about how these processes operate in the same organism, analysis of the H₂ cycle demonstrates the need to quantify the efficiency of these processes, particularly in comparison to other diazotrophs.

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REFERENCES

- Berman-Frank, I., A. Quigg, Z. V. Finkel, A. J. Irwin, and L. Haramaty. 2007. Nitrogen-fixation strategies and Fe requirements in cyanobacteria. *Limnol. Oceanogr.* **52**:2260–2269.
- Bothe, H., J. Tenggkeit, G. Eisbrenner, and M. G. Yates. 1978. The hydrogenase-nitrogenase relationship in the blue-green alga *Anabaena cylindrica*. *Planta* **133**:237–242.
- Bothe, H., E. Distler, and G. Eisbrenner. 1978. Hydrogen metabolism in blue-green algae. *Biochimie* **60**:277–289.
- Capone, D. G. 1993. Determination of nitrogenase activity in aquatic samples using the acetylene reduction procedure, p. 621–631. In P. F. Kemp, B. F. Sherr, E. B. Sherr, and J. J. Cole (ed.), *Handbook of methods in aquatic microbial ecology*. Lewis Publishers, Boca Raton, FL.
- Chen, Y.-B., J. P. Zehr, and M. Mellon. 1996. Growth and nitrogen fixation of the diazotrophic filamentous nonheterocystous cyanobacterium *Trichodesmium* sp. IMS101 in defined media: evidence for a circadian rhythm. *J. Phycol.* **32**:916–923.
- Church, M. J., C. M. Short, B. D. Jenkins, D. M. Karl, and J. P. Zehr. 2005. Temporal patterns of nitrogenase gene (*nifH*) expression in the oligotrophic North Pacific Ocean. *Appl. Environ. Microbiol.* **71**:5362–5370.
- Conrad, R. 1988. Biogeochemistry and ecophysiology of atmospheric CO and H₂. *Adv. Microb. Ecol.* **10**:231–283.
- Eisbrenner, G., and H. Bothe. 1979. Modes of electron transfer from molecular hydrogen in *Anabaena cylindrica*. *Arch. Microbiol.* **123**:37–45.
- Grabowski, M. N. W., M. J. Church, and D. M. Karl. 2008. Nitrogen fixation rates and controls at Stn ALOHA. *Aquat. Microb. Ecol.* **52**:175–183.
- Hihara, Y., K. Sonoike, M. Kanehisa, and M. Ikeuchi. 2003. DNA microarray analysis of redox-responsive genes in the genome of the cyanobacterium *Synechocystis* sp. strain PCC 6803. *J. Bacteriol.* **185**:1719–1725.
- Howard, J. B., and D. C. Rees. 2006. How many metals does it take to fix N₂? A mechanistic overview of biological nitrogen fixation. *Proc. Natl. Acad. Sci.* **103**:17088–17093.
- Howarth, D. C., and G. A. Codd. 1985. The uptake and production of molecular hydrogen by unicellular cyanobacteria. *J. Gen. Microbiol.* **131**:1561–1569.
- Hyman, M. R., and D. J. Arp. 1987. Quantification and removal of some contaminating gases from acetylene used to study gas-utilizing enzymes and microorganisms. *Appl. Environ. Microbiol.* **53**:298–303.
- Kolber, Z., O. Prášil, and P. G. Falkowski. 1998. Measurements of variable chlorophyll fluorescence using fast repetition rate techniques: defining methodology and experimental protocols. *Biochim. Biophys. Acta* **1367**:88–106.
- Lowe, D. J., and R. N. F. Thorneley. 1984. The mechanism of *Klebsiella pneumoniae* nitrogenase action. *Biochem. J.* **224**:877–909.
- Min, H., and L. A. Sherman. 2010. Hydrogen production by the unicellular, diazotrophic cyanobacterium *Cyanothece* sp. strain ATCC 51142 under conditions of continuous light. *Appl. Environ. Microbiol.* **76**:4293–4301.
- Mitsui, A., and S. Suda. 1995. Alternative and cyclic appearance of H₂ and O₂ photoproduction activities under non-growing conditions in an aerobic nitrogen-fixing unicellular cyanobacterium *Synechococcus* sp. *Curr. Microbiol.* **30**:1–6.
- Montoya, J. P., C. M. Carolyn, J. P. Zehr, A. Hansen, T. A. Villareal, and D. G. Capone. 2004. High rates of N₂ fixation by unicellular diazotrophs in the oligotrophic Pacific Ocean. *Nature* **430**:1027–1031.
- Pennebaker, K., K. R. M. Mackey, R. M. Smith, S. B. Williams, and J. P. Zehr. 2010. Diel cycling of DNA staining and *nifH* gene regulation in the unicellular cyanobacterium *Crocospaera watsonii* strain WH8501 (*Cyano-phyta*). *Environ. Microbiol.* **12**:1001–1010.
- Punshon, S., and R. M. Moore. 2008. Aerobic hydrogen production and dinitrogen fixation in the marine cyanobacterium *Trichodesmium erythraeum* IMS101. *Limnol. Oceanogr.* **53**:2749–2753.
- Rao, K. K., and D. O. Hall. 1996. Hydrogen production by cyanobacteria: potential, problems and prospects. *J. Mar. Biotechnol.* **4**:10–15.
- Rupperecht, J., B. Hankamer, J. H. Mussgnug, G. Ananyev, C. Disumkes, and O. Kruse. 2006. Perspectives and advances of biological H₂ production in microorganisms. *Appl. Microbiol. Biotechnol.* **72**:442–449.
- Saino, T., and A. Hattori. 1982. Aerobic nitrogen fixation by the marine non-heterocystous cyanobacterium *Trichodesmium (Oscillatoria)* spp.: its protective mechanisms against oxygen. *Mar. Biol.* **70**:251–254.
- Scranton, M. I., P. C. Novelli, A. Michaels, S. G. Horrigan, and E. J. Carpenter. 1987. Hydrogen production and nitrogen fixation by *Oscillatoria thiebautii* during in situ incubations. *Limnol. Oceanogr.* **32**:998–1006.
- Seiler, W., and U. Schmidt. 1974. Dissolved non-conservative gases in seawater, p. 219–243. In E. Goldberg (ed.), *The sea*, vol. V. Wiley Interscience, New York, NY.
- Smith, L. A., S. Hill, and M. G. Yates. 1976. Inhibition by acetylene of conventional hydrogenase in nitrogen-fixing bacteria. *Nature* **262**:209–210.
- Staal, M., S. te Lintel-Hekkert, F. Harren, and L. J. Stal. 2001. Nitrogenase activity in cyanobacteria measured by acetylene reduction assay: a comparison between batch incubation and on-line monitoring. *Environ. Microbiol.* **3**:343–351.

28. **Strickland, J. D. H., and T. R. Parsons.** 1972. A practical handbook of seawater analysis. Fisheries Research Board of Canada, Ottawa, Ontario.
29. **Suggett, D. J., C. M. Moore, A. E. Hickman, and R. J. Geider.** 2009. Interpretation of fast repetition rate (FRR) fluorescence: signatures of phytoplankton community structure versus physiological state. *Mar. Ecol. Prog. Ser.* **376**:1–19.
30. **Tamagnini, P., R. Axelsson, P. Lindberg, F. Oxelfelt, R. Wünschiers, and P. Lindblad.** 2002. Hydrogenases and hydrogen metabolism of cyanobacteria. *Microbiol. Mol. Biol. Rev.* **66**:1–20.
31. **Tuit, C., J. Waterbury, and G. Ravizza.** 2004. Diel variation of molybdenum and iron in marine diazotrophic cyanobacteria. *Limnol. Oceanogr.* **49**:978–990.
32. **van der Oost, J., W. A. Kannevorff, K. Krab, and R. Kraayenhof.** 1987. Hydrogen metabolism of three unicellular nitrogen-fixing cyanobacteria. *FEMS Microbiol. Lett.* **48**:41–45.
- 32a. **Walker, C. C., and M. G. Yates.** 1978. The hydrogen cycle in nitrogen-fixing *Azotobacter chroococcum*. *Biochimie.* **60**:225–231.
33. **Waterbury, J. B., and J. M. Willey.** 1988. Isolation and growth of marine planktonic cyanobacteria. *Methods Enzymol.* **167**:100–105.
34. **Wilson, S. T., R. A. Foster, J. P. Zehr, and D. M. Karl.** 2010. Hydrogen production by *Trichodesmium erythraeum*, *Cyanothece* sp., and *Crocospaera watsonii*. *Aquat. Microb. Ecol.* **59**:197–206.
35. **Zehr, J. P., M. T. Mellon, and S. Zani.** 1998. New nitrogen-fixing microorganisms detected in oligotrophic oceans by amplification of nitrogenase (*nifH*) genes. *Appl. Environ. Microbiol.* **64**:3444–3450.
36. **Zehr, J. P., J. B. Waterbury, P. J. Turner, J. P. Montoya, E. Omoregie, G. F. Steward, A. Hansen, and D. M. Karl.** 2001. Unicellular cyanobacteria fix N₂ in the subtropical North Pacific Ocean. *Nature* **412**:635–638.