



# Hydrogen Dynamics in *Trichodesmium* Colonies and Their Potential Role in Mineral Iron Acquisition

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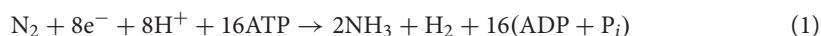
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N<sub>2</sub>-fixing cyanobacteria mediate H<sub>2</sub> fluxes through the opposing processes of H<sub>2</sub> evolution, which is a by-product of the N<sub>2</sub> fixation reaction, and H<sub>2</sub> uptake, which is driven by uptake hydrogenases. Here, we used microelectrodes to characterize H<sub>2</sub> and O<sub>2</sub> dynamics in single natural colonies of the globally important N<sub>2</sub> fixer *Trichodesmium* collected from the Gulf of Eilat. We observed gradually changing H<sub>2</sub> dynamics over the course of the day, including both net H<sub>2</sub> evolution and net H<sub>2</sub> uptake, as well as large differences in H<sub>2</sub> fluxes between individual colonies. Net H<sub>2</sub> uptake was observed in colonies amended with H<sub>2</sub> in both light and dark. Net H<sub>2</sub> evolution was recorded in the light only, reflecting light-dependent N<sub>2</sub> fixation coupled to H<sub>2</sub> evolution. Both net H<sub>2</sub> evolution and H<sub>2</sub> uptake rates were higher before 2 pm than later in the day. These pronounced H<sub>2</sub> dynamics in the morning coincided with strong net O<sub>2</sub> uptake and the previously reported diel peak in N<sub>2</sub> fixation. Later in the afternoon, when photosynthesis rates determined by O<sub>2</sub> measurements were highest, and N<sub>2</sub> fixation rates decrease according to previous studies, the H<sub>2</sub> dynamics were also less pronounced. Thus, the observed diel variations in H<sub>2</sub> dynamics reflect diel changes in the rates of O<sub>2</sub> consumption and N<sub>2</sub> fixation. Remarkably, the presence of H<sub>2</sub> strongly stimulated the uptake of mineral iron by natural colonies. The magnitude of this effect was dependent on the time of day, with the strongest response in incubations that started before 2 pm, i.e., the period that covered the time of highest uptake hydrogenase activity. Based on these findings, we propose that by providing an electron source for mineral iron reduction in N<sub>2</sub>-fixing cells, H<sub>2</sub> may contribute to iron uptake in *Trichodesmium* colonies.

**Keywords:** *Trichodesmium*, colony, N<sub>2</sub> fixation, H<sub>2</sub> evolution, uptake hydrogenase, O<sub>2</sub> fluxes, iron acquisition, dust

## INTRODUCTION

Marine primary productivity is often limited by the availability of dissolved organic or inorganic nitrogen (Moore et al., 2013). Diazotrophic cyanobacteria can access an additional nitrogen source, dinitrogen (N<sub>2</sub>) gas, and reduce it to ammonia, thereby making it available for other phytoplankton. As part of the N<sub>2</sub> fixation reaction catalyzed by nitrogenase, an equimolar amount of hydrogen (H<sub>2</sub>) is produced:



Since the reduction of protons ( $H^+$ ) consumes both reducing equivalents and ATP,  $H_2$  evolution contributes to the high energy costs related to  $N_2$  fixation. However, all  $N_2$ -fixing cyanobacteria analyzed so far have either uptake hydrogenases or bidirectional hydrogenases that allow them to recycle some of this  $H_2$  (Tamagnini et al., 2002). As these hydrogenases can feed electrons from  $H_2$  into the respiratory electron transport chain, the uptake of  $H_2$  provides a mechanism for recycling reducing equivalents.

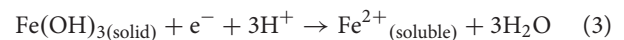
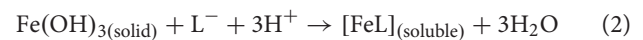
The cyanobacterium *Trichodesmium* is one of the major marine  $N_2$  fixers and has been estimated to contribute up to 50% to total nitrogen fixation in some areas (e.g., Karl et al., 1997; Dore et al., 2002). A range of different protective mechanisms has been proposed to shield the  $O_2$ -sensitive nitrogenase from photosynthetically evolved  $O_2$  in *Trichodesmium*. Firstly, spatial separation of  $N_2$  fixation and photosynthesis was suggested for the colony-level (Paerl and Bebout, 1988) as well as the single-cell level ( $N_2$ -fixing cells termed diazocytes; Bergman and Carpenter, 1991; Berman-Frank et al., 2001). Additionally, nitrogenase activity was shown to be strictly regulated over the diel cycle by daily synthesis of the nitrogenase pool in the morning and inactivation by post-translational modification in the afternoon, allowing for a relatively narrow peak of activity around midday (Zehr et al., 1993; Berman-Frank et al., 2001). A down-regulation of photosynthesis during this midday peak in  $N_2$  fixation was suggested as a mechanism to protect nitrogenase from  $O_2$  (Berman-Frank et al., 2001). Regarding the  $H_2$  metabolism of *Trichodesmium*, the genome of *Trichodesmium erythraeum* IMS101 encodes for the uptake hydrogenase *hupSL*<sup>1</sup> and previous studies on its  $H_2$  metabolism showed that it can recapture ca. 70% of the evolved  $H_2$  (Wilson et al., 2010, 2012).

In natural systems, *Trichodesmium* often forms colonies that host a wide range of epibiotic bacteria with diverse functions (Hewson et al., 2009; Hmelo et al., 2012; Lee et al., 2017; Frischkorn et al., 2018). Apart from providing a substrate for bacteria, colonies can induce formation of distinct chemical microenvironments. Early studies suggested anoxic microenvironments in *Trichodesmium* colonies to facilitate  $N_2$  fixation in colonies (Paerl and Bebout, 1988), potentially including the Knallgas reaction (i.e., the reaction of  $H_2$  with  $O_2$  to form  $H_2O$ ) as a mechanism to reduce  $O_2$  concentrations (Saino and Hattori, 1982). However, more recent studies showed that during day-time in the light, when photosynthesis is active,  $O_2$  concentrations in colonies can increase to as much as 200% of air saturation, which would impose a considerable challenge to the  $O_2$ -sensitive nitrogenase in *Trichodesmium* (Eichner et al., 2017, 2019). In view of this colony-specific potential impediment to  $N_2$  fixation, it becomes all the more relevant to identify the role of colony formation.

Recent studies have suggested colony formation to facilitate iron acquisition from dust, as *Trichodesmium* colonies were shown to efficiently capture dust particles, move them to the colony center and enhance iron dissolution from the dust (Rubin et al., 2011). The physiological and biochemical mechanisms of dust particle translocation to the colony center and iron dissolution from dust by *Trichodesmium* are still under

investigation. In a parallel study (Eichner et al., submitted), we examined whether chemical gradients within colonies provide beneficial conditions for iron dissolution in the colony center. Measuring  $O_2$  and pH gradients within *Trichodesmium* colonies with microsensors, we found that the respiration-induced decreases in  $O_2$  and pH in colonies are not large enough to significantly enhance iron dissolution. Hence, the active centering of dust particles in the colony must have a different reason.

The release of soluble iron from minerals, where it is generally present in the form of Fe(III) oxide or oxyhydroxide, can be facilitated by ligand (L) binding (Eq. 2) and/or the reduction of  $Fe^{3+}$  to  $Fe^{2+}$  (Eq. 3):



Mineral iron dissolution by *Trichodesmium* has been suggested to involve ligand-promoted dissolution (Basu et al., in press) and/or reductive dissolution (Rubin et al., 2011). While *Trichodesmium* has been demonstrated to reduce dissolved iron (Roe and Barbeau, 2014; Lis et al., 2015), direct measurements of mineral iron reduction by this organism are still lacking. Reductive dissolution of mineral iron most likely occurs in the vicinity of the cells in the colony center, where the minerals are concentrated. One possibility is electron transfer to Fe(III) directly on the cell surface, using electrons derived from photosynthesis or respiration, by a yet unidentified mechanism. Alternatively, accumulation of a reductant in the colony microenvironment might enable reductive dissolution. Here, we propose that the uptake of  $H_2$ , which is produced during  $N_2$  fixation, might play a previously unrecognized role in supplying reducing power for iron acquisition. Specifically, we hypothesized that  $H_2$  can act as a reductant for mineral-bound Fe(III), which is then liberated from the dust matrix for uptake and assimilation.

To address this hypothesis, we firstly used microelectrodes to characterize  $H_2$  fluxes and their dynamics in relation to  $O_2$  fluxes over the diel cycle in the microenvironment of single colonies collected in the Gulf of Eilat. In a second step, we used this data to analyze the kinetics of  $H_2$  uptake under different light conditions and stages of the diel cycle. Finally, to evaluate the potential of  $H_2$  as a reductant for mineral iron dissolution and acquisition, we investigated whether addition of  $H_2$  affects the uptake of mineral iron by *Trichodesmium* colonies.

## MATERIALS AND METHODS

### Colony Sampling

*Trichodesmium* colonies were sampled from the Gulf of Eilat/Aqaba in the Red Sea over a period of 2 months from March to May 2018. Colonies were collected with a 200  $\mu\text{m}$  net, either placed statically at ca. 1–2 m depth on a pole extended from the pier (3–4 m bottom depth) for approx. 2 h, or by vertical net tows from 20 m to the surface carried out from a boat at ca. 300 m

<sup>1</sup>[http://genome.jgi-psf.org/finished\\_microbes/trier/trier.home.html](http://genome.jgi-psf.org/finished_microbes/trier/trier.home.html)

bottom depth in the Gulf. Puff-shaped colonies were then hand-picked with Pasteur pipettes and washed with trace metal free filtered sea water (cleaned using ion exchange resin Chelex 100). Colonies were collected multiple times during the day and kept in filtered seawater under stable light conditions (ca. 130  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) at 25°C for ca. 1–5 h until measurements were started. Assuming that the diel rhythm of metabolic activity by the colonies is maintained under these conditions, rate measurements over the diel cycle are reported relative to the time of measurement rather than sample collection time.

## Microsensor Measurements

Microsensor measurements were performed on single colonies. The colonies were placed in filtered seawater in Petri dishes of approx. 50 ml volume, and held in position with a thin glass needle above a nylon mesh to ensure unperturbed diffusion in all directions. The seawater was slowly mixed during measurements by an air flow over the water surface produced with a pipette connected to an aquarium air pump. Measurements were performed at approx. 25°C and 350–450  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , unless specified otherwise.

Clark-type  $\text{O}_2$  microelectrodes with ca. 10  $\mu\text{m}$  tip diameter were made at Max Planck Institute for Marine Microbiology as described earlier (e.g., Kühl and Revsbech, 2000).  $\text{O}_2$  sensors were calibrated with seawater bubbled with  $\text{N}_2$  gas (0% reading) and with air-equilibrated seawater (21% reading; 212  $\mu\text{mol L}^{-1} \text{ O}_2$  at 23°C and salinity of 40) and corrected for changes in electrode performance over the course of the experiments by assuming that  $\text{O}_2$  concentrations in the bulk seawater surrounding the colonies (i.e., outside of the microenvironment affected by cellular  $\text{O}_2$  fluxes) was in equilibrium with the room air, which was facilitated by the large surface area of the Petri dish used for measurements.  $\text{H}_2$  microelectrodes (Unisense) had a tip size of 50  $\mu\text{m}$  and minimum detection limit below 50  $\text{nmol L}^{-1}$  and were calibrated with solutions of known  $\text{H}_2$  concentrations prepared by dilution of  $\text{H}_2$ -saturated seawater (solubility of 652  $\mu\text{mol L}^{-1}$  at 26°C and salinity of 40) assuming a linear response of the electrode. Calibrations were performed daily to account for changes in electrode sensitivity over the duration of the study.

For recording depth profiles, microelectrodes were moved toward and through the colonies at 50–100  $\mu\text{m}$  step size with a micromanipulator (VT-80, Micos) driven by a motor (Faulhaber minimotor, SA) while observing the colony at ca. 5 $\times$  magnification in a stereomicroscope (SMZ1500, Nikon with DMC G5, Panasonic camera). For simultaneous measurements of  $\text{O}_2$  and  $\text{H}_2$  profiles on the same colony, the two sensors were mounted in a 90° angle (Figure 1A) on two separate micromanipulators. Each colony was photographed for determination of colony dimensions and calculation of volume and surface area assuming spherical geometry.

Net  $\text{O}_2$  and  $\text{H}_2$  fluxes in and out of the colonies were calculated from the steady state gradients at the colony surface (Figure 1B) according to Fick's first law of diffusion:

$$J = -D(\Delta C/\Delta r) \quad (4)$$

where  $J$  represents the interfacial  $\text{O}_2$  or  $\text{H}_2$  flux,  $D$  the diffusion coefficient ( $2.2 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$  for  $\text{O}_2$  and  $4.3 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$  for  $\text{H}_2$  at 25°C and salinity 40; Broecker and Peng, 1974), and  $\Delta C$  the concentration difference measured over the respective distance,  $\Delta r$ , at the colony surface. Interfacial flux was converted to the volume-normalized rate using estimates of surface area and volume for each colony. The surface of the colony was defined visually based on measured profiles as the depth where gradients in  $\text{O}_2$  concentrations were steepest. Note that this was typically the boundary of the relatively dense colony core (as indicated in Figure 1), i.e., excluding single filaments protruding outward from the core area.

For detecting not only net  $\text{H}_2$  evolution but also potential net  $\text{H}_2$  uptake rates,  $\text{H}_2$  profiles were recorded under artificially elevated  $\text{H}_2$  concentrations. To this end,  $\text{H}_2$  was added by pipetting  $\text{H}_2$ -saturated seawater into the measurement container, reaching final concentrations from below detection limit up to 13  $\mu\text{mol L}^{-1}$  ( $6 \pm 12 \mu\text{mol L}^{-1}$ ).

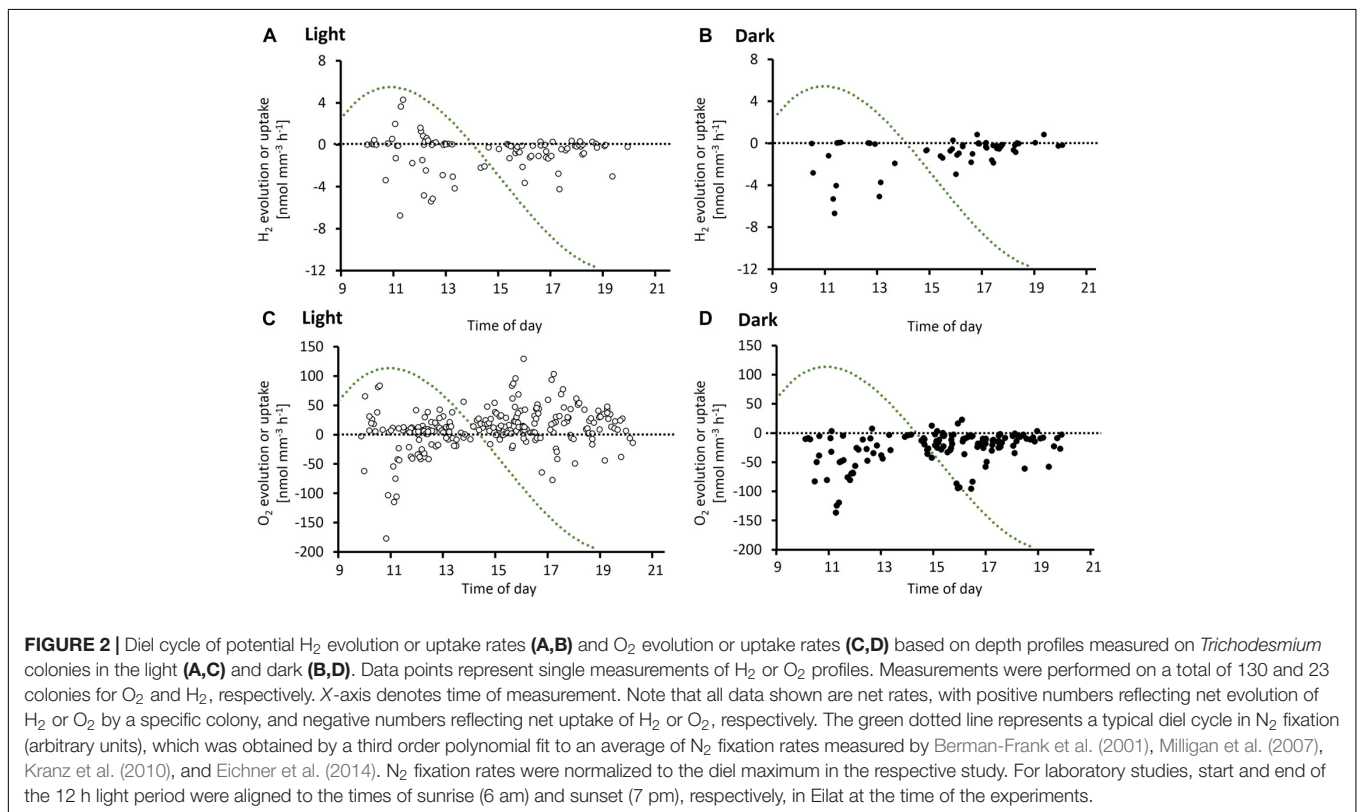
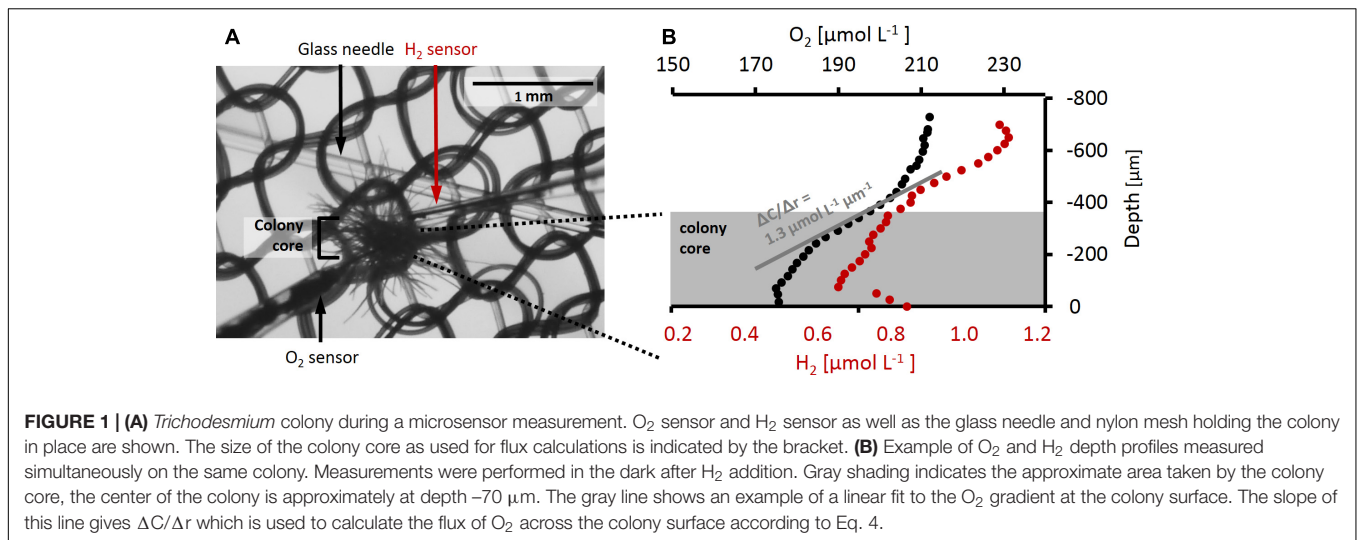
## Iron Uptake Measurements

Iron uptake from radioactively labeled  $^{55}\text{Fe}$ -ferrihydrite colloids by natural colonies was determined as described by Basu and Shaked (2018). Briefly, ca. 50 colonies were placed in 5 ml acid-cleaned glass vials and  $^{55}\text{Fe}$ -ferrihydrite was added to a final concentration of 100  $\text{nmol L}^{-1}$ . Vials were then amended with  $\text{H}_2$ -saturated seawater to reach a final  $\text{H}_2$  concentration of  $45 \pm 34 \mu\text{mol L}^{-1}$  (18–102  $\mu\text{mol L}^{-1}$ ), closed without headspace and incubated at ca. 300  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  and 25°C for 3–12 h. Control samples without added  $\text{H}_2$  were prepared for each experiment and  $\text{H}_2$  concentrations in all vials were determined at the end of incubations with  $\text{H}_2$  microelectrodes. At the end of the incubations, samples were transferred into Ti-EDTA-citrate solution (20 min) to ensure removal of absorbed  $^{55}\text{Fe}$ -ferrihydrite and effective detachment of bacteria, and subsequently filtered on 8  $\mu\text{m}$  polycarbonate filters to retain *Trichodesmium* biomass.  $^{55}\text{Fe}$  internalized by *Trichodesmium* was determined by  $\beta$ -counting of the filters in a Tri-carb 1600 CA liquid scintillation counter (Packard).

## RESULTS

### $\text{H}_2$ Dynamics Over the Diel Cycle – Relation to $\text{O}_2$ Fluxes

Microsensor measurements revealed a large variability in  $\text{H}_2$  and  $\text{O}_2$  fluxes between single colonies of *Trichodesmium* analyzed under similar conditions at the same time of the day (Figure 2). Both net evolution and net uptake of  $\text{H}_2$  were observed in the presence of added  $\text{H}_2$  (Figures 2A,B). The range of net  $\text{H}_2$  and  $\text{O}_2$  fluxes was clearly dependent on light and showed a distinct pattern over the diel cycle. Net  $\text{H}_2$  uptake was observed in both light and dark, whereas net  $\text{H}_2$  evolution was observed only in the light. Both net  $\text{H}_2$  evolution and net  $\text{H}_2$  uptake were highest during morning and midday. In the afternoon, there was no net  $\text{H}_2$  evolution and  $\text{H}_2$  uptake rates were relatively low. The peak in net  $\text{H}_2$  evolution and uptake in the late morning/midday coincided with a peak in  $\text{O}_2$  uptake

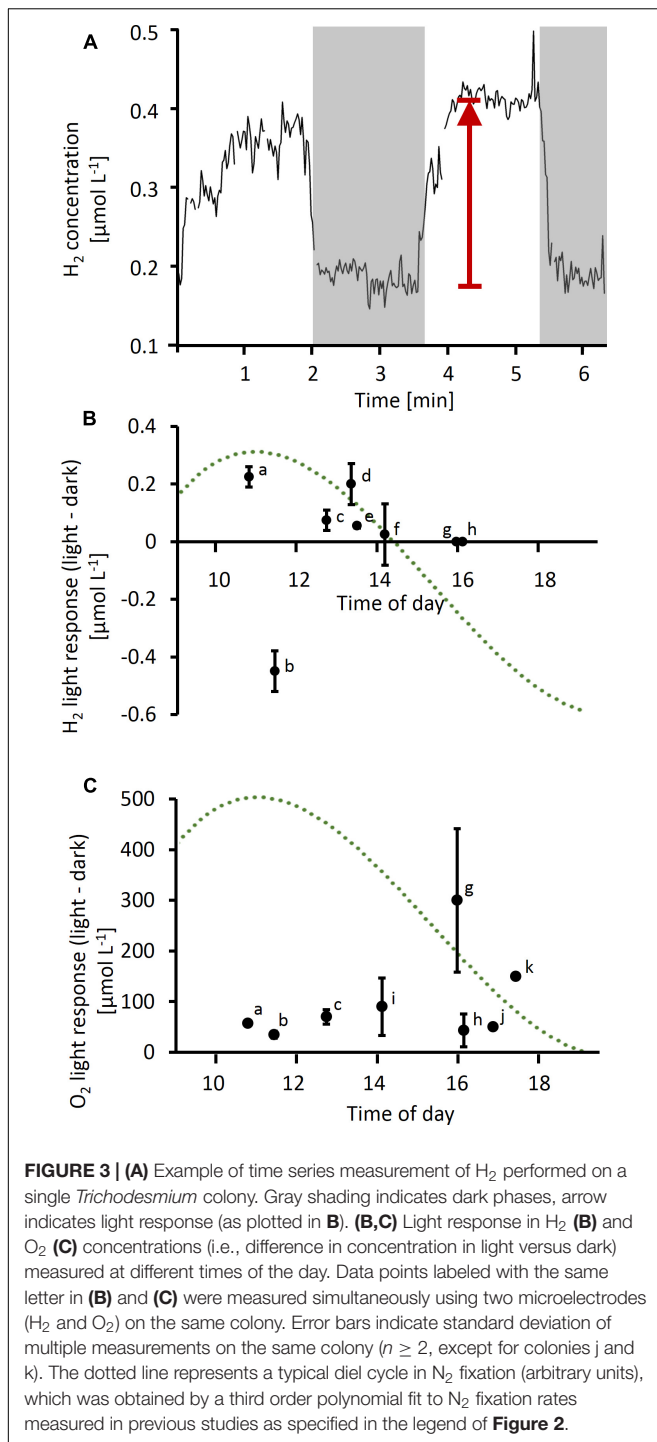


(respiration) rates (**Figures 2C,D**) as well as the previously reported daily maximum in N<sub>2</sub> fixation rates in *Trichodesmium*, ca. 5 h after beginning of the light period (e.g., Berman-Frank et al., 2001; Levitan et al., 2007; Milligan et al., 2007; Kranz et al., 2010; Eichner et al., 2014). Following a transient decrease in O<sub>2</sub> fluxes (**Figures 2C,D**) after midday, respiration and photosynthesis increased again. Photosynthesis rates reached their daily maximum in the late afternoon, when there was no net H<sub>2</sub> evolution, moderate H<sub>2</sub> uptake and N<sub>2</sub> fixation rates were reported to be low (**Figure 2C**; Berman-Frank et al., 2001;

Levitan et al., 2007; Milligan et al., 2007; Kranz et al., 2010; Eichner et al., 2014).

In a separate experiment, O<sub>2</sub> and H<sub>2</sub> concentrations were followed over time at the same position close to the center of a colony during consecutive light and dark phases (**Figure 3A**). An increase in H<sub>2</sub> concentrations by up to 200 nmol L<sup>-1</sup> was observed when the lights were switched on in four out of eight colonies tested (**Figure 3B**). One colony tested in the morning showed the opposite response to light, i.e., higher H<sub>2</sub> concentrations in the dark than in the light (colony b, **Figure 3B**).





The response to light was strongest around midday, as observed for absolute fluxes of H<sub>2</sub> (**Figure 2**), and in concert with the previously reported peak in N<sub>2</sub> fixation. Two colonies tested in the afternoon, i.e., outside of the N<sub>2</sub> fixation period, showed no light response in H<sub>2</sub> fluxes, although the same colonies were clearly active as indicated by their strong response in O<sub>2</sub> concentrations to light (colonies g and h, **Figures 3B,C**). O<sub>2</sub> responses to light showed an opposite diel pattern compared to

H<sub>2</sub> responses, with stronger responses in the afternoon than at morning/midday (**Figure 3C**).

Simultaneous measurements of H<sub>2</sub> and O<sub>2</sub> gradients performed by using two microelectrodes on the same colony showed a positive correlation ( $R^2 = 0.67$ ) between net O<sub>2</sub> and H<sub>2</sub> uptake when measured in the afternoon in the dark, with a ratio of ca. 50 mol O<sub>2</sub> per mol H<sub>2</sub> taken up (**Figure 4A**). In light, when photosynthesis was active, O<sub>2</sub> fluxes were not strongly correlated with H<sub>2</sub> uptake ( $R^2 = 0.2$ ; **Figure 4B**).

## H<sub>2</sub> Dynamics Over the Diel Cycle – Reaction Kinetics

Aiming for a more systematic understanding of the kinetics of H<sub>2</sub> uptake and evolution in *Trichodesmium*, we analyzed the dependence of H<sub>2</sub> fluxes across the colony surface on the concentration of added H<sub>2</sub> under different conditions (**Figure 5**). This approach of measuring H<sub>2</sub> evolution under different H<sub>2</sub> concentrations as well as light conditions over the diel cycle allowed us to explore the regulation of uptake hydrogenase activity on different levels, separating the roles of substrate availability, instantaneous light intensity and the underlying diel rhythm. To evaluate the relative importance of each of these parameters for H<sub>2</sub> uptake, we firstly focused on H<sub>2</sub> fluxes under conditions when we expected no H<sub>2</sub> evolution, i.e., measurements carried out in the dark and in the afternoon, when nitrogenase is presumably inactive (**Figures 5A–C**). Under these conditions, the rate of H<sub>2</sub> uptake can be simply described as

$$\text{H}_2 \text{ uptake} = k \times [\text{H}_2], \quad (5)$$

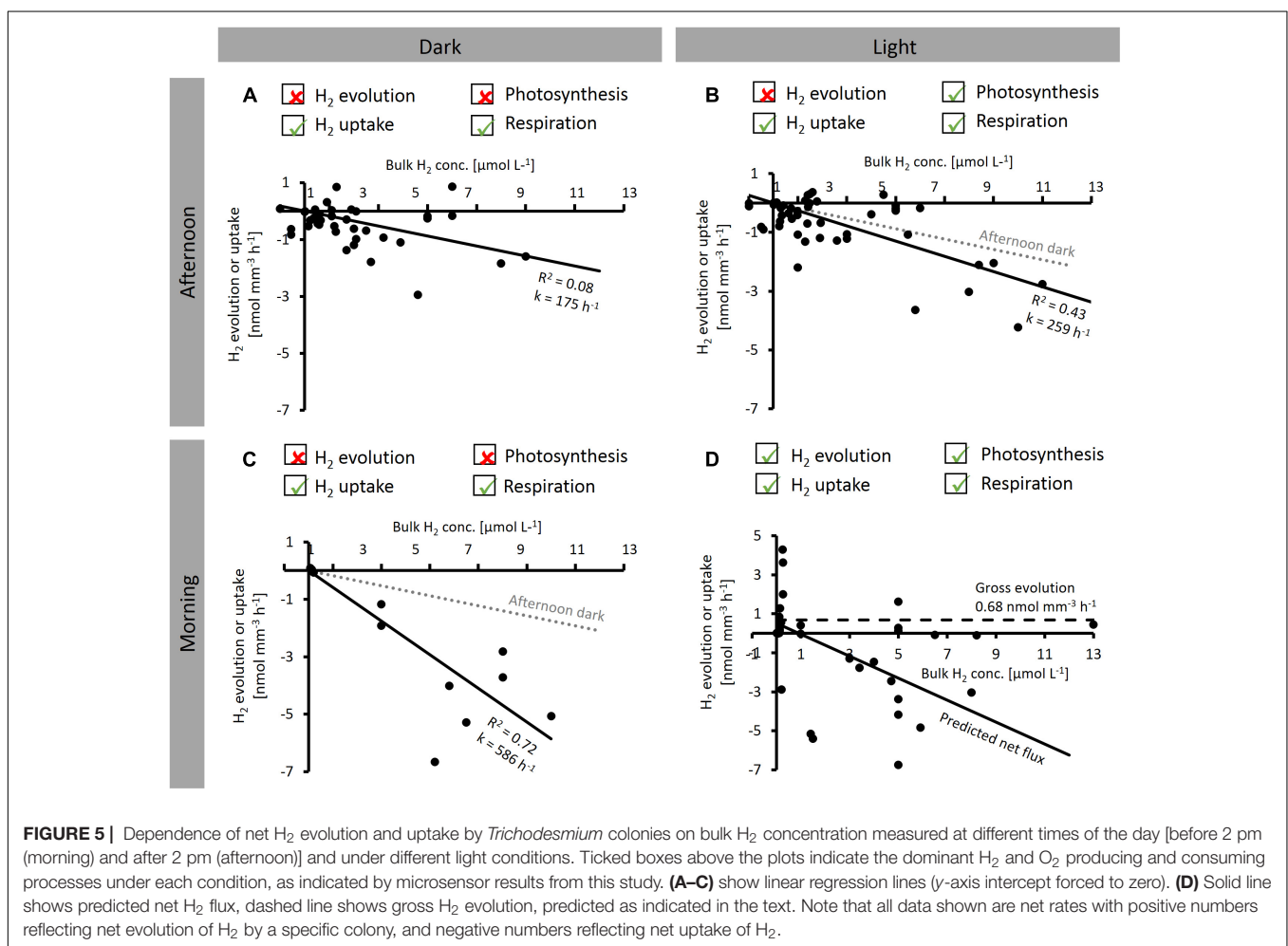
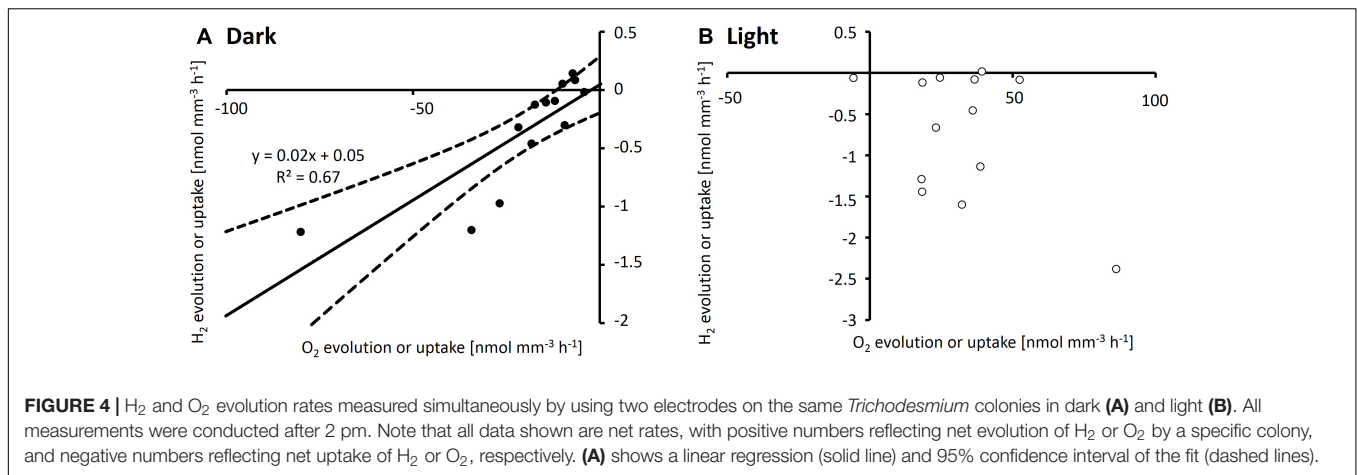
where  $k$  is a pseudo first order kinetic rate constant with units of  $\text{h}^{-1}$ , which appears as the slope of the regression lines in **Figures 5A–C**.

In the afternoon, H<sub>2</sub> uptake was not significantly different between light and dark ( $F$ -test,  $p > 0.05$ ; **Figures 5A,B**), with  $k$  values of 175 and 259  $\text{h}^{-1}$  for dark and light, respectively. On the other hand, comparison of the dark  $k$  value determined in the afternoon to that determined in the morning shows that H<sub>2</sub> uptake was strongly affected by the time of the day, with a threefold stimulation in the morning ( $k = 586 \text{ h}^{-1}$ ;  $F$ -test,  $p < 0.05$ ; **Figure 5C**).

To estimate the implications of our findings for H<sub>2</sub> fluxes under natural conditions, we furthermore used our experimental data to predict the average H<sub>2</sub> flux under typical bulk H<sub>2</sub> concentrations in natural systems. Under N<sub>2</sub>-fixing conditions, when H<sub>2</sub> evolution (by nitrogenase) and H<sub>2</sub> uptake (by hydrogenase) co-occur (**Figure 5D**), the net H<sub>2</sub> flux can either be positive (net evolution) or negative (net uptake), depending on the bulk H<sub>2</sub> concentration. The rate of net H<sub>2</sub> evolution under these conditions can be described as the difference between gross H<sub>2</sub> evolution and H<sub>2</sub> uptake, which is, in turn, given by the product of  $k$  and [H<sub>2</sub>] (cf. Eq. 5):

$$\text{Net H}_2 \text{ evolution} = \text{Gross H}_2 \text{ evolution} - k \times [\text{H}_2] \quad (6)$$

Given that H<sub>2</sub> uptake across the colony surface cannot persist when there is no H<sub>2</sub> present in the bulk seawater, the rate



of gross H<sub>2</sub> evolution was estimated from net H<sub>2</sub> evolution rates measured at the lowest bulk H<sub>2</sub> concentrations in our experiments (average net H<sub>2</sub> evolution below 0.5  $\mu\text{mol L}^{-1}$  bulk H<sub>2</sub>). Please note that this concept of “gross H<sub>2</sub> evolution” refers to fluxes across the colony surface, whereas significantly larger fluxes may occur *within* the colony. Assuming that nitrogenase

activity is not dependent on external H<sub>2</sub> concentrations, the gross H<sub>2</sub> evolution rate would be constant across all H<sub>2</sub> concentrations at 0.68  $\text{nmol mm}^{-3} \text{h}^{-1}$  (dashed line in **Figure 5D**). Since light had only a minor effect on  $k$  (**Figure 5B**), we assumed  $k$  determined in the morning in dark to be valid also under light conditions. The resulting predicted net H<sub>2</sub> flux is plotted

in **Figure 5D** (solid line). Based on the  $x$ -axis intercept of this line, our data suggest net  $H_2$  evolution to occur below a bulk  $H_2$  concentration of ca.  $1 \mu\text{mol L}^{-1}$ , whereas at higher  $H_2$  concentrations,  $H_2$  uptake is dominant.

## Response of Mineral Iron Uptake to $H_2$ Addition

Mineral iron uptake by *Trichodesmium* colonies in incubations without added  $H_2$  was similar across five experiments conducted at different times of the day (**Figure 6**), with average uptake rates of  $14 \pm 3 \text{ fmol Fe colony}^{-1} \text{ day}^{-1}$ . In the presence of  $H_2$ , mineral iron uptake was strongly elevated in three out of five experiments, including both puff-shaped colonies (experiments #2 and #3; **Figure 6**) and tuft-shaped colonies (experiment #1; **Figure 6**). Over a whole day-night cycle, iron uptake was increased by more than a factor of 2 in the presence of  $H_2$  (experiment #1, **Figure 6**). In shorter incubations, the response in uptake to  $H_2$  was dependent on the time of the day. The strongest response was observed in incubations that were started at midday (experiments #2 and 3, **Figure 6**), and thus included the previously reported maximum in  $N_2$  fixation rates. In incubations lasting from the end of the  $N_2$  fixation period till the evening (experiments #4 and 5, **Figure 6**), there was no response to  $H_2$ .

## DISCUSSION

### $H_2$ Uptake and Evolution by Individual Colonies

Our microsensor measurements of  $H_2$  and  $O_2$  gradients in single, field-collected *Trichodesmium* colonies demonstrate significant variability in  $H_2$  metabolism between individual colonies, which resulted in net  $H_2$  fluxes directed either in or out of the colony. Single *Trichodesmium* colonies thus provide distinct and highly diverse microenvironments with regard to  $H_2$  concentrations. Our experimental approach does not distinguish between the contribution of *Trichodesmium* and its associated bacteria to the  $H_2$  fluxes. However, the fact that *Trichodesmium* colonies in our study were always oxic ( $>100 \mu\text{mol L}^{-1} O_2$ ) precludes activity of many of the known  $H_2$ -consuming taxa, such as anoxygenic phototrophs and sulfate reducing bacteria, and  $H_2$ -producers such as fermenting cyanobacteria. It should be noted that gross  $H_2$  fluxes may be significantly larger than the net  $H_2$  fluxes determined in this study, particularly in the confined space of a colony microenvironment with close spatial coupling of sources and sinks.

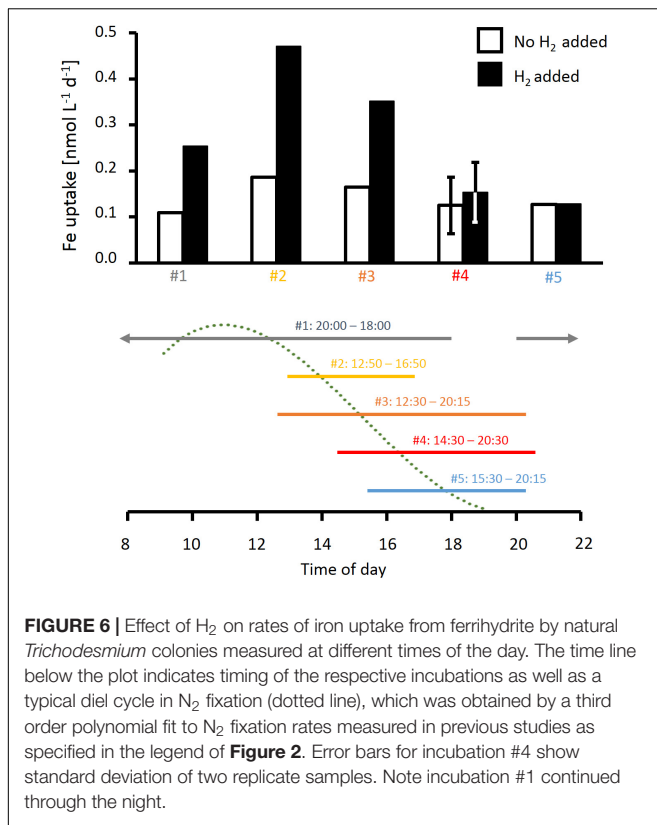
Previous studies have reported a wide range of  $H_2$  evolution rates by *Trichodesmium*. Field studies in different locations across the North Atlantic and Caribbean showed  $H_2$  evolution rates ranging between  $0.002$  and  $0.05 \text{ nmol colony}^{-1} \text{ h}^{-1}$  (Scranton, 1983, 1984; Scranton et al., 1987). The maximum net  $H_2$  evolution rate per colony in our study ( $0.08 \text{ nmol colony}^{-1} \text{ h}^{-1}$ ) strongly exceeds these previous field-based estimates. For laboratory cultures grown in single filaments under ca.  $50 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , net  $H_2$  evolution rates in the order of  $1 \text{ nmol } (\mu\text{g chl } a)^{-1} \text{ h}^{-1}$  and up to  $3 \text{ nmol } (\mu\text{g chl } a)^{-1} \text{ h}^{-1}$

were reported (Wilson et al., 2010, 2012). Using a chlorophyll  $a$  content per colony determined previously in the North Pacific Subtropical Gyre ( $14 \text{ ng colony}^{-1}$ ; Eichner et al., 2017) to normalize  $H_2$  fluxes, our maximum estimate also exceeds net  $H_2$  evolution determined in the laboratory by approx. a factor of two (Wilson et al., 2010). Similarly, maximum net  $H_2$  uptake in our study [ $9 \text{ nmol } H_2 (\mu\text{g chl } a)^{-1} \text{ h}^{-1}$ ] exceeds previous estimates of  $H_2$  uptake for *Trichodesmium* (estimated as the effect of the hydrogenase inhibitor carbon monoxide on net  $H_2$  evolution; Saino and Hattori, 1982) by approx. a factor of two. Aside from potential effects of experimental conditions among these studies, these comparisons demonstrate that  $H_2$  fluxes in and out of the microenvironment of single colonies can be significantly larger (at their diel maximum) than average  $H_2$  fluxes determined in incubations of several colonies or homogenous cultures.

In contrast to the previous reports of net  $H_2$  evolution, the average across all our measurements in light was a net  $H_2$  uptake rate of  $0.01 \pm 0.03 \text{ nmol colony}^{-1} \text{ h}^{-1}$ . While experimental conditions in previous laboratory as well as field studies (such as different light intensities, colony formation and different species) most probably contribute to the differences between ours and previous results, possibly the most important difference was that our measurements were performed under micromolar  $H_2$  concentrations, while in natural waters  $H_2$  concentrations are typically in the low nanomolar range (e.g., Scranton, 1983; Wilson et al., 2013). Assuming a linear relation between net  $H_2$  evolution/uptake and bulk  $H_2$  concentration (**Figure 5D**), we extrapolated our data to lower  $H_2$  concentrations, and predicted that under bulk  $H_2$  concentrations below ca.  $1 \mu\text{mol L}^{-1}$ ,  $H_2$  evolution would exceed  $H_2$  uptake. Under natural  $H_2$  conditions, the net  $H_2$  flux would thus – on average – be directed out of the colony, implying net  $H_2$  evolution in line with previous observations. However, our measurements also demonstrate that substantial variability in  $H_2$  fluxes (as observed within and between previous studies) can even be manifested on the level of single colonies sampled over a short period of time at the same location.

### Dependence of $H_2$ Fluxes on Light and the Diel Cycle

Measuring the steady-state  $H_2$  gradients on the colony surface against a background of added  $H_2$  enabled us to estimate the capacity of *Trichodesmium* colonies for  $H_2$  uptake under various conditions. Since the  $H_2$  uptake rates were measured under artificially elevated background concentrations of  $H_2$ , the rates we report should be considered as potential rates. The capacity for  $H_2$  uptake was not uniform over the diel cycle and dependent on light (**Figures 2, 3**), indicating that uptake hydrogenase is not only regulated by the availability of its substrate  $H_2$ , in line with previous findings (Tamagnini et al., 2002). The fact that both net evolution and net uptake of  $H_2$  were observed reflects the delicate balance between the two highly active, opposing processes of  $H_2$  evolution by nitrogenase and  $H_2$  uptake by uptake hydrogenase. However, considering only conditions where nitrogenase is presumably inactive (afternoon and/or dark) allowed us to separate these process and indicated



that light itself had only a minor effect on uptake hydrogenase (**Figures 5A–C**). Consequently, the light effects on net H<sub>2</sub> fluxes in our study (**Figures 2, 3**) were most likely driven by the light response of nitrogenase activity, whereas uptake hydrogenase was not directly affected by light. The strong stimulation of dark H<sub>2</sub> uptake in the morning compared to the afternoon (**Figure 5C**), in turn, indicates that H<sub>2</sub> uptake is regulated over the diel cycle, potentially via an endogenous rhythm. The links between H<sub>2</sub> metabolism, respiratory and photosynthetic electron transport in cyanobacteria make for a complex regulatory system. O<sub>2</sub> fluxes measured at the same time and/or on the same colonies thus provided an important reference for gaining insights into the physiological mechanisms driving the H<sub>2</sub> dynamics we observed in *Trichodesmium* colonies. In the following paragraph, we discuss these potential physiological mechanisms.

## Underlying Physiological Mechanisms

### Processes Involved in the Dark

In the dark, O<sub>2</sub> uptake by respiration and H<sub>2</sub> uptake by hydrogenase were correlated (**Figure 4A**), in line with previously suggested links between these processes (e.g., Appel and Schulz, 1998). Uptake hydrogenases have been reported to be located on either the thylakoid membrane or the cytoplasmic membrane and to feed electrons into the plastoquinone (PQ) pool of the respiratory electron transport chain (ETC), potentially via a cytochrome-like anchoring unit that binds the H<sub>2</sub>-oxidizing subunit to the membrane and facilitates electron transport to the respiratory ETC (Appel and Schulz, 1998; Khanna et al., 2016).

Electrons originating from H<sub>2</sub> could then be transported from PQ to cytochrome b<sub>6</sub>f, and further to a terminal oxidase where they are used to reduce O<sub>2</sub>. The ratio of O<sub>2</sub> to H<sub>2</sub> uptake observed in the dark in the afternoon (**Figure 4A**) suggests that, under these conditions, 1 out of 50 electrons reducing O<sub>2</sub> comes from H<sub>2</sub>, with the remainder originating from carbohydrates.

### Processes Involved in the Light

In the light, O<sub>2</sub> and H<sub>2</sub> fluxes are determined by photosynthesis and N<sub>2</sub> fixation in addition to respiration and H<sub>2</sub> uptake. Net H<sub>2</sub> evolution was observed in light but not in dark (**Figure 2**), which we attribute to the light-dependence of nitrogenase activity observed in previous studies on *Trichodesmium* (e.g., Rabouille et al., 2006; Staal et al., 2007). Activation of nitrogenase by light was also reflected in the instantaneous increase in H<sub>2</sub> concentrations when the light was switched on during the time of highest N<sub>2</sub> fixation (**Figure 3**). These findings are in agreement with previous studies showing instantaneous decreases in net H<sub>2</sub> evolution in response to addition of NH<sub>4</sub><sup>+</sup> (which inhibits nitrogenase), DCMU (an inhibitor of PSII), and darkness in *Trichodesmium* (Wilson et al., 2010, 2012), as well as a direct dependence of H<sub>2</sub> evolution on PSI activity in *Cyanobacteria* (Min and Sherman, 2010; Skizim et al., 2012). Notably, the interaction between O<sub>2</sub> evolution, nitrogenase activity and H<sub>2</sub> uptake can be modulated by the reduction state of the photosynthetic electron transport chain (Wilson et al., 2012), suggesting that the quantitative relationship between these processes is strongly dependent on the instantaneous light intensity. Due to the O<sub>2</sub>-sensitivity of uptake hydrogenase (e.g., Arp and Burris, 1981; Houchins and Burris, 1981; Axelsson and Lindblad, 2002), the high levels of photosynthetic O<sub>2</sub> evolution in the afternoon (**Figure 2C**) might impede not only H<sub>2</sub> evolution by nitrogenase but also H<sub>2</sub> uptake, leading to lower levels of H<sub>2</sub> uptake in the afternoon than in the morning (**Figure 2B**), and causing a loss of the correlation between H<sub>2</sub> and O<sub>2</sub> fluxes in the light (**Figure 4B**).

### Regulation Over the Diel Cycle

The timing of highest variability in net H<sub>2</sub> fluxes coincided with the time of highest net O<sub>2</sub> uptake as well as the midday peak in N<sub>2</sub> fixation reported previously (e.g., Berman-Frank et al., 2001). The down-regulation in net O<sub>2</sub> evolution during this part of the day is believed to act as a mechanism to protect nitrogenase from O<sub>2</sub> (e.g., Berman-Frank et al., 2001) and might additionally benefit uptake hydrogenase, which is also O<sub>2</sub>-sensitive (e.g., Houchins and Burris, 1981; Axelsson and Lindblad, 2002; Tamagnini et al., 2007). H<sub>2</sub> evolution and N<sub>2</sub> fixation rates have been previously shown to be correlated over a range of conditions (Tamagnini et al., 2002 and references therein), yet most previous studies on H<sub>2</sub> evolution by *Trichodesmium* did not resolve H<sub>2</sub> fluxes over the diel cycle. A laboratory study on *Trichodesmium* cultures grown in single filaments under a 12:12 h light-dark cycle at significantly lower intensities (44 μmol photons m<sup>-2</sup> s<sup>-1</sup>) did not show the same diel pattern in net H<sub>2</sub> evolution (Wilson et al., 2010), potentially due to feedbacks of colony formation and/or the range of light intensities in our field study.

During the morning, when N<sub>2</sub> fixation and thus H<sub>2</sub> evolution were presumably highest, elevated H<sub>2</sub> concentrations might



elicit an increase in the activity of uptake hydrogenase. The fact that we observed high *net* H<sub>2</sub> evolution as well as uptake at this time of the day may reflect a mismatch in timing between the activation of nitrogenase and uptake hydrogenase – a slight offset in timing would result in strong net fluxes, while a balanced system with a constant fraction of H<sub>2</sub> recycling would result in minimal net fluxes. Interestingly, H<sub>2</sub> uptake was highest at morning/midday even in dark measurements (**Figure 2B**) where nitrogenase was presumably inactive, suggesting that uptake hydrogenase activity is regulated not only by H<sub>2</sub> availability (i.e., by nitrogenase activity) or by light, but potentially also via an endogenous rhythm, as shown for nitrogenase in *Trichodesmium* (Chen et al., 1998). Both, uptake hydrogenases and nitrogen metabolism, are regulated by the transcriptional regulator NtcA (e.g., Tamagnini et al., 2002; Khanna et al., 2016), which was suggested to be under the control of a circadian rhythm in *Cyanothece* (Bradley and Reddy, 1997). In the afternoon, H<sub>2</sub> fluxes were less pronounced (**Figures 2A,B**) and did not respond to light (**Figure 3B**) as strongly as in the morning, even though colonies were clearly active and generally responsive to light as indicated by the high O<sub>2</sub> evolution in the light (**Figures 2C, 3C**). H<sub>2</sub> evolution by nitrogenase can presumably not respond to light at this time of the day since nitrogenase becomes inactivated by post-translational modification in the afternoon (Zehr et al., 1993). The fact that also H<sub>2</sub> uptake did not respond strongly to light (**Figure 3**) and uptake rates were low despite addition of H<sub>2</sub> (**Figure 2**) at this time of the day suggests that uptake hydrogenase might be down-regulated in the afternoon in a similar way as nitrogenase.

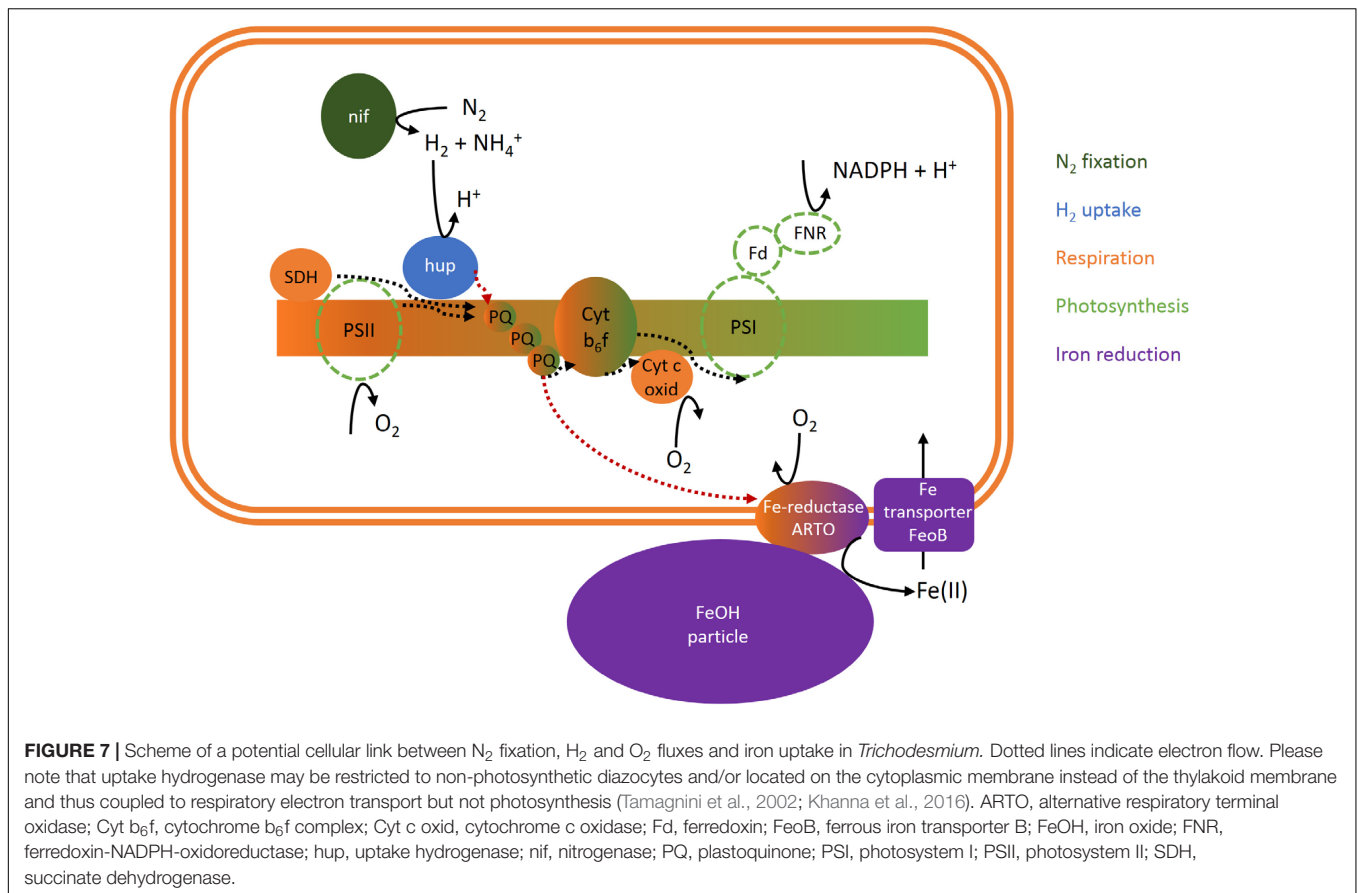
## The Potential Role of H<sub>2</sub> in Iron Acquisition

Using a recently optimized radiotracer method for measuring iron uptake from mineral iron by *Trichodesmium* colonies (Basu and Shaked, 2018), we were able to investigate the effects of H<sub>2</sub> on iron uptake under conditions that closely resembled the acquisition of iron from dust under natural conditions. Interestingly, these experiments revealed that the uptake of mineral iron was strongly stimulated by the presence of H<sub>2</sub> (**Figure 6**). This effect was observed across different colony morphologies, which are generally assumed to represent different species (e.g., Hynes et al., 2012). Also the timing of H<sub>2</sub> uptake and iron uptake was synchronized: Both H<sub>2</sub> uptake and evolution as well as the response of iron uptake to H<sub>2</sub> were strongest around midday, whereas in the afternoon, both H<sub>2</sub> uptake and iron uptake responded less strongly to H<sub>2</sub>. Taken together, these findings suggest a potential mechanistic link between iron uptake and H<sub>2</sub> metabolism. While the exact mechanisms of mineral iron acquisition by *Trichodesmium* are yet to be elucidated, there are several indications for a reductive step involved in the process, such as the slow-down of iron uptake from ferrihydrite in the presence of the Fe(II) ligand ferrozine (**Supplementary Figure 1**). The electron source for this reduction has not been identified. Based on the large H<sub>2</sub> fluxes in colonies in combination with

the stimulation of iron uptake by H<sub>2</sub> and indications for co-regulation of uptake hydrogenase and iron uptake over the diel cycle, we propose that H<sub>2</sub> may function as an electron source for mineral iron reduction. While the quantitative importance of this potential mechanism needs to be investigated in future studies under H<sub>2</sub> concentrations that more closely mimic natural conditions, we focus here on identifying a potential pathway of electron flow that might provide such a link between H<sub>2</sub> and mineral iron reduction on the cellular level (**Figure 7**).

The link between uptake hydrogenase and the respiratory electron transport chain has been reported previously (e.g., Appel and Schulz, 1998; Wilson et al., 2010), and was reflected in the correlation between H<sub>2</sub> and O<sub>2</sub> uptake in our study (**Figure 4**). In *Synechocystis*, mineral iron reduction was recently suggested to be facilitated by the alternative respiratory terminal oxidase ARTO (Kranzler et al., 2014). ARTO is also expressed and regulated in response to iron availability in *Trichodesmium* (Polyviou et al., 2018). As it accepts electrons from PQ (Lea-Smith et al., 2016), it could potentially serve as a link between the respiratory electron transport chain and iron reduction. Since on average, cellular iron uptake rates are several orders of magnitude lower than respiration rates (e.g., iron uptake of ca.  $1-9 \times 10^{-18}$  mol Fe cell<sup>-1</sup> d<sup>-1</sup>, Basu and Shaked, 2018, as compared to respiration of ca.  $3 \times 10^{-12}$  mol O<sub>2</sub> cell<sup>-1</sup> d<sup>-1</sup>, Eichner et al., 2017), only a small fraction of electrons in the respiratory pathway would need to be channeled to iron reduction to meet cellular iron demands, while the remaining electrons would reduce O<sub>2</sub>. Nevertheless, a coupling of H<sub>2</sub> uptake to iron acquisition via the respiratory ETC (i.e., electron flow from uptake hydrogenase via the PQ pool to ARTO) could explain the stimulation of iron uptake by H<sub>2</sub> observed in this study (**Figure 6**) during the time of the day when high rates of H<sub>2</sub> uptake were observed (**Figures 2, 3**). The reduced activity of uptake hydrogenase in the afternoon/evening (**Figures 2, 3**), potentially caused by a down-regulation/post-translational modification of the protein, in turn, could explain the lack of a response in iron uptake to H<sub>2</sub> at this time of the day (**Figure 6**).

Previous studies on heterocystous, filamentous cyanobacteria suggested that uptake hydrogenase is present only in heterocysts (e.g., Tamagnini et al., 2007). Such a restriction of uptake hydrogenase to N<sub>2</sub>-fixing cells would provide not only a tight spatial coupling between H<sub>2</sub> evolution and uptake, but also the benefit of reduced O<sub>2</sub> concentrations that would protect both nitrogenase and uptake hydrogenase from oxidative damage. In *Trichodesmium*, it has been suggested that only 10–20% of cells in each filament, the diazocytes, express nitrogenase and actively fix N<sub>2</sub> (e.g., Bergman and Carpenter, 1991; Berman-Frank et al., 2001). Notably, those cells would have a significantly higher iron requirement associated to synthesis of the iron-rich nitrogenase than vegetative cells (19–53% of cellular iron was estimated to be bound in nitrogenase on average across all cell types; Kustka et al., 2003). The pathway of iron reduction coupled to H<sub>2</sub> uptake we propose here could provide a mechanism of surplus iron reduction to fuel the enhanced iron demand in these cells. The restriction of hydrogenase to diazocytes would also imply that H<sub>2</sub> uptake and photosynthesis



are separated on a single-cell level, and thus do not share the same electron transport chain, potentially explaining why light did not have strong direct effects on  $H_2$  uptake (at the time when nitrogenase was presumably inactive; **Figure 5**). Furthermore, assuming that the  $H_2$  fluxes we detected as an average of all cells in a single colony were actually catalyzed by only 10–20% of the cells, it should be noted that the ratio of  $H_2:O_2$  uptake in those cells would be 5–10 $\times$  larger than the average ratio we measured (**Figure 4**). Particularly since photosynthetic water splitting is presumably not available as an electron source in diazocytes,  $H_2$  as an alternative electron source might be relatively more important in these cells. In summary, although the contribution of  $H_2$  to total electron flow in the ETC (as estimated from the ratio of  $H_2:O_2$  uptake; **Figure 4**) was relatively low when averaged over all cells and determined outside of the reported peak in  $N_2$  fixation, we suggest that in specialized  $N_2$ -fixing cells at the time of active  $N_2$  fixation, a significant part of the electron flux in the respiratory electron transport chain might be channeled from  $H_2$  to iron. Future studies should thus aim to confirm the physiological mechanism and quantitative importance of this pathway in *Trichodesmium*. Complementary molecular biological studies on the metabolic potential of associated bacteria in *Trichodesmium* colonies will bring further insights into the potential contribution of these bacteria to both hydrogen uptake and iron reduction within colonies.

## CONCLUSION

By high-resolution measurements of  $H_2$  and  $O_2$  gradients on single colonies, we were able to demonstrate highly variable  $H_2$  microenvironments in colonies of the important  $N_2$  fixer *Trichodesmium*. We found that the net  $H_2$  flux could be directed either in or out of the colony.  $H_2$  uptake was regulated not only by  $H_2$  availability and by light, but also over the diel cycle, potentially by an endogenous clock. Both  $H_2$  uptake and evolution were generally most active in the morning at the time of highest  $N_2$  fixation, reflecting the tight link between nitrogenase and uptake hydrogenase activity. The large variability in  $H_2$  fluxes between individual colonies analyzed under similar conditions furthermore highlights that single colonies may provide diverse micro-niches differing strongly in  $H_2$  metabolism, including systems characterized by either net  $H_2$  evolution or net  $H_2$  uptake. Given that the experimental approaches used in previous studies (measuring bulk  $H_2$  evolution rates under natural  $H_2$  concentrations) do not allow for detecting such potential hot-spots of net  $H_2$  uptake, our findings highlight the need for more broadly applying (1) methods that allow for quantifying  $H_2$  uptake rather than only net evolution (such as  $^3H_2$  assays; Wilson et al., 2013), and (2) high-resolution measurements that can reveal small-scale environmental variability (such as microsensors).

Combining H<sub>2</sub> measurements with radiotracer experiments, we furthermore observed a strong stimulation of mineral iron uptake in the presence of H<sub>2</sub>, indicating a previously unrecognized link between H<sub>2</sub> and acquisition of iron. We propose a potential pathway of electron flow linking N<sub>2</sub> fixation, H<sub>2</sub> uptake, respiration and iron reduction on the cellular level, which should be the subject of further physiological investigations. This link between H<sub>2</sub> uptake and mineral iron acquisition adds a new component to the complex network of nutrient acquisition mechanisms employed by *Trichodesmium* in the colony microenvironment.

## DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

## AUTHOR CONTRIBUTIONS

ME, YS, and DB designed the study. ME performed the microsensor measurements with help by DB and analyzed the data. SB performed the iron uptake experiments and analyzed the data. ME drafted the manuscript. All authors contributed to the data interpretation and writing the manuscript, and read and approved the final version of the manuscript for submission.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.01565/full#supplementary-material>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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