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Re-evaluation of nitrogenase oxygen-protective mechanisms in the planktonic marine cyanobacterium *Trichodesmium**

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ABSTRACT: There were no apparent differences in distribution of photosystems (PS) I and II between central cells and tip cells of trichomes of *Trichodesmium thiebautii* colonies collected in the eastern Caribbean Sea. Single cell absorption spectra, observations of phycoerythrin fluorescence via epifluorescence microscopy, and enhanced DCMU chlorophyll *a* fluorescence were essentially identical for both internal and peripheral regions, thus indicating that nitrogenase is not protected from O_2 via the absence of PS II in the central region of the colony. The calculated DCMU-induced Fluorescence Response Index (FRI) indicates that both central and tip cell areas are similar (FRI range 0.55 to 0.65), and active in photosynthesis. Our microelectrode measurements of O_2 within and outside of healthy colonies did not indicate a low O_2 region in the colony center relative to concentrations at the surface. A theoretical model of oxygen diffusion and respiration in the colony indicated that respiration alone cannot account for the low O_2 microzones previously observed by others. The clear sensitivity of *T thiebautii* nitrogenase to O_2 indicates that there must be other, probably intracellular, mechanisms of protecting nitrogenase from O_2 inactivation.

INTRODUCTION

Species within the genus *Trichodesmium* (Anagostides & Komarek 1988) are important in the euphotic zone of tropical seas because of their ability to fix atmospheric nitrogen (Carpenter & Price 1977). *Trichodesmium* spp. do not possess heterocysts, specialized cells which lack PS II pigments and within which nitrogenase is protected from oxygen deactivation. It has been proposed (Carpenter & Price 1976) that the center of the colony of *Trichodesmium* spp. acts in much the same manner as a heterocyst in protecting nitrogenase from oxygen. According to this hypothesis, one possible mechanism is that the central cells in trichomes which pass through the middle of the colony lack PS II pigments, and hence oxygenesis.

There is abundant evidence which suggests that the central region of the *Trichodesmium* spp. colony is the site of nitrogen fixation. For example, the cytoplasm of these cells is highly reduced, chemically, as shown by the localized deposition of formazan crystals (Paerl & Bland 1982). In addition, several studies (Carpenter & Price 1976, Bryceson & Fay 1981) have shown that the physical integrity of the colony is essential for maintaining high rates of nitrogen fixation. If the colony is disrupted by agitation, as could result from wave action, its ability to fix nitrogen diminishes. Presumably, disruption of the colony permits oxygen to enter the central region. If oxygen is sparged from seawater prior to agitation there is no decrease in rate of N_2 fixation indicating that oxygen deactivation is a primary cause of the decrease in nitrogenase activity (Carpenter & Price 1976). As is the case in heterocysts, carboxylation does not occur in the central cells of *Trichodesmium* spp. colonies. Using ^{14}C autoradiography, Carpenter & Price (1976) found no short-term (ca 2 h) $^{14}CO_2$ incorporation in this region. The lack of carbon fixation in central cells was substantiated by Bryceson & Fay (1981) who also noted the absence of carboxysomes in these cells. Carboxysomes were found in peripheral cells on the outside

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of the colony. However, Gantt et al. (1984), in contrast to Bryceson & Fay (1981), found no obvious differences in cell microstructure between central and peripheral cells in electron micrographs. Using immuno-gold antibodies to nitrogenase, Bergman & Carpenter (1990) noted that nitrogenase is present in about 20 to 40 % of all trichomes and that these trichomes are randomly distributed in the colony. All cells in these trichomes contained nitrogenase.

Some investigations indicate that cellular processes are of major importance in protecting nitrogenase from oxygen. For example, Saino & Hattori (1982) have proposed that uptake hydrogenases in *Trichodesmium* spp. play a major role in protecting nitrogenase from oxygen. They note substantial acetylene reduction (AR) among separated (colony disrupted) trichomes and suggest that these free trichomes are a significant source of fixed N in the sea. However, they also observed that acetylene reduction per trichome was several-fold higher in intact than in separated colonies.

The purpose of our study was: (1) to determine whether cells in the center of a colony are differentiated and protect nitrogenase in a manner similar to heterocysts, and (2) to explore a number of other possible mechanisms of protecting nitrogenase from oxygen deactivation. The theory of compartmentalization of nitrogenase into cells in the central region seems tenable, and field observations appear to support it, yet the exact mechanism of protection of nitrogenase remains unknown.

MATERIALS AND METHODS

Research was carried out in the Caribbean Sea on the RV 'Columbus Iselin' between February 23 and March 15, 1988 and also November 4 and 25, 1988 (Table 1). Colonies of *Trichodesmium thiebautii* were collected using a 1 m diameter plankton net (256 µm mesh), which was towed at 1 knot for 15 min at 25 m depth. Colonies were then isolated using either a plastic inoculation loop or a wide bore glass Pasteur pipette. For acetylene reduction assays (Hardy et al. 1968), 10 colonies were gently placed in 5 ml of glass fiber-filtered seawater in 9 ml wide-mouth serum bottles. Acetylene was generated from calcium carbide, and 1 cc was injected into each serum bottle. Previous research has validated these collection, assay and incubation methods (Carpenter et al. 1987). After a 1 to 2 h incubation, on deck at 60 % ambient sunlight (ca 1000 µE m^{-2} s^{-1}) and temperature of 27 to 28 °C, 100 µl of the gas phase was withdrawn, and the ethylene content was assayed using a Shimadzu mini-II flame ionization gas chromatograph having either a 4 m Porapak R column or a 3 m Unibead (Alltech) column.

Table 1. Locations and dates of collection of *Trichodesmium thiebautii* for physiological measurements

Date (1988)	Location
Mar 3	12°15′ N, 61°48′ W
Mar 5	12°48′ N, 61°48′ W
Mar 6	14°41′ N, 64°00′ W
Mar 7	16°18′ N, 69°12′ W
Mar 13	24°00′ N, 74°10′ W
Nov 18	12°26′ N, 80°33′ W
Nov 21	20°21′ N, 73°25′ W

All cell counts of *T. thiebautii* in serum bottle incubations were done by dispersing a known number of colonies in a vortex mixer, then counting cells in transects at 400× in a Sedgwick-Rafter cell. In one experiment, rate of AR was measured under either white light or red (> 600 nm) with light from a 150 W cool-white spotlight (450 µE m^{-2} s^{-1}). Fructose (5 µM) was added to observe the effect of a supplemental carbon source.

Microscale oxygen distributions in and around colonies were determined with microelectrodes (Revsbech & Jorgensen 1986). We used cathode-type electrodes having tip diameters of 5 and 3 µm. Single *Trichodesmium thiebautii* colonies were placed into small plastic Petri dishes containing seawater and a glass-fiber filter. A few teased up glass fibers of the filter pad immobilized the colony during oxygen determinations. Electrodes were positioned with a micromanipulator (Brinkman model MM5) while the specimen and probe position were observed under a binocular microscope. The micromanipulator, microscope, and Petri dish were mounted on a gimballed table. A Diamond General model 1201 Chemical Microsensor supplied the electrode polarization voltage and displayed the output current, which was calibrated against air (100 % atmospheric equilibrium saturation of O_2) and N_2 (zero O_2) bubbled seawater (saturation was typically 4.9 ml l^{-1}). Irradiance (0 to 200 µE m^{-2} s^{-1}) was supplied with a fiber optic light source (Fiber Lite model 9645-00, Cole Parmer Instrument Company) and PAR (photosynthetically active radiation) intensity was measured with a Li-Cor light sensor. Absorption and fluorescence emission spectra of single cells were measured using a Zeiss Universal microscope adapted for quantitative microspectrophotometry and fluorometry. The scanning procedure was automated through an IBM PC/XT computer linked to the microscope with a Zeiss Microscope Photometer Control (MPC 64). Various shutter manipulations and data acquisition routines were controlled by a Zeiss Lambda Scan program. A tungsten lamp (12V, 60W) was used as the light source for absorption spectra, and an H-10 monochrometer (Instruments SA, Inc.) was mounted in the light path. Absorption spectra

are presented as the particle absorption efficiency factor, $Q_a(\lambda)$, which can be calculated from

$$Q_a(\lambda) = 1 - \left[\frac{I_s(\lambda)}{I_o(\lambda)}\right]$$

where $I_s(\lambda)$ = the light intensity measured when a sample cell is placed under the objective at a certain wavelength λ; $I_o(\lambda)$ = the light intensity when a blank region on the slide is measured under the same condition. For each sample the Q_a value at 750 nm of each sample was subtracted from the wavelengths spanning the entire spectrum in order to compensate for light lost due to scattering (Iturriaga et al. 1988).

Fluorescence emission spectra were obtained using an Hg burner (HBO 50W/3 DC) with the excitation wavelength ranging from 450 to 490 nm. Zeiss filter set 48 77 09 was used for epifluorescence measurement, but the original LP520 was replaced by LP510 as the barrier filter. The emission spectra were corrected by comparing the spectrum of the tungsten lamp at 12V obtained on our system to the standard spectrum for 3300 K color temperature provided by Zeiss.

For both absorbance and fluorescence emission measurements, fresh *Trichodesmium thiebautii* colonies were placed either in a Palmer Maloney or a Sedgwick-Rafter counting chamber and covered with a # 1 glass coverslip. Air vesicles, which produce significant light scattering, were collapsed by placing colonies in a sealed (capped luer-lock) syringe and then pressurizing it in a bench vise.

The metabolic inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) was used to induce fluorescence among individual cells in *Trichodesmium thiebautii* colonies. DCMU inhibits photosystem II electron transport; hence cells lacking PS II should exhibit no induced fluorescence due to this compound (Samuelsson & Oquist 1977). Prior to the measurement of emission spectra, colonies were kept in the dark for at least 10 min. After control spectra were measured, 3 drops of 50 µM DCMU solution were added to the Palmer Maloney cell, and colonies were incubated in the dark for 20 to 40 min before the DCMU-induced spectra were measured. Preliminary tests indicated that this was a suitable incubation period.

RESULTS

Pigments

There were no measurable qualitative differences in pigment composition between the central and the peripheral cells (Fig. 1). Absorption spectra of single cells indicated that peripheral cells contained phycoerythrin in concentrations similar to those found in

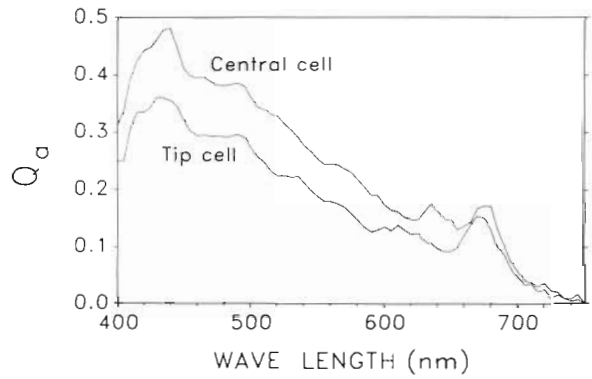

Fig. 1 *Trichodesmium thiebautii*. Absorption spectra of central and tip cell in trichomes of cells collected and analyzed on March 12, 1988

central cells, thus suggesting the presence of PS II pigments in both locations. Absorption characteristics of phycoerythrin in *Trichodesmium thiebautii* were similar to that of phycoerythrin found in red algae (Shimura & Fujita 1975), having 3 major absorption bands at 495, 547 and 562 nm. Our observations indicate a peak at 490 nm and a shoulder at about 570 nm, and these may result from phycoerythrin.

DCMU-induced fluorescence emission spectra were not different between central and peripheral cells (Fig. 2). Peaks in phycoerythrin and chlorophyll *a* fluorescence were present at 565 and 680 nm, respectively. The ratio of fluorescence at 680 nm with DCMU to that without DCMU addition was similar for tip and central cells.

Epifluorescence microscopy of colonies viewed under blue excitation light indicated that phycoerythrin is present, as shown by orange fluorescence, in both central and peripheral cells (Fig. 3). Among 50 colonies examined, there appeared to be no detectable differences in phycoerythrin fluorescence between regions of the colonies. In some colonies, red fluorescence from chlorophyll *a* was observed (see center of Fig 3); however, this fluorescence can be attributed to associated microalgae, particularly diatoms, which inhabited the center of radiate colonies and appeared to cling to trichomes.

Colony size vs N_2 fixation

Since rates of AR in small vs large colonies of *Trichodesmium thiebautii* totally overlap (Fig. 4), it is apparent from simple inspection that colony size was not related to per-cell AR rate. Mean size of large colonies averaged 216, 209 and 191 $\times 10^3$ cells on March 5, 6, and 7, respectively, while small colonies averaged 25.5, 26.5, and 32.0 $\times 10^3$ cells colony^{-1}. As expected, larger colonies reduced acetylene at a greater rate on a per-

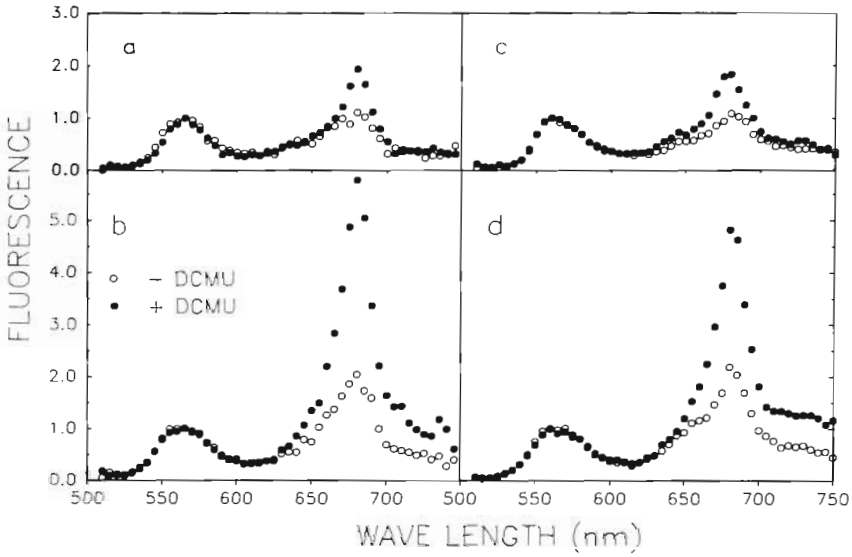

Fig. 2. *Trichodesmium thiebautii*. Fluorescence emission spectra of (a, c) spherical and (b, d) fusiform colonies in (a, b) tip and (c, d) central cells with (●) and without (○) DCMU addition. Cells were collected and analyzed on March 7, 1988

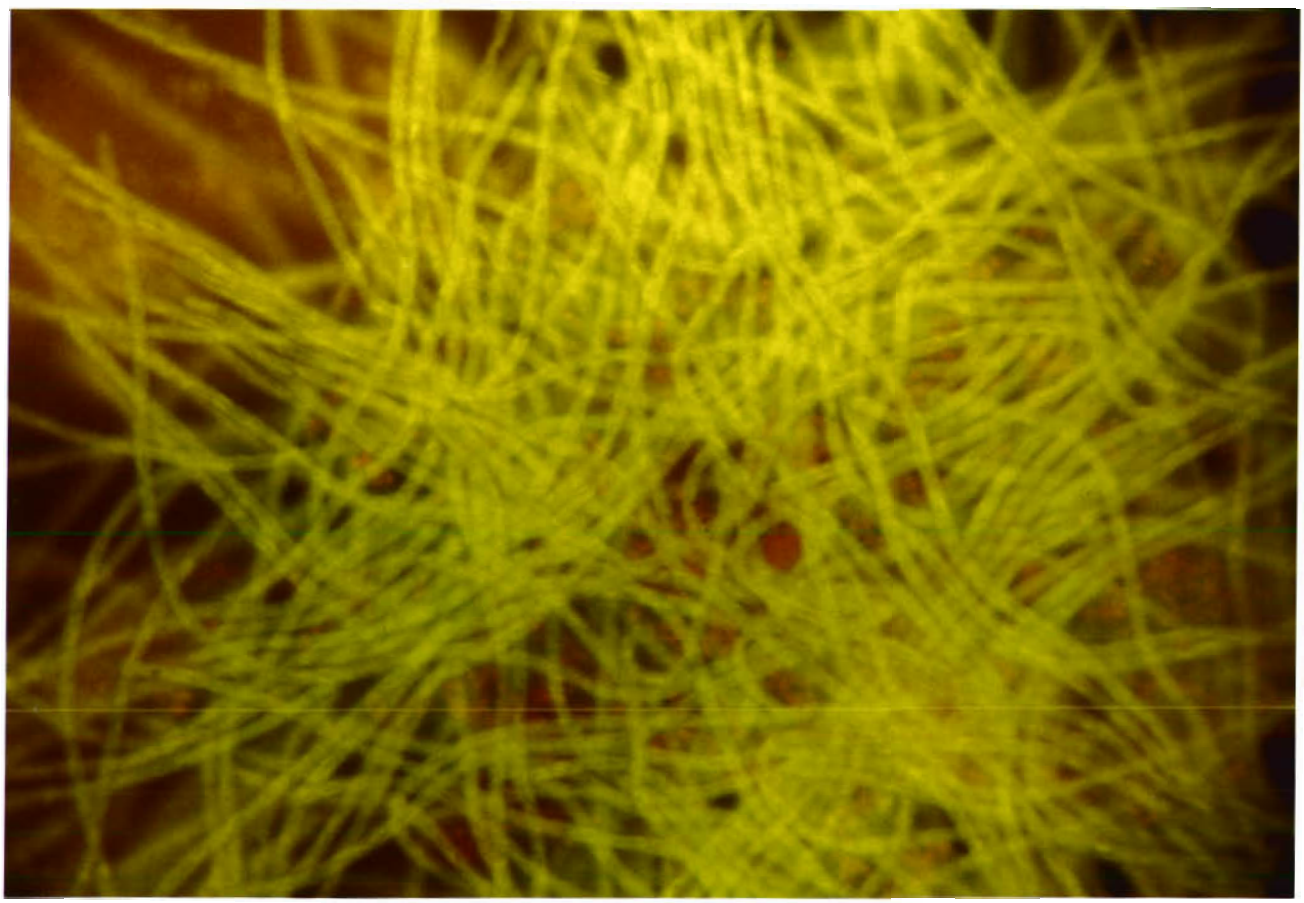

Fig. 3. *Trichodesmium thiebautii*. Epifluorescence micrograph of central and peripheral cells under blue excitation light showing uniform orange fluorescence among central and tip cells

colony basis than smaller ones. On a colony basis, rates averaged 0.106 (SD = 0.13) nmol N h^{-1} for large, and 0.012 (SD = 0.007) nmol N h^{-1} for small. Mean rate for small colonies was 4.52 (SD = 2.53) fmol N $cell^{-1}$ h^{-1} while for larger ones it was 5.18 (SD = 6.23) fmol N $cell^{-1}$ h^{-1} for all light levels and data combined. For the light intensities tested (range 80 to 1500 μE m^{-2} s^{-1}), there were no significant differences in AR rates.

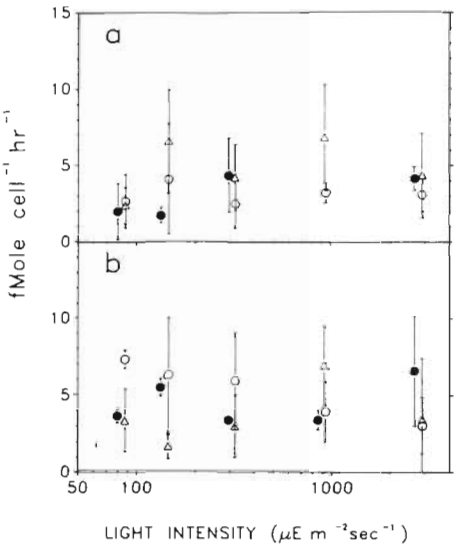

Fig. 4. *Trichodesmium thiebautii*. Nitrogen fixation (fmol N fixed cell^{-1} h^{-1}) plotted for (a) large and (b) small colonies collected on March 5 (○), 6 (●) and 7 (△), 1988. Error bars give one standard deviation. Replicate AR (acetylene reduction) measurements, n = 4

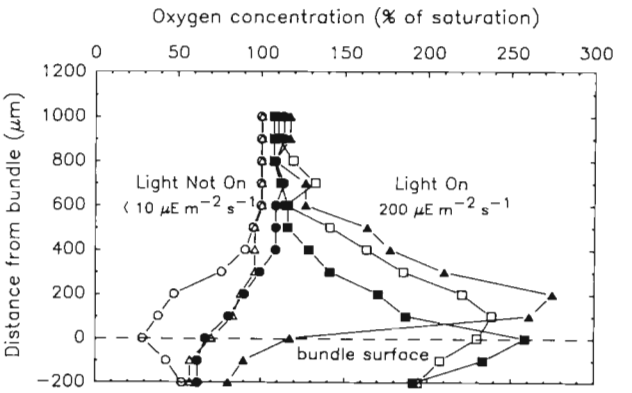

Fig. 5. *Trichodesmium thiebautii*. Oxygen concentration (% of saturation) inside and outside of colonies at high und low light intensities. (●, ○, △) 10 μE m^{-2} s^{-1}; (■, □, ▲), 200 μE m^{-2} s^{-1} Saturating O_2 = 4.95 ml^{-1}

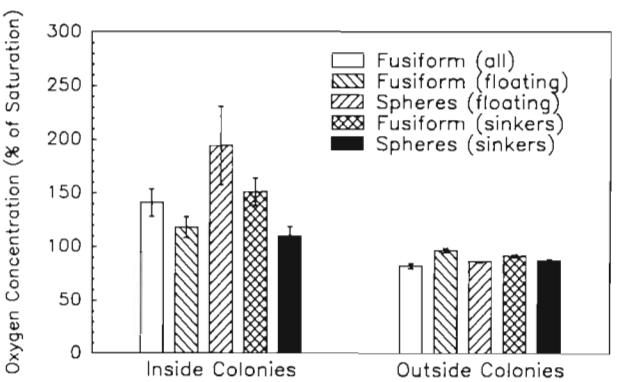

Fig. 6. *Trichodesmium thiebautii*. Oxygen concentrations inside and outside colonies. Illumination at 200 μE m^{-2} s^{-1}

Oxygen content

Photosynthestic oxygen production resulted in high concentrations of oxygen at the surface of the colonies at a PAR intensity of 200 μE m^{-2} s^{-1} (Fig. 5). At low light (10 μE m^{-2} s^{-1}) the O_2 in the colony center dropped to almost half of the saturation value. These measurements indicate the dynamic nature of interstitial O_2 in the colony When exposed to 200 μE m^{-2} s^{-1}, the central region of colonies often had O_2 concentrations above that of saturation seawater values (Fig. 6). These results are in contrast to the low oxygen regions typically found in the center of *Trichodesmium* spp. colonies by Pearl & Bebout (1988). Differences may have been due to the different physiological states between the colonies examined by us and previously by Paerl & Bebout (1988). Colony morphology did not appear to be a factor in affecting internal oxygen concentrations (Fig. 6).

There was no difference in rates of AR between colonies incubated in white light as compared with light greater than 600 nm (Fig. 7). Similarly, addition of fructose did not affect acetylene reduction rates under either light condition.

DISCUSSION

From these observations, we conclude that there is no differentiation, as regards pigments, between internal and peripheral region trichomes in the *Trichodesmium thiebautii* colony. The DCMU fluorescence induction measurements indicated that tip cells and central cells have equally active PS II systems. The fluorescence response index (FRI) as defined by Cullen & Renger (1979) ([DCMU-induced – control fluorescence]/DCMU-induced fluorescence) was 0.57 and 0.65 for tip and central cells, respectively, in spherical colonies and 0.58 and 0.55 for tip and central cells, respectively, in fusiform colonies. These values are typical of healthy, growing phytoplankton (Cullen & Renger 1979) and suggest the presence of an active PS II in the central region of the colony. Furthermore, when colonies were incubated under red light, thus preventing activity of phycoerythrin, and white light, there were no differences in rates of acetylene reduction (Fig. 7). Since phycoerythrin appears to be present in central cells in the colony, and is involved in oxygenesis, the fact that N_2 fixation was not enhanced when phycoerythrin activity ceased (under red light) suggested that there must be intracellular mechanisms for protection of nitrogenase from oxygen. Fructose, provided as reductant for N_2 fixation (Fig. 7), did not

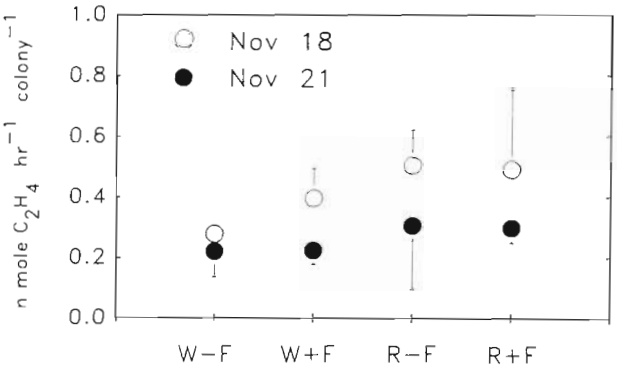

Fig. 7. *Trichodesmium thiebautii*. Rate of acetylene reduction in white light (W), and red light (R) at a wavelength > 600 nm, with (+F) and without (−F) addition of fructose at 5×10^{-4} M on November 18 and 21, 1988

stimulate nitrogenase activity, thus suggesting that sufficient reductant existed.

While it appears that the center of the colony is not differentiated as regards PS II pigments, it is possible that a microzone of low O_2 concentration could exist in the center as a result of respiration in the absence of photosynthetic O_2 production. One approach to determining whether one large microzone could exist in the colony is to examine a simple model of O_2 consumption via respiration and O_2 introduction through diffusion from outside. The study of Paerl & Bland (1982) showing high rates of tetrazolium dye reduction by internal regions of *Trichodesmium* spp. bundles suggests the possibility of relatively high respiration rates located in these regions. However, if shading were a valid protective mechanism, then one would suppose that larger colonies would be more efficient, on a per-cell basis, than smaller ones in fixing N_2. This, in fact, was not true (Fig. 4), and there was no difference in rates of N_2 fixation between colonies. We can determine whether this low O_2 zone could exist as a result of respiratory activity via simple theoretical calculations.

For example, let us assume a spherical colony in seawater at a dissolved oxygen concentration equal to air-saturated equilibrium (C_{sat}) and a sink provided by cells respiring at a rate (R) within the colony. The inner, non-photosynthetic region of the colony has a radius (b). Under steady-state conditions the oxygen concentration (C_r) at a point (r) somewhere within the non-photosynthetic part of the colony is given by the equation (Jørgensen 1977),

$$\frac{\partial C_r}{\partial t} = D_s \nabla^2 C_r - R = 0 \qquad (2)$$

where D_s = oxygen diffusivity

The diffusion of oxygen into the colony is balanced by consumption (respiration) of oxygen within it. For the spherical colonies the system has radial symmetry, thus,

$$\nabla^2 C_r = \frac{1}{r^2} \frac{\partial}{\partial r} \left[r^2 \frac{\partial C_r}{\partial r} \right] \qquad (3)$$

When we substitute this into the differential equation we get

$$D_s \frac{1}{r^2} \frac{d}{dr} \left[r^2 \frac{dC_r}{dr} \right] - R = 0 \qquad (4)$$

Integrating and solving for the boundary conditions gives an oxygen profile which follows the equation

$$C_r = C_{sat} - \frac{R}{6 D_s} (b^2 - r^2) \qquad (5)$$

This equation can be used to calculate the oxygen concentration at the center of the colony if we know oxygen concentration outside the colony, and have reasonable estimates for the diffusivity of oxygen within the colony and the respiration rate of *Trichodesmium thiebautii*.

Assuming that respiration is 50 % of P_{max}, we can use the above equation to calculate oxygen within the colony center. A reasonable value for P_{max} is 0.05 µg C $colony^{-1}$ h^{-1} (Li et al. 1980). Therefore, a conservative estimate for the rate of respiration is 0.002 µmol C $colony^{-1}$ h^{-1}. If we make the simplifying assumption that the moles of carbon respired equals the moles of oxygen consumed, we find that the oxygen demand by respiration is 0.064 µg O_2 $colony^{-1}$ h^{-1}. A typical radius for the whole colony is about 0.065 cm, therefore the calculated rate of oxygen utilization per volume is 1.5×10^{-2} µg O_2 cm^{-3} s^{-1}.

Based on the assumption that the central region of the colony does not fix carbon, we can calculate the concentration of oxygen that would be found at the center of the colony from Eq. (5), using $C_{(sat)} = 5$ µg O_2 cm^{-3} (Jørgensen 1977); $D_s = 1.6 \times 10^{-5}$ cm^{-2} s^{-1} (Jørgensen 1977); $R = 1.5 \times 10^{-2}$ µg O_2 cm^{-3} s^{-1}; b = 0.025 cm (from microscopic measurements); r = 0.0 cm (at the center of colony). Thus: $C_{r=0} = 4.9$ µg O_2 cm^{-3}.

The value given for D_s, taken from Jørgensen (1977), is calculated for a bacterial film. The value assigned for R is based on a respiration rate equal to 50 % of P_{max}. These calculations indicate that the oxygen concentration in the center of the colony is virtually the same as the saturating value for seawater and is identical to ambient O_2 outside the colony. Oxygen diffusivity (D_s) in tightly interwoven mats of *Nostoc parmelioides* has been measured by W.K. Dodds (Montana State University pers. comm.) and was observed to be 6×10^{-7} cm^{-2} s^{-1}. Using this value and Eq. (5), $C_{r=0}$ is 2.5 µg O_2 cm^{-3}. Given the fact that O_2 production via photosynthesis is not even considered in Eq. (5), it is clear that low oxygen conditions could not be supported by simple respiration alone.

Our results appear different from those of Paerl & Bebout (1988) in that our measurements indicated that

the centers of colonies typically had oxygen concentrations as high or higher than at the surface of the colonies. While we did not observe O_2 microzones, their existence has been documented. It is possible that microzones of oxygen in *Trichodesmium* spp. are highly variable in size, and dependent on the time of day, exposure to light, the attached bacterial population, and physiological state of the colony. Furthermore it is possible that small microzones exist which surround trichomes responsible for N_2 fixation. Thus the position of the microelectrode is critical in determining the location of the microzone. Recently, Bergman & Carpenter (1990), using immuno-gold antibody localization techniques, determined that nitrogenase was present in only about 20 to 40 % of the trichomes present in a colony. These trichomes were randomly distributed, and all cells in an N_2 fixing trichome possessed nitrogenase, thus suggesting the possibility that very small microzones exist around these nitrogen-fixing trichomes.

These observations on pigments and oxygen concentrations indicate that the oxygen-protective mechanism for *Trichodesmium thiebautii* may well be associated with an intracellular enzymatic process. However, the fact that nitrogenase activity decreases dramatically when the colony is disrupted indicates the importance of the aggregate form. It would appear that there is no single oxygen protective mechanism, but that several processes (inter- and intracellular) act together to protect nitrogenase. There are several possible oxygen-scavenging enzymes which could produce microzones within the cell for the protection of nitrogenase. Saino & Hattori (1982) have proposed uptake hydrogenases as a possible process. The role of these uptake hydrogenases in removing O_2 has yet to be examined in *Trichodesmium* spp. The net production of hydrogen has been investigated by Scranton et al. (1987) for *T. thiebautii,* and rates of H_2 production in situ were found to be 2 to 3 orders of magnitude slower than those of N_2 fixation. However, there could be high production of H_2 intracellularly by nitrogenase and consumption by a unidirectional uptake hydrogenase which can oxidize hydrogen and use oxygen as a terminal acceptor. Intracellular O_2 could be scavenged, thus protecting nitrogenase, yet there would be little net production of H_2. The fact that several workers have noted high ratios of acetylene reduction to nitrogen fixation in *Trichodesmium* spp. supports this hypothesis. If there were no hydrogen production by nitrogenase, then the ratio of reduced acetylene to dinitrogen would be 3.0. However, Carpenter & Price (1977) obtained an average ratio of 6:1, Mague et al. (1974) had ratios as high as 50:1, and Scranton et al. (1987) obtained a mean ratio of 4.1:1.

Another oxygen protective mechanism could involve superoxide dismutase. These enzymes are widespread among phytoplankton and convert OH, O_2^- and singlet oxygen to H_2O_2 which is then destroyed by peroxidase (Puppo & Rigaud 1986). Superoxide dismutase has been implicated in protecting nitrogenase in *Anabaena cylindrica* (Landis et al. 1978) and *Azotobacter vinelandii* (Dingler & Oelze 1987) and may also function in *Trichodesmium* spp. To fully understand how this species is able to protect its nitrogenase it is important that we determine whether these enzymes are operative in *Trichodesmium* spp. and provide protection of nitrogenase. Our results indicate that there is no centrally located region with heterocyst-like cells in the *T. thiebautii* colony. Differentiated cells or trichomes may still exist, and they could be scattered throughout the colony, but they appear not to be clustered in the central region as previously proposed by Carpenter & Price (1976).

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