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Response to "Another look at green *Trichodesmium* colonies"

Orcutt et al. provide an alternative interpretation of the data presented on green *Trichodesmium* colonies in Neveux et al. (2006). Neveux et al. (2006) suggested two hypotheses for the green *Trichodesmium* found in the Coral Sea: a new species or an ecotype of already known species photoacclimated or photoadapted to low light environment. Orcutt et al. based their work on five colonies and noted that the puff forms of the green colonies had DNA amplicons, indicating that they were *Trichodesmium thiebautii*. This clearly requires a closer examination, and we support their efforts in this regard. As we noted in the text, our speculations on whether this was a new species required molecular confirmation. Orcutt et al.'s data are a good initial step in this regard. Their comments on the need for multiple lines of evidence to characterize cyanobacteria species are on the mark, and we are glad they found a forum for their thoughts.

However, we cannot agree with the physiological interpretation they apply to their PCR results. Their examination of tuft morphologies of three green *Trichodesmium* yielded no amplicons, whereas the two puff samples did. On the basis of this, they put forward a hypothesis that green colonies may have been *T. thiebautii* in a state of senescence. The only argument in support of this was the lack of N_2 fixation (acetylene reduction) by the green colonies (four experiments, no ethylene production, D. G. Capone, pers. comm.). For comparison, three experiments on "normal" brown *Trichodesmium* incubated under the same conditions (1% illumination) yielded no fixation in one of three experiments (D. G. Capone, pers. comm.). Orcutt et al. then suggest that since *Trichodesmium* has been observed to fix nitrogen in the presence of nitrate in laboratory culture (albeit at reduced rates), this supports the notion that the green colonies were in state of senescence. They have no further affirmative or rate data on green *Trichodesmium*. We suggest that this line of reasoning is flawed since (1) the isolates used in the laboratory cultures are not the strain seen in the Coral Sea and possibly not even the same species, (2) there is no assessment of how acclimation to low light or reduced temperature interacts with N_2 fixation in *Trichodesmium* other than N_2 fixation decreases with depth (Holl et al. 2007) and general N_2 fixation decreases with temperature (Staal et al. 2007), and (3) the general literature on N_2 fixation in cyanobacteria has long supported the idea that supplies of dissolved inorganic nitrogen (DIN) reduce or eliminate N_2 fixation (Bothe 1982). Differences between ammonium and nitrate in repressing nitrogenase have been well addressed by Bergman et al. (1997). At this time, we suggest that the literature on DIN effects on *Trichodesmium* is too limited to address the point of what the lack of N_2 fixation means.

The Berman-Frank et al. (2004) paper is a valuable description of programmed cell death (PCD) in *Trichodesmium*; however, the data must be interpreted in its full context. They note specifically that cell death was characterized by "degradation of thylakoids, carboxysomes, gas vesicles, and cyanophycin granules" (p. 998). Significant nuclease activity was detected concurrent with (but not before) greatly reduced $F_v:F_m$ values (p. 1002; note that $F_v:F_m$ from the fast repetitive rate fluorometry and pulse amplitude modulation [PAM] are not the same). This is not consistent with our data on green *Trichodesmium*. However, PAM data from surface slicks of *Trichodesmium* on our cruise were consistent with light-induced PCD as reported by Berman-Frank et al.'s (2004) data in that there was a near-zero $F_v:F_m$ value and no response to increasing actinic illumination. We are confident that what we observed was not the same phenomenon reported by Berman-Frank et al. (2004).

It is also difficult to refute an undefined condition. Senescence is a general term. However, Orcutt et al. used it to describe conditions that resulted in degenerate DNA. It is hard to interpret that as meaning anything other than dead or dying cells, particularly if PCD is invoked. Our data do not support this, and, in fact, the PAM data are the most compelling evidence that the colonies were not in senescence.

Orcutt et al. interpret the low rETR data in Neveux et al.'s (2006) fig. 7 as indicative of an impaired electron transport system. The rETR is a derived term that is a function of both $F_v:F_m$ (the measured parameter) and light. When the original $F_v:F_m$ data are plotted for both brown (Fig. 1A) and green *Trichodesmium* (Fig. 2A), it is apparent that green *Trichodesmium* has an extremely high $F_v:F_m$ (yield) of 0.7. This is an absolute measure of the rate of water splitting in photosystem (PS) II. The complex processes that produce high $F_v:F_m$ values require intact membranes and vigorous electron transport systems. Low $F_v:F_m$ values indicate less robust conditions or (as in many cyanobacteria) an initial fluorescence with a component less active in transferring electrons to the reaction centers. There is an extensive literature (*see* U. Schreiber and coworkers) on the use of $F_v:F_m$ to detect stress. High $F_v:F_m$ is not associated with degenerate systems, although it may be found in cells with reduced growth rates due to nutrient limitation (which is not senescence) (Parkhill et al. 2001). When we compared the initial dark $F_v:F_m$ value of green puffs ($n = 15$) and green tufts ($n = 33$), there was no significant difference between them (means $\pm$ SD, 0.65 $\pm$ 0.07 and 0.68 $\pm$ 0.06, respectively). The rETR data are presented as the second panel in both figures (Figs. 1B, 2B). This value is the product of $F_v:F_m$, light, and some constants; hence, low light levels will always produce low rETR. In comparing Figs. 1B and 2B, we note that the rETR values for both green and brown *Trichodesmium* are very similar to approximately 75–100 μmol quanta m^{-2} s^{-1}. While not identical, it is reasonable evidence that

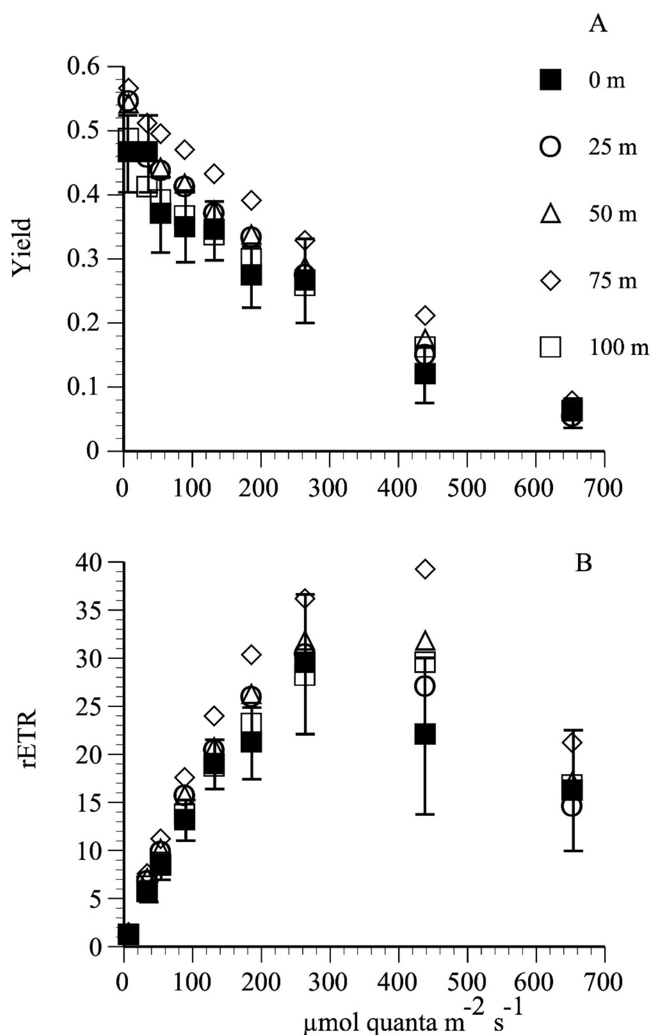

Fig. 1. PAM fluorometry data from brown *Trichodesmium* colonies. (A) $F_v : F_m$ values (yield) from colonies collected at different depths. The error bar (95% confidence interval) is shown for only one curve for clarity and represents the general degree of variability seen in colonies. (B) Relative electron transport rates (rETR) for the same colonies as in A. Error bars are as in panel A.

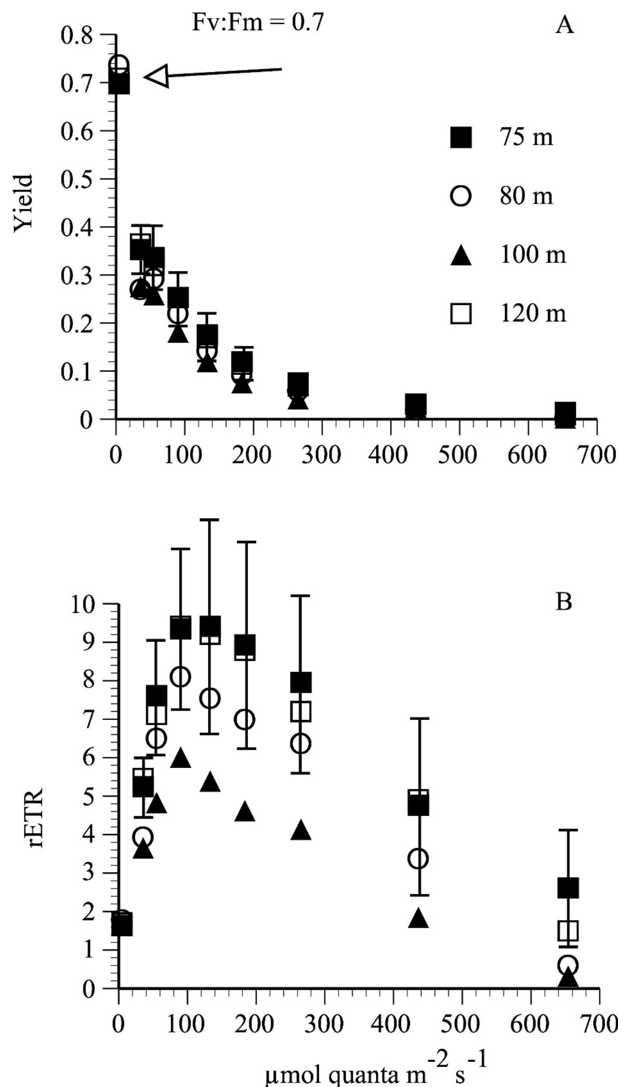

Fig. 2. PAM fluorometry data from green *Trichodesmium* colonies. Error bars are as in Fig. 1A. (A) $F_v : F_m$ values (yield) from colonies collected at different depths. The arrow indicates the high $F_v : F_m$ value noted in dark-adapted colonies (error bar is less than the size of the symbol). (B) Relative electron transport rates (rETR) for the same colonies as in panel A.

the differences noted between the two types of colonies at higher light levels are the result of physiological differences, not a degradation unique to green colonies. The decrease in rETR in green *Trichodesmium* is a well-known physiological process of down-regulation due to nonphotochemical quenching mechanisms (Campbell et al. 1998; Ralph et al. 2002). This is the process by which excess photochemical energy is bled off to sinks other than electron transport between PS II and PS I. This was described in a classic paper by White and Critchley (1999). In their paper, the figures clearly illustrate that active changing of F_o' during a rapid light curve is associated with the short-term acclimation process. Stressed, photoinhibited cells show a flat F_o' profile, the exact opposite of what we observed. While their work was on peas (which are clearly not cyanobacteria), the important point is the differential dynamic regulation of the photosystem that is typical of photacclimation and short-term responses to light.

The fact that green colonies were not in senescence appeared still most obvious in the raw fluorescence data that generate the instantaneous yield ($F_v : F_m$) values for both green and brown *Trichodesmium* (Fig. 3). In this figure, the initial chlorophyll fluorescence value (F_o, zero PAR value of F_o') prior to the saturation flash is shown with the concurrent value for the saturation flash (F_m, zero PAR value of F_m'). These are the data that generate $F_v : F_m$ for the various actinic light values during the rapid light curve. There are two processes occurring here. First, the initial, low F_o (Q totally oxidized) is increasing as the actinic light increases. This is due to increased reradiation of energy as fluorescence as Q becomes increasing reduced

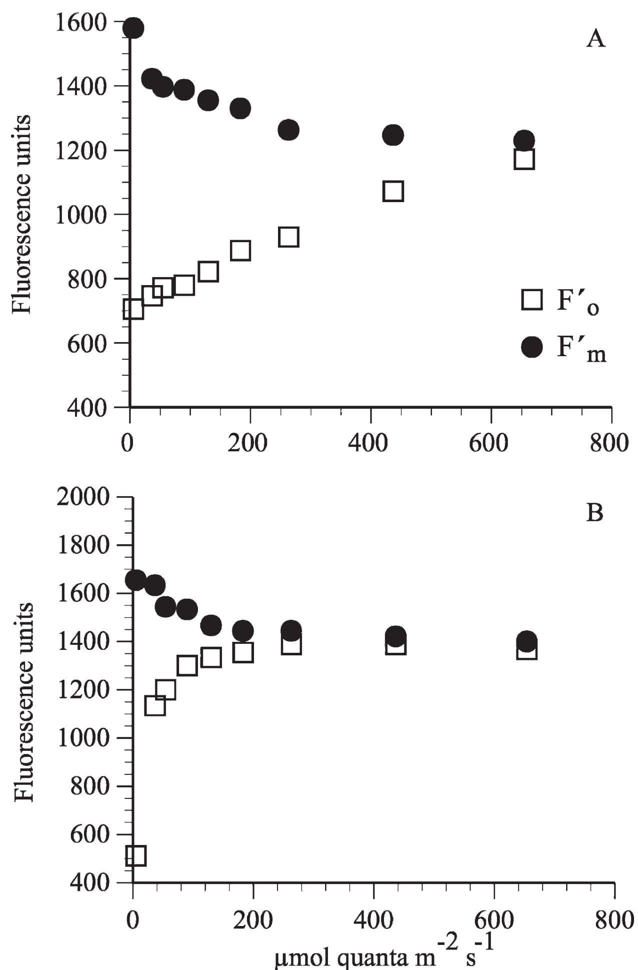

Fig. 3. Raw fluorescence data from a single colony. In each graph, the zero PAR value is actually F_o or F_m, not F_o' or F'_m. (A) Brown *Trichodesmium*, 01 November 1999, 15 m. (B) Green *Trichodesmium* 28 November 1999, 120 m.

and reaction centers close. The light intensity at which this happens is quite low in green *Trichodesmium*, an observation that is consistent with a low light acclimated form. It is a more gradual process in the higher light-adapted brown *Trichodesmium*. Second, and more to the point, the F_m steadily decreases in both forms as nonphotochemical quenching (mechanisms that divert energy from photochemical work) are induced. This is shown by the increasing difference between the maximum F_m and the instantaneous F_m (noted as F'_m) as the actinic light increases. This is a physiological mechanism that quenches excess photochemical energy. The consistent pattern seen in multiple colonies would not be found if thylakoids were undergoing different degrees of regulation degradation. These data are positive, affirmative evidence that the green colonies were not degenerate. It is unfortunate that space limitations precluded including this data in the original paper. Additionally, when green colony was exposed during 10 min under continuous light intensity of 186 μE m^{-2} s^{-1} before to run as a new rapid light curve, the cells recovered very quickly from the high light exposure after a 40-s dark period (yield = 0.64). This confirms that green *Trichodesmium* was in good physiological state.

The initial dark $F_v:F_m$ values noted in green *Trichodesmium* are high, much higher than typically seen in cyanobacteria. Typically, phycobilisomes contribute to F_o but not to F_m; hence, $F_v:F_m$ values are lower (as seen in brown *Trichodesmium* traces). Higher $F_v:F_m$ is consistent with the loss of phycobilisomes as noted in cyanobacteria mutants (Campbell et al. 1998) and in *Prochlorococcus* (Six et al. 2007). Our data could not address this, but they suggest that in green *Trichodesmium*, the existing phycobilins are more tightly coupled to photosynthesis than in brown *Trichodesmium*.

As a last note on the PAM data, each colony had to be examined and centered on the microscope state. Unlike the Orcutt et al. samples, we visually examined each colony under the microscope and verified the integrity of the colony. Orcutt et al. used samples left over from our experiments that had been picked only by eye from net tows and rinsed in filtered seawater. It is possible, of course, that they picked three dead green *Trichodesmium* tufts by sheer chance. We cannot attest to the integrity of the colonies they used.

Furthermore, Neveux et al. (2006) presented the high phycourobilin : phycoerythrobilin (PUB : PEB) ratio (2 : 1) of phycoerythrin in green *Trichodesmium* as an argument that these colonies were photoacclimated or photoadapted to low light conditions. This pigment has an absorption maximum at wavelengths that penetrate the deepest in the oligotrophic oceans. Orcutt et al. noted that this relatively high PUB : PEB ratio was also reported (1.7–2.1) in *Trichodesmium* populations collected from the surface waters (15 m) of the Caribbean Sea (Subramaniam et al. 1999). However, the latter observation concerned only absorption spectra of extracts obtained on mixtures of *Trichodesmium erythraeum* and *T. thiebautii*. Several arguments lead us to suspect some bias in this ratio related to the extraction procedure (or more generally to sample treatment) used by Subramaniam et al. (1999). First, in this paper, information on in vitro– or in vivo–corrected fluorescence excitation spectra of phycoerythrin was not presented, and action spectra for photosynthetically oxygen evolution, which concerned living colonies of each species (*T. thiebautii* or *T. erythraeum*), were not in favor of a high PUB : PEB ratio. Oxygen evolution indeed was higher or similar at the PEB absorption peak than at the PUB one. The tentative explanation was the low efficiency of energy transfer from PUB to PS II, a significant fraction being reemitted at 565 nm as fluorescence. However, 565 nm is generally considered as the peak of PEB fluorescence emission, energy absorbed by PUB being transferred with high efficiency to PEB before transfer to phycocyanin, allophycocyanin, and PS II chlorophyll. Considering that energy losses by fluorescence related to PUB itself was not reported in the Subramaniam et al.'s (1999) paper, we see no reason to explain how transfer to PS II would be less efficient when energy is absorbed by PUB or directly by PEB. Second, our results on populations of the

southwest tropical Pacific and north Australian waters did not show diel variations of the PUB : PEB ratio either in *T. thiebautii* (fig. 6 in Neveux et al., 2006) or in *T. erythraeum*. These results contrast with absorption spectra from extracts reported by Subramaniam et al. (1999). Third, previous literature on the phycoerythrin absorption spectra of *T. thiebautii* showed generally PUB : PEB ratios around 1 (Fujita and Shimura 1974; Haxo et al. 1987) in accordance with our corrected fluorescence excitation spectra (Neveux et al. 2006, fig. 1). Only one exception was reported on filaments (provisionally identified as *T. thiebautii* but not apparently confirmed by more recent information) isolated in marine coastal waters at Qingdao (China) that contained a phycoerythrin without PUB (Haxo et al. 1987). This discussion assumes that *T. thiebautii* and *T. erythraeum* have the same phycoerythrin in the Caribbean Sea than the same species in the southwest Pacific Ocean, an assumption that seems reasonable to us. Environmental conditions in surface waters of these two regions do not seem different enough to explain this phenotypic difference. Finally, the PE we observed in green *Trichodesmium* not only had a high PUB : PEB ratio but also presented shifts of the excitation maxima of PUB and PEB (Neveux et al. 2006, fig. 1) that were not reported in the Caribbean Sea.

Complementary information is certainly required to conclude definitively if green colonies represent a photoacclimated or photoadapted form of *Trichodesmium*. In the absence of a true measure of absorption of the light excitation by the different colonies (narrow bandwidth centered at 470 nm), the rapid fluorescence light curve did not allow to conclude that green *Trichodesmium* was photosynthetically more or less efficient than brown *Trichodesmium* at low light level. The ^{14}C-photosynthesis-light curves at simulated or natural ambient light intensities could resolve this point. However, the rapid light curves showed that the green population did not tolerate ambient high light intensity, an observation consistent with their distribution in relatively deep waters (Neveux et al. 2006).

In conclusion, Orcutt et al. have no quantitative arguments to demonstrate senescence of the green colonies, whereas our PAM results show that they have physiologically active photosystems and that they respond to light conditions differently than did brown colonies of *T. thiebautii*.

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References

BERGMAN, B., J. R. GALLON, A. N. RAI, AND L. J. STAL. 1997. N_2 fixation by non-heterocystous cyanobacteria. FEMS Microbiol. Rev. **19:** 139–185.

BERMAN-FRANK, I., K. D. BIDLE, L. HARAMATY, AND P. G. FALKOWSKI. 2004. The demise of the marine cyanobacterium, *Trichodesmium* spp., via an autocatalyzed cell death pathway. Limnol. Oceanogr. **49:** 997–1005.

BOTHE, H. 1982. Nitrogen fixation, p. 87–104. *In* D. W. Carr and B. A. Whitton [eds.], The biology of cyanobacteria. University of California Press.

CAMPBELL, D., V. HURRY, A. K. CLARKE, P. GUSTAFSSON, AND G. OQUIST. 1998. Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. Micro. Mol. Biol. Rev. **62:** 667–683.

FUJITA, Y., AND S. SHIMURA. 1974. Phycoerythrin of the marine blue-green algae *Trichodesmium thiebautii*. Plant Cell Physiol. **15:** 939–942.

HAXO, F. T., R. A. LEWIN, K. W. LEE, AND M. LI. 1987. Fine structure and pigments of *Oscillatoria* (*Trichodesmium*) aff. *thiebautii* (Cyanophyta) in culture. Phycologia **26:** 443–456.

HOLL, C. M., T. A. VILLAREAL, C. D. PAYNE, T. CLAYTON, J. P. MONTOYA, AND C. HART. 2007. Characterization of a bloom of the diazotrophic marine cyanobacterium *Trichodesmium* sp.: Implications for N cycling in the Gulf of Mexico. Limnol. Oceanogr. **52:** 2249–2259.

NEVEUX, J., M. M. B. TENÓRIO, C. DUPOUY, AND T. A. VILLAREAL. 2006. Spectral diversity of phycoerythrins and diazotroph abundance in tropical waters. Limnol. Oceanogr. **51:** 1689–1698.

PARKHILL, J. P., G. MAILLET, AND J. J. CULLEN. 2001. Fluorescence-based maximal quantum yield for PSII as a diagnostic of nutrient stress. J. Phycol. **37:** 517–529.

RALPH, P. J., R. GADEMANN, A. W. D. LARKUM, AND M. KUHL. 2002. Spatial heterogeneity in active chlorophyll fluorescence and PSII activity of coral tissues. Mar. Biol. **141:** 639–646.

SIX, C., Z. V. FINKEL, A. J. IRWIN, AND D. A. CAMPBELL. 2007. Light variability illuminates niche-partitioning among marine picocyanobacteria. PLoS ONE **2:** e1341, doi:10.1371.

STAAL, M., AND OTHERS. 2007. Nitrogen fixation along a north-south transect in the eastern Atlantic Ocean. Limnol. Oceanogr. **52:** 1305–1316.

SUBRAMANIAM, A., E. J. CARPENTER, D. KARENTZ, AND P. G. FALKOWSKI. 1999. Bio-optical properties of the diazotrophic cyanobacteria *Trichodesmium* spp. I. Absorption and photosynthetic action spectra. Limnol. Oceanogr. **44:** 608–617.

WHITE, A. J., AND C. CRITCHLEY. 1999. Rapid light curves: A new fluorescence method to assess the state of the photosynthetic apparatus. Photosyn. Res. **59:** 63–72.

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