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Growth, Nitrogen Fixation, and Spectral Attenuation in Cultivated *Trichodesmium* Species

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Physiological studies of *Trichodesmium* species have been hindered by difficulties in maintaining actively growing, nitrogen-fixing cultures. Previous cultivation successes have not been widely duplicated. We present here a simple modified seawater medium and handling techniques which have been used to maintain actively growing *Trichodesmium thiebautii* in laboratory culture for over 1 year. The cultured population, isolated from coastal Atlantic waters, has a growth rate of 0.23 division day^{-1} and exhibits light-dependent nitrogen fixation during exponential growth. Various morphologies, including solitary trichomes, and aggregates (spherical puffs and fusiform tufts) are present during growth. Spectral and scalar irradiance were measured within naturally occurring (coastal Atlantic) aggregates with small (diameter, 50 to 70 μm) spherical fiber-optic sensors. In contrast to naturally occurring puffs, cultivated *Trichodesmium* aggregates exhibited spectral properties consistent with low-light adaptation. Cultivated puff-type aggregates were also examined by using oxygen microelectrodes. The simple medium and approach used for cultivation should be easily reproducible and amenable to further manipulations and modifications useful for physiological studies of *Trichodesmium* spp. in culture.

Nitrogen fixation in the nonheterocystous filamentous cyanobacterium *Trichodesmium* (*Oscillatoria*) spp. has been observed and documented for several decades (6, 30). Previous studies have shown that N_2 fixation in *Trichodesmium* spp. is light mediated, showing a pattern similar to photosynthetic activity (20, 27). Oxygen produced during photosynthesis readily inhibits and degrades nitrogenase, the enzyme responsible for nitrogen fixation (7). Therefore, this pattern of contemporaneous nitrogen and carbon fixation has been widely observed only in cyanobacteria which form specialized non-oxygen-evolving cells, called heterocysts, in which nitrogen fixation is localized. *Trichodesmium* species are among a small group of cyanobacteria which can conduct these two processes concurrently without forming heterocysts. Although several potential explanations for this unusual ability of the *Trichodesmium* species have been proposed, no definitive explanation has yet emerged.

The paucity of *Trichodesmium* cultures available for physiological study has been a hindrance to furthering our understanding of the enigmatic abilities of these species. Although several laboratories have reported success at establishing cultures (9, 18, 24), to date only the cultures of Ohki and Fujita have been documented to grow (18) and fix (17) nitrogen. Unfortunately, transfer and establishment of these and/or other *Trichodesmium* isolates to other laboratories has been difficult. Our observations suggest that special handling procedures, described here, are important for successful cultivation. Using these techniques, we have maintained an N_2-fixing culture of *Trichodesmium* originally isolated from North Carolina Atlantic coastal waters for over 1 year in the laboratory (Fig. 1 and 2). We have tentatively identified this population as *Trichodesmium thiebautii* on the basis of size (trichome width, 8 to 10 μm; cell length, 4 to 6 μm), and observed aggregate morphologies (2, 29). However, because of uncertainties concerning the reliability of these characteristics as taxonomic indicators, we shall refer to this population as a "*Trichodesmium*" sp. pending further identification. Other populations of *Trichodesmium* spp. from these waters (October 1990, June 1992) were also amenable to cultivation and exhibited morphologies similar to those of the population with which we have worked more extensively (September 1991).

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MATERIALS AND METHODS

Medium preparation. The medium used for *Trichodesmium* culture is prepared from oligotrophic offshore seawater collected in 20-liter, 0.01 N HCl-rinsed, polycarbonate carboys. The seawater (800 to 900 ml) is diluted with distilled deionized water (100 to 200 ml) to a volume of 1 liter at 32‰ salinity. After filtering the diluted seawater solution through a Whatman GF/F glass fiber filter, phosphate, magnesium, calcium, and iron additions are made from stock solutions listed in Table 1. Stock solutions are previously made up in deionized water and kept refrigerated after autoclave sterilization. Tricine buffer (Sigma Chemical Co.) is then added to the seawater solution (25 mg liter^{-1}). The pH of the solution is adjusted to 8.17 with 0.1 N HCl and 0.1 N NaOH solutions while continuously stirring. The seawater solution is then filter sterilized through a Whatman GF/F prefilter and a 0.2-μm-pore-size Metricel membrane filter (Gelman Filtration Products) to produce the working medium (*Trichodesmium* medium [TMstd]). Alternatively, tricine-buffered, pH-adjusted seawater solution may be autoclaved and stock additions may be made aseptically. This second method, however, is more susceptible to precipitation, which renders the medium unusable. Medium is aseptically dispensed into sterile Erlenmeyer flasks (50 to 500 ml), to a level just above their widest diameter. Two variations of this medium are

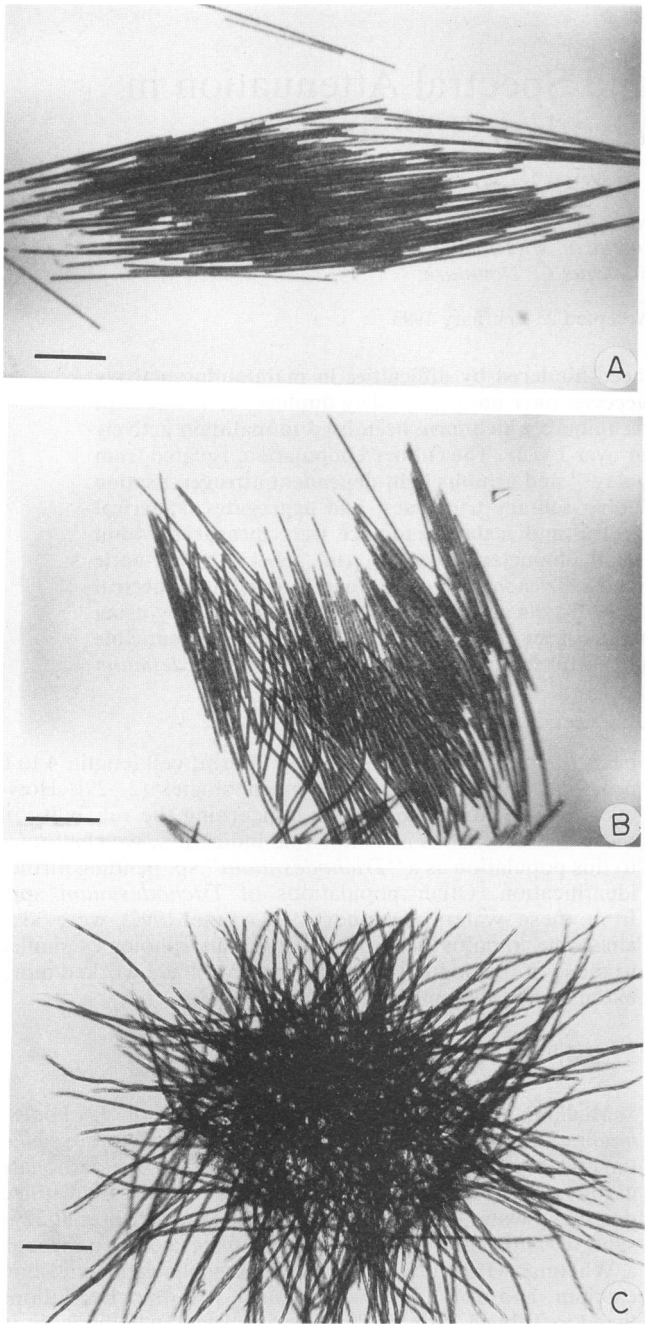

FIG. 1. Cultivated *Trichodesmium* aggregates from North Carolina coastal Atlantic waters. (A) Cultivated *T. thiebautii* fusiform (tuft) aggregate. (B) Several tuft colonies arranged in parallel. (C) Spherical (puff) cultivated *Trichodesmium* aggregate; note the loosely bound, random arrangement of filaments and the open spaces visible between them. Bars, 100 μm.

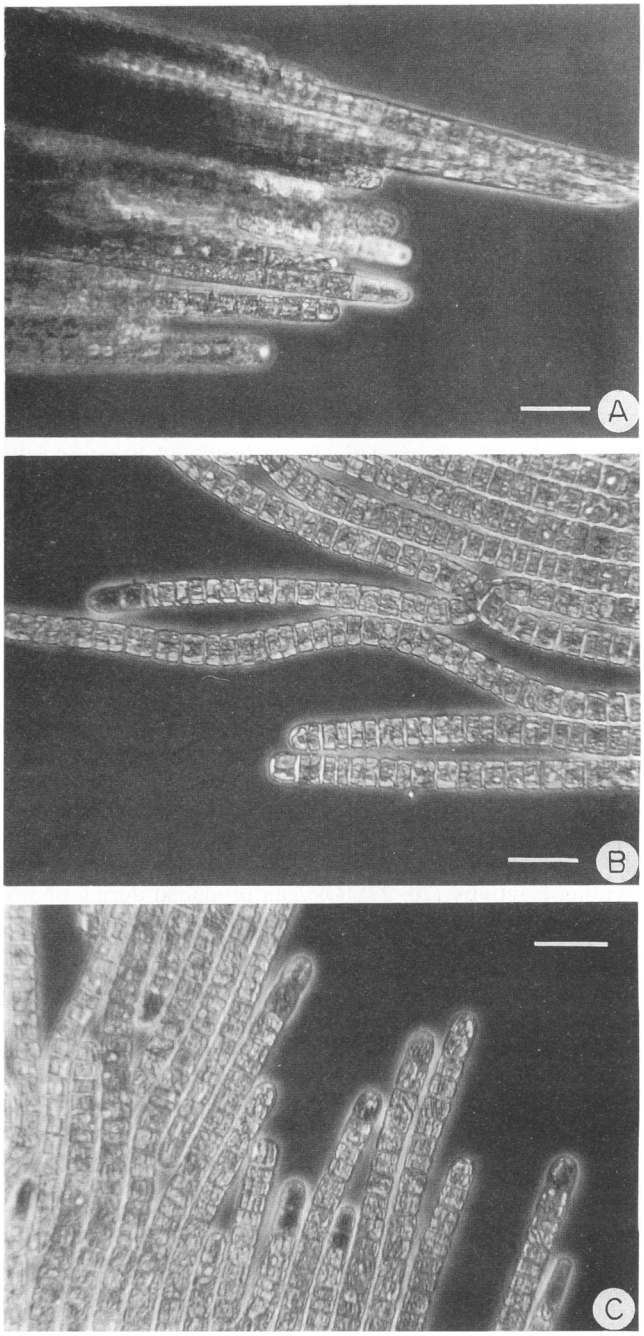

FIG. 2. High-magnification views of cultivated *Trichodesmium* filaments located in aggregates. Note the presence of gas vacuoles (highly reflective regions) in individual cells. (A) Tip of a tightly aggregated fusiform tuft. (B) Tip of a loosely aggregated fusiform tuft. (C) Peripheral region of a spherical puff. Bars, 20 μm.

derived by aseptically adding Guillard and Ryther's F medium vitamin solution (1, 8), in either F/4 concentration (TM.5V) or F/2 concentration (TMV), from a filter-sterilized stock solution (Table 1).

Generating unialgal Cultures from natural populations. Generation of actively growing unialgal *Trichodesmium* spp. in culture required frequent monitoring and/or treatment and transfer during initial isolation stages. Intact aggregates (small fusiform tufts and spherical puffs) free of macroscopically visible fouling were selected from a plankton tow sample and transferred by using a 1.0-μl polystyrene inoculating loop (Elkay Division of Labsystems) into fresh TMV medium. To rid colonies of eukaryotic organisms, 50 to 100 mg of cycloheximide liter^{-1} (Sigma Chemical Co.) was added. Cycloheximide proved effective at killing eukaryotic

TABLE 1. Trichodesmium growth media[a]

Addition	Stock (per liter)	Amount
Tricine		25.0 mg
K_2HPO_4	8.7 g	1.0 ml[b]
$MgSO_4 \cdot 7H_2O$	49.3 g	1.0 ml
$MgCl_2 \cdot 6H_2O$	40.6 g	1.0 ml
$CaCl_2 \cdot 6H_2O$ or $CaCl_2 \cdot 2H_2O$	43.8 g 29.4 g	2.0 ml
Disodium EDTA and $FeCl_3 \cdot 6H_2O$	7.4 g 1.1 g	0.2 ml
F/2 vitamin stock solution		1.0 ml (TMV) 0.5 ml (TM.5V) 0.0 ml (TMstd)
Thiamine HCl	100 mg	
Biotin	500 μg	
B_{12}	500 μg	

[a] To 1 liter of 32‰ oligotrophic seawater add 25 mg of tricine buffer. While stirring to prevent precipitation adjust pH to between 8.1 and 8.2. Stock solutions may be added and medium filter may be sterilized (0.2 μm Metricel membrane filter), or seawater with tricine may be autoclaved and stock additions may be made asceptically.

[b] This addition yields a final concentration of 50 μM. Lower levels may be used, i.e. 0.1 to 0.5 ml corresponding to 5 and 25 μM final concentrations, respectively. See text for further comments.

cells; however, after 4 to 8 days, treated flasks often contained both *Trichodesmium* spp. and picoplanktonic organisms. These unidentified picoplankton appeared as paired small spherical cells 2.5 to 3.0 μm in diameter. Once the picoplankton were macroscopically visible (either a gold-green cloudiness in the media or a bright green mass attached to a *Trichodesmium* aggregate), the *Trichodesmium* filaments soon lysed. Apparently, as eukaryotic cells die, cycloheximide-treated populations are exposed to higher nutrient concentrations which may not favor *Trichodesmium* spp. (which fix N_2 and are typically found in oligotrophic waters) but are apparently suitable for the contaminating picoplankton.

Picoplankton contamination was eradicated by treating populations with cycloheximide for a shorter time period (24 to 36 h) and then transferring aggregates by loop to a fresh TMstd medium, cycloheximide-free wash, in which they were swirled to rid them of detritus and elevated nutrient concentrations. After washing, aggregates were transferred to fresh cycloheximide-free TM.5V medium, again with inoculating loops to minimize transfer of media and contaminants. Populations were then monitored closely for signs of eukaryote or picoplankton contamination. *Trichodesmium* aggregates which did not show eukaryotic or picoplankton contamination after 1 week were transferred to TMV medium.

Maintenance and handling. *Trichodesmium* stock cultures in TMV medium were maintained in a temperature-controlled incubator (Precision model 815) at 25°C. Illumination was provided by a 50/50 mixture of Sylvania cool white and Gro-Lux fluorescent lamps at 55 to 65 microeinsteins m^{-2} s^{-1} PAR flux on a 14-h light/10-h dark cycle. Larger colonies tended to aggregate in the meniscus of the medium, along the wall of the culture vessel. Smaller colonies and single filaments remained suspended in the medium to a greater extent. Evaporation of medium caused meniscus-associated colonies to attach to the vessel wall. To slow the rate of evaporation while permitting gas (i.e., O_2, CO_2) exchange, we inserted a layer of polyvinyl chloride (PVC) film (Reynolds food wrap) between the foam plug and the flask. The addition of a layer of PVC film also minimized fluctuations in salinity and ionic strength due to evaporation. *Trichodesmium* sensitivity to such fluctuations has been reported by others (24) and was observed for this culture as well. Cultures were swirled every 2 to 3 days to minimize attachment of filaments to the vessel wall along the meniscus and subsequent fouling. Associated eubacterial populations remained low (not visible by light microscopy) as long as filaments were not allowed to become attached to the vessel wall or to reach late exponential growth. Transfers were made during exponential growth when fouling bacteria were at lowest densities relative to *Trichodesmium* biomass.

Growth rate determination. The following procedure was used to inoculate 45 replicate 20-ml batch cultures. Aliquots (10 ml) of sterile TMstd medium were added to 50-ml Erlenmeyer flasks. An inoculum was prepared from unialgal *Trichodesmium* colonies of various sizes from four midexponential-phase 100-ml cultures. The culture contents were combined and filtered onto a sterile 5.0-μm-porosity Nuclepore filter (Nuclepore Corp.). Care was taken to filter these filaments gently, while rinsing them with additional sterile medium to resuspend them twice more. The filter was never allowed to become totally dry during this process. The filter holding the filaments was then touched to the surface of a small volume of medium contained in a sterile petri plate to dislodge the filaments into the medium. This concentrated suspension of filaments was transferred to a flask and diluted to a volume of 500 ml with TMstd to generate the inoculum. Vitamins were added to the inoculum so that a 1:1 inoculum-to-sterile-medium dilution provided a vitamin concentration equivalent to F/2. The filtration and transfer steps acted to gently break up aggregates of various sizes into relatively uniform small bundles of one to four filaments each, which greatly facilitated reproducibility in the amount of biomass transferred during inoculation. Each Erlenmeyer flask was given two 5-ml additions of inoculum. The inoculum flask was gently swirled often to maintain homogeneity in trichome distribution during transfers. Inoculated flasks were then stoppered with PVC film evaporation barriers and foam plugs. The 45 replicate 20-ml batch cultures were incubated under standard maintenance conditions as described above. They were swirled every other day during lag phase, daily during early exponential phase, and twice a day during late exponential phase.

At five time points of the growth curve, six replicates were arbitrarily selected and assayed for nitrogenase activity (NA) and then filtered and saved for chlorophyll *a* analysis. Contents of each flask (20 ml of culture) were swirled and poured into a 38-ml serum vial. Three vials of uninoculated TMV medium served as blanks. Vials were capped with gas-tight rubber stoppers. Three milliliters of air was removed from each vial, and 3 ml of CaC_2-generated acetylene was added. Triplicate vials were wrapped in foil for dark incubations. The vials were then returned to the incubator. Nitrogenase activity was measured for a 3- to 4-h period beginning 1 h after the onset of the incubator light phase under which the cultures had been grown. At the end of the incubation period, 0.3-ml headspace samples were analyzed by gas chromatography, using a Shimadzu GC-9A chromatograph (flame ionization detector) equipped with a 2-m Poropak-T stainless steel column at 80°C. Ultra-high-purity N_2 served as the carrier gas. Vial contents were then gently filtered onto 25-mm Whatman 934 AH filters. Two drops of concentrated $MgCO_3$-saturated seawater were added to each filter to preserve chlorophyll *a* intact. All filters were wrapped in foil, quick-frozen in liquid N_2, and stored frozen

for later analysis of chlorophyll *a* content by the trichromatic method (23) using a Milton Roy Spectronic 1201 spectrophotometer. Culture filtrate of the six replicates used for NA assessment was pooled. Ten milliliters of the pooled filtrate was preserved with 0.4 ml of phenol in preparation for later NH_4^+ analysis using a modification of the phenol-hypochlorite method (23). The remainder of the filtrate was frozen for later PO_4^{3-} analysis by the molybdate blue method (23) and NO_3^- by a colorimetric method after reduction by cadmium shaking (10).

Irradiance and oxygen measurements. Visual observations of our culture and natural populations of *Trichodesmium* spp. suggested that some shift in pigment composition (photoadaptation) had occurred during isolation and laboratory culture. To examine this possibility, we used recently developed light microsensors (13) to examine the light absorption characteristics of cultivated and natural *Trichodesmium* colonial aggregates. Cultivated aggregates were sampled from batch cultures in late exponential growth stage in which larger (diameter, ca. 400 to 800 μm) aggregates had formed. Naturally occurring aggregates were collected from 50 km offshore in plankton tows and returned to the laboratory for same-day (8 to 10 h after collection) measurements of spectral properties.

For optical measurement, *Trichodesmium* colonies were placed into a submerged shallow petri dish contained in a temperature-controlled aquarium. The petri dish was lined with black velvet. The black color served to reduce back-scattered light, and the cloth fibers served to immobilize the aggregates. Colonies also were held in place by embedding them in 1% agar in seawater. The aquarium was filled with either offshore seawater or nonsterile TMstd and maintained at 26°C. Spectral scalar irradiance was measured with spherical fiber-optic sensors (13). These sensors had diffusing spheres (50 to 70 μm wide). Each fiber-optic microsensor used had an isotropic (±10%) response to light at angles from −160° to +160°. Sensors were connected via an optical coupling (F-915t; Newport Corp.) to a detector system (Spectroscopy Instruments, O-SMA) consisting of a spectrograph (Monospec 18; Jarrel Ash) and an optical multichannel analyzer (Princeton Eiry detector, 1,024 channels). The detector system was wavelength calibrated by using the line spectra from Ne and Hg lamps. Incident light, i.e., the downward scalar irradiance (E_{od}), was measured with the scalar irradiance microsensor positioned over a light trap consisting of a black cylinder. Ambient light was about 5% higher than incident light because of back-scattering from the surroundings. Scalar irradiance measurements within colonies (E_o) could be measured at the same position relative to the light field as the external irradiance measurements, by virtue of a rotating cylinder mounted in the aquarium. The cylinder contained three equidistant wells; one was used as the light trap, another contained the petri dish holding the aggregate to be studied, and the third contained a photon irradiance meter (LiCor Li 185 A).

Depth distributions of O_2 in and around aggregates were measured by a Clark type O_2 microelectrode with a 90% response time of 0.3 s and a 6-μm-wide measuring tip (25). Oxygen and fiber-optic sensors were attached to a motor driven micromanipulator to control their placement and movement. The exact position of the microelectrode and fiber-optic sensor relative to the aggregate surface was determined with a dissecting microscope. The surface of the aggregate was defined as the position where the trichomes formed a dense laterally coherent surface with a distinct shape with only single trichomes protruding from it. Sensors positioned at an angle of 45° to the light beam were advanced into the colony at 50-μm depth increments.

RESULTS

Growth of *Trichodesmium* spp. in culture was documented as an increase in chlorophyll *a* concentration over time (Fig. 3). A semi-log plot of chlorophyll *a* concentration (in micrograms $liter^{-1}$) versus time (in days) yielded a K_{10} value of 0.07 with an R^2 of 0.94. Converting this value to divisions per day (log base 2 increase per day) yielded an increase in chlorophyll *a* content of 0.23 divisions day^{-1}. The chlorophyll-based growth curve (plotted as average chlorophyll values), along with the light and dark NA, is shown in Fig. 3A. Concentrations of PO_4^{3-}, NO_3^-, and NH_4^+ in filtrate remaining after removal of *Trichodesmium* biomass are shown for each sampling date (Fig. 3B). Dark NA was undetectable at all times. Light NA increased noticeably between day 16 and day 27. After day 27 the light NA activity began to decrease sharply, and by day 34 it was the same as dark levels. Chlorophyll *a* values, however, continued to increase over this time period. Analysis of nutrient concentrations in the medium filtrate showed consistently low nitrate concentrations throughout the growth curve experiment. Phosphate concentrations remained relatively constant between 45 and 55 μM until later in the experiment when concentrations decreased slightly. Ammonium concentrations were low (<0.1 μM) for days 16, 24, and 27 but increased on day 31 (0.85 μM) and more than doubled by day 34 (2.26 μM).

Spectral attenuation was measured in the centers of cultured late-exponential-stage puff-type spherical aggregates and in oceanic spherical aggregates that were collected the same day in an offshore plankton tow. We had previously observed that cultured *Trichodesmium* spp. appeared dark reddish brown whereas the natural population from which they were isolated was pale golden brown. The 29 June 1992 tow sample *Trichodesmium* population was very similar in coloration and aggregate morphologies exhibited to the initial population from which our culture was isolated in September 1991. Figure 4 shows a representative attenuation spectrum for both cultured and natural *Trichodesmium* spherical aggregates. Several spectra were collected for each type. For comparison, spectra (one from the culture and one from the offshore tow) were selected in which the ratios of attenuation at 675 and 700 nm, respectively, were similar, indicating that the two bundles have similar light-scattering properties so that differences observed in the spectra can be attributed to differences in light absorption (i.e., pigment content differences). The oceanic aggregate had notable absorption in the 450- to 500-nm region, indicating relatively high carotenoid content (β-carotene, echinenone, myxoxanthophyll). Absorption in this region, along with low attenuation in the green region, also indicates a higher content of phycourobilin relative to phycoerythrobilin chromophores. This pattern was previously observed in naturally occurring *Trichodesmium* spp. by Lewis et al. (14). Conversely, the cultured colony had absorption peaks at approximately 492, 545, and 567, with the first peak being dominant. This pattern was quite similar to the triple peaks (495, 547, 562) measured (using an opal-glass method) on intact *Trichodesmium* filaments for photosynthetically active phycoerythrin in waters off Kuroshio, Japan (28). We presume that the peak at 625 to 627 is phycocyanin.

Irradiance attenuation measurements showed evidence of self-shading occurring in a cultivated aggregate. The maxi-

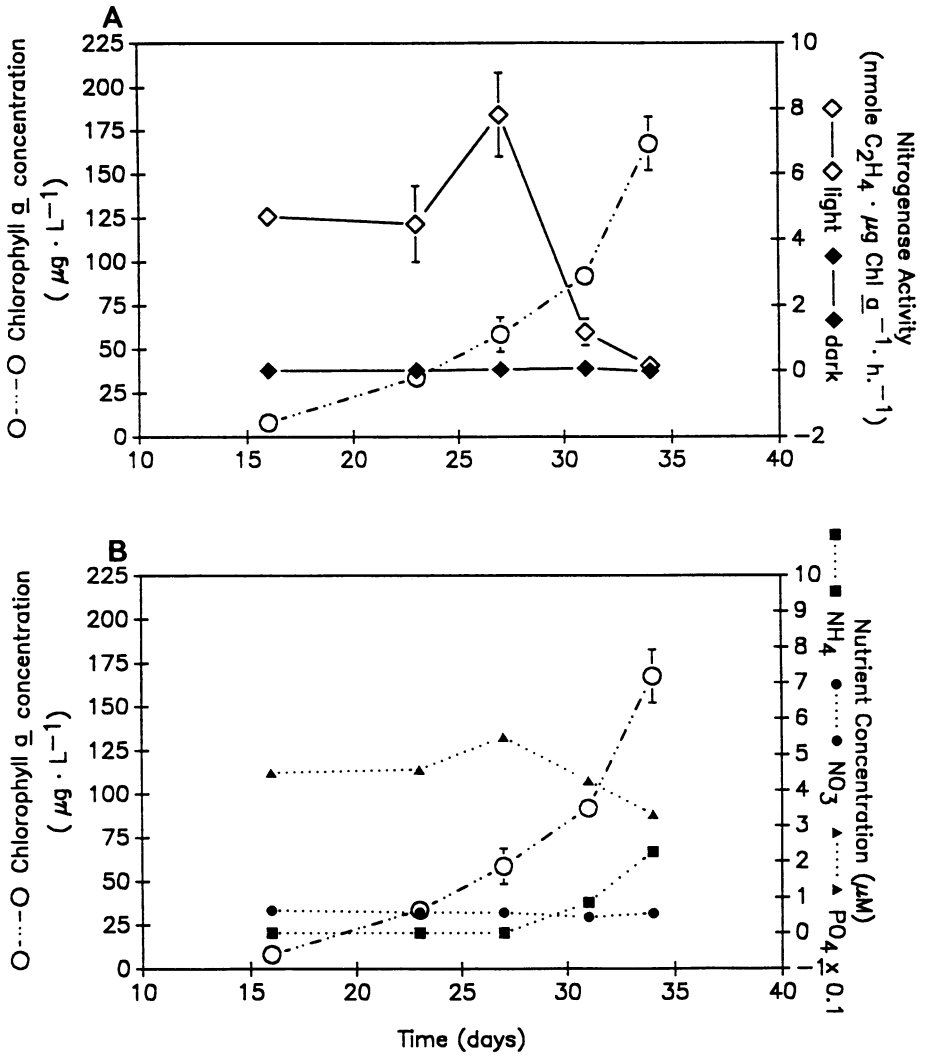

FIG. 3. Chlorophyll *a* content, nitrogenase activity, and nutrient content of medium versus time. (A) Chlorophyll *a* content values are averages of five to six replicates (bars indicate standard errors of the mean). Light and dark NA are averages of three replicates (bars indicate standard errors of the mean). (B) Nutrient concentrations in pooled medium filtrate for six replicates.

mum attenuation measured in an ellipsoid puff-type cultivated aggregate (dimensions, 400 by 600 μm) is shown in Fig. 5. In a unidirectional light field the lowest light intensity is not measured in the center but slightly below it on the side farthest from the light source. Less than 40% of ambient light for all wavelengths in the visible PAR range remained in this area of the aggregate.

Figure 6 shows the steady-state distribution of O_2 in an ellipsoid culture-grown aggregate (dimensions, 400 by 800 μm) at different incident irradiance intensities. From the O_2 gradient in the diffusive boundary layer above the surface, the net production of O_2 can be calculated. The O_2 compensation point for this bundle was close to 16 microeinsteins m^{-2} s^{-1}.

DISCUSSION

Requirements for cultivation. Since we used an amended seawater medium, exact final concentrations of all chemical constituents are not precisely known. The medium described here was adapted from one used for the heterocystous freshwater cyanobacterium *Aphanizomenon flos-aquae* (16), which also forms fusiform-type aggregates, by adding the same major cations and anions to a buffered seawater base. Medium and incubation modifications were based on growth, N_2 fixation, and morphological responses to each modification of medium composition or technique. The vitamins cyanocobalamin, biotin, and thiamine, which are required by many marine phytoplankton (15), were added. Calcium concentrations were determined to be critical for survival and optimal growth of cultured *Trichodesmium* species by Ohki and Fujita (18). Omitting the calcium stock addition from our formulation resulted in lysed *Trichodesmium* filaments within a few days of transfer. This effect is puzzling, since the Ca^{2+} enrichment (0.4 mM) is relatively small in relation to the large amount of Ca^{2+} already present from the 900 ml of seawater (approximately 9 mM). Perhaps the complexation of the Ca^{2+} in this medium renders it more readily available to *Trichodesmium* spp.

The phosphate content of TMstd is much higher than that used by Ohki and Fujita (18), who found trace metal con-

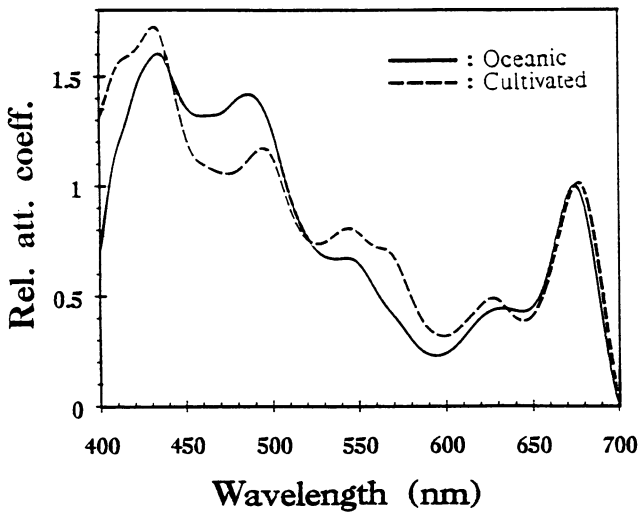

FIG. 4. Attenuation coefficients for the scalar irradiance, K_o, in a spherical puff aggregate from culture (dashed line) and from coastal Atlantic oceanic waters (solid line). Attenuation coefficients are calculated as the natural logarithm of the ratio between ambient light and the light in the center of the bundles. The spectra are normalized to the 675-nm peak.

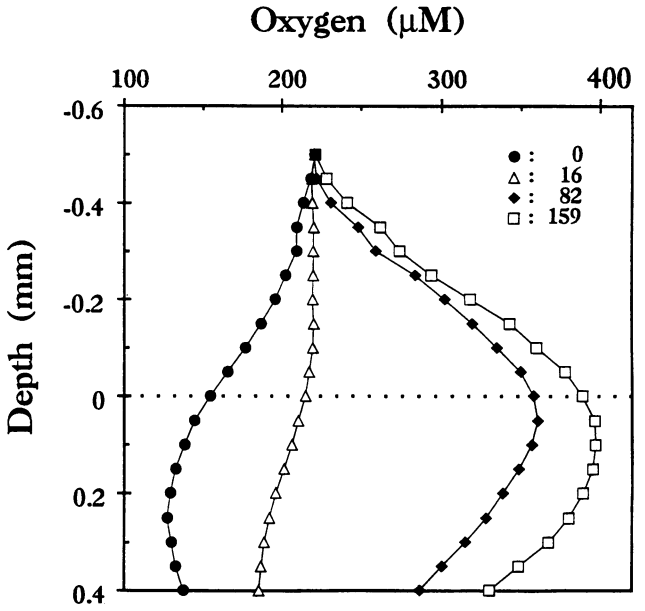

FIG. 6. Steady-state distribution of O_2 in a cultivated *T. thiebautii* puff aggregate, under four different incident irradiance levels (values in microeinsteins $meter^{-2}$ $second^{-1}$) noted in figure.

taminants in ultrapure grade phosphate reagents to be inhibitory to *Trichodesmium* growth. Using a chelex column to purify their phosphate stock solution, they were able to increase the final phosphate concentration in their media to 13 μM, resulting in enhanced growth. However, even with chelex cleaning, concentrations of above 32 μM still caused growth inhibition in their culture. We used reagent grade phosphate stocks (Baker), with no special cleaning steps, and fortuitously did not see inhibitory effects on this *Trichodesmium* culture even at high concentrations (ca. 25 to 50 μM). The concentrations of phosphate left in the growth curve medium filtrate suggest, however, that the medium phosphate level is in excess of that required and could be lowered substantially. Omitting the iron addition resulted in cell lysis in a few days. Vitamin enrichment appeared to promote more vigorous growth and aggregate formation. The tricine buffer that we used did not appear to cause any of the problems reported by others for Tris (18). The value of 8.17 as the standard maintenance pH was empirically derived from observations of the pH of the most robust cultures in early trials of media. Subsequent observations revealed good growth between pH 7.7 and pH 8.2.

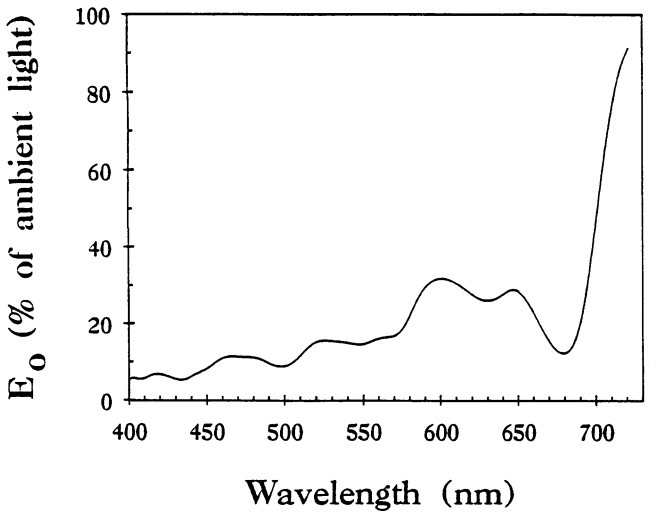

FIG. 5. Spectral scalar irradiance, E_o, in the center of a 500-μm (average diameter), cultivated *T. thiebautii* puff aggregate.

After removal of eukaryotic grazers and contaminating picoplankton, providing a stable, consistent chemical environment (such as a buffered solution in which evaporative moisture loss and concomitant salinity and ionic strength fluctuations are minimized) is required for *Trichodesmium* growth in culture. Use of PVC film was advantageous in this regard. Cotton or foam stoppers promote high rates of evaporation, while solid stoppers or caps will not provide adequate gas exchange. Gentle agitation by occasionally swirling flasks is also beneficial. Incubating cultures on standard shaker tables caused cell lysis. Aeration by bubbling air into cultures resulted in cells being thrown onto vessel walls as bubbles burst and was additionally disadvantageous since evaporation rates were increased. A logical next step for further studies would be to establish cultures in chemostats.

Growth rate and characteristics in batch culture. The growth rate observed for cultured *T. thiebautii* is comparable to growth rates reported for other cultures (9, 18) and for natural populations in North Atlantic waters (14). The rates of NA in this culture have been measured only under low (60 microeinsteins m^{-2} s^{-1}) irradiance levels to date. Among literature-based rates normalized for chlorophyll *a* content, these rates appear higher than those of the culture of Ohki and Fujita under approximately 45 microeinsteins m^{-2} s^{-1} irradiance (17) and lower than the natural rates reported by Saino and Hattori under sunlight irradiance of approximately 250 microeinsteins m^{-2} s^{-1} (27). Since these various growth rates were measured under different irradiance levels, fur-

ther comparison of our culture with other cultures and naturally occurring populations is warranted.

We observed an increase in medium NH_4^+ concentration occurring simultaneously with decreasing biomass-specific NA during late exponential growth. Low bacterial population densities and close association of light with measurable NA strongly suggest that *Trichodesmium* spp. themselves are responsible for the bulk of the N_2 fixation in culture. Chlorophyll *a* levels continued to increase exponentially from day 31 to day 34 even though nitrogen fixation rates dropped significantly. This may reflect a shift away from N_2 fixation to N recycling to support the continued rate of growth. If so, the mechanisms triggering this shift are not clear. Phosphate concentrations in the media were beginning to decline at this time, but a substantial amount of phosphate was still present. Iron availability was not determined; hence, the possibility that iron limitation may have been forcing the shift could not be tested on this culture, though it has been suggested to occur in nature (26). Subsequent work (in progress) has shown that when inoculated into TMV media lacking the Fe addition, this *Trichodesmium* culture exhibits a loss of chlorophyll *a* and no NA after 11 days of incubation, whereas the addition of Fe (0.5 μM and 1.0 μM) plus EDTA at the beginning of the 11-day incubation enhances chlorophyll *a* and NA.

Ammonium accumulating in the culture medium was presumably derived from *Trichodesmium* spp., since these are the sole source of newly fixed N in what initially was N-depleted medium. Why *Trichodesmium* spp. would release ammonium or perhaps other dissolved N forms when N is required to support carbon fixation and growth is unclear. Perhaps ammonium appearing in the medium results from leakage during intracellular conversion of fixed nitrogen reserves (i.e., phycoerythrin). Suppression of NA in *Trichodesmium* cultures on addition of various nitrogen sources has been observed (19).

Morphological variability in cultivated and natural populations. During growth in batch culture, this *Trichodesmium* population showed morphological changes similar to those observed in other *Trichodesmium* cultures (17). Mechanical disruption was used at the beginning of the experiment to facilitate homogeneity in the inoculation step. This resulted in single filaments and small needle-shaped bundles of one to four filaments arranged in parallel being the dominant morphologies. During the transition from lag to exponential phase, morphologies shifted to a mix of small needles and medium fusiform bundles, with the occasional appearance of a radially arranged puff-type colony. During late exponential growth, larger fusiform bundles and puff-type aggregates consisting of randomly, rather than radially, oriented trichomes appeared. Our results showed the highest rates of NA per chlorophyll *a* occurring during mid-exponential growth when small and medium size fusiform aggregates (bundles) were dominant. The larger puff-type aggregates did not appear until NA rates had dropped to negligible levels, in agreement with observations of Ohki and Fujita for cultured *Trichodesmium* spp. (17). Because of the low rates of NA associated with aggregate formation in this culture, NA responses to morphological changes and various degrees of aggregation were not rigorously examined. Preliminary observations indicated no significant impact of bundle disaggregation on the low rates of NA observed in a 1-month-old batch culture.

This result appears contrary to work on natural populations which indicated higher rates of NA associated with larger puff- and tuft-shaped aggregates relative to smaller ones (20) or in aggregates relative to single trichomes (5). It should be noted that the late-stage puff-type aggregates seen in this culture were only loosely held together and did not appear to have the cohesive, tightly compacted inner cores often observed in natural puff aggregates (4, 22). Figures 1C (cultivated puff-type aggregate) and 7 (natural puff-type aggregates) illustrate these differences. Generally, filaments in these late-stage cultured puffs appeared randomly arranged, whereas naturally occurring puffs are often, in

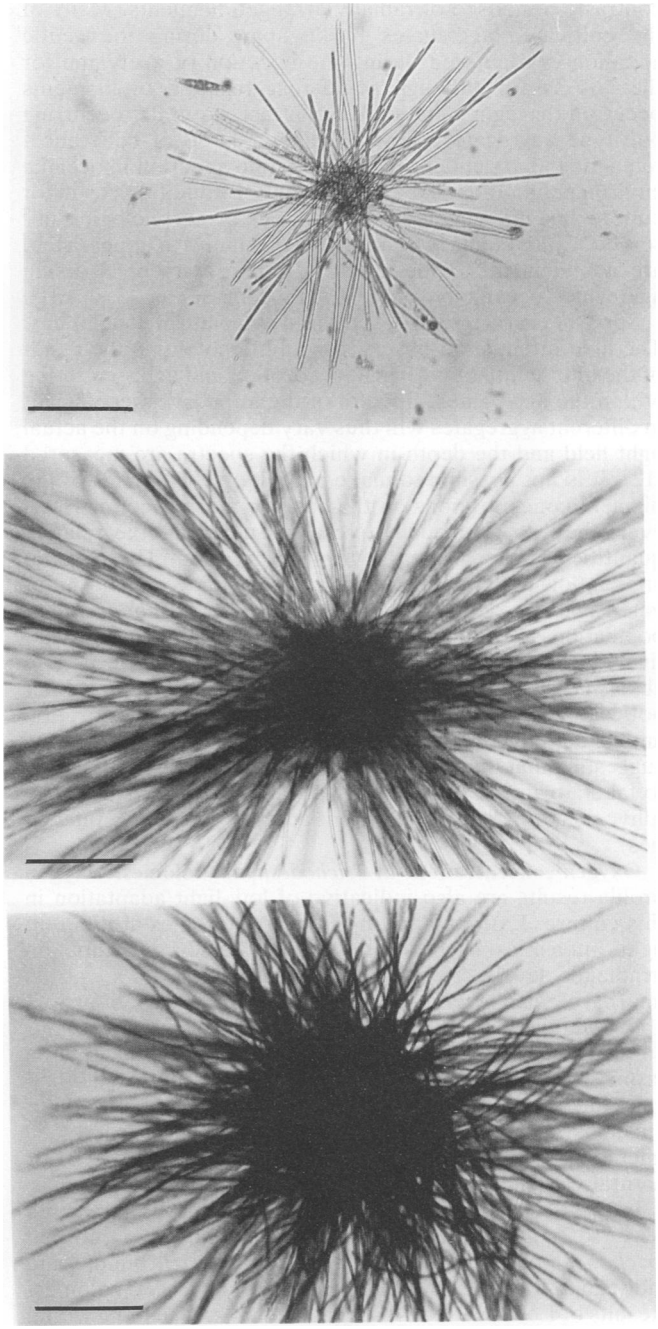

FIG. 7. Naturally occurring *T. thiebautii* puff-type aggregates of various sizes from North Carolina coastal Atlantic waters. Note the radial arrangement and the tight association of filaments in the center of the puff-type aggregates. Bar, 100 μm.

contrast, composed of radially arranged filaments. Many of the cultivated aggregates broke apart during the gentle agitation encountered during the injection of acetylene for the NA assay, although some reaggregation of filaments occurred during incubation. In contrast, naturally occurring puff-type aggregates show a higher degree of coherence, remaining intact under mild to moderate agitation in oceanic environments. It appears that the growth conditions used for culture had an effect on the aggregate morphologies observed. Cultivated aggregates, though similar in appearance, are not identical to the majority of the actively N_2-fixing aggregated oceanic types previously documented (3, 20, 21).

Spectral characteristics of cultured and natural populations. The light attenuation observed within a colony is the result of the interaction of both light absorption and light scattering within the aggregates. Spectra of the attenuation coefficient in different aggregates will thus vary depending on the actual light field and the depth in which the spectra are measured (12). It is not possible to make quantitative estimates of the pigment concentrations on the basis of these spectra. However, they do give qualitative assessments of the relative quantities of the pigments within aggregates. The spectral attenuation patterns seen inside an oceanic versus a cultivated aggregate (Fig. 4) indicate that oceanic *Trichodesmium* populations are high-light adapted by virtue of their relatively high absorbance in the 450- to 500-nm carotenoid absorption region. Cultivated *Trichodesmium* spp. exhibit noticeably lower carotenoid and phycourobilin absorption and higher phycoerythrin absorption than the oceanic forms, suggesting that they have adapted their pigment composition to take advantage of the low light regimen in the laboratory (phycoerythrin is highly efficient at lower irradiance intensities).

Oxygen concentrations in *Trichodesmium* colonies. Oxygen profile results are also indicative of low-light adaptation in this culture. Extrapolation from oxygen profiles at different light intensities suggest that at our standard incubation irradiance levels (55 to 65 microeinsteins m^{-2} s^{-1}), the internal oxygen level seen in this aggregate is only about 30% above ambient. For smaller, more abundant bundles in our culture where diffusional exchange would be expected to be faster, O_2 supersaturation probably rarely occurs. The oxygen compensation appears to occur around 16 to 20 microeinsteins m^{-2} s^{-1}. By contrast, a much higher value (280 microeinsteins m^{-2} s^{-1}) has recently been calculated for natural Caribbean *Trichodesmium* populations (11).

Interaction of light availability, colonial morphology, and NA. Adaptation to low light may explain why cultured *Trichodesmium* spp. show higher NA in small bundle and individual trichome morphologies than in larger colonies. Results of Lewis et al. (14) suggested that self-shading within intact colonies will reduce the specific absorption per unit of pigment. Since NA is light mediated, under low light (in this case ca. 60 microeinsteins m^{-2} s^{-1}), single trichomes and small bundles could be better able to collect sufficient light (energy) to support N_2 fixation than would shaded trichomes in larger bundles, where trichomes could actually become light limited. Under low-light conditions where both C and N_2 fixation pathways are competing for light energy, formation of aggregates would be highly disadvantageous. Perhaps the bundling behavior observed during late exponential growth of this culture occurs in part as a result of a metabolic shift to N recycling rather than light-mediated N_2 fixation to meet N requirements. If, as it appears, cells are leaking nitrogenous compounds, it may be advantageous for filaments to clump together in an attempt to recapture as much of these dissolved N forms as possible or, alternatively, to facilitate heterotrophic bacterial remineralization of other needed nutrients (i.e., Fe or CO_2), as long as the clumping does not prevent adequate light absorption to support carbon fixation. In the high irradiance (500- to 2,000-microeinstein m^{-2} s^{-1} range) levels occurring in upper oceanic water, clumping behavior is more likely to be a protective shading mechanism. The spectral absorption patterns observed in both our cultivated and natural populations appear similar to those reported by others for various *Trichodesmium* populations. This new technique offers advantages in that spectra can be determined without extraction procedures, filtration, or colony integrity disruption. Because of the size of the probes, however, use of this technique is currently limited to larger aggregates.

We are currently attempting to expose cultivated filaments to higher light levels in an attempt to induce altered aggregate morphologies and pigment compositions, similar to naturally occurring aggregates. In future work, cultures will be established in chemostats in which the natural nutrient cycling and turbulence can be better replicated. New offshore populations should also be brought into culture and maintained from the onset under PAR fluxes similar to those experienced in nature. The relatively simple methods we have described here for purifying and maintaining *T. thiebautii* in culture should be helpful in reaching these and other goals so that some of the remaining mysteries of the *Trichodesmium* species may be solved.

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REFERENCES

1. **Andersen, R. A., D. M. Jacobson, and J. P. Sexton.** 1991. Provasoli-Guillard Center for Culture of Marine Phytoplankton: catalog of strains. Provasoli-Guillard Center for Culture of Marine Phytoplankton, West Boothbay Harbor, Maine.
2. **Borstad, G. A., J. F. R. Gower, and E. J. Carpenter.** 1992. Development of algorithms for remote sensing of *Trichodesmium* blooms, p. 193–210. *In* E. J. Carpenter, D. G. Capone, and J. G. Rueter (ed.), Marine pelagic cyanobacteria: *Trichodesmium* and other diazotrophs. Kluwer Academic Press, Dordrecht, The Netherlands.
3. **Capone, D. G., J. M. O'Neil, J. Zehr, and E. J. Carpenter.** 1990. Basis for diel variation in nitrogenase activity in the marine planktonic cyanobacterium *Trichodesmium thiebautii*. Appl. Environ. Microbiol. **56**:3532–3536.
4. **Carpenter, E. J.** 1983. Physiology and ecology of marine planktonic *Oscillatoria* (*Trichodesmium*). Mar. Biol. Lett. **4**:69–85.
5. **Carpenter, E. J., and C. C. Price.** 1976. Marine *Oscillatoria* (*Trichodesmium*): explanation for aerobic nitrogen fixation without heterocysts. Science **191**:1278–1280.
6. **Dugdale, R. C., D. W. Menzel, and J. H. Ryther.** 1961. Nitrogen fixation in the Sargasso Sea. Deep-Sea Res. **7**:298–300.
7. **Fay, P.** 1992. Oxygen relations of nitrogen fixation in cyanobacteria. Microbiol. Rev. **56**:340–373.
8. **Guillard, R. R. L.** 1975. Culture of phytoplankton for feeding

marine invertebrates, p. 26–60. *In* W. L. Smith and M. H. Chanley (ed.), Culture of marine invertebrate animals. Plenum Press, New York.

9. **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.
10. **Jones, M. N.** 1984. Nitrate reduction by shaking with cadmium, alternative to cadmium columns. Water Res. **5:**643–646.
11. **Kana, T. M.** 1992. Oxygen cycling in cyanobacteria with special reference to oxygen protection in *Trichodesmium* spp., p. 29–41. *In* E. J. Carpenter, D. G. Capone, and J. G. Rueter (ed.), Marine pelagic cyanobacteria: *Trichodesmium* and other diazotrophs. Kluwer Academic Publishers, Dordrecht, The Netherlands.
12. **Lassen, C., H. Ploug, and B. B. Jørgensen.** 1992. Microalgal photosynthesis and spectral scalar irradiance in coastal marine sediments of Limfjorden, Denmark. Limnol. Oceanogr. **37:**760–772.
13. **Lassen, C., H. Ploug, and B. B. Jørgensen.** 1992. A fibre-optic scalar irradiance microsensor: application for spectral light measurements in sediments. FEMS Microbiol. Ecol. **86:**247–254.
14. **Lewis, M. R., O. Ulloa, and T. Platt.** 1988. Photosynthetic action, absorbtion, and quantum yield spectra for a natural population of *Oscillatoria* in the North Atlantic. Limnol. Oceanogr. **33:**92–98.
15. **McLachlan, J.** 1973. Growth media—marine, p. 25–52. *In* J. R. Stein (ed.), Handbook of phycological methods: culture methods and growth measurements. Cambridge University Press, Cambridge.
16. **O'Flaherty, L. M., and H. K. Phinney.** 1970. Requirements for the maintenance and growth of *Aphanizomenon flos-aquae* in culture. J. Phycol. **6:**95–97.
17. **Ohki, K., and Y. Fujita.** 1988. Aerobic nitrogenase activity measured as acetylene reduction in the marine non-heterocystous cyanobacterium *Trichodesmium* spp. grown under artificial conditions. Mar. Biol. **98:**111–114.
18. **Ohki, K., J. G. Rueter, and Y. Fujita.** 1986. Cultures of the pelagic cyanophytes *Trichodesmium erythraeum* and *T. thiebautii* in synthetic medium. Mar. Biol. **91:**9–13.
19. **Ohki, K., J. P. Zehr, P. G. Falkowski, and Y. Fujita.** 1991. Regulation of nitrogen-fixation by different nitrogen sources in the marine non-heterocystous cyanobacterium *Trichodesmium* sp. NIBB1067. Arch. Microbiol. **156:**335–337.
20. **Paerl, H. W., and B. M. Bebout.** 1988. Direct measurement of O_2-depleted microzones in marine *Oscillatoria*: relation to N_2 fixation. Science **241:**442–445.
21. **Paerl, H. W., and B. M. Bebout.** 1992. Oxygen dynamics in *Trichodesmium* spp. aggregates, p. 43–60. *In* E. J. Carpenter, D. G. Capone, and J. G. Rueter (ed.), Marine pelagic cyanobacteria: *Trichodesmium* and other diazotrophs. Kluwer Academic Press, Dordrecht, The Netherlands.
22. **Paerl, H. W., B. M. Bebout, and L. E. Prufert.** 1989. Bacterial associations with marine *Oscillatoria* sp. (*Trichodesmium* sp.) populations: ecophysiological implications. J. Phycol. **25:**773–784.
23. **Parsons, T. R., Y. Maita, and C. M. Lalli.** 1984. A manual of chemical and biological methods for seawater analysis. Pergamon Press, New York.
24. **Ramamurthy, V. D.** 1972. Procedures adopted for the laboratory cultivation of *Trichodesmium erythraeum*. Mar. Biol. **14:**232–234.
25. **Revsbech, N. P.** 1989. An oxygen microelectrode with a guard cathode. Limnol. Oceanogr. **34:**474–478.
26. **Rueter, J. G.** 1988. Iron stimulation of photosynthesis and nitrogen fixation in *Anabaena* 7120 and *Trichodesmium* (Cyanophyceae). J. Phycol. **24:**249–254.
27. **Saino, T., and A. Hattori.** 1978. Diel variation in nitrogen fixation by a marine blue-green alga, *Trichodesmium thiebautii*. Deep-Sea Res. **25:**1259–1263.
28. **Shimura, S., and Y. Fujita.** 1975. Phycoerythrin and photosynthesis of the pelagic blue-green alga *Trichodesmium thiebautii* in the waters of Kuroshio, Japan. Mar. Biol. **31:**121–128.
29. **Siddiqui, P. J. A., E. J. Carpenter, and B. Bergman.** 1992. *Trichodesmium*: ultrastructure and protein localization, p. 9–28. *In* E. J. Carpenter, D. G. Capone, and J. G. Rueter (ed.), Marine pelagic cyanobacteria: *Trichodesmium* and other diazotrophs. Kluwer Academic Publishers, Dordrecht, The Netherlands.
30. **Taylor, B. F., C. C. Lee, and J. S. Bunt.** 1973. Nitrogen-fixation associated with the marine blue-green alga, *Trichodesmium*, as measured by the acetylene-reduction technique. Arch. Mikrobiol. **88:**205–212.