APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1993, p. 1495–1503 Vol. 59, No. 5
0099-2240/93/051495-09$02.00/0

Identification of the Sources of Energy for Nitrogen Fixation and Physiological Characterization of Nitrogen-Fixing Members of a Marine Microbial Mat Community

BRAD M. BEBOUT,†* MATTHEW W. FITZPATRICK, AND HANS W. PAERL

Institute of Marine Sciences, University of North Carolina at Chapel Hill, 3431 Arendell Street, Morehead City, North Carolina 28557

Received 30 November 1992/Accepted 1 March 1993

Experimental manipulations of a microbial mat community were performed to determine sources of energy and reductant used for nitrogen fixation and to physiologically characterize the responsible diazotrophs. The dominant photolithotrophic members of this community were nonheterocystous cyanobacteria, but other potential nitrogen-fixing microorganisms were also present. Pronounced diel variability in rates of acetylene reduction was observed, with nighttime rates a factor of three to four higher than daytime rates. Acetylene reduction measured at night was dependent upon the occurrence of oxygenic photosynthesis the preceding day; mats incubated in the dark during the daytime reduced acetylene at rates comparable to those of light-incubated mats but were not able to reduce acetylene at the normally high rates the following night. The addition of various exogenous carbon compounds to these dark-incubated mats did not elicit nighttime acetylene reduction. Nighttime acetylene reduction apparently proceeds under anoxic conditions in these mats; the highest rates of acetylene reduction occur late at night. Additions of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (an inhibitor of oxygenic photosynthesis) to mats resulted in a pronounced stimulation of acetylene reduction during the day, but acetylene reduction the next night proceeded at greatly reduced rates (relative to untreated mats). This daytime stimulation, under the 3-(3,4-dichlorophenyl)-1,1-dimethylurea-induced anoxic conditions in the experimentally treated mats, was light dependent. These results suggest that nitrogen fixation in these mats may be attributed to the activities of nonheterocystous cyanobacteria utilizing storage products of oxygenic photosynthesis under anoxic conditions at night.

Biological nitrogen fixation (diazotrophy) is a characteristic feature of many benthic photosynthetic communities (6, 31, 33, 40, 43, 51). Extremely high rates of oxygenic photosynthesis are also common in these communities and may result in high oxygen concentrations in the photic zone during daytime (3, 30, 34, 35). Nitrogen fixation is considered to be an anaerobic process (37), and the nitrogen-fixing enzyme (nitrogenase) from cyanobacteria is irreversibly damaged by oxygen in cell extracts (13). Nitrogen-fixing microorganisms in benthic photosynthetic communities therefore necessarily employ a number of mechanisms by which the oxygen-sensitive enzyme is protected. These mechanisms include sequestering the nitrogenase into heterocysts (specialized cells which do not evolve oxygen and are relatively impermeable to it [52]), respiratory protection of the nitrogenase (localized consumption of oxygen [15]), a temporal separation of photosynthesis and nitrogen fixation (23, 50), and protective conformational changes of the enzyme (45; for a more detailed review of these mechanisms, see references 12 and 17).

While nitrogen fixation in benthic photosynthetic communities is generally attributed to the cyanobacteria in the surface layers of the community (31, 40, 43, 49), this is difficult to demonstrate conclusively. The circumstantial evidence for the importance of cyanobacteria in microbial mat nitrogen fixation is, briefly, as follows. (i) In a number of systems (particularly in benthic microbial communities dominated by heterocystous cyanobacteria [19, 33, 51] but also in some systems dominated by nonheterocystous cyanobacteria [19, 31, 40]), nitrogen fixation has been demonstrated to be light dependent. (ii) In salt marsh environments, the highest rates of nitrogen fixation occur in areas of the sediments colonized by cyanobacteria (4, 18). (iii) Profiles of rates of nitrogen fixation often show maximum rates in the cyanobacterial layers (41). Finally, (iv) immunochemical screening of microbial mats has revealed the presence of nitrogenase in some of the most common mat-building cyanobacteria, e.g., a *Lyngbya* sp. (29) and *Oscillatoria limosa* (42). Associated with the photosynthetic members of the mat community, however, are diverse heterotrophic, chemolithotrophic, and chemoorganotrophic bacterial genera, many of which are known to fix nitrogen (8, 25, 53). Nitrogen fixation in microbial mats is usually temporally separated from oxygenic photosynthesis, with the highest rates of nitrogen fixation occurring at night (1, 3, 9, 43). This has led some (3, 26) to speculate that nitrogen fixation in these systems may be attributable not only to the cyanobacteria but also to the associated bacterial populations.

While a temporal separation of oxygenic photosynthesis and nitrogen fixation appears to be a general feature of microbial mats dominated by nonheterocystous cyanobacteria, at least two different patterns of acetylene reduction have been described, and these patterns have been related to oxygen concentrations within the mats. Stal et al. (43) and Villbrandt et al. (49) have reported patterns of nitrogen fixation in established mats characterized by two peaks of activity, one at sunrise and one at sunset. Nitrogen fixation rates were low both at midday (because of oxygen inhibition) and during the middle of the night (because of the energy limitations of anaerobic metabolism in the anoxic mats at

* Corresponding author.
† Present address: Max-Planck-Institut für Marine Mikrobiologie, Fahrenheitstrasse 1, W-2800 Bremen 33, Germany.

night, in the opinion of these authors). Low light intensities during the transitions between light and dark periods were hypothesized to have provided energy for nitrogen fixation during periods of a relatively low oxygen concentration. In sediments newly colonized by cyanobacteria studied by these same authors, high rates of acetylene reduction were sustained throughout the night and attributed to the availability of oxygen in these sediments throughout the dark period (48). In the well-developed microbial mat studied by Bebout et al. (3) and in mats located in Baja California (2), however, oxygen was not available at night, and yet high rates of acetylene reduction were sustained. This observation led to speculation that the high rates of nitrogen fixation at night were not exclusively attributable to the activity of cyanobacteria but possibly also to facultatively anaerobic heterotrophic bacteria associated with the cyanobacteria (3, 26). Therefore, three light and oxygen concentration scenarios conducive to nitrogen fixation have been identified in the literature (in different microbial mats): (i) anoxic and dark conditions, (ii) oxic and dark conditions, and (iii) low-light and low-oxygen conditions.

The present work is an attempt to identify the sources of energy for nitrogen fixation at night under anoxic conditions in mats similar to those studied by Bebout et al. (3) and to physiologically characterize the nitrogen-fixing members of this marine microbial mat community. Experimental manipulations of the energy- and reductant-generating processes in intact microbial mat communities were performed in an attempt to determine their effects on the normally high rates of nitrogen fixation at night. Evidence is presented that anaerobic metabolism by mat-building nonheterocystous cyanobacteria plays an important role in the use of photosynthetically generated storage compounds for nitrogen fixation at night. Photosystem I (PS I) activity may be an important source of energy for nitrogen fixation at certain times of the day.

MATERIALS AND METHODS

Field site. Microbial mats were located on Bird Shoal, a dredge spoil island located in coastal North Carolina. The mats are intertidal; exposure at low tide generally lasts 1 to 2 h per 12-h tidal cycle. They grow on coarse sand (average diameter of sand grains, 200 μm). Although community composition is quite variable seasonally, the dominant cyanobacterial members usually include *Microcoleus chthonoplastes*, *Lyngbya* spp., and *Oscillatoria* spp. Diatoms (*Amphora* sp. and *Navicula* sp.) are generally present near the mat surface. A distinct layer of purple photosynthetic bacteria is infrequently present. Dark sandy sediments which smell strongly of sulfide underlie the mats.

Light measurement. Photosynthetically active radiation (PAR) was measured in all experiments with a Li-Cor LI-1000 data logger coupled to a Li-Cor quantum irradiance sensor placed ~20 cm from the microbial mat. PAR measurements were averaged and recorded at 10-min intervals.

Experimental manipulations. Results from several diel experiments conducted under similar conditions are presented here. On the basis of prior observations of the heterogeneity of these mat systems (1, 3), it was necessary to replicate not only the experimental treatments within a diel experiment ($n = 3$) but the diel experiments as well (generally, $n = 3$ to $n = 5$) to have confidence in the patterns reported. A single diel experiment consisted of measurements made over the course of a 24-h period (0600 to 0600 h). Eight incubation periods were used for measurements of rates of acetylene reduction and uptake of added $NaH^{14}CO_3$ over the course of each diel period. The incubation intervals were 0600 to 0800, 0800 to 1000, 1000 to 1200, 1200 to 1400, 1400 to 1700, 1700 to 2030, 2030 to 0000, and 0000 to 0600 h. Previous experimentation had revealed that the rates of acetylene reduction were linear for at least 6 h.

Microbial mats were collected and transported to the Institute of Marine Sciences (~7 km) on the day prior to an experiment by using acrylic coring devices (14-cm diameter by 10-cm height). The mats were then cut into sections (6 by 6 by 2 cm deep) with a scalpel and placed into shallow (~3-cm-high) polyethylene pans. Pans containing mats were submerged in a running seawater bath under natural illumination until sundown. The pans were then positioned in the bath on concrete blocks so that the running seawater came to within 1 cm of the tops of the containers. The pans containing the mats were therefore isolated at this point from the running seawater, but effective temperature control of the experimental pans was achieved because of the continued flow of seawater in the bath. At this time, the water in the pans was exchanged with 100 ml of fresh seawater. Pans containing mats to be incubated under dark conditions were also covered with loose-fitting aluminum foil covers at this time. Three replicate pans were used for each experimental treatment. The pans were incubated independently from each other (no mixing between pans) in the water bath, and each pan was subsampled for the determination of acetylene reduction or $^{14}CO_2$ uptake for each experimental incubation.

We note that the protocol used for these diel experiments is different from the format of previously published experiments. "Natural-incubated mat" refers to a mat which was subjected to the normal diel pattern of light and dark conditions. "Dark-incubated mat" refers to a mat that was not exposed to PAR (sunlight) during an entire daylight portion of a diel cycle, not just the period of time used for the acetylene reduction incubation. The experiments described here were designed to test the effects of manipulations of the energy- and reductant-generating processes which occurred on a diel time scale, not the shorter-term effects which occur over the period of time used to make an acetylene reduction rate measurement (2 to 6 h).

Inhibitors. Inhibitors of oxygenic photosynthesis [3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU); final concentration, 2×10^{-5} M] and/or sulfate reduction (sodium molybdate; final concentration, 20 mM) were added to the pans containing mats and 100 ml of seawater at sunset. Acetylene reduction and $^{14}CO_2$ uptake incubations began at 0600 h the next day. As mentioned above, these experimental conditions differ from our previously reported results in that the effect of the inhibitor over an entire diel period, not an experimental incubation, is being tested.

Exogenous carbon additions. The effects of a variety of exogenous carbon sources on rates of acetylene reduction were tested by adding these compounds to the pans containing mats at a final concentration of 10 mM. The compounds tested included D-glucose, acetate (as sodium acetate), ethanol, and glycolate (as glycolic acid [pH 8.1]). These carbon sources were added at the beginning of the light period to simulate their hypothesized release from photosynthetically active cyanobacteria. Mats were therefore subjected to the effects of the exogenous sources of carbon over the daytime and nighttime portions of one diel cycle.

Acetylene reduction techniques. Nitrogen fixation was assayed by the acetylene reduction technique (47). Small (1.15-cm^2) cores of microbial mat communities were obtained by using a cut-off 5-ml syringe. These cores were cut

off at a depth of 1 cm below the mat-water interface and placed into serum bottles (total volume, 38 ml) containing 20 ml of freshly collected seawater. The serum bottles were then capped with gas-tight rubber stoppers (Wheaton sleeve stoppers). By using a 5-ml syringe, 2 ml of the 18-ml headspace was removed from the sealed serum bottle, and 5 ml of acetylene (generated by adding calcium carbide to water) was then injected through the stopper into the aqueous phase to start the incubation. The bottles were incubated in the running seawater bath for temperature control and exposed to natural illumination (or covered with aluminum foil in the case of the dark-incubated mats). Incubations were terminated by shaking serum bottles by hand for 10 s. The headspace of the serum bottles was then sampled by using a 10-ml syringe and injected into an evacuated serum bottle (total volume, 9.1 ml) to be analyzed by gas chromatography.

Acetylene and ethylene were quantified in a Shimadzu GC-9A gas chromatograph with a flame ionization detector. A 2-m stainless-steel Poropak T filled column held at 80°C was used to separate the gases. High-purity nitrogen was the carrier gas. Rates of ethylene production were calculated on an areal basis.

$^{14}CO_2$ incorporation. Incorporation of radioactively labeled CO_2 (added as $NaH^{14}CO_3$) was used as an index of primary productivity. Because of the difficulties associated with measuring the proportions of radioactively labeled bicarbonate and nonlabeled bicarbonate (specific activity) available to, and being taken up by, the community of interest, this technique does not allow direct measurement of photosynthetic rates (34). The rates of incorporation of label were used here to elucidate the relative differences in $^{14}CO_2$ incorporation between experimentally manipulated microbial mats.

A small core (1.15 cm^2) of microbial mat was obtained by using a plastic cut-off 5-ml syringe. This core was cut off at a depth of 0.5 cm by using a scalpel and placed into a borosilicate glass scintillation vial containing 20 ml of seawater. A 0.2-ml aliquot of $NaH^{14}CO_3^-$ (specific activity, 68 $\mu Ci \cdot mol^{-1}$) was then added to the vial. A polypropylene-lined cap was used to seal the vials, which were then placed on their sides for the duration of the incubation period. Incubation periods generally were either 2 or 3 h. At the termination of the incubation, mats were processed and counted as previously described (3, 26).

Diel measurements of acetylene reduction in a cyanobacterial culture. Measurements of diel patterns of acetylene reduction and addition of metabolic inhibitors were carried out in essentially the same fashion described for microbial mats in a culture of a *Lyngbya* sp., a cyanobacterium isolated from the mat. This culture was not axenic but exhibited extremely low numbers of bacteria (no bacteria could be detected through microscopic examination during the logarithmic phase of growth). Cultures on a 12-h-light–12-h-dark light cycle were subdivided into 100-mm petri dishes containing medium (ASN III [36]) on the night before the experiment was to begin. Three separate petri dishes were used for each treatment. At each time point, forceps were used to separate about 1 g (wet weight) of *Lyngbya* culture from the treatment containers for acetylene reduction and $^{14}CO_2$ uptake incubations.

Statistical analyses. Rates of acetylene reduction in various experimental treatments were compared by using a one-way analysis of variance. A Student-Newman-Keuls multiple-comparison test was used to discern treatments significantly different from each other at the alpha equals 0.05 level. All statistical analyses were performed by using the SAS software package on an IBM-compatible personal computer.

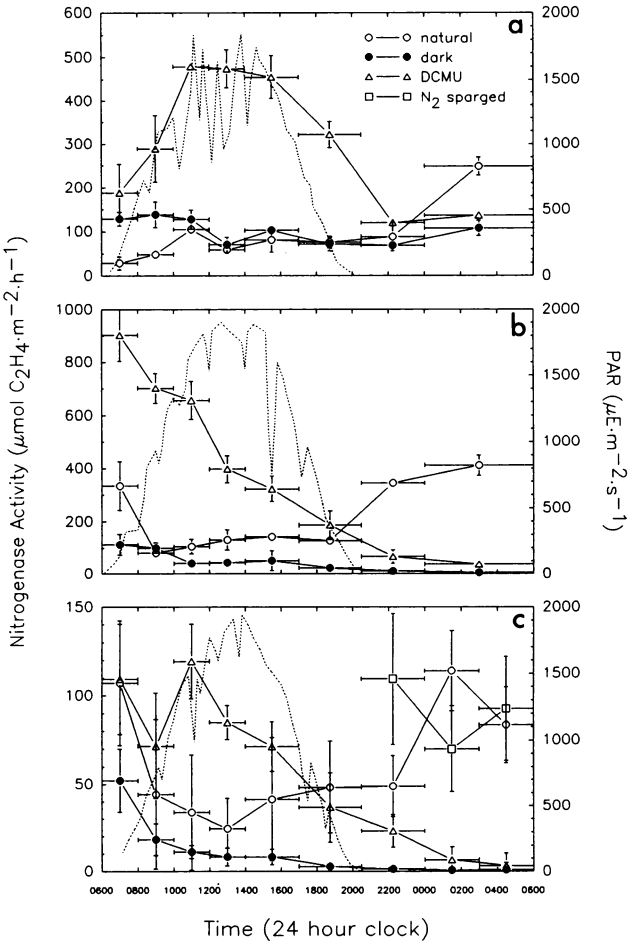

FIG. 1. Diel patterns of acetylene reduction (nitrogenase activity) in the Bird Shoal marine microbial mat community. The pattern of acetylene reduction under natural illumination was altered by incubating mats under conditions of darkness and by adding the PS II inhibitor DCMU at a concentration of 2×10^{-5} M. Three separate diel periods are shown, i.e., 13 May 1992 (a), 1 July 1992 (b), and 29 July 1992 (c). Panel c illustrates the effect of N_2-sparging the incubation flask at night. Horizontal bars span the time intervals used for acetylene reduction incubations, and vertical error bars indicate the standard deviations of three replicates. The dotted line indicates the pattern of PAR. μE, microeinsteins.

RESULTS

Acetylene reduction under natural and dark-incubated conditions. Strong diel variability in acetylene reduction was observed in mats exposed to a normal diel irradiance curve (illuminated during the day, dark at night). A nighttime maximum in acetylene reduction was observed, with rates approximately three to four times higher than daytime rates (Fig. 1). Experimental manipulations of the normal diel pattern of irradiance caused the mats to deviate from this pattern of activity. Rates of acetylene reduction of natural- and dark-incubated mats were generally not significantly different from each other during daytime. During some daylight incubations, however, there were significant differ-

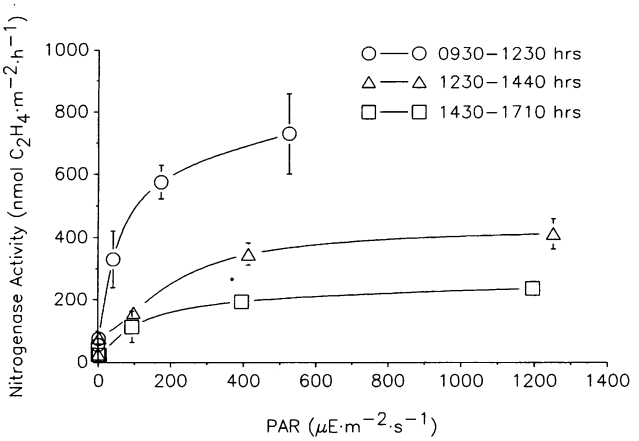

FIG. 2. Effect of irradiance (PAR) on DCMU-stimulated acetylene reduction. Neutral density screening was used to attenuate natural illumination to the levels shown. Microbial mats were exposed to DCMU for the entire daylight portion of the diel cycle, and three separate incubations were conducted on that day: 0930 to 1230 h, 1230 to 1440 h, and 1430 to 1710 h. The vertical error bars indicate the standard deviations of three replicate incubations. μE, microeinsteins.

ences, and in these cases the light-incubated (natural) rates of acetylene reduction were invariably higher than those of the dark-incubated mats (Fig. 1). There was always a significant difference in the rates of acetylene reduction between natural-incubated and dark-incubated mats the next night. Microbial mats kept in the dark for an entire photoperiod had very low rates of acetylene reduction the next night relative to mats which had been incubated in the light (Fig. 1).

Effects of anoxic conditions on rates of acetylene reduction. Acetylene reduction at night was occurring under anoxic conditions. Oxygen microelectrode profiles in dark-incubated mats revealed them to be completely anoxic at the surface (data not shown), and there was no stirring in the incubation vessels to bring in oxygen from the overlying water. Mats incubated in N_2-sparged water did not exhibit rates of nitrogen fixation significantly different from mats incubated in aerated seawater, with the exception of one datum point in one experiment (2030 to 0000 h, the first incubation after the transition of the mat from light to dark conditions; Fig. 1c).

Effects of an inhibitor of oxygenic photosynthesis (DCMU) on acetylene reduction. Treatment of mats with the inhibitor DCMU resulted in greatly increased rates of acetylene reduction in light-incubated mats during the day. Mats treated with DCMU did not, however, exhibit the high rates of acetylene reduction the following night that are characteristic of untreated mats. DCMU-stimulated acetylene reduction during the day was light dependent, loosely following the pattern of the irradiance curves in two of the three experiments depicted in Fig. 1. A separate experiment confirmed the relationship between DCMU-stimulated acetylene reduction and irradiance (Fig. 2). A linear relationship between these two variables existed at low irradiances, but acetylene reduction saturated at about 400 to 500 microeinsteins · m^{-2} · s^{-1}. This relationship held throughout the day, but the overall rates declined dramatically from the morning to the afternoon (Fig. 2).

Effects of an inhibitor of sulfate reduction on DCMU-stimulated acetylene reduction. Mats treated with both

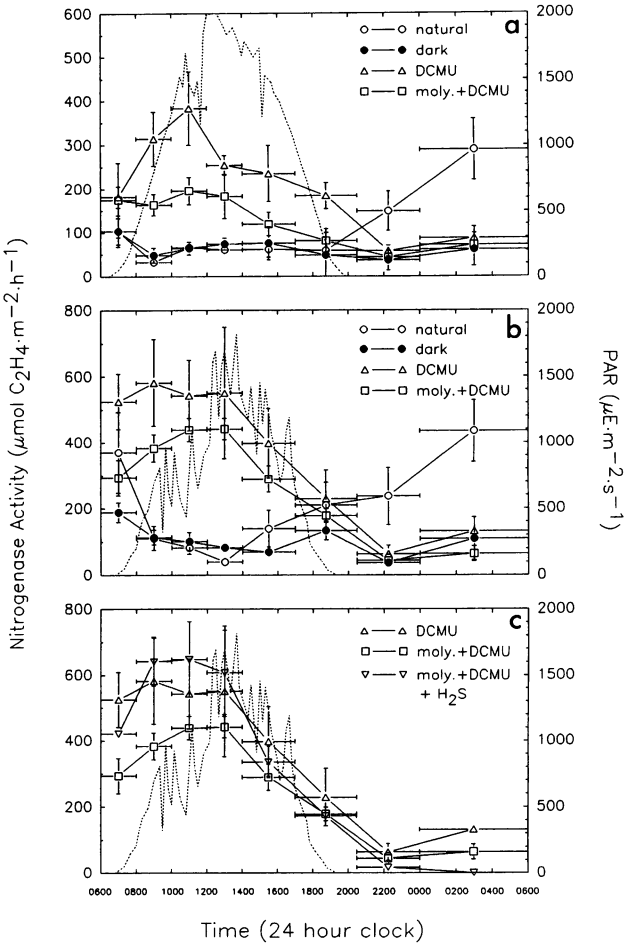

FIG. 3. Effect of sodium molybdate (an inhibitor of sulfate reduction) on rates of DCMU-stimulated acetylene reduction. Treatment of mats with sodium molybdate at a concentration of 20 mM and DCMU at 2×10^{-5} M resulted in lower rates of acetylene reduction than treatment of mats with DCMU alone (a and b). Adding H_2S to the DCMU- and sodium molybdate-treated mats apparently counteracted the molybdate suppression (c). Two separate diel experiments are shown, i.e., 25 August 1992 (a) and 15 September 1992 (b and c). Dotted lines indicate the pattern of PAR. Horizontal bars span the time intervals used for acetylene reduction incubations, and vertical error bars indicate the standard deviations of three replicate determinations of acetylene reduction. Panels b and c contain data from the same diel period, separated for clarity. μE, microeinsteins.

DCMU and sodium molybdate (an inhibitor of sulfate reduction) exhibited lower rates of acetylene reduction than mats treated with DCMU alone (and higher rates of acetylene reduction than untreated mats) during the daytime (Fig. 3). The degree to which DCMU-stimulated acetylene reduction was inhibited by the sodium molybdate was variable. In one diel experiment, we obtained an approximately 50% reduction in DCMU-stimulated acetylene reduction; in others, the effect was less pronounced (Fig. 3). Additions of sodium sulfide to the mats resulted in a recovery of molybdate-inhibited rates of DCMU-stimulated acetylene reduction (Fig. 3).

Effects of exogenous carbon compounds on acetylene reduction. The addition of low-molecular-weight carbon com-

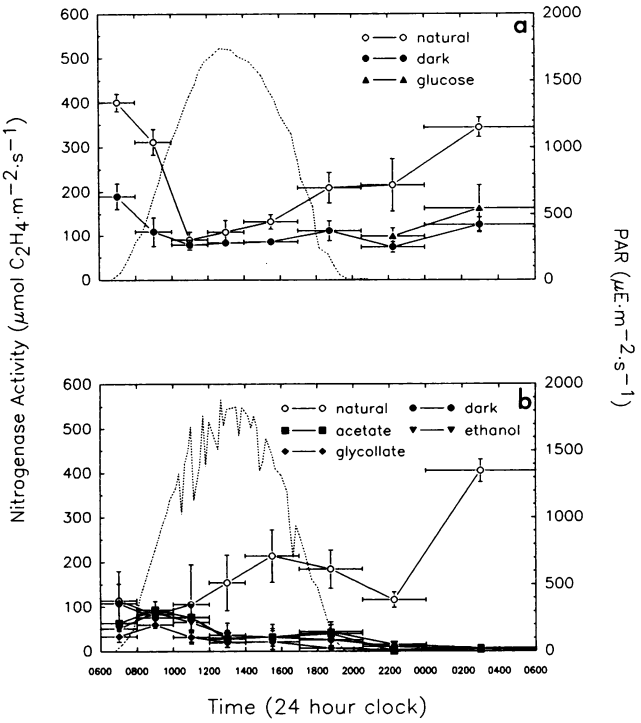

FIG. 4. Effect of nighttime additions of exogenous carbon on rates of acetylene reduction in light-deprived mats. Mats incubated under conditions of darkness during the day were treated with various exogenous sources of carbon (glucose, acetate, glycolate, and ethanol; all at 10 mM) the next night. Patterns of acetylene reduction in mats incubated in the dark with no carbon additions and under natural illumination conditions are also depicted. Two separate diel periods are shown, i.e., 1 September 1992 (a) and 9 September 1992 (b). Dotted lines indicate the pattern of PAR. Horizontal bars span the time intervals used for acetylene reduction incubations, and vertical error bars indicate the standard deviations of three replicate determinations of acetylene reduction. μE, microeinsteins.

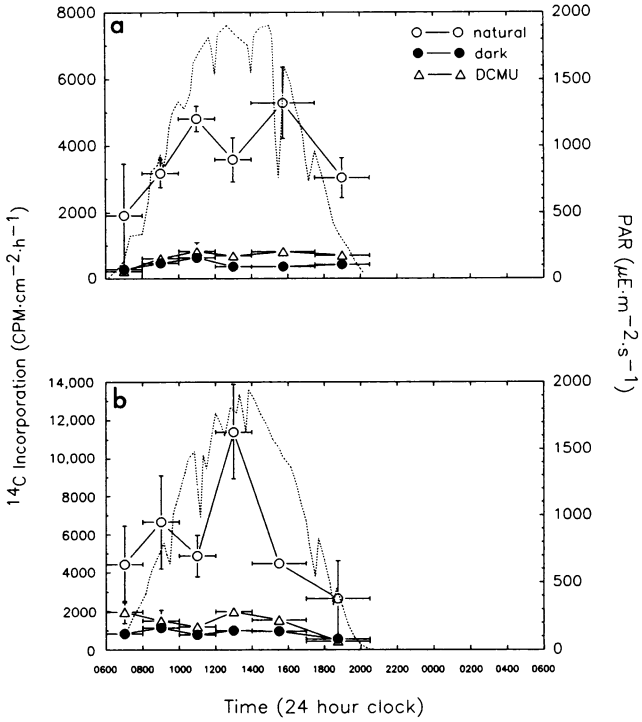

FIG. 5. Diel patterns of uptake of $^{14}CO_2$ by the Bird Shoal microbial mat community. Mats were incubated under conditions of natural irradiance, darkness, and under natural irradiance with the PS II inhibitor DCMU. Two separate diel periods are shown, i.e., 1 July 1992 (a) and 29 July 1992 (b). Horizontal bars span the time intervals used for the $^{14}CO_2$ uptake incubations, and vertical bars indicate the standard deviations of three replicate incubations. The dotted line shows the pattern of PAR to which the mats were exposed. μE, microeinsteins.

pounds (glucose, acetate, ethanol, and glycolate) to the dark-incubated mats did not result in rates of acetylene reduction significantly higher than the rates obtained in untreated dark-incubated mats (Fig. 4).

$^{14}CO_2$ incorporation. Incorporation of $^{14}CO_2$ by the light-incubated mat community followed the pattern of incident irradiation (Fig. 5). Mats incubated in the dark did not incorporate significantly more $^{14}CO_2$ than killed controls (data not shown). Mats treated with DCMU, and incubated in the light, exhibited greatly reduced rates of $^{14}CO_2$ incorporation relative to untreated light-incubated mats. However, DCMU-treated and light-incubated mats incorporated significantly more $^{14}CO_2$ than dark-incubated mats at some points in the diel cycle (Fig. 5).

Acetylene reduction in a cyanobacterium isolated from the mats. A culture of a nonheterocystous cyanobacterium isolated from the Bird Shoal mat had the same characteristic pattern of nitrogen fixation as the mat from which it was isolated (Fig. 6). Exposure of this culture of *Lyngbya* sp. to light (12-h-light–12-h-dark cycle) was necessary for nitrogen fixation the next dark period. Treatment of the organism with DCMU during the light period eliminated the nighttime nitrogen fixation. Stimulation of daytime light-dependent nitrogen fixation by DCMU, such as we had observed in the mats, was, however, not seen in the culture.

DISCUSSION

Diel patterns of acetylene reduction and occurrence of anaerobic acetylene reduction. The results presented here corroborate our earlier measurements of patterns of nitrogen fixation in North Carolina microbial mats (1, 3) but differ from the patterns reported by Stal et al. (43) and Villbrandt et al. (49) for North Sea island microbial mats. We observed high rates of acetylene reduction in microbial mats at night in the absence of free oxygen. Oxygen microelectrode measurements made on a number of previous occasions in these mats (data not shown) indicate that oxygen is absent from the surface layers, even with vigorous stirring of the overlying aerated water. Attempts to experimentally reduce the oxygen concentrations by nitrogen sparging the seawater in the incubation bottles had no effect on rates of nitrogen fixation during nighttime incubations (Fig. 1c).

Physiological characterization of the nitrogen-fixing microorganism(s) within the mat. High nighttime rates of acetylene reduction were dependent upon the occurrence of oxygenic photosynthesis during the previous light period. Microbial mats kept in the dark during the day, and mats treated with the inhibitor DCMU, did not reduce acetylene at the normally high rates the next night (Fig. 1). From these observations, we conclude that the nitrogen-fixing (acetylene-reducing) microorganisms within this mat utilize the accumulated products of oxygenic photosynthesis for acetylene reduction in the dark under anoxic conditions. The

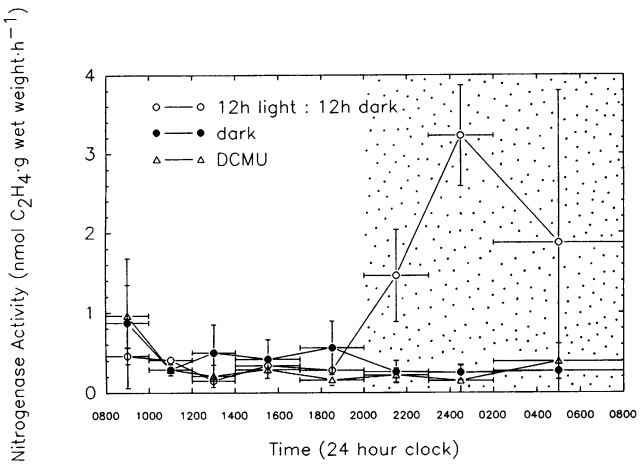

FIG. 6. Diel patterns of acetylene reduction in a culture of *Lyngbya* sp. isolated from the Bird Shoal microbial mat. The culture was on a 12-hour-light–12-h-dark (stippled) light cycle. Rates of acetylene reduction are shown for *Lyngbya* sp. exposed to the 12-h-light–12-h-dark cycle, both with no additions and with additions of the PS II inhibitor DCMU at 2×10^{-5} M, as well as dark-incubated *Lyngbya* sp. Horizontal bars span the time intervals used for acetylene reduction incubations, and vertical bars indicate the standard deviations of three replicate incubations.

coupling between oxygenic photosynthesis and nitrogen fixation is apparently very tight; mats denied the light energy from a single photoperiod were unable to reduce acetylene at the characteristically high rates the next night. The list of organisms potentially responsible for nighttime acetylene reduction can then thus be narrowed to (i) the oxygenic phototrophs (cyanobacteria) or (ii) heterotrophic organisms dependent upon photosynthate (derived from oxygenic phototrophs) from the photoperiod immediately preceding a dark period.

To investigate the possibility that nighttime acetylene reduction could be attributed to heterotrophic activity, we used additions of substrates metabolized by a variety of nitrogen-fixing microorganisms, including some compounds identified as being important in the cyanobacterially mediated stimulation of other groups of microorganisms (14). The requirement for daytime oxygenic photosynthesis in supporting nighttime acetylene reduction could not be supplanted with any of the exogenous carbon sources we tested. Although the list of substrates added is not very extensive, treatment of mats that had been deprived of light throughout the day with a variety of low-molecular-weight carbon compounds (Fig. 4) did not stimulate acetylene reduction above that measured in nontreated mats. A similar experimental result was reported previously (31).

Although the possibility has not been excluded definitively, it seems highly unlikely that a community of bacteria exists which would exclusively utilize cyanobacterial exudate to fix nitrogen at night (presumably storing it from the daytime period) and not possess the capacity to utilize one of the experimental exogenous sources of carbon tested here. Such a hypothetical community of bacteria would be considered to be obligate symbionts of the cyanobacteria and would seem to be maladapted to this microbial mat ecosystem, an ecosystem characterized by rapid (time scale of days to weeks) changes in the dominant cyanobacterial members. A culture of *Lyngbya* sp. isolated from the mat revealed patterns of nitrogen fixation very similar to patterns seen in the mats (Fig. 6), and this culture contained no microscopically visible attached heterotrophic population. We conclude, then, that nonheterocystous cyanobacteria are the most likely source of the acetylene reduction measured at night.

The importance of previous light history in supporting dark nitrogen fixation in cyanobacteria is well known (10, 11, 32). Utilization of stored carbon compounds is the ultimate source of reducing potential for nitrogen fixation, even in cyanobacteria which are able to conduct oxygenic photosynthesis and nitrogen fixation simultaneously (5, 7, 21, 22, 39). Cyanobacteria are capable of sequestering a variety of storage products and extracting energy from these products through a variety of metabolic pathways (36). Glycogen, in particular, has been found to be important in supporting nitrogen fixation in cyanobacteria (10, 16). A few cyanobacteria have been shown to be capable of nitrogen fixation under dark anoxic conditions, and these cultures were able to utilize their own reserve carbon (44, 45). The cyanobacteria in the Bird Shoal microbial mat are also apparently able to utilize photosynthate at night under anoxic conditions to drive the energetically expensive process of nitrogen fixation.

The finding that exogenous sources of carbon could not supplant the requirement for oxygenic photosynthesis for nighttime nitrogen fixation appears to be in conflict with previous reports that additions of dissolved organic carbon (DOC) result in a stimulation of acetylene reduction in a number of nitrogen-fixing communities, including planktonic aggregates (marine snow), detrital aggregates composed of vascular plant detritus, and microbial mats (25–28). The earlier experiments were performed by using a bioassay approach in which microbial mat (and other potential nitrogen-fixing) communities were treated for several hours to several days with elevated concentrations (5 to 20 mM) of DOC. The higher rates of acetylene reduction in DOC-amended bioassay treatments could be due to either a utilization of the added substrate as an energy and/or reductant source for nitrogen fixation or a lowering of oxygen tensions caused by the respiratory consumption of the substrate and oxygen by both diazotrophs and nondiazotrophs. In addition, longer-term (several-day) bioassays could result in enrichment and selection for certain groups of microorganisms. In some of these bioassay experiments, natural sources of DOC were added to the microbial communities of interest. Differences in experimental design (bioassay versus nighttime substrate additions) and in the nature of the added DOC (natural DOC versus the relatively few simple substrates added here) preclude direct comparison of these earlier bioassay experiments with the present results. We are not directly addressing the effect of added DOC on acetylene reduction in otherwise similarly treated microbial mats. The experiments presented here are designed instead to test the null hypothesis that exogenous sources of DOC can substitute for the occurrence of daytime oxygenic photosynthesis to supply energy and/or reductant for naturally occurring nighttime acetylene reduction. The results of these experiments clearly indicate that the sources of DOC tested cannot do this.

Although our findings indicate a very tight energetic coupling of the temporally separated processes of oxygenic photosynthesis and nitrogen fixation, it is possible that in other microbial mats, or possibly at some stages in the seasonal or developmental cycle of this mat, that the heterotrophic contribution to nitrogen fixation could be more

important. Heterotrophic nitrogen-fixing microorganisms have been isolated from these mats (20, 26). Substantial acetylene reduction was measured throughout the day in mats incubated in both the light and the dark (Fig. 1). There was generally no significant difference between rates of acetylene reduction in light- and dark-incubated mats during the daytime. Where there were significant differences in daytime rates of acetylene reduction in these two treatments, however, the mats incubated in the light invariably exhibited rates higher than those of dark-incubated mats. This is an indication that light is an important source of energy or reductant for nitrogen fixation in spite of the much higher oxygen tensions which exist in the light-incubated mats. The Bird Shoal microbial mat is an extremely heterogeneous system, however, and is constantly changing in response to environmental variables. A week-long period of time in which low tides coincide with high irradiance, for example, results in greatly reduced cyanobacterial biomass. The differences we observed in the absolute rates of acetylene reduction in dark-incubated mats in the daytime (from 0 to 100 μmol of $C_2H_4 \cdot m^{-2} \cdot h^{-1}$) may reflect the changing importance of the heterotrophic contribution to whole-mat rates of acetylene reduction. The daily integrated acetylene reduction not directly coupled to oxygenic photosynthesis (dark-incubated samples in Fig. 1, 3, and 4) ranged from 9.5 to 85.2% (mean value, 36.2%) of the integrated daily acetylene reduction in the samples exposed to natural diel patterns of irradiance.

Significance of DCMU-stimulated acetylene reduction. A dramatic stimulation of acetylene reduction was observed in mats treated with the inhibitor DCMU (Fig. 1 and 3). Stimulation of acetylene reduction by DCMU has been reported previously in short-term incubations of many microbial mat systems (3, 40, 43) as well as in cultures of cyanobacteria isolated from mats (29, 46). It is generally concluded that this stimulation results from an alleviation of the oxygen inhibition of nitrogenase. The diel patterns of DCMU-stimulated activity, and consequences of the DCMU treatment for acetylene reduction at night, had not been previously investigated. Although the DCMU-stimulated acetylene reduction activity was correlated with irradiance, the rates decreased over the course of the day (Fig. 2). The reasons for this decrease are not completely clear, but some hypotheses can be suggested.

PS I activity may support acetylene reduction through any one of three different mechanisms. The first mechanism is utilized by photosynthetic sulfur bacteria as well as cyanobacteria. Light is used to fix CO_2 by using H_2S as a source of electrons, and nitrogen fixation is accomplished by using reducing power and energy generated from catabolism of the products of photosynthesis. In the second mechanism, electrons donated by sulfide are channeled through PS I and eventually used to reduce nitrogenase without the fixation of CO_2. Although this mechanism has been reported previously in cyanobacteria (24), it is not well characterized. In the third mechanism, NADH and NADPH produced by the metabolic breakdown of previously fixed carbon is channeled through PS I at the plastoquinone site and eventually used to reduce ferredoxin. This is the pathway common in heterocysts (39). The first mechanism seems an unlikely candidate to explain the high rates of DCMU-stimulated acetylene reduction in this mat because of the low rates of $^{14}CO_2$ uptake in mats under treatment with DCMU (Fig. 5) and the absence of high rates of nighttime acetylene reduction in DCMU-treated mats (Fig. 1 and 3). Reducing power generated through PS I activity is not being used for CO_2 fixation, and this first mechanism of DCMU stimulation of acetylene reduction may be rejected. Sulfide-dependent and NADH-dependent acetylene reduction will be considered in greater detail.

(i) Sulfide-dependent acetylene reduction. Acetylene reduction dependent upon sulfide oxidation through PS I activity (channeling electrons from sulfide to dinitrogen) would seem to be an excellent strategy for nitrogen fixation in microbial mats, and this pathway has been reported previously in at least one cyanobacterium (24). In a recent review article, Fay (12) commented that it was surprising that more organisms possessing this capability had not been reported. To investigate whether this process could explain the DCMU-stimulated acetylene reduction in the Bird Shoal mat, we attempted to manipulate (decrease) the sulfide concentrations in the mats by using sodium molybdate, an inhibitor of sulfate reduction (Fig. 3).

Additions of sodium molybdate and DCMU to microbial mats did result in a decrease in the DCMU-stimulated acetylene reduction activity (Fig. 3). It is unlikely that the reduction in activity was due to inhibition of acetylene reduction by sulfate-reducing bacteria because of the observed strong light dependence of the DCMU-stimulated activity. A light enhancement of rates of sulfate reduction has been reported recently in a microbial mat (14), but the mechanism of this light stimulation (use of glycolate excreted by photorespiring cyanobacteria) would not have been possible in the presence of DCMU. The data presented here, therefore, may indicate a possible role for sulfide in the observed DCMU-stimulated acetylene reduction.

We made no direct measurements of sulfide concentrations ($[H_2S]$) within the mat, and cannot say to what extent sodium molybdate decreased the $[H_2S]$. It is possible that the sulfide inventory in the mat was enough to support some sulfide-dependent acetylene reduction (the rates did not go to zero with the addition of sodium molybdate) even in the absence of sulfide production from sulfate reduction. We experimentally tested the hypothesis that the effect of molybdate on acetylene reduction was to decrease the sulfide concentrations in the mat by adding sodium sulfide to the mat (enough sodium sulfide was added to result in a final H_2S concentration of 200 μM, if no other sulfide had been present). Unfortunately, the partially oxygenated water to which the sodium sulfide solution was added, as well as complexes formed by the sodium molybdate and the sodium sulfide, prevented us from determining the final sulfide concentrations in the experimentally treated mats. We were, however, able to recover all of the sodium molybdate-induced decreases in DCMU-stimulated acetylene reduction with sulfide additions (Fig. 3).

Further evidence for sulfide-dependent nitrogen fixation may be obtained by comparing the results of the experiments by using DCMU-treated cyanobacterial cultures with the results obtained from the whole-mat community. Cultures did not contain sulfide in appreciable amounts and did not exhibit DCMU-stimulated acetylene reduction.

(ii) NADH- and NADPH-dependent acetylene reduction. It has been shown (in cell-free systems obtained from heterocysts) that NADH and NADPH generated from glycolysis, the tricarboxylic acid cycle, and the oxidative pentose phosphate pathway are channeled through PS I and eventually used to reduce ferredoxin, the ultimate electron donor to nitrogenase (38, 39). It is possible that NADH and NADPH could also be used to reduce nitrogenase in the nonheterocystous cyanobacteria present in many microbial mats. The operation of this pathway would provide an alternative (to sulfide depletion) explanation for the reduction in DCMU-

stimulated acetylene reduction over the course of the day. If a carbon source necessary for NADH and NADPH generation (e.g., storage glucan) was becoming depleted over the course of the day, a similar pattern might result.

The mechanism responsible (either sulfide or NADPH and NADH donation to PS I) for the observed dramatic stimulation of acetylene reduction in DCMU-treated mats remains unresolved. Clearly, studies of the relative importance of these mechanisms would be better accomplished by using pure cultures of mat microorganisms to decrease the complex interactions between the various members of the mat community. Although the conditions responsible for the DCMU-stimulated acetylene reduction are artificial, mat microorganisms are exposed to high levels of sulfide and low levels of light at least twice in every diel cycle. Indeed, some cyanobacteria in the deeper layers of the mat are exposed to these conditions for most of the diel cycle. Sulfide-dependent nitrogen fixation would appear to represent an ideal strategy for nitrogen fixation under these conditions. Although we can only speculate, early mornings would be particularly suited to sulfide-dependent nitrogen fixation throughout most of the mat, and it is possible that this mechanism, in addition to or instead of the mechanisms previously suggested (dependence of nitrogen fixation on oxygen-requiring respiration), could be responsible for the high rates of acetylene reduction which occur in many mats at that time.

ACKNOWLEDGMENTS

We thank L. J. Stal for many helpful discussions. The comments of N. Blair, F. Garcia-Pichel, and two anonymous reviewers greatly improved the manuscript.

Financial support was provided by NSF grants OCE 901246, OCE 9115706, and BSR 8917880 to H.W.P.

REFERENCES

1. **Bautista, M. F., and H. W. Paerl.** 1985. Diel N_2 fixation in an intertidal marine cyanobacterial mat community. Mar. Chem. **16**:369–377.
2. **Bebout, B. M.** 1992. Interactions of nitrogen and carbon cycling in microbial mats and stromatolites. Ph.D. dissertation. University of North Carolina at Chapel Hill, Chapel Hill.
3. **Bebout, B. M., H. W. Paerl, K. M. Crocker, and L. E. Prufert.** 1987. Diel interactions of oxygenic photosynthesis and N_2 fixation (acetylene reduction) in a marine microbial mat community. Appl. Environ. Microbiol. **53**:2353–2362.
4. **Bergstein, T., Y. Henis, and B. Z. Cavari.** 1981. Nitrogen fixation by the photosynthetic sulfur bacterium *Chlorobium phaeobacterioides* from Lake Kinneret. Appl. Environ. Microbiol. **41**:542–544.
5. **Bothe, H., and E. Loos.** 1972. Effect of far red light and inhibitors on nitrogen fixation and photosynthesis in the blue-green alga *Anabaena cylindrica*. Arch. Microbiol. **86**:241–254.
6. **Carpenter, E. J., C. D. Van Raalte, and I. Valiela.** 1978. Nitrogen fixation by algae in a Massachusetts salt marsh. Limnol. Oceanogr. **23**:318–327.
7. **Cox, R. M., and P. Fay.** 1969. Special aspects of nitrogen fixation by blue-green algae. Proc. R. Soc. Lond. B Biol. Sci. **172**:357–366.
8. **Dalton, H.** 1980. Chemoautotrophic nitrogen fixation, p. 177–195. *In* W. D. P. Stewart and J. R. Gallon, (ed.), Nitrogen fixation. Academic Press Ltd., London.
9. **Diaz, M. R., J. E. Corredor, and J. M. Morell.** 1990. Nitrogenase activity of *Microcoleus lyngbyaceus* mat communities in a eutrophic, tropical marine environment. Limnol. Oceanogr. **35**:1788–1795.
10. **Ernst, A., H. Kirschenlohr, J. Diez, and P. Boger.** 1984. Glycogen content and nitrogenase activity in *Anabaena variabilis*. Arch. Microbiol. **140**:120–125.
11. **Fay, P.** 1976. Factors influencing dark nitrogen fixation in a blue-green alga. Appl. Environ. Microbiol. **31**:376–379.
12. **Fay, P.** 1992. Oxygen relations of nitrogen fixation in cyanobacteria. Microbiol. Rev. **56**:340–373.
13. **Fay, P., and R. P. Cox.** 1967. Oxygen inhibition of nitrogen fixation in cell-free preparations of blue-green algae. Biochim. Biophys. Acta **143**:562–569.
14. **Fründ, C., and Y. Cohen.** 1992. Diurnal cycles of sulfate reduction under oxic conditions in cyanobacterial mats. Appl. Environ. Microbiol. **58**:70–77.
15. **Gallon, J. R.** 1989. The physiology and biochemistry of N_2 fixation by non-heterocystous cyanobacteria. Phykos **28**:18–46.
16. **Gallon, J. R., S. M. Perry, T. M. A. Rajab, K. A. M. Flayeh, J. S. Yunes, and A. E. Chaplin.** 1988. Metabolic changes associated with the diurnal pattern of N_2 fixation in *Gloeothece*. J. Gen. Microbiol. **134**:3079–3087.
17. **Gallon, J. R., and L. J. Stal.** 1992. N_2 fixation in non-heterocystous cyanobacteria: an overview, p. 115–139. *In* E. J. Carpenter, D. G. Capone, and J. Rueter (ed.), Marine pelagic cyanobacteria: *Trichodesmium* and other diazotrophs. Kluwer Academic Publishers, Amsterdam.
18. **Jones, K.** 1974. Nitrogen fixation in a salt marsh. J. Ecol. **62**:553–565.
19. **Jones, K.** 1992. Diurnal nitrogen fixation in tropical marine cyanobacteria: a comparison between adjacent communities of non-heterocystous *Lyngbya* sp. and heterocystous *Calothrix* sp. Br. Phycol. J. **27**:107–118.
20. **Kirshtein, J. D., H. W. Paerl, and J. Zehr.** 1991. Amplification, cloning, and sequencing of a nifH segment from aquatic microorganisms and natural communities. Appl. Environ. Microbiol. **57**:2645–2650.
21. **Lyne, R. L., and W. D. P. Stewart.** 1973. Emerson enhancement of carbon fixation but not acetylene reduction (nitrogenase activity) in *Anabaena cylindrica*. Planta **109**:27–38.
22. **Maryan, P. S., R. R. Eady, A. E. Chaplin, and J. R. Gallon.** 1986. Nitrogen fixation by *Gloeothece* sp. PCC 6909: respiration and not photosynthesis supports nitrogenase activity in the light. J. Gen. Microbiol. **132**:789–796.
23. **Mullineaux, P. M., J. R. Gallon, and A. E. Chaplin.** 1981. Acetylene reduction (nitrogen fixation) by cyanobacteria grown under alternating light-dark cycles. FEMS Microbiol. Lett. **10**:245–247.
24. **Padan, E., and Y. Cohen.** 1982. Anoxygenic photosynthesis, p. 215–235. *In* N. G. Carr and B. A. Whitton, (ed.), The biology of the cyanobacteria. Blackwell Scientific Publications Ltd., Oxford.
25. **Paerl, H. W.** 1990. Physiological ecology and regulation of N_2 fixation in natural waters. Adv. Microb. Ecol. **11**:305–344.
26. **Paerl, H. W., B. M. Bebout, and L. E. Prufert.** 1989. Naturally occurring patterns of oxygenic photosynthesis and N_2 fixation in a marine microbial mat: physiological and ecological ramifications, p. 326–341. *In* Y. Cohen and E. Rosenberg (ed.), Microbial mats: physiological ecology of benthic microbial communities. American Society for Microbiology, Washington, D.C.
27. **Paerl, H. W., K. M. Crocker, and L. E. Prufert.** 1987. Limitation of N_2 fixation in coastal marine waters: relative importance of molybdenum, iron, phosphorus, and organic matter availability. Limnol. Oceanogr. **32**:525–536.
28. **Paerl, H. W., and L. E. Prufert.** 1987. Oxygen-poor microzones as potential sites of microbial N_2 fixation in nitrogen-depleted aerobic marine waters. Appl. Environ. Microbiol. **53**:1078–1087.
29. **Paerl, H. W., L. E. Prufert, and W. W. Ambrose.** 1991. Contemporaneous N_2 fixation and oxygenic photosynthesis in the nonheterocystous mat-forming cyanobacterium *Lyngbya aestuarii*. Appl. Environ. Microbiol. **57**:3086–3092.
30. **Parker, B. C., G. M. Simmons, Jr., R. A. Wharton, Jr., K. G. Seaburg, and F. G. Love.** 1982. Removal of organic and inorganic matter from antarctic lakes by aerial escape of bluegreen algal mats. J. Phycol. **18**:72–78.
31. **Pearson, H. W., R. Howsley, C. K. Kjeldsen, and A. E. Walsby.**

1979. Aerobic nitrogenase activity associated with a non-heterocystous filamentous cyanobacterium. FEMS Microbiol. Lett. **5**:163–167.

32. **Post, A. F., J. G. Loogman, and L. R. Mur.** 1986. Photosynthesis, carbon flows and growth of *Oscillatoria agardhii* Gomont in environments with a periodic supply of light. J. Gen. Microbiol. **132**:2129–2136.
33. **Potts, M., and B. A. Whitton.** 1977. Nitrogen fixation by blue-green algal communities in the intertidal zone of the Lagoon of Aldabra Atoll. Oecologia **27**:275–283.
34. **Revsbech, N. P., B. B. Jørgensen, and O. Brix.** 1981. Primary production of microalgae in sediments measured by oxygen microprofile, $H^{14}CO_3^-$ fixation, and oxygen evolution methods. Limnol. Oceanogr. **26**:717–730.
35. **Revsbech, N. P., J. Nielsen, and P. K. Hansen.** 1988. Benthic primary production and oxygen profiles, p. 69–83. *In* T. H. Blackburn and J. Sorensen (ed.), Nitrogen cycling in coastal marine environments. John Wiley & Sons Ltd., Tiptree, Great Britain.
36. **Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Stanier.** 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. **111**:1–61.
37. **Robson, R. L., and J. R. Postgate.** 1980. Oxygen and hydrogen in biological nitrogen fixation. Annu. Rev. Microbiol. **34**:183–207.
38. **Scherer, S., E. Sturzl, and P. Böger.** 1982. Interaction of respiratory and photosynthetic electron transport in *Anabaena variabilis* Kutz. Arch. Microbiol. **132**:333–337.
39. **Schrautemeier, B., H. Bohme, and P. Böger.** 1984. In vitro studies on pathways and regulation of electron transport to nitrogenase with a cell-free extract from heterocysts of *Anabaena variabilis*. Arch. Microbiol. **137**:14–20.
40. **Skyring, G. W., R. M. Lynch, and G. D. Smith.** 1988. Acetylene reduction and hydrogen metabolism by a cyanobacterial/sulfate-reducing bacterial mat ecosystem. Geomicrobiol. J. **6**:25–31.
41. **Stal, L. J.** 1985. Nitrogen fixing cyanobacteria in a marine microbial mat. Ph.D. dissertation. Rijksuniversiteit Groningen, Groningen, The Netherlands.
42. **Stal, L. J., and B. Bergman.** 1990. Immunological characterization of nitrogenase in the filamentous non-heterocystous cyanobacterium *Oscillatoria limosa*. Planta **182**:287–291.
43. **Stal, L. J., S. Grossberger, and W. E. Krumbein.** 1984. Nitrogen fixation associated with the cyanobacterial mat of a marine laminated microbial ecosystem. Mar. Biol. **82**:217–224.
44. **Stal, L. J., and H. Heyer.** 1987. Dark anaerobic nitrogen fixation (acetylene reduction) in the cyanobacterium *Oscillatoria* sp. FEMS Microbiol. Ecol. **45**:227–232.
45. **Stal, L. J., and W. E. Krumbein.** 1981. Aerobic nitrogen fixation in pure cultures of a benthic marine *Oscillatoria* (cyanobacteria). FEMS Microbiol. Lett. **11**:295–298.
46. **Stal, L. J., and W. E. Krumbein.** 1985. Nitrogenase activity in the non-heterocystous cyanobacterium *Oscillatoria* sp. grown under alternating light-dark cycles. Arch. Microbiol. **143**:67–71.
47. **Stewart, W. D. P., G. P. Fitzgerald, and R. H. Burris.** 1967. *In situ* studies on N_2 fixation using the acetylene reduction technique. Proc. Natl. Acad. Sci. USA **58**:2071–2078.
48. **Villbrandt, M., W. E. Krumbein, and L. J. Stal.** 1991. Diurnal and seasonal variations of nitrogen fixation and photosynthesis in cyanobacterial mats. Plant Soil **137**:13–16.
49. **Villbrandt, M., L. J. Stal, and W. E. Krumbein.** 1990. Interactions between nitrogen fixation and oxegenic photosynthesis in a marine microbial mat. FEMS Microbiol. Ecol. **74**:59–72.
50. **Whitney, D. E., G. M. Woodwell, and R. W. Howarth.** 1975. Nitrogen fixation in Flax Pond: a Long Island salt marsh. Limnol. Oceanogr. **20**:640–643.
51. **Whitton, B. A., and M. Potts.** 1982. Marine littoral, p. 515–542. *In* N. G. Carr and B. A. Whitton (ed.), The biology of cyanobacteria. Blackwell Scientific Publications Ltd., Oxford.
52. **Wolk, C. P.** 1982. Heterocysts, p. 359–386. *In* N. G. Carr and B. A. Whitton (ed.), The biology of the cyanobacteria. Blackwell Scientific Publications Ltd., Oxford.
53. **Yates, M. G., C. D. P. Partridge, C. C. Walker, A. N. van der Werf, F. O. Campbell, and J. R. Postgate.** 1980. Recent research into the physiology of heterotrophic, non-symbiotic nitrogen-fixing bacteria, p. 161–176. *In* W. D. P. Stewart and J. R. Gallon (ed.), Nitrogen fixation. Academic Press Ltd., London.