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Diel Interactions of Oxygenic Photosynthesis and N_2 Fixation (Acetylene Reduction) in a Marine Microbial Mat Community

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Diel variations in N_2 fixation (acetylene reduction), CO_2 fixation, and oxygen concentrations were measured, on three separate occasions, in a marine microbial mat located on Shackleford Banks, North Carolina. Nitrogenase activity (NA) was found to be inversely correlated with CO_2 fixation and, in two of the three diel periods studied, was higher at night than during the day. Oxygen concentrations within the top 3 mm of the mat ranged from 0 to 400 μM on a diel cycle; anaerobic conditions generally persisted below 4 mm. NA in the mat was profoundly affected by naturally occurring oxygen concentrations. Experimentally elevated oxygen concentrations resulted in a significant depression of NA, whereas the addition of the Photosystem II inhibitor 3(3,4-dichlorophenyl)-1,1-dimethylurea decreased oxygen concentrations within the mat and resulted in a significant short-term enhancement of NA. Mat N_2-fixing microorganisms include cyanobacteria and heterotrophic, photoautotrophic, and chemolithotrophic eubacteria. Measured (whole-mat) NA is probably due to a combination of the NA of each of these groups of organisms. The relative contributions of each group to whole-mat NA probably varied during diel and seasonal (successional) cycles. Reduced compounds derived from photosynthetic CO_2 fixation appeared to be an important source of energy for NA during the day, whereas heterotrophic or chemolithotrophic utilization of reduced compounds appeared to be an important source of energy for NA at night, under reduced ambient oxygen concentrations. Previous estimates of N_2 fixation calculated on the basis of daytime measurements may have seriously underestimated diel and seasonal nitrogen inputs in mat systems.

Cyanobacterial mat systems often occur in environments characterized by nutrient-depleted waters (1, 3, 9, 24, 38). An extensive mat community exists on Shackleford Banks, North Carolina. This community is regularly flooded with N-depleted coastal water and generally exhibits significant and persistent rates of N_2 fixation (nitrogenase activity [NA]) (2; M. F. Bautista, M.S. thesis, University of North Carolina, Chapel Hill, 1984). Chronic nitrogen limitation in adjacent coastal planktonic and subtidal benthic habitats appears to be a feature of ecosystems in which cyanobacterial mats occur (20; L. E. Prufert and H. W. Paerl, manuscript in preparation). High rates of N_2 fixation and photosynthesis within these systems may provide substantial contributions to productivity and nitrogen budgets of shallow water environments (10, 11, 24, 37). Accordingly, elucidation of environmental factors regulating "new" nitrogen inputs via N_2 fixation is of central importance in our understanding of production potentials of these communities.

Cyanobacterial mat communities can be found in numerous other habitats, including shallow seas (32), arctic lakes (21), sewage treatment plants (22), and hot springs (7), and are a common feature of coastal marine environments, such as mangrove swamps (38), temperate and tropical mud flats (38), lagoons (1), intertidal areas (10), and salt marshes (3, 11, 37).

Several reports have elucidated the dynamic nature of diverse metabolic processes that occur in cyanobacterial mats (12–14, 16). In particular, use of microelectrode techniques has facilitated the resolution of metabolic processes on spatial and temporal scales not previously possible in mats (25). Recent work conducted in N-depleted waters adjacent to the Shackleford mat has shown that planktonic N_2 fixation is primarily regulated by ambient O_2 concentration and the availability of oxidizable organic matter (18a). The goal of this study was to determine N_2 and CO_2 fixation potentials and to identify their regulating factors in the benthic cyanobacterial mat community on Shackleford Banks. We investigated diel patterns in N_2 and inorganic CO_2 fixation in relation to irradiance, as well as temporal and spatial changes in O_2 distribution within the mat.

MATERIALS AND METHODS

Field site. Shackleford Banks is a barrier island forming part of North Carolina's coastal Outer Banks system. An extensive area of microbial mat (approximately 60 ha) occurs in an intertidal lagoon, located at the western tip of the island (Fig. 1). The mat is protected from wave action and is flooded irregularly by high tides. This physically stable habitat supports year-round mats (C. Polemini, M.S. thesis, University of North Carolina, Chapel Hill, 1976) composed of filamentous cyanobacteria (*Lyngbya aestuarii*, *Microcoleus chthonoplastes*, and *Phormidium* spp.), diatoms (*Nitzschia closteria*, *Nitzschia filiformis*, *Navicula complanata*, *Rhopaloidia* sp., and *Amphora coffeiformis*), and heterotrophic, photoautotrophic, and chemoautotrophic bacteria, as well as diverse protozoans and invertebrates (nematodes, crustacean larvae, harpactacoid copepods, and snails) (Fig. 2). Microbial mat examined during the diel studies was collected at the Shackleford Banks study site on three occasions: 2 June 1986 (experiment DI), 18 June 1986 (experiment DII), and 28 June 1986 (experiment DIII).

Diel studies. Mat was collected during low tide (no overlying water). A 1-m^2 section was cut from the surrounding mat with a scalpel and a thin sheet of plywood was inserted horizontally beneath the mat at a depth of ca. 4 cm. The 1-m^2 section was lifted from the underlying sand and transported

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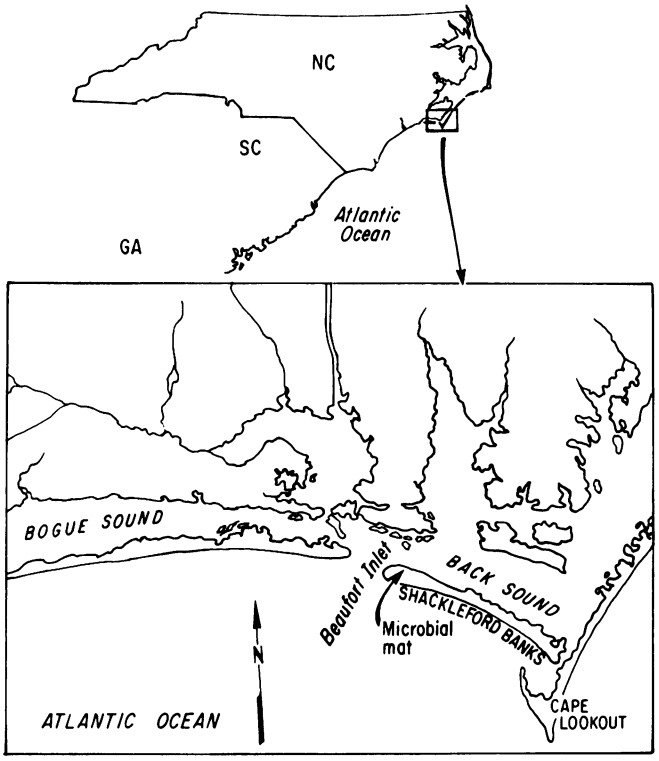

FIG. 1. Location of the Shackleford Banks cyanobacterium-dominated microbial mat. The mat is situated in a lagoon on the western end of the island. This region is periodically flooded by high tides.

to the laboratory. The mat was then placed into a small (2-m^2) pool and submersed by ca. 4 cm of circulating seawater (flow rate, ca. 1 liter m^{-1}). The experimental system was maintained outside under full sunlight and in situ temperature conditions (ca. 23°C). The mat was acclimated to the pool for 24 h prior to the experimental period.

For primary productivity and NA measurements, ca. 25-cm^2 sections were excised from the larger mat sample at the beginning of each of seven time intervals and subsectioned into 1-cm^2 replicates. Another 25-cm^2 section of mat was carefully removed from the pool and placed under a small amount of water from the pond for determining vertical O_2 microprofiles.

Mat primary productivity was determined by measuring $^{14}CO_2$ assimilation. Approximately 15 min prior to each incubation period, 12 1-cm^2 pieces were randomly assigned to dark and light treatments. Each 1-cm^2 piece was placed in a 25-ml Erlenmeyer flask which was subsequently filled with fresh seawater from the pool and capped with a rubber serum stopper. A 0.25-ml aliquot of sterile [^{14}C]$NaHCO_3$ solution (specific activity, 58 mCi $mmol^{-1}$; total activity, 7.75 μCi ml^{-1}; ICN Radiochemicals, Inc.), was injected through the serum stopper. Flasks were then lightly shaken to distribute the ^{14}C and placed, with the mat surface facing up, in the pool. Dark-incubated flasks were covered with three layers of aluminum foil during the incubation period. Circulating seawater in the pool was maintained at 23°C (in situ temperature) during incubation.

Since incubation periods lasted for up to 3 h, we conducted preliminary experiments aimed at examining the linearity of ^{14}C assimilation by mat material during this time span. During these experiments ^{14}C incubations were terminated at 0.5-h intervals following isotope addition (six replicates per interval). Incubations were terminated by carefully removing the ^{14}C-inoculated seawater from the flasks and gently rinsing the mat pieces twice with unlabeled seawater. Mat pieces were dried at 75°C, ground into a fine powder, fumed over concentrated HCl for 30 min (to remove residual inorganic ^{14}C), and then vented in air for at least 3 h to remove the HCl. The residual powder was then assayed for ^{14}C activity by liquid scintillation spectrometry (Beckman LS-7000), using Scintiverse scintillation cocktail (Fisher Scientific Co.). Internal unquenched [^{14}C]hexadecane standards (calibrated by New England Nuclear Corp.) were used to determine counting efficiencies among 1-cm^2 mat pieces. These efficiences ranged from 85 to 95%.

When calculating CO_2 fixation with the ^{14}C method, it is necessary to determine the concentration of dissolved inorganic carbon in the experimental system. Because seawater is nearly saturated with dissolved inorganic carbon (35), it was assumed to be reasonably representative of dissolved inorganic carbon concentration in the mat. At the beginning of each sampling interval, pool water was collected and analyzed for dissolved inorganic carbon by infrared analyses. Triplicated 0.25-ml pool water samples were directly injected into a reservoir (containing 10 ml of 50% H_3PO_4) continuously sparged with Ar which served as a carrier gas, sweeping evolved CO_2 through a Drierite ($CaSO_4$; W. A. Hammond Drierite Co.) desiccator column into an infrared analyzer (Beckman model 864). A linear calibration curve was obtained with standards prepared from Na_2CO_3. Carbon assimilation rates were calculated as per Strickland and Parsons (34), modified for areal rather than volumetric estimates.

NA was measured by using a modification of the acetylene reduction technique (33). The enzyme nitrogenase catalyzes the reduction of C_2H_2 to C_2H_4, which can be detected by gas chromatography. Replicate (n = 6) 1-cm^2 sections of mat were placed in 25-ml Erlenmeyer flasks. A 20-ml aliquot of overlying pool water was poured into each flask, and a serum stopper was inserted, leaving a 10-ml headspace. Purified acetylene (4 ml; Matheson Scientific, Inc.) was injected into the aqueous phase (seawater) of inverted flasks. Dark-incubated flasks were covered with aluminum foil, and all samples were incubated for 3 h in the pool. Care was taken to assure that mat sections were oriented properly relative to incoming sunlight. Following incubation, flasks were removed from the pool, shaken vigorously for 7 s to liberate C_2H_4 produced in the mat, and vented to achieve equilibrium with atmospheric pressure by inserting a 25-gauge syringe needle through the serum stopper. A 0.3-ml subsample of the headspace was then 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 (Matheson Scientific) served as the carrier gas. Flasks containing pool water without mat were used to correct for background C_2H_4 and C_2H_4 contamination in acetylene.

Oxygen profiles in the mat were made by using 0.7-mm-diameter minielectrodes (Diamond Electrotech no. 760) having 45° beveled tips. The sturdy steel casings of these electrodes allowed for repeated penetration into the sandy mats, whereas more fragile glass microelectrodes had previously proven highly susceptible to breakage. However, the relatively large diameter of these mini-electrodes permitted less spatial resolution; therefore, readings were made at intervals of 1 mm (+2 and +1 mm above the surface, at the surface, and into the mat to a depth of 8 mm). A reference

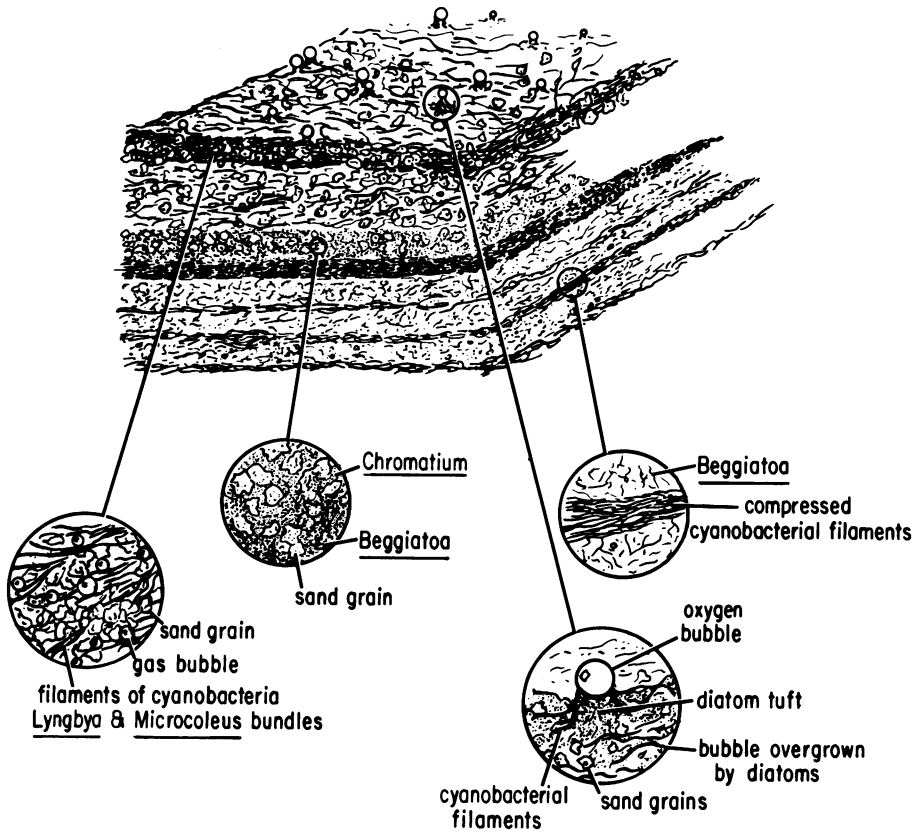

FIG. 2. Schematic representation of a cross sectional view of the microbial mat community.

electrode was immersed in pool water near the minielectrode and a picoammeter (Keithley model 485) was used to measure the electrode output signal. The electrodes were lowered through the mat with a micromanipulator, pausing at 1-mm intervals until a stable reading could be obtained with the picoammeter (ca. 10 s). To calibrate the electrodes, two seawater samples were taken from the incubation pool approximately 0.5 h before the profiles were taken and sparged with air and N_2. Either before or after profiles were taken, the electrode and reference were placed in each of the gas-sparged solutions and picoammeter readings were recorded. The solutions were then titrated (4, 6) to determine O_2 content. Temperature and salinity were measured so that Winkler determinations of the O_2-saturated (air-sparged) standard could be compared with theoretical saturation values (36). Picoammeter readings from the electrodes were then corrected for drift and compared with an oxygen standard regression to get micromolar O_2 values. Readings were not taken in the water overlying the mat during the first diel study (DI). In all cases, six profiles were taken at haphazardly determined locations at each time point.

The flux of photosynthetically active radiation (PAR) at the surface of the water was monitored at 10-min intervals throughout the diel periods with a Li-Cor (model 192) quantum sensor coupled to a Li-550B printing integrator.

Effects of O_2 on mat N_2-fixing potentials. To investigate the effect O_2 had on NA in the mat independent of the influences of PAR and CO_2 fixation, the following experiments were performed. Mat pieces (1 cm^2) were cut and placed in 25-ml flasks containing 20 ml of seawater. Flasks were sealed with serum stoppers and connected to a vacuum manifold by inserting needles through the stoppers. Air containing a range of O_2 enrichments was rapidly (within 1 min) flushed through each flask, yielding aqueous O_2 concentrations equivalent to 100 (unenriched air), 150, and 200% of atmospheric equilibrium saturation (n = 3). This range of O_2 concentrations commonly occurs in the Shackleford mat and water immediately overlying the mat (Paerl, in press). The impacts of different oxygen concentrations ($[O_2]$) on NA were examined immediately after flushing, using 1- to 2-h acetylene reduction assays with dark and light treatments.

To determine the effect of photosynthetically derived O_2 on mat NA, a sample of the mat was treated with 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). This potent inhibitor of photosynthetic O_2 evolution, while not affecting respiration, has been shown to effectively and rapidly lower mat O_2 tension (18a). Accordingly, DCMU was added prior to acetylene reduction assays to determine if photosynthetically evolved O_2 in the mat suppressed NA during illuminated (daylight) periods. The DCMU additions were made during mid- to late-morning hours, when photosynthetic oxygen production was maximal. Mat pieces (1 cm^2) were cut and placed into flasks containing seawater to which DCMU was added to a final concentration of 2×10^{-5} M (Paerl et al., in press). Flasks were then sealed, mildly shaken, and assayed, in triplicate, for NA. Incubation periods ranged from 0.5 to 2 h.

Effects of ammonium on mat NA. To investigate the effects of ammonium concentration ($[NH_4^+]$) on mat NA, the following experiment was performed. Mat pieces (1 cm^2) were placed into 25-ml Erlenmeyer flasks and covered with 20 ml of freshly collected seawater. A sample of the seawater was assayed for ammonium concentration, using the phenol-hypochlorite method (29), and found to be below the limits of

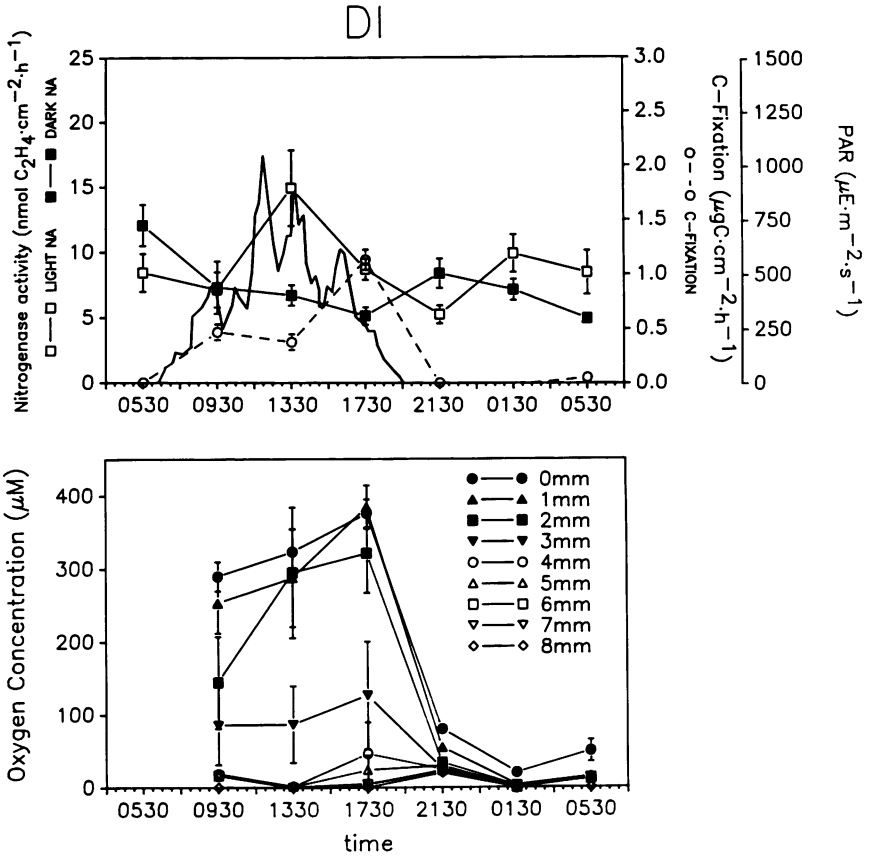

FIG. 3. Patterns of surface PAR (solid line), CO_2 fixation (○), NA in the light (□), NA in the dark (■), and the vertical distribution of dissolved O_2 in the Shackleford Banks marine microbial mat community obtained in the first diel study (DI). Data points for NA and CO_2 fixation are plotted at the midpoint of each 3-h incubation period. Missing oxygen data points at 0530 are due to sulfide poisoning of the electrodes, which would indicate low oxygen concentrations. Error bars represent one standard error above and below the mean (n = 6 for NA and $[O_2]$; n = 3 for CO_2 fixation).

detection for this method (<5 μg of N liter^{-1}). A stock solution of NH_4Cl was prepared with this seawater and added to flasks to yield final ammonium concentrations of 0 (no stock solution), 1,000, 2,000, and 4,000 μg of N liter^{-1}. Flasks containing seawater, and no mat, at each of the final ammonium concentrations served as controls for adsorption and uptake of ammonium by planktonic organisms in the seawater. Flasks were incubated for 3 h under natural illumination and temperature conditions. The flasks were then sealed and assayed for NA as previously described.

RESULTS

Irradiance. PAR generally became detectable at approximately 0600 (dawn), increased until ca. 1300, and decreased to undetectable levels by ca. 2000 (Fig. 3, 4, and 5). A smooth bell-shaped curve was obtained for the second diel study (DII), a bright cloudless day (Fig. 4). Partly cloudy conditions resulted in irregular PAR patterns during the first and third diel studies (DI and DIII, respectively) (Fig. 3 and 5). An afternoon thunderstorm period resulted in substantial depression of PAR in DI. Maximum PAR flux values recorded were ca. 1,000 microeinsteins m^{-2} s^{-1} for DI and ca. 1,500 microeinsteins m^{-2} s^{-1} for DII and DIII.

CO_2 fixation. Photosynthetic CO_2 fixation commenced shortly after daybreak, resulting in the sharpest rate increases between 0530 and 0930 (Fig. 3, 4, and 5). In all three experiments, rates of CO_2 fixation decreased to undectable levels by 2130. Maximum rates of CO_2 fixation were 2.25 μg of C cm^{-2} h^{-1} for DII and DIII and ca. 1.0 μg of C cm^{-2} h^{-1} for DI. These maxima were recorded in the early morning in DII and late afternoon in DI and DIII.

Cumulative $^{14}CO_2$ fixation generally appeared linear during the first 1.5 h of incubation, regardless of PAR intensity (Fig. 6). During incubations in early to mid-morning hours, linearity generally persisted throughout the entire 3-h incubation, indicating that the added $^{14}CO_2$ incurred no detectable physical-chemical barriers to its diffusion into the mat. Linearity was apparently established within 0.5 h of $^{14}CO_2$ addition. During midday and afternoon incubations, linearity often ceased after 1.5 to 2 h. The loss of linearity probably reflected inhibition of $^{14}CO_2$ fixation, possibly due to excessive O_2, photorespiration, nutrient limitation, or a combination of these factors.

NA. NA in the mat was highest at night in two of the three diel periods (Fig. 4 and 5). During DI, NA was maximal at 1330 (Fig. 3). During daylight hours, NA was usually higher in light-incubated samples (nonoverlapping standard error bars) or not different between light- and dark-incubated samples. At night, there was generally a smaller difference between light-and dark-incubated samples. A morning (0930 to 1330) increase in NA from predawn rates, associated with a decrease in CO_2 fixation, was observed in all three diel experiments. NA decreased during the next time interval

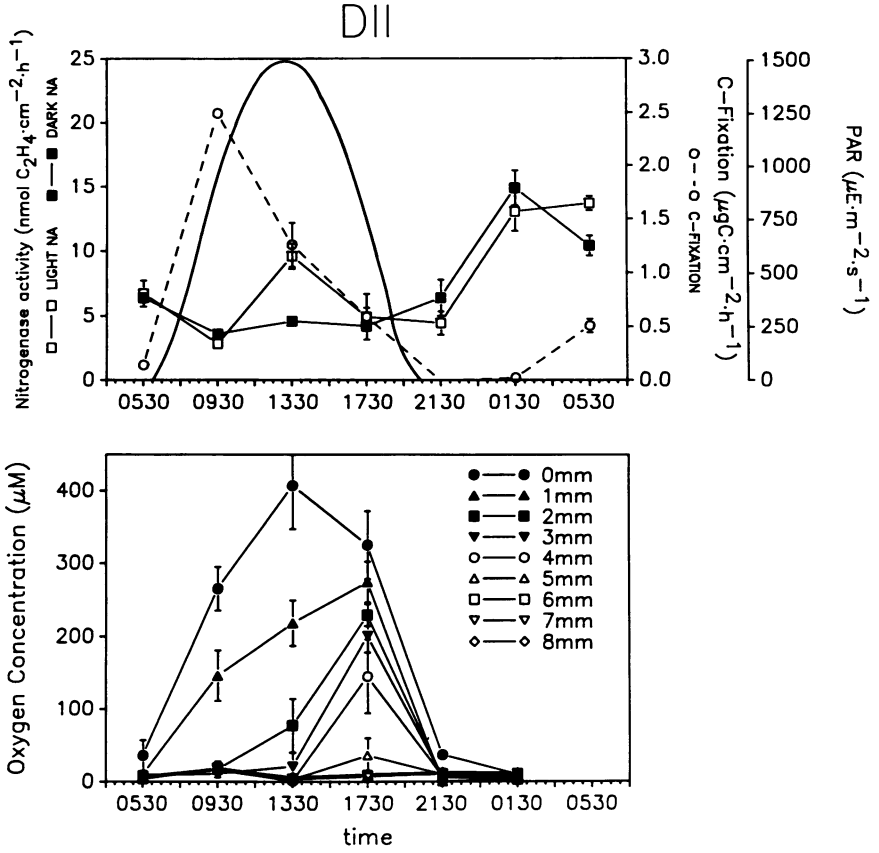

FIG. 4. Patterns of surface PAR (solid line), CO_2 fixation (○), NA in the light (□), NA in the dark (■), and the vertical distribution of dissolved O_2 in the Shackleford Banks marine microbial mat community obtained in the second diel study (DII). Data points for NA and CO_2 fixation are plotted at the midpoint of each 3-h incubation period. Missing oxygen data points at 0530 are due to sulfide poisoning of the electrodes, which would indicate very low oxygen concentrations. Error bars represent one standard error above and below the mean (n = 6 for NA and $[O_2]$; n = 3 for CO_2 fixation).

(1330 to 1730), and in DI and DII this decrease in NA was associated with an increase in CO_2 fixation. In DII, CO_2 fixation continued to decline during this time interval. Maximum rates of NA for all three diel experiments were 15 to 20 nmol of C_2H_4 cm^{-2} h^{-1}.

Oxygen distribution. Oxygen profiles revealed that dissolved $[O_2]$ in the mat rose sharply between 0400 and 0800 (Fig. 3, 4, 5, and 7). This increase was clearly associated with the commencement of photosynthetic CO_2 fixation. Anaerobic conditions returned throughout the mat between 1600 and 0000. The onset of anaerobic conditions at the mat surface was also indicated by the appearance of the motile obligately microaerophilic bacterium *Beggiatoa* sp., which is found at the aerobic/anaerobic interface. Oxygen concentrations in the mat varied between 0 and 400 μM (saturation, ca. 250 μM) (Fig. 3, 4, and 5). Oxygen concentrations in the 2 mm of water immediately overlying the mat ranged from 0 to 300 μM (Fig. 7). Higher O_2 concentrations were measured in DII than in the other two experiments. Oxygen concentrations were maximum in the mat at 1600 in all three experiments. Maximum $[O_2]$ were generally at the mat surface, although a subsurface maximum was present on two ocassions (1600, DI; 1200, DIII) (Fig. 7). Oxygen concentrations in excess of 100% saturation did not occur below a depth of 3 mm, and anaerobic conditions persisted throughout the diel period in mat below a depth of 4 mm in DI and DIII (Fig. 3 and 5) and below 5 mm in DII (Fig. 4). The net diffusive flux of O_2 at the mat-water interface was generally into the mat but was out of the mat when maximum rates of O_2 evolution occurred.

DISCUSSION

There is an inverse correlation between both CO_2 fixation and integrated oxygen concentrations with NA in the Shackleford Banks mat community (least-squares regression, all three experiments pooled, means of each time interval; Fig. 8). Both of these correlations were found to be significant ($P < 0.05$, t test). High rates of CO_2 fixation were observed variously in the early morning and late afternoon, whereas the highest rates of NA occur late at night or in the early morning. This contrasts strongly with diel patterns in NA reported previously for cyanobacterium-dominated planktonic systems. Nitrogenase activity in these systems is generally found to be closely associated with CO_2 fixation (23, 26). A temporal separation of CO_2 fixation and NA has been reported in some planktonic systems (15, 17, 19), but NA is rarely observed to be important at night (17) and has not been reported to occur at rates higher than daytime rates. In contrast, higher nighttime NA was reported in the Shackleford mat previously by Bautista (M.S. thesis) and Bautista and Paerl (2), in a cyanobacterial mat on the North Sea (30), and in a culture of the nonheterocystous cyanobacterium *Oscillatoria* sp. isolated from the North Sea mat (31).

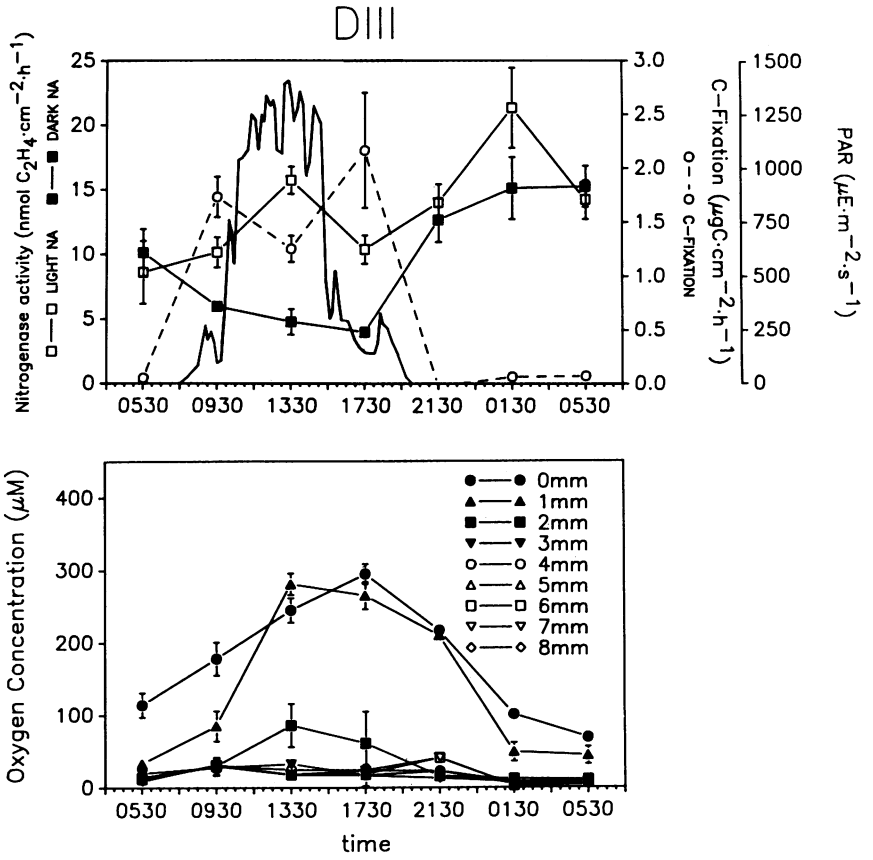

FIG. 5. Patterns of surface PAR (solid line), CO_2 fixation (○), NA in the light (□), NA in the dark (■), and the vertical distribution of dissolved O_2 in the Shackleford Banks marine microbial mat community obtained in the third diel study (DIII). Data points for NA and CO_2 fixation are plotted at the midpoint of each 3-h incubation period. Error bars represent one standard error above and below the mean (n = 6 for NA and $[O_2]$; n = 3 for CO_2 fixation).

Perhaps higher nighttime rates of NA are characteristic of organisms which experience periods of low $[O_2]$ on a diel cycle.

The PAR data obtained during the three diel studies, together with the frequent sampling intervals, enabled us to determine the effects of short-term (3- to 4 h) changes in PAR on CO_2 fixation and NA in the mat. In general, and not surprisingly, CO_2 fixation responded rapidly to changes in light intensity (Fig. 3, 4, and 5). The fixation of CO_2 appeared to be photoinhibited, or suppressed by high $[O_2]$, early in the day in all three diel studies. Decreasing light intensities during afternoon hours gave rise to secondary peaks in CO_2 fixation in DI and DII, possibly indicating a recovery from photoinhibition of O_2 suppression. In all three studies, NA increased concomitantly with decreases in CO_2 fixation. What is not clearly delineated, however, is whether the observed inverse correlation of NA with CO_2 fixation is the result of competition for energy (reduced carbon compounds) between the two metabolic processes, inhibition of NA by photosynthetically produced O_2 in the mat, or some other factor which varies with PAR. Both CO_2 fixation and O_2 content of the mat were inversely correlated with rates of NA ($P < 0.05$) (Fig. 8). Clearly, these correlations are complicated by the close physiological relationship of O_2 evolution and CO_2 fixation.

Comparison of the relative rates of NA in light- versus dark-incubated samples provides an indication of the dependence of mat N_2 fixation on light energy. In general, NA in light- and dark-incubated samples was nearly the same (Fig. 3, 4, and 5). At 1330 in all three experiments, however, NA was much higher in light-incubated samples. At this point in the diel cycle, light availability played a role in regulating NA. Samples incubated in the light were apparently able to obtain the necessary energy to support the peak in NA measured at this time, whereas dark-incubated samples were not. During DI, low and variable PAR due to cloudy conditions gave rise to relatively low rates of CO_2 fixation (half of

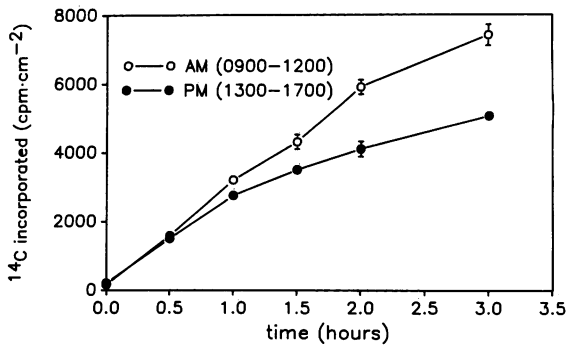

FIG. 6. Photosynthetic uptake of $[^{14}C]NaHCO_3$ by the Shackleford microbial mat over time. Linearity of uptake is compared for morning and afternoon periods. Error bars represent one standard error above and below the mean (n = 3).

the rates measured in DII) and late night/early morning peaks in NA, similar to the those observed in DII and DIII, did not occur. High rates of CO_2 fixation during daylight seem to be necessary for supporting NA at night.

Effect of oxygen on NA. Energy limitation alone cannot produce all of the observed diel variability in NA. For example, late-night maxima in NA during DII and DIII indicated that sufficient energy was available. The rapid increases in NA after sunset and the subsequent nighttime maxima probably indicate an absence of suppression by photosynthetically produced O_2. The potential for O_2 suppression of NA in the mat certainly exists. The enzyme nitrogenase is degraded rapidly in vitro by O_2 (39), and elevated ambient oxygen concentrations have been shown to inhibit NA in a number of natural systems (20, 23, 27). Nitrogen-fixing microorganisms living within the Shackleford mat community (nonheterocystous cyanobacteria and eubacteria) exhibit no morphological barriers to ambient oxygen concentrations. Organisms living within the upper 3 mm of the mat are exposed to oxygen concentrations that range from 50 to 400 μM (ca. 20 to 200% of atmospheric equilibrium) on a diel basis. The effects of increased $[O_2]$ on NA in the mat can be shown experimentally. When oxygen concentrations within the mat were increased, in the absence of changes in PAR and CO_2 fixation, a significant ($P < 0.05$) inhibition of NA resulted (Fig. 9).

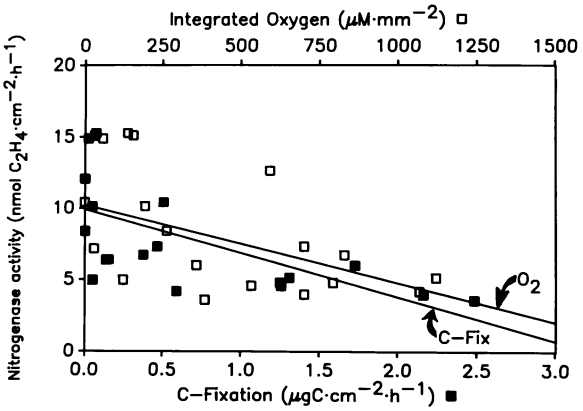

FIG. 8. Relationship of CO_2 fixation and integrated oxygen concentrations on NA in the Shackleford microbial mat. Data were pooled from three diel studies for the analyses. Both regression lines were found to be significantly different from zero ($P < 0.05$ t test). Correlation coefficients were −0.617 for NA versus C fixation and −0.456 for NA versus integrated oxygen.

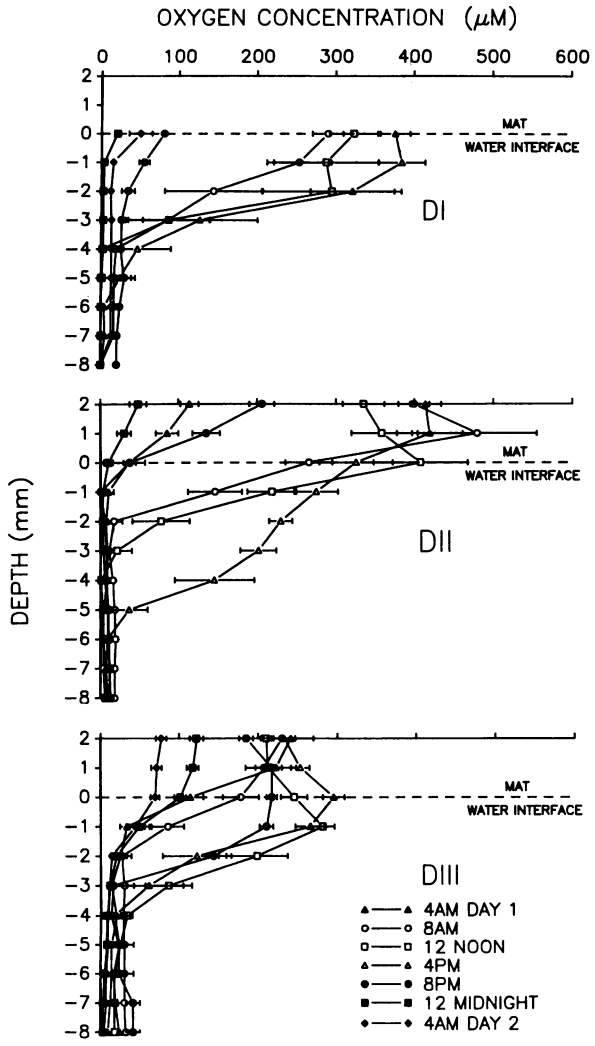

FIG. 7. Vertical distribution of oxygen within the Shackleford microbial mat for three diel cycles. Error bars represent one standard error above and below and mean of six O_2 minielectrode profiles.

The addition of DCMU to the mat during experiment DIII resulted in a significant ($P < 0.05$) stimulation of NA during mid- to late-morning hours (Fig. 10). It is somewhat difficult to unequivocally interpret the results of DCMU experiments because, in addition to lowering oxygen concentrations within the mat (Fig. 11), DCMU causes a decoupling of light gathering from energy-producing processes, resulting in decreased availability of reduced carbon compounds needed to support NA. However, the observed rapid positive effects of DCMU addition on NA and simultaneous negative effects on $[O_2]$ (Fig. 11) lead us to conclude that $[O_2]$ is a strong determinant in the observed suppression of NA. If uncoupling of energy-producing processes (by DCMU) had been an important factor regulating NA, we should have observed a decrease in NA upon DCMU additions, instead of a significant increase. Accordingly, we conclude that suppression of NA by $[O_2]$ is important, particularly during mid- to late-morning hours when photosynthetic O_2 production is maximal.

It has been shown that organisms lacking structural de-

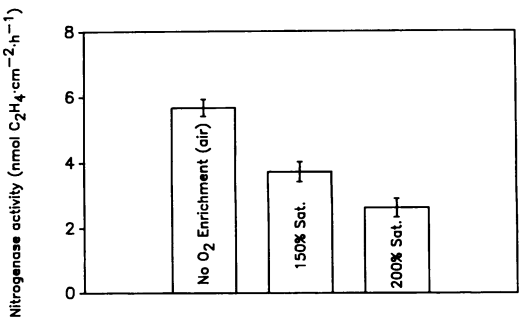

FIG. 9. Effect of experimentally elevated dissolved O_2 concentrations (150 and 200% of atmospheric concentrations) on NA in the Shackleford mat. Error bars represent one standard error above and below the mean ($n = 3$).

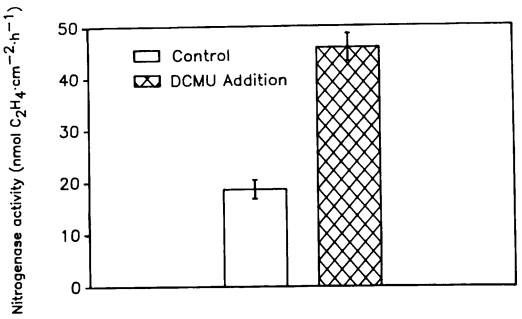

FIG. 10. Effect of the Photosystem II inhibitor DCMU (at 2×10^{-5} M) on NA in the Shackleford microbial mat. The experiment was conducted with mat collected in the morning. Error bars represent one standard error above and below the mean of three samples.

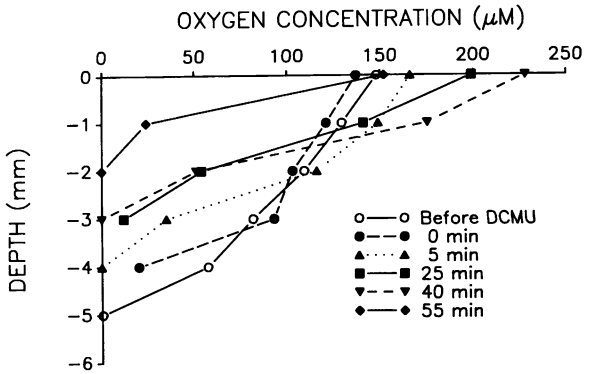

FIG. 11. Dissolved O_2 concentrations, obtained with O_2 mini electrodes, within the mat before and after the addition of the Photosystem II inhibitor DCMU, showing the resulting lowered O_2 tension in the mat.

fenses against oxygen (e.g., heterocysts) use other strategies to avoid O_2 stress on the N_2-fixing enzyme nitrogenase (5, 18, 27). Microorganisms in the Shackleford mat may use one or more of these strategies to combat the great variations in oxygen concentration which they experience on a diel cycle. The temporal separation of O_2-evolving photosynthesis and NA, as suggested by Paerl and Kellar (19), Bautista (M.S. thesis), and Mitsui et al. (18) is probably important. Clumping or aggregation of filaments may also play a important role in the contemporaneous optimization of CO_2 and N_2 fixation, as originally suggested by Carpenter and Price (3).

A number of additional mechanisms have been offered to explain diel variations in NA in other systems. Ammonium regulates NA in natural systems via the strong suppressive impact that this end product of N_2 fixation exerts on the enzyme nitrogenase (39). Interstitial ammonium concentrations were not initially measured during this study. Therefore, we conducted subsequent experiments to investigate the possibility that diel variations in ammonium concentration could be responsible for diel changes in NA. Diel fluctuations in ammonium concentration may be caused by differential rates of decomposition (i.e., ammonification) and ammonium uptake under fluctuating O_2 conditions in the mat. We found mat NA to be unaffected by ammonium concentrations as high as 2,000 μg of N $liter^{-1}$ (Fig. 12). Ammonium concentrations in porewater obtained from the mat by centrifugation or filtration never exceeded 200 μg of N $liter^{-1}$. It is therefore unlikely that diel variations in ammonium concentration could be responsible for the large variations observed in NA.

It has been shown recently that NA in the unicellular cyanobacterium *Synechococcus* sp. is regulated by an endogenous mechanism which periodically lowers daylight photosynthetic (and hence O_2 evolution) rates, thereby enabling cells to minimize O_2 inhibition of nitrogenase (18). Although we have no evidence to refute or support the existence of such a mechanism among the array of potential cyanobacterial N_2 fixers in the mat community, we consider it unlikely that this constitutes an important regulatory mechanism for the community as a whole. The close inverse correlation of both oxygen concentrations and CO_2 fixation with NA, as well as the fact that the observed rates of NA in the mat are probably due to the combined effort of a group of microorganisms, favors an explanation based on environmental regulatory mechanisms.

It is rather difficult to ascertain which organisms in the Shackleford mat community are responsible for the observed NA. Daylight NA may be due to photoautotrophic cyanobacteria and purple sulfur bacteria, while nighttime NA is most likely attributable to heterotrophic and chemoautotrophic eubacteria. The ability to use organic matter as an energy source for NA has been demonstrated in numerous planktonic cyanobacteria (9, 28). The scarcity of reducible carbon compounds in these systems, however, probably prevents heterotrophic N_2 fixation from making an important contribution to the total amount of N_2 fixed (28). Heterotrophic N_2 fixation, by eubacteria as well as cyanobacteria, is much more likely to be important in the Shackleford mat community, due to the presence of substantial quantities of organic debris which can be made available to microorganisms by degradative and fermentative processes active within the mat. We have isolated N_2-fixing heterotrophic eubacteria and photoautotrophic cyanobacteria from the Shackleford mat. Sulfate-reducing bacteria and the purple sulfur bacterium *Chromatium* sp., an obligate anaerobic phototroph, both capable of fixing N_2 (39), have also been observed. It seems likely that the rates of NA we measured reflected the collective N_2-fixing potentials of this diverse group of microorganisms. The depth to which photosynthet-

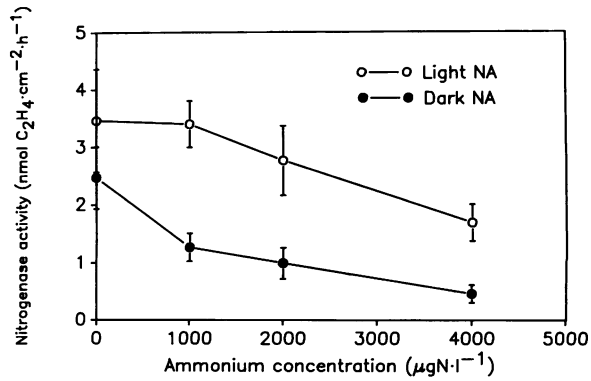

FIG. 12. Effect of experimentally elevated NH_4^+ concentrations on NA in the Shackleford microbial mat. Naturally occurring NH_4^+ concentrations have not been observed to be above 200 μg of N $liter^{-1}$. The rates of NA in this experiment were much lower than those recorded in the diel experiments because the mat was collected in the fall, a time when NA in the Shackleford mat is low (Paerl et al., unpublished data). Error bars represent one standard error above and below the mean (n = 3).

ically produced O_2 penetrates into the mat during the day is an important factor in determining the proportion of whole-mat NA attributable to photoautotrophic, heterotrophic, and chemoautotrophic organisms. The relative abundances, as well as the relative contribution of each member of the mat community to measured (whole-mat) NA, probably vary on diel and seasonal (successional) cycles.

Our finding of higher rates of NA at night in DII and DIII has profound implications for the extrapolation of one-time acetylene reduction measurements to an entire diel period, as is commonly done in the construction of nitrogen budgets (3, 20, 37). For example, if an estimate of N_2 fixed was based on midday rates of NA, daily, and seasonal, nitrogen inputs attributable to this process would be seriously underestimated. It is likely, therefore, that annual nitrogen inputs due to N_2 fixation in mat systems may be much higher than previously assumed.

Regulation of NA on a whole-mat basis is probably best explained by the combined controlling influences of rates and histories of CO_2 fixation and O_2 tension. Heterotrophic or chemoautotrophic NA may make an important contribution at night when O_2 tension is low, whereas light-mediated NA is more important to whole-mat NA in the daytime.

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