Microscale Vertical Profiles of N_2 Fixation, Photosynthesis, O_2 , Chlorophyll *a*, and Light in a Cyanobacterial Assemblage

WALTER K. DODDS

Department of Biology, Montana State University, Bozeman, Montana 59717-0001

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Profiles of $^{15}N_2$ fixation, O_2 production (gross photosynthesis), O_2 concentration, chlorophyll *a* concentration, and photon fluence rates were measured with 50- μm resolution in colonies of the heterocyst-forming cyanobacterium *Nostoc parmelioide*s. Microelectrode measurements were made after 20 h of incubation under $^{15}N_2$ gas. Colonies were frozen, and 50- μm sections were prepared by using a freezing microtome and analyzed for ^{15}N enrichment and chlorophyll *a* concentration. Colonies exhibited steep spatial gradients in rates of gross photosynthesis, O_2 concentration, and irradiance, with the highest values generally occurring at the surface. O_2 concentration, photosynthesis, and irradiance all showed positive correlations, but chlorophyll *a* concentrations varied independently of photosynthesis and irradiance. Forty-four percent of the variation in ^{15}N incorporation was explained by gross photosynthesis (a positive correlation) when incorporation of ^{15}N was expressed per unit of biomass (chlorophyll *a*).

The enzyme nitrogenase is sensitive to O_2 ; this sensitivity has led to speculation that O_2 -depleted microzones may be extremely important in regulating N_2 fixation in pelagic environments (13) and in cyanobacterial mats or layers (1, 10, 11). Recent advances in microelectrode technology have allowed measurement of steep gradients of O_2 (1, 4, 10, 11, 14, 15, 17), photosynthesis (14-16), and light (8) in microbial assemblages. Despite the importance of microenvironmental factors to N_2 fixation, N_2 fixation rates in microbial mats have not been measured at high resolution. In this study, environmental parameters and microbial processes which may influence N_2 fixation were measured on a submillimeter scale and the extent to which they are correlated to actual rates of $^{15}N_2$ incorporation was determined.

MATERIALS AND METHODS

*Nostoc parmelioide*s was the test organism. This N_2 -fixing cyanobacterium is commonly found in streams in the United States (2, 3, 21; W. Dodds, J. Phycol., in press). The cyanobacterium forms ear-shaped colonies approximately 1 cm in diameter and 0.5 mm thick that attach to rocks in flowing water. The colonies are leathery and essentially unialgal. Rocks with colonies attached were collected from Hyalite Creek, Gallatin County, Mont. (45°32' N, 111°0' W), and used within 2 h.

Three colonies were removed from rocks and held in place with fine needles, flat side up, on an agar surface. The immobilized colonies were then placed in a chamber with recirculated stream water, and current velocity was set at 0.5 $cm\ s^{-1}$ as measured by a thermistor current meter (9) 2 mm above the colonies. The chamber was sealed and purged for 30 min with He gas to remove $^{14}N_2$ gas; a 202-ml headspace of 99 atom% $^{15}N_2$ (Monsanto Corporation), O_2 , and CO_2 (80:20:1) was then added, followed by a 20-h incubation at 19°C under a high-intensity quartz-halogen light at 1,380 microeinsteins $m^{-2}\ s^{-1}$ (measured with a LiCor 190S quantum sensor).

Colonies were removed from the chamber, and profiles of O_2 and photosynthesis (O_2 production) were measured with an O_2 microelectrode (15) under a cool halogen fiberoptic light source at 550 microeinsteins $m^{-2}\ s^{-1}$. Before gross

photosynthesis was measured with an O_2 microelectrode, the colony was illuminated and allowed to attain a steady state O_2 concentration. In this state, O_2 production by photosynthesis was equal to the loss of O_2 resulting from O_2 consumption and mass transport away from the assemblage. The rate of decline in O_2 concentration was measured within 2 s after illumination was terminated, and the rate of decline (consumption plus mass transport) was equivalent to the O_2 production rate in the illuminated steady state (14-16). This technique measures only gross photosynthesis (14-16).

The diameter of the tip of the O_2 electrode was 10 μm ; the electrode produced a 0.03-nA current in N_2 -sparged stream water and a 4.79-nA current at 0.24 mM O_2 when a polarizing voltage of -0.75 V was applied. Because the outer envelope of *N. parmelioide*s colonies is extremely tough, the tip of a 26-gauge hypodermic needle was used to slit the envelope of each colony before electrode insertion. Microscopic examination revealed that the slit closed around the inserted electrode. Irradiance profiles were measured with a flat-tipped fiberoptic light probe (8). Incoming light was provided by a halogen fiberoptic light which was either unfiltered or filtered with a red, green, or blue filter. The red filter cut off wavelengths shorter than 600 nm, the green filter had a peak transmission at 535 nm and a 1/2 bandwidth (50% transmission) of 50 nm, and the blue filter had a peak transmission at 470 nm and a 1/2 bandwidth of 70 nm.

Colonies were then placed between two glass microscope slides, frozen, and sectioned on a Reichert-Jung 2800 Frigocut N freezing microtome at -30°C. This freezing procedure did not change colony thickness by more than 5% (measured directly with a micrometer). The colonies were mounted for sectioning in O.C.T. embedding medium (Miles Scientific, Div. Miles Laboratories, Inc.). Embedding medium was not included in samples to prevent nitrogen contamination (inorganic or organic). However, analysis of the N content of the O.C.T. medium with a Carlo Erba 1106 elemental analyzer showed <10⁻³% N by weight, making contamination unlikely.

Whole *N. parmelioide*s colonies were naturally oriented (flat side up) and sectioned from top to bottom. The sectioning plane was parallel to the top surface of the colony.

FIG. 1. O_2 concentration profiles in three *N. parmelioide* colonies. The symbols used on this graph represent three different colonies. The same symbol denotes the same colony in Fig. 1 to 4 and 6 to 9.

Sections (3 by 6 by 0.05 mm) were dried at 50°C for 12 h and then analyzed for percent enrichment with ^{15}N by emission spectrometry (20). Chlorophyll *a* was extracted from sections (1 by 1 by 0.05 mm) by adding 0.2 ml of dimethylsulfoxide (6), freezing and thawing the sections three times, and then adding 10 ml of acetone-water (9:1). The phaeophytin-corrected chlorophyll *a* concentration was determined fluorometrically (19).

RESULTS

There were steep gradients of O_2 in individual *N. parmelioide* colonies. Profiles of all three illuminated colonies

FIG. 2. Profiles of photosynthetic O_2 production in three *N. parmelioide* colonies. The same symbol denotes the same colony in Fig. 1 to 4 and 6 to 9. The *x* axis represents photosynthesis.

FIG. 3. Profiles of chlorophyll *a* in three *N. parmelioide* colonies. The same symbol denotes the same colony in Fig. 1 to 4 and 6 to 9.

showed surface O_2 concentrations from 1.3 to 1.9 times the saturation concentration (0.24 mM O_2). These values dropped to 0.68 to 1.0 times the saturation concentration at the bottom of the colonies, away from the light (Fig. 1). However, as depth increased there was a slight initial O_2 increase in one colony.

There were also steep gradients of O_2 production (gross photosynthesis) throughout each individual colony. Profiles showed very low rates of photosynthesis at the bottom of the colonies and higher rates at the colony surface (Fig. 2). Thus, assuming that photosynthesis translates into cyanobacterial growth, most growth probably occurs on the upper surface of the *N. parmelioide* colony.

FIG. 4. Profiles of unfiltered light in three *N. parmelioide* colonies. The same symbol denotes the same colony in Fig. 1 to 4 and 6 to 9.

FIG. 5. Profiles of unfiltered, red, green, and blue light through an *N. parmelioidea* colony. These data are from the colony represented by the open square ($\square$) in all of the other figures. Symbols: $\circ$, unfiltered light; $\triangle$, red light; $\square$, green light; and $\diamond$, blue light.

The chlorophyll profiles showed high heterogeneity between colonies (Fig. 3). There was also heterogeneity within colonies, but this was the result of small changes between sections which translated into fairly smooth profiles. The heterogeneity of tissue within colonies of *N. parmelioidea* has previously been documented (3).

Light was attenuated rapidly in all three colonies, with 91 to 95% of surface irradiance being depleted at the bottom of colonies (Fig. 4). The colony with the highest chlorophyll *a* concentration attenuated the most light (Fig. 3). The nonlinearity of the plots of light versus depth (Fig. 4) shows the uneven distribution of biomass throughout the colony, as do the chlorophyll profiles. If the colonies were homogeneous in terms of light absorption (pigment composition and scattering), then they would provide linear plots when data are presented on a log scale, as in Fig. 4.

All wavelengths were not attenuated to the same degree through the colonies (Fig. 5); 4% of the total irradiance passed through the colonies. Of the red, green, and blue light, 6, 9, and 11%, respectively, passed through. The high levels of light absorption at all wavelengths at the surface correlate with the low levels of photosynthesis recorded at the bottom of the colonies (Fig. 2). The slight increase in unfiltered light between 200 and 300 μm is probably a result of backscatter from the agar surface, where the colony being measured was held. Measurements did not include estimates of backscattered light (8).

Regions with high levels of gross photosynthesis also had high O_2 concentrations (Fig. 6A). There was also rough correspondence between light and photosynthesis (Fig. 6B). The relationship between chlorophyll and photosynthesis was not well defined (Fig. 6C). These data illustrate that O_2 concentration, light, and photosynthesis are interdependent even at submillimeter scales.

Individual colonies had very different rates of $^{15}\text{N}_2$ incorporation under illumination, which was reflected in percent ^{15}N enrichment (Fig. 7). The colony with high ^{15}N enrichment also had high chlorophyll concentrations (Fig. 8). Therefore, for comparison between colonies, ^{15}N enrich-

FIG. 6. Relationship between photosynthesis, O_2 , light, and chlorophyll *a* in *N. parmelioidea* colonies at different depths within the three colonies. The same symbol denotes the same colony in Fig. 1 to 4 and 6 to 9. The y axes in panels B and C and the x axis in panel A represent photosynthesis. In panel B, the proportion of total light is equal to (light at the depth of measurement)/(light at the *N. parmelioidea* surface).

ment was normalized to chlorophyll *a* concentration. When multiple regression analysis was used to ascertain factors correlated to biomass-corrected $^{15}\text{N}_2$ incorporation, gross photosynthesis was positively correlated to incorporation (Fig. 9) and explained 44% of the variance ($P < 0.01$). O_2 concentration and light explained 7% (a negative correlation) and 4% (a positive correlation) of the variance, respectively, but these correlations were not significant ($P > 0.05$).

DISCUSSION

The method presented above for obtaining profiles of N_2 fixation has benefits and limitations. Calculating actual rates of N_2 incorporation by *N. parmelioidea* is problematic because an estimate of cellular N is required. Therefore, N_2

FIG. 7. Profiles of N_2 fixation through three *N. parmelioide* colonies. The same symbol denotes the same colony in Fig. 1 to 4 and 6 to 9.

fixation is expressed as atom percent ^{15}N enrichment or chlorophyll *a*-corrected ^{15}N enrichment. *Nostoc* colonies have areas with large amounts of mucopolysaccharide gel, which has a significant N content (5) and which could also contain many non- N_2 -fixing bacteria. Therefore, N content cannot be used to estimate the biomass of physiologically active cells because N contained in gel is always included in organic N measurements. For example, if the data were presented as an incorporation rate of N based on organic N present, an area with a large number of cells, each of which had low activity, would be undistinguishable from an area with a large amount of gel and a few cells that were actively fixing N_2 . Since the mucopolysaccharide gel also contains proteins, total protein cannot be used to normalize rates. Furthermore, N analysis requires a fairly large sample, which was not available given the size of the *N. parmelioide* colonies used and the amount needed for ^{15}N and chlorophyll *a* analysis.

Because *N. parmelioide* colonies are small, and because a 3- by 6- by 0.05-mm section was needed (10 μg of N) for

FIG. 8. Atom percent ^{15}N enrichment (excess) of *N. parmelioide* colonies as related to chlorophyll *a* concentrations. The same symbol denotes the same colony in Fig. 1 to 4 and 6 to 9.

FIG. 9. Relationship between chlorophyll *a*-corrected ^{15}N incorporation and photosynthesis for three *N. parmelioide* colonies. The same symbol denotes the same colony in Fig. 1 to 4 and 6 to 9. The *x* axis represents photosynthesis.

emission spectroscopy, only one section was obtained at each depth for $^{15}N_2$ and chlorophyll analyses. Therefore, measurements within each colony could not be replicated, and because some samples were lost during preparation there are some datum points missing from Fig. 7. Missing data would be less of a problem with larger cyanobacterial assemblages, in which samples could be replicated. If more N per unit volume was present or if thicker slices were taken, then patches of smaller area could have been used, allowing for sample duplication.

Finally, freezing water in samples can cause expansion (ice is approximately 9% less dense than water). If pressure is applied during freezing, expansion is horizontal. With the sample freezing technique that I used on *N. parmelioide*, there was no more than 5% shrinkage (due to compression during freezing). If each section is 5% too small, then six sections into the colony there is a position error of 30%. In addition, there were six 50- μm sections in the colonies that were 300 μm thick (measured in the microelectrode profiles), suggesting a maximum position error of 17% per section.

The technique of measuring $^{15}N_2$ incorporation directly in intact cyanobacterial assemblages yielded the N_2 fixation measurements with the highest resolution (50 μm) of which I am aware. Similar data could not be generated by using acetylene reduction. The steep gradients in light, O_2 , and photosynthesis would be difficult to duplicate in a system which was dissected into component layers before N_2 fixation measurement by acetylene reduction. Furthermore, acetylene can affect other factors, such as denitrification (18), and organisms which oxidize ethylene can interfere with measurement of acetylene reduction.

Use of a freezing microtome to take high-resolution samples for analysis of chlorophyll *a* and uptake of $^{15}N_2$ allows simultaneous measurement of two important biological factors on ecologically relevant scales. The freezing and sectioning technique presented above can now be added to the repertoire of techniques applied by ecologists examining interrelationships between microbial processes in microhabitats. The sectioning technique may be useful for making other measurements in microbial assemblages, such as locating radiolabeled substrates for growth and analyzing biological molecules other than chlorophyll, because freezing during preparation preserves the samples.

Microbial processes are interdependent on a small scale. The relationships between photosynthesis, O_2 , and irradi-

ance are related to the fact that light drives photosynthesis and O_2 is produced by photosynthesis. Incorporation of $^{15}N_2$, scaled by chlorophyll *a* concentrations, was dependent upon rates of photosynthesis and was slightly negatively correlated to O_2 concentrations. This occurred despite a strong positive relationship between O_2 concentrations and photosynthesis. Systems which require the presence of low O_2 tensions for N_2 fixation may include environments in which the predominant N_2 fixers are bacteria or non-heterocyst-forming cyanobacteria (e.g., see references 1, 10, 12, and 13). Since O_2 concentrations were not highly correlated to N_2 fixation, bacteria may not be important N_2 fixers within *N. parmelioides* colonies.

Rates of ^{15}N fixation were measured only in the light. In systems which are not dominated by heterocyst-forming cyanobacteria, dark $^{15}N_2$ fixation can be extremely important (1). However, it has been shown that stream-inhabiting *Nostoc* colonies exhibit the highest rates of N_2 fixation in daylight (7), and acetylene reduction by *Nostoc pruniforme* is also lower under reduced irradiance (5). Therefore, understanding N_2 fixation in illuminated colonies is crucial to explaining factors which have the greatest ecological relevance to total N_2 fixation by *N. parmelioides*.

The data presented here confirm that microbial processes are influenced by the microhabitat measured on a scale of <1 mm, even in a unialgal assemblage. Fine sectioning of $^{15}N_2$ -enriched cyanobacterial assemblages in concert with microelectrode measurements of O_2 , photosynthesis, and light clarified the relationships between N_2 fixation and microenvironmental conditions. N_2 fixation varied over 100- μm intervals in a natural benthic algal community, and this variation was related to gradients of gross photosynthesis on the same scale.

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