On-Line Monitoring of Nitrogenase Activity in Cyanobacteria by Sensitive Laser Photoacoustic Detection of Ethylene†

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A new and extremely sensitive method for measuring nitrogenase activity through acetylene reduction is presented. Ethylene produced by nitrogenase-mediated reduction of acetylene is detected by using laser photoacoustics (LPA). This method possesses a detection limit making it 3 orders of magnitude more sensitive than traditional gas chromatographic analysis. Photoacoustic detection is based on the strong and unique absorption pattern of ethylene in the CO₂ laser wavelength region (9 to 11 μ m). The high sensitivity allowed on-line monitoring of nitrogenase activity in a culture of the heterocystous cyanobacterium *Nodularia spumigena*, which was isolated from a water bloom in the Baltic Sea. This setup makes it unnecessary to take subsamples from the culture and avoids long incubations in sealed vials. The fast response of the LPA technique allows measurement of real-time dynamic changes of nitrogenase activity. The method was used to analyze in vivo saturation of nitrogenase by acetylene in *N. spumigena*. It is demonstrated that 20% acetylene does not saturate nitrogenase and that the degree of saturation depends on light intensity. With concentrations of acetylene as low as 2.5% it is possible to assess the degree of saturation and to extrapolate to total nitrogenase activity. In *N. spumigena* nitrogenase activity becomes independent of light intensity above 20 to 80 μ mol of photons m⁻² s⁻¹ at 20% O₂.

The fixation of dinitrogen (N₂) by microorganisms is the only process in nature that counteracts the losses of combined nitrogen from the environment by denitrification. Except for a few highly specialized prokaryotic species that are capable of using molecular nitrogen as their source of nitrogen for growth, all living organisms depend on one or another source of combined nitrogen, such as nitrate (NO₃⁻), ammonium (NH₄⁺), or organic nitrogen (e.g., amino acids). Often, these nutrients are short in supply, and in natural waters primary productivity usually is limited by the availability of nitrogen, except when diazotrophic (N₂-fixing) cyanobacteria are the predominant primary producers (14). Together with photosynthesis, nitrogen fixation ranks among the most vital processes on Earth. Hence, its study is well deserved.

The acetylene reduction method is the method most widely used for the measurement of nitrogenase activity. This method is based on the property of nitrogenase to reduce compounds with a triple bond (5, 6). In addition to reducing N₂ to NH₄⁺, nitrogenase reduces acetylene to ethylene. Both of these gases can be measured with high sensitivity by gas chromatography with flame ionization detection. The acetylene reduction method is easy and inexpensive. Notwithstanding its sensitivity, the acetylene reduction technique requires incubations which take from minutes when very active dense cell suspensions are used to several hours for material that contains less activity (i.e., in dilute suspensions or in cell suspensions containing low biomass). Often it is necessary to concentrate natural samples or even cultures and incubate them for considerable periods of

time, which impairs the temporal resolution of these measurements. Moreover, long incubations and the use of concentrated cell suspensions in sealed vials will induce artifacts like changes in conditions such as oxygen and carbon dioxide concentrations or pH, all of which strongly affect nitrogenase activity. In phototrophic organisms, dense cell suspensions will also influence the local light intensity in the suspension due to strong self-shading effects. Usually, acetylene reduction assays are carried out by supplying 10 to 20% acetylene in the gas phase (22). These concentrations are considered to saturate nitrogenase. Because the affinity of nitrogenase for acetylene is much higher than that for dinitrogen, at these acetylene concentrations little or no nitrogen is fixed (23). Hence, the organism may experience this situation as a nitrogen limitation to be counteracted by an increase of the level of nitrogenase. Prolonged incubations during the acetylene reduction assay are therefore problematic (15, 18).

With the development of powerful lasers and sensitive microphones, gas detection by conversion of light into acoustical signals (laser photoacoustics [LPA]) became very sensitive. For instance, ethylene can be measured with a detection limit of 6 ppt (by volume) (4, 7). The LPA detection of ethylene is based on its strong absorption in the infrared (IR) region, at specific laser lines of a CO₂ laser. IR detection of ethylene has been used by Ashtakala et al. (1) to measure nitrogenase activity, with a sensitivity comparable to that of gas chromatography, in the parts-per-billion (by volume) range. Due to its much lower detection limit, the LPA technique can be used to measure on-line the acetylene reduction of small amounts of biomass and to monitor small changes in nitrogenase activity in real time.

LPA was applied here to investigate nitrogen fixation in the heterocystous cyanobacterium *Nodularia spumigena*. This cyanobacterium forms water blooms in the Baltic Sea (14). The Baltic Sea is located in Northern Europe and represents the

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FIG. 1. LPA setup. Components: 1, grating to select wavelengths; 2, chopper; 3, water-cooled CO₂ laser tube; 4, focusing lens; 5, photoacoustic cell; 6, front mirror; 7, gas flow system.

largest body of brackish water in the world. It is a largely enclosed basin and is connected via only a narrow channel to the North Sea. The Baltic Sea receives considerable input of nutrients via runoff and wet deposition. In recent years this has led to increased phytoplanktic blooms (12). In summer primary productivity becomes nitrogen limited, resulting in the development of diazotrophic blooms of *Aphanizomenon flos-aquae* and *N. spumigena*. These blooms contribute considerably to the nitrogen input of the Baltic Sea, but no precise estimates of this input are available. The LPA method allows precise mea-

surements and monitoring of nitrogen fixation, with high temporal resolution.

MATERIALS AND METHODS

LPA detection of ethylene. The detection of ethylene is based on its strong IR absorption at specific laser lines of a CO₂ laser in the 9- to 11- μ m-wavelength region. Other molecules, such as NH₃, O₃, and H₂O, also absorb in this wavelength region and can be detected similarly by the photoacoustic technique (8, 17).

The LPA setup consists of a high-power CO₂ laser (100 W for an acoustic cell placed inside the laser cavity [Fig. 1]). The gas from the outlet of the sample cuvette flows continuously through the acoustic cell at atmospheric pressure. At well-chosen laser wavelengths the IR radiation is absorbed by trace gas molecules, and as a consequence the molecules are excited to a higher internal energy level. The absorption wavelength corresponds to a rotational-vibrational excitation. Molecular de-excitation (i.e., collision relaxation) increases the kinetic energy and temperature of the gas surrounding the excited molecules. Since this process occurs in an acoustic cell of constant volume, it will cause an increase in pressure. When the light source is chopped at an audio frequency, pressure fluctuations of the same frequency occur inside the acoustic cell. The pressure changes are detected by a sensitive microphone mounted in the center of the acoustic cell and amplified by a lock-in amplifier.

Ethylene possesses a distinct fingerprint-like absorption spectrum in the CO₂ laser wavelength region (2). The strongest ethylene absorption is at the 10P14 CO₂ laser line (wavelength, 10.53 μ m; absorption strength, 30.4 atm⁻¹ cm⁻¹, and a much weaker absorption is observed on the 10P12 line (10.51 μ m, 4.8 atm⁻¹ cm⁻¹). During one concentration measurement, the corresponding microphone signals on both laser lines are determined. The difference yields the concentration corrected for contributions from other gases and from window absorption. The resulting detection limit for ethylene is 6 parts ethylene in 10¹² parts air (7, 8). The time response is determined by the time needed to switch the grating between the two laser lines. In the present setup the sampling rate is 20 s, and with steady-state concentrations of 20 to 600 ppb, we were able to detect changes of less than 0.5 ppb.

Incubation chamber. All experiments were carried out with a temperature-controlled gas-tight incubation chamber (Fig. 2), with a well-defined light regimen (light field, 1.5 cm) and a volume of 12 ml filled with 6 ml of sample (a modification of the system described in reference 3). A gas flow was led over the watery sample, which was vigorously mixed by a magnetic stirrer in order to accomplish fast gas exchange. A Clark-type oxygen electrode was placed in the incubation chamber to continuously monitor the oxygen concentration during the experiment. A slide projector (250-W halogen lamp) was used as light source. Different light intensities were obtained by positioning slides with neutral-density filters in the light beam. The incubation chamber was connected to the photoacoustic cell by polystyrene tubing (Fig. 1). This setup allowed on-line monitoring

FIG. 2. Incubation chamber. Components: 1, oxygen electrode; 2, magnetic stirrer; 3, water cooling inlet and outlet; 4, gas inlet and outlet. The culture volume used for incubations was 8 ml.

FIG. 3. Acetone trap. Components: 1, water inlet; 2, water outlet; 3, height-adjustable glass tube to regulate water level in the trap; 4, safety water outlet; 5, floating valve to close the gas outlet when the water level becomes too high; 6, gas inlet; 7, hole where the acetone-free gas enters the central tube that leads the gas outlet; 8, gas outlet.

of nitrogenase activities in the culture at different light, acetylene, and oxygen concentrations.

Gas mixtures. Clinical air (mixture of N_2 and O_2 [20%], low CO_2) or mixtures of nitrogen and oxygen were mixed with acetylene by using mass flow controllers (Brooks model 5850E). Gases were obtained from Hoek-Loos (Dieren, The Netherlands). Gas mixtures were prepared with 0 to 20% acetylene. The contaminating ethylene concentration present in the acetylene increased proportionally with the acetylene concentration in the gas mixture.

At high acetylene levels the photoacoustic signal increased dramatically at both laser lines due to the presence of acetone, which served as the solvent of acetylene in the gas cylinder. In particular, this became a problem when the pressure in the gas cylinder decreased during the course of the measurements. Because these acetone concentrations caused intolerably high noise levels, an acetone trap was introduced into the system (Fig. 3). The acetone trap consisted of a water chamber with a continuous water flow through which the gas was bubbled. The trapping process is based on the difference in the solubilities of acetone, acetylene, and ethylene in water. A water flowthrough system was chosen to avoid acetone saturation of the water volume of the trap. The trap also scavenged ethanol, which was an additional interfering gas because it was used as a solvent for 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, an inhibitor of photosystem II (13). During the determination of the acetylene saturation curves, the trap was placed between the incubation chamber and the LPA cell (Fig. 1). This position of the trap allowed the accurate regulation of acetylene concentrations in the incubation chamber by the mass flow controllers. Due to the low solubility of ethylene in water, no significant losses of ethylene occurred. This was confirmed by injecting known amounts of ethylene into the gas flow before and after the trap. However, the trap increased the instrument response time of the system from 1.8 to 5.8 min. If fast response measurements were required, the trap was placed in front of the incubation chamber. Since some acetylene is also retained in the trap, the actual acetylene concentration in the incubation chamber was measured separately by gas chromatography (22).

The rate of gas flow over the culture was kept as high as possible in order to obtain a fast time response. For most experiments the optimal flow rate was determined to be 2.5 liters h^{-1} , resulting in C_2H_2 concentrations in the range of 20 to 600 ppb and allowing detection of small changes in nitrogenase activity. The measured rates of ethylene production in the gas phase are described by

$$P_t = P(1 - e^{-t/D}) + P_0 e^{-t/D}; t \geq 0 \quad (1)$$

Here P is the rate of ethylene production (nanoliters $hour^{-1}$) by the cyanobacteria. P_0 , P_t , and P are the production rates at or before time zero, at time t , and at steady state, respectively. The steady-state production rate P equals P_0 plus ΔP . ΔP is assumed to be constant. D is the gas dilution constant (hours $^{-1}$).

Culture. The heterocystous cyanobacterium *N. spumigena* was used in this study. This strain was provided by P. Hayes (Bristol University, Bristol, United

Kingdom), who isolated it from a cyanobacterial bloom in the Baltic Sea. *N. spumigena* was grown in a mixture of one part artificial seawater medium ASN 3 and two parts freshwater medium BG 11 (21). The medium was devoid of any source of combined nitrogen, and therefore, the organism grew diazotrophically. The salinity of the medium was approximately 9‰. The cultures were grown as batch cultures in aerated Kluver flasks at 20°C with continuous illumination at an intensity of 15 μmol of photons $m^{-2} s^{-1}$. For experiments, exponentially growing cultures were used. The chlorophyll (Chl) contents of the samples used in the experiments were in the range of 0.7 to 1.66 μg of Chl a ml^{-1} .

RESULTS

Acetylene saturation of nitrogenase activity in vivo. Figure 4 shows ethylene production rates (P_t) during the stepwise increase of the acetylene concentration from 0 to 20% during dark incubation (Fig. 4A) and with illumination at 80 μmol of photons $m^{-2} s^{-1}$ (Fig. 4B). Each acetylene concentration was applied for at least five volume changes (0.5 h) until a steady-state C_2H_4 photoacoustic signal was reached. The experimental data were fitted to equation 1, and production rates were used for further analyses. A complete set of measurements at a single light intensity, therefore, took 7 to 12 h. Five different levels of illumination were examined (Table 1). From these results, acetylene saturation curves were derived by using a Michaelis-Menten equation:

$$P_s = \frac{P_{max} \times [S]}{(K_m + [S])}$$

where P is the velocity of ethylene production (micromoles $hour^{-1}$ milligram of Chl a^{-1}), P_{max} is the maximum ethylene production rate (micromoles $hour^{-1}$ milligram of Chl a^{-1}), K_m is the apparent affinity constant (percent C_2H_2), and $[S]$ is the substrate concentration (percent C_2H_2).

As shown in Fig. 4B, incubation of the culture at a constant light intensity of 80 μmol of photons $m^{-2} s^{-1}$ for 10 h resulted in an increase of nitrogenase activity. At the end of this experiment, after 11 and 12 h of incubation, the acetylene concentration was lowered to 4.8 and 1%, respectively. The ethylene production increased linearly over time by 0.11 $nl h^{-2}$. This increase may have been the result of (i) growth, (ii) higher nitrogenase content, or (iii) higher specific activity of the enzyme. Due to the presence of acetylene, nitrogenase would reduce less N_2 , and consequently, the organism may become nitrogen depleted. This nitrogen depletion may increase nitrogenase activity, e.g., by increasing the flow of energy and reducing equivalents to the enzyme. This increase was not observed when the assay was carried out in the dark (data not shown). The rates of acetylene reduction used for Michaelis-Menten fits were corrected for this increase in activity during the course of the experiment; the data of Fig. 5 correspond to the initial nitrogenase activity at $t = 0$.

The nitrogenase activities (P_s) as a function of acetylene concentration were used to construct acetylene saturation curves (Fig. 5). The acetylene saturation curves measured in the dark and with illumination at 80 μmol of photons $m^{-2} s^{-1}$ showed good fits to the Michaelis-Menten enzyme kinetics equation up to acetylene concentrations of 10% (Fig. 5A). The acetylene saturation curves measured at light intensities of 9, 34, and 450 μmol of photons $m^{-2} s^{-1}$ fit very well to the Michaelis-Menten equation at all acetylene concentrations tested (Fig. 5B). The experiments shown in Fig. 5A and B differed in their gas handling. The results depicted in Fig. 5A were obtained when acetylene was mixed with air, and consequently, O_2 (and N_2) concentrations decreased significantly at higher applied acetylene concentrations. No corrections for this effect were made (Fig. 5A). The data depicted in Fig. 5B

FIG. 4. Rates of ethylene production (P_t) by *N. spumigena* at different acetylene concentrations plotted against time. Arrows indicate when steady-state values are reached at the acetylene concentrations noted above the arrows. (A) Acetylene saturation curve measured in the dark; (B) acetylene saturation curve measured at 80 μmol of photons $\text{m}^{-2} \text{s}^{-1}$.

were obtained with mixtures of C_2H_2 , N_2 , and O_2 in which the concentration of O_2 was kept constant at 20%. Thus, the apparent disagreement between the Michaelis-Menten fit and the experimental data of Fig. 5A at high acetylene concentrations stems from an experimental artifact, i.e., that the O_2

concentration was not kept constant during these measurements.

The results shown in Fig. 5 are summarized in Table 1. The degrees of saturation [$(P_s/P_{\text{max}}) \times 100\%$] of nitrogenase activity at 2.5 and 20% acetylene are derived from the Michaelis-

TABLE 1. Apparent K_m and $V_{\max}$ values for acetylene reduction in *N. spumigena* at different light intensities together with degree of saturation at 2.5 or 20% acetylene in the gas flow

I (μmol of photons $\text{m}^{-2} \text{s}^{-1}$)	K_m (%) ^a	$V_{\max}$ (μmol h^{-1} mg of $\text{Chl } a^{-1}$) ^a	r^2	Degree of saturation (%) ^b with:	
				2.5% C_2H_2	20% C_2H_2
Dark	1.8	1.04	0.989	58	92
9	5.1	4.17	0.993	33	80
30	5.6	6.93	0.992	31	78
80	6.9	4.70	0.821	26	74
450	7.4	3.67	0.999	25	73

^a Fitted with the Michaelis-Menten kinetic model.

^b Defined as $(P_s/P_{\max}) \times 100\%$.

Menten fit. Remarkably, even at 20% C_2H_2 , in vivo nitrogenase activity in *N. spumigena* was not saturated. The highest degrees of saturation were obtained for dark conditions, i.e., 58 and 92% saturation at 2.5 and 20% acetylene in the gas phase, respectively. For light intensities higher than 9 μmol of photons $\text{m}^{-2} \text{s}^{-1}$, the degree of saturation appeared to be only slightly influenced by the specific light intensity applied. Under illumination, the average degrees of saturation were 30 and 78% at 2.5 and 20% acetylene, respectively. The apparent K_m increased slightly with increasing light intensity (in the dark and at 450 μmol of photons $\text{m}^{-2} \text{s}^{-1}$, $K_m = 1.8$ and 7.4% C_2H_2 , respectively). This allowed us to perform the acetylene reduction assay at, e.g., 2.5% C_2H_2 , since a correction for partial saturation can be applied.

Light saturation of in vivo nitrogenase activity. Figure 6 shows ethylene production rates (P_t) for an experiment in which *N. spumigena* was first incubated for 30 min in the dark, then kept for 3 h at 50 μmol of photons $\text{m}^{-2} \text{s}^{-1}$, and subsequently returned to dark conditions. The experiment was carried out with 2.5% acetylene in the gas phase. In the dark the rate of ethylene production was 14.5 nmol h^{-1} . When switched to light conditions, the cyanobacteria responded instantaneously; the ethylene production jumped to 44 nmol h^{-1} (the delayed response is due to the dilution rate). Subsequently, the rate of acetylene reduction slowly increased further by 5.5 nmol h^{-2} . After 3 h of illumination, the change to dark conditions resulted in a decrease of the ethylene production rate from 55 to 34 nmol h^{-1} . Thus, after 3 h of incubation in light, the dark acetylene reduction rate was considerably higher than the 14.5 nmol h^{-1} observed at $t = 0.5$ h. The increase per hour in the dark was calculated to be 6.6 nmol h^{-2} , slightly higher than the increase per hour observed during illumination (5.5 nmol h^{-2}). When these values were corrected for the difference in degrees of saturation of nitrogenase in the dark and in the light (57 and 30%, respectively), the increase in nitrogenase activity became 50% higher in the light than in the dark (18 and 12 nmol h^{-1} , respectively).

The observed light-induced increase of acetylene reduction is most likely due to an increase in specific nitrogenase activity. The rate of increase was too high to be explained by growth of the organism (growth rate, $>36 \text{ h}^{-1}$).

Figure 7A depicts the ethylene production rates (P_t) measured during a series of irradiations with 2.5% C_2H_2 . The data were fitted to equation 1 to obtain steady-state values, P_r , which were then plotted versus irradiance (Fig. 7B). The data of Fig. 7B were fitted with a light saturation model (10, 26) which is frequently used for carbon fixation or oxygen production measurements:

$$P_t = P_{\max}[1 - e^{-\alpha I/P_{\max}} + P_{\text{dark}}] \quad (2)$$

where P_t is the steady-state ethylene production rate (micromoles hour^{-1} milligram of $\text{Chl } a^{-1}$) at a specific irradiance I , $P_{\max}$ is the maximum light-induced ethylene production rate (micromoles hour^{-1} milligram of $\text{Chl } a^{-1}$), α is the nitrogen fixation efficiency [micromoles hour^{-1} milligram of $\text{Chl } a^{-1}$ (micromoles $\text{meter}^{-2} \text{second}^{-1})^{-1}$], P_{dark} is the ethylene production rate in the dark (micromoles hour^{-1} milligram of $\text{Chl } a^{-1}$), and I is irradiance (micromoles of photons $\text{meter}^{-2} \text{second}^{-1}$).

This experiment was carried out twice (results from only one of these are shown in Fig. 7). The rates of maximum total acetylene reduction ($P_{\max} + P_{\text{dark}}$) for *N. spumigena* in these two experiments were estimated to be 2.2 and 3.5 μmol of C_2H_4 mg of $\text{Chl } a^{-1} \text{h}^{-1}$. Because of the differences in specific nitrogenase activity between the cultures, the light intensities at which these maximum rates were obtained were likewise different and amounted to 18 and 80 μmol of photons $\text{m}^{-2} \text{s}^{-1}$, respectively. The dark rates (P_{dark}) in the two experiments were 1.2 and 2.2 μmol of C_2H_4 mg of $\text{Chl } a^{-1} \text{h}^{-1}$, and the affinities (α) were estimated to be 0.18 and 0.06 μmol of C_2H_4 mg of $\text{Chl } a^{-1} \text{h}^{-1}$ (μmol of photons $\text{m}^{-2} \text{s}^{-1})^{-1}$. The $P_{\max}$ (defined as the total maximum rate of acetylene reduction minus P_{dark}) values were virtually equal and were estimated to be 1.1 and 1.3 μmol of C_2H_2 h^{-1} mg of $\text{Chl } a^{-1}$. From these values of $P_{\max}$ and α , I_k ($I_k = P_{\max}/\alpha$) was calculated as 6 and 21 μmol of photons $\text{m}^{-2} \text{s}^{-1}$, respectively. These low values of I_k demonstrate that the efficiency of *N. spumigena* for light-mediated diazotrophy is high. The low light irradiances at which nitrogenase is saturated indicate that light is not likely to be the limiting factor for nitrogen fixation under normal light conditions. Moreover, *N. spumigena* showed a high rate of dark nitrogen fixation compared to the maximum activity in the light.

DISCUSSION

The acetylene reduction assay is one of the most frequently used methods to measure nitrogenase activity. Combined with gas chromatographic determination of acetylene and ethylene, it is a fast, inexpensive, sensitive, and easy method. However, the method usually requires incubation in sealed vials with either relatively long incubation periods or high concentrations of biomass, or even both, when material possessing low activity is investigated. Extended batch incubations are known to interfere with these measurements (15, 18).

Rarely, the acetylene reduction assay has been used to monitor on-line nitrogenase activity in cultures (24). Without exception, such cultures were of high density and high acetylene concentrations were applied.

Haystead et al. (9) reported saturation of cell-free preparations of nitrogenase from heterocystous cyanobacteria at a $p\text{C}_2\text{H}_2$ of 0.02 atm. In cultures, a 10-fold-higher concentration (20%) is usually considered sufficient to saturate nitrogenase (22, 23). One reason for this difference is that unlike with cell-free preparations, in vivo assays require the diffusion of acetylene into the interior of the cell. This may pose a problem, particularly for heterocystous cyanobacteria. In this group of cyanobacteria, nitrogenase is located in special cells, the heterocysts, in which the enzyme is protected against inactivation by oxygen (27). The heterocyst has a thick cell envelope which serves as a gas diffusion barrier (25) and also limits the entry of acetylene into this cell.

The higher nitrogenase activity observed in the light would result in a more rapid depletion of acetylene in the heterocyst, leading to a steeper gradient of acetylene and the requirement for higher concentrations outside the cell. This would explain

FIG. 5. Acetylene saturation curves at different light intensities. Each point is a result of a fit to experimental results, P_s (Fig. 4), with equation 1, correction for background and a slow increase of specific nitrogenase activity over time. (A) ■, dark; ●, 80 $\mu\text{mol of photons m}^{-2} \text{s}^{-1}$. Acetylene was mixed with air, resulting in a significant decrease in oxygen concentration at higher acetylene concentrations. (B) □, 9 $\mu\text{mol of photons m}^{-2} \text{s}^{-1}$; △, 34 $\mu\text{mol of photons m}^{-2} \text{s}^{-1}$; ▽, 450 $\mu\text{mol of photons m}^{-2} \text{s}^{-1}$. Mixtures of C_2H_2 , O_2 , and N_2 in which the concentration of oxygen was kept constant were used.

the observed increase of the apparent K_m . Another factor that may influence the apparent K_m and $V_{\max}$ is the availability of light that provides ATP and reduced ferredoxin, cosubstrates for nitrogenase. In this paper it has been demonstrated that even 25% acetylene in the gas phase gives only 93% saturation

of nitrogenase in *N. spumigena* incubated in the dark. Knowledge of the level of saturation of the enzyme at different light intensities is important when rates of acetylene reduction are converted to rates of nitrogen fixation. However, as shown in this paper, it must also be taken into account when dark and

FIG. 6. Rates of ethylene production (P_t) by *N. spumigena* in the dark (■) and with $50 \mu\text{mol}$ of photons $\text{m}^{-2} \text{s}^{-1}$ (□), with 5% C_2H_2 in the gas flow. ▨, background ethylene measurements without a *Nodularia* sample. The slow increase observed after switching to light incubation reflects an increasing specific nitrogenase activity.

light activities are compared. The theoretical ratio of C_2H_2 reduction to N_2 fixation is 4:1, which is based on the following reactions catalyzed by nitrogenase: $\text{C}_2\text{H}_2 + 2[\text{H}] \rightarrow \text{C}_2\text{H}_4$ and $\text{N}_2 + 8[\text{H}] \rightarrow 2\text{NH}_3 + \text{H}_2$. Comparison of ^{15}N fixation and acetylene reduction (11, 19) has confirmed this ratio. The use of the stable isotope ^{15}N for the determination of nitrogen fixation has been limited, particularly because it was less sensitive than the acetylene reduction assay (22). Only recently, Montoya et al. (19) improved the procedure for measuring nitrogen fixation by ^{15}N incorporation considerably and obtained a sensitivity which approached that of acetylene reduction as measured by gas chromatography. Despite this greatly increased sensitivity of the ^{15}N method, low temporal resolution still poses a problem, and incubations of 3 to 6 h are required (17). It is very well possible that the highly variable ratios of acetylene reduction and ^{15}N fixation found during measurements in natural ecosystems (20) can partly be attributed to insufficient and unknown degrees of acetylene saturation.

In the setup described in this paper, the incubation time depends on the time needed for ethylene to reach equilibrium for gas exchange between the gas and water phases rather than on the sample rate and dilution coefficient. The strength of the method comes from its extreme sensitivity, which allows measurements in dilute suspensions at relatively short incubation times and the application of low concentrations of acetylene. The on-line monitoring also allows measurements under constant conditions, which eliminates artifacts caused by changes in concentrations of oxygen or carbon dioxide due to respiration or photosynthesis during the incubations.

It was shown that the saturation of nitrogenase by acetylene was different in the dark and in the light. However, above a very low light intensity level, saturation values were independent on the level of irradiance applied in the experiments. This was attributed to the fact that the light-response curve for

nitrogenase activity in *N. spumigena* showed an extremely high efficiency (α) (Fig. 7B). The higher degree of saturation of nitrogenase by acetylene in the dark was most likely due to the fact that under these conditions, the flow of reducing equivalents from the neighboring vegetative cells to the heterocyst becomes limiting, resulting in lower rates of acetylene reduction.

An interesting phenomenon was observed when acetylene was mixed with air rather than with mixtures of N_2 and O_2 . When air was used as the gas to be mixed with acetylene, the oxygen concentration necessarily decreased with increasing acetylene concentrations. This resulted in significantly lower rates of acetylene reduction than predicted by Michaelis-Menten kinetics, both in the light and in the dark. However, when O_2 concentrations were kept constant at 20%, this phenomenon was not observed. This means that an O_2 level lower than that in air resulted in a lower nitrogenase activity in *N. spumigena*. In the dark, nitrogenase activity is totally dependent on respiratory energy generation, but the finding that oxygen is also required for optimal nitrogenase activity in the light was unexpected. It is speculated that respiratory oxygen uptake by the heterocyst also occurs in the light and contributes to nitrogen fixation. Respiration has been shown to be an important source for energy for nitrogen fixation in the light in the nonheterocystous cyanobacterium *Gloeothecae* sp. (16).

In this paper we have shown that LPA determination of ethylene can be used as an alternative to traditional gas chromatographic assays. Determination of nitrogenase activity by the acetylene reduction method with LPA is 3 orders of magnitude more sensitive and can therefore be used on-line for cultures of diazotrophic organisms. The sensitivity of the method allows real-time nitrogenase measurements. LPA thus can replace the gas chromatographic assay of acetylene reduction when a high sensitivity and/or fast response is required.

FIG. 7. Rates of ethylene production (P_t) by *N. spumigena* at different light intensities. (A) Experimental values. Incubations were carried out with 2.5% C_2H_2 at 21°C , as follows: 0 h 4 min, start of dark; 1 h 23 min, start of $5 \mu\text{mol of photons m}^{-2} \text{s}^{-1}$; 1 h 45 min, start of $9 \mu\text{mol of photons m}^{-2} \text{s}^{-1}$; 2 h 52 min, start of $18 \mu\text{mol of photons m}^{-2} \text{s}^{-1}$; 3 h 18 min, start of $34 \mu\text{mol of photons m}^{-2} \text{s}^{-1}$; 3 h 49 min, start of $88 \mu\text{mol of photons m}^{-2} \text{s}^{-1}$; 4 h 10 min, start of $133 \mu\text{mol of photons m}^{-2} \text{s}^{-1}$; 4 h 36 min, start of $315 \mu\text{mol of photons m}^{-2} \text{s}^{-1}$; 5 h 3 min, start of dark. The arrows indicate the steady-state production values at the different light intensities, denoted by the numbers above the arrows (micromoles of photons meter $^{-2}$ second $^{-1}$). (B) Fitted ethylene production rates obtained from the experimental curve in panel A by use of equation 1. The resulting data were fitted with equation 2 to obtain photosynthetic parameters α and I_k . Error bars indicate the standard errors, as calculated by the curve fit program.

Without the sensitive LPA technique that allows on-line monitoring of acetylene reduction at constant and dilute cell suspensions, it would have been difficult to obtain the satisfactory acetylene saturation and light-response curves presented here.

Presently, LPA is available only as an experimental setup for use in the laboratory, but a robust version of this apparatus which can be used, e.g., on research vessels is being constructed.

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