Nitrogen Fixation (Acetylene Reduction) by Epiphytes of Freshwater Macrophytes

LINDA R. FINKE† AND H. W. SEELEY, JR.*

*Department of Microbiology, College of Agriculture and Life Sciences, Cornell University,
Ithaca, New York 14853*

The involvement of epiphytic microorganisms in nitrogen fixation was investigated in a shallow freshwater pond near Ithaca, N.Y. The acetylene reduction technique was used to follow diel and seasonal cycles of nitrogen fixation by epiphytes of *Myriophyllum spicatum*. Acetylene-reducing activity was maximal between noon and 6 p.m., but substantial levels of activity relative to daytime rates continued through the night. Experiments with the seasonal course of activity showed a gradual decline during the autumn months and no activity in January or February. Activity commenced in May, with an abrupt increase to levels between 0.45 and 0.95 nmol of ethylene formed per mg (dry weight) of plant per h. Through most of the summer months, mean rates of acetylene reduction remained between 0.15 and 0.60 nmol/mg (dry weight) per h. It was calculated from diel and seasonal cycles that, in the pond areas studied, epiphytes were capable of adding from 7.5 to 12.5 µg of N per mg of plant per year to the pond. This amount is significant relative to the total amount of nitrogen incorporated into the plant. Blue-green algae (cyanobacteria), particularly *Gloeotrichia*, appeared to bear prime responsibility for nitrogen fixation, but photosynthetic bacteria of the genus *Rhodopseudomonas* were isolated from *M. spicatum* and shown to support high rates of acetylene reduction.

In recent years, nitrogen fixation studies have been conducted in a variety of aquatic habitats. In marine and brackish waters, the activities of phytoplankton (7, 25, 37) and of nitrogen-fixing organisms associated with seaweeds (6, 17), nearshore grasses and surrounding sediments (12, 27), and decaying plant materials (13, 16) have been investigated. In freshwaters, studies have concentrated on nitrogen fixation by phytoplankton (14, 41) and sediment organisms (4, 21, 24). Although the importance of macrophytes colonizing the littoral region of freshwater environments is well documented (30, 43) and primary productivity of associated epiphytes has been investigated (1, 29), few reports dealing with the nitrogen-fixing activities of epiphytes have appeared (34). It is known, however, that bacteria and blue-green algae (cyanobacteria) capable of nitrogen fixation are found in the epiphyte community (1, 14, 32). Furthermore, studies of wetlands and tundra pools have indicated that active nitrogen-fixing organisms are associated with vegetation (3, 15, 39).

It was the purpose of the experiments reported here to determine whether or not nitrogen fixation is commonly associated with aquatic macrophytes. Because such activity became apparent, further studies at one site were undertaken to examine seasonal and diel variations in the levels of nitrogen fixation. Some nitrogen-fixing epiphytes were isolated and further characterized. Results of these studies suggest that in bodies of water with extensive areas colonized by macrophytes, attached populations of algae and bacteria may contribute significantly to nitrogen inputs.

MATERIALS AND METHODS

Study sites. Plant materials used for a preliminary survey of epiphytic nitrogen fixation were obtained from several bodies of water near Ithaca, N.Y., including Dryden Lake, Cayuta Lake, and ponds of an experimental pond site administered by the Department of Agronomy, Cornell University, Ithaca.

Dryden Lake, in southeastern Tompkins County, is a highly eutrophic lake of 47.6 hectares and a maximum depth of 4 m. The lake bottom is soft muck, colonized by dense populations of rooted submerged plants, including *Elodea*, *Myriophyllum*, and *Potamogeton* species.

Cayuta Lake, covering 150 hectares in eastern Schuyler County, is also highly eutrophic and is completely overgrown at the southern end by *Myriophyllum spicatum*, the dominant macrophyte.

Experimental pond site no. 2 was constructed in 1962 and includes 50 square ponds, each of 400 m², and

† Present address: Department of Biology, Xavier University, Cincinnati, OH 45207.

a 5-hectare pond with a depth of less than 1.5 m. The latter drains approximately 100 to 150 hectares of woodland and abandoned agricultural land. The flora of the ponds varies according to the plant species originally introduced for study purposes; the 5-hectare pond is dominated by *M. spicatum* introduced from nearby Cayuga Lake, with the floating-leaved species *Potamogeton natans* a minor component of the flora.

Acetylene reduction measurements. Rates of nitrogen fixation by epiphytes of plants were estimated by the acetylene reduction technique (12, 36).

Plant segments were excised and transferred underwater to 30-ml serum bottles (actual capacity, about 38 ml). The bottles were plugged with sleeve-type rubber stoppers, and excess water pressure was vented through a syringe needle. For assays done in the laboratory, samples were returned at this stage for further manipulations. Samples for in situ assay at the pond site were prepared on location.

Twenty milliliters of water was removed from the sample bottles, the bottles were restoppered, and 2 ml of air was withdrawn and replaced by 2 ml of acetylene (Matheson Gas Products, East Rutherford, N.J.) to give an atmosphere of 10% acetylene before equilibration of this gas with the water phase. Bottles were routinely shaken gently in a short arc five times to hasten solution of the gas but to avoid dislodging attached organisms from the plants. The control for each experiment was a sample of lake or pond water without deliberately introduced plant material, treated as above. Upon occasion, controls were added which contained plant samples incubated without acetylene. No endogenous ethylene generation was ever noted.

Samples for in situ incubation were suspended at a depth of 10 cm or as noted. The duration of the assay was routinely 3 h, except in the case of preliminary experiments, where longer periods were used. At the conclusion of in situ assays, 4 ml of water was injected into the bottle before withdrawal of 4 ml of gas phase into a 4-ml Vacutainer (Becton, Dickinson & Co., Rutherford, N.J.). It was determined in preliminary experiments that such a disturbance of the water phase of an unshaken assay bottle released significant amounts of apparently unequilibrated ethylene into the gas phase. Gas samples were analyzed for ethylene content the same day or, at the most, within 2 days of collection.

In laboratory assays, plants were incubated with acetylene as described above at room temperature (23 to 26°C) under a double bank of fluorescent lighting (warm-white and daylight) at about 6,000 lx of light intensity. Portions (0.4 ml) of the gas phase were removed from samples for analysis at desired times.

Concentrations of ethylene in the gas phase were determined on a 1.2-m column of Porapak R (100 to 120 mesh) in a Hewlett-Packard 402 high-efficiency gas chromatograph with a flame ionization detector. Helium was the carrier gas at a flow rate of 50 ml/min, and the temperature was 60°C. Standard curves of peak heights for ethylene diluted in air to concentrations of 1 nmol to 1.6 μmol/0.4 ml were made at frequent intervals.

Dry weights of plants and associated epiphytes were determined by filtering samples through Millipore HA

membrane filters of 0.45-μm pore size or the analogous Gelman GN-6 filters which had been dried to constant weight at 95°C. After filtration and drying, corrections were applied for weight loss of water extractable substances from the filters.

Field studies. Preliminary experiments showed that acetylene reduction rates were greater, on a dry weight basis, for plants more heavily colonized by epiphytes. In studies in the 5-hectare pond, *M. spicatum* segments of nearly the same length and including three whorls of leaves were routinely collected from an area undergoing rapid spread of these plants. For the study of seasonal variation in acetylene reduction rates in this body of water, five plant segments, including those in all stages of colonization, were picked to gain an overall estimate of a year's activity by epiphytes. Samples were collected and assayed at weekly intervals during the warm months and at longer intervals when weather conditions were harsh.

For studies concerning diel cycles of acetylene reduction activity, an attempt was made to minimize variation due to this colonization factor by selection of *M. spicatum* segments estimated visually to be at a "moderate" stage of colonization. Samples were collected at 2-h intervals over a 24-h period, and samples collected during the hours of darkness were manipulated with the aid of dim light (less than 50 lx of intensity). Light intensity measurements were made with a Weston model 756 sunlight illumination meter.

Laboratory experiments. To assess the effects of combined nitrogen on acetylene reduction rates, segments of *M. spicatum* about 0.3 m long were held in battery jars in the laboratory at 23 to 26°C, illuminated by fluorescent lighting. Enough KNO$_3$ to produce a final concentration of 20 mM was added to one jar at day zero; NH$_4$Cl was added each day to three others, to produce a concentration of 1.0, 0.5, or 0.1 mM. A fifth jar of plants was not treated with nitrogen. The pH of the solutions remained between 7.6 and 8.6 during the treatment period. At intervals, triplicate portions of plant segments from each jar were removed for acetylene reduction assay as described above. For dark and light bottle experiments in the laboratory, one set of samples was shielded from light by a wrapping of aluminum foil.

Counting, enrichment, and isolation of nitrogen-fixing epiphytes. Total plate counts for chemoheterotrophic bacteria associated with *M. spicatum* were performed by blending leaves in sterile phosphate-buffered water, pH 7.2. Spread plates of dilutions were made on standard plate count agar medium (Difco Laboratories, Detroit, Mich.) or on Taylor medium (38). For estimates of numbers of nitrogen-fixing chemoheterotrophs, spread plates were made on a mannitol medium modified by Werner et al. (42) from that of Hino and Wilson (18). Purple nonsulfur bacteria were selected for in pour plates of a medium containing 10 mM L-malate as carbon source, modified from that of Gibson (11) by the omission of (NH$_4$)$_2$SO$_4$.

Anaerobic incubations were performed in anaerobic jars that were repeatedly evacuated and flushed with prepurified N$_2$. For incubation of purple nonsulfur plates or enrichment cultures, fluorescent lighting was supplemented by incandescent lighting. Incubations were at room temperature, 23 to 26°C.

The enrichment media for nitrogen-fixing chemoheterotrophs and photoheterotrophs were the broth forms of the agar media described above. For routine growth of isolated purple nonsulfur bacteria, the broth medium was supplemented with 40 mg of yeast extract per liter.

Blue-green algae were enriched for and purified on medium containing (in milligrams per liter): K_2HPO_4, 150; $MgSO_4 \cdot 7H_2O$, 200; $CaCl_2 \cdot 2H_2O$, 25; $Na_2SiO_3 \cdot 5H_2O$, 43; $FeCl_3 \cdot 6H_2O$, 2; $MnCl_2 \cdot 4H_2O$, 0.4; $Na_2MoO_4 \cdot 2H_2O$, 0.4; H_3BO_3, 0.6, $CuSO_4 \cdot 5H_2O$, 0.04; $ZnSO_4 \cdot 7H_2O$, 0.024. Ionagar 2S was added at a concentration of 1.2% where appropriate.

Acetylene reduction assays of enrichments and isolates. Assays for acetylene reduction by bacterial isolates were performed in 10-ml serum bottles (actual capacity, 13.3 ml). Anaerobic conditions were established by flushing the vessels for 3 to 5 min with prepurified N_2, scrubbed of O_2 by passage through a heated reduced-copper catalyst in the case of anaerobic chemoheterotophic assays. A suitable volume of the gas phase was replaced with acetylene to give a C_2H_2 content of 10%. For the estimate of maximal acetylene-reducing activity by *Rhodopseudomonas gelatinosa*, the isolate was grown in a 1-liter Roux bottle continuously sparged with prepurified N_2 and held in a water bath at 28 to 30°C. The water bath was lighted by a double bank of fluorescent lights as well as an incandescent lamp. Triplicate samples of 3.3 ml each were removed at intervals for assay, and assay bottles were also suspended in the water bath.

Algal acetylene reduction assays were performed in a 250-ml Erlenmeyer flask sealed with a rubber bung fitted with a rubber serum stopper. A volume of algal suspension equal to about half the total volume of the flask was used, with the appropriate volume of acetylene added to produce a partial C_2H_2 pressure before equilibration of 0.1 atm. Assay flasks were incubated at about 4,000 lx of fluorescent light on a slow shaker to promote equilibration of gases; 0.5-ml aliquots of the gas phase were removed at intervals for analysis. In calculating the total amount of ethylene produced, the partial pressure present in the gas phase as determined by gas chromatographic analysis was used to estimate the amount of the gas dissolved in the aqueous phase at equilibrium.

Dry weights of algal and bacterial suspensions used for acetylene reduction assays were determined by filtration of the samples through dried, weighed Sartorius 12806 polyvinyl chloride membrane filters of 0.45-μm pore size (Beckman Instruments, Inc., Mountainside, N.J.). Filters were soaked in 30% isopropanol immediately before filtration and were dried both before and after filtration in a desiccator over P_2O_5.

RESULTS

Surveys of lakes and ponds for acetylene reduction. All plant samples collected from lakes and ponds near Ithaca, N.Y., showed acetylene reduction activity to some extent (Table 1). Both in situ and laboratory assays were performed in some cases. Microscopic inspection of plant materials showed the presence of heterocystous blue-green algae. Prominent in active samples were hemispherical colonies of *Gloeotrichia*; also common were species of *Anabaena* and trichomes of basally attached *Calothrix*.

Study of seasonal changes in acetylene reduction rates. The large pond at the experimental pond site and its dense population of *M. spicatum* were chosen for more extensive study. In situ assays of acetylene reduction by epiphytes of this plant were begun in August 1974, and sampling was done on a regular basis from October 1974 to November 1975 (Fig. 1). Activity, expressed on a dry-weight basis, gradually declined during the autumn months, with slight activity still noted in December under an ice cover. Activity was absent in January and February under ice, but traces of acetylene reduction were detected in March and April of 1975. An abrupt burst of activity occurred in early May, representing a 25-fold increase over the mid-April rate. Water temperatures rose rapidly in May 1975, being 6, 12, and 18°C on 16 April, 7 May, and 9 May, respectively. Mean acetylene reduction activities for the summer months remained between 0.15 and 0.60 nmol of ethylene produced per mg (dry weight) per h. Final high levels of activity occurred in October, after which a gradual decline occurred, as in the previous year. Except for the high rates detected in May and October and the low levels of the winter months, variations in levels of activity appeared to reflect random variations in the mean dry weights of the samples collected, with higher activities correlated with higher mean dry weights.

Daily cycles of acetylene-reducing activity. Three experiments were performed to track diel changes in acetylene reduction rates (Fig. 2), with the center graph including data for light intensity changes during the period of the assay. Variations in weather conditions may be reflected in individual curves, though a general similarity prevails. In all experiments, activities were greatest during afternoon incubation periods (points shown were at starting times of 3-h incubation periods). The exceptionally high reading of 3 p.m. on 2 August is the mean of three determinations, of 0.54, 0.92, and 1.72 nmol of ethylene produced per mg (dry weight) per h. As light decreased there was a gradual drop to lowest activity levels in late evening. With the return of light in the morning, but lagging behind this slightly, an increase in acetylene reduction rates was noted.

An interesting aspect of all three studies is that activity occurred during the night, the rates attained in complete darkness being comparable to those of many daytime incubation periods. In each case, an actual transient rise in activity was noted during the night. Random variations in

TABLE 1. *Occurrence of acetylene reduction associated with macrophytes of lakes and ponds near Ithaca, N.Y.*

Source	Date (1973)	Plant	Incubation conditions[a]	Total ethylene production (nmol/mg, dry wt)	Comments
Cayuga Lake	30 July	*Myriophyllum*	L	4.8	2 heavily colonized leaves
	30 July	*Myriophyllum*	L	2.8	1 node of 4 leaves
	30 July	*Myriophyllum*	L	0.50	Terminal 1.5-inch (ca. 3.8-cm) segment, lightly colonized
Dryden Lake	31 Aug.	*Lemna* and *Wolffia*	L	0.62	Collected from lake margins
	31 Aug.	*Elodea*	L	4.0	Lightly colonized
	31 Aug.	*Myriophyllum*	L	5.2	Lightly colonized
	31 Aug.	*Myriophyllum*	L	0.20	Terminal segment, no visible colonization
	16 Aug.	*Elodea*	IS	0.77, 2.2, 6.9, 12.7	4 samples, lightly colonized
	16 Aug.	*Myriophyllum*	IS	9.0, 12.1	2 samples, lightly colonized
	16 Aug.	*Myriophyllum*	IS	61.0	Heavily colonized by *Gloeotrichia*
Experimental site, pond 208	25 July	*Chara*	IS (3 h)	5.8 ± 2.8[b]	6 samples, 1.5 inch (ca. 3.8 cm) each
	30 March	Artificial "leaves"[c]	L	40.3 ± 18.2[b]	10 samples
Experimental site, 5-hectare pond	15 Aug.	*Myriophyllum*	IS	0.43, 0.75	2 lightly colonized samples
	15 Aug.	*Myriophyllum*	IS	1.8	Moderately colonized
	15 Aug.	*Myriophyllum*	IS	7.7	Heavily colonized

[a] Incubation conditions: 24 h (unless otherwise noted); L, laboratory, artificial lighting; IS, in situ, ambient lighting conditions.

[b] Mean ± 1 standard deviation.

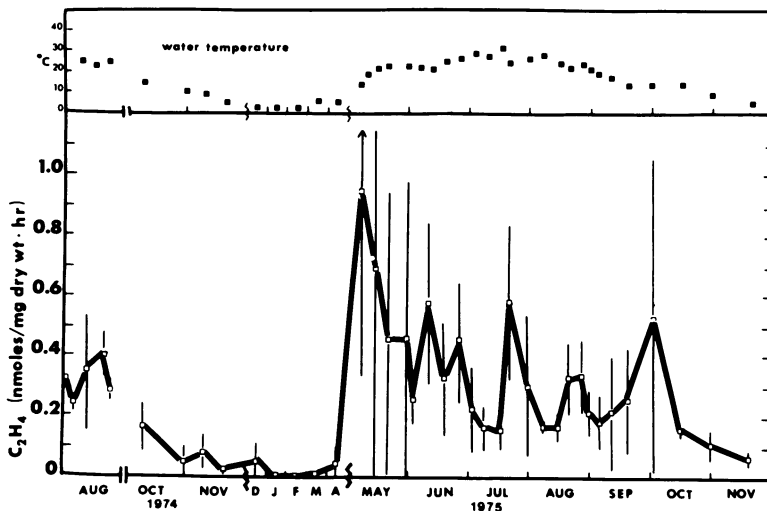[c] Polyethylene strips incubated 6 weeks in situ; epiphyte material removed for dry weight determination.

FIG. 1. *Mean acetylene reduction rates of epiphytes on M. spicatum in the 5-hectare pond, from August 1974 to November 1975. Each point represents the mean of five replicates, with ±1 standard deviation indicated by the vertical bars. Assays were performed in situ.*
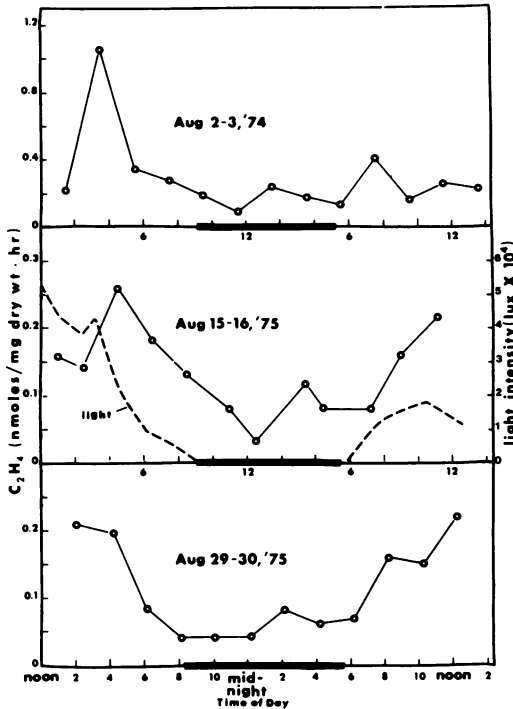
FIG. 2. *Diel variations in mean acetylene reduction rates for epiphytes of M. spicatum collected from and assayed in the 5-hectare pond on three occasions. Points shown are means of three replicates in the 1974 study and five replicates in both 1975 studies. Light intensity readings are indicated for the study period of 15 to 16 August 1975.*

the mean dry weights of samples did not appear to account for the differences noted during the night. During the course of the 15 to 16 August 1975 experiment, *Gloeotrichia* colonies and other epiphytic materials were scraped from the undersides of *P. natans* leaves at 7:00 p.m., 10:45 p.m., and 1:25 a.m. and assayed for acetylene reduction in the normal manner. Mean acetylene reduction rates for duplicate samples were, respectively, 0.38, 0.31, and 0.26 nmol of ethylene formed per mg (dry weight) per h. These data again demonstrated that there was no abrupt cessation of acetylene reduction when light was gone.

Five replicate samples of *M. spicatum* were assayed for their ability to reduce acetylene at different depths in the pond in open water. Equivalent rates of about 0.25 nmol of ethylene formed per mg (dry weight) per h were supported at all levels from the surface to the pond bottom, a total depth of 0.6 m. In addition, four samples incubated at a depth of 10 cm below a surface layer of densely tangled *M. spicatum* fronds showed a mean rate of 0.29 ± 0.13 nmol

of ethylene formed per mg (dry weight) per h relative to a rate of 0.37 ± 0.24 for a control group incubated in open water.

Overall, these experiments suggest that factors which may restrict light availability to epiphytes—crowding of plants, dim light periods during the day, or depth—do not have as great an effect on acetylene-reducing activity as one might anticipate.

Effect of nitrogen amendments. To verify that the acetylene-reducing activity associated with *M. spicatum* was a biological response by nitrogen-fixing epiphytes to nitrogen limitation in their environment, nitrogen amendments were supplied to vessels containing the plants, and the effects on acetylene reduction were determined. Activity was lost within 3 days by epiphytes supplied with 1.0 or 0.5 mM ammonium (Fig. 3). With 0.1 mM ammonium, a somewhat slower drop in acetylene reduction rates occurred. Loss of activity was even slower in samples amended with 20 mM nitrates and was incomplete. Twenty to thirty percent of the original activity was maintained for up to 7 days.

Laboratory light and dark experiments. Laboratory investigations were performed to determine the relative effects of light and dark conditions on acetylene reduction rates. Plants were collected from one of the ponds in mid-morning and held under laboratory lighting during subsequent manipulations. In some cases, *Gloeotrichia* colonies were individually picked from the plants. One set of serum bottles (three or four replicates) was held in the light, whereas the other set was wrapped in aluminum foil. Gas samples were removed at intervals for up to 6
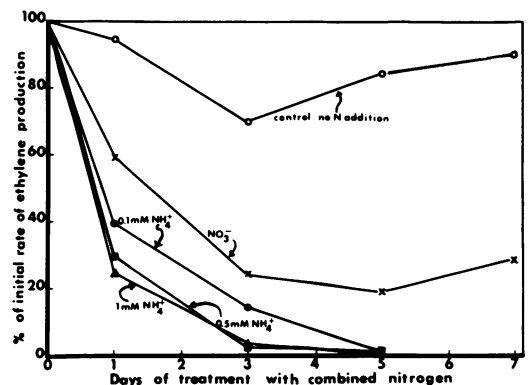

FIG. 3. *Effect of ammonium and nitrate on acetylene reduction by epiphytes of M. spicatum assayed in the laboratory. Points shown are means of three determinations and represent the rate of acetylene reduction at the time indicated (3-h assay) relative to the rate at day zero, before introduction of combined nitrogen.*

h. Results of several such experiments are shown in Fig. 4. Typically, dark activity fell to one-half to one-third of light activity within 1 to 2 h. By 4 h, the rates sustained by dark samples were less than one-fifth of those of lighted samples in all cases. Actual amounts of ethylene produced by *Gloeotrichia* colonies in these experiments were low, possibly due to damage to the colonies during removal from the plants.

Investigations of acetylene reduction by algae. Microscopic examinations of plants active in acetylene reduction from all sites revealed the presence of heterocystous blue-green algae in most cases. *Gloeotrichia* colonies were especially noticeable, and further study of this epiphyte was pursued. *Gloeotrichia* colonies removed carefully from the macroalga *Chara* reduced acetylene in the laboratory at a mean rate of 4.2 nmol of ethylene formed per mg (dry weight) per h. By comparison, *Chara* samples with their epiphytic populations intact showed a rate of 0.57. Numbers of the hemispherical colonies typical of *Gloeotrichia* and associated with 1- to 1.5-inch (ca. 2.5- to 3.8-cm) *Chara* segments ranged from 24 to 49.

Because of such findings, primary isolation of this *Gloeotrichia* sp. was attempted. Colonies were removed from *Chara* and cultivated in a nitrogen-free salts medium and repeatedly streaked on the solid medium. A unialgal culture was obtained with a low level of bacterial con-

tamination. Two-day-old unialgal cultures were assayed in the light for acetylene reduction activity, with results shown in Fig. 5. Linear rates of acetylene reduction for the two trials equaled 0.81 and 0.62 nmol of ethylene formed per mg (dry weight) per min (49 and 37 nmol/mg per h, respectively).

Studies with chemoheterotrophic epiphytes. Attempts to isolate nitrogen-fixing bacteria from among the epiphytes were preceded by plate count determinations of total numbers of heterotrophs. Table 2 shows results of plate counts for several types of heterotrophs of interest. It is evident from the numbers of bacterial colonies developing on the Hino and Wilson medium that nitrogen-fixing chemoheterotrophs were not a significant fraction of the total chemoheterotrophic population. Furthermore, it is likely that many of the colonies isolated on such a plate-counting medium were actually those of nitrogen-scavenging bacteria utilizing adsorbed NH_3. Attempts to pick colonies from both aerobic and anaerobic plates into nitrogen-free broth resulted in poor or no growth upon transfer. Previous enrichment attempts on the original Hino and Wilson medium, containing sucrose rather than mannitol, had proved similarly unsuccessful.

Table 2 discloses that organisms capable of growth on a malate medium with only traces of nitrogen were numerous. Although not all colonies developing under a nitrogen atmosphere in the light on this medium were pigmented, nevertheless, the *Rhodospirillaceae* were quickly apparent in the development of reddish turbidity in enrichments from *M. spicatum* leaves. Isolates of two species were purified and identified by morphological, cultural, and physiological
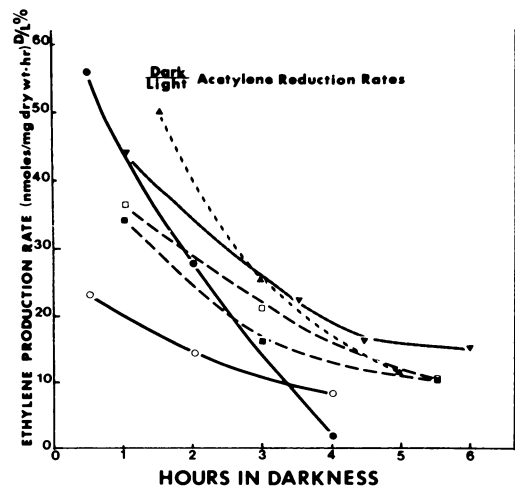

FIG. 4. *Loss of acetylene-reducing activity in the dark by several plant-epiphyte associations and by Gloeotrichia colonies separated from host plants. Each point indicates the mean rate of reduction by three or four samples held in darkness relative to the rate sustained by similar samples held in the light. Symbols: ○, Chara sp. 1975; ●, colonies from Chara 1975; □, Chara sp. 1974; ■, colonies from Chara 1974; ▼, M. spicatum 1974; ▲, Chara sp. 1973.*
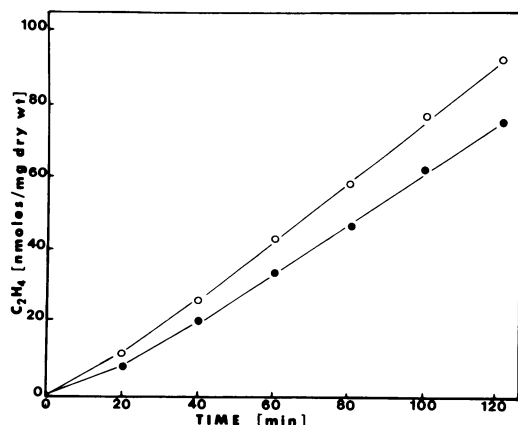

FIG. 5. *Acetylene reduction by 2-day-old unialgal cultures of Gloeotrichia sp. isolated from Chara, assayed in the laboratory.*

characteristics and by pigment spectra as *R. gelatinosa* and *R. palustris*. *R. gelatinosa* was grown in a large batch culture sparged with nitrogen and assayed at intervals for nitrogenase activity. Results of this experiment are shown in Fig. 6. The organism grew with a doubling time of 15 h under nitrogen-fixing conditions. Acetylene reduction activity was greatest in midlogarithmic phase, and maximum mean activity for duplicate samples was 16 nmol of ethylene formed per mg (dry weight) per min.

DISCUSSION

It is not surprising that acetylene reduction was associated universally with plant samples collected from eutrophic lakes and ponds. Algae and bacteria capable of nitrogen fixation are frequently associated with vegetation, both terrestrial and aquatic (15, 31, 32). The large populations of bacteria present at the macrophyte surface (about 10^6/mg [dry weight], see Table 2)

would find organic carbon available as leachate from the plant and attached algae (40, 44), but competition for combined nitrogen would be keen. As a result, any nitrogen-fixing potential of organisms suited to the epiphytic niche is likely to be expressed. The experiment describing the effect of combined nitrogen amendments demonstrated the nitrogen limitation extant in the samples.

Seasonal variation is great in the nitrogen fixation rates associated with phytoplankton (14, 20, 36), and, in at least one case, a rapid bloom of planktonic *Gloeotrichia* was responsible for a sudden increase in nitrogen-fixing activity (10). No comparable descriptions of blooms of nitrogen-fixing epiphytes exist, although Allen (1) described the course of colonization of macrophytes by a mixed flora occasionally including *Gloeotrichia*. The controlling influence of temperature has been demonstrated in some nitrogen fixation studies (16, 26) and is likely to be

TABLE 2. *Numbers of organisms per milligram (dry weight) of M. spicatum from the 5-hectare pond, as enumerated by plate-counting methods, using the media indicated*[a]

Date	No. of heterotrophs (medium)				
	Total		Photosynthetic	On N-free medium	
	Aerobic	Anaerobic		Aerobic	Anaerobic
7/3/74	9.2×10^5 (T)	—[b]	—	—	—
10/1/74	1.9×10^6 (T)	1.6×10^6 (T)	2.2×10^6 (PNS)	2.0×10^4 (HW)	2.7×10^4 (HW)
5/9/75	9.8×10^5 (PCA)	—	5.3×10^5 (PNS)	—	—
7/8/75	3.0×10^5 (PCA)	—	1.0×10^5 (PNS)	—	—

[a] Media: T, Taylor medium (38); PNS, malate purple nonsulfur medium; HW, Hino and Wilson mannitol medium (18); PCA, plate count agar (glucose-tryptone-yeast extract).
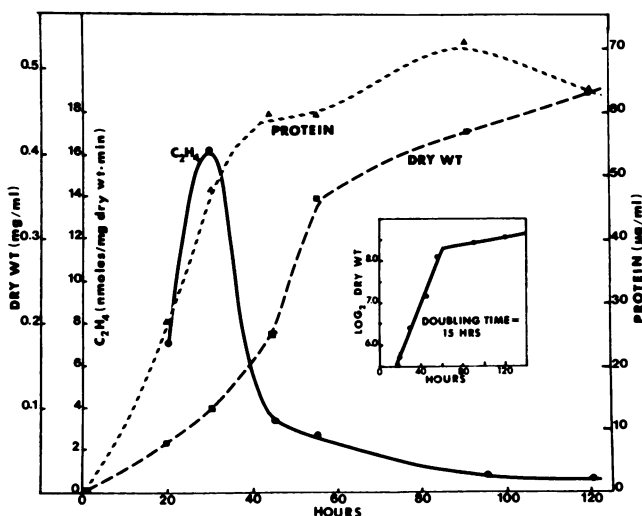
[b] —, Plating not done.

FIG. 6. *Growth in a temperature-controlled batch culture of a strain of R. gelatinosa isolated from M. spicatum, showing changes in mean acetylene reduction rates during the course of growth of the culture.*

responsible for the rapid onset of activity observed in early May in the present study. Since the pond was shallow and the ice cover had been gone for some time, it is unlikely that a sudden availability of nutrients as with lakes showing a spring and autumn overturn could be the major controlling factor. However, blue-green algae with heterocysts were not readily apparent among the epiphytes in early May, and relatively few of the 5×10^5 colonies developing on media selective for purple nonsulfur bacteria were typical of these nitrogen-fixing species. Therefore, at present the organisms actually responsible for this "first flush" of activity are unknown. Within 2 weeks, both *Tolypothrix* and *Gloeotrichia* with heterocysts were observed among the epiphytes, and it is possible that trichomes of the former were overlooked in previous inspections.

Unlike the findings in phytoplankton studies, nitrogen fixation rates associated with salt-marsh *Spartina* stems remain relatively steady throughout the summer (16), as was observed in the present study. It is likely that the dense populations of microorganisms (Table 2) colonizing such plants are a stabilizing influence for dissolved oxygen, CO_2, nutrient, and pH changes, as suggested by Ruinen for terrestrial leaves (32). In particular, a moderation of fluctuations in dissolved O_2 and CO_2 levels by intense microbial respiration should be of benefit for nitrogen fixation (23). This is made evident by the fact that afternoon depressions in phytoplankton activity have been attributed to high dissolved oxygen concentrations (36, 41), whereas in the present study maximal acetylene-reducing rates and dissolved oxygen levels both occurred in mid- to late afternoon.

Experimental results have differed on the question of the ability of nitrogen-fixing blue-green algae to maintain such activity in dim light and darkness (8, 14, 33, 35, 41). However, Horne (19) has noted a diel cycle of acetylene reduction by lotic freshwater *Nostoc* which is similar to that reported in the present study, where brief nighttime maxima occurred between 1 and 4 a.m. after both bright and overcast days. Recovery of activity in the morning lagged behind the return of light, supporting Horne's conjecture that replacement of internal reductant pools depleted in nighttime metabolism may be required (19).

Laboratory experiments with freshly collected materials incubated under light and dark conditions did not provide much support for in situ findings that substantial rates of acetylene reduction are supported throughout the night. The activity of both plants and separated algae decreased rapidly in the dark. However, in in situ experiments, *Gloeotrichia* and other epiphytes scraped from plants in twilight or in dark hours showed good activity. It should be noted that samples collected for the laboratory work were obtained in midmorning and held in the laboratory at light levels (about 5,000 lx) which were considerably less than those of the environment. These conditions may have combined to restrict acquisition of reductant levels necessary to maintain good rates of acetylene reduction in the dark. In preliminary experiments, *Gloeotrichia* isolates have shown some ability to continue reducing acetylene after the light is switched off (L. Finke, unpublished observations).

Although recovery of acetylene reduction in the morning lagged behind the return of light, it was rapid and apparently complete even on a dull day. Furthermore, incubation at up to 0.62 m below the pond surface or beneath a dense surface mat of tangled *M. spicatum* did not seem to result in restriction of acetylene reduction rates. These findings may reflect acclimation of epiphytic blue-green algae to shading commonly encountered in their niche. Reports indicate that blue-green algae reach saturating light intensities for nitrogen fixation between 20 and 30 klx (1,860 to 2,800 footcandles) (5), intensities which are likely to be present even at the bottom of the pond on a bright day and at the surface on a dim day.

Work reported by Fay (9) suggests that there may be a greater heterotrophic potential among freshwater blue-green algae than had been previously suspected. If so, the ability to assimilate external carbonaceous compounds for growth and N_2 fixation in the dark should be especially prominent among blue-green algae typically growing as epiphytes. The heterotrophic abilities of *Gloeotrichia*, the principal blue-green algal component in these studies, have not been reported. However, the heterotrophic abilities of other genera of the family *Rivulariaceae*, notably *Calothrix*, are well documented (22).

A *Gloeotrichia* sp. appeared to be the major blue-green algal epiphyte in all habitats studied, but *Anabaena* spp. were frequently observed as well throughout the season, and a *Tolypothrix* sp. was conspicuously present in late spring samples of *M. spicatum* from the large pond. Only the activity of *Gloeotrichia* was further investigated. In unialgal culture, *Gloeotrichia* reduced acetylene at rates of 0.62 and 0.81 nmol of ethylene formed per mg (dry weight) per min, a value somewhat higher than reported by Stewart et al. (35) for freshly collected planktonic *Gloeotrichia*.

At the experimentally determined rates, and

based on the average dry weight per colony of *Gloeotrichia* calculated from experimental data, it is theoretically possible for a few such colonies to account for all of the acetylene reduction activity associated with aquatic macrophytes. However, the presence of nitrogen-fixing epiphytes other than the blue-green algae is indicated by experimental results. The purple nonsulfur bacteria are apparently as well represented among the epiphytes of living aquatic plants as they have been shown to be among decomposing plant materials (16). Due to their ability to metabolize organic acids, they could be expected to be active where photosynthetic products are leached from living plants (40, 44) as well as where such organic acids are formed by decomposition. In the present study, *Rhodospirillaceae* appeared rapidly in enrichment cultures with *M. spicatum* leaves as the inoculum and malate as the *R. palustris* and *R. gelatinosa* appeared to be most common, as has been found in other studies of aquatic *Rhodospirillaceae* (2). The maximal rate of acetylene reduction by *R. gelatinosa* under laboratory conditions was approximately 20 times that of *Gloeotrichia*, on a dry-weight basis. Calculations from this experiment and from preliminary experiments showed that *R. gelatinosa* was capable of reducing acetylene at a rate of 12 nmol/10^8 cells per h. If 50% of the 2.2×10^6 colonies per mg of plant developing on the selective malate medium (Table 2) were *Rhodopseudomonas* spp., a rate of acetylene reduction of 0.12 nmol/mg (dry weight) of plant per h could be sustained at the maximal rate. In early Ocotber 1974, when these counts were obtained, the noontime acetylene reduction rate in situ was 0.14 nmol of ethylene per mg (dry weight) per h. It is therefore within the capabilities of the purple nonsulfur bacteria to contribute significantly to observed rates of acetylene reduction in nature. The extent of actual in situ activities of these organisms, however, is unknown. Such factors as the availability of organic carbon and particularly the oxygen tension in the microzones where such bacteria exist would be of great importance.

Despite the failure of attempts to isolate chemoheterotrophic nitrogen fixers from among the epiphytes, it seems unlikely that they would be totally absent. There are reports of association of free-living, nitrogen-fixing bacteria, particularly *Azotobacter*, with marine, freshwater, and terrestrial plants (17, 31, 32). Some colonies were found on nitrogen-free enrichment plates, although in low numbers relative to total numbers of heterotrophs. The nighttime pulse of activity noted in these studies could, theoretically, result from a lowering of oxygen tensions by respiration to the point where nitrogen-fixing bacteria inhibited by higher tensions could become active.

An estimate of overall nitrogen contributions to the 5-hectare pond by epiphytes cannot be made, due to the concentrated sampling in one area and the lack of data for $^{15}N_2$ incorporation. However, it is conceivable that nitrogen fixed by epiphytes may have been helpful to *M. spicatum* in its rapid spread across the pond, which was constructed in nitrogen-poor subsoil and with no major sources of agricultural or residential pollution. Calculations from seasonal and diel variations in acetylene reduction rates yield an estimate of 1.34 total μmol of ethylene produced per mg (dry weight) of plant material per year.

Conversion of this figure to the equivalent amount of nitrogen fixed is made difficult by the fact that experimentally derived conversion factors (4.2 to 4.8 in a recent study of blue-green algae by Peterson and Burris [28]) differ somewhat from that derived theoretically, a value of 3. If one takes the actual factor to be between 3 and 5, the outer limits for annual nitrogen incorporation by epiphytic activity may be calculated to be between 7.5 and 12.5 μg of N fixed per mg (dry weight) per year. Elemental analysis of *M. spicatum* from the pond showed the plant to be 2.5% N, by dry weight (J. Peverly, personal communication), or 25 μg of N per mg (dry weight). By this kind of analysis, it can be seen that the amount of nitrogen added by fixation by a plant's epiphytes in one season may equal 30 to 50% of the amount of nitrogen accumulated by the plant. Though this study cannot give figures for actual epiphytic nitrogen fixation, it does suggest an important role for the attached epiphyton of some bodies of freshwater, a role investigators would be well advised to consider in future studies of nitrogen input.

LITERATURE CITED

1. **Allen, H. L.** 1971. Primary productivity, chemoorganotrophy, and nutritional interactions of epiphytic algae and bacteria on macrophytes in the littoral of a lake. Ecol. Monogr. 41:97–127.
2. **Biebl, H., and G. Drews.** 1969. Das in-vivo-Spektrum als taxonomisches Merkmal bei Untersuchungen zur Verbreitung von *Athiorhodaceae.* Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 2 123:425–452.
3. **Blasco, J. A., and D. C. Jordan.** 1976. Nitrogen fixation

in the muskeg ecosystem of the James Bay Lowlands, Northern Ontario. Can. J. Microbiol. **22:**897-907.

4. **Brooks, R. H., Jr., P. L. Brezonik, H. D. Putman, and M. A. Keirn.** 1971. Nitrogen fixation in an estuarine environment: the Waccasassa on the Florida Gulf Coast. Limnol. Oceanogr. **16:**701-710.

5. **Burris, R. H.** 1976. Nitrogen fixation by blue-green algae of the Lizard Island area of the Great Barrier Reef. Aust. J. Plant Physiol. **3:**41-51.

6. **Carpenter, E. J.** 1972. Nitrogen fixation by a blue-green epiphyte on pelagic *Sargassum.* Science **178:**1207-1208.

7. **Carpenter, E. J., and J. J. McCarthy.** 1975. Nitrogen fixation and uptake of combined nitrogenous nutrients by *Oscillatoria (Trichodesmium) thiebautii* in the western Sargasso Sea. Limnol. Oceanogr. **20:**389-401.

8. **Dugdale, V. A., and R. C. Dugdale.** 1962. Nitrogen metabolism in lakes. II. Role of nitrogen fixation in Sanctuary Lake. Limnol. Oceanogr. **7:**170-177.

9. **Fay, P.** 1976. Factors influencing dark nitrogen fixation in a blue-green alga. Appl. Environ. Microbiol. **31:**376-379.

10. **Findley, D. L., D. I. Findley, and J. R. Stein.** 1973. Surface nitrogen and plankton in Skaha Lake, British Columbia (Canada). Freshwater Biol. **3:**111-122.

11. **Gibson, J.** 1975. Uptake of C_4 dicarboxylates and pyruvate by *Rhodopseudomonas spheroides.* J. Bacteriol **123:**471-480.

12. **Goering, J. J., and P. L. Parker.** 1972. Nitrogen fixation by epiphytes on sea grasses. Limnol. Oceanogr. **17:**320-323.

13. **Gotto, J. W., and B. F. Taylor.** 1976. N_2 fixation associated with decaying leaves of the red mangrove (*Rhizophora mangle*). Appl. Environ. Microbiol. **31:**781-783.

14. **Granhall, U., and A. Lungren.** 1971. Nitrogen fixation in Lake Erken. Limnol. Oceanogr. **16:**711-719.

15. **Granhall, U., and H. Selander.** 1973. Nitrogen fixation in a subarctic mire. Oikos **24:**8-15.

16. **Green, F., and J. Edmisten.** 1974. Seasonality of nitrogen fixation in Gulf Coast salt marshes, p. 113-126. *In* H. Lieth (ed.), Phenology and seasonality modeling. Springer-Verlag New York, Inc., New York.

17. **Head, W. D., and E. J. Carpenter.** 1975. Nitrogen fixation associated with the marine macroalga *Codium fragile.* Limnol. Oceanogr. **20:**815-823.

18. **Hino, S., and P. W. Wilson.** 1958. Nitrogen fixation by a facultative Bacillus. J. Bacteriol. **75:**403-408.

19. **Horne, A. J.** 1975. Algal nitrogen fixation in Californian streams: diel cycles and nocturnal fixation. Freshwater Biol. **5:**471-477.

20. **Horne, A. J., and C. R. Goldman.** 1972. Nitrogen fixation in Clear Lake, California. I. Seasonal variation and the role of heterocysts. Limnol. Oceanogr. **17:**678-692.

21. **Keirn, M. A., and P. L. Brezonik.** 1971. Nitrogen fixation by bacteria in Lake Mize, Florida, and in some lacustrine sediments. Limnol. Oceanogr. **16:**720-731.

22. **Khoja, T., and B. A. Whitton.** 1971. Heterotrophic growth of blue-green algae. Arch. Mikrobiol. **79:**280-282.

23. **Lex, M., and W. D. P. Stewart.** 1973. Algal nitrogenase, reductant pools, and photosystem I activity. Biochim. Biophys. Acta **292:**436-443.

24. **Macgregor, A. N., and D. R. Keeney.** 1973. Acetylene reduction assay of anaerobic nitrogen fixation by sediment of selected Wisconsin lakes. J. Environ. Qual. **2:**438-440.

25. **Mague, T. H., N. M. Weare, and O. Holm-Hansen.** 1974. Nitrogen fixation in the North Pacific Ocean. Mar.

Biol. **24:**109-119.

26. **Marsho, T. V., R. P. Burchard, and R. Fleming.** 1975. Nitrogen fixation in the Rhode River estuary of Chesapeake Bay. Can. J. Microbiol. **21:**1348-1356.

27. **Patriquin, D., and R. Knowles.** 1972. Nitrogen fixation in the rhizosphere of marine angiosperms. Mar. Biol. **16:**49-58.

28. **Peterson, R. B., and R. H. Burris.** 1976. Conversion of acetylene reduction rates to nitrogen fixation rates in natural populations of blue-green algae. Anal. Biochem. **73:**404-410.

29. **Pieczynska, E., and W. Szczepanska.** 1966. Primary productivity in the littoral of several Mansurian lakes. Int. Ver. Theor. Angew. Limnol. **16:**372-379.

30. **Rich, P. H., R. G. Wetzel, and N. V. Thuy.** 1971. Distribution, production, and role of aquatic macrophytes in a southern Michigan marl lake. Freshwater Biol. **1:**3-21.

31. **Ruinen, J.** 1961. The phyllosphere. I. An ecologically neglected milieu. Plant Soil **15:**81-109.

32. **Ruinen, J.** 1974. Nitrogen fixation in the phyllosphere. Front. Biol. **33:**121-167.

33. **Rusness, D., and R. H. Burris.** 1970. Acetylene reduction (nitrogen fixation) in Wisconsin lakes. Limnol. Oceanogr. **15:**808-813.

34. **Silver, W. S., and A. Jump.** 1975. Nitrogen fixation associated with vascular aquatic macrophytes, p. 121-125. *In* W. D. P. Steward (ed.), Nitrogen fixation by free-living micro-organisms. Cambridge University Press, New York.

35. **Stewart, W. D. P., G. P. Fitzgerald, and R. H. Burris.** 1967. *In situ* studies on nitrogen fixation using the acetylene reduction technique. Proc. Natl. Acad. Sci. U.S.A. **58:**2071-2078.

36. **Stewart, W. D. P., T. Mague, G. P. Fitzgerald, and R. H. Burris.** 1971. Nitrogenase activity in Wisconsin lakes of differing degrees of eutrophication. New Phytol. **70:**497-509.

37. **Taylor, B. F., C. C. Lee, and J. S. Bunt.** 1973. Nitrogenfixation associated with the marine blue-green alga, *Trichodesmium,* as measured by the acetylene reduction technique. Arch. Mikrobiol. **88:**205-212.

38. **Taylor, C. B.** 1940. Bacteriology of freshwater. I. Distribution of bacteria in English Lakes. J. Hyg. **40:**616-640.

39. **Tjepkema, J. D., and H. J. Evans.** 1976. Nitrogen fixation associated with *Juncus balticus* and other plants of Oregon wetlands. Soil Biol. Biochem. **8:**505-509.

40. **Tukey, H. B.** 1971. Leaching of substances from plants. p. 67-80. *In* T. F. Preece and C. H. Dickinson (ed.), Ecology of leaf surface microorganisms. Academic Press Inc., New York.

41. **Vanderhoef, J. N., P. L. Liebson, R. J. Musil, C. Huang, R. E. Fiehwig, J. W. Williams, D. L. Wackwitz, and K. T. Mason.** 1975. Diurnal variation in algal acetylene reduction (nitrogen fixation) *in situ.* Plant Physiol. **55:**273-276.

42. **Werner, D., H. J. Evans, and R. J. Seidler.** 1974. Facultatively anaerobic nitrogen-fixing bacteria from the marine environment. Can. J. Microbiol. **20:**59-64.

43. **Wetzel, R. G.** 1964. Primary productivity of aquatic macrophytes. Int. Ver. Theor. Angew. Limnol. Part 1 **15:**426-436.

44. **Wetzel, R. G.** 1969. Factors influencing photosynthesis and excretion of dissolved organic matter by aquatic macrophytes in hard water lakes. Int. Ver. Theor. Angew. Limnol. **17:**72-85.