ABSTRACT

NITROGEN FIXATION AND PRODUCTIVITY IN A EUTROPHIC HARD-WATER LAKE: *IN SITU* AND LABORATORY STUDIES

By

Tran Phuoc Duong

Nitrogen fixing activity by planktonic organisms in a shallow eutrophic hard-water lake in southwest Michigan was measured at midday each week during the summer of 1970 and 1971 (July-September, 1970 and June-August, 1971) using the acetylene reduction technique. Samples were obtained from 0, 1, 2, and 3 m and incubated with acetylene at their original depth for 1 hr. Acetylene reducing activity was confined to the aerobic 0-3 m zone where heterocyst-bearing *Aphanizomenon* and *Anabaena* were dominant in 1970 and 1971, respectively. Acetylene reduction was not detectable in the 4-5 m anaerobic zone where *Thiopedia* and green sulfur bacteria dominated the bacterial population. The population of *Aphanizomenon* was more dense (3,197 x 10^3 filaments/l) than *Anabaena* (355 x 10^3 filaments/l) but less efficient in reducing acetylene (3 x 10^{-9} and 2 x 10^{-7} µmole/filament/hr, respectively.

Both algae reduced significant amounts of acetylene at night with Aphanizomenon reducing more at night than during the day. The peak activity during the day occurred the third week of July both years.

From the weekly studies of acetylene reduction, it was estimated that reduction in the water column to 3 m was approximately 3,000 μmole acetylene/m^2/day in 1971 assuming a fixation period of 12 hr. Using the conversion ratio 3 ethylene formed to 2 ammonia produced, this represents an estimated quantity of 2 g/m^2 ammonia produced during the 1971 bloom period.

An association between the blue-green algae Anabaena, Calothrix, and Dichothrix and the duckweeds Spirodela and Lemna was shown to be active in reducing acetylene. Dissection and microscopy revealed that microcolonies of the above algae were located in the lower mesophyll and in the pockets of the duckweeds. The acetylene reducing activity was associated with the fronds (leaves, rather with the roots) of the plants and was light dependent. The association reduced an average of 63 mμmole acetylene/g wet wt/hr when the assay was conducted in the lake.

Weekly primary productivity and excretion of organic carbon were also measured at 1 m intervals from June to October, 1971 using ^{14}C methods. Net primary productivity (light minus dark CO_2 uptake) ranged from 300.0 to 2,762.5 mg C/m^2/day, being mostly due to the activity of blue-green algae which dominated the aerobic zone (0-2 m). Dark CO_2 uptake averaged 322.4 mg C/m^2/day or 21.4% of net primary productivity. Total excretion of newly fixed carbon was about 3.5% of the net primary productivity. The excretion was highest in the aerobic zone.

Strains of *Rhodopseudomonas* and *Rhodomicrobium* isolated from this lake grew well on a variety of organic carbon compounds such as organic acids, alcohols, carbohydrates, and carbohydrate polymers in nitrogen-free medium. *Rhodomicrobium* produced angular structures similar to cysts in old cultures. The growth and acetylene reduction by *Rhodomicrobium* were optimum at 30 C under 300 ft-c. Acetylene reduction by this organism was suppressed by as low as 0.0005% combined nitrogen and the induction of the acetylene reducing activity was higher in argon or helium than in nitrogen atmospheres.

In situ acetate uptake was highest at 3 m where *Thiopedia* and green sulfur bacteria represented 90% of the bacterial population. In the laboratory, a 10 ml sample of the natural population of these bacteria photodegraded to CO_2 about 2.4% of 100 μg of uniformly labeled acetate/hr at 29 C under 600 ft-c.

Tran Phuoc Duong

In situ and laboratory data suggest that photosynthetic bacteria are capable of participating in all steps of degradation of organic carbon but were not important nitrogen fixers in Wintergreen Lake.

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By

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To the Vietnamese countryman to whom
I am in debt

To my fathers and mothers
To my wife, Dai, Dan, Dong, and Dien
To professor Pham hoang Ho who inspires me

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I. INTRODUCTION

Photosynthetic phytoplankton and photosynthetic bacteria constitute a great part of the photosynthetic producing capacity in lakes (Hutchinson, 1967; Kondrat'eva, 1963; Wetzel, 1964; Culver and Brunskill, 1969). These plankton serve as food for zooplankton and bottom fauna which in turn are devoured by fish and so on through the food chain (Prowse, 1955; Kondrat'eva, 1963).

The objective of this study was to evaluate the productive capacity of the planktonic organisms in a eutrophic lake. The work focused mainly on the primary productivity, atmospheric nitrogen fixation, and the major limnological parameters which affect these activities. The studies were done both *in situ* and under laboratory conditions.

II. THE LAKE

A. Location

Wintergreen Lake (Fig. 1) which was used in this study is a part of the Kellogg Bird Sanctuary which was established and donated to Michigan State University by Mr. W. K. Kellogg (Manny, 1971). Wintergreen Lake, T.la, R 9 W, is located in Kalamazoo County, Michigan about twelve miles northwest of Battle Creek and fifteen miles northeast of Kalamazoo.

B. Description of the Lake

1. Topography. Wintergreen Lake is situated in a low ridge lying between Cooper Ridge and Kalamazoo River. The ridge contains a chain of lakes which run from northeast to southwest, the largest of which is Gull Lake. The basin of Wintergreen Lake is of glacial origin, probably formed during the last ice invasion (Taube and Bacon, 1952; Chandler, 1963). The soils of the area are of the Gray Brown Podzolic group which have a thin organic covering and a illuvial horizon (Whiteside *et al.*, 1968; Millar *et al.*, 1966). The lake is surrounded by loamy soils (Fig. 2). These soils are characterized by a porous,

Figure 1. Bathymetric map of Wintergreen Lake showing location of sampling stations A and B used in this study (figure courtesy of Dr. B. A. Manny).

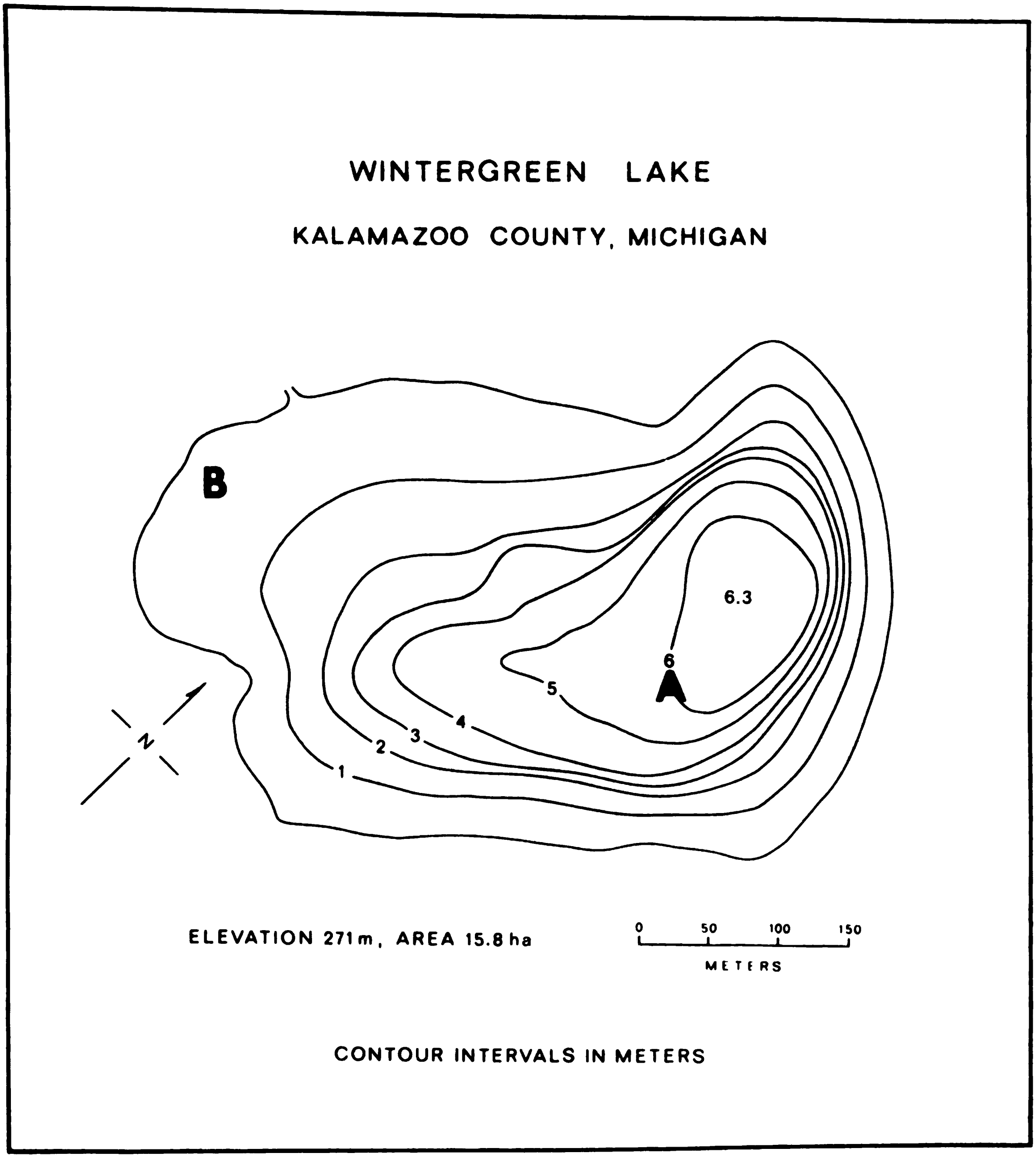
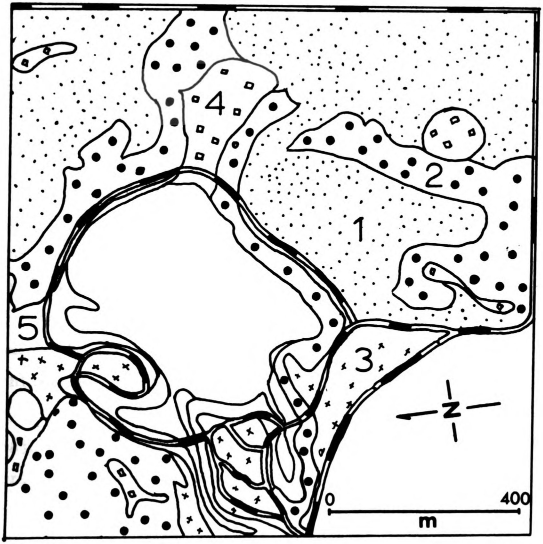

WINTERGREEN LAKE
KALAMAZOO COUNTY, MICHIGAN
B
A
6.3
6
5
4
3
2
1
N
ELEVATION 271m, AREA 15.8 ha
0
50
100
150
METERS
CONTOUR INTERVALS IN METERS

Figure 2. Soil map of the Wintergreen Lake watershed area. Fox loam or silt loam (1); Fox sandy loam (2); Oshtemo sandy loam (3); Washtenaw loam (4); and Brady loam (5). The map also shows small road running around the lake (this map was adapted from the Use Capability Map prepared in 1963 by Soil Conservation Service, U.S. Department of Agriculture).


1
2
3
4
5
N
0
400
m

calcareous subsoil. The underdrainage is excellent, except for the Washtenaw loam (4) which has poor drainage (Perkin and Tyson, 1926; Whiteside, personal communication). The limey, porous soils probably contribute greatly to the hardness of the water in Wintergreen Lake. Nearly 80% of the soils of the watershed area is cultivated or used for grazing cattle. The remainder is covered by oak, hickory, maple, and aspen trees (Manny, 1971).

2. Morphometry. The morphometry of Wintergreen Lake as described by Manny (1971) is given in Table 1. The lake is more or less rectangular. The area covered by shallow water is more than 50% of the basin and is dotted with marl deposits (Schneibner, 1958). The southwest submerged shore is covered by plant remains (20-40 cm thick); the opposite end has a thinner organic deposit. The rest of the submerged shore is solid and sandy. The major water sources are surface runoff and pipe drainage originating from a dairy feed-lot. In the spring, water overflows a small dam in Midland Park into Gull Lake.

C. Climate

The climate of the Gull Lake area alternates between continental and semi-marine (Eichmeier). When there is little or no wind, the climate becomes

Table 1. Morphometric parameters of Wintergreen Lake*

Parameters	
Basin	type 35
Length (m)	544
Width (m)	375
Area (ha)	14.98
Volume (m^3)	530,584
Maximum depth (m)	6.3
Mean depth (m)	3.54
Relative depth (%)	1.44
Volume development	1.69
Shore development	1.15

*From Manny (1971)

continental. A strong wind from Lake Michigan transforms the weather into the semi-marine type.

The annual mean air temperature (9.11 C) has not changed in the last 46 years; the range is -20 C to 30 C. Annual precipitation and snowfall in the Gull Lake area averages 88.62 and 170.50 cm, respectively (USCOMM-NOAA, 1971).

III. HYDROGRAPHY

A. Materials and Methods

Sampling was conducted each Monday. Temperature, dissolved oxygen, pH, and transparency of the entire water column were measured at one meter intervals from July to November, 1970, and from June through October, 1971. In 1971 alkalanity, conductivity, and light were also measured. Temperature was measured *in situ* using a thermistor (Model 43 TB, Yellow Springs Instr. Co., Yellow Springs, Ohio). Incident and reflected light were measured with a custom underwater photometer (Rich and Wetzel, 1969). Other physical and chemical analyses of water obtained with a three-liter Van Dorn sampler were performed within one hour of sampling. Oxygen was estimated by a modified Winkler technique (Mackereth, 1963); pH was measured using a Beckman pH meter (Model 76-A). Total alkalinity was determined by titration using H_2SO_4 and a mixture of bromcresol green and methyl red as the indicators. Conductivity was measured with a conductive bridge (Model 31, Yellow Springs Instr. Co., Yellow Springs, Ohio). The transparency of water was followed with a white secchi disc.

B. Results and Discussion

1. Temperature. Wintergreen Lake is temperate, dimictic, and second order (Hutchinson, 1957; Ruttner, 1960; Manny, 1971). Turnover is completed within 2-5 days in October-November and April. Figure 3 shows the distribution of temperature of air and water during the summer stratification; the thermocline stretches from 2.0 to 3.0 m (see also Fig. 45). In both years the mud reached a maximum of 12.8 C in early October, then was cooled during the fall mixing. The diurnal variation of temperature and pH on 4-5 Aug., 1970, are shown in Table 2. The nocturnal isotherm reported by Manny and Hall (1969) in Lake Michigan, did not occur in Wintergreen; this was probably due to the weak wind which failed to mix the epilimnion. Temperature data for the diurnal measurement on 27-28 July, 1971, is shown in Figure 7. Temperature increased after sunrise, reached a maximum in the afternoon, then decreased to a minimum in the morning of the next day.

2. Light and Water Transparency. Figure 4 shows the per cent transmittance of total surface incident light (A) and reflected light (B). More than 50% of total surface incident light was absorbed by the first 1 m of water. The quantity of light absorbed by the water column varied with the wavelengths. Figure 5 shows that

Figure 3. Distribution of temperature in Wintergreen Lake in the summer of 1970 and 1971 (A) and (B): Air temperature 0.5 m above water. (C) and (D): Temperature isopleths of water.

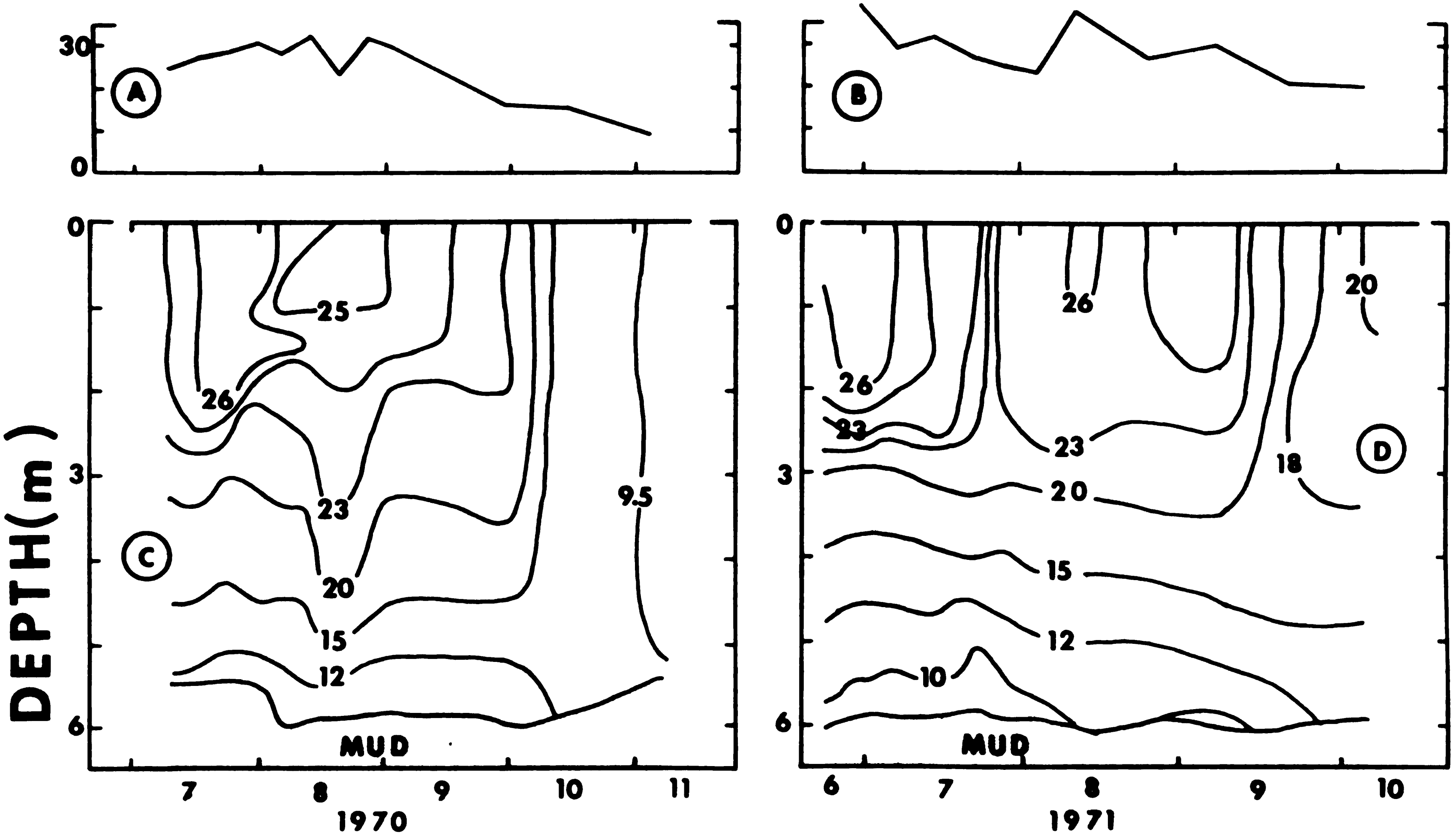

30
0
A
B
0
3
6
DEPTH(m)
C
D
25
26
23
20
15
12
9.5
26
23
23
20
18
20
15
12
10
MUD
MUD
7
8
9
10
11
1970
6
7
8
9
10
1971

Table 2. Diurnal distribution of temperature and pH on 4-5/8 1970

Depth	PM		AM			PM		
(m)	8.15	11.30	5.15	8.02	10.53	2.05	5.0	8.0
				Temperature				
air	19.5	16.5	14.0	16.0	21.0	26.8	23.5	22.0
0	26.0	25.0	24.0	24.0	25.5	26.0	26.8	26.5
1	26.0	25.5	24.5	25.5	25.0	25.0	25.1	25.3
2	24.5	24.5	24.5	24.5	24.5	23.5	24.0	24.2
3	21.5	21.0	21.0	21.2	21.5	20.8	20.7	22.0
4	17.0	17.0	17.0	17.5	17.5	16.5	17.1	17.5
5	13.0	13.0	13.0	13.0	14.0	13.0	13.0	13.0
mud (6)	11.0	11.0	11.0	10.5	11.0	10.5	10.5	11.0
				pH				
0	9.30				9.40		9.50	9.40
1	9.50				9.60		9.70	9.65
2	9.50				9.50		9.70	9.35
3	8.70				8.12		8.38	8.75
4	7.60				7.70		7.64	7.50
5	7.30				7.22		7.25	7.30

Figure 4. Percentage of total surface light at various depths in the water column during the summer of 1971.
(A) Incident light
(B) Reflected light

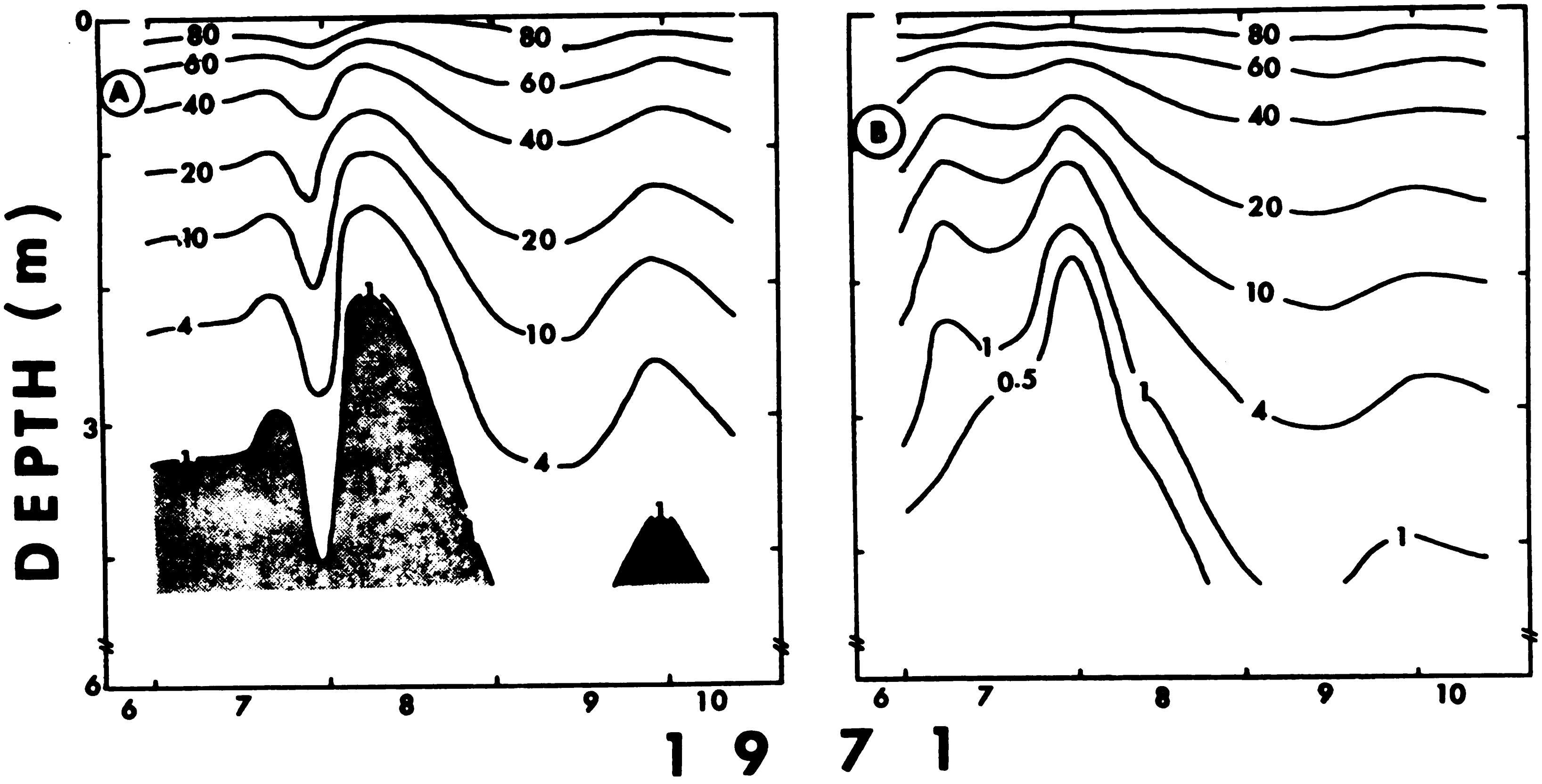
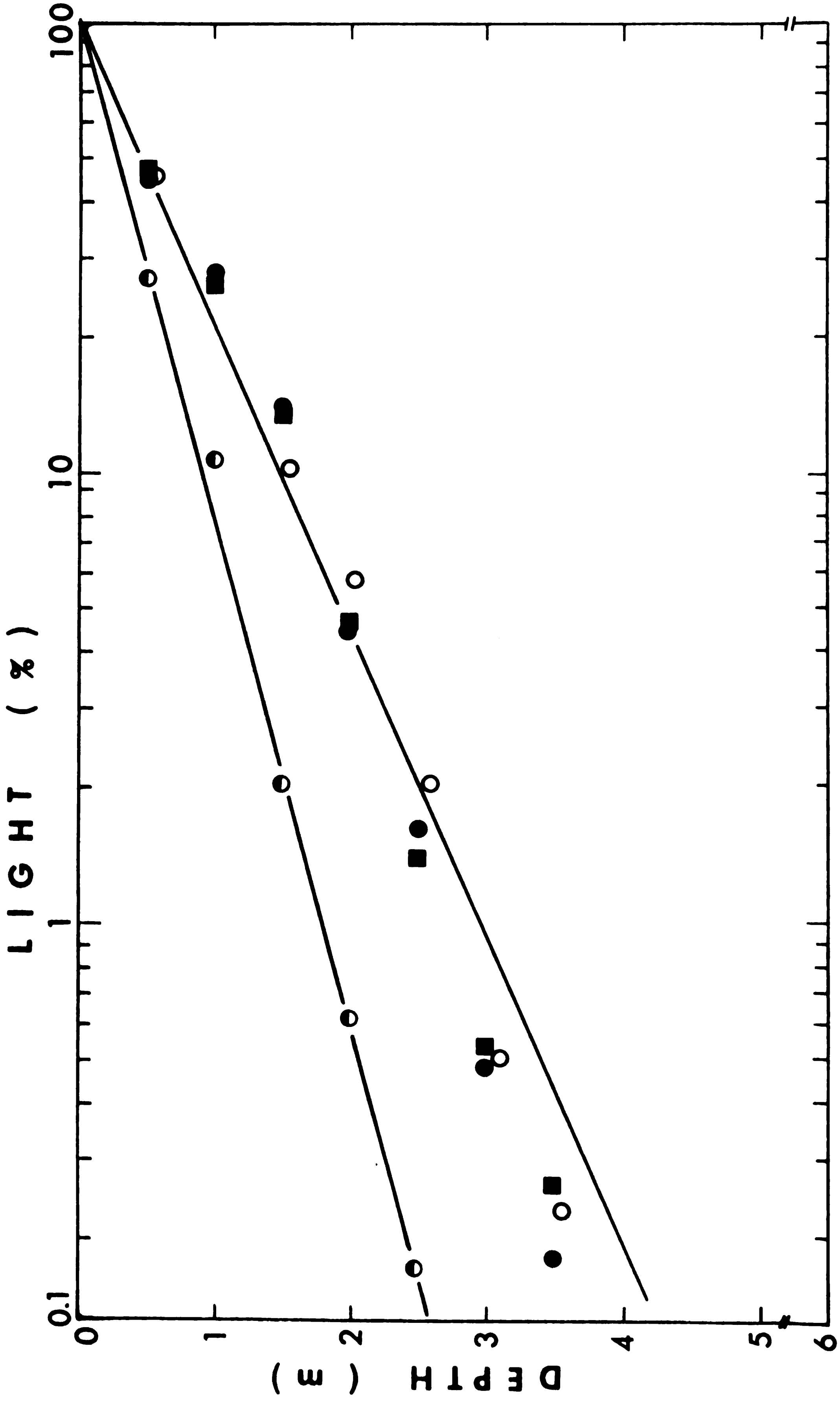

DEPTH (m)
0
3
6
A
B
80
60
40
20
10
4
1
0.5
6
7
8
9
10
1971

Figure 5. Light transmittance in the water column of Wintergreen Lake in July, 1971. White light (○);red (●) ; green (■); blue (◓).

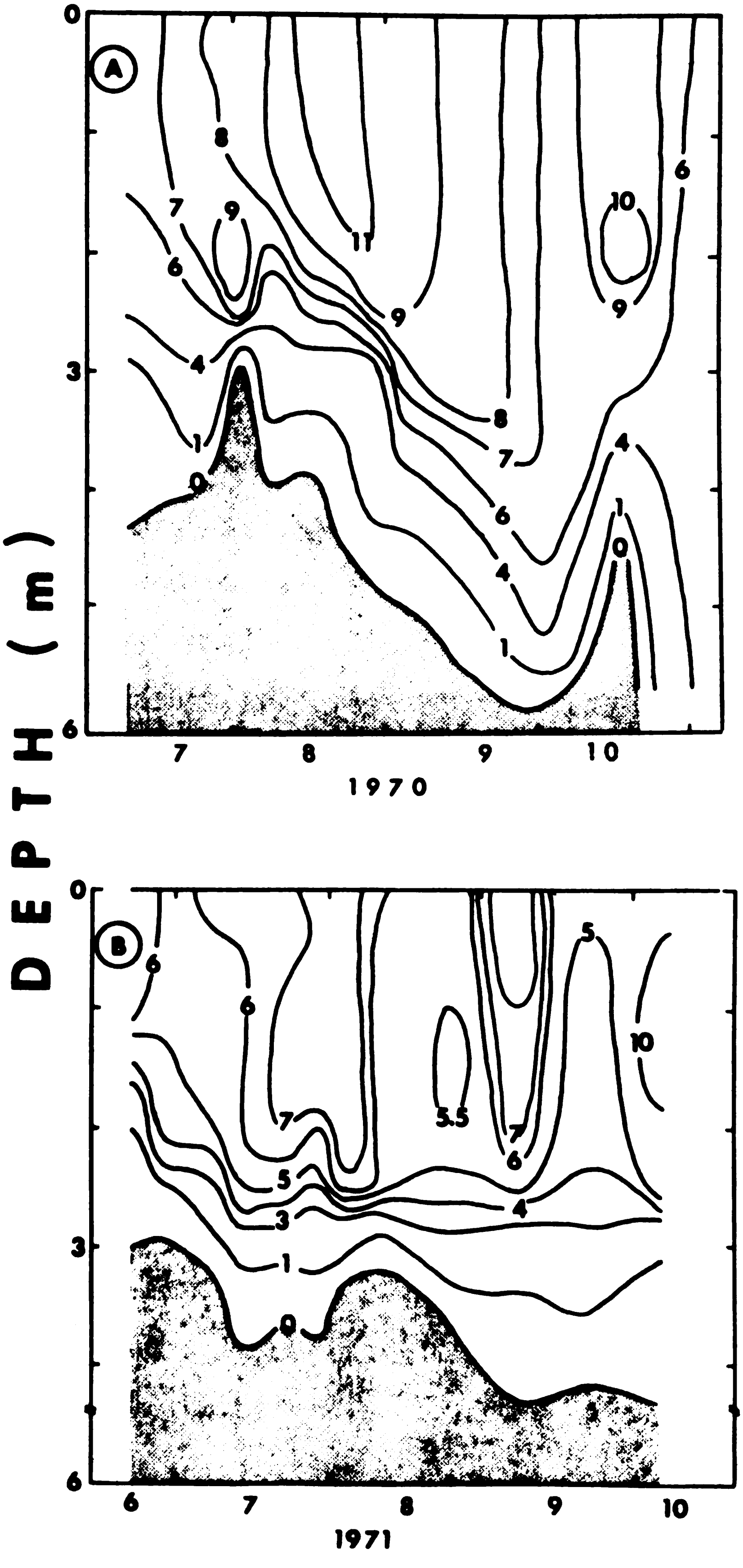

LIGHT (%)
100
10
1
0.1
DEPTH (m)
0
1
2
3
4
5
6

more than 90% of the incident blue light was absorbed by 1.0 m of surface water. This strong absorption was probably due to the high organic matter content of the water (Hutchinson, 1957; Ruttner, 1960). The absorption of red and green light was of the same magnitude as that of white light. Thus high absorption of incident light by the water column appeared to be due to the high plankton density, organic matter, and probably colloidal suspension of $CaCO_3$ (Wetzel, 1966).

The color of the water varied from a light blue in winter and early spring to a yellow-green in summer. In general the secchi disc transparency was inversely proportional to primary productivity and acetylene reduction (Fig. 8). However, the lowest values for transparency occurred during die-offs of algal blooms (3/8/70, 6/8/71, Fig. 8, 15, and 20) and did not coincide with maximum acetylene reduction, primary productivity, or chlorophyll concentration. Thus, in Wintergreen Lake, secchi disc transparency only served as an indicator of the concentration of organic matter and not necessarily of the activity of a biotic community.

3. Dissolved Oxygen. During the fall and spring mixing periods, the oxygen distribution was uniform throughout the lake profile (Fig. 6). The development of a clinograde distribution of oxygen was rapid and

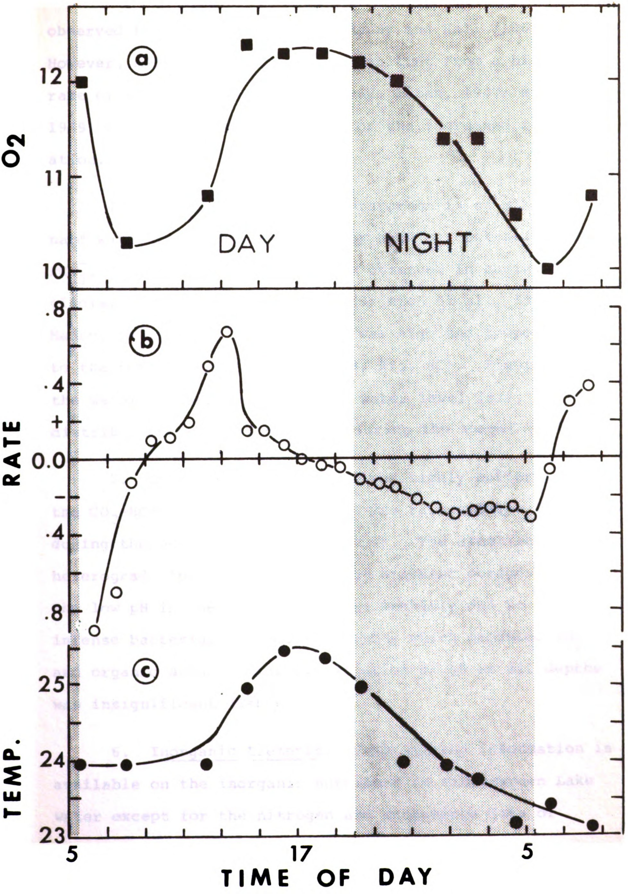
Figure 6. Dissolved oxygen concentration (mg/l) isopleths during the summer of 1970 and 1971. The shaded area indicates the anaerobic zone.


A
0
3
6
8
7
9
6
11
9
10
6
9
4
1
0
8
7
6
4
4
1
0
1
7
8
9
10
1970
DEPTH (m)
B
0
3
6
6
6
10
5
7
5.5
7
6
5
4
3
1
0
6
7
8
9
10
1971

typical of a eutrophic lake. The depletion of oxygen in the hypolimnion (within 3-5 weeks) following stratification was probably due to loss of oxygen to lake sediment (Hutchinson, 1957) and bacterial respiration but the later is believed to be the main cause of the oxygen deficit in the hypolimnion of this lake. Figure 44 shows the clinograde distribution of oxygen during the stagnant period. The high oxygen concentration at 2 m was related to photosynthesis.

Figure 7 shows the concentrations of oxygen (a), the rates of change of oxygen (b), and the diurnal variation of temperature (c) during the period 27-28 July, 1971. The concentration of oxygen decreased during the night to reach a minimum value at 8 AM, then began increasing to obtain a maximum at 5:30 PM. The curve for the rate of change of oxygen was constructed from the diurnal oxygen curve using Odum's method (Odum, 1956). The curve shows a high rate of O_2 production (photosynthesis) in the morning as indicated by the positive slope, passed an equivalence point (O_2 produced was equal to O_2 consumed) at 9 AM and reached a maximum at noon. In the afternoon, the amount of O_2 respired was greater than the O_2 produced, causing a negative slope. This was probably due to a stimulative effect of increasing temperature (Fig. 7c) on the respiration of the entire community. The stimulation of respiration by temperature was also

Figure 7. Oxygen concentration (mg/l) a; rate of change in oxygen concentration (mg O_2/l/hr) b; and temperature (C) c during the diurnal sampling period of 27-28/7/71.


a
DAY
NIGHT
O2
12
11
10
b
RATE
.8
.4
+
0.0
-
.4
.8
c
TEMP.
25
24
23
5
17
5
TIME OF DAY

observed by Verduin (1956) and Manny and Hall (1969). However, metabolite secretions resulting from a higher rate of photosynthesis (Fogg, 1962; Lucas, 1947; Wetzel, 1969) may be the causal factor for the increased respiration.

4. Total Alkalinity. Wintergreen is an alkaline hard water lake, characterized by high concentrations of HCO_3^-, Ca, and Mg, as is commonly observed in basins of glacial origin (Wetzel, 1966-1969; Rich *et al.*, 1971; Manny, 1971). The conductivity was high and proportional to the total alkalinity (Table 3, Fig. 8C). Figure 8 shows the water transparency (A, B_1), water level (B2), and the distribution of alkalinity (C) during the summer 1971.

5. pH. Wintergreen water was highly buffered by the CO_2-HCO_3^--$CO_3^=$ system. The pH was rarely below 7 during the period of study (Fig. 9). The alkaline heterograde developed only during stagnant periods. The low pH in the hypolimnion was probably due to intense bacterial metabolic activity which produced CO_2 and organic acids. Diurnal variation of pH at all depths was insignificant (Table 2).

6. Inorganic Elements. Very limited information is available on the inorganic nutrients in Wintergreen Lake water except for the nitrogen and phosphorus data of

Table 3. Conductivity (μ ohms) of Wintergreen Lake water during the summer of 1971

Depth (m)	22/6	6/7	20/7	3/8	24/8	7/9	21/9	5/10
0	270	268	270	258	270	281	305	291
1	280	273	271	261	273	280	310	299
2	298	280	274	264	271	283	308	303
3	330	310	319	298	308	313	310	321
4	370	355	365	360	418	358	312	350
5	372	430	430	422	483	460	505	437

Figure 8. Water transparency, water level, and alkalinity.
(A) and (B_1): Transparency of the Secchi disc (depth at which disc disappears from view) during the summer of 1970 and 1971.
(B_2): Water level (m)
(C): Total alkalinity in the water column during the summer of 1971 expressed as mg/l of $CaCO_3$. The shaded area indicates the anaerobic zone.

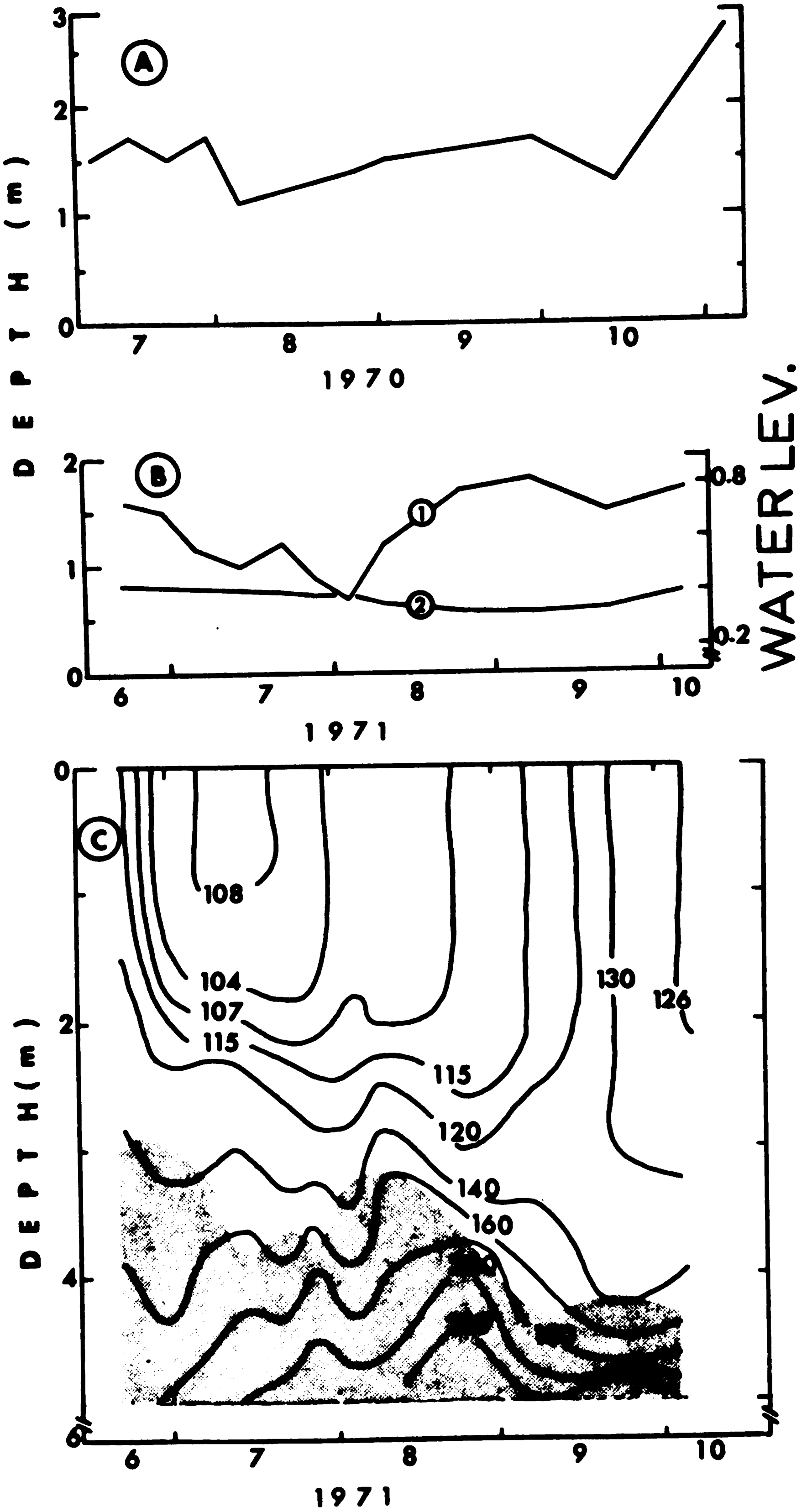
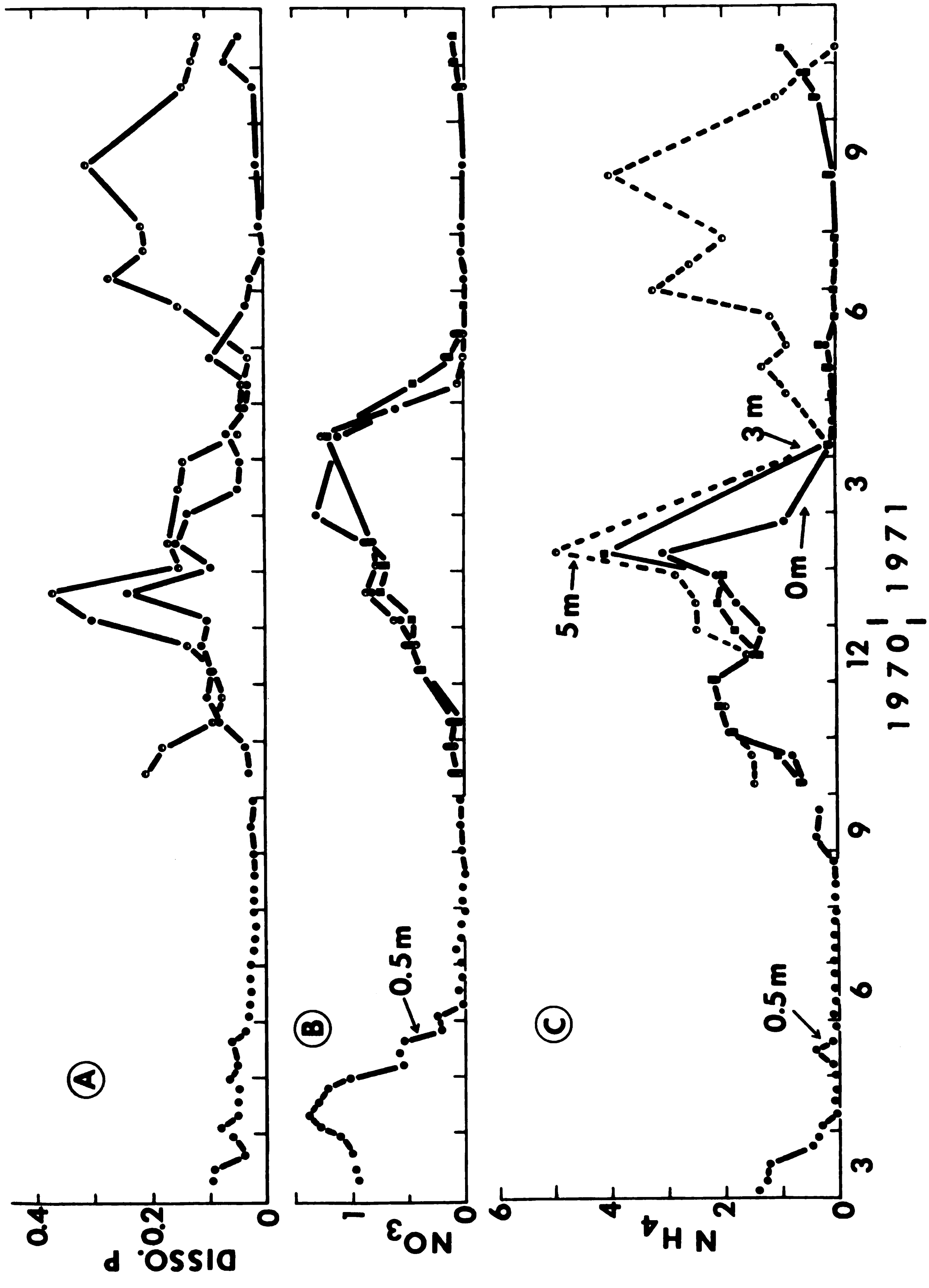

Ⓐ
DEPTH (m)
3
2
1
0
7
8
9
10
1970
Ⓑ
2
1
0
0.8
0.2
WATER LEV.
①
②
6
7
8
9
10
1971
Ⓒ
DEPTH(m)
0
2
4
6
108
104
107
115
115
120
140
160
130
126
6
7
8
9
10
1971

Figure 9. pH of Wintergreen Lake water at various depths during the summer of 1970 and 1971.

Manny (1971). The following discussion is based on his data for the last three years. Ammonium and nitrate had similar seasonal distribution patterns (Fig. 10). The concentrations of ammonium and nitrate at 0.0 m and 0.5 m increased following the fall turnover and reached their maxima in winter. Apparently the low temperature did not totally inhibit nitrification during the winter, as some of the ammonium freed by the fall turnover was eventually oxidized to nitrate. Romanova (1957) reported that the distribution of nitrifying bacteria in Lake Baikal followed a yearly cycle. Their numbers increased from spring to summer, reaching a maximum in fall. The lag of the nitrate maximum in winter (Fig. 10B) may be due to slow-growing nitrite oxidizers (Peck, 1968; Alexander, 1967) and are more sensitive to high concentrations of ammonium in an alkaline environment (Alexander, 1967). Nakos (1969) found the activity of Nitrobacter to be suppressed by as low as 1.0 mg/l of ammonium; the concentrations of ammonium in Wintergreen were greater than 1.0 mg/l from November to February (Fig. 10C). The low levels of nitrate and ammonium in the epilimnion in June-August (0.0-19.0 μg/l and 0.0-36.0 μg/l respectively) coincided with the highest nitrogen fixation (Fig. 18, 19) by the blue-green algal bloom. The total Kjeldahl nitrogen and total organic nitrogen per unit algal cell volume

Figure 10. The concentration (mg/l) of dissolved phosphorous (A), nitrate (B), and ammonium (C) in Wintergreen Lake water from March, 1970, to October, 1971.
Symbols: 0.0 or 0.5 m (●); 3.0 m (■); 5.0 m (◓).
(All data were provided by Dr. B. A. Manny of Kellogg Biological Station, Michigan State University.)


Ⓐ
DISSO. P
0.4
0.2
0
Ⓑ
NO$_3$
1
0
0.5m
Ⓒ
NH$_4$
6
4
2
0
0.5m
5m
3m
0m
3
6
9
12
3
6
9
1970
1971

(Manny, 1971, Fig. 17 and Fig. 19) were also low (about 300 μg/l) during this time.

Dissolved phosphorus (filtered water) and total phosphorus (unfiltered water) (Fig. 10A and Manny, 1971, Fig. 9) had similar annual distribution patterns to ammonium and nitrate. Their concentrations increased to reach a maximum in winter and decreased in summer.

As soon as the stratification began in early summer, the odor of H_2S was detected at 5 m and soon moved up to 4-4.5 m where it remained for the duration of the stagnant season.

Table 4 summarizes the concentrations of the other elements in Wintergreen Lake as reported by Manny (1971).

In summary, the physical and chemical properties of Wintergreen Lake are those of a dimictic, alkaline, hard water, eutrophic lake.

Table 4. Annual range in concentrations of various chemical components in Wintergreen Lake water from 1 m in 1970-71*

Parameter	Concentration (mg/l)
UV-labile DON	0.50 -1.37
UV-refractory DON	0.22 -0.52
TDON	0.62 -1.60
NO_3	0.005-1.34
NO_2	0.012-0.040
NH_4	0.005-2.32
Total dissolved P	0.02 -0.13
Total dissolved C	6.0 -9.1
Ca	10.0 -48

*From Manny (1971)

IV. THE BIOTA

A. Introduction

The producers which utilize light energy in Wintergreen Lake are composed of three physiologically distinct groups--macrophytes, algae, and photosynthetic bacteria--which occupy different habitats within the lake basin. In the following study, all effort was concentrated on these communities although others are equally important in cycling matter in the lake.

B. Materials and Methods

No attempt was made to measure quantitatively the macrophyte population and activity. Study of their distribution and identification of genera (sometimes species) was achieved by sporadic field observations. Water samples for algal enumeration and photosynthetic pigment analysis were taken directly from the water used for acetylene reduction assays or primary production measurements.

1. Algal Enumerations. Algal samples were preserved immediately with Lugol's solution in brown polyethylene bottles and stored at 10 C. Blue-green algal

filaments (trichomes) and heterocysts were counted microscopically in a one-ml Sedgewick-Rafter counting chamber under a 10X objective; reported data are the average of 20 fields. *Microcystis* were counted as number of colonies.

2. *Photosynthetic Pigments*. (All laboratory work in this section was conducted by Dr. Wetzel's laboratory.) The samples for pigment analysis were prepared as follows: 40 to 200 ml of water (depending on the photosynthetic plankton density) were filtered onto Millipore HA membrane filters under low vacuum (1/3 atm). The filters were then homogenized in a Teflon glass homogenizer (Model 4288-C, A. H. Thomas Co., Philadelphia, Pa.) in 90% basic acetone. The extracts were centrifuged and the absorption of the supernatants were measured in a spectrophotometer (Hitachi-Perkin Elmer, Model UV-VIS 139). Concentrations of chlorophylls were calculated by using the trichromatic equations of Parson and Strickland (1963) as modified by Westlake (1969); chlorophyll *a* also was corrected for pheopigments using the modified equation (Wetzel and Westlake, 1969) of Parson and Strickland's (1963).

3. *Multivariate Statistical Analysis*. The relationship between living organisms and their environment is complex (Lindeman, 1942). Therefore it is

difficult to single out and weigh the effect of each factor (Singh, 1955; Saunders, 1963; Wetzel, 1966; Brown, 1971) on others.

As an approach to this problem, multivariate statistical analysis was used (Green, 1971). A computer program was used which permits stepwise deletion of independent variables from a least squares solution until some stopping criterion is fulfilled (Rafter and Ruble, 1966). The criterion used was a minimum significance probability (Sig F_{bi}) of 10% for the regression coefficient for the current candidate for deletion (MINSIG = .10).

By this sequential process, independent variables were eliminated which probably ($p > .10$) did not account for variation in the dependent variable (above that accounted for by the remaining independent variables and the overall mean of the dependent variable). The final solution printed out, after this criterion was met, included statistics whereby the relative importance of independent variables retained can be estimated: constant (b_o), regression coefficients (b_i), F_{bi}, Sig F_{bi}, partial correlation coefficients, and "R^2 deletes." The last statistic is the coefficient of multiple determination when the independent variable under consideration is left out of the solution. The "R^2 delete" for each independent variable can be compared to R^2 for the solution which includes the variable.

The difference R^2 - "R^2 delete$_i$" is an estimate of the percentage of total variation in the dependent variable which was associated with the independent variable under consideration.

The facilities of the Michigan State University Computer Laboratory were used.

C. Results and Discussion

Macrophytes were composed largely of free floating duckweeds: (two species of *Lemna*, *Spirodela*, and Wolffia), submerged *Ceratophyllum demersum* L., two species of *Potamogeton*, *Myriophyllum*, *Najas*, and emergent *Nuphar advena* Aiton, two species of *Nymphaea*. The emergent macrophytes occupied approximately 72% (Manny, 1971) of the Wintergreen basin. Vigorous growth of macrophytes occurred in the period from April to June when the temperature increased. Submerged leaves became heavily coated with organic matter, epiphytes, and white materials, presumably marl deposits during the stagnant period. The population of planktonic algae in Wintergreen Lake, as in other aquatic habitats (Olivier, 1955; Singh, 1955; Vertebnaya, 1957; Wetzel, 1964; Caspers, 1964; Hrbáček, 1964; Billaud, 1968; Stewart *et al.*, 1971) followed a seasonal cycle. The development of these algae was limited to 0-3 m water layer during stratified periods. According to Manny (1971), the algal population during the 1970-1971 period was dominated by Chlorophyta in the cold months (November-April).

Data collected during the summers 1970-1971 indicated that the algal population of Wintergreen Lake water was also subjected to a shift of species (Table 5). Aphanizomenon and Microcystis dominated the algal population in the summer and early fall of 1970. However, in summer, 1971, Aphanizomenon developed earlier (May-June) then died off in late June. The Aphanizomenon-Microcystis bloom was succeeded by an Anabaena-Microcystis bloom which reached its maximum in late July. Photographs of representative algae are shown in Figure 11. The Anabaena-Microcystis bloom died off in late August, 1971, to give way to a short-lived bloom of green filamentous algae which developed at 3-4 m. At this time, the thermocline was below 4 m.

Table 5 and Figure 12 illustrate total numbers of algae, concentration of chlorophyll a, and carotenoids per liter during summer of 1971.

The results of the statistical analysis are shown in Table 6 and 7. In general, the chlorophyll a concentration (algal mass) in Wintergreen during the period June-September, 1971, was correlated to the combination of total alkalinity, temperature, pH, and dissolved phosphorus. The combination of these parameters explained about 81% of the variation of chlorophyll a

Table 5. Total algae population (filaments + colonies/l x 10^3) during the summer of 1971*

Depth (m)	22/6	29/6	6/7	13/7	20/7	27/7	3/8	10/8	7/9	21/9	5/10
0	1,425	722	570	432	318	836	380	114	88	80	76
1	1,159	247	399	603	674	917	399	247	80	38	90
2	663	1,321	494	380	608	494	380	190	83	56	130
3	1,558	589	38	38	456	209	247	266	22	28	98
4	456	19	0	228	76	19	-	114	360**	26	42
5	94	133	0	-	57	19	19	76	198**	50	30

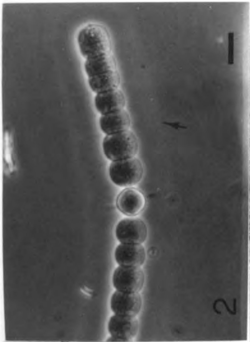
*6/7: *Aphanizomenon* + *Anabaena* + *Microcystis* dominant
6/7-3/8: *Anabaena* + *Microcystis* dominant

**Short-lived green filamentous algae

Figure 11. Photographs of representative algae in Wintergreen Lake:

(1): *Aphanizomenon* showing heterocyst (arrow)
(2): *Anabaena* showing sheath (arrow) and heterocyst
(3): *Anabaena*
(4): Semi-epiphytic green algae (*Oedogonium*) developed in June.

Scale: Bars represent 10 µ



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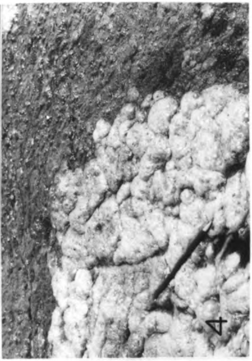

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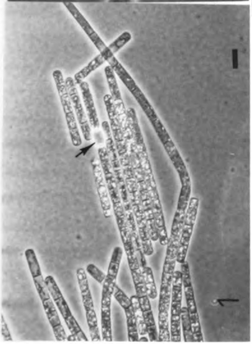

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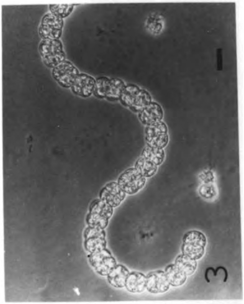


3

Figure 12. The concentration (μg/l) of chlorophyll *a* (●) and carotenoids (◓) at 0 m (A), 1 m (B), 2 m (C), and 3 m (D) in Wintergreen Lake during the summer of 1971.

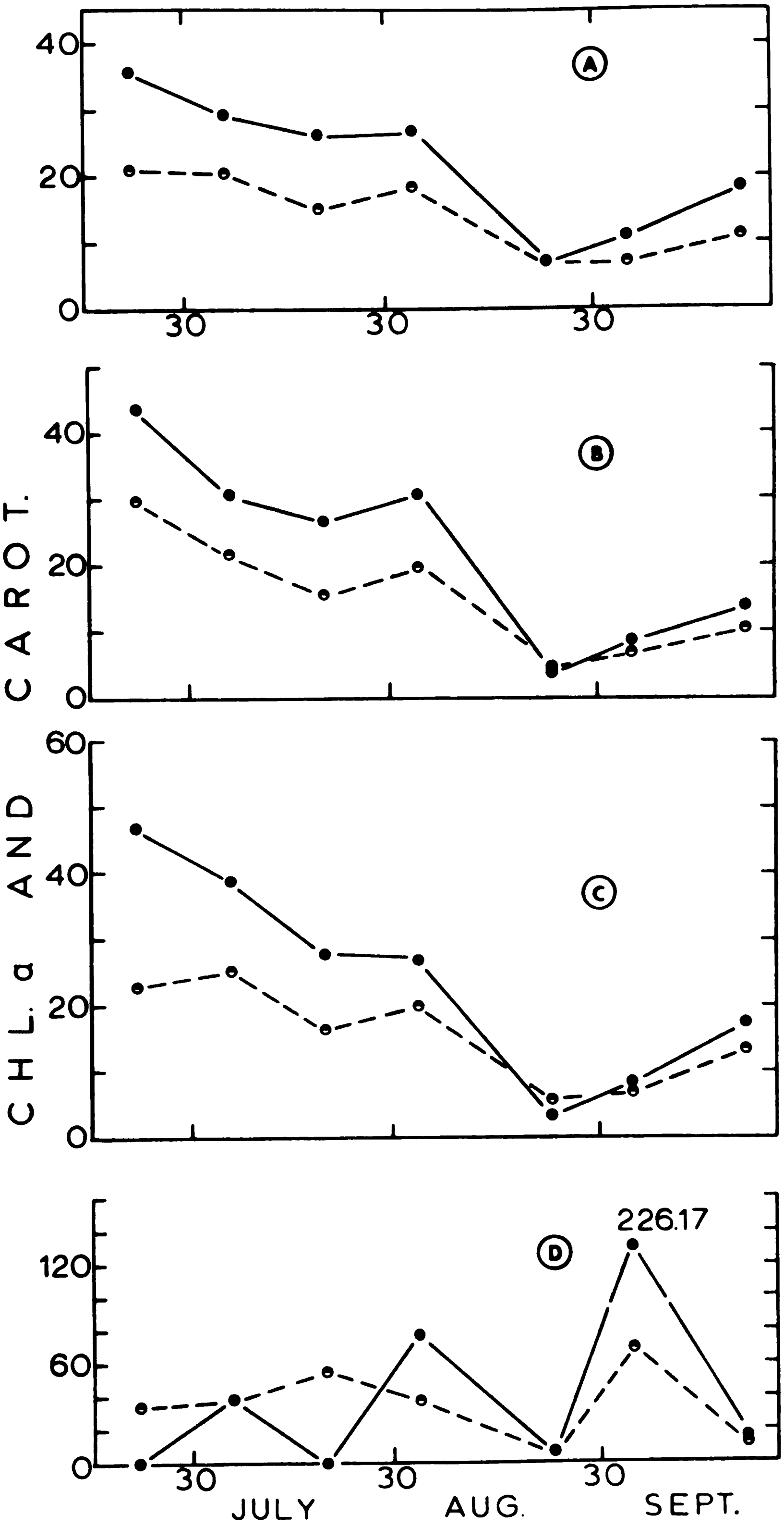

A
B
C
D
CHL. a AND CAROT.
0
20
40
60
120
226.17
30
30
30
JULY
AUG.
SEPT.

Table 6. Multivariate statistical analysis*

Dependent variable Y: chl a (0-2m)

$$Y = -8008.084 - 1.680X_1 - 7.090X_2 + 1937.899X_3 - 112.388X_3^2 + 2.371X_4$$

(a) Overall Regression

	Sum of Squares	df	Mean of Squares	F	Sig.
Regression (about mean)	2741.457	5	548.291	12.585	<0.0005
Error	609.938	14	43.567		
Total (about mean)	3351.396	19			

(b) Multiple Correlation Coefficients

Mean = 23.461 µg/l $R^2 = 0.812$ R = 0.904

	Reg. Coef.	Sig. Level	Partial Corr. Coef.	R^2 Deletes
Constant	-8008.084	0.011		
Alkalinity (X_1)	-1.680	0.023	-0.562	0.734
Temperature (X_2)	-7.090	0.001	-0.761	0.567
pH (X_3)	1937.899	0.008	0.636	0.694
(X_3^2)	-112.388	0.008	-0.668	0.693
Diss. P (X_4)	2.371	<0.0005	0.823	0.437

---

*Factors tested: Net primary productivity, available light, total alkalinity, temperature, pH, nitrate, ammonium, phosphate, carotenoids, and oxygen.

Table 7. Multivariate statistical analysis*

Dependent variable Y: Carotenoids (0–2m)

$$Y = 338.323 - 0.657\ X_1 - 3.308\ X_2 - 1.063\ X_3^2 + 1.529\ X_4 - 0.013\ X_5$$

(a) Overall Regression

	Sum of Squares	df	Mean of Squares	F	Sig.
Regression (about mean)	837.359	5	167.472	12.135	<0.0005
Error	193.206	14	13.800		
Total (about mean)	1030.566	19			

(b) Multiple Correlation Coefficients

Mean = 15.474 µg/l

	Reg. Coef.	Sig. Level	Partial Corr. Coef.	R^2 Deletes
Constant	338.323	0.003		
Alkalinity (X_1)	-0.657	0.001	-0.732	0.596
Temperature (X_2)	-3.308	0.001	-0.726	0.604
$(pH)^2$ (X_3^2)	-1.063	0.083	-0.445	0.766
Diss. P (X_4)	1.529	<0.0005	0.838	0.371
Light (X_5)	-0.013	0.037	-0.525	0.741

---

*<u>Factors tested</u>: Net primary productivity, available light, total alkalinity, temperature, pH, nitrate, ammonium, phosphate, chlorophll <u>a</u>, and oxygen.

concentration. Table 6, part (b), column 4 also indicates that the concentration of chlorophyll a was negatively related to total alkalinity and temperature. It also shows that at certain values of pH, the chlorophyll a concentration was inversely proportional to pH. The most significant factor in this group was the dissolved phosphorus which when depleted from the group, caused the R^2 value to drop in half ($R^2 = 0.437$).

The concentration of carotenoids was also correlated to light, total alkalinity, temperature, pH, and dissolved phosphorus (Table 7). Again carotenoid concentration was inversely related to total alkalinity, temperature, pH, and available light. The relationship between carotenoid and pH was nonlinear. Dissolved phosphorus was highly correlated to carotenoid concentration, thus it seems that dissolved phosphorus was the most decisive factor which limited the development of blue-green algae.

Photosynthetic bacteria in the hypolimnion (3m-mud) were composed mainly of *Thiopedia* (purple sulfur bacteria), *Chromatium*, *Chlathrochloris* sp., and *Prosthecochloris* sp. These organisms constituted 90% of the bacterial population above the mud (Caldwell, unpublished data, MSU). These blooms also exhibited an annual cycle. The blooms disappeared each year during fall turnover probably due to the inhibitive effect of oxygen on these obligate anaerobes. Some of these bacteria probably survived the oxygenated period buried in the sediment. As discussed in Chapter III, after the spring overturn, the hypolimnion rapidly became anaerobic which favored the development of various anaerobic bacteria. Small but significant numbers of *Thiopedia* can be seen microscopically as early as late May. The newly established anaerobic conditions also favored the development of heterotrophic anaerobic bacteria which fermented organic matter leading to the formation of fatty acids, alcohols, etc., which could serve as substrates for the photosynthetic and methane bacteria.

As the stratification of the water column intensified, the metalimnion was permanently established at 2-3 m which limited oxygen movement from the surface water into the hypolimnion during the entire stagnant period (June-August). During this time, the hypolimnion was continuously supplied with organic compounds synthesized in the metalimnion and epilimnion. In the hypolimnion some of those organic compounds were degraded by a bacterial population presumably consisting of photosynthetic, acid-forming (Toerien *et al*., 1967), methanogenic (Barker, 1956), nitrate-reducing and sulfate-reducing bacteria.

V. PRIMARY PRODUCTIVITY

A. Introduction

The problems of relative primary productivity of terrestrial and marine ecosystems have been of continuing interest to limnologists and oceanographers. Odum (1963, 1971) believed that terrestrial and marine habitats might be equally productive. According to Odum (1963):

> . . . basic primary productivity is not necessarily a function of the kind of producer organism or the kind of medium (whether, air, fresh water, or salt water), but is controlled by local supply of raw materials, sun energy, and the ability of local communities as a whole (and including man) to utilize and regenerate materials for continuous reuse.

In this part of the study, the primary productivity of phytoplankton in Wintergreen Lake was measured. To avoid confusion, the terminology of productivity used in this chapter will be briefly discussed and defined.

B. Definition

The definitions of productivity adopted here are based on the definitions of Odum (1971) but have been adopted to conform with this study. The primary productivity of phytoplankton is defined as the rate at which light energy is stored by photosynthetic activity in the form of organic substances. It is important to distinguish the terms in the photosynthetic production process:

a. Gross productivity is the total rate of photosynthesis, including materials used in respiration and excreted by phytoplankton during the measurement period.

b. Net primary productivity of phytoplankton or net light CO_2 fixation is the rate of storage of organic cellular materials in excess of the respiratory utilization and excretion by phytoplankton during the measurement period.

C. <u>Materials and Methods</u> (All laboratory work was done by Dr. R. G. Wetzel's laboratory.)

Primary productivity of photosynthetic plankton was measured by a modification (cf. Wetzel, 1964; Goldman, 1960; Goldman, 1963) of the ^{14}C technique of Steemann Nielsen (1951). All calibrations were done in terms of absolute activity as determined by gas-phase analysis, to eliminated errors associated with counting of radioactivity (Goldman, 1968). The concentrations of absolute activity were determined by Dr. R. G. Wetzel (3.24 or 3.665 μC_i/ml). The sampling schedule coincided with that described in Chapter III for 1971. The primary productivity was measured at 1 m intervals throughout the entire water column. Water of each depth was taken using an opaque 3-liter Van Dorn sampler and transferred to two clear and one opaque (with black polyethylene tape) ground-glass-stoppered Pyrex bottles (125 ml). A correction was made for the variation in bottle volumes. Remaining water was collected into brown polyethylene bottles for pigment analysis. All of the sample bottles were kept in a black box to prevent physiological changes caused by direct sun light. A 1-ml solution of $^{14}CO_2$, predominantly $NaH^{14}CO_3$, was injected into each bottle. The dark bottles were secured then with an extra layer of aluminum foil. The samples were then resuspended at their original depths. Time of sampling was carefully planned so that the incubation could begin at around 10 AM to ensure maximum photosynthesis (Vollenweider and Nauwerck, 1961) and uniform timing. After a 3-hour incubation period, samples were placed in a black box and taken immediately to the laboratory. Usually about 10-15 min elapsed between sample removal and laboratory analysis. A 25 ml sample from each bottle was filtered through a Millipore filter (HA, 0.45 μ) on a multiple millipore filter unit (a vacuum of 1/3 atm was used). A 10 ml sample from each bottle was filtered through the same type of Millipore filter (HA, 0.45 μ) attached to a syringe and the filtrate was collected and acidified to approximately pH 2 with 0.4 ml of 3% H_3PO_4 and flushed with nitrogen for 2 min to drive off dissolved $^{14}CO_2$.

Photosynthetic Millipore filters and $^{14}CO_2$-free filtrates (duplicated) were dried and exposed to fumes of HCl for 10 min to remove any $^{14}CO_2$ contaminant. Radioactivity was determined (with a minimum of 1,000 counts) by a gas-flow Geiger-Mueller counter (Nuclear Chicago, Model 6919, with micromil window D-47). The net primary production was calculated following the standardized procedures of Goldman _et_ _al_., (1969). A correction of 6% for isotopic discrimination was made in the net primary productivity data. A diurnal factor was calculated from the total solar energy and the portion received during the _in_ _situ_ incubation period (Belfort pyrheliometer, Belfort Instr. Co., Baltimore, Md.). Total available inorganic carbon was calculated from total carbonate alkalinity and pH measured at the same time (see Chapter III).

The radioactivity of the filter and filtrate were plotted versus depth. The daily net primary productivity and net excretion were calculated using a planimeter to estimate plot area and the diurnal factor.

D. Results and Discussion

The ^{14}C method is considered one of the best available techniques to measure photosynthesis, although there are uncertainties inherent to it, such as:

(a) There is no way to determine whether this technique is measuring gross production, net production, or some value between these two (Steemann Nielsen, 1963; Vollenweider and Nauwerck, 1961). The radioactivity measured may not represent the true photosynthesis due to assimilation of dissolved organic materials by algae (Wright, 1964; Pearce and Carr, 1967, 1968).

(b) Interferences result in the loss of ^{14}C, due to lowering or inhibition of photoactivity by nutrient exhaustion (Talling, 1957); alteration of the chemical composition of the enclosed water (Gessner and Pannier, 1958), etc., could be great during prolonged incubation periods (> 8 hr, Vollenweider and Nauwerck, 1961).

(c) Enclosure of water sample eliminates turbulence (Verduin *et al.*, 1959) causing sedimentation of algae at the bottom of the bottles.

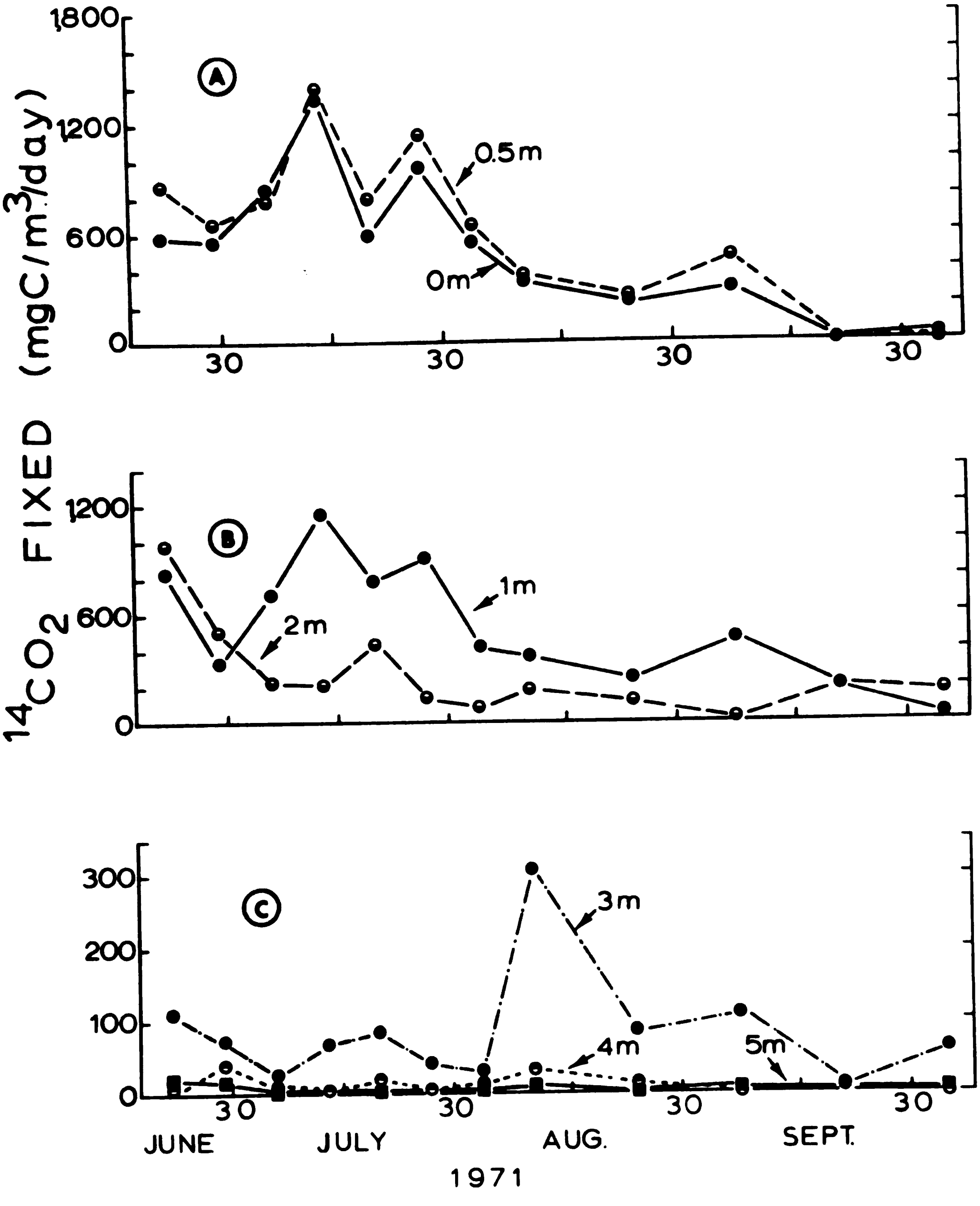
There is sufficient available information which indicates that photoassimilation of organic matter by algae is negligible because of an exceedingly small amount of available organic carbon in lake water (Wright and Hobbie, 1965; Wetzel, 1967, 1968). Other possible errors were minimized by incubating for a short period (3 hr, Vollenweider and Nauwerck, 1961) at a fixed daily time.

The net primary production rate reported here was the radioactivity computed from CO_2 fixation by light minus dark samples with the assumption that the above errors were negligible.

Net primary productivity is shown in Figure 13. Net photofixation rates of CO_2 in the anaerobic zone , 3-5 m, were negligible, as compared to those in the epilimnion. This low primary productivity was observed elsewhere in marl lakes, but where the mineralization of organic matter is almost completed in the aerobic zones (Kuznetsov, 1956), the productivity may be higher (Culver and Brunskill, 1969). Culver and Brunskill reported that primary production of green sulfur bacteria in deep water of Green Lake, New York, accounted for almost 83% of the annual productivity (290 gC/m^2/year) of the lake. Total annual primary productivity including planktonic organisms and macrophytes in Wintergreen Lake would rank far above its neighboring Lawrence Lake (Rich *et al*., 1971) and most of the lakes studied so far (see Wetzel, 1964, Table 10).

The weekly fluctuation of net CO_2 fixation (Fig. 13) was probably due to (1) vertical movement of algae and (2) daily fluctuation of solar radiation received. The first hypothesis is justified by the diurnal vertical density variations of *Aphanizomenon* (1970), or *Anabaena* and *Microcystis* (1971) shown in Figures 22 and 26. Similar observations were reported by Talling (1957), who observed that *Anabaena flos-aquae* Born & Flahvar. *intermedia* f. *spiroides* Woron accumulated in the surface water during the day but was evenly distributed in the

Figure 13. Net primary productivity at 0 m, 0.5 m (A); 1 m, 2 m (B); 3 m, 4 m, and 5 m (C) in Wintergreen Lake during the summer of 1971.


1800
1200
600
0
A
0.5m
0m
30
30
30
30
B
1200
600
0
1m
2m
C
300
200
100
0
3m
4m
5m
JUNE
JULY
AUG.
SEPT.
1971
$^{14}CO_2$ FIXED (mgC/m^3/day)

water column during the nocturnal mixing. Both Anabaena and Microcystis seemed to display diurnal distributions (Fig. 24, 26) in Wintergreen Lake. It should be noted that heterocyst density was consistently higher during the day than the night. The dynamics and mechanism of the movement of algae were not studied. There is evidence which indicates that gas vacuoles found in procaryotic organisms (bacteria and blue-green algae, Cohen-Bazire *et al.*, 1969) probably play a major role in the daily ascent of algae (Talling, 1957) against turbulence (Gessner, 1948) and passive sinking which tend to drive the algae to lower zones. Oxygen bubbles formed by intense photosynthesis also play an important role in keeping algae in the trophogenic zone. Visible bubbles of oxygen can be seen attached to filamentous algae on a quiet day. Increasing body density of algae resulting from accumulation of photosynthetic products may play an important role in the nocturnal downward movement of algae.

The sudden high net primary productivity at 3 m on August 10 (Fig. 13, C) coincided with the loss of the previous floating capability of the algae which now accumulated at 3 m. At this time the majority of algae was probably dying; filaments began to break into shorter segments.

Figure 13 supported the arguments that favorable temperature and high available solar radiation in the

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summer are not necessary for the highest biological activities. But, the combination of a whole set of chemical, physical, and biological factors determine the activity and composition of the algal population. The multivariate statistical analysis shown in Table 8 indicates a significant correlation between the net primary productivity in the epilimnion (0-2 m) and the combination of available light, ammonium, and chlorophyll a. The net photosynthetic rate was positively correlated to an increasing amount of available light up to a certain level, however, strong light inhibited photometabolism of CO_2. Available light during the study period (1971) varied from 277.94 to 648.53 g cal per cm^2 per day (at the Kellogg Gull Lake Laboratories). No attempt, however, was made to determine the saturation value of light. The general effect of light on CO_2 uptake can be visualized from the graphs of net CO_2 uptake versus depth (Fig. 14). The CO_2 uptake was seemingly inhibited by the strong light at the surface. This partial inhibition of photosynthesis at the surface by strong solar radiation has been commonly observed during the growing season (Edmondson, 1956; Verdium, 1956; Talling, 1957; Goldman, 1960; Goldman and Wetzel, 1963; Wetzel, 1964; Schindler and Holmgren, 1971). The photoinhibition of the photosynthetic apparatus by strong light probably involves photoinactivation of their enzymes (Steemann Nielsen, 1962; Steeman Nielson and Jørgensen, 1962).

Table 8. Multivariate statistical analysis*

Dependent variable Y: Net primary productivity (0-2 m)

$Y = -179.402 + 2.219X_1 - 0.002X_1^2 - 1.646X_2 + 14.559X_3$

(a) Overall Analysis

	Sum of Squares	df	Mean of Squares	F	Sig.
Regression (about mean)	1073933.145	4	268483.286	9.26	<0.001
Error	435007.176	15	29000.478		
Total (about mean)	1508940.321	19			

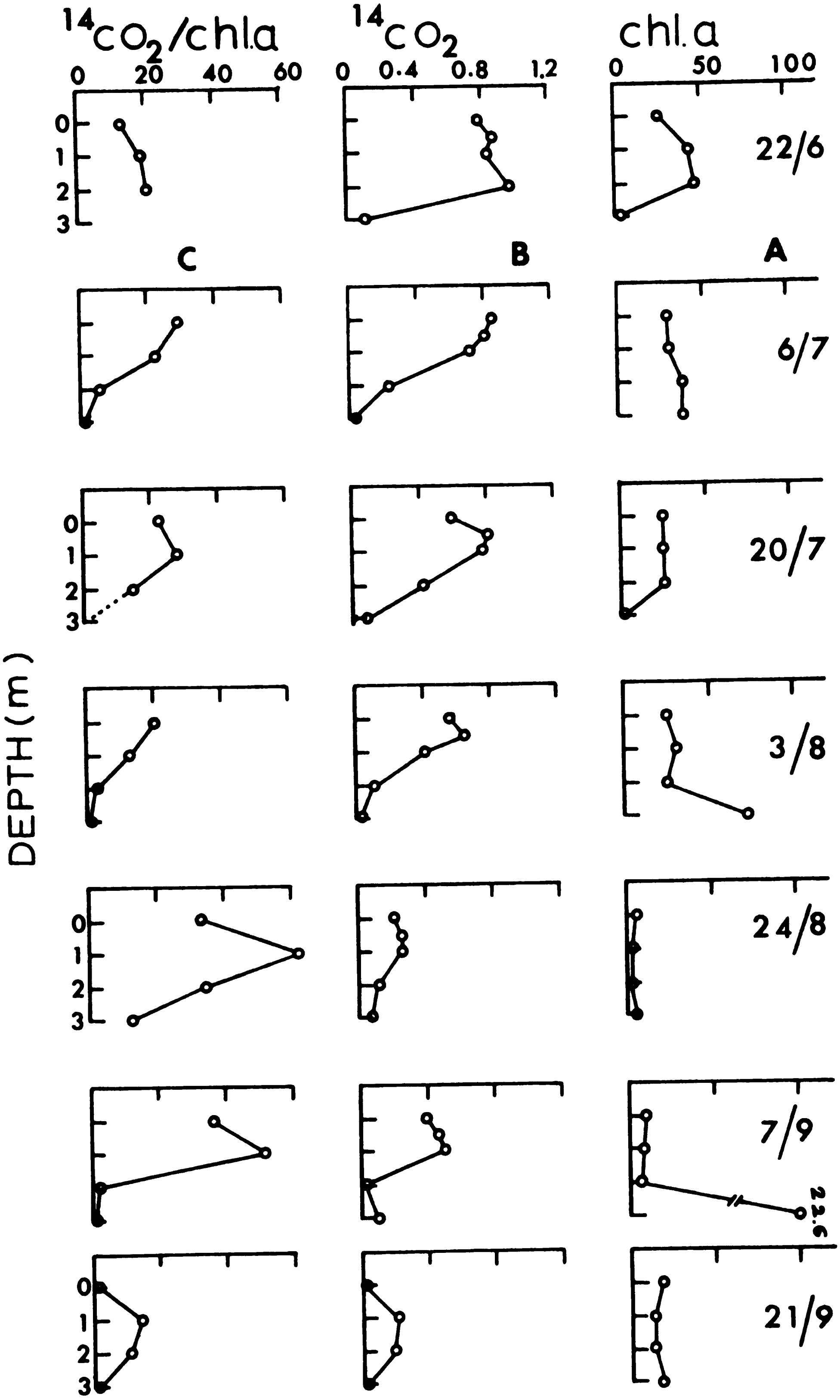
(b) Multiple Correlation Coefficients

Mean = 448.067 mg C/m^3/day $R^2 = 0.712$ $R = 0.844$

	Reg. Coef.	Sig. Level	Partial Corr. Coef.	R^2 Deletes
Constant	-179.402	0.239		
Light (X_1)	2.219	0.018	0.564	0.577
X_1^2	-0.002	0.070	-0.499	0.639
Ammonium (X_2)	-1.646	0.554	-0.154	0.705
Chl _a_ (X_3)	14.559	<0.0005	0.773	0.282

*_Factors tested_: Available light, alkalinity, temperature, pH, nitrate, ammonium, total dissolved P, chl _a_, carotenoids, oxygen, and depth.

Figure 14. The distribution with depth of chlorophyll *a* (μg/l), (A); net primary productivity (mg C/l/day), (B); and net primary productivity per unit chlorophyll *a* (mg C/mg chl *a*/day), (C) during the summer of 1971.


$^{14}CO_2$/chl.a
$^{14}CO_2$
chl. a
0
20
40
60
0
0.4
0.8
1.2
0
50
100
0
1
2
3
22/6
C
B
A
6/7
20/7
3/8
24/8
7/9
22.6
21/9
DEPTH (m)

The effect of the selective absorption of blue light by the water column (Fig. 5) on photometabolism of CO_2 by *in situ* organisms was not studied. However, Findernegg (1964) reported that in a eutrophic lake, by the time algal blooms occurred, the decline of CO_2 uptake did not conform to the decreased transmittance of some wavelengths. It was, however, correlated with the total energy of the whole photosynthetic active spectrum. But recently, Kaushik and Kumar (1970) found that green and blue light showed very little promotion of growth of *Anabaena doliolum* and *Fischerella mucicola* as compared to yellow, red, or white light. Wallen and Geen (1971a, 1971b) also found that light of different spectral quality, but the same intensity, produced differences in cell growth rates, CO_2 assimilation rates, photosynthetic products, concentrations of various photosynthetic pigments, proteins, DNA, and RNA in two species of marine plankton algae. The same authors (1971c) subsequently found qualitative and quantitative differences in photosynthetic carbon products between surface algal samples and deeper algal samples in a lake even though these algae were subjected to considerable vertical transport. They suggested that the observed changes were in response to light quality rather than intensity.

Table 9 shows that net primary productivity at 3-5 m was highly correlated with available light, total

Table 9. Multivariate statistical analysis*

Dependent variable: Net primary productivity (3-5 m)

$$Y = 190.513 + 1.623X_1 - 0.171X_2 - 24.500X_3 + 0.981X_3^2 - 70.895X_4 + 18.928X_4^2$$

(a) Overall Analysis

	Sum of Squares	df	Mean of Squares	F	Sig.
Regression (about mean)	28217.716	6	4702.953	41.37	<0.0005
Error	2273.770	20	113.688		
Total (about mean)	30491.486	26			

(b) Multiple Correlation Coefficients

Mean = 26.91 mg C/m^3/day $R^2 = 0.925$ $R = 0.9620$

	Reg. Coef.	Sig. Level	Partial Corr. Coef.	R^2 Deletes
Constant	190.513	0.003		
Light (X_1)	1.623	<0.0005	0.755	0.826
Alkalinity (X_2)	-0.171	0.062	-0.483	0.910
Temperature (X_3)	-24.500	0.003	-0.599	0.883
X_3^2	0.981	0.001	0.658	0.868
Oxygen (X_4)	-70.895	<0.0005	-0.710	0.849
(X_4^2)	18.928	0.003	0.597	0.884

*Factors tested: Net primary productivity, available light, alkalinity, temperature, pH, oxygen, and depth.

alkalinity, temperature, and oxygen. Carbon dioxide uptake was positively correlated with available light and inversely correlated with oxygen concentration and total alkalinity. Temperature became inversely related to light CO_2 fixation at certain high values of temperature.

It is surprising that there was no significant correlation between concentration of dissolved phosphorus and net primary productivity (Table 8). This lack of correlation seems contrary to the general belief that phosphorus is probably important biogenic element limiting primary productivity (Brock, 1966; Rich et al., 1971; Kalff, 1971).

Table 10b shows dark CO_2 fixation which could be due to algal dark metabolism (Holm-Hansen, 1962), heterotrophic bacteria (Wood and Stjernholm, 1962), chemolithotrophic bacteria (Elsden, 1962) and animals.

During the period 22/6 to 5/10, 1971, dark CO_2 fixation averaged 322.40 mg C per m^2 per day or 21.40% of total CO_2 photofixation. Similarly significant rates of dark CO_2 fixation were reported by Kuznetsov (1956) and Sorokin (1961) in stratified reservoirs.

Total chemosynthesis (dark fixation) rates were fairly constant during the beginning of summer until the sudden die-off of the blue-green algal bloom in late August. Intense degradation of organic debris

Table 10a. Distribution of net primary productivity in Wintergreen water column during the summer of 1971 (mg C/m^3/day)

Depth (m)	22/6	29/6	6/7	13/7	20/7	27/7	3/8	10/8	24/8	7/9	21/9	5/10
0	586.50	560.10	583.80	1342.20	592.50	982.80	560.90	344.90	227.80	396.90	0.00	10.50
0.5	862.20	659.10	797.70	1391.20	804.10	1153.10	646.90	366.80	253.90	471.60	0.00	0.00
1	824.80	334.90	711.00	1149.70	776.90	902.80	417.50	387.90	255.10	488.60	205.40	53.20
2	982.40	498.10	233.20	200.90	429.50	141.60	92.54	180.60	119.60	7.40	199.00	173.70
3	111.20	71.80	27.50	64.00	82.50	40.10	28.90	305.90	84.50	109.20	4.40	58.80
4	0.40	40.30	11.80	4.90	19.70	7.40	15.40	33.10	15.20	0.00	12.50	0.00
5	20.80	11.80	1.20	0.80	0.00	1.10	2.44	9.80	0.00	4.70	1.80	3.40
Total	2526.10	1517.00	1838.50	2762.50	1901.10	2075.80	1764.60	1262.50	702.20	1006.80	423.10	299.60

Table 10b. Chemosynthesis (dark bottle, mg C/m^3/day)

Depth (m)	22/6	29/6	6/7	13/7	20/7	27/7	3/8	10/8	24/8	7/9	21/9	5/10
0	55.10	31.80	35.30	44.90	50.40	46.20	35.60	26.20	21.60	23.20	3.20	2.40
0.5	63.70	29.20	38.90	43.30	51.00	51.60	31.30	31.70	21.80	6.40	11.20	9.40
1	76.30	27.80	42.20	42.70	51.60	61.10	28.50	39.70	22.40	2.00	55.00	40.80
2	51.10	140.90	39.10	29.50	33.60	72.30	25.70	22.70	12.10	4.50	24.90	287.00
3	78.60	64.20	111.90	194.30	65.20	48.90	74.60	64.90	12.70	16.70	2.40	41.60
4	133.40	69.10	25.30	25.90	11.70	24.30	28.20	82.30	45.30	352.80	31.30	78.00
5	13.10	112.40	22.90	18.80	48.50	23.20	15.30	30.20	38.50	42.30	42.40	97.20
Total	407.60	446.20	277.40	399.40	261.00	276.00	207.90	266.00	152.60	448.00	170.30	556.40

following the algal die-off seemingly stimulated dark CO_2 fixation, which exceeded photosynthesis in early October. A similar observation following algal die-off was reported by Kuznetsov (1958).

Kuznetsov (1956) rejected the chemosynthesis idea attributed to photosynthetic bacteria. He found no connection between natural populations of photosynthetic bacteria and anaerobic chemosynthesis in reservoirs. He also found that the productions of sulfate-reducing and iron bacteria were negligible. On the contrary, he found high chemosynthesis in the anaerobic zone due to the activity of hydrogen sulfide-oxidizing bacteria (Thionic bacteria) which were found mostly at the interface between the hydrogen sulfide and oxygenated layers (Kuznetsov, 1968).

The metabolism of single carbon compounds has been recently reviewed by Ribbons _et_ _al_. (1970). Methane-oxidizing bacteria are known to oxidize methane to CO_2 and then assimilate it _via_ the carboxydismutase reaction. Thus the presence of methane-oxidizing bacteria could contribute to the chemosynthetic activity in oxygenated zones of natural waters. Sorokin (1961) observed the coincidence between the highest population of methane-oxidizing bacteria and the sudden drop of methane concentration at the boundary of the aerobic and anaerobic zones in a stratified reservoir. A similar distribution of methane was also observed by Tiedje (unpub.) during the summers 1970 and 1971 in Wintergreen Lake. Methane bacteria (see reviews by Stadtman, 1967 and Toerien and Hattingh, 1969) may also be important CO_2 fixers in the anaerobic zone.

There was no significant correlation between dark CO_2 uptake from 0 to 5 m with any single or any combination of the following factors tested: available light, temperature, oxygen, alkalinity, depth, net light CO_2 uptake, and pH.

Excretions of the ^{14}C assimilated are shown in Table 11. For convenience, excretion in the transparent bottles is called light excretion and that in the dark bottles is called dark excretion. Light excretion rates were highest in June and lowest in September-October within the study period. Light excretion rates averaged 60.63 mg C per m^2 per day or about 3.5% of that of the total net CO_2 uptake in the transparent bottles.

Most of the available information concerning excretion of newly fixed carbon came from the works of Fogg (1952), Wetzel and his coworkers (1969, 1971). Data collected by Fogg (1952) from pure cultures of *Anabaena* showed that extracellular non-nitrogenous organic compounds were almost equal to extracellular nitrogenous compounds. These nitrogenous substances were composed mainly of peptides and amides. In his

Table 11a. Distribution of rate of organic excretions in Wintergreen Lake during the summer of 1971 (transparent bottle, mg $C/m^3/day$)

Depth (m)	22/6	6/7	13/7	20/7	27/7	3/8	10/8	24/8	7/9	21/9	10/5
0	39.16	19.0	22.50	19.30	32.70	17.20	15.60	9.8	13.20	3.20	2.80
0.5	39.20	36.70	26.30	19.90	33.20	20.9	3.30	8.60	10.90	3.0	3.20
1	49.20	18.10	18.70	21.90	26.30	12.10	10.20	8.45	5.30	5.40	6.40
2	32.50	9.50	8.50	12.20	6.80	4.2	8.65	5.40	4.2	4.0	12.70
3	6.60	6.80	5.10	5.75	4.90	4.50	9.10	3.7	6.10	2.35	3.10
4	9.80	6.40	8.00	1.40	4.30	5.70	5.70	6.50	3.50	3.40	4.50
5	4.90	5.40	5.50	3.50	6.90	5.20	7.10	5.80	7.20	6.70	6.0
Total	142.10	65.70	68.3	64.0	81.9	49.0	56.35	39.65	39.5	25.0	35.50
Percentage	4.80	3.10	2.20	3.0	3.50	2.50	3.70	4.60	2.70	4.20	4.10

Table 11b. Dark excretion (dark bottle, mg $C/m^3/day$)

Depth (m)	22/6	6/7	13/7	20/7	27/7	3/8	10/8	24/8	7/9	21/9	10/5
0	21.20	6.60	6.15	5.55	5.35	5.40	3.30	2.80	4.30	3.30	4.40
1	16.0	3.30	3.00	3.10	2.10	2.60	3.10	2.50	3.10	3.50	5.20
2	6.10	4.20	3.90	2.35	3.20	2.20	6.20	2.40	4.40	2.50	4.50
3	--	6.80	4.50	3.40	1.40	4.00	8.10	2.40	3.70	2.90	5.10
4	4.60	6.60	6.70	2.20	2.90	2.50	4.80	6.40	3.40	2.60	6.20
5	4.40	8.30	6.00	3.30	3.10	6.20	6.70	3.90	7.30	6.90	7.20
Total	52.30	35.80	30.25	19.90	18.00	22.90	32.20	20.40	26.20	21.70	32.60
Percentage	12.80	12.90	7.60	7.60	6.50	11.00	12.10	13.40	5.80	12.70	5.86

study, young cultures excreted the highest per cent of nitrogen fixed (50%). The per cent excreted decreased toward the end of the exponential phase then increased to a lesser extent in stationary phase.

The growth of the *Anabaena* bloom in Wintergreen Lake followed, more or less, the classic growth curve (Fig. 15). However, the organic carbon excretion pattern *in situ* shown in Figure 16 and Table 11a was different from that observed by Fogg (1952) in pure cultures of *Anabaena*. This difference may be due to a number of reasons: (1) Fogg (1952) measured in detail only the excretion of nitrogenous substances, the present study measured total substances excreted. The excretion of *Microcystis* which were also present in large numbers may cause this different pattern. (2) Extra-cellular organic carbon measured *in situ* could represent the amount left behind after intense metabolic activities of epiphytic bacteria (Odum, 1957; Wetzel, 1969; Allen, 1971; also cf. Wetzel, 1968), free-living bacteria (Wright and Hobbie, 1965, 1966; Hobbie and Wright, 1965; Wetzel, 1967) or by algae themselves (Khoja and Whitton, 1971; also cf. Danforth, 1962).

The total amount of carbon excreted (3.5% of the total net CO_2 fixed) in Wintergreen Lake was higher than the 1.5% observed by Fogg (1958, cited by Fogg, 1962) in a Swedish lake when *Gloeotrichia* and *Aphanizomenon*

Figure 15. The density of *Aphanizomenon* plus *Anabaena* populations (filaments/l x 10^5, ●) and total heterocysts (numbers/l x 10^5, ◒) at 0 m (A), 1 m (B), 2 m (C), and 3 m (D) during the summer of 1971.

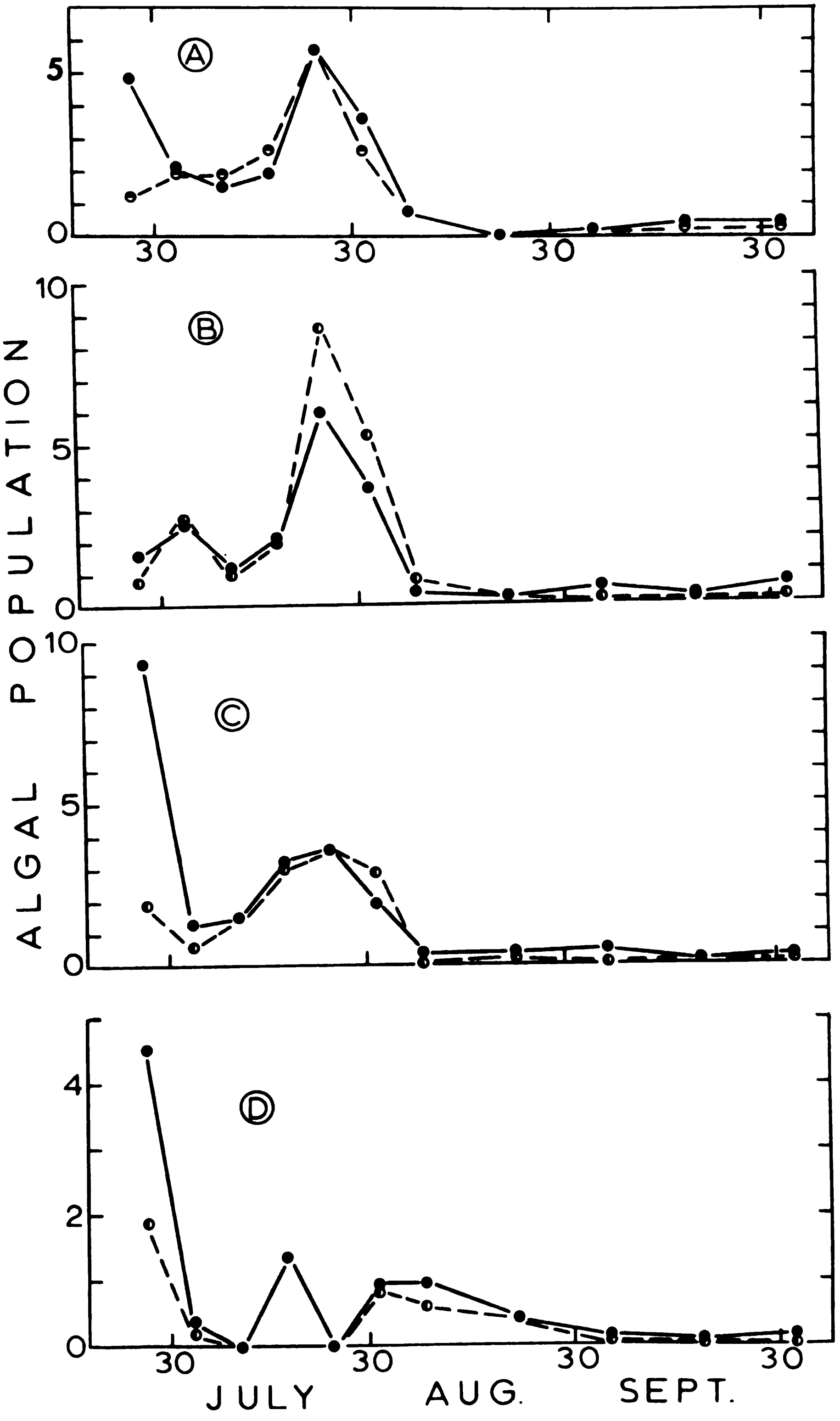
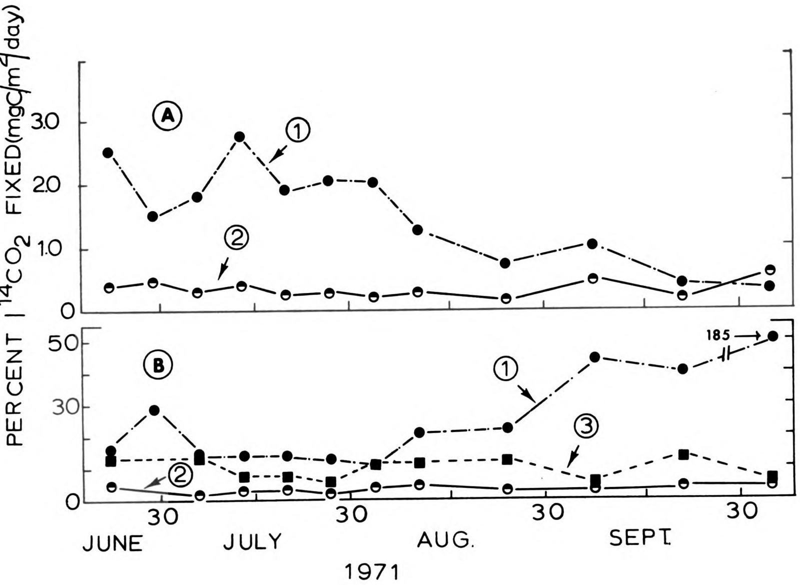

A
B
C
D
ALGAL POPULATION
0
5
10
4
2
30
JULY
AUG.
SEPT.

Figure 16. Production of 1971

(A_1): Total net primary productivity (mg C/m^2/day x 10^3)
(A_2): Total dark CO_2 fixation (mg C/m^2/day x 10^3)
(B_1): Total dark fixation as a percentage of total net primary productivity
(B_2): Total light excretion as a percentage of total net primary productivity
(B_3): Total dark excretion as a percentage of total dark fixation


PERCENT | $^{14}CO_2$ FIXED(mgC/m²/day)
A
B
①
②
③
3.0
2.0
1.0
0
50
30
0
185 →
30
30
30
30
JUNE
JULY
AUG.
SEPT.
1971

were the predominant plankton. It is reasonable that the percentage of newly fixed carbon that was excreted also varies with the type of algal bloom, lake environment, and analytical procedure. The excretion of newly fixed carbon also has been shown to be influenced by environmental conditions (Fogg, 1952). Fogg and Nalewajko (unpub. cited by Fogg, 1962) found that the liberation of extracellular products amounted to 90% of the total carbon fixed in Lake Windermere, English Lake District, in which diatoms were the most abundant planktonic algae. Miller (1971) reported that the mean annual secretion of dissolved organic carbon by phytoplankton in a near-by marl lake (Lawrence Lake) was 5.8% of net production. However, this lake did not have blue-green algal blooms similar to those in Wintergreen Lake.

Extracellular products measured from 3-5 m probably resulted from the activity of both anaerobic and aerobic bacteria in this zone. Total light excretion (in transparent bottles) sometimes exceeded net primary productivity (Tables 11 and 10). The excretion of organic carbon by bacteria has been reviewed by Wilkinson (1958). Extracellular polysaccharides may exceed cellular dry weight in certain conditions. The mean dark excretion by planktonic organisms was 28.4 mg C per m^2 per day (8.8% of total carbon fixed in the dark and 46.8% of total extracellular products in transparent bottles). Thus, overall, dark excretion and light excretion were almost equal. However, the vertical distribution of these excreted products was ununiform (Table 11). Furthermore the excretion in transparent bottles minus that in dark bottles (9.92 mg C/m^2/day) in the epilimnion (1-2 m) was higher than dark excretion (4.78 mg C per m^2 per day). The excretion in transparent bottles minus that in dark bottles (0.67 mg C per m^2 per day) in the lower region (3-5 m) was, however, lower than dark excretion (4.68 mg C/m^2/day).

Results of statistical analysis of excretion data are shown in Table 12 which indicates that the excretion of newly fixed carbon in transparent bottles (0-2 m) was highly correlated with the combination of oxygen, available light, pH,depth, and net primary productivity. Among these factors, pH was inversely related to excretion at certain high pH values. The correlation between available light and total excretion in transparent bottles was also not linear. This excretion was, however, highly correlated with net primary productivity.

There was no significant correlation between total excretion in transparent bottles in the 3-5 m zone and any one or combination of the tested factors such as net primary productivity, oxygen concentration, total alkalinity, available light, temperature, pH, and depth.

Table 12. Multivariate statistical analysis*

Dependent variable: Excretion in transparent bottles (0-2 m)

$$Y = 4813.105 + 2.143\ X_1 + 0.035\ X_2 - 1135.703\ X_3 + 66.548 X_3^2 + 7.378\ X_4 + 0.020\ X_5$$

(a) Overall Analysis

	Sum of Squares	df	Mean of Squares	F	Sig.
Regression (about mean)	2942.164	6	490.360	13.96	<0.0005
Error	702.491	20	35.124		
Total (about mean)	3644.655	26			

(b) Multiple Correlation Coefficients

Mean = 15.883 mg C/m^3/day $R^2 = 0.8073$ $R = 0.8985$

	Reg. Coef.	Sig. Level	Partial Corr. Coef.	R^2 Deletes
Constant	481.105	0.008		
Oxygen (X_1)	2.143	0.015	0.513	0.738
Light (X_2)	0.035	0.012	0.527	0.732
pH_2 (X_3)	-1135.703	0.007	-0.554	0.721
(X_3^2)	66.548	0.007	0.558	0.719
Depth (X_4)	7.378	0.029	0.466	0.753
Light CO_2 Fixed (X_5)	0.020	<0.0005	0.749	0.560

*Factors tested: Light net CO_2 uptake, oxygen, alkalinity, available light, temperature, pH, and depth.

It is concluded that the production of planktonic organisms in Wintergreen Lake during the period 22/6 to 5/10, 1971, had the following characteristics:

1. Net primary productivity of planktonic communities was high, ranging from 300.0 to 2,762.5 mg C per m^2 per day and was mostly due to the activity of blue-green algae in the epilimnion (0-2 m).

2. Net primary productivity in the 1-2 m region was correlated significantly with the combination of available light and chlorophyll a concentration. The net primary productivity of the 3-5 m region was correlated with a set of factors: available light, total alkalinity, temperature, and oxygen concentration.

3. Dark CO_2 fixation averaged 322.40 mg C per m^2 per day or 21.40% of the net primary productivity (net CO_2 fixation). There was no correlation between dark fixation and physical, chemical, and biological parameters measured at all depths.

4. Total excretion averaged 60.60 mg C per m^2 per day or 3.5% of total net carbon fixed. Excretion is believed to be from algae and bacteria, however, algae were probably the most important contributors because of their higher biomass.

5. Total net productivity including net primary productivity, dark fixation, and excretion in Wintergreen

Lake was 1,890.00 mg C per m^2 per day or about 150 tons of organic carbon produced during the period of 22/6 to 5/10, 1971, in the lake.

VI. *IN SITU* NITROGEN FIXATION

A. Introduction

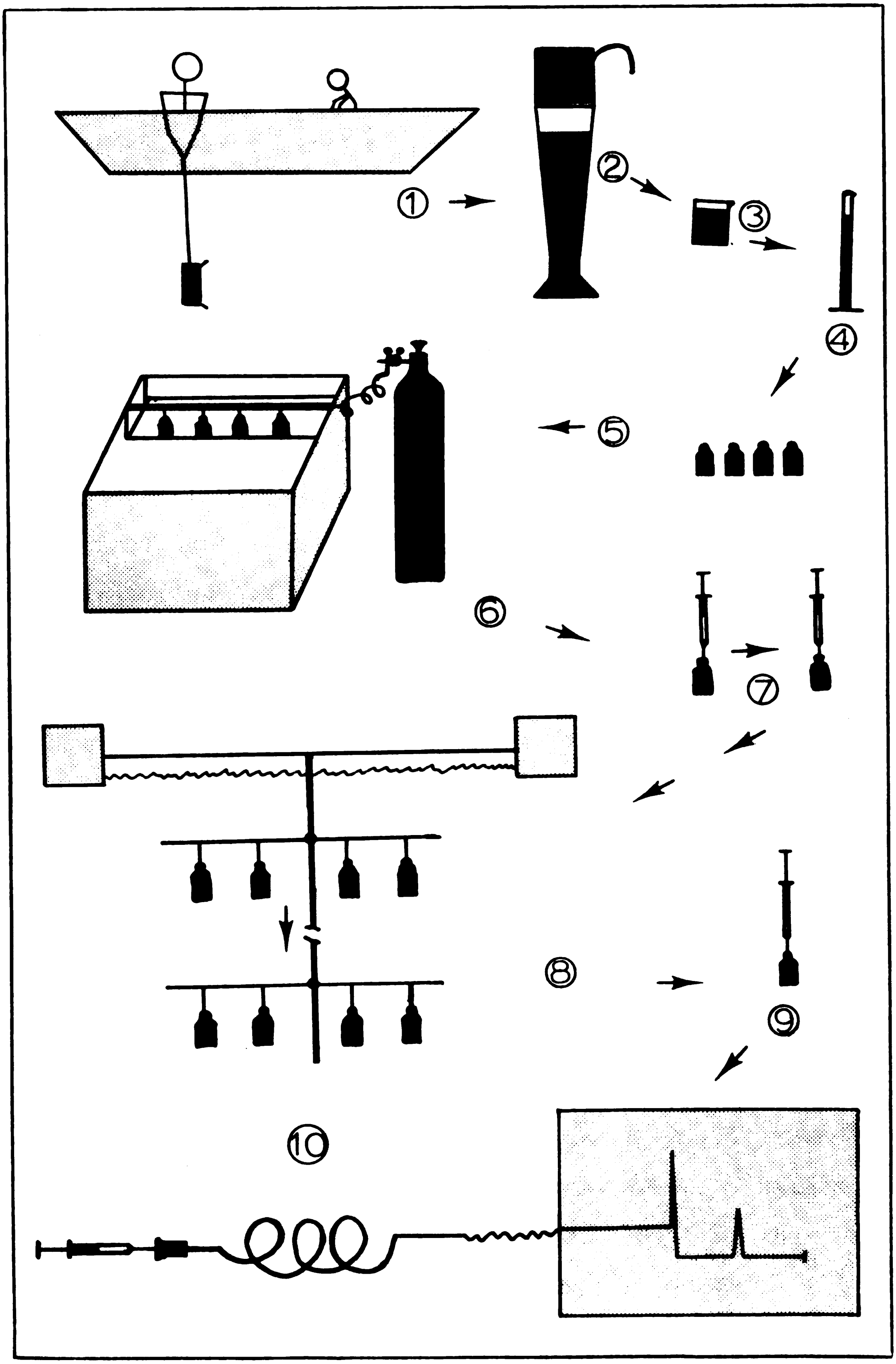
Blue-green algae (planktonic, benthic, and epiphytic) together with bacteria, e.g. *Clostridium*, *Azotobacter*, photosynthetic bacteria, etc., are the most likely organisms to contribute to the nitrogen income of the aquatic environment. Estimation of the rates of atmospheric nitrogen fixation by these organisms *in situ* is difficult. The measurements based on total nitrogen change (Bremner, 1965; Nishigaki and Shioiri, 1959), or on ^{15}nitrogen uptake (Dugdale *et al*., 1959; Dugdale and Dugdale, 1962; Dugdale *et al*., 1964; Goering and Neess, 1964; Stewart, 1970a; Horne and Fogg, 1970), have certain limitations. The first technique is not sufficiently sensitive and is indirect, thus incorporating many serious errors. The ^{15}nitrogen technique is time consuming, expensive, and requires sophisticated equipment (mass spectrometer). In the last five years, the study of biological nitrogen fixation both *in situ* and *in vitro* has progressed rapidly following the discovery by Scholhorn and Burris (1966, 1967) and by Dilworth (1966) that nitrogenase also reduced acetylene to ethylene.

Hence, the measurement of the rate of reduction of acetylene may be used as an index for the rate of nitrogen fixation. This method has been used widely by biologists, oceanographers, soil scientists, limnologists etc. to monitor biological nitrogen fixation (Stewart *et al.*, 1967, 1968; Hardy *et al.*, 1968; Brezonik and Harper, 1969; Jewell and Kulasooriya, 1970; Elleway *et al.*, 1971; Stewart *et al.*, 1971; Macgregor and Johnson, 1971). The advantages of this technique are that it is sensitive (10^3 times more sensitive than ^{15}N, 10^6 times more sensitive than Kjeldahl analysis), cheap and rapid (Hardy *et al.*, 1968). The main disadvantage, however, is that it is indirect. However, previous studies comparing the ^{15}N and acetylene reduction methods showed a consistent correlation, thus supporting the validity of the latter.

B. Materials and Methods In Situ

The technique used for this study was adopted from that of Hardy, Holsten, Jackson, and Burns (1968). The samples obtained for acetylene reduction were taken at the same time as those for limnological and productivity studies (Chapters III, V). Sporadic checks indicated that water without phytoplankton and from below 3 m, did not reduce acetylene in both the summer of 1970 and 1971. Therefore, the routine acetylene reduction assay was limited to the depths 0, 1, 2, and 3 m. The major steps for preparation of the sample for the acetylene reduction assay are summarized in Figure 17. Water was collected at station A (Fig. 1) with a 3-liter Van Dorn sampler (1). A 1 liter sample (0.5 liter if phytoplankton density was high) was filtered through a flour sifter (top 2) in which the agitating part was removed and the bottom screen was lined with a nylon netting of 20 μ square pore size (Nitex, Tobler, Ernst Traber, Inc., New York, N.Y.). The filter retained more than 95% of the algal population as revealed by microscopic examination. The reason for not using a finer netting was to avoid lengthy filtration periods and thus prolonged exposure of algae to the surface environment. Algae retained on the filter were carefully poured into a 50-ml beaker (3) and the volume was brought to 40 ml with filtrate from the same depth. A 10-ml sample (4) was transferred to each of four 26-ml serum bottles (A. H. Thomas Co., Philadelphia, Pa.). In the summer of 1970, the samples were not flushed with a nitrogen-free gas mixture, however, in the summer of 1971, they were flushed with a gas mixture containing 0.04% CO_2, 22% O_2, and 78% argon (Matheson Gas Products, Joliet, Ill.) (6). Immediately after flushing the bottles were capped with rubber serum stoppers (A. H. Thomas Co., Philadelphia, Pa.); excess pressure was released by a quick puncture with a needle. All of the bottles

Figure 17. Procedure for *in situ* acetylene reduction assay in the lake.


①
②
③
④
⑤
⑥
⑦
⑧
⑨
⑩

prepared in this manner were stored in a dark box while preparing ones from other depths. A portion of the head space gas was taken out (1 or 2 ml) prior to addition of 1 ml (1970) or 2 ml (1971) of acetylene (Matheson Gas Products, Joliet, Ill.) (7). The algae in one of the four bottles from each depth were killed immediately after addition of acetylene by injecting 0.2 ml of 2% $HgCl_2$. This bottle served as the control. The sample preparation for the four depths took about 40 min. Sample bottles were incubated at 11-12 AM at their original depth (8). The reduction reaction was stopped by injecting 0.2 ml of 2% $HgCl_2$ after one hour of incubation in the lake (9). Samples were kept in an ice chest during transportation from the lake to the laboratory. Samples were allowed to equilibrate at room temperature and 0.5 ml of the headspace gas was injected into a gas chromatograph (10) equipped with an H_2-flame ionization detector (Model 600-D, Varian Aerograph, Walnut Creek, Calif.) and interfaced with a Varian recorder (Model 20). The flow rates were: nitrogen carrier gas (Hi-pure), 25 ml/min; H_2,25 ml/min; and compressed air, 300 ml/min. All of these gases were purchased from General Dynamics (Detroit, Mi.). The column was packed with Porapak N (100-120 mesh, Waters Assoc. Inc., Framingham, Mass.). The length of the columns varied, depending on the oven temperature

(3mm x 1.3 m at 85 C in 1970 or 3mm x 1.0 m at 45 C in 1971). It was found that good separation of peaks could be achieved at low temperatures with the shorter column even when the samples contained large quantities of methane gas. The quantity of ethylene produced in the sample was determined from a standard curve relating peak height to millimicromoles ethylene. Certified standards of 47 ppm, 830 ppm ethylene in nitrogen were obtained (Matheson, Co., Joliet, Ill.) to prepare the standard curve. Standards were run at each analysis period to correct for changes in detector sensitivity.

The effect of changes of light intensity and temperature on the rate of acetylene reduction by natural populations of algae was done as follows: a water sample was taken from 1 m at midnight (18/7/71), brought to the laboratory, and the acetylene reduction assay was carried out immediately. The samples were incubated in a constant temperature aquarium described in Chapter VIII at 10, 20, 30, and 35 C under 100, 300, 600, and 1,000 ft-c, which was provided by one fluorescent tube and four tungsten flood-light bulbs.

The effect of different light quality on the rate of acetylene reduction was done using a unialgal culture of *Aphanizomenon* (Durham strain received from Dr. J. H. Gentile, National Marine Water Laboratory, West Kingston, R.I.) grown in nitrogen-free ASM-1 medium (Gorham *et al.*,

1964) under fluorescent light (about 250 ft-c). Subsamples of this culture were incubated with acetylene (20%) in 26-ml bottles covered with white, green, and red cellophane papers. Triplicate samples were incubated at 25 C on a multi-purpose rotator (Model 150V, Scientific Industries Inc., Queens Village, N.Y.) to ensure even distribution of light.

To check the effect of phosphate on the rate of acetylene reduction, various amounts of K_2HPO_4 were added to 10 ml of concentrated algae samples from 2 m to have final amended concentrations of 0.0, 10, 50, 100, and 1000 µg/l. The incubation was 1 hr at 2 m. The experiment was repeated twice on 3/7 and 16/7/71 (*Anabaena* was dominant).

C. Results and Discussion

The annual nitrogen income of Wintergreen Lake consists of combined nitrogen and gaseous nitrogen.

1. Combined Nitrogen Sources:

These sources are migrating Canada geese and ducks, local geese and ducks, effluent from a dairy feed-lot lagoon, wastes from the near-by school, fertilizer and soil nitrogen run-off from the surrounding cropped lands, underground water, and aerial precipitation (rain and snow).

The contribution of nitrogen by local and migrating waterfowl to Wintergreen Lake is being studied by

Manny and Johnson. The nitrogen input by waterfowl during winter alone was estimated to be about 5.0 mg NO_3^-, 36.1 mg organic nitrogen per m^2 (Manny, 1971).

There were about 300 resident waterfowl (Manny, 1971) and several thousand Canada geese and ducks which spent a few days before flying south during late fall and early winter. Fresh droppings of local waterfowl contained on the average of 1.6 - 2.0% (dry weight) nitrogen (microKjeldahl analysis). Such a high nitrogen content of the excreta compared to the 0.15% reported by Sylvester and Anderson (1964, cited by Manny, 1971), might be explained by the nitrogen rich diet provided by the sanctuary personnel and visitors to the waterfowl.

The concentrations of total nitrogen in the effluent water from the dairy lagoon and the school lagoon (1.6 - 4.9 mg/l) were always higher than those in open water (1.5 - 2.2 mg/l) in Wintergreen Lake during March-April periods in 1970 (Manny, 1971).

The contribution of nitrogen by fertilizer run-off or by underground water could be important particularly in the spring. The downward movement of nitrate becomes significant under irrigated conditions and during the rainy season (Murphy and Gosch, 1970) especially in porous soils (Linville and Smith, 1971) like those which surround Wintergreen Lake.

Aerial precipitation is also an important source of nitrate and ammonia (Delwiche, 1970). Manny (1971) estimated that freshly fallen snow from Wintergreen Lake contained (μg/l) 1124 nitrate, 16.3 nitrite, and 724 ammonia. The nitrogen content of rain water received in Wintergreen Lake was, however, not measured.

2. Fixation of Atmospheric Nitrogen: Measurable acetylene reduction in Wintergreen Lake water was confined to the euphotic zone (0-3 m, Fig. 18 and 19). The acetylene reduction by photosynthetic bacteria and other indigenous flora in the hypolimnion was undetectable (see Chapter IX for details). The reduction of acetylene by mud flora was, however, not studied.

The acetylene reduction of Wintergreen Lake water was significant only during the period of the blue-green algal bloom in both 1970 and 1971. The fixation was maximum in July then decreased gradually to undetectable levels in mid-August. A dense *Aphanizomenon* population returned in late November and early December after the fall turnover (1970). This later bloom of *Aphanizomenon* was more dense (approx. 5 times more filaments/l) than that in the summer. The late algal bloom usually accumulated in a surface layer approximately 5 m wide and about 0.5 - 1.0 cm thick, near the southwest end of the lake. The trichomes appeared old and clumped together forming visible bundles. This phenomenon was also observed by Horne and Fogg (1970) in Windermere

Figure 18. Rates of acetylene reduction at 0, 1, 2, and 3 m in Wintergreen Lake in 1970 when *Aphanizomenon* was dominant.

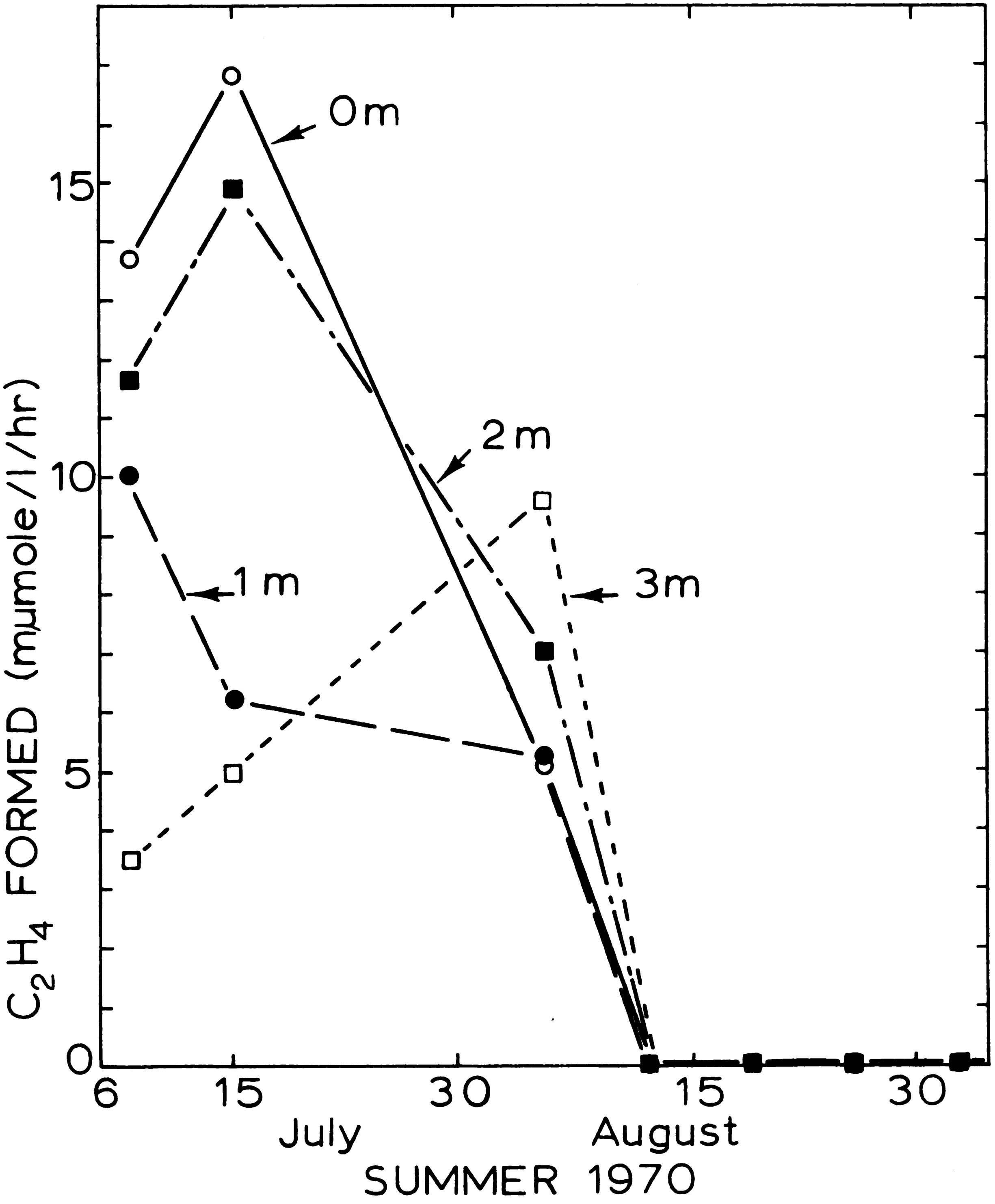
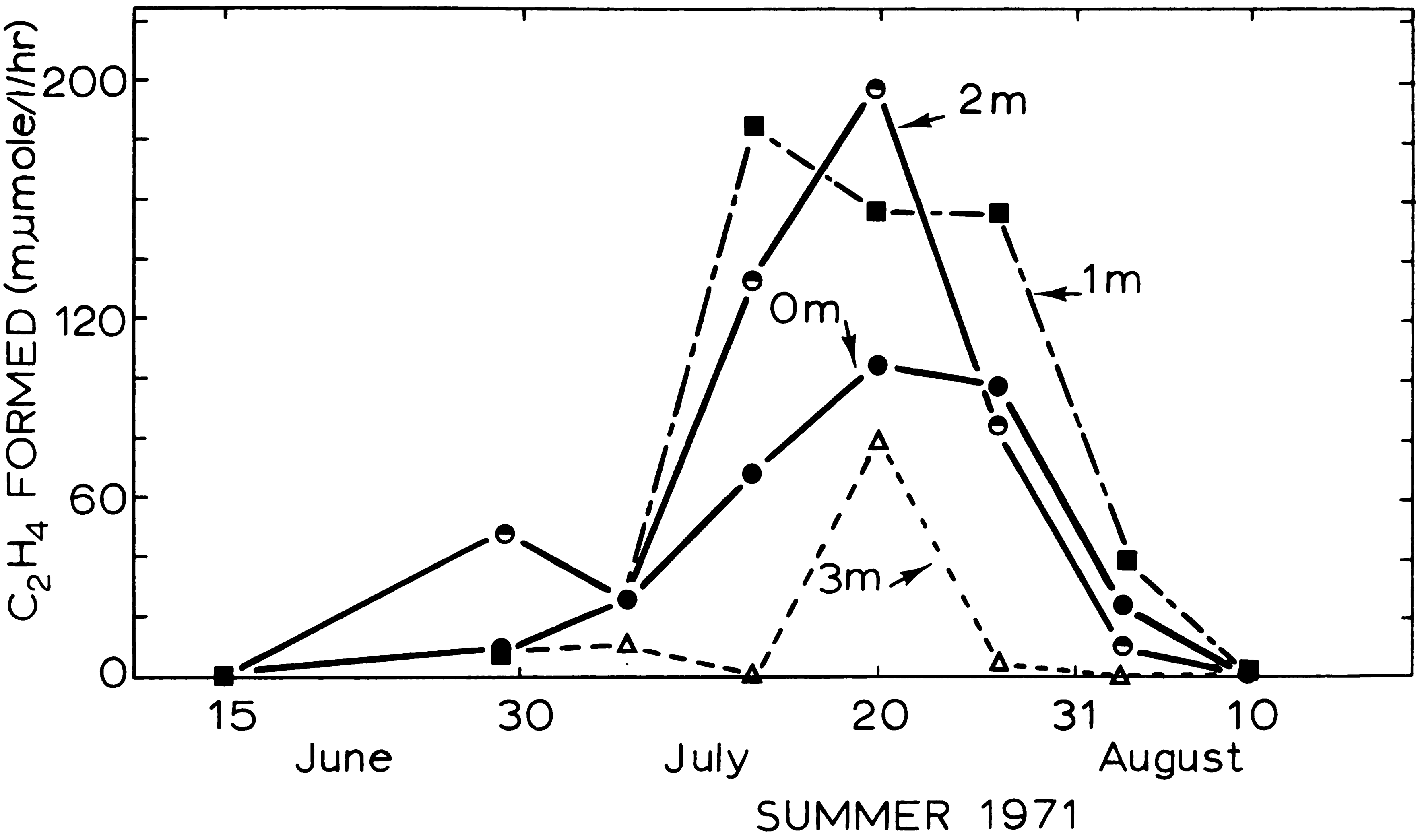

0m
2m
1m
3m
C_2H_4 FORMED (mμmole/l/hr)
0
5
10
15
6
15
30
15
30
July
August
SUMMER 1970

Figure 19. Rates of acetylene reduction at 0, 1, 2, and 3 m in Wintergreen Lake in 1971 when *Anabaena* was dominant.


C_2H_4 FORMED (mμmole/l/hr)
200
120
60
0
15
30
20
31
10
June
July
August
SUMMER 1971
2m
1m
0m
3m

(England) in 1966 where the $^{15}N_2$ fixation per volume was the highest observed in the lake. The acetylene reduction rate by the windblown bloom in Wintergreen was, however, undetectable. Microscopic observation showed very few heterocycts but a large number of gonidia.

The rate of acetylene reduction by Wintergreen Lake water during the summer bloom of 1971 was almost 5 times that in 1970, although the number of filaments and heterocysts of *Aphanizomenon* (Fig. 20) per volume (1970) was almost 10 times greater than that of *Anabaena* (Fig. 15) in 1971. The lower fixation rate in 1970 may have been due to the presence of nitrogen gas in the incubation bottles, but Stewart *et al*. (1971) observed that N_2 did not measurably affect the acetylene reduction by *Aphanizomenon* in Wisconsin lakes because N_2 is less soluble in water than acetylene. This inefficiency of *Aphanizomenon* in reducing acetylene (Stewart *et al*., 1967) and in N_2 fixation (Stewart *et al*., 1968) was observed in both unialgal cultures and in algal samples taken from Lake Monona, Mendota, and Kegonsa, Wisconsin.

The multivariate statistical anslysis (Table 13) indicates that the weekly rate of acetylene reduction (1971) was significantly correlated with the combination of light, oxygen, alkalinity, depth, filament density, heterocyst density, and pH.

Figure 20. Density (numbers/l x 10^5) of the *Aphanizomenon* filaments (●) and heterocysts (◓) in 1970 at 0 m (A), 1 m (B), 2 m (C), and 3 m (D). The data for 5/8 is from the diurnal sampling.

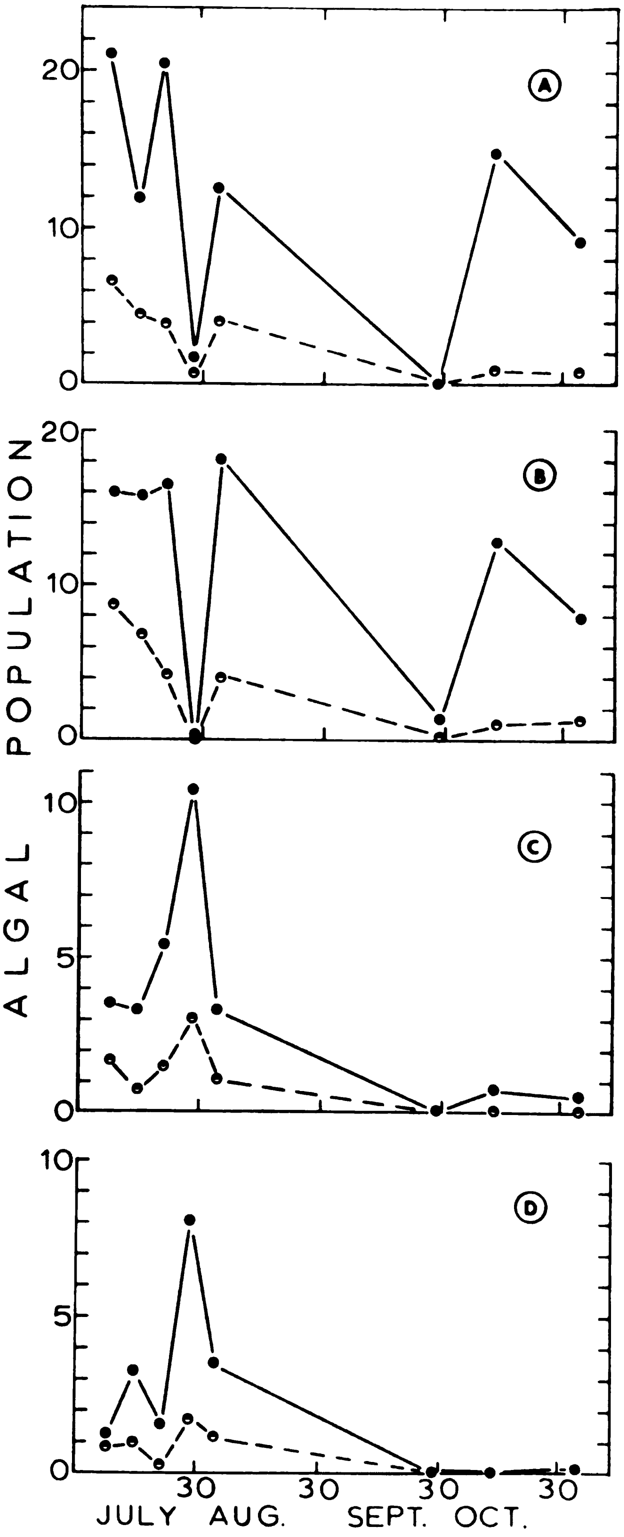

A
B
C
D
ALGAL POPULATION
20
10
0
20
10
0
10
5
0
10
5
0
30
30
30
30
JULY
AUG.
SEPT.
OCT.

Table 13. Multivariate statistical analysis*

Dependent variable: Rate of acetylene reduction (0-2 m) in the summer of 1971

$$Y = -1290.98 + 52.87X_1 - 2.88X_1^2 + 243.97X_2 - 15.43X_2^2 + 8.03X_3 + 99.54X_4 - 0.13X_5 + 0.34X_6 - 9.15X_7$$

(a) Overall Analysis

	Sum of Squares	df	Mean of Squares	F	Sig.
Regression (about mean)	79706.83	9	8856.31	15.03	<0.0005
Error	6582.75	11	589.34		
Total (about mean)	86189.58	20			

(b) Multiple Correlation Coefficients

Mean = 68.21 mμmole C_2H_4/l/hr $R^2 = 0.92$ $R = 0.96$

	Reg. Coef.	Sig. Level	Partial Corr. Coef.	R^2 Deletes
Constant	-1290.98	0.055		
Light (X_1)	52.87	0.001	0.79	0.80
X_1^2	-2.88	0.001	-0.88	0.79
Oxygen (X_2)	243.97	0.001	0.88	0.79
X_2^2	-15.43	0.002	-0.77	0.81
Alkalinity (X_3)	8.03	0.050	0.55	0.89
Depth (X_4)	99.54	0.003	0.74	0.83
Filaments (X_5)	-0.13	0.004	-0.74	0.83
Heterocysts (X_6)	0.34	<0.0005	0.89	0.63
pH (X_7^2)	-9.15	0.003	-0.75	0.82

*Factors tested: Available light, oxygen, alkalinity, net primary productivity, temperature, pH, depth, heterocyst and heterocyst-bearing algae.

(a) Heterocyst and Filament Densities. The reduction of acetylene in Wintergreen Lake, as in the Wisconsin Lakes (Goering and Neess, 1964; Stewart *et al.*, 1967; Stewart *et al.*, 1968; Stewart *et al.*, 1971), the English Lake district (Horne and Fogg, 1970), Santuary Lake, Pennsylvania (Dugdale and Dugdale, 1962), Sargasso and Arabian Sea (Dugdale *et al.*, 1964), Yellowstone hot spring (Stewart, 1970a), algal crust in the Arizona desert (Macgregor and Johnson, 1971), and flooded rice soils (Nishigaki and Shiori, 1959), was associated with the presence of heterocyst-bearing blue-green algae (Fig. 11). Heterocysts are speculated to be the site of nitrogen fixation (Stewart *et al.*, 1968; Ogawa and Carr, 1969; Wolk, 1970; Jewell and Kulasooriya, 1970; Nelson *et al.*, 1971; Wolk and Wojciuch, 1971). In Wintergreen Lake, however, the efficiency (acetylene reduction/heterocyst) varied considerably with depth, month, and time (Table 14) although a positive correlation (R = 0.89) between the weekly rates of acetylene reduction and the frequency of heterocysts was found.

More direct evidence that heterocyst-bearing *Aphanizomenon* and *Anabaena* were the agents catalyzing the reduction and not epiphytic bacteria associated with the algae, nor the planktonic bacteria, is that the filtrate going through 20-μ netting did not reduce acetylene and the reduction of acetylene by an algal

Table 14. Diurnal variation of rate of ethylene produced per heterocyst

(mμ mole/heterocyst/hr x 10^{-3})

Ethylene/Heterocyst

(1) On 4-5/8/1970

Depth	8:15 PM	11:30 PM	5:15 AM	8:02 AM	10:53 AM	2:05 PM	8:00 PM
0	0.018	0.024	0.020	0.016	0.004	0.008	0.008
1	0.016	0.015	0.018	0.008	--	0.008	0.009
2	0.017	0.024	0.015	0.010	0.005	0.003	0.006
3	--	--	0.012	0.021	0.006	0.010	--

(2) On 5-6/7/71

	1:45 PM	7:10 PM	12:15 AM	3:40 AM	8:00 AM	2:10 PM	8:15 PM	2:35 AM
0	0.141	0.422	0.542	0.436	0.572	0.146	0.328	0.845
1	0.169	0.676	0.141	0.172	0.181	0.102	0.185	1.408
2	0.113	0.058	0.141	0.521	0.389	0.478	1.351	0.197
3	0.080	0.560	--	0.563	--	0.563	0.282	0.282

(3) On 27-28/8/71 at 1 m

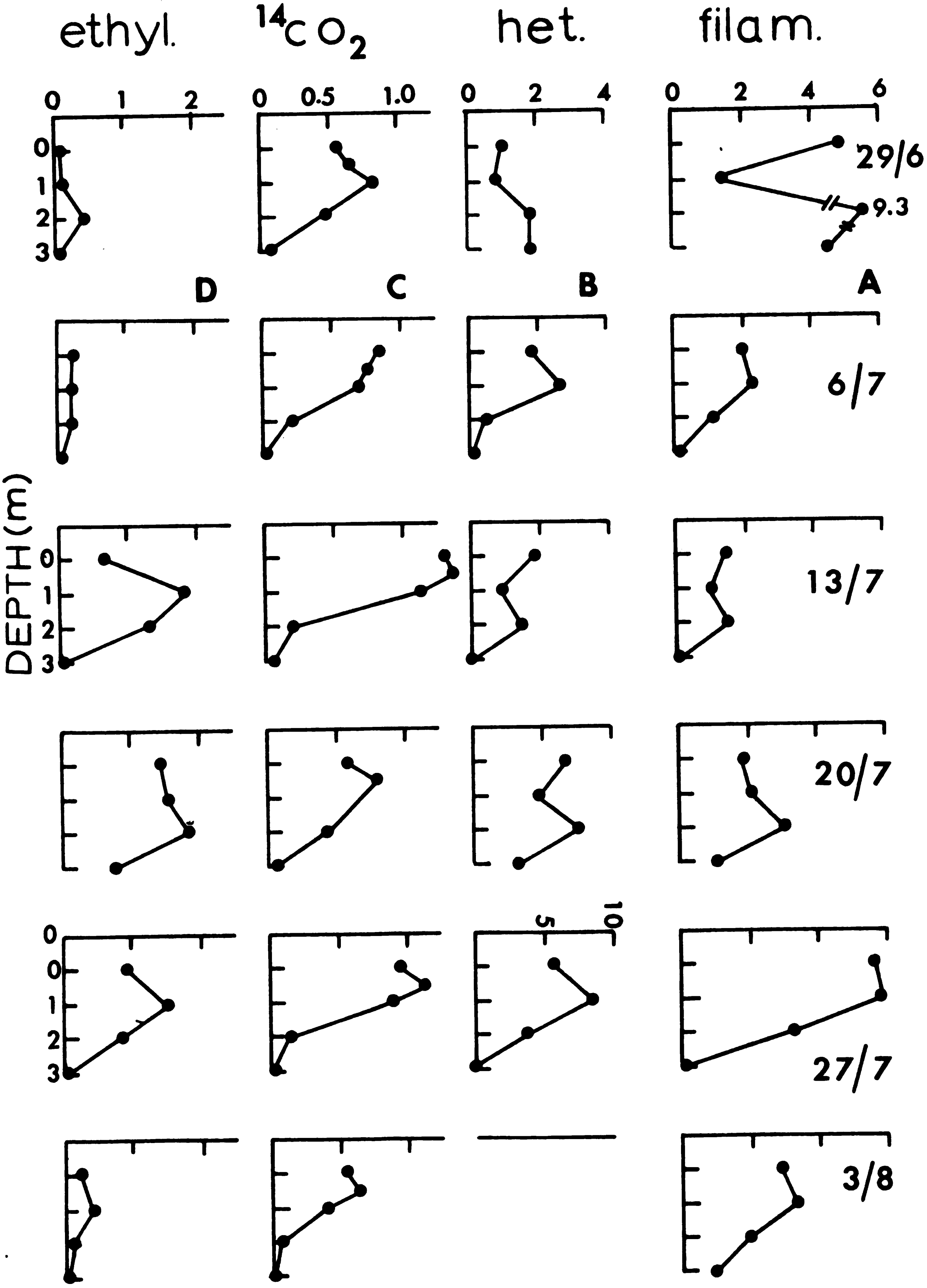
Time	Ethylene/Heterocyst	Time	Ethylene/Heterocyst
5:50 AM	0.046	8:30 PM	0.312
7:45	0.204	10:35	0.100
10:20	0.182	12:50 AM	0.148
12:25 PM	0.776	2:35	0.209
2:30	0.460	4:38	0.233
4:30	0.371	6:35	0.209
6:35	0.554	8:40	0.596

sample which was forced through 25 G x 5/8" hypodermic needle (to break algal cells) was undetectable. This is in agreement with data reported by Stewart *et al.* (1971) that the concentration of algal sample from Lake Mendota up to 4,000 times by filtering did not affect the rate of acetylene reduction per water sample unit. Also, non-heterocystous blue-green algae, for example *Oscillatoria* (Horne and Fogg, 1970) or *Cladophora* and *Microcystis* (Stewart *et al.*, 1967), presumably loaded with epiphytic bacteria taken from the same water at the same time with nitrogen-fixing *Aphanizomenon* and *Anabaena*, did not fix $^{15}N_2$ (Horne and Fogg, 1970) or reduce acetylene (Stewart *et al.*, 1967). The negative correlation between the rate of acetylene reduction and filaments (trichomes) density was not expected, though it is consistent with available information that filaments without heterocysts do not reduce acetylene.

(b) *Available Light*. The correlation between available light and weekly rate of acetylene reduction was not linear (Table 13) apparently because high available light became partially inhibitive to the rate of acetylene reduction. The pattern of this relationship may be seen in Figure 21, where strong light intensity on the water surface apparently inhibited acetylene reduction, but at 3 m the acetylene reduction was negligible due to the low light intensity. The

Figure 21. Distribution with depth and date in 1971 of the following:

(A) Heterocystous algae (filaments/l x 10^5)
(B) Heterocysts (number/l x 10^5)
(C) Net primary productivity (mg C/l/day)
(D) Rate of acetylene reduction (mμ mole/l/hr x 10^2)


ethyl.
$^{14}CO_2$
het.
filam.
0
1
2
0
0.5
1.0
0
2
4
0
2
4
6
29/6
9.3
D
C
B
A
6/7
DEPTH (m)
0
1
2
3
13/7
20/7
5
10
27/7
3/8

response of the natural population of algae taken from Wintergreen at midnight (18/7/71, 23.1 C) to various temperatures and light intensities was mixed (Table 15a). At 10 C, the reduction rate was highest at 100 ft-c, but at 35 C, the highest reduction rate was obtained at 1,000 ft-c.

In general, the reduction rate at a given light intensity increased with the increasing temperature. The reason for sampling at night was to eliminate as much as possible the available carbon and light-induced ATP. Samples were kept dark and aerobic until assay which lasted 30 min.

The transmittance of light of different wavelengths was not the same through Wintergreen water. As shown in Figure 5, less blue light than red or green light was present in the water below the surface. Red and green lights are not optimum for all pigments of blue-green algae (cf. Brody and Brody, 1962; Bogorad, 1962; O'heocha, 1962), thus probably limiting photosynthesis as well as nitrogen fixation.

In the present study, the relative rates of acetylene reduction by Anabaena under different quality lights were: white, 154; red, 141; and green, 138.5 (mm ethylene peak per sample per hr). Red and green light were less effective than white; but the difference was small.

Table 15a. Effect of light, temperature on rate of acetylene reduction by a natural population of blue-green algae obtained at midnight. Data expressed in mμmoles of ethylene produced per l per hr

Ft-c \ Temp. (C)	10	20	30	35
100	12.30	31.00	32.60	29.40
300	5.70	30.50	23.50	42.80
600	8.80	27.00	37.50	44.00
1000	9.10	27.30	39.60	44.40

Table 15b. Effect of added phosphorous on rate of acetylene reduction

Added Phosphate (μg/l)	Exp. 1 (3/7/71)	Exp. 2 (16/7/71)
	Ethylene produced (mm peak height/ l/hr)	Ethylene produced (mm peak height/ l/hr)
0	44.70	47
10	82.80	36
50	101.75	31.6
100	75.50	29.2
1000	---	33.0

(c) Phosphorous. The ecological importance of this element in the development of algal blooms has been discussed briefly in Chapters IV, V. Stewart et al. (1970) reported that additions of phosphate would stimulate the rate of acetylene reduction by laboratory grown blue-green alga and that this technique might be used to measure available phosphate in natural waters. However, in this study the effect of added phosphorous on the rate of acetylene reduction by natural population was inconclusive (Table 15b). In the first experiment, the rate of acetylene reduction increased with increasing K_2HPO_4. But in the second experiment the rate of acetylene reduction in the presence of added phosphate was lower than that of the control. It should be noted that the concentration of phosphorus during the first experiment was higher than that during the second experiment (Fig 10A). Added phosphorous may change the delicate ecological balance of the community through a number of ways, (1) change of pH of the water (see review by Kuhl, 1962) (2) precipitation of iron as ferric phosphate (Hutchinson, 1970), which is essential for algal growth and known to be present at very low concentrations during the growing season (McMahon, 1969), (3) stimulation of growth of dominant Microcystis and their associated bacteria (Shapiro et al., 1969), causing other elements to become limiting to the nitrogen-fixing algae,

(4) inhibitive effect of added phosphorous to photosynthesis of algae such as that which occurred in Cayuga Lake, New York (Hamilton, 1969). The change of water pH by added phosphate is unlikely because the pH of all bottles including the control did not change after incubation.

The effect of added phosphorus on the activity of natural population deserves more discussion. Similar inhibition of added phosphorus on CO_2 uptake was also observed by Wetzel (1966); Goldman and Armstrong (1969); Kalff (1971); Sakamoto (1971) in short-term experiments. However, Goldman and Armstrong (1969) and Sakamoto (1971) found that CO_2 uptake responded to the increasing phosphorus added if the incubation of samples with phosphorus lasted longer than two days. It seems that the uncertain response of algae to added phosphorus related the phosphorus pool in cells. Algae can accumulate phosphorus more than they actually need and use it later during environmental stress (cf. O'Kelley, 1968). When phosphorus was supplied to phosphorus starved algae, it may be used for synthesis of the nucleic acids necessary for the reproduction, therefore reducing CO_2 uptake or acetylene reduction. The increase in CO_2 uptake in long-term experiments was probably due to a demand for carbon skeleton by the newly formed cells. Cell division stimulated by a moderate amount of phosphate was observed by Goldman and Armstrong (1969) in long-term experiments in Lake Tahoe.

Thus the resistance of the biological system against temporary environmental stress may pose some problems in interpretation of nutrient-limiting experiments, thus may be misleading. Three approaches may be used to monitor limiting factors which affect the development of algae and their productivity. (1) Changing a factor in question by adding nutrients or manipulating physical factors, then monitoring the response of a natural population or pure culture. This method has been used widely by limnologists (Gerloff and Skoog, 1954; 1957; Goldman, 1960, 1964; Wetzel, 1966; Goldman and Armstrong, 1969; Stewart *et al*., 1970; Kalff, 1971; Sakamoto, 1971). (2) Measurement of chemical, physical, and biological parameters then analysis by the multivariate statistical method to determine limiting factor(s). (3) Analysis of cellular composition and then compared with critical composition determined under laboratory conditions (Gerloff and Skoof, 1954).

The last approach is difficult and time consuming hence it is not practical. The first method is convenient and fast but has some inherent uncertainties due to the resistance of the populations to change as discussed above. The second method requires long-range study and a great number of sampling times and also has an inherent limitation in that the values measured by available technology may not be the true value due to the mobility of elements. According to Pomeroy (1960):

> Measurement of the concentration of dissolved phosphate in natural waters gives a very limited indication of phosphate availability. Much or virtually all of phosphate in the system may be inside organism at any given time, yet it may be overturning every hour with the result that there will be a constant supply of phosphate for organisms able to concentrate it from a very dilute solution.

The advantage of this method is that one is able to analyze a large number of factors at the same time.

The use of any one of the above approaches to study a complex ecosystem such as Wintergreen Lake would be shortcoming due to the inherited uncertainties discussed above. The combination of the first two approaches would be desirable.

(d) Oxygen. It is well known that oxygen inhibits nitrogenase activity of whole cells (Stewart, 1969; Stewart and Lex, 1970; Stewart and Pearson, 1970; Drozd *et al*., 1970). In the present study, the *in situ* data indicate that low levels of oxygen did not appear to inhibit the rate of acetylene reduction (Table 12), though higher levels may.

(e) Combined Nitrogen. The periods of measurable acetylene reduction and large numbers of heterocysts in

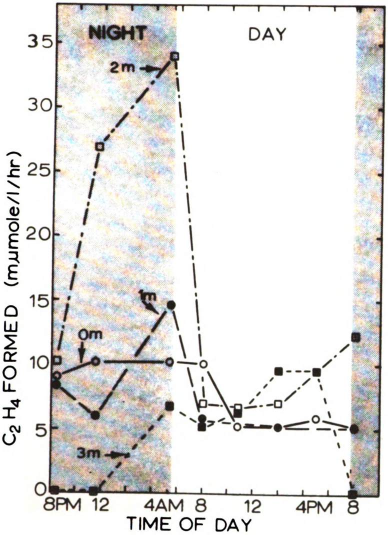
1970-1971 coincided with the lowest concentrations of nitrate and ammonium (Fig. 10, B, C) in Wintergreen Lake water. This inverse correlation between nitrogen fixation or number of heterocysts and combined nitrogen under laboratory and *in situ* conditions is well documented. Ogawa and Carr (1969) reported that ammonium has the strongest suppressive effect on heterocyst formation and that heterocyst production was evident 24 hr after transferring *Anabaena variabilis* from nitrate containing medium to a nitrogen-free medium. According to Horne and Fogg (1970), $^{15}N_2$ fixation maxima in surface water (0-5 m) in Windermere and Esthwaite Water (England) coincided with the lowest values of nitrate-N concentration (200 μg/l). The concentration of ammonium-N rarely exceeded 20 μg/l, but it rose sharply after the fixation peak; probably due to algal excretions or lysis (Fogg, 1952; Fogg and Westlake, 1955). The concentrations of nitrate at the fixation maxima were even much lower (1 μg/l) in Lake Mendota (Stewart *et al.*, 1971) and undetectable in Sanctuary Lake, Pennsylvania (Dugdale and Dugdale, 1962). The observation that combined nitrogen inhibits nitrogen fixation by living cells is well accepted but the mechanism of the inhibition is not certain. Stewart *et al.* (1968) found that ammonium and nitrate did not have an immediate effect on acetylene reduction of several pure, unialgal cultures, or natural populations of blue-green algae. They speculated that combined nitrogen did not inhibit nitrogenase activity but suppressed nitrogenase synthesis. This argument is in line with the findings of Hardy *et al*. (1968) that the nitrogenase preparation of *Azotobacter* was not sensitive to low levels of ammonium but rather was inhibited by high concentrations of NH_4^+ ions as well as that of Na^+ ions. Jewell and Kulasooriya (1970) found that the rate of acetylene reduction by blue-green algae grown on a nitrogen-free medium declined after a maximum value until most of the newly fixed nitrogen in the cellular pool was used for cellular synthesis, after which the rate of reduction increased again. These authors concluded that the complex pattern of nitrogen fixation observed, depended on the internal nitrogen pool, age, and possibly newly excreted organic nitrogen. Nelson and his coworkers (1971) also found that the internal nitrogen pool could regulate nitrogenase activity; furthermore, they found that upon transferring from a nitrate-containing medium to a nitrogen-free medium, *Anabaena sp*. produced more heterocysts and nitrogenase under an argon-CO_2 atmosphere than under a N_2-CO_2 atmosphere. Thus, it seems that N_2 does not directly control nitrogenase synthesis nor does combined nitrogen control its activity (*via* classical allostery). Rather, combined nitrogen in the medium and/or the cellular pool as well as other physiological parameters of the cell seem to turn on or off the nitrogenase synthesizing activity. When nitrogen becomes available from newly fixed nitrogen or from an external source, the cell may, in addition to stopping new nitrogenase synthesis, shut off fixation by diverting energy and the limited reducing power from nitrogenase. The delayed inhibition (60 min) of acetylene reduction by combined nitrogen observed by Stewart and his coworkers (1968) on Nostoc is consistent with the above explanation since the heterocysts could carry out fixation with ATP provided by photosystem I in the heterocyst (Fay et al., 1968) until available reducing power and/or carbon skeleton already pumped in from vegetative cells is exhausted, hence, nitrogen fixation ceases.

The relatively smooth curves for acetylene reduction for 1970 and 1971 (Fig. 18, 19), as compared to Figure 1 of Jewell and Kulasooriya (1970), could be due to a number of factors, (1) the long spacing of time between samplings in this study, therefore the turning points were missing, (2) the algal population in Wintergreen Lake water was in a steady state in which sinking filaments were replaced by young active filaments, (3) the excreted organic nitrogen was utilized immediately by the non-nitrogen-fixing organisms, (4) any combination of the above factors.

(f) Other Factors. The positive correlation between acetylene reduction and depth is understandable since only data from 0-2 m were analyzed and, as Figure 11 indicated, higher rates of acetylene reduction occurred at 1 and 2 m. There was a relationship between total alkalinity and acetylene reduction; however, this correlation was not very significant. The non-linear negative correlation between pH and acetylene reduction indicates that most of the time, high pH was inversely related to acetylene reduction. The lack of correlation between acetylene reduction and CO_2 uptake or chlorophyll a was probably due to the interference of the *Microcystis*.

(g) Diurnal Acetylene Reduction. The diurnal acetylene reduction measurement was done once in 1970 and twice in 1971 during the period of high acetylene reduction. Both the bloom of *Aphanizomenon* (1970) and *Anabaena* (1971) had the capacity to reduce acetylene at night although the pattern and the significance of the reduction by the two blooms were different. Figures 22 and 23 show the variation of acetylene reduction rate, algal population, and heterocysts at each depth during a 24-hr period on 4-5/8/70. The reduction rates, at

Figure 22. Diurnal rates of acetylene reduction (left) and *Aphanizomenon* filament densities (right) during the diurnal sampling period of 4-5/8/70.


NIGHT
DAY
C_2H_4 FORMED (mμmole/l/hr)
35
30
25
20
15
10
5
0
2m
1m
0m
3m
8PM
12
4AM
8
12
4PM
8
TIME OF DAY

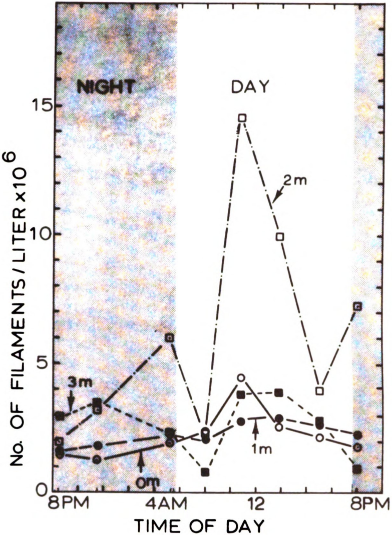
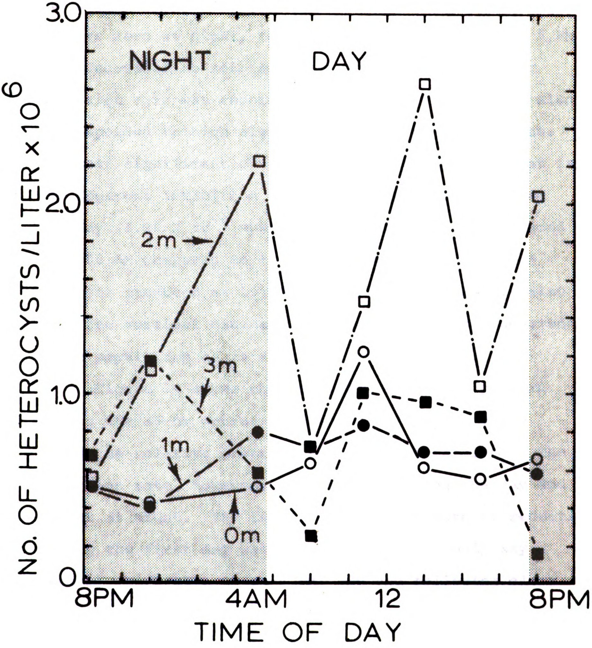

NIGHT
DAY
No. OF FILAMENTS / LITER x10^6
15
10
5
0
2m
3m
1m
0m
8PM
4AM
12
8PM
TIME OF DAY

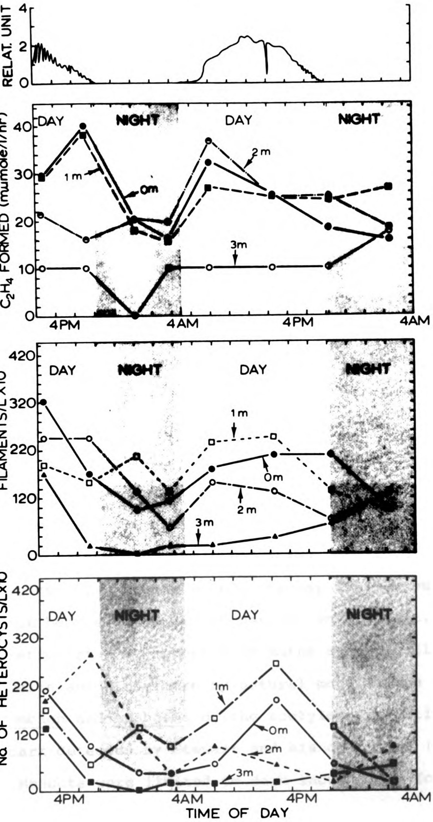
Figure 23. Densities of Aphanizomenon heterocysts during the diurnal sampling period of 4-5/8/70.


NIGHT
DAY
2m
3m
1m
0m
3.0
2.0
1.0
0
No. OF HETEROCYSTS/LITER x 10^6
8PM
4AM
12
8PM
TIME OF DAY

all depths measured, except at 3 m, were higher during the night than during the day. The reduction rate at 3 m was zero at night, reached the maximum value at 5 PM then decreased to zero again at sunset. The highest reduction rate was recorded at sunrise at 2 m which also corresponded to high algal and heterocyst populations. The most significant observation from this experiment is the apparent inhibition of acetylene reduction during the day (5.20×10^{-6} mμmoles ethylene/filament produced at 5:00 AM compared to 1.13×10^{-6} mumoles ethylene/filament produced at 10:50 AM). Figures 22 and 23 also show the vertical diurnal distribution of the blue-green algae population as is discussed in Chapter V.

Figure 24 shows the acetylene reduction pattern during the 36-hr period of 5-6/7/71. The top curve shows the relative distribution of light received. The reduction rate, however, was highest during the day and lowest at night. The lag in the maximum rate of reduction during the first day was correlated to a cloudy sky (storm) which cleared at 6 PM. On the following clear day (6/7/71), the reduction rates reached a maximum in the early morning (8 AM) then decreased while the algal population did not change. This low acetylene reduction (0-2 m) may have been due to the high light intensity and/or excess oxygen from photosynthesis (Stewart and Pearson, 1970).

Figure 24. Diurnal solar radiation received (relative units, top), rates of acetylene reduction, *Aphanizomenon* plus *Anabaena* population densities, and total heterocyst densities during the diurnal sampling period of 5-6/7/71.

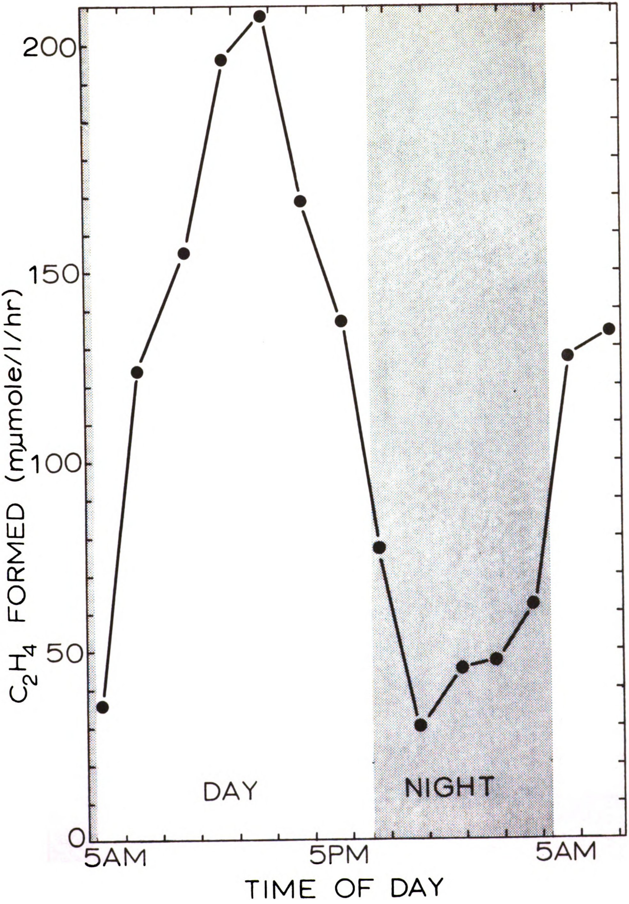

RELAT. UNIT
0
2
4
C_2H_4 FORMED (mμmole/l/hr)
0
10
20
30
40
DAY
NIGHT
DAY
NIGHT
1 m
0m
2 m
3m
4PM
4AM
4PM
4AM
FILAMENTS/L X10^3
0
120
220
320
420
DAY
NIGHT
DAY
NIGHT
1 m
0m
2 m
3m
No. OF HETEROCYSTS/Lx10^3
0
120
220
320
420
DAY
NIGHT
DAY
NIGHT
1m
0m
2m
3m
4PM
4AM
4PM
4AM
TIME OF DAY

Another 28-hour sampling period (27-28/7/71) at 1 m was designed to follow more closely the diurnal variation of acetylene reduction. The sky was clear except for clouds at 11 AM (27/7/71) and at 9 AM (28/7/71). The lag in the reduction maximum (about 2 PM), Figure 25, was probably related to the high turbidity of the water; only 35% of the surface light reached 1 m depth and the secchi disc transparency was near the lowest observed value (0.9 m). The reduction rate was lowest at 10 PM then increased rapidly after sunrise. Here again, *Anabaena* and *Microcystis* apparently moved up during the daytime and sank at night (Fig. 26).

The double peaks (10 AM and 5-6 PM) of the rate of acetylene reduction by *Anabaena* blooms reported by Stewart *et al*. (1971) in Green Bay were not observed in any diurnal measurement at Wintergreen Lake.

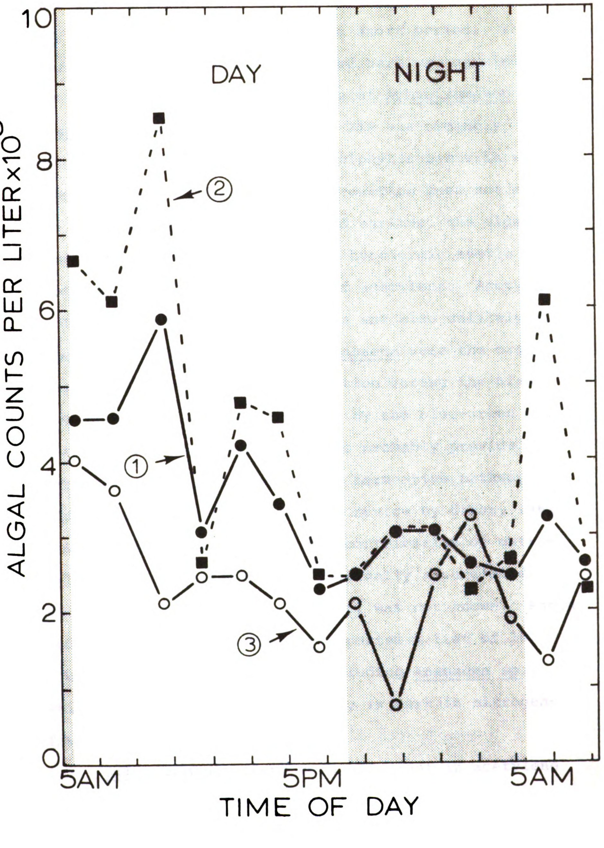
The capacity of blue-green algae to grow heterotrophically in the dark in nitrogen-free medium has been reported by Watanabe (1967) and Fay (1965), but it has not been studied in the natural environment. The dark fixation of $^{15}N_2$ reported by Horne and Fogg (1970) in Esthwaite and Windermere by natural populations of *Aphanizomenon* and *Anabaena* or the acetylene reduction in the dark detected by Stewart and his coworkers (1967) in Lake Mendota were limited to dark incubations for a short period after light. The light energy stored as

Figure 25. Diurnal rates of acetylene reduction at 1 during the diurnal sampling period of 27-28/7/71 when *Anabaena* was dominant.



C_2H_4 FORMED (mμmole/l/hr)
200
150
100
50
0
DAY
NIGHT
5AM
5PM
5AM
TIME OF DAY

Figure 26. Densities of *Anabaena* filaments (1), *Anabaena* heterocysts (2), and *Microcystis* colonies (3) at 1 m during the diurnal sampling period of 27-28/7/71.


10
8
6
4
2
0
ALGAL COUNTS PER LITER × 10^5
DAY
NIGHT
②
①
③
5AM
5PM
5AM
TIME OF DAY

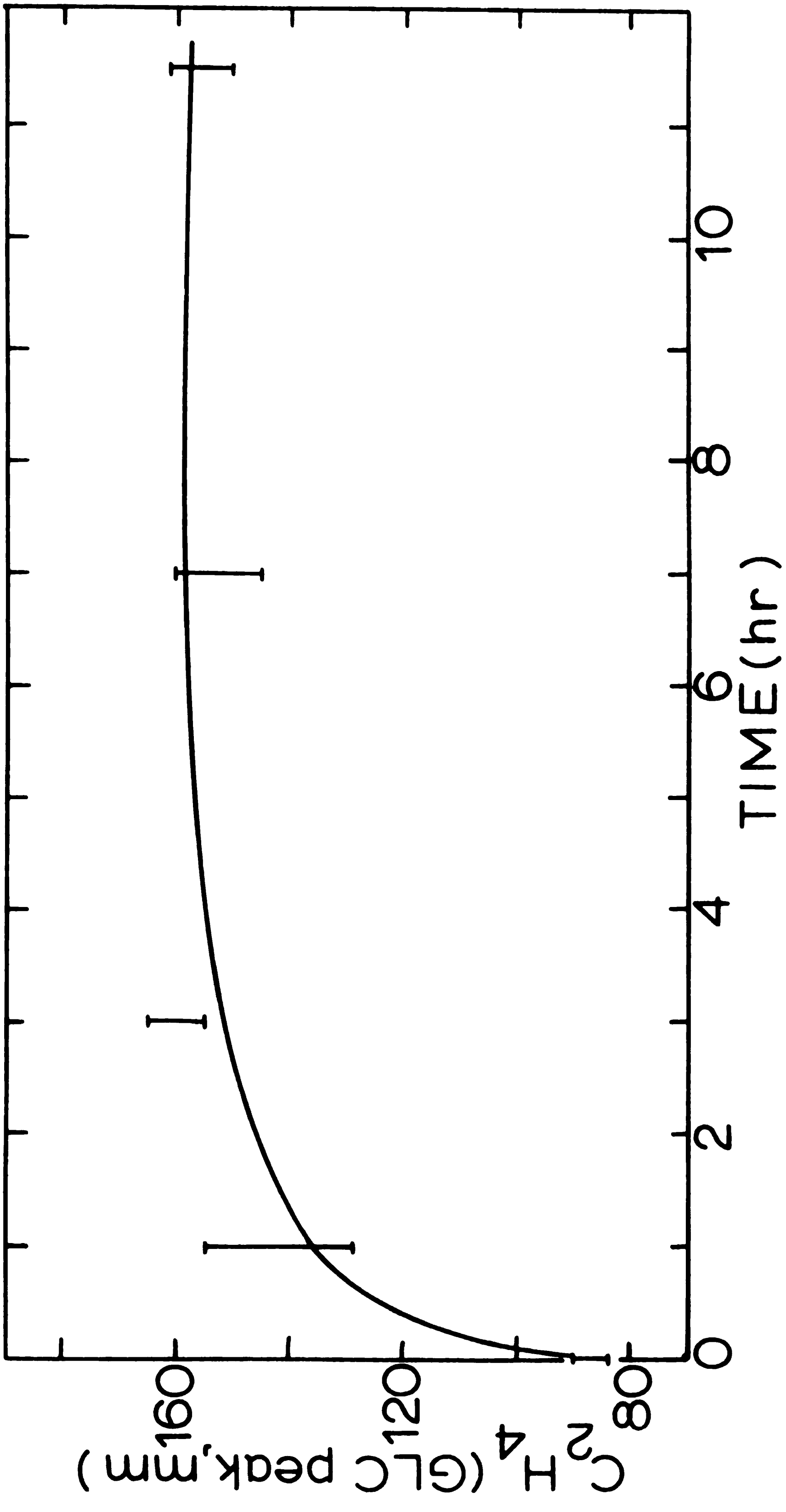
ATP could be sufficient to allow algae to fix nitrogen or to reduce acetylene in such a short period. The dark acetylene reduction reported here was carried out by long-term light starved cells of *Aphanizomenon* and *Anabaena* in which light-induced ATP was probably exhausted. The argument that epiphytic bacteria were responsible for the acetylene reduction does not appear to be true because, as mentioned earlier, the algal sample forced through 25 G 5/8" hypodermic needle reduced insignificant amounts of acetylene. Acetylene reduction by planktonic bacteria was also unlikely; therefore, *Aphanizomenon* and *Anabaena* were the organisms responsible for acetylene reduction during the night. The energy and reductant needed by the blue-green algae for dark acetylene reduction was probably provided through several respiratory and fermentive pathways (Leach and Carr, 1970, also see review by Gibbs, 1962). The relative importance of the contribution of extracellular organic carbon and internally accumulated photosynthetic products, however, was not known. Khoja and Whittman (1971) recently reported a list of 10 species of blue-green algae including *Anabaena sp.*, which can grow heterotrophically in dark in nitrogen-free medium.

Aphanizomenon seems more efficient in acetylene reduction at night and at low light intensity (sunrise) than under bright day light (Fig. 22). The acetylene reduction at sunrise was almost 400% that at full light. This hypothesis was supported by a laboratory study in which a unialgal culture of *Aphanizomenon* was grown and assayed for acetylene reduction at a light intensity of either 300 or 1,200 ft-c. *Aphanizomenon* reduced more acetylene at the low light intensity than at the high light intensity (140 mm ethylene at 1,200 ft-c versus 166 mm at 300 ft-c).

The reverse situation was, however, obtained with the *Anabaena* bloom which reduced more acetylene in the day than at night. The acetylene reduction at night by the bloom of *Anabaena* accounted for approximately 20% of that reduced in the day (Fig. 25).

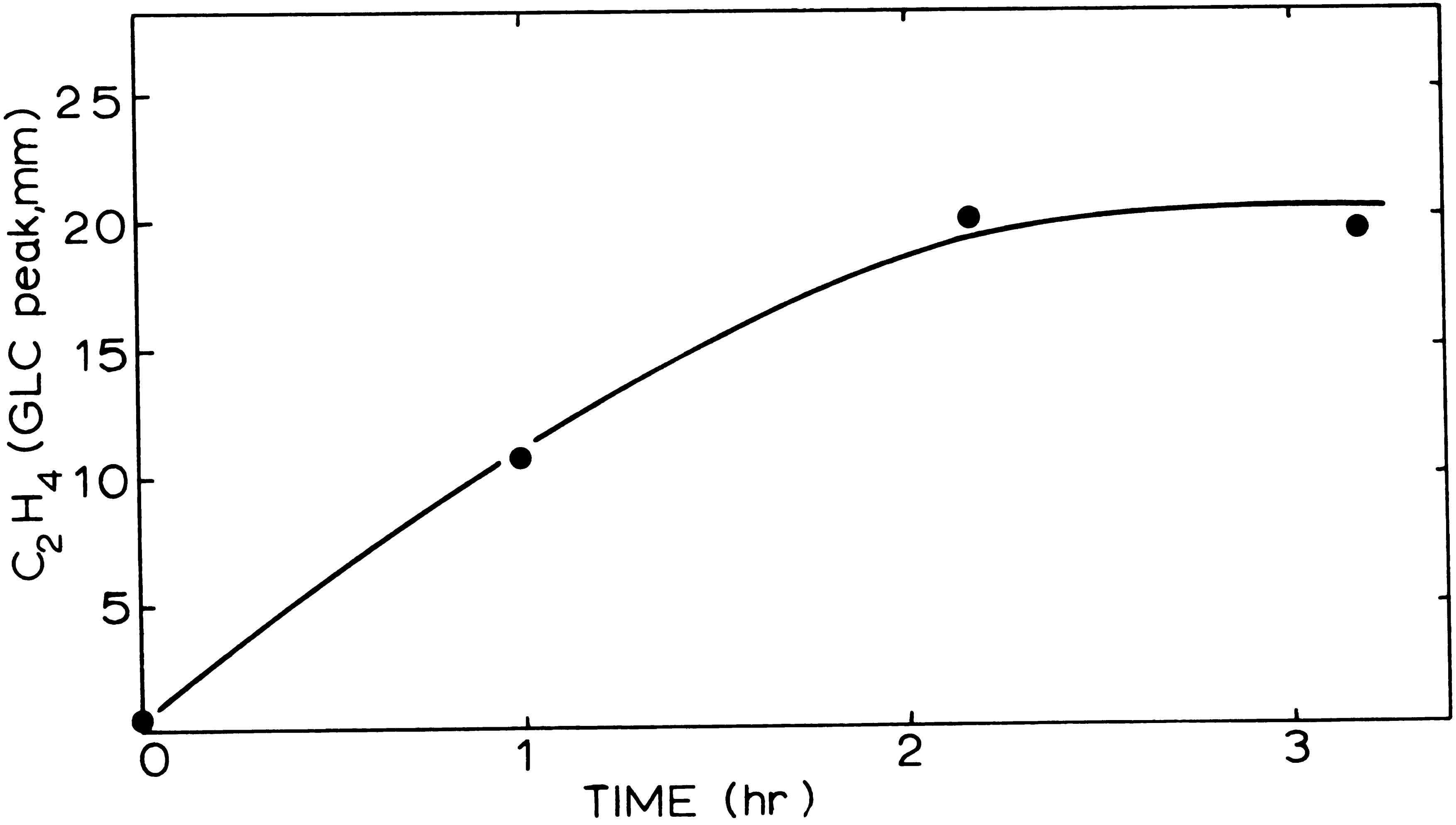
The recovery of light-starved *Aphanizomenon* and *Anabaena* was rapid. When a unialgal *Aphanizomenon* culture was assayed at the end of a 10-hr dark-growing cycle, the reduction rate was saturated within two hr after the light was turned on (Fig. 27). A similar recovery in a sample of *Anabaena*, taken from Wintergreen Lake (7/19/71) and kept in dark for about 17 hr, was obtained within 2 hr (Fig. 28). These results may explain the early daily maximum of the rate of acetylene reduction at sunrise by *Aphanizomenon* and by *Anabaena* at 8 AM.

Figure 27. Recovery of acetylene reduction ability in a light-starved culture of *Aphanizomenon*. The figure shows rates of ethylene production (mm ethylene peak/sample/hr) after light was turned on (0 hr).



C_2H_4 (GLC peak, mm)
80
120
160
0
2
4
6
8
10
TIME (hr)

Figure 28. Recovery of acetylene reduction ability in a light-starved *Anabaena* taken from Wintergreen Lake at 1:00 AM and kept in the dark for approximately 17 hr. Figure shows rates of ethylene produced (mm ethylene peak/sample/hr, at 20 C and 500 ft-c of fluorescent light) after light was turned on (0 hr).


C_2H_4 (GLC peak,mm)
25
20
15
10
5
0
1
2
3
TIME (hr)

Thus the diurnal data indicate that acetylene reduction at night was significant. How light-starved cells acquired the ATP required for acetylene reduction remains puzzling. It could be provided by respiration or fermentation of photosynthetic reserves.

The combination of laboratory and *in situ* data over the two-year period leads to the following conclusions:

(1) Heterocyst bearing *Aphanizomenon* (1970) and *Anabaena* (1971) were the main organisms responsible for the acetylene reduction measured. In general, acetylene reduction rates were correlated with heterocyst number. There is no evidence that planktonic or epiphytic bacteria associated with phytoplankton reduced acetylene.

(2) The period of acetylene reduction and presence of heterocyst bearing blue-green algae coincided with the period when the concentration of combined nitrogen (nitrate and ammonium) was at low levels.

(3) The acetylene reduction was limited to the euphotic zone (0-3 m). The effects of light on acetylene reduction rate were related to depth and water transparency.

(4) Both *Aphanizomenon* and *Anabaena* in Wintergreen Lake were capable of reducing acetylene both at day and night. Total acetylene reduction averaged 3,083.5

μmole/m^2/day assuming a 12-hr reduction period. Using the theoretical conversion factor of 1.5 (ethylene produced: ammonia formed) the total nitrogen fixed by planktonic algae in Wintergreen Lake was estimated about 315 Kg ammonia per season or 21 Kg ammonia per hectare per season.

VII. ACETYLENE REDUCTION BY NITROGEN-FIXING ORGANISMS ASSOCIATED WITH DUCKWEEDS

A. Introduction

Duckweeds or Lemnaceae are the smallest aquatic flowering plants. Their distribution is world wide (Daubs, 1965). Rao (1953) reported that thick duckweed communities presumably modified the physical and chemical properties of the water underneath favoring the development of blue-green algae over other algae, although any direct effect of duckweeds on the growth of these blue-green algae has not been elucidated. On the other hand Bottomley (1919) reported that exudates of nitrogen-fixing organisms and other bacteria promoted growth of *Lemna*. In this part of the study, the composition and the distribution of the duckweed community in Wintergreen Lake and in the small lagoons and shallow ponds connected with it were conducted. The nitrogenase activity of organisms associated with duckweeds and the morphological aspect of this interaction were also investigated.

B. Materials and Methods

Duckweeds were identified using Daubs' Monograph of Lemnaceae (Daubs, 1965) as a guide. For the acetylene reduction assay, water containing duckweeds was collected at station B (see Fig. 1) and passed through a flour sifter with a metal screen of 1.5-mm square openings. The sifter retained Lemna and Spirodela. The filtrate which contained Wolffia was passed through a second modified sifter that was equipped with nylon netting of 35-μ pore size (Nitex, Tobler, Ernst Traber, Inc., New York), which retained all Wolffia. Water passing this filter was called the last filtrate. Since it was too time consuming to separate Lemna from Spirodela they were always assayed together for acetylene reduction. Lemna and Spirodela or Wolffia were removed from the sifter and dried briefly by pressing them gently between towel paper. One gram of each category was immediately weighed and transferred to a 26-ml serum bottle. The last filtrate was used to bring the total volume in each bottle to 16 ml. The details of preparation and analysis for acetylene reduction were identical to those in Chapter VI. Triplicate samples of the last filtrate, Wolffia and the mixture of Lemna and Spirodela, were incubated with 10% acetylene for one hour at the lake water surface. The reduction was stopped by injecting 0.5 ml of 2% $HgCl_2$ into the bottles. One bottle from each category which had $HgCl_2$ added at the beginning of the incubation served as a control.

The effect of light intensities and temperatures on the rate of acetylene reduction by duckweeds was studied as follows: samples of duckweeds collected from Wintergreen Lake were prepared as described above and incubated in the constant-temperature aquarium under light of 100, 300, 600, and 1,000 ft-c intensities. Temperatures tested were 10, 25, and 35 C.

Axenic cultures of Lemna perpusilla Torrey and Lemna gibba Limnaeus obtained from Dr. J. A. D. Zeevaart (Plant Biology Laboratory, Michigan State University) were also tested for acetylene reduction. They were grown in Hutner's medium with or without combined nitrogen. The carbon source was either 1% sucrose (w/v) or 0.1% $NaHCO_3$. They were grown in a temperature-controlled growth chamber (Model CEL 25-7, Sherer-Gillett Co., Marshal, Michigan) under 600 ft-c (when supplied with sucrose) or 1,200 ft-c (when CO_2 was carbon source) of fluorescent and tungsten light. Growth temperature was 20-22 C. The acetylene assay was performed with 5- or 7-day-old cultures. The details of the preparation for acetylene reduction were the same as those described above.

Fresh duckweeds were examined under a stereomicroscope at 100X magnification to locate "algal colonies." The colonized leaves were sliced vertically into thin sections by hand using razor blades and examined immediately under the Leitz phase microscope. Leaves were also pressed to release the blue-green algal colonies from duckweed.

C. Results

1. *Composition of Duckweeds*. The community of duckweeds was composed exclusively of *Lemna*, *Spirodela polyrhiza*, and *Wolffia columbia*, although the percentage of each species varied with time. Species of *Lemna* were not identified. During the early summer (June), *Lemna* and *Spirodela* dominated the population (ratio between them was 1:1) with very few *Wolffia* observed. *Wolffia* became more prevalent in late July and finally overgrew the others in late September (the ratio between *Lemna* and *Spirodela* was about 1:2 at that time). *Lemna* and *Spirodela* appeared to be struggling for survival; their leaves were smaller and turion formation was obvious.

2. *Distribution of Duckweeds*. Duckweeds, at their maximum development in July and August, formed a dense layer covering the shallow lagoons completely, the dairy lagoon, and the Duck pond. In Wintergreen Lake itself, duckweeds formed a broad irregular marginal band 5-15 m wide along the shores. They were entangled with emerged

Ceratophyllum, *Myriophyllum*, *Potagometon*, *Najas*, and *Nuphar* along the west and south-west sides. Duckweeds were also piled up in a thick layer 3-8 m wide by the prevailing south to north wind at the north end during early July. Their distribution was reduced to the very edges of the lake at the end of the summer. The remaining duckweeds were eaten by ducks, and other birds migrating from Canada (October-December) or died when the water froze.

3. *Nitrogen Fixation*. *Wolffia*, the water surrounding the duckweed or axenic *Lemna* did not reduce acetylene. *Lemna* and *Spirodela*, with associated microflora, from the lake did. Table 16a shows the quantities of ethylene produced by duckweeds collected in the late summer; turions were present in all samples. Photoreduction of acetylene by *Lemna* and *Spirodela* *in situ* was five times as much as dark reduction (Table 16b). The acetylene reducing activity seemed to be associated with the leaves rather than the roots (Table 16b). The higher rate of activity of the root-free plants than the whole plants may be explained as follows: the root-free leaves may release some organic materials which enhanced the activity of the associated nitrogen-fixing organisms. Also, the higher availability of light may have been responsible for this discrepancy since only 0.5 g of wet weight of root-free leaves was assayed. To determine

Table 16a. *In situ* rate of acetylene reduction by whole plants of *Lemna* and *Spirodela* collected from Wintergreen Lake in 1971

Date	C_2H_4 Formed (mμmole/gwet wt/hr)
24/8	93.08
1/9	46.80
7/9	90.00
21/9	27.20

Table 16b. Effect of plant part and light on *in situ* acetylene reduction by plants of *Lemna* and *Spirodela* (samples taken from Wintergreen Lake on 1/9/71).

Conditions	C_2H_4 Formed (mμmole/gwet wt/hr)
Whole plants (light)	46.80
Excised roots (light)	4.20
Leaves only (light)	92.16
Shaken water (light)	2.13
Water (light)	0.00
Whole plants (dark)	10.08

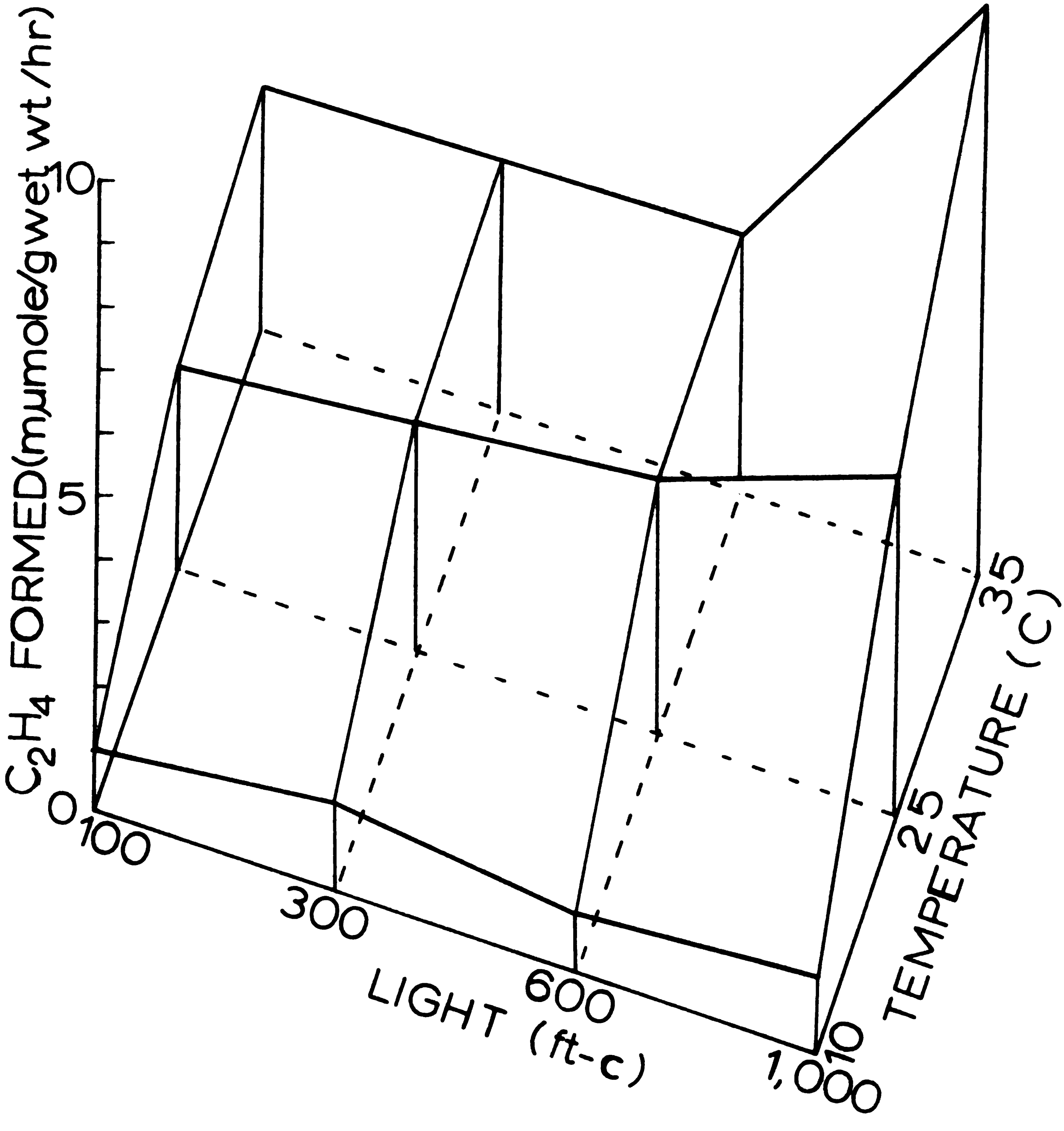
how firmly nitrogen-fixing organisms were attached to duckweeds, water containing the Lemnaceae was shaken by hand for five minutes and *Wolffia* in the first filtrate was picked up and discarded. The filtrate prepared in this way (without *Wolffia*) reduced very little acetylene (Table 16b).

The reduction of acetylene by freshly collected *Lemna* and *Spirodela* from Wintergreen Lake which were incubated in a temperature controlled aquarium increased with increasing temperature and light intensity (Fig. 29). The low acetylene reduction in the laboratory study may have been due to the prolonged darkness during transportation from the lake to the laboratory.

Axenic cultures of *Lemna perpusilla* or *Lemna gibba* did not reduce acetylene. Leaves of *Lemna* in all nitrogen-free cultures turned yellowish within 10 days due to nitrogen deficiency. Upon introduction of a growing unialgal culture of *Aphanizomenon*, the dying *Lemna* recovered within a week and reproduced normally for 6 months; the other cultures that did not receive *Aphanizomenon* eventually died. The surviving *Lemna* depended completely on blue-green algae for its nitrogen.

4. *Anatomy Study*. The anatomy of *Lemna* and *Spirodela* leaves (fronds) was similar to that of the floating leaves of more complex aquatic flowering plants. The leaf was made up mainly by spongeneous mesophyll with

Figure 29. Effect of light and temperature on the rate of acetylene reduction by natural samples of Lemna and Spirodela incubated in the laboratory. Samples were taken from Wintergreen Lake on 4/9/71.


C_2H_4 FORMED(mμmole/gwet wt/hr)
10
5
0
100
300
600
1,000
LIGHT (ft-c)
10
25
35
TEMPERATURE (C)

large lacunae (Jacob, 1947). The upper epidermis had a thick cuticune and the lower was bare but protected by a thin mucilagenous layer. Each leaf had two pockets in which flowers, fruits, or turions were formed. Three species of heterocyst bearing blue-green algae were found to attach to *Lemna* and *Spirodela* leaves. Two species had a tapering filamentous form and belonged to the genera *Calothrix* and *Dichothrix* (Fig. 30). Both had sheaths and base heterocysts. The other species was fragile, formed irregular colonies, and belonged to the genus *Anabaena* (Fig. 30). *Calothrix*, *Dicothrix*, and *Anabaena* were found mostly inside the pockets of mature *Lemna* and *Spirodela* plants or in the lower mesophyll of older leaves which had turned yellow.

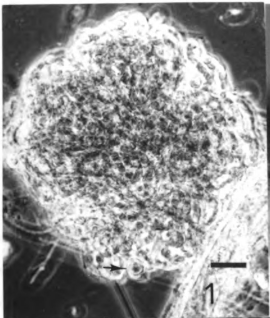
D. *Discussion*

From the above observations and experimental data, it may be concluded that heterocyst bearing blue-green algae tightly associated with *Lemna* and *Spirodela* plants were capable of reducing acetylene. The seasonal succession of species in the natural environment may be restated to emphasize the role of available nitrogen and other nutrients. The symptoms of nitrogen deficiency described by Jacobs (1947) were observed on *Lemna* and *Spirodela*, but not on *Wolffia*, when available nitrogen concentration in the water was lowest. *Wolffia*, which

Figure 30. Algae associated with *Lemna* and *Spirodela*.

(1) *Anabaena* colony which developed on lower mesophyll of *Spirodela polyrhiza*. The figure also shows heterocysts (arrow).
(2) Small colony of *Calothrix* (arrow) entangled with organic matter inside *Spirodela polyrhiza* pocket.
(3) Blue-green algae separated from lower mesophyll of *Lemna* showing heterocysts (arrow).

Scale: Bars represent 10 µ.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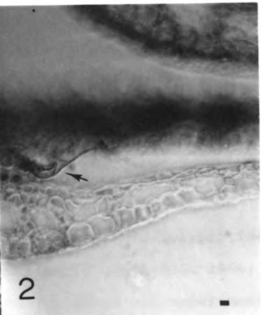
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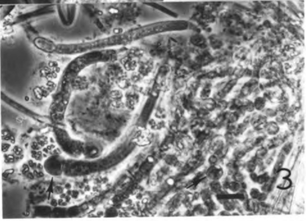
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apparently could not compete well with other duckweeds for nutrients in the early summer, outgrew *Lemna* and *Spirodela* when nitrogen, phosphate, and possibly other elements were depleted.

There are several possibilities for the nature of the interaction between the duckweeds and the algae. Symbiosis is unlikely for the following reasons: *Lemna* and *Spirodela* showed a nitrogen deficiency while associated blue-green algae fixed nitrogen actively. Blue-green algae were reported to secrete nitrogenous substances which were made up mainly of ring structured polypeptides which may not be usable by duckweeds (Fogg, 1952, 1966; and Bottomley, 1919). These observations and experimental results are not contrary to the laboratory data. Nitrogen starved *Lemna* grown with *Aphanizomenon*, showed signs of recovery when *Aphanizomenon* became old, and presumably some filaments lysed; also the number of bacteria originally associated with this alga was high at this time (about 10^6 cells per ml). Thus it is possible that *Lemna* utilized nitrogenous compounds resulting from bacterial degradation of excreted polypeptides. Thus duckweed might not behave like a partner of symbiosis with blue-green algae but rather assimilate nitrogen from lysed cells or from byproducts of bacterial degradation. The nitrogen deficiency of duckweeds indicated that they were not obtaining sufficient nitrogen from the associated blue-green algae.

Commensalism is probably more descriptive of this association. Blue-green algae as well as other microorganisms may find duckweed leaves a good place for physical support, protection against direct sun light, and an excellent source of carbohydrates and growth factors. No heterocyst bearing blue-green algae were ever detected attached to the upper leaf surface or the root system. Wetzel and Manny (1972, in press) reported that about 0.02-0.07% of CO_2 uptake by Lemna perpusilla was excreted under laboratory conditions. Masses of blue-green algae entangled with mucilagenous substances in the pockets (Fig. 30, 2) and the lower mesophyll of duckweeds were observed under the microscope by using India ink. These substances were possibly secreted by both the algae and duckweeds.

Thus heterocyst bearing blue-green algae associated with Lemna and Spirodela appeared to be epiphytes and active nitrogen fixers in the natural environment when available nitrogen was limited. The contribution of this association to the nitrogen income of Wintergreen Lake and its associated lagoons was estimated at 37 kg NH_3 per season. Thus total nitrogen fixed by planktonic algae and duckweed-algae association during nitrogen fixation season was 352 kg NH_3. The economic implication of this loose association and the food quality of Lemnaceae in cattle feeding are very important.

The present finding of the association between duckweeds and blue-green algae which fix atmospheric nitrogen add to the possible economic potential of these plants. It was an old practice of the Vietnamese farmers to fertilize their small backyard vegetable garden with *Azolla* and duckweeds to improve yield. Since duckweeds have a great capacity to tolerate pollution (Coler and Gunner, 1970), they can be grown in carbon-enriched ponds or lakes for mass production. Muzafarov *et al.* (1968) found that *Lemna minor* L. contained 30-32% of albumins, 30-35% of starch plus vitamins. Recently duckweeds have been used to feed cattle since they are nutritious, easy to grow in mass, and fast growing (Abdulaev, 1969). Poultry has been shown to prefer *Lemna* and both yield and quality of meat increased when poultry was fed with *Lemna* (Muzafarov *et al.*, 1968). These workers found that the amount of vitamin A and carotene in the liver and yolk of tested poultry increased 2-2.3 times over the controls.

The duckweed-algae association also offers some potential for solving problems of recycling elements in closed environments such as long-term space travel, if higher plants have to be used as proposed by Whatley (1971). The advantage of this association is that it can provide oxygen and human food; it needs a very small space for growth, and can grow on liquid waste, and is apparently more palatable than many algae.

More study of physical, physiological relationship between duckweeds and their associated communities as well as the contribution of duckweed-blue-green algae association is needed.

VIII. PHOTOSYNTHETIC BACTERIA

A. Introduction

The photosynthetic bacteria are among the most primitive, currently living forms (Losada *et al.*, 1960) which are widespread in nature. They are comprised of green sulfur, purple sulfur, and purple non-sulfur bacteria. The photosynthetic bacteria are characterized by their ability to grow under anaerobic conditions when exposed to light and in the presence of an oxidizable substrate, the nature of which varies with the species (van Niel, 1931-1971). The photometabolism of CO_2, organic acids, alcohols, etc., which are believed to be the major fermentative products, by photosynthetic bacteria has been studied extensively in several laboratories (van Niel, 1931-1971; Muller, 1933; Foster, 1940; Gest *et al.*, 1950; Glover *et al.*, 1952; Eisenberg, 1953; Kornberg and Lascelles, 1960; Losada *et al.*, 1960; Stanier *et al.*, 1959; Hurlbert and Lascelles, 1963; Nesterov *et al.*, 1965; Anderson and Fuller, 1967; Evans *et al.*, 1966; Stokes and Hoare, 1969; Schick, 1971c, also cf. Wiessner, 1970). The metabolism of CO_2 and simple organic acids by photosynthetic bacteria grown anaerobically in the light could occur through several alternative pathways; the reductive pentose phosphate pathway (Losada _et al_., 1960), the reductive tricarboxylic acid cycle (Evans _et al_., 1966), the forward tricarboxylic acid cycle, and the glyoxylate cycle (Losada _et al_., 1960), etc. The importance of each cycle for any one organism is dictated by its nutritional history, the composition of the carbon source, and the incubation conditions of the experiment. It has been shown that photosynthetic bacteria prefer to assimilate simple organic acids over CO_2 (Anderson and Fuller, 1967). In most cases, the simultaneous assimilation of CO_2 and organic acids results in a decrease or complete suppression of fixation of the exogenous CO_2. Several species of photosynthetic bacteria have also been shown to have the ability to fix atmospheric nitrogen when grown in nitrogen-free media, in the light, and under anaerobic conditions (Kamen and Gest, 1949; Lindstrom _et al_., 1950; Lindstrom _et al_., 1951; Gest _et al_., 1950; Wall _et al_., 1952; Newton and Wilson, 1953; Okuda and his coworkers, 1960-1961; Arnon _et al_., 1961; Winter and Arnon, 1970; Evans and Smith, 1971; Schick, 1971a, 1971b; also cf. Stewart, 1966).

The purpose of this research was to isolate photosynthetic bacteria from Wintergreen Lake and to study their capacity to reduce acetylene and the various factors which affect this activity.

B. Materials and Methods

1. van Niel's Modified Medium. For the enrichment cultures a standard modification of van Niel's mineral medium (1944) was used consisting of 0.5 g $(NH_4)_2SO_4$, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$, 0.2 g NaCl, 3.0 g $NaHCO_3$, 1.0 g yeast extract in 900 ml distilled water. The medium was adjusted to pH 6.8 with 5% H_3PO_4. Three grams of sodium acetate was dissolved in 100 ml of distilled water. The salt and acetate solutions were autoclaved separately and mixed together when they cooled to room temperature.

2. Medium "98." Van Niel's modified medium gave low cell yields when yeast extract was omitted. Unless otherwise noted, the following medium "98" was used in all experiments with pure cultures of photosynthetic bacteria. Solution 1: 1 l of deionized water with 40.0 g KH_2PO_4 and 60.0 g K_2HPO_4. Solution 2: 1 l of deionized water with 2.0 g $MgSO_4.7H_2O$, and 0.5 g $CaCl_2$. Solution 3: (Pfennig and Lippert, 1966) 500 mg of EDTA (ethylene diamine tetraacetic acid) was dissolved in 100 ml deionized water. The solution was adjusted to pH 6.8 with 10% NaOH. EDTA was then mixed with other salt solutions consisting of 800 ml deionized water with 200 mg $FeSO_4.7H_2O$, 10 mg $ZnSO_4.7H_2O$, 3 mg $MnCl_2.4H_2O$,

2 mg $NiCl_2 \cdot 6H_2O$, and 3 mg $Na_2MoO_4 \cdot 2H_2O$. This solution was diluted to 1 l with deionized water.

The final mineral salt solution was prepared by adding 15 ml of solution 1, 100 ml of solution 2, and 10 ml of solution 3 to 500 ml deionized water, mixed. This solution was diluted to 900 ml with deionized water. The pH of the final mineral solution was 6.7-6.9. The organic substrate and 2.0 mg of yeast extract were dissolved in 100 ml of deionized water. The final mineral salt and organic substrate solutions were autoclaved separately, cooled to room temperature under repurified nitrogen gas then mixed together.

3. Isolation. All isolations of photosynthetic bacteria were done in the summer of 1970. Sediments or partly degraded algal mats which floated to the surface of the lake were collected into 120 ml screw-caped bottles; the bottles were then filled with surface lake water. The algal mats were decaying residue from the lake bottom which had migrated to the surface. These mats were often dotted with red spots of colonies of *Rhodopseudomonas*, *Rhodospirillum* on the surface layer, and pink colonies of *Thiopedia* underneath. Portions of lake water from the anaerobic zone were also collected into other bottles which were then filled with van Niel's modified medium. All bottles were incubated under a 60-watt tungsten bulb at room temperature. Visual development of purple color

occurred within 4-7 days in the bottles containing the algal mat, 15-20 days in those containing sediment, and 5-10 days in those containing hypolimnion water. This primary enrichment was subcultured several times in the above medium. Cell suspensions from these secondary enrichments were diluted in sterile deionized water from which a loopful was transferred to one of a series of test tubes (15 X 150 mm) containing 15 ml of nitrogen-free medium "98" with 1.7% agar, kept at 45 C in a water bath. Each inoculated tube was shaken using a test tube mixer (Fisher Mini-Shaker Model 58, Fisher Scientific Co., Chicago, Ill.). A loop of agar from this tube was then transferred to the second molten agar tube, mixed and so on, to give a total of four dilutions of agar shake tubes. Tubes were flushed with nitrogen gas (Hi pure, 99.9998% pure) and capped with rubber stoppers. All tubes were incubated at room temperature under 60-watt incandescent light. Irregular colonies of *Rhodomicrobium* began to develop within 10-15 days, in the bottom half of the test tubes. *Rhodopseudomonas* developed earlier (5-7 days) and formed spindle colonies throughout the agar column. The tubes and then the agar column were broken and the individual colonies exposed at the agar break were picked and suspended in oxygen-free sterile deionized water to repeat the shake tube isolation. *Rhodopseudomonas* cells were usually trapped inside colonies of *Rhodomicrobium* by the branched network of the latter. Therefore, shake tube isolation and subculturing in liquid medium were repeated until pure cultures were obtained.

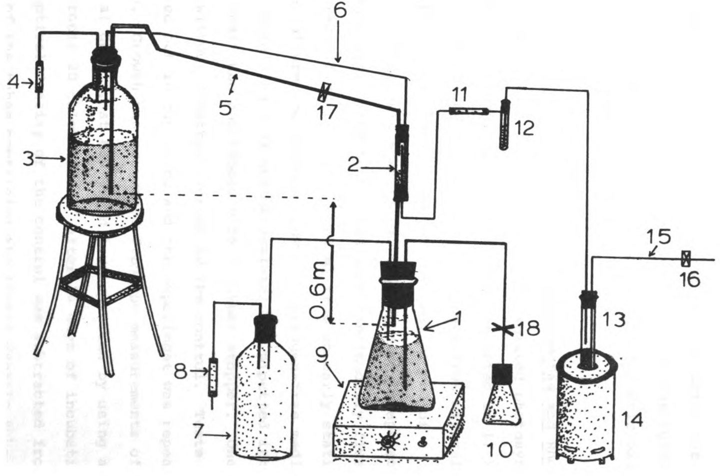
Rhodopseudomonas strains were isolated using either the above shake-tube technique or by streaking a cell suspension on agar slants that were prepared in widemouth bottles under a nitrogen atmosphere (Hi pure). *Rhodopseudomonas* developed faster than *Rhodomicrobium*; therefore they could be isolated easily using the latter technique.

4. *Preparations for Electron Microscope Studies*. For morphological studies, cell suspensions were diluted in deionized water to about 10^3 cell/ml. A drop of this suspension was transferred to an electron microscope grid (200 mesh) which had been previously coated with a thin layer of 0.05% collodion (for negative stain) or 0.25% formvar (for carbon shadow). The grid with the cell suspension was allowed to sit for about 2 min, then it was drained and air dried. Air dried cells on grids were coated with gold-palladium #46 at 35 or 45°. The negative stain was done with 0.5% of phosphotunsgtic acid in water. The prepared grids were examined with a Hitachi electron microscope (HU-11, Hitachi, Ltd., Tokyo) or a Philips EM-300 electron microscope (T.M.N.V. Philips of Holland).

5. Anaerobic Gas Flushing System. Unless otherwise noted, all work on photosyntehtic bacteria, such as transferring, preparation of media, nitrogen fixation assay, etc., was done under repurified nitrogen or argon. Traces of oxygen in these gases were removed by the Hungate technique (Hungate, 1950) as modified by Bryant and Robinson (1961). The anaerobic flushing system (Fig. 31) composed of a vertical furnace (Sargent-Welch Scientific Co., Detroit, Mi.), maximum temperature of about 500 C, which contained a quartz tube (homemade, 2.5 X 3.0 cm) filled to two-thirds of its length with cupric oxide wire (CuO, Fisher Scientific Co., Fair Lawn, N.J.). The tube was plugged with a rubber stopper. The gas source was connected to a one-quarter-inch copper tube which extended through the stopper and cupric oxide and almost reached the bottom of the quartz tube. The scrubbed gas exited through a similar copper tube which was connected to four rubber tubing lines. Each line was attached to a glass syringe (5-ml) filled with cotton and equipped with a large stainless steele needle (B-D Yale Luer-lok, 18 gauge 31/2," Becton, Dickinson and Co., Rutherford, N.J.). The syringe could be taken off the line and autoclaved. The needle was bent to make a hook of about 45 degrees. During aseptic work, the needle was sterilized by heating over a bunsen flame prior to inserting into the container mouth. The flow of gas

Figure 31. Continuous culture of Rhodomicrobium

Culture vessel (1) was stirred continuously by magnetic stirrer (9); the vessel was connected to sampling line (10) and waste container (7). Mineral salt container (10 liter) containing nitrogen free medium "98" at full strength (3) was connected to the flow regulator (2) by two lines (5) and (6). It also had one air outlet (4) and one refill line. The medium flow regulator (2), made from glass tubing (25 x 120 mm), was connected with gas source (11). It was constructed so that the medium coming in by the inlet (5) flows down to the culture vessel; extra medium was forced to go back through line (6) to the medium container (3) by gas (nitrogen) pressure built up in the regulator. The nitrogen gas or medium coming from the flow regulator (2) forced extra culture in the culture vessel to the waste outlet (7). The flow rate of medium from (3) to (2) was adjusted at (17) by a screw clamp. It was critical to move the medium container up or down so that the pressure head in the vessel (1) was about 60 cm below that in the container (3) to assure the medium flow. The entire system was flushed continuously by nitrogen gas which was repurified by reduced cupric oxide (13) heated by a furnace (14). Growth was regulated by the rate of fresh medium addition.


6
4→
5
17
11
12
3→
2→
15
16
0.6m
13
1
18
8→
9
14
7→
10

was adjusted by the tank regulator. Before use, the cuprix oxide wire in the tube was heated to about 500 C, then reduced by flushing with hydrogen gas until the wire turned pink. It was necessary to keep the furnace heating all the time and to regenerate the cupric oxide once a week.

6. Photometabolism of Rhodopseudomonas and Rhodomicrobium. Tests for growth of the isolated photosynthetic bacteria on organic substrates were conducted in 17 X 150 mm test tubes which contained 9 ml nitrogen-free mineral medium "98" plus 0.2% (unless otherwise noted) of one of a variety of organic substrates. During media preparation and inoculation the test tubes were continuously flushed with repurified nitrogen. One ml from an early stationary phase culture (cells were grown in nitrogen-free medium "98" containing 0.3% sodium acetate) was pipetted into each test tube and capped with a rubber stopper. One tube without substrate served as the control. Tests were carried out in duplicate and the experiment was repeated twice. Growth was estimated from the measurements of the optical density at 680 mμ in 1-cm cuvettes by using a Spectronic 20 colorimeter. After 10 days of incubation the optical density of the control was subtracted from that of the tubes containing the tested organic substrates to obtain the final growth measurement. The tubes were supported by a circular wood frame which held

the tubes equidistant from a 100-watt tungsten bulb. The incubation temperature was about 27 C and the light intensity was 300 ft-c at the tubes.

7. Acetylene Reduction Assay. *Rhodomicrobium* was used to study the ability of this genus to reduce acetylene. For convenience, *Rhodomicrobium* was grown in a continuous culture apparatus which was constructed from a 1.5 l erlenmeyer flask (Fig. 31). The culture was stirred continuously by a magnetic stirrer and illuminated with a 60-watt tungsten bulb (about 150 ft-c at the surface).

The culture could be kept at a late log phase by this system. Cells used for preparations of all experiments described below were taken from this continuous culture.

Acetylene reduction assays were done 18 to 20 hours (log phase) after subculturing to broth medium from the continuous culture. Unless otherwise indicated, all cells used for acetylene reduction were grown in nitrogen-free medium "98" containing 0.0002% yeast extract, 0.3% acetate, stirred by magnetic stirrer and incubated under an atmosphere of repurified nitrogen, and in the presence of continuous tungsten light.

To determine the optimum acetate concentration, time of incubation and quantity of acetylene for the assay, cells were grown at about 30 C, 300 ft-c, harvested in log phase (for optimum acetate concentration experiment, culture was centrifuged at 3,000 x g for ten minutes, resuspended in fresh medium) and assayed for acetylene reduction under a repurified argon atmosphere.

For studies on the effect of light intensities and temperatures, cells were grown in side-arm flasks (Nephelo culture flask, screw cap, 500 ml, Belco Glass, Inc., Vineland, N.J.) placed in an aquarium (0.31 X 0.32 X 0.77 m) in which the water temperature could be controlled, at four different light intensities (100, 200, 300, 400 ft-c) which were obtained by varying the length of the light path in the aquarium. The water was maintained at desired temperatures of 25, 30, 37 C by a constant temperature circulator (Model 20 CTC, Bronwill Scientific Inc., Rochester, N.Y.). Cultures were constantly stirred by magnetic stirrers placed underneath the aquarium. Continuous light was provided by a row of four tungsten flood-light bulbs (75-150 watts). Light received at the walls of the individual flasks was measured using Tri-Lux-Footcandle meter (Kling Photo Corp., New York, N.Y.). Growth was followed by measuring optical density at 680 mμ. Acetylene assays were done with 16 ml of suspensions of cells taken directly from the culture flasks without resuspending in new medium. The cells were incubated under 10% acetylene in argon in 26-ml serum bottles at the same temperature and light intensity at which they were grown.

The effect of various combined nitrogenous compounds on acetylene reduction was also studied. In this study, cells were grown under 300 ft-c at about 30 C in 500-ml side-arm flasks containing 400 ml medium "98," stirred continuously by a magnetic stirrer. The acetylene reduction assay was done at log phase without resuspending cells in new medium. Ten ml of the culture was pipetted into each of a series of 26-ml serum bottles into which 1 ml of solutions of various concentrations of yeast extract, ammonium chloride, or sodium nitrate was added to have the final concentrations indicated. Cells then were incubated under 10% acetylene in a repurified argon atmosphere at 30 C and 300 ft-c. A half ml of head space gas in the bottle was injected into the gas chromatograph at the indicated times. A control (no combined nitrogen added) was always used.

Induction of acetylene reduction activity was measured using cells grown in medium "98" with 0.1% NH_4Cl and 0.0002% yeast extract in a nitrogen atmosphere. The cells were harvested at log phase, centrifuged at 3,000 x g for 10 min and resuspended in nitrogen-free medium "98" in 500-ml flasks and the flasks were flushed continuously with repurified nitrogen, argon, or helium (about 100 ml/min). Samples for the acetylene reduction assay (triplicate) were done at the times indicated in 26-ml serum bottles. Bottles were incubated for 1 hr on the disc of a multi-purpose rotator (Model 150 V, Scientific Products, Inc., Queens Village, N.Y.) to insure uniform light distribution (300 ft-c, 28 C). The experiment for each type of gas was repeated twice.

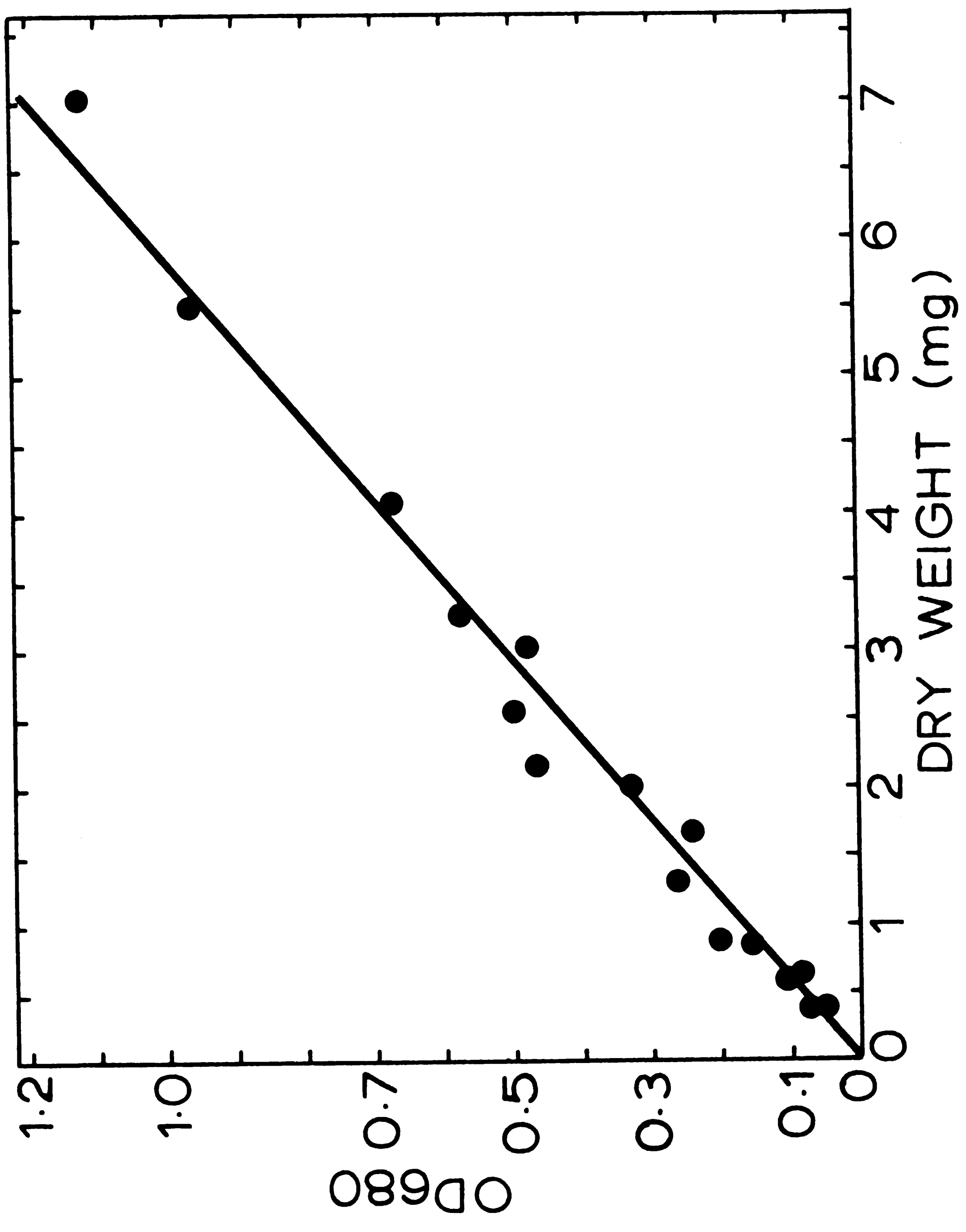
Since the quality of light affects bacterial photosynthesis (Pfennig, 1967), it should similarly affect nitrogen fixation which derives its necessary energy from photosynthesis. This implies that when some wave lengths of light are selectively eliminated, the acetylene reduction rate could be affected. This was done by wrapping white, red, yellow (amber), and blue transparent cellophane paper around the cultures. The absorption spectrum of each color of paper was determined on paper which had been wrapped around a 1-cm cuvette and placed in a scanning Beckman grating spectrophotometer (Model DB-G, Beckman Instruments, Inc., Fullerton, Calif.). The transmission of white cellophane was practically unchanged. The red cellophane absorbed all wavelengths up to 570 mμ but began to transmit light which reached maximum transmittance from 625 to 750 mμ then the percent transmittance decreased again. With the yellow cellophane, maximum transmittance was obtained with the wavelengths greater than 550 mμ and to lesser extent with wavelengths shorter than 400 mμ; maximum absorption occurred in the range of

400-500 mμ. Finally, the light blue cellophane transmitted maximally in the regions of 375-475 mμ and 700-750 mμ. In summary, white cellophane supplied all of the tungsten light wavelengths. The yellow cellophane and to a lesser degree the blue cellophane supplied most of the wavelengths which are absorbed by both carotenoids and bacterial chlorophyll. Finally the red cellophane transmitted wavelengths longer than 600 mμ.

In the first experiment, cells were grown under tungsten light in 500-ml side-arm flasks; samples were wrapped with transparent colored cellophane paper (as indicated) when incubated with acetylene. In the second experiment, cells were grown in flasks wrapped with white, red, yellow, and light blue cellophane papers. These flasks were placed at an equal distance from a 100-watt tungsten bulb (about 300 ft-c at flask wall). Samples for acetylene assay were also wrapped with the same colored cellophane paper as grown and incubated at 300 ft-c.

A standard curve (Fig. 32) relating optical density at 680 mμ to dry weight was prepared as follows. Cells were grown in nitrogen-free medium under nitrogen gas at 300 ft-c and 30 C, harvested at log phase, centrifuged, resuspended, and diluted to various optical densities (Spectronic 20, at 680 mμ) using the original supernatant. Triplicate cell suspensions (10 ml) were dried in an oven at 80 C for 24 hr, then weighed. A correction was

Figure 32. Standard curve relating optical density at 680 mμ of culture to dry weight of <u>Rhodomicrobium</u>.


OD680
1.2
1.0
0.7
0.5
0.3
0.1
0
DRY WEIGHT (mg)
0
1
2
3
4
5
6
7

was made for the weight of salts in the suspensions. In all experiments this curve was used to obtain dry weight values.

C. Results and Discussion

1. Enrichment and Isolation. The frequency of the development of photosynthetic bacteria in the enrichment cultures varied depending on the inoculum source. Visible color development occurred in all bottles inoculated with algal mats, in 50-70% of the bottles inoculated with mud and in 80-100% of the bottles inoculated with water. The composition of photosynthetic bacterial community also depended on inoculum source. Water samples taken from 3-5 m always supported the development of *Thiopedia*. *Thiopedia* usually appeared first followed by *Chromatium* domination after 10 to 20 days. The intense decomposition could have produced a high concentration of H_2S which is thought to inhibit *Thiopedia* and favor *Chromatium*. It is worth noting that *Thiopedia* was able to grow on the wall of bottles containing sediment taken from deep water which were kept in the refrigerator at 10 C in the dark. Visible development occurred only above the sediment. The fermentation of organic material at this temperature is expected to be extremely slow, hence H_2S released was also low. The energy source in this particular condition is not known; it is probable that

light was admitted by the occasional opening of the refrigerator. Several attempts to isolate *Thiopedia* and *Chromatium* by using various media containing bicarbonate, or various organic carbon sources with added H_2S, however, failed.

Thiopedia and *Rhodospirillum* developed first in samples containing the algal-mat, however, *Rhodospirillum* rapidly overgrew *Thiopedia*. *Rhodospirillum* occasionally constituted 98% of the total bacterial population in the enrichment cultures. Upon sitting for some time or transferring to medium "98," *Rhodopseudomonas* became dominant and *Rhodospirillum* eventually disappeared. *Rhodomicrobium* usually appeared in an old culture when *Rhodospirillum* had almost disappeared; the ratio between *Rhodomicrobium* and *Rhodopseudomonas* was about 2:1 after the disappearance of *Rhodospirillum*.

Enrichment cultures containing mud taken from shallow water, which appeared to have less freshly deposited organic material, usually supported the growth of *Rhodopseudomonas* first, then later *Rhodomicrobium*.

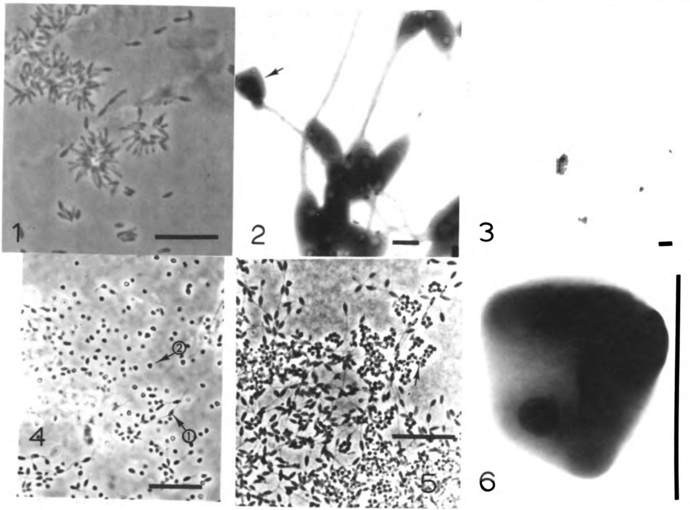
All attempts to isolate *Rhodospirillum* also failed. *Rhodomicrobium* and ten strains of *Rhodopseudomonas* were isolated; these genera were apparently less sensitive to oxygen than the others. The isolates were all budding bacteria containing bacterial chlorophyll, and grew on acetate as the sole carbon source in a nitrogen-free medium under anaerobic conditions and light. Some strains, presumably belonging to the species *Rhodopseudomonas palustris*, required p-aminobenzoic acid (Hutner, 1946) for maximum growth. All isolates grew well when supplied with yeast extract as low as 0.0002% (w/v) in nitrogen-free medium.

2. *Some Characteristics of the Isolated Rhodomicrobium*. This organism was isolated for the first time by Duchow and Douglas (1949) and was classified in the family Hyphomicrobiaceae under the name of *Rhodomicrobium vannielii* (Duchow and Douglas, 1949; Douglas, 1957). This organism reproduces by budding and has morphology similar to *Hyphomicrobium*, however, it is an anaerobic, photosynthetic, non-sulfur bacterium. Mature cells of the isolate had an oval to rod shape (1-1.2 x 1.5-2.5 μ); cells grown in nitrogen-free medium were smaller. Round young cells were formed at the tips of "stalks." These newly formed cells were motile when separated from motor cells (Fig. 33, 3). Each motor cell can bear as much as 4-5 stalks which originated from its body and grew in different directions. Two to four stalks sometimes appeared to join without a mature cell at the joining point. Mature cells in cultures about 1-2 weeks old supplied with acetate had a refractile granule near each end (Fig. 33, 4). These granules were probably poly-β-hydroxy butyrate which was previously reported

Figure 33. Isolated photosynthetic bacteria. (Cells were grown in nitrogen-free medium "98.")

(1) *Rhodopseudomonas*
(2) Negative stained *Rhodomicrobium* showing cyst (arrow) and vegetative cells of stationary phase culture supplied with acetate.
(3) Gold-palladium shadowed motile cell producing stalk and young cell.
(4) 15-day-old culture grown in acetate (phase microscope) showing PHB granules (arrow 1) and cysts (arrow 2).
(5) 20-day-old culture grown in mannitol showing cyst (arrow) and vegetative cells without PHB.
(6) Negative stained cyst.

Scale: Bars in pictures 1, 4, and 5 represent 10 μ, those in pictures 2, 3, and 6 represent 1 μ.


1
2
3
4
②
①
5
6

by Hoare and Gibson (1964) in Rhodomicrobium vannielii grown with acetate as the carbon source. These granules were, however, not observed when cultures were supplied with other organic carbon such as mannitol, starch, etc.

Rapid and extensive growth of Rhodomicrobium was achieved with medium "98" in the presence or absence of combined nitrogen under light. Exogenous CO_2 was not required when organic carbon served as the carbon source. This result seems contrary to the earlier observation by Duchow and Douglas (1949) and Trentini and Starr (1967) that Rhodomicrobium required exogenous CO_2 for anaerobic heterotrophic growth. However, in the present study significant CO_2 was produced during photoheterotrophic growth. Increasing quantities of CO_2 in the head space of an acetate grown culture was observed by head space analysis with a Carle Basic gas chromatograph equipped with a thermoconductivity detector (Model 8000, Carle Instrument, Inc., Fullerton, Calif.) or by trapping $^{14}CO_2$ when uniformly labeled ^{14}C acetate was the substrate. Photodegradation of organic acids has been shown to supply both sufficient reducing power and CO_2 for heterotrophically growing cultures of other purple photosynthetic bacteria (Muller, 1933; Gest *et al.*, 1950; Glover *et al.*, 1952; Losada *et al.*, 1960).

Attempts to grow Rhodomicrobium under the light in nitrogen-free medium containing CO_2 as the sole carbon source and hydrogen as the reductant has failed so far although Pfennig (1969) and Hoare and Hoare (1969) succeeded in growing this organism autotrophically in the presence of hydrogen and combined nitrogen.

Old cultures of *Rhodomicrobium* isolated from Wintergreen Lake formed a cyst-like structure (0.5-1.0 x 0.8-1.0 μ) which had an angular shape under the phase microscope (Fig. 33, 4 and 5). Negatively stained preparations examined by electron microscope (Fig. 33, 2 and 6) also showed an irregular angular shape; some had the appearance of a corn grain, some had an angular egg shape. All were nonmotile. These peculiar structures were observed by Murray and Douglas (1950) in old cultures but were ignored until Gorlenko (1969) who isolated a budding photoheterotrophic bacterium, physiologically and morphologically similar to *Rhodomicrobium*, from Lake Bolshoi Kichier, reported that the angular-shaped structure had characteristics of a true spore. He called these structures "spores" because they stained positive with specific spore stains and they survived heating at 100 C for 30 min. The tests for heat survival of the angular structures from the present *Rhodomicrobium* isolated was, however, inconclusive. Therefore, these structures are subsequently referred to as "cysts." The nitrogen or carbon source did not affect cyst formation. Old colonies in agar shake tubes contained about 50% or more cysts which appeared in pairs thus giving an appearance of dividing cells but without visible connecting stalk between the two cells.

Gorlenko (1969) reported that he observed some germinating "spores" which stained like vegetative cells and formed stalks before producing young cells at the stalk tips. Some of his pictures showing germinating "spores" were, however, seemingly old vegetative cells which were split away from the branched network by mechanical factors as usually observed in the present study. Nonetheless, observations in the present study are in agreement with Gorlenko in that the angular structures were produced in old cultures and these structures produced vegetative cells under optimum nutritional conditions. Information accumulated so far does not permit any speculation on the physiological nature or the ecological role of the cysts.

3. *Photometabolism of Organic Compounds*. The growth of *Rhodomicrobium* on a variety of organic substrates in nitrogen-free medium "98" supplied with 0.0002% yeast extract (as well as *Rhodopseudomonas*) is presented in Table 17. In general, *Rhodomicrobium* was capable of using a wider range of organic substrates than *Rhodopseudomonas*. *Rhodomicrobium* metabolized all sugars tested except ribose. This result is contrary to the report of Pfennig (1969) that *Rhodomicrobium vannielii* did not

Table 17. Growth of *Rhodomicrobium* and *Rhodopseudomonas* on a variety of organic compounds

Organic Substrate**	Net Growth*		Organic Substrate**	Net Growth*	
	Rhodomicrobium	*Rhodopseudomonas*		*Rhodomicrobium*	*Rhodopseudomonas*
d(+)-Lactose	2	0	Acetate	3	2
d(-)-Mannitol	3	0	Lactate	3	3
d(+)-Cellobiose	3	0	Fumarate	1	2
Sucrose	3	0	Succinate	1	0
Bacto dextrose	3	0	Citrate	0	0
Rhamnose	1	0	Pyruvate	3	3
D-Galactose	2	0	Glutamate	3	0
Ribose	0	1	Malate	3	3
Arabinose	0	0	Ethyl acetate	2	3
Sorbitol	1	0	Glutarate	1	0
Starch	3	0	Salicylate	1	I
Resorcinol[1]	2	I	Benzoate	I	I
Chitin	3	0	Galacturonate	1	0
Inulin	3	0	Propionate	1	2
Gelatine	1	0	Aspartate	1	0
Glycerol	2	2	Asparagin	3	0
Toluene	0	1	Leucine	2	3
Inositol[2]	I	0	Cystein[8]	1	0
Naphthol[3]	3	2	Glycine	0	0
Methanol[4]	1	1	DL-Alanine	0	0
Ethanol[5]	2	1	Thiosulfate	1	-
Butanol[6]	1	2			
Propanol[7]	2	0			

*Net increase in O.D. over the control which had no substrate added: 0 = 0 O.D. unit increase, 1 = 0.03 to 0.1, 2 = 0.1 to 0.5, 3 = greater than 0.5; I the growth was inhibited.

**All substrate concentrations were 0.2% except for: 1,2 = 0.05%, 3, 4, 5, 6, 7 = 0.02%, 8 = 0.01%.

utilize glucose, fructose, and mannitol. Table 17 also indicates that Rhodomicrobium can metabolize carbohydrate polymers such as starch, chitin, and inulin. This surprising metabolic capacity to utilize sugar may be explained by the fact that acetate left over in the inoculum may have facilitated or initiated the metabolism.

This explanation is in line with the observation of van Niel (1931) that the development of representatives of Athiorhodaceae in media containing sugars was greatly enhanced by contamination with lactate. Rhodomicrobium and Rhodopseudomonas did not grow well when alcohol served as the carbon source. This was probably due to lack of CO_2 (Qadri and Hoare, 1968) which was probably for alcohol metabolism.

Rhodomicrobium and Rhodopseudomonas utilized most of the simple organic acids tested particularly those of the tricarboxylic cycle with the exception of citrate which may be impermeable to the cell (Eisenberg, 1953).

It is necessary to emphasize again that CO_2 and reducing power were not provided by external sources in this experiment therefore they must have originated from the degradation of the organic compounds or from the acetate contamination (Muller, 1933; Ormerod, 1956).

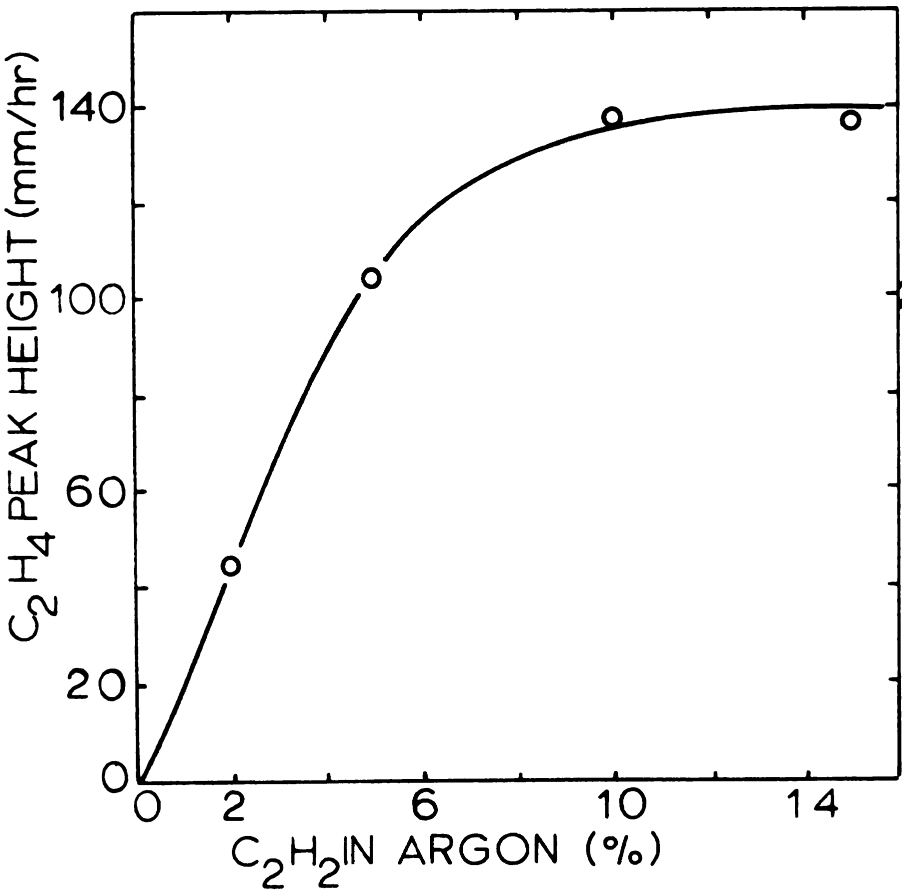
4. Acetylene Reduction. Rhodomicrobium was chosen for studies on acetylene reduction because:

(1) nitrogen fixation in this organism has not been studied in detail since Lindstrom _et al_. (1951) showed that this organism can grow in nitrogen-free medium and fix $^{15}N_2$, (2) the effect of polymorphism observed by Douglas and Wolfe (1959) and Gorlenko (1969) on the efficiency of nitrogen fixation by this organism could be pronounced, and (3) the growth of this organism in the absence of exogenous CO_2 has not been studied.

The maximum acetylene reduction rate was obtained with 0.3% (w/v) or more of acetate. This concentration of acetate also supported maximum growth of this organism. The maximum rate of acetylene reduction was achieved with 10% acetylene in an argon atmosphere (Fig. 34, left). The ethylene formation leveled off after one hour of incubation (Fig. 34, right). The initial lag in acetylene reduction shown in Figure 34, right, was presumably due to the low solubility of acetylene in water (Morrisson and Boyd, 1966, p. 239) or to the effect of transfer (Hardy _et al_., 1968). Both growth and acetylene reduction were optimum in a pH range of 6.5-7.1.

Ethylene production and growth in a nitrogen-free medium by _Rhodomicrobium_ were light dependent (Fig. 35) as is the case for other purple photosynthetic bacteria (Kamen and Gest, 1949; Newton and Wilson, 1953; Arnon _et al_., 1961; Evans and Smith, 1971; Schick, 1971a,

Figure 34. Acetylene reduction by *Rhodomicrobium* versus acetylene concentration (left), time course of ethylene production (right), both in assay bottles with argon as the dominant head-space gas.


C_2H_4 PEAK HEIGHT (mm/hr)
0
20
60
100
140
0
2
6
10
14
C_2H_2 IN ARGON (%)

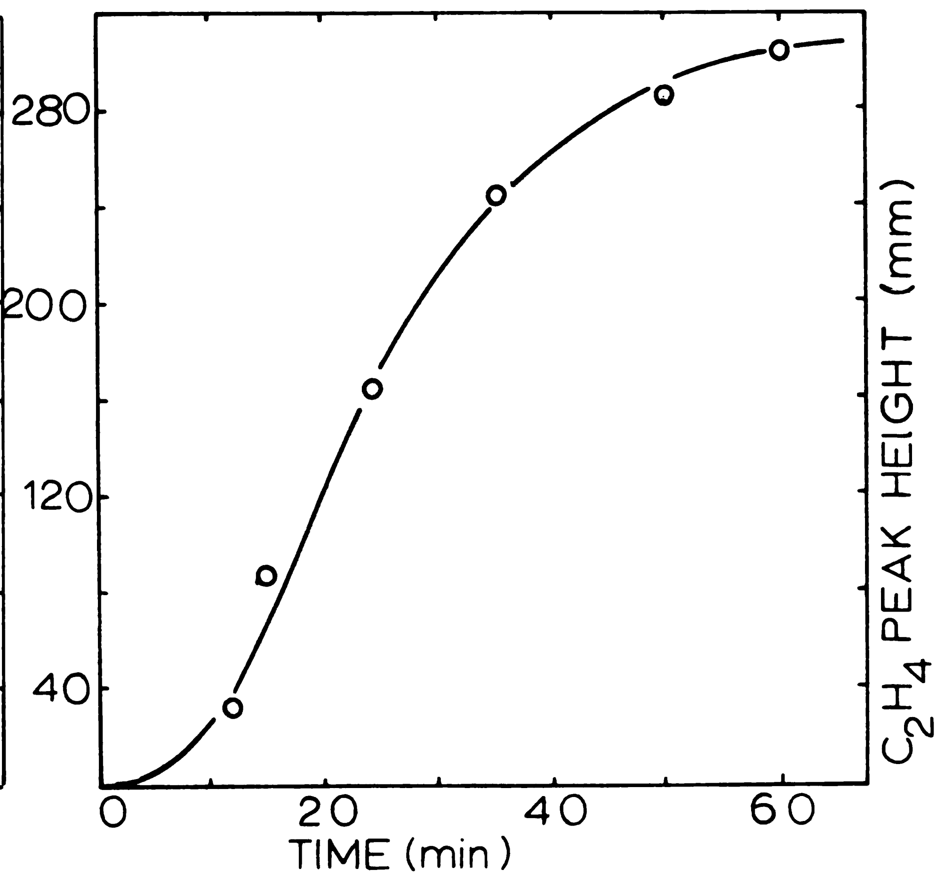
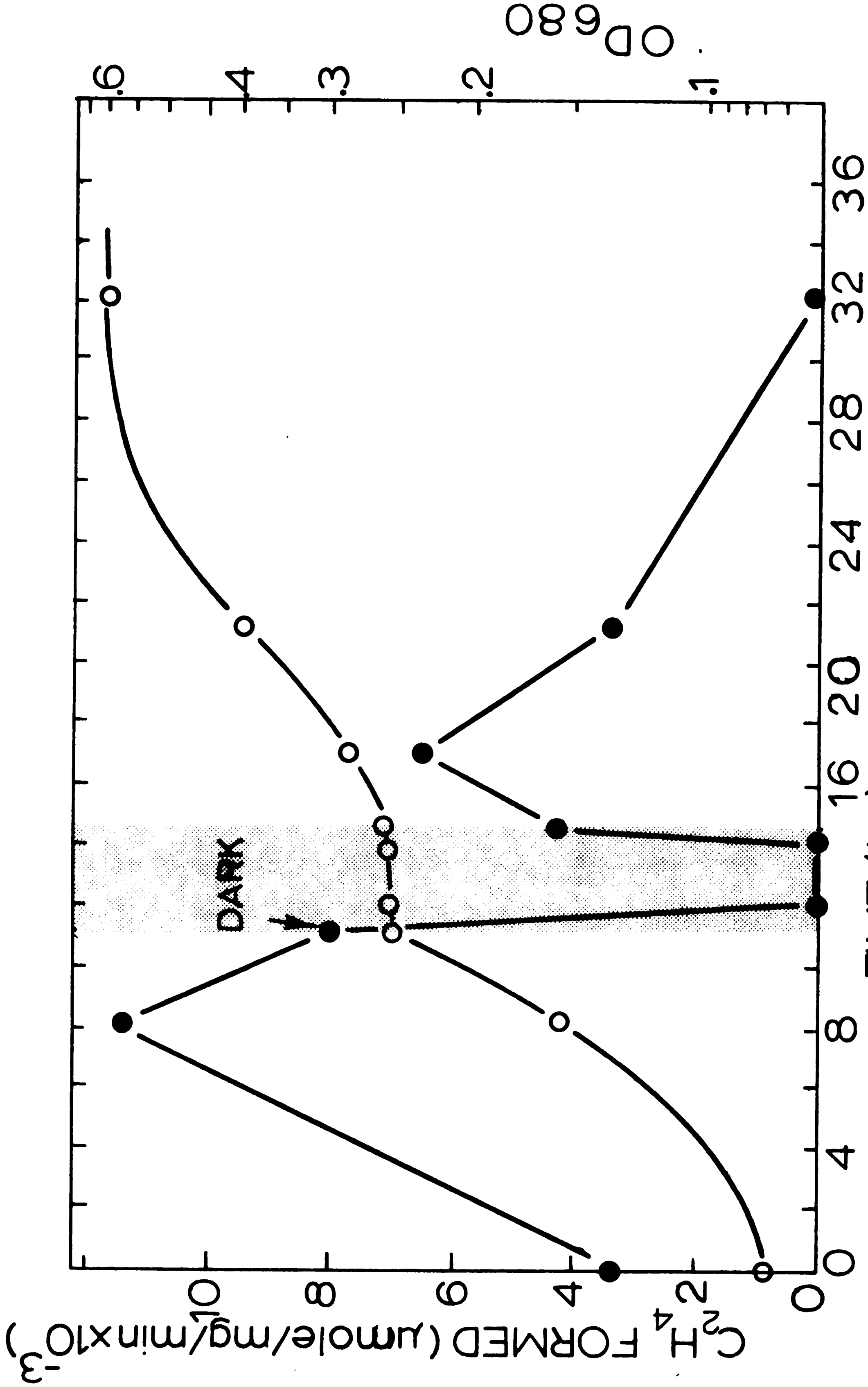

40
120
200
280
0
20
40
60
TIME (min)
C_2H_4 PEAK HEIGHT (mm)

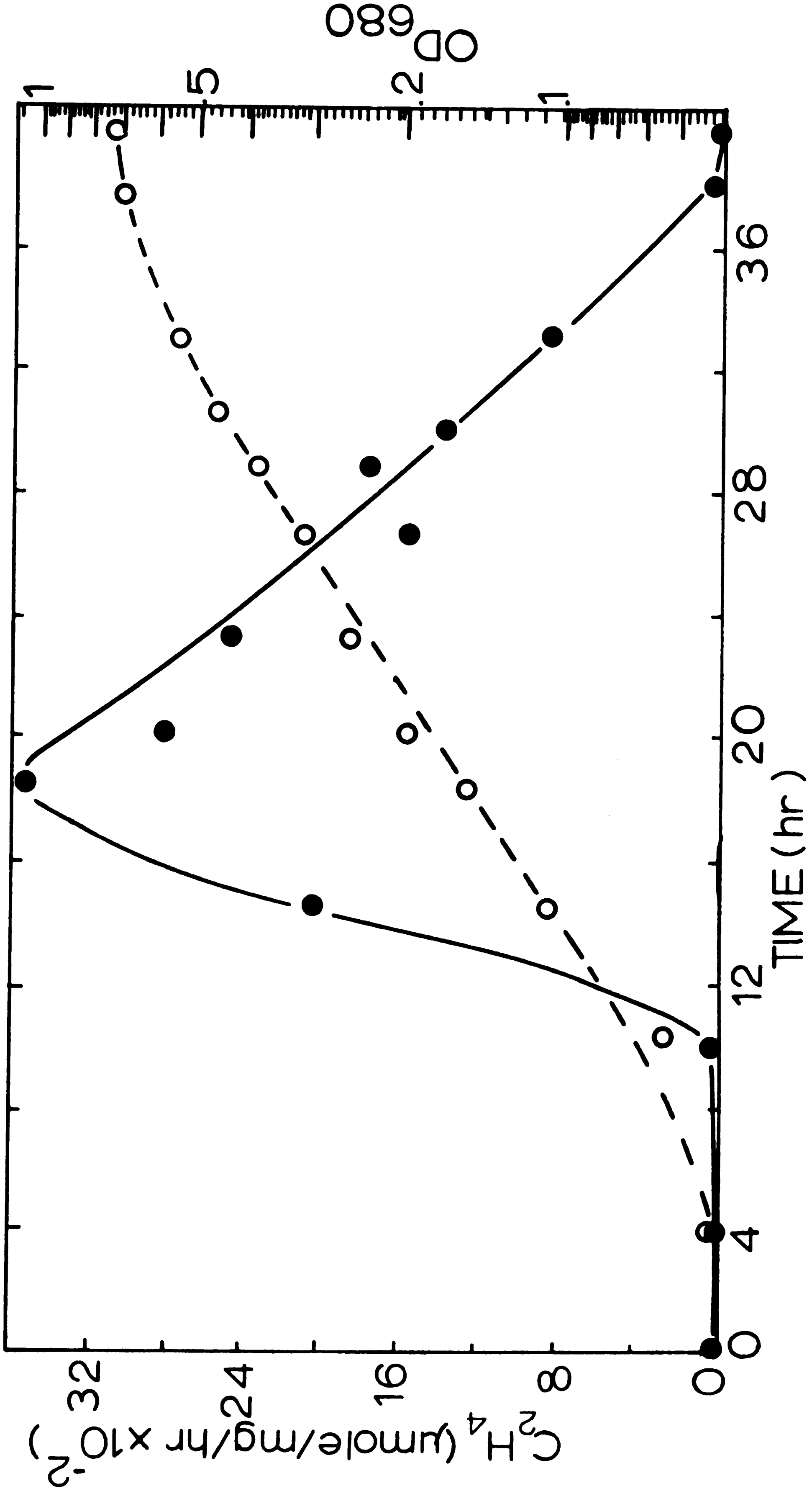
Figure 35. Effect of a dark period on the rate of acetylene reduction (●) and growth (○) by *Rhodomicrobium*.


C_2H_4 FORMED (µmole/mg/min x10^{-3})
0
2
4
6
8
10
TIME (hr)
0
4
8
16
20
24
28
32
36
DARK
OD_{680}
.1
.2
.3
.4
.6

1971b). The continuation of ethylene production at a lower rate, after the light was turned off as is shown in Figure 35 (arrow) indicates that acetylene reduction in Rhodomicrobium was a dark reaction and not related to the light-dependent process of ATP production and mobilization of reducing power (Arnon et al., 1961) which were necessary for acetylene reduction. Dark nitrogen fixation by light-grown cells of Rhodospirillum rubrum (Schick, 1971a) and Chloropseudomonas ethylicum (Evans and Smith, 1971) has been reported. Schick (1971a) found that nitrogen fixation continued but at a lower rate for 20 min after the light was turned off. The low but significant rate of acetylene reduction in the dark reported here probably was due to the accumulation of ATP, reducing power, and early products of carbon metabolism while in the light but which were exhausted after the cells were subjected to darkness for one hour (Fig. 35). Acetylene reduction resumed again when the light was turned on, however, the rates of reduction were much lower than those before the light was turned off. This appears to be due not to the dark period (4 hr) but to the older age of the culture. This explanation is supported by the growth and acetylene reduction data shown in Figure 36.

The effect of polymorphism on the acetylene reduction was studied in a culture inoculated with cells

Figure 36. Rates of acetylene reduction (●) and growth (○) observed in a *Rhodomicrobium* culture.


C_2H_4 (µmole/mg/hr x10^{-2})
0
8
16
24
32
TIME (hr)
0
4
12
20
28
36
OD_{680}
.1
.2
.5
1

harvested by centrifuging from a stationary-phase culture previously grown with 0.10% of ammonium chloride in the medium. The culture was continuously flushed with repurified nitrogen gas. Samples assayed for acetylene reduction were taken at the times indicated. The growth was slower in this experiment when the flushing rate exceeded 100ml/min, presumably because of the flushing out of newly formed CO_2 which is necessary for acetate photometabolism (Stanier et al., 1959). The acetylene reduction curve in Figure 36 indicates three distinct stages. The first stage (0-10 hr) which was the initial lag in acetylene reduction. This could be due to a number of factors: (1) classical growth lag in new medium (Lamanna and Mallette, 1965), (2) effect of dissolved oxygen in newly prepared medium, (3) large endogenous nitrogen pool in inoculum which suppressed nitrogenase synthesis, (4) or the composition of the polymorphic population (relative percentages of cysts, motile, and non-motile vegetative cells). The first three factors are unlikely. Growth in new media (Fig. 35) and induction of acetylene reduction using a log phase inoculum (Fig. 41) occurred only in a short time after transfer. The preparation of medium was done under a strictly anaerobic atmosphere. However, microscopic observation supported explanation (4) since the culture contained about 55% non-motile branched cells, 45% cysts and very small number of motile vegetative cells in stage one (0-10 hr). When the acetylene reduction rate became more significant in stage 2 (10-20 hr), the motile cells and young branched network (which had vegetative cells without PHB granules) became more dominant and the cyst population diminished. At the end of stage two (approximately at 20 hr) the population of the culture was comprised of approximately 70% branched cells and 30% motile cells with a small number of cysts.

In stage 3 (> 20 hr), the percentage of motile cells decreased and cyst forms increased. Angular structures could readily be seen at the end of phase 3. Branched cells began to accumulate visible granules of PHB at the beginning of the stationary phase. Thus, acetylene reduction activity appeared to correlate with the presence of motile cells and young network and it diminished when cells were preparing to form cysts. The small fluctuation of acetylene reduction rates in phase 3 appears to be due to experimental errors. However, the acetylene reducing system may be suppressed by an increase of nitrogen in the internal nitrogen pool as was observed in blue-green algae by Jewell and Kulasooriya (1970) or by the extracellular organic nitrogen excreted or released by lysing cells (Okuda _et al._, 1961a) or a combination of these three.

Literature concerning nitrogen fixation or acetylene reduction by spores of *Clostridium* or cysts of *Azotobacter* is nil. Recently Hitchins (personal communication) found that cysts of *A. vinelandii* reduced only acetylene upon germination. It is expected that this observation also holds for cysts of *Rhodomicrobium* even though the conditions for cyst formation in the two organisms are quite different. Cyst formation in *Azotobacter* is stimulated by β-hydroxybutyrate, crotonate, or butanol (Lin and Sadoff, 1968; Hitchin and Sadoff, 1970) while cyst formation in *Rhodomicrobium* was apparently triggered by nutrient exhaustion.

The optimum light intensity for *Rhodomicrobium* growth was 300-400 ft-c, regardless of the temperature tested as shown in Table 18. The growth rate was the same regardless of whether ammonium or atmospheric nitrogen was the nitrogen source since the generation time of a culture supplied with 0.1% (w/v) of ammonium chloride was 10.50 hr as compared to 10.66-12.56 hr for cultures in nitrogen-free medium (Table 18, column 4). A similar generation time of 10.50 hr was reported by Trentini (1967) who grew *Rhodomicrobium vannielii* in a medium containing bicarbonate, lactate, and ammonium sulfate under 700 ft-c light. However, the best growth rate (5.10 hr) was obtained when he supplied cultures with a mixture of 10 vitamins and aspartic acid.

Table 18. Effect of light and temperature on growth and rate of acetylene reduction by *Rhodomicrobium*

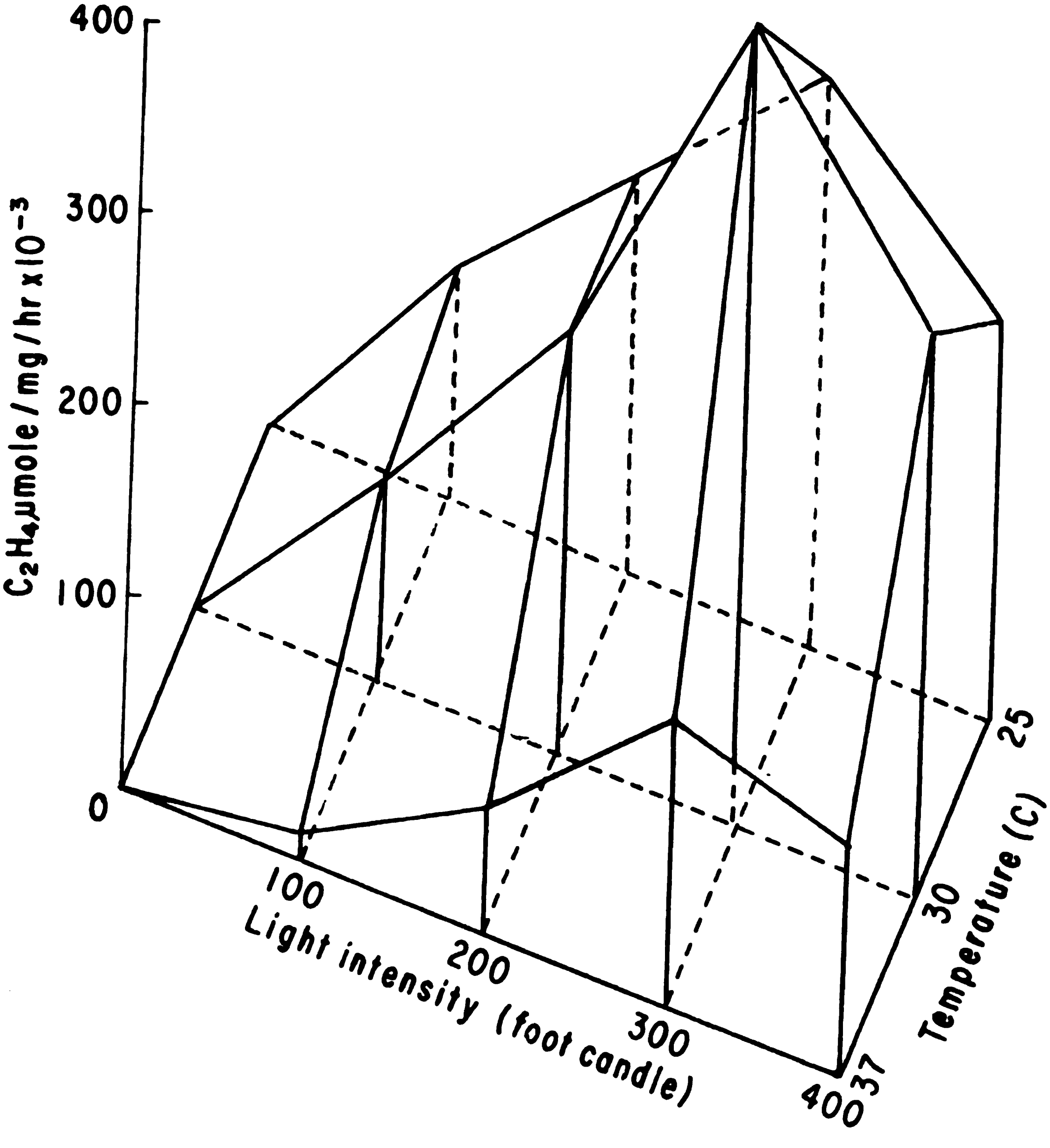
Temperature (C)	Light (ft-c)	Final Dry Weight (mg)	G* (hr)	C_2H_4 mμmole/sample/ hr	Rate mμmole/mg dry weight/hr
25	400	1.55	10.66	396.0	255.0
	300	1.22	11.23	330.0	300.0
	200	1.75	23.92	360.0	206.0
	100	1.37	33.12	172.0	125.5
30	400	1.36	12.56	400.0	294.0
	300	0.90	11.63	340.0	378.0
	200	0.90	30.49	202.0	224.4
	100	0.55	41.15	60.0	109.0
37	400	1.70	11.07	204.7	120.0
	300	1.55	12.89	230.0	148.0
	200	1.37	15.29	84.0	61.3
	100	0.95	83.33	9.0	10.0

*Generation time

Note: G was calculated during period immediately prior to acetylene reduction assay

The rates of acetylene reduction per cell dry weight are shown in Table 18 and Figure 37. It appears that under these conditions, the rate of acetylene reduction was highest under 300 ft-c at 30 C. Acetylene reduction did not correlate with final cell yield (Table 18, column 5 and column 3). At 37 C under 400 ft-c fast growth and a high dry weight did not result in a high acetylene reduction rate (120 mμmole C_2H_4 per mg dry wt per hr at 37 C and 400 ft-c as compared to 206 mμmole C_2H_4 per mg dry wt per hr at 25 C under 200 ft-c. The self-shadow regulation is unlikely when all final yield and reduction rates are considered (Table 18, columns 3 and 6). Larger filaments were observed at 37 C under 300-400 ft-c (as compared to those under the same light conditions at lower temperatures) which could account for the low rate of reduction since Conti and Hirsch (1965) and Morita and Conti (1963) observed that the stalks of *Rhodomicrobium* had little or no bacterial chlorophyll. It may follow that stalks were not active in acetylene reduction hence they could cause the inconsistency between dry weight and acetylene reduction. The age of the culture at the time the assay was done could also be important because of the narrow optimum for acetylene reduction as shown in Figure 36. The formation of abnormal stalks which were nearly as wide as the mature cells reported by Trentini and Starr

Figure 37. Effect of light and temperature on the rate of acetylene reduction by *Rhodomicrobium*.


400
300
200
100
0
C_2H_4 µmole / mg / hr x 10^{-3}
100
200
300
400
Light intensity (foot candle)
25
30
37
Temperature (C)

(1967) at 37 C was not found in the present study; however, filaments at 37 C were a little larger than those formed at lower temperatures.

All previously studied nitrogen-fixing organisms use N_2 only if there is no other utilizable nitrogen source present (Kamen and Gest, 1949; Gest et al., 1950; Ormerod et al., 1961; Sorger, 1969; and Schick, 1971a). The effect of combined nitrogen on acetylene reduction by Rhodomicrobium is shown in Figures 38, 39, and 40. The reduction was suppressed by as little as 0.0005% (w/v) of combined nitrogen; however, the effective suppression time was different depending upon the nitrogen source and the cell concentration: (a) Yeast extract (Fig. 38): 0.0005% (w/v) of yeast extract did not suppress the acetylene reduction activity of the whole cells. The initial high quantity of ethylene produced in samples containing 0.005% and 0.05% yeast extract was probably a lag effect due to stimulation by the yeast extract. (b) Sodium nitrate (Fig. 39): 0.0005% of sodium nitrate suppressed nitrogenase activity for only 20 min before the reduction rate returned to values similar to the control. The addition of 0.05% of nitrate inactivated nitrogenase system only for 60 min. (c) Ammonium chloride (Fig. 40): 0.0005% of ammonium chloride suppressed acetylene reduction for only 5-10 min. However, concentrations of 0.005% and 0.05% of ammonium chloride inhibited reduction for 80 and 140 min respectively.

Figure 38. Effect of different concentrations of yeast extract on the rate of acetylene reduction by *Rhodomicrobium*.

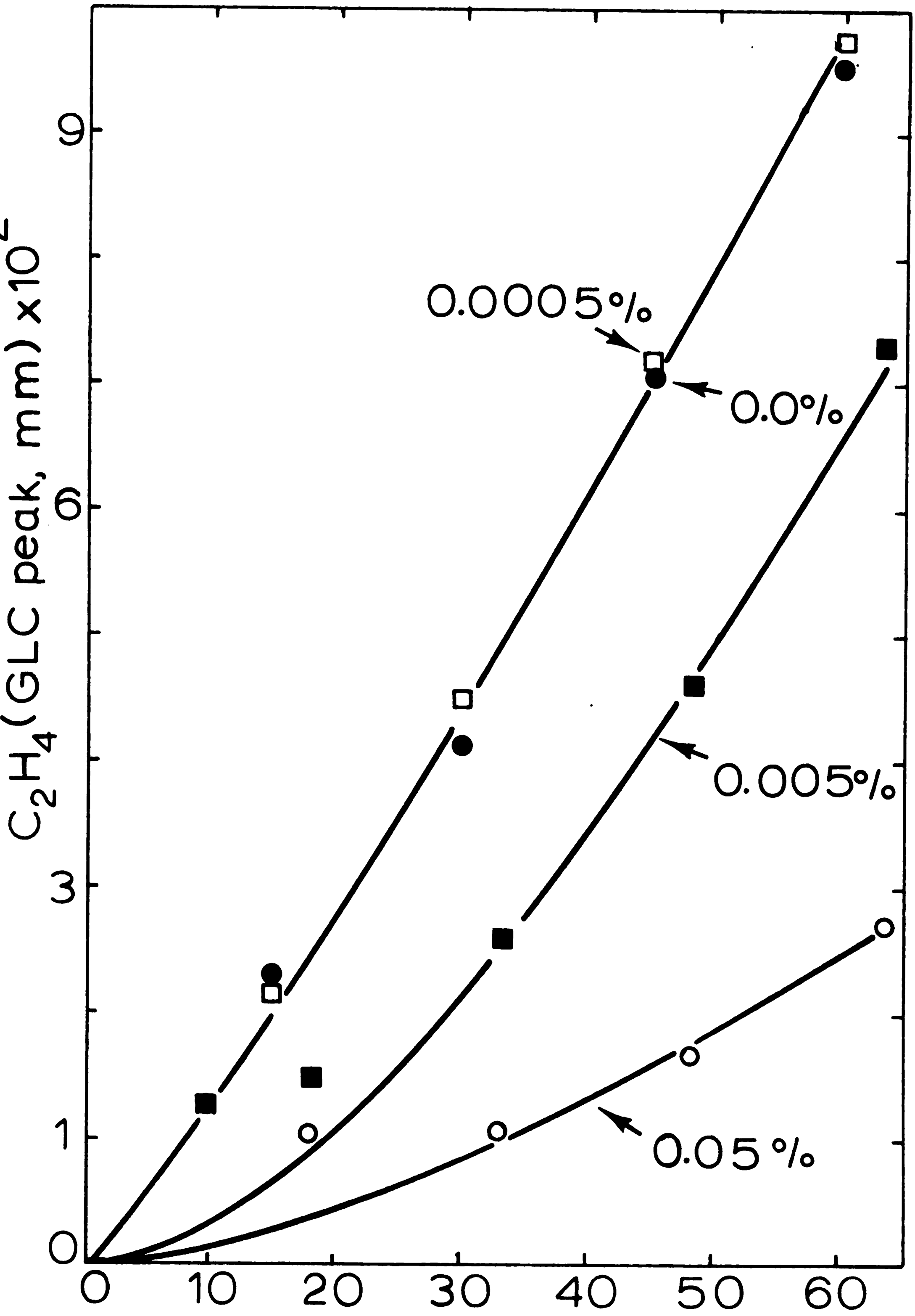

C_2H_4(GLC peak, mm) x10^2
TIME (min)
0
1
3
6
9
0
10
20
30
40
50
60
0.0005%
0.0%
0.005%
0.05%

Figure 39. Effect of different concentrations of sodium nitrate on the rate of acetylene reduction by *Rhodomicrobium*.

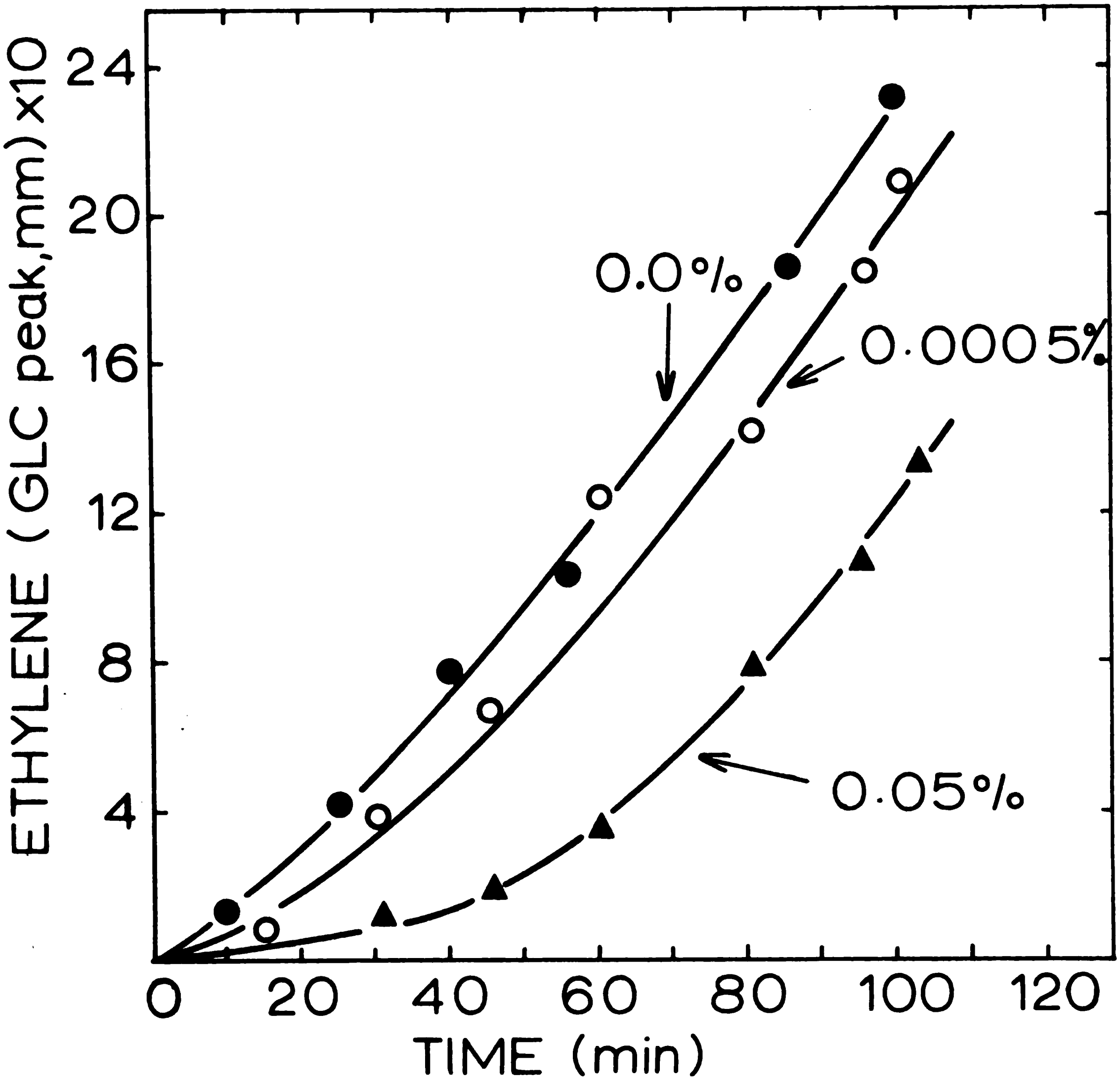
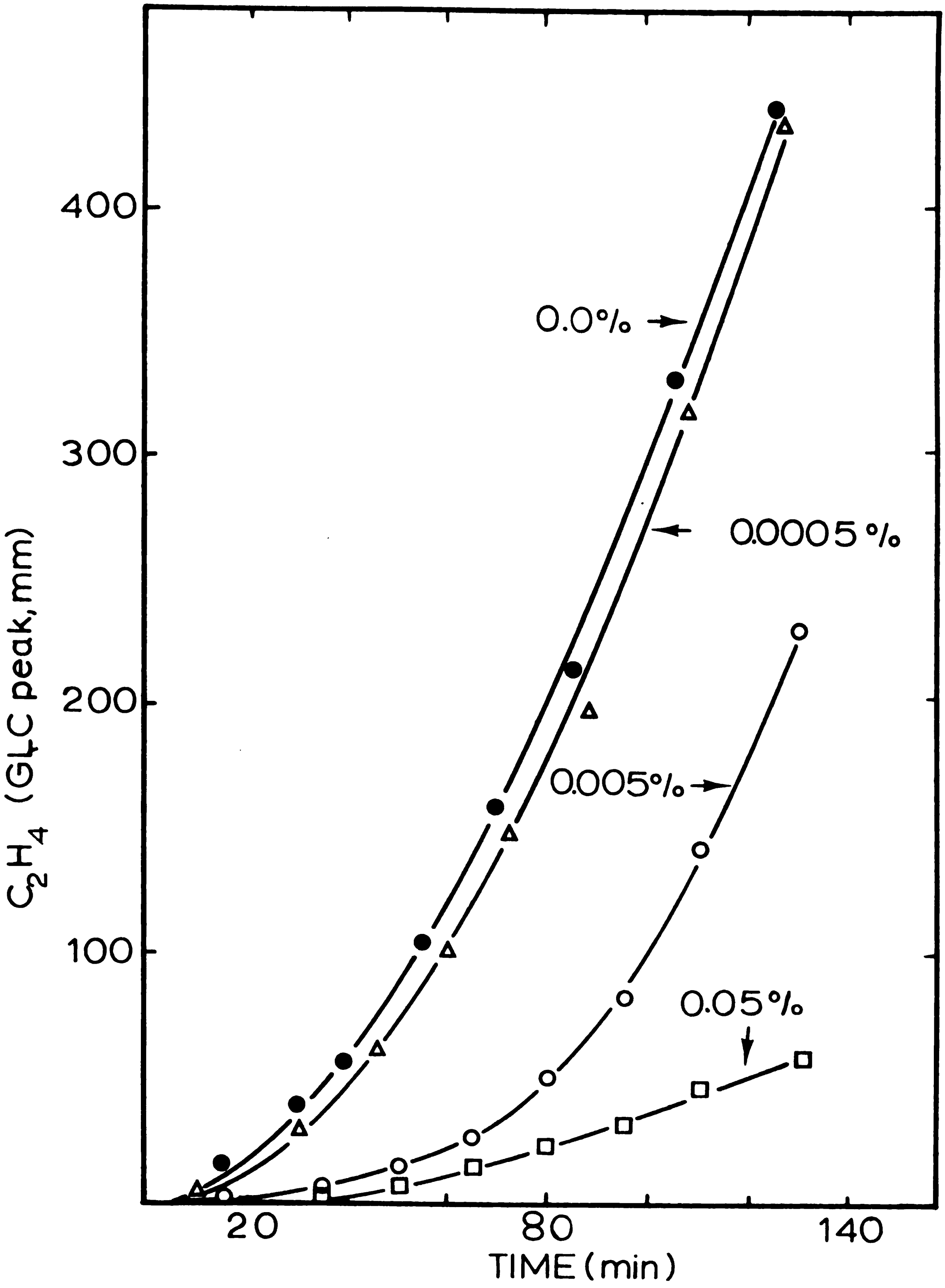

ETHYLENE (GLC peak, mm) x10
TIME (min)
0
20
40
60
80
100
120
4
8
12
16
20
24
0.0%
0.0005%
0.05%

Figure 40. Effect of different concentrations of ammonium chloride on the rate of acetylene reduction by <u>Rhodomicrobium</u>.

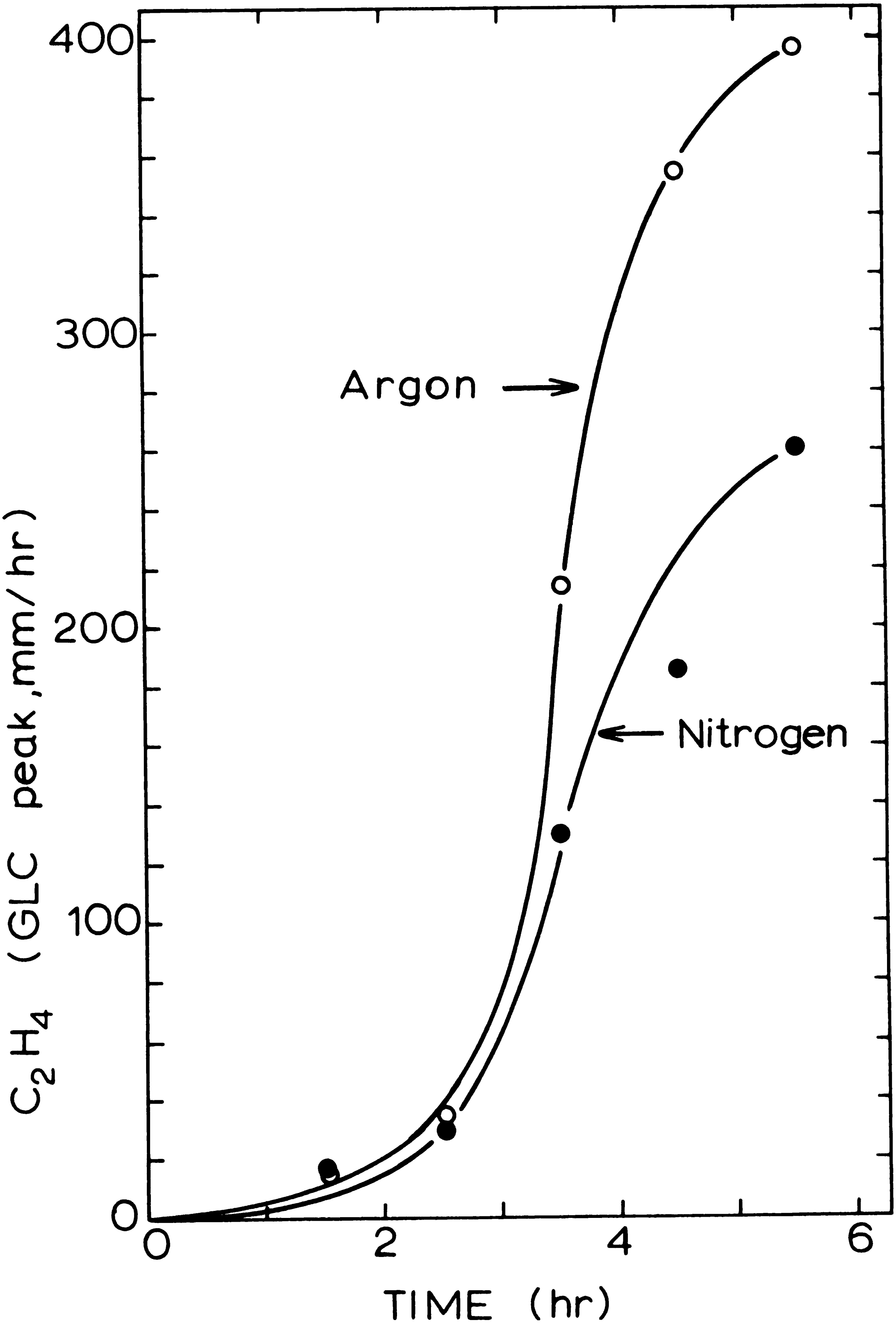

400
300
200
100
20
80
140
TIME (min)
C_2H_4 (GLC peak, mm)
0.0%
0.0005%
0.005%
0.05%

All concentrations of combined nitrogen tested have failed to stop completely the activity of nitrogenase system. These results further support the idea (see Chapter VI) that combined nitrogen did not act directly (allosteric control) on the nitrogenase system but rather the cells divert the limited energy and reducing power to the other processes such as ammonium assimilation which require lesser quantities of energy and reductant for synthesis of cellular materials. The shift of metabolic process concerning nitrogen metabolism would not stop completely the flow of available electrons and energy to the already synthesized nitrogenase and nitrogen fixation would still proceed, but at a lesser rate until nitrogenase was diluted by cell division.

The immediate suppression of acetylene reduction by nitrate as is shown in Figure 39 seems contrary to the observations by Taniguchi and Kamen (1963) in which *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* did not use nitrate as a nitrogen source until after being grown in the presence of nitrate as the only available nitrogen source. Since the metabolism of nitrate by *Rhodomicrobium* was not studied in detail, any conclusion drawn from this limited information would be premature.

Figure 41 shows the induction of acetylene reducing activity when cells grown on combined nitrogen were transferred to nitrogen-free medium and incubated

Figure 41. The induction of nitrogenase synthesis by argon or nitrogenase in Rhodomicrobium (Ethylene produced by Rhodomicrobium cells following transfer from a combined-nitrogen medium to a nitrogen-free medium—grown cells of Rhodomicrobium was incubated under an argon or nitrogen atmosphere).


400
300
200
100
0
0
2
4
6
TIME (hr)
C_2H_4 (GLC peak, mm/hr)
Argon
Nitrogen

under nitrogen or argon gas. The rate of acetylene reduction in an argon atmosphere was much higher than that in the sample in the nitrogen atmosphere. Each point was the average of three samples. When the experiment was repeated using a helium atmosphere, the results were similar to data for the argon atmosphere. The high nitrogenase synthesis under argon and helium was also observed by Nelson _et_ _al_. (1971) with _Anabaena_ sp. which did not grow during the experimental period because of the lack of nitrogen (argon gas contained less than 5 ppm N_2 by volume). The specifications for the argon used in this experiment indicated no more than 10 ppm N_2 was present. Thus it is unlikely that N_2 was the specific inducer for nitrogenase synthesis, however, the cell apparently develops a greater nitrogen reducing ability under conditions of nitrogen starvation.

Table 19 shows the effects of light quality on the acetylene reduction by _Rhodomicrobium_ grown under tungsten white light but assayed under colored light. Since the yellow cellophane transmitted most of the wavelengths which are selectively absorbed by bacterial photosynthetic pigments, its high rate of acetylene reduction is predictable. The effect was pronounced, however, with red and blue light, which absorbed, respectively, all and a portion of wavelengths available to the existing carotenoids. However, when _Rhodomicrobium_ was grown

Table 19. Effect of light quality on acetylene reduction by <u>Rhodomicrobium</u>*

Light Conditions	C_2H_4 Formed/ Sample/hr (GLC peak, mm)
White	115.70
Red	94.00
Yellow (amber)	123.50
Light blue	94.25

*Culture grown at 30 C under 300 ft-c continuous white tungsten light. Samples were wrapped with corresponding colored cellophane papers when incubated with acetylene under the same light and temperature as grown. Each value is the average of duplicates.

in cellophane-wrapped cultures, yellow light supported growth as well as did white light, but the highest yield was obtained under blue and red light (Table 20, column 2).

It is necessary to note that the results of these experiments only indicate the qualitative effects of light quality on growth and acetylene reduction by *Rhodomicrobium*, because the amount of light received by the cultures was not the same after going through colored cellophane papers.

5. *Conclusion*. From information collected in this part of the study, it can be concluded that: (1) Several isolates of *Rhodomicrobium* and *Rhodopseudomonas* can grow on numerous organic carbon compounds as the sole carbon source in the presence or absence of combined nitrogen. (2) The acetylene reduction activity of whole cells was suppressed by utilizable combined nitrogen. (3) *Rhodomicrobium* had the ability to synthesize nitrogenase in the absence of combined nitrogen under N_2, argon, or helium atmosphere. (4) Elimination of certain light wavelengths affected the rate of acetylene reduction. (5) *Rhodomicrobium* appeared to undergo a change of form (life cycle) that under the laboratory conditions, can be depicted as follows (page 205). This "life cycle" was based only on microscopic observation and yet to be proved by further studies.

Table 20. Effect of wavelength on growth and acetylene reduction by Rhodomicrobium*

Color Paper	Final Dry Weight (mg)	C_2H_4 Formed (mμmole/mg dry weight/hr)
White	1.90	134.20
Red	1.90	192.20
Yellow (amber)	1.85	113.80
Light blue	1.70	200.00

*Cultures were started at the same dry weight per volume, incubated at 28 C under 300 ft-c continuous white tungsten light and correspondent colored cellophane papers. Acetylene reduction assay was done under the same conditions as grown. Each value is the average of duplicates.

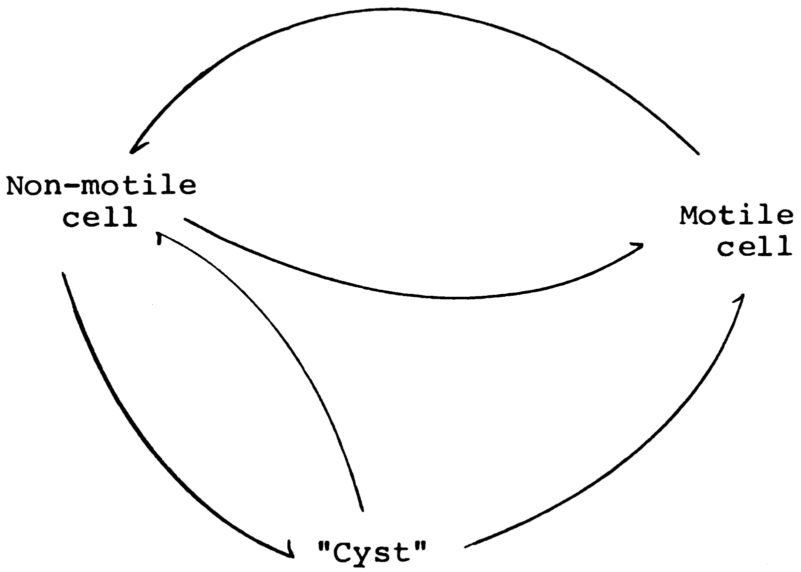


Figure 42. Suggested life cycle of *Rhodomicrobium*.

IX. PHOTOMETABOLISM BY NATURAL POPULATIONS OF PHOTOSYNTHETIC BACTERIA

A. Introduction

The function of natural populations of photosynthetic bacteria in the environment has been ignored, although they were discovered in the nineteenth century (Kondrat'eva, 1963). Some of the reasons for this lack of progress are: (1) they are difficult to work with *in situ* due to the lack of portable instrumentation to provide a thoroughly anaerobic system, (2) the most prevalent of these organisms in nature have not been grown in pure culture.

The purpose of this study was to gain some insight about the activity of natural populations of photosynthetic bacteria.

B. Materials and Methods

Samples for the photosynthetic pigment study were taken at station A (10/7/71) with a 3-l Van Dorn sampler, stored in 1-l brown polyethylene bottles and brought to the laboratory immediately. The qualitative extraction of pigments was done as follows. Approximately 100 ml water sample from 2 m and 50 ml from 3 m, 4 m, and 5 m were collected on Millipore filters (GS 0.22 µ) under low vacuum (about 1/3 atm). The filters were then sonicated for 10 sec at maximum power (3/8" diameter tip, Biosonik III, Bronwill Scientific, Inc., Rochester, N.Y.) in a 7:2 (v/v) acetone methanol mixture (Cohen-Bazire *et al.*, 1957). Sonicated samples were centrifuged at 10,000 xg for 10 min and the supernatant was scanned in a 1-cm cuvette with a scanning spectrophotometer (Model DB-G, Beckman Instruments, Inc., Fullerton, Calif.). The extraction lasted about 20 min.

The *in situ* assimilation of acetate was measured as follows. Water samples from various depths were collected by a 3-l Van Dorn sampler and transferred to a 500-ml flask which was continuously flushed with argon. Ten ml of the sample was pipetted into each of two 26-ml clear serum bottles which were continuously flushed with argon (Fig. 17, step 6). A one-half ml aliquot of a stock solution of $^{14}C_1$-acetate (made to specific activity of 1 µc per 100 µg per ml—New England Nuclear, Boston, Mass.) was injected into each bottle. The bottles were capped and incubated at the original depth for 0.5 hr. Metabolic activity was stopped by adding 0.2 ml of Lugol's solution. Samples were filtered onto Millipore filters (GS 0.22 µ) under low vacuum. Adsorption was corrected for by subtracting the radioactivity found in filters which were from samples which had Lugol's solution added immediately after the acetate addition. Filters were dried under low vacuum in a desiccator overnight then dissolved in 19 ml of Bray's cocktail containing 4% cab-o-sil. (Bray's modified cocktail contained 60 g Naphthalene, 4 g PPO [2,5-Diphenyloxazole], 200 mg Dimethyl POPOP 1,4 bis-[2-(4-methyl-5-phenyloxazolyl)]-Benzen, 100 ml Methanol, 20 ml Ethylene glycol and made to 1 l with p-dioxane). The samples were counted in a Tri-carb liquid scintillation spectrometer (Model 3310, Packard Instrument, Inc., Downers Grove, Ill.).

Laboratory studies of photodegradation of acetate by the natural population of photosynthetic bacteria was done as follows. Water was pumped at night into a 5-gallon bottle which was flushed with excess lake water to displace residual oxygen, capped, and brought to the laboratory. Ten ml duplicates of the samples were incubated with 100 μg of uniformly labeled plus unlabeled acetate (New England Nuclear, Boston, Mass.). The samples were incubated for 1 hr under light intensities and temperatures indicated, in a constant temperature aquarium described in Chapter VIII. Duplicate bottles were incubated in the dark at each temperature as the controls. At the end of the incubation, the bacteria were killed with 0.2 ml of 2% $HgCl_2$. The pH of the sample was adjusted to 11 with 1 N NaOH to trap $^{14}CO_2$ in solution. After two hours the samples were transferred to 125-ml flasks which were stoppered with rubber stoppers through which a 0.8 X 12 cm glass tubes penetrated. The outside end of the glass tubes was capped with a serum cap; a two-ml plastic cup containing 1 ml of 1 N NaOH was hung near the other end of the glass tube which was inside the flask and above the liquid level. One ml of 1 N HCl was injected into the flask to lower the pH of the sample to below 3. The flasks were gently shaken at room temperature for 10 hr. One-half ml of the NaOH from the plastic cup was transferred to 19 ml of Bray's solution containing 4% Cab-O-Sil and counted. Errors due to photodegradation by high light intensity or volitalization of acetate accounted for less than 0.5% of the radioactivity trapped.

In situ acetylene reduction by anaerobic water was assayed by filling 120-ml clear serum bottles with water taken from 4 and 5 m by a Van Dorn sampler. Excess water was flushed through the bottle to assure the removal of oxygen. Each bottle was capped with serum stoppers which were pierced by a 18 G 1 1/2" needle to release the small amount of air trapped in the inner rim of the stopper. This needle was then connected to a 10-ml plastic syringe, and a second needle which was connected to an argon source was inserted. Ten ml of water was withdrawn by the syringe with the volume being immediately filled with argon. Both needles were then removed. One ml of argon from the prepared bottles was taken out and replaced by 1 ml of acetylene. Bottles were suspended at the original depths for 2 hr after which the metabolic activity was stopped by adding 0.2 ml of 2% $HgCl_2$.

C. Results and Discussion

During the stratification period, Wintergreen Lake water was divided into three different regions: aerobic zone 0-3 m, intermediate zone 3 m, and anaerobic zone 4 m-bottom. *Thiopedia* occupied the oxidized-reduced intermediate metalimnion (3-3.5 m) and the green sulfur bacteria were found from 3.0 m to near the bottom.

The population of photosynthetic bacteria was estimated in the summer of 1970 by using the inverted microscope technique. The population of *Thiopedia* was about 10^5-10^7 plates/ml, and that of green sulfur bacteria between 10^4-10^6 cells/ml. The green sulfur bacteria (*Chlathrochloris* sp. and *Prosthecochloris* sp., but not *Chromatium*) developed in small clumps; therefore the above figure for the green sulfur bacterial population was only a rough estimation. Caldwell (1971 unpub. MSU) estimated that the green sulfur bacteria accounted for 90% of the total bacterial population in the region between 4-5 m.

Figure 43 shows the absorption spectra of pigments extracted by the acetone-methanol mixture. The maximum absorption peak at 660 mμ was due to the chlorophyll a of blue-green algae. The maximum absorption peak at 650 mμ was characteristic of chlorobium chlorophyll (Clayton, 1963). The absorption peak at 765 mμ corresponded to that of bacteriochlorophyll (Clayton, 1963) probably from Thiopedia.

The low absorption peak of the 2 m sample was probably due to the short duration of the extraction.

The amount of bacteriochlorophyll extracted was surprisingly low (Fig. 43, 3 m sample) although the population of Thiopedia was large enough to give the water from 3 m a pink color. Perhaps Thiopedia relied on their carotenoids which are photosynthetically active (Pfennig, 1967) as their principal light trapping pigments. No attempt, however, was made to extract the carotenoids.

The amount of chlorobium chlorophyll (660 mμ peak) appeared high although its presence at 3 m was visually masked by the pink color of Thiopedia. Figure 44 shows the relative acetate uptake by natural bacterial populations assayed in situ. The maximum uptake at 3 m corresponded with the presence of a dominate population of Thiopedia. The acetate uptake by dark and light bottles was similar. The possible explanation for the

Figure 43. Absorption spectra of photosynthetic pigments from natural populations extracted by a mixture of acetone and methanol (7:2, v/v). The figure shows absorption maxima of chl *a* (660 mμ of blue-green algae) and those of BChl (765 mμ, *Thiopedia*) and chlorobium Chl (650 mμ, green sulfur bacteria). Samples were taken on 10/7/71.

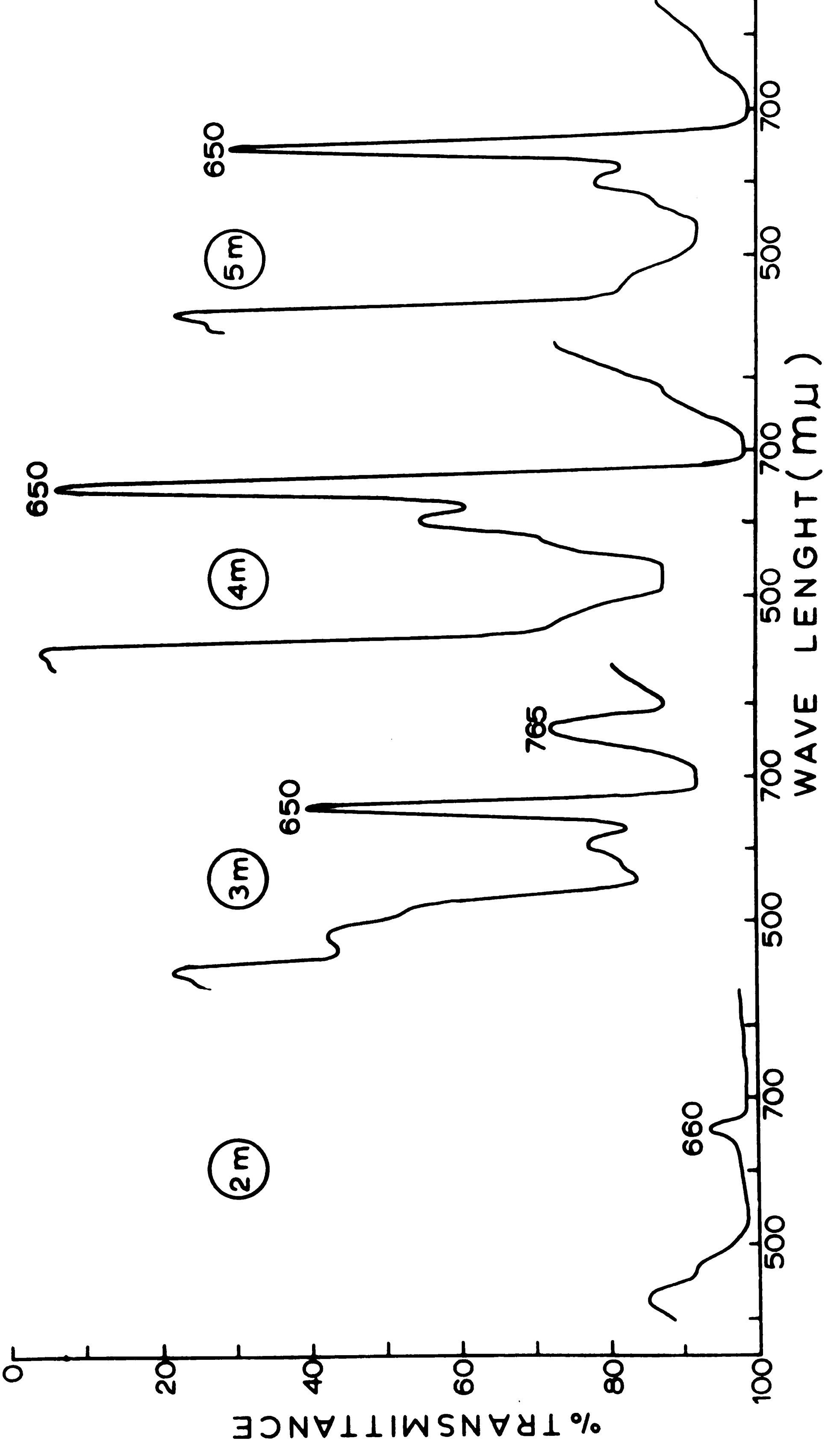
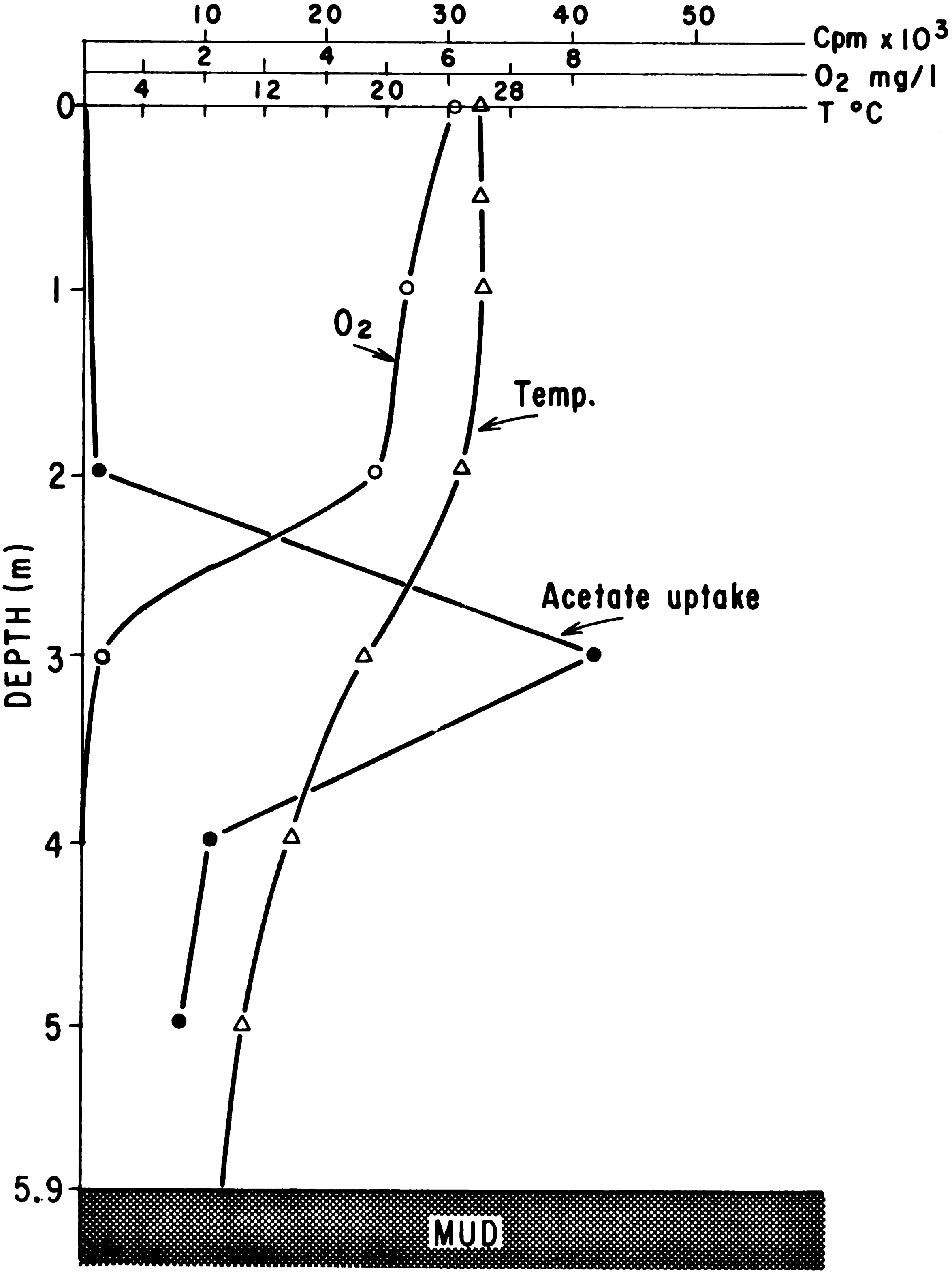

% TRANSMITTANCE
0
20
40
60
80
100
WAVE LENGHT (mμ)
2m
3m
4m
5m
500
700
660
650
765

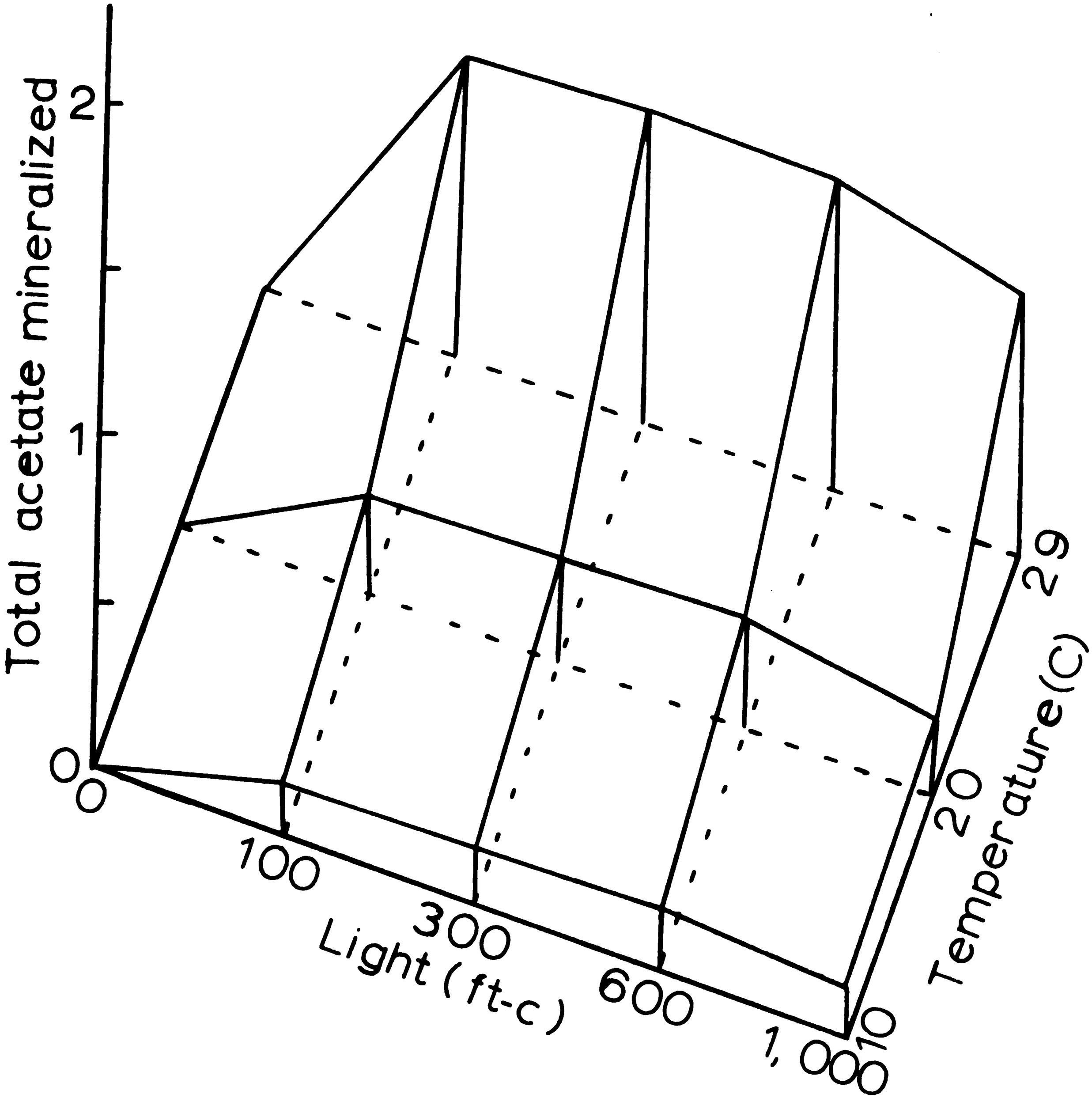
Figure 44. $^{14}C_1$-Acetate assimilated in 30 min by 10 ml of the natural microbial population (10^5 cpm = approx. 1.6 μg acetate). The oxygen and temperature profiles on the day of the experiment (3/7/71) are also shown.


10
20
30
40
50
Cpm x 10³
2
4
6
8
O₂ mg/l
4
12
20
28
T °C
0
1
2
3
4
5
5.9
DEPTH (m)
O₂
Temp.
Acetate uptake
MUD

absence of a light effect are: (1) photosynthetic bacteria assimilated acetate in the dark (Uffen and Wolfe, 1970), (2) organisms other than photosynthetic bacteria were responsible for the uptake, (3) reserve ATP pools were sufficient for a short dark assimilation of acetate (incubation was 0.5 hr). No attempt was made *in situ* to verify any of these hypotheses. However, experimental results shown in Figure 45 indicate that mineralization of assimilated acetate by a natural population (dominated by *Thiopedia*) assayed in the laboratory was light dependent. Figure 45 shows the rate of photodegradation of acetate to CO_2 by 10 ml of photosynthetic bacteria per hr. The highest degradation occurred at 100-600 ft-c and at 29 C where trapped CO_2 represented 2.4% of the added acetate. Dark degradation at best accounted for about 40% of the total degradation. The considerable dark CO_2 evolution could be due to the activity of methane-forming bacteria which also metabolize the fermentative end-products to CO_2 (cf. Toerien and Hattingh, 1969).

Various representatives of photosynthetic bacteria have been shown to grow effectively utilizing light and fatty acids or a variety of other fermentative end-products (Van Niel, 1931-1971; Muller, 1933; Stanier *et al.*, 1959; Thiele, 1968; Pfenning, 1969; also see Chapter VIII) under laboratory conditions. Certain organic compounds were converted completely into cellular

Figure 45. Effect of light and temperature on mineralization of added acetate by a natural population of photosynthetic bacteria taken from 3.5 m in Wintergreen Lake on 10/8/71 (μg acetate degraded/ 10 ml sample/hr). The population was dominated by *Thiopedia* and green sulfur bacteria.


Total acetate mineralized
2
1
0
0
100
300
600
1,000
Light (ft-c)
10
20
29
Temperature (C)

material (Muller, 1933) while very little exogenous CO_2 was consumed (Losada et al., 1960). There is no reason why this photosynthetic mode of life did not occur in the hypolimnion of Wintergreen Lake where light was available (Fig. 4). The high methane concentration in the hypolimnion (Tiedje, unpub.) indicates that the fermentative end-products, which are substrates for the methane-forming bacteria and photosynthetic bacteria, were abundant in this region.

The processes of methane production in lakes are poorly understood although they are very important in the recycling of carbon. Information on these processes has accumulated mostly through studies in rumen (Hungate, 1966) and sewage digesters and with pure cultures (Siebert and Hattingh, 1967; Hattingh et al., 1967; Toerien and Siebert, 1967; Toerien et al., 1968; Siebert et al., 1968; Toerien and Hattingh, 1969; Toerien, 1970). Methanogenic bacteria appear to utilize either H_2 and CO_2 or organic fermentative end-products, primarily acetate. Photosynthetic bacteria are known to utilize similar substrates as well as more complex organics. The present studies on the assimilation of organic compounds and mineralization of acetate suggest that the photosynthetic bacteria may be important in all steps of recycle of the organic carbon in natural water.

Several attempts to measure acetylene reduction by samples from 4-5 m gave negative results. This could be due to: (1) suppression of nitrogenase by high concentrations of combined nitrogen present in the hypolimnion, (2) inability to reproduce lake conditions in the assay bottle (particularly anaerobiosis).

REFERENCES

Abdullaev, D. A. 1969. The use of common duckweed as green food for chickens. Uzb. Viol. Zh. (Aka. Nauk Uzb. SSR, Ser. Biol) 13:42-43. (in Russ.).

Alexander, M. 1967. Introduction to soil microbiology. John Wiley & Sons, New York.

Allen, H. L. 1971. Primary productivity, chemo-organotrophy, and nutritional interactions of epiphytic algae and bacteria on macrophytes in the littoral of a lake. Ecol. Monogr. 41: 97-127.

Anderson, L. and R. C. Fuller. 1967. Photosynthesis in *Rhodospirillum rubrum*. II. Photoheterotrophic carbon dioxide fixation. Plant Physiol. 42: 491-496.

Arnon, D. I., M. Losada, M. Nozaki, and K. Tagawa. 1961. Photoproduction of hydrogen, photofixation of nitrogen and a unified concept of photosynthesis. Nature (London) 190: 601-606.

Barker, H. A. 1956. Bacterial fermentations. John Wiley & Sons, New York.

Billaud, V. A. 1968. Nitrogen fixation and the utilisation of other inorganic nitrogen sources in a subarctic lake. J. Fish. Res. Bd. Can. 25: 2101-2110.

Bogorad, L. 1962. Chlorophylls, pp. 385-408. In R. A. Lewin (ed.) Physiology and Biochemistry of Algae. Academic Press, New York.

Bottomley, W. B. 1919. The effect of nitrogen-fixing organisms and nucleic acid derivatives on plant growth. Proc. Roy. Soc. Ser. B. 91: 83-95.

Bremner, J. M. 1965. Total nitrogen, pp. 1149-1178. In C. A. Black, D. D. Evans, J. L. White, L. E. Ensminger and F. E. Clark (ed.) Methods of Soil Analysis, Agronomy 9, part 2. American Society of Agronomy, Publisher, Madison, Wisconsin.

Brezonik, P. L. and C. L. Harper. 1969. Nitrogen fixation in some anoxic lacustrine environments. Science 164: 1277-1279.

Brock, T. D. 1966. Principles of microbial ecology. Prentice-Hall, Englewood Cliffs, New Jersey.

Brody, M. and S. S. Brody. 1962. Light reactions in photosynthesis, pp. 3-23. In R. A. Lewin (ed.) Physiology and biochemistry of algae. Academic Press, New York.

Brown, A. L. 1971. Ecology of fresh water. Heinemann Educational Books Ltd., London.

Bryant, M. P. and I. M. Robinson. 1961. An improved non-selective culture medium for ruminal bacteria and its use in determining diurnal variation in members of bacteria in the rumen. J. Dairy Sci. 44: 1446-1456.

Caspers, H. 1964. Characteristics of hypertrophic lakes and canals in cities. Verh. int. Ver. Limnol. 15: 631-638.

Chandler, D. C. 1963. Michigan, pp. 95-116. In D. G. Frey (ed.) Limnology in North America. The University of Wisconsin Press, Madison, Wisconsin.

Clayton, R. K. 1963. Absorption spectra of photosynthetic bacteria and their chlorophylls, pp. 495-500. In H. Gest, A. San Pietro, and L. P. Vernon (ed.) Bacterial photosynthesis. The Antioch Press, Yellow Springs, Ohio.

Cohen-Bazire, G., W. R. Sistrom, and R. Y. Stanier. 1957. Kinetic studies of pigment synthesis by non-sulfur purple bacteria. J. Cell. Comp. Physiol. 49: 25-68.

________, R. Kunisawa, and N. Pfennig. 1969. Comparative study of the structure of gas vacuoles. J. Bacteriol. 100: 1049-1961.

Coler, R. A. and H. B. Gunner. 1970. Laboratory measure of an ecosystem response to a sustained stress. Appl. Microbiol. 19: 1009-1012.

Conti, S. F. and P. Hirsch. 1965. Biology of budding bacteria. III. Fine structure of *Rhodomicrobium* and *Hyphomicrobium* sp. J. Bacteriol. 89: 503-512.

Culver, D. A. and G. J. Brunskill. 1969. Fayetteville Green Lake, New York. V. Studies of primary production and zooplankton in a meromictic marl lake. Limnol. Oceanogr. 9: 862-873.

Danforth, W. F. 1962. Substrate assimilation and heterotrophy, pp. 99-123. In R. A. Lewin (ed.) Physiology and biochemistry of algae. Academic Press, New York.

Daubs, E. H. 1965. A monograph of Lemnaceae. Ill. Biol. Monogr. No. 34. The University of Illinois Press, Urbana, Illinois.

Delwich, C. C. 1970. The nitrogen cycle, pp. 71-80. In A Scientific American Book: The Biosphere. W. H. Freeman and Company, San Francisco.

Dilworth, M. 1966. Acetylene reduction by nitrogen-fixing preparation from *Clostridium pasteurianum*. Biochem. Biophys. Acta 127: 285-294.

Douglas, H. C. 1957. Hyphomicrobiales, Bergey's mannual of determination of bacteriology, 7th ed. The Williams & Wilkins Company. Baltimore.

________ and R. S. Wolfe. 1959. Motility of *Rhodomicrobium vannielii*. J. Bacteriol. 78: 597-598.

Drozd, J. and J. R. Postage. 1970. Interference by oxygen in the acetylene-reduction test for aerobic nitrogen-fixing bacteria. J. Gen. Microbiol. 60: 427-429.

Duchov, E. and H. C. Douglas. 1949. *Rhodomicrobium vannielii*, a new photoheterotrophic bacterium. J. Bacteriol. 58: 409-416.

Dugdale, R., V. Dugdale, J. Neess and J. Goering. 1959. Nitrogen fixation in lakes. Science 130: 859-860.

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

Dugdale, R. C., J. J. Goering, and J. H. Ryther. 1964. High nitrogen fixation rates in the Sargasso Sea and the Arabian Sea. Limnol. Oceanogr. 9: 507-510.

Edmonson, W. T. 1956. The relation of photosynthesis by photoplankton to light in lakes. Ecology 37: 161-174.

Eichmeier, A. H. Climate of Gull Lake, Michigan, Climatography of US No. 20-20. USCOMM, US Gov. Print. Off., Washington, D.C.

Eisenberg, M. A. 1953. The tricarboxylic acid cycle in *Rhodospirillum rubrum*. J. Biol. Chem. 203: 815-836.

Elleway, R. F., J. R. Sabine, and D. J. D. Nicholas. 1971. Acetylene reduction by rumen microflora. Arch. Mikrobiol. 76: 277-291.

Elsden, S. R. 1962. Photosynthesis and lithotrophic carbon dioxide fixation, pp. 1-40. In I. C. Gunsalus and R. Y. Stanier (ed.) The bacteria, vol. III: Biosynthesis. Academic Press, New York.

Evans, M. C. W., B. B. Buchanan, and D. I. Arnon. 1966. A new ferrodoxin-dependent carbon reduction cycle in a photosynthetic bacterium. Proc. Natl. Acad. Sci. U.S.A. 55: 928-934.

________ and R. V. Smith. 1971. Nitrogen fixation by the green photosynthetic bacterium *Chloropseudomonas ethylicum*. J. Gen. Microbiol. 65: 95-98.

Fay, P. 1965. Heterotrophy and nitrogen fixation in *Chlorogloea fritschii*. J. Gen. Microbiol. 39: 11-20.

________, W. D. P. Stewart, A. E. Walsby, and G. E. Fogg. 1968. Is the heterocyst the site of nitrogen fixation in blue-green algae? Nature (London) 220: 810-812.

Findenegg, I. 1964. Type of planktonic primary production in the lakes of the Eastern Alps as found by the radioactive carbon method. Verh. int. Ver. Limnol. 15: 352-359.

Fogg, G. E. 1952. The production of extracellular nitrogenous substances by a blue-green alga. Proc. Roy. Soc. Ser. B. 139: 372-379.

Fogg, G. E. and D. F. Westlake. 1955. The importance of extracellular products of algae in freshwater. Verl. int. Ver. Limnol. 15: 219-232.

________. 1962. Extracellular products, pp. 475-489. In R. A. Lewin (ed.) Physiology and biochemistry of algae. Academic Press, New York.

________. 1966. The extraproducts of algae. Oceanogr. Mar. Biol. Rev. 4: 195-212.

Foster, J. W. 1940. The role of organic substances in photosynthesis of purple bacteria. J. Gen. Physiol. 24: 123-134.

Gerloff, G. C., G. P. Fitzgerald, and F. Skoof. 1950. The isolation, purification, and culture of blue-green algae. Amer. J. Bot. 37: 216-218.

________ and G. Skoof. 1954. Cell contents of nitrogen and phosphorous as a measure of their availability for growth of *Microcystis aeruginosa*. Ecology 35: 348-353.

________ and F. Skoog. 1957. Nitrogen as limiting factor for the growth of *Microcystis aeruginosa* in southern Wisconsin lakes. Ecology 38: 556-561.

Gessner, F. 1948. The vertical distribution of phytoplankton and the thermocline. Ecology 29: 386-389.

________ and F. Pannier. 1958. Influence of oxygen tension on respiration of phytoplankton. Limnol. Oceanogr. 3: 478-480.

Gest, N., M. D. Kamen, and H. M. Bregoff. 1950. Studies on the metabolism of photosynthetic bacteria. V. Photoreduction of hydrogen and nitrogen fixation by *Rhodospirillum rubrum*. J. Biol. Chem. 182: 153-170.

Gibbs, M. 1962. Respiration, pp. 61-90. In R. A. Lewin (ed.) Physiology and biochemistry of algae. Academic Press, New York.

Glover, J., M. D. Kamen, and H. V. Genderen. 1952. Studies on the metabolism of photosynthetic bacteria. XII. Comparative light and dark metabolism of acetate and carbonate by *Rhodospirillum rubrum*. Arch. Biochem. Biophys. 35: 384-409.

Goering, J. J. and J. C. Neess. 1964. Nitrogen fixation in two Wisconsin lakes. Limnol. Oceanogr. 9: 535-539.

Goldman, C. R. 1960. Molybdenum as a factor limiting primary productivity in Castle Lake, California. Science 132: 1016-1017.

________. 1963. The measurement of primary productivity and limiting factors in fresh water with carbon-14. In M. S. Doty (ed.) Proc. of Conf. Primary Productivity Measurement, Marine and Fresh Water, 1961. U.S. Atomic Energy Commission TID-76, 33, 101-113.

________ and R. G. Wetzel. 1963. A study of the primary productivity of Clear Lake, Lake County, California. Ecology 44: 283-294.

________. 1964. Primary productivity and micronutrient limiting factors in some North American and New Zealand lakes. Verh. int. Ver. Limnol. 15: 365-374.

________. 1968. The use of absolute activity for eliminating serious errors in the measurement of primary production with C^{14}. J. Cons. Int. Explo. Mer. 32: 172-179.

________ and R. Armstrong. 1969. Primary productivity studies in Lake Tahoe, California. Vehr. int. Ver. Limnol. 17: 49-71.

________, E. Steemann Nielson, R. A. Vollenweider, and R. G. Wetzel. 1969. The ^{14}C light and dark bottle technique, pp. 70-73. In R. A. Vollenweider (ed.) A manual on methods for measuring primary production in aquatic environments. Blackwell Scientific Publication, Oxford.

Gorham, P. R., J. McLachlan, U. T. Hammer, and W. K. Kim. 1964. Isolation and culture of toxic strains of *Anabaena flos-aquae* (lyngb.) de Breb. Verh. int. Ver. Limnol. 15: 796-804.

Gorlenko, V. M. 1969. Spore formation in a budding photoheterotrophic bacterium. Microbiology 38: 126-134. (Engl. trans.).

Green, R. H. 1971. A multivariate statistical approach to the Hutchinsonian niche: bivalve molusks of Central Canada. Ecology 52: 543-556.

Hamilton, I. R., R. H. Burris, and P. W. Wilson. 1964. Hydrogenase and nitrogenase in a nitrogen fixing bacterium. Proc. Natl. Acad. Sci. U.S.A. 52: 637:641.

Hamilton, D. H. 1969. Nutrient limitation of summer phytoplankton growth in Cayuga Lake. Limnol. Oceanogr. 9: 579-590.

Hardy, R. W. F., R. D. Holsten, E. K. Jackson, and R. C. Burns. 1968. The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiol. 43: 1185-1207.

Hattingh, W. H. J., J. P. Kotze, P. G. Thiel, D. F. Toerien, and M. L. Siebert. 1967. Biological changes during the adaptation of an anaerobic digester to a synthetic substrate. Water Res. 1: 255-277.

Hillman, W. S. 1961. The Lemnacaea, or duckweeds. A review of the descriptive and experimental literature. Bot. Rev. 27: 221-287.

Hitchin, V. M. and H. L. Sadoff. 1970. Morphogenesis of cyst in *Azobacter vinelandii*. J. Bacteriol. 104: 492-498.

Hoare, D. S. and J. Gibson. 1964. Photoassimilation of acetate and the biosynthesis of amino acids by *Chlorobium thiosulphatophilum*. Biochem. J. 91: 546-559.

________ and S. L. Hoare. 1969. Hydrogen metabolism by *Rhodomicrobium vannelii*. J. Bacteriol. 100: 1124-1125.

Hobbie, J. E. and R. T. Wright. 1965. Bioassay with bacterial uptake kinetics: Glucose in fresh water. Limnol. Oceanogr. 10: 471-474.

Holm-Hansen, O. 1962. Assimilation of carbon dioxide, pp. 25-45. In R. A. Lewin (ed.) Physiology and biochemistry of algae. Academic Press, New York.

Horne, A. J. and G. E. Fogg. 1970. Nitrogen fixation in some English lakes. Proc. Roy. Soc. Ser. B. 175: 351-366.

Hrbáček, J. 1964. Contribution to the ecology of water-bloom-forming blue-green algae—*Aphanizomenon flos-aquae* and *Microcystis aeruginosa*. Verh. int. Ver. Limnol. 15: 837-846.

Hungate, R. E. 1950. The anaerobic mesophillic cellulolytic bacteria. Bacteriol. Rev. 14: 1-49.

________. 1966. The rumen and its microbes. Academic Press, New York.

Hurlbert, R. E. and J. Lascelles. 1963. Ribulose diphosphate carboxylase in Thiorhodaceae. J. Gen. Microbiol. 33: 445-458.

Hutchinson, G. E. 1957. A treatise on limnology, vol. 1. John Wiley & Sons, New York.

________. 1967. A treatise on limnology, vol. 2. John Wiley & Sons, New York.

________. 1970. The biosphere, pp. 3-11. In a scientific American Book: The Biosphere. W. H. Freeman and Company, San Francisco.

Hutner, S. H. 1946. Organic growth essentials of the aerobic nonsulfur photosynthetic bacteria. J. Bacteriol. 52: 213-221.

Jacob, D. L. 1947. An ecological life-history of Spirodela polyrhiza (greater duckweed) with emphasis on the turion phase. Ecol. Monogr. 17: 438-469.

Jewell, W. J. and S. A. Kulasooriya. 1970. The relation of acetylene reduction to heterocyst frequency in blue-green algae. J. Exp. Bot. 21: 874-880.

Kalf, J. 1971. Nutrient limiting factors in an arctic tundra pond. Ecology 52: 655-659.

Kamen, M. D. and H. Gest. 1949. Evidence for a nitrogenase system in the photosynthetic bacterium, Rhodospirillum rubrum. Science 109: 560.

Kaushik, M. and H. D. Kumar. 1970. The effect of light on growth and development of two nitrogen fixing blue-green algae. Arch. Mikrobiol. 74: 52-57.

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

Kondrat'eva, E. N. 1963. Photosynthetic bacteria. USAEC and Natl. Sci. Found., Washington, D.C. (Engl. trans.).

Kornberg, H. L. and J. Lascelles. 1960. The formation of isocitratase by Athiorhodaceae. J. Gen. Microbiol. 23: 511-517.

Kuhl, A. 1962. Inorganic phosphorous uptake and metabolism, pp. 211-229. In R. A. Lewin (ed.) Physiology and biochemistry of algae. Academic Press, New York.

Kuznetsov, S. I. 1956. Application of radioactive isotopes to the study of processes of photosynthesis and chemosynthesis in lakes. Proc. First Int. Conf. Peaceful Uses of Atomic Energy, Geneva, 1956. U.N., New York, pp. 368-376.

________. 1958. A study of the size of bacterial populations and of organic matter formation due to photosynthesis and chemosynthesis in water bodies of different types. Verh. int. Ver. Limnol. 13: 156-169.

________. 1968. Recent studies on the role of microorganisms in the cycling of substances in lakes. Limnol. Oceanogr. 13: 211-223.

Lamanna, C. and M. F. Mallette. 1965. Basic bacteriology, 3rd. ed. The Williams & Wilkins Company, Baltimore.

Leach, C. K. and N. G. Carr. 1970. Electron transport and oxidative phosphorylation in the blue-green alga _Anabaena_ _verabilis_. J. Gen. Microbiol. 64: 55-70.

Lin, L. P. and H. L. Sadoff. 1968. Encystment and polymer production by _Azobacter_ _vinelandii_ in the presence of β-hydroxybutyrate. J. Bacteriol. 95: 2336-2343.

Lindeman, R. L. 1942. The trophic-dynamic aspect of ecology. Ecology 23: 399-418.

Lindstrom, E. S., S. R. Tove, and P. W. Wilson. 1950. Nitrogen fixation by the green and purple sulfur bacteria. Science 112: 196-198.

________, S. M. Lewis, and M. J. Pinsky. 1951. Nitrogen fixation and hydrogenase in various bacterial species. J. Bacteriol. 61: 481-487.

Linville, K. W. and G. E. Smith. 1971. Nitrate content of soil cores from corn plots after repeated nitrogen fertilization. Soil Sci. 112: 249-255.

Losada, M., A. V. Trebst, S. Ogata, and D. I. Arnon. 1960. Equivalence of light and adenosine triphosphate in bacterial photosynthesis. Nature (London) 186: 753-760.

Lucas, C. E. 1947. The ecological effects of external metabolites. Biol. Rev. Cambridge Phil. Soc. 22: 270-295.

Macgregor, A. N. and D. E. Johnson. 1971. Capacity of desert algal crusts to fix atmospheric nitrogen. Soil Sci. Soc. Amer. Proc. 35: 834-835.

Mackereth, F. J. H. 1963. Some methods of water analysis for limnologists. Freshwater Biol. Stat. Scit. Pub. No. 21.

Manny, B. A. and C. A. S. Hall. 1969. Diurnal changes in stratification and dissolved oxygen in the surface waters of Lake Michigan. Proc. 12th Conf. Great Lakes Res., Int. Ass. Great Lakes Res., pp. 622-634.

________. 1971. Interactions of dissolved and particulate nitrogen in lake metabolism. Ph.D. Thesis, Michigan State University.

Marsh, H. V., J. M. Galmiche, and M. Gibbs. 1965. Effect of light on the tricarboxylic acid cycle in Scenedesmus. Plant Physiol. 40: 1013-1022.

McMahon, J. W. 1969. The annual and diurnal variations in the vertical distribution of acid-solute ferrous and total iron in a small dimictic lake. Limnol. Oceanogr. 14: 357-367.

Millar, C. E., L. M. Turk, and H. D. Foth. 1966. Fundamentals of soil science. John Wiley & Sons, New York.

Miller, M. C. 1971. Primary production and secretion by photoplankton and their role in carbon cycling in lakes. Ph.D. Thesis, Michigan State University.

Morita, S. and S. F. Conti. 1963. Localization and nature of the cytochromes of Rhodomicrobium vannielii. Arch. Biochem. Biophys. 100: 302-307.

Morrison, R. T. and R. N. Boyd. 1966. Organic chemistry, 2nd ed. Allyn and Bacon, Boston.

Muller, F. M. 1933. On the metabolism of the purple sulfur bacteria in organic media. Arch. Mikrobiol. 4: 131-255.

Murphy, L. S. and J. W. Gosch. 1970. Nitrate accumulation in Kansas ground water. Completion report to Office of Water Resources Research, Dept. Int. Kansas Water Resour. Res. Inst.

Murray, R. G. E. and H. C. Douglas. 1950. The reproductive mechanism of Rhodomicrobium vannielii and the accompanying nuclear changes. J. Bacteriol. 59: 157-167.

Muzafarov, A. M., T. Taubayev, and M. Abdiyev. 1968. The use of Lemna minor L. for poultry feeding. Uzb. Biol. Zh. (Aka. Nauk Uzb. SSR Ser. Biol.) 12: 42-43. (In Russ.).

Nakos, G. G. 1969. Cultural studies and effects of pesticides in nitrifying systems. Ph.D. Thesis. Michigan State University.

Nelson, A., R. Rippka, and R. Kunisawa. 1971. Heterocyst formation and nitrogenase synthesis in Anabaena sp. Arch. Mikrobiol. 76: 139-150.

Nesterov, A. I., I. N. Gogstov, and E. N. Kondrat'eva. 1965. Significance of light intensity for the utilization of various carbon compounds by photosynthesizing bacteria. Microbiology 30: 163-167. (Engl. trans.).

Newton, J. W. and P. W. Wilson. 1953. Nitrogen fixation and photoproduction of molecular hydrogen by Thiorhodaceae. Antonie van Leeuwenhoek; J. Microbiol. Serol. 19: 71-77.

Nishigaki, S. and M. Shioiri. 1959. Nitrogen cycles in the rice field soil. I. The effect of the blue-green algae on the nitrogen fixation of atmospheric nitrogen in the water-logged rice soils. Soil and Plant Food (Tokyo) 5: 36-39.

O'hEocha, C. 1962. Phycobilins, pp. 421-435. In R. A. Lewin (ed.) Physiology and biochemistry of algae. Academic Press, New York.

O'Kelley, J. C. 1968. Mineral nutrition of algae. Annu. Rev. Plant Physiol. 19: 89-112.

Odum, E. P. 1963. Ecology. Holt, Rinehart and Winston, New York.

________. 1971. Fundamentals of Ecology. Third ed. W. B. Saunders Company, Philadelphia.

Odum, H. T. 1956. Primary production in flowing waters. Limnol. Oceanogr. 1: 102-117.

________. 1957. Trophic structure and productivity of Silver Springs, Florida. Ecol. Monogr. 27: 55-112.

Ogawa, R. E. and J. F. Carr. 1969. The influence of nitrogen on heterocyst production in blue-green algae. Limnol. Oceanogr. 9: 342-351.

Okuda, A., M. Yamaguchi, and M. Kobayashi. 1960. Nitrogen-fixing microorganisms in paddy soils. V. Nitrogen fixation in mixed culture of photosynthetic bacteria (*Rhodopseudomonas capsulatus* species) with other heterotrophic bacteria. Soil Plant Food (Tokyo) 6: 35-39.

________, ________, and ________. 1961a. Nitrogen fixing microorganisms in paddy soils. VII. Products in mixed culture of *Rhodopseudomonas capsulatus* with *Azobacter vinelandii*. Soil Sci. Plant Nutr. (Tokyo) 7: 115-118.

________, ________, ________, and T. Katayama. 1961b. Nitrogen-fixing microorganisms in paddy soils: VIII. Nitrogen fixation in mixed culture of photosynthetic bacteria (*Rhodopseudomonas capsulatus* species) with other heterotrophic bacteria. Soil Sci. Plant Nutr. (Tokyo) 7: 146-151.

________ and M. Kobayashi. 1962. Nitrogen-fixing microorganisms in paddy soils. IX. Products in the mixed culture of *Rhodopseudomonas capsulatus* and *Azobacter vinelandii*. Soil Sci. Plant Nutr. (Tokyo) 8: 1-6.

Olivier, S. R. 1955. Contribution to the limnological knowledge of the Salada Grand Lagoon. 2. Plankton seasonal variations and some correlation with physical-chemical factors. Verl. int. Ver. Limnol. 12: 302-308.

Omerod, J. G. 1956. The use of radioactive carbon dioxide fixation in *Rhodospirillum rubrum*. Biochem. J. 64: 373-380.

Ormerod, J. G., K. S. Ormerod, and H. Gest. 1961. Light-dependent utilization of organic compounds and photoproduction of molecular hydrogen by photosynthetic bacteria, relationship with nitrogen metabolism. Arch. Biochem. Biophys. 94: 449-463.

Parsons, T. R. and J. D. H. Strickland. 1963. Discussion of spectrophotometric determination of marine-plant pigments, with revised equations for ascertaining chlorophylls and carotenoids. J. Mar. Res. 21: 155-163.

Pearce, J. and N. G. Carr. 1967. The metabolism of acetate by the blue-green algae, *Anabaena variabilis* and *Anacystis nidulans*. J. Gen. Microbiol. 49: 301-313.

________ and ________. 1968. The incorporation and metabolism of glucose by *Anabaena variabilis*. J. Gen. Microbiol. 54: 451-462.

Peck, H. D. 1968. Energy-coupling mechanisms in chemolithotrophic bacteria. Annu. Rev. Microbiol. 22: 489-518.

Perkins, S. O. and J. Tyson. 1926. Soil survey of Kalamazoo county, Michigan. U.S. Dept. Agril., Bureau of Soils. U.S. Gov. Print. Off., Washington, D.C.

Pfennig, N. and K. D. Lippert. 1966. Über das Vitamin B_{12}-Bedürfnis phototropher Schwefelbakterien. Arch. Mikrobiol. 55: 258-266.

________. 1967. Photosynthetic bacteria. Annu. Rev. Mikrobiol. 21: 285-324.

________. 1969. *Rhodopseudomonas acidophila*, sp. n., a new species of the budding purple nonsulfur bacteria. J. Bacteriol. 99: 597-602.

Pomeroy, L. R. 1960. Residence time of dissolved phosphate in natural waters. Science 131: 1731-1732.

Provasoli, L. 1964. Effects of external metabolites on algae. Verh. int. Ver. Limnol. 15: 804-805.

Prowse, G. A. 1955. The role of phytoplankton in studies on productivity. Verh. int. Ver. Limnol. 12: 159-163.

Qadri, S. M. H. and D. S. Hoare. 1968. Formic hydrogenlyase and the photoassimilation of formate by a strain of Rhodopseudomonas palustris. J. Bacteriol. 95: 2344-2357.

Rafter, M. E. and W. L. Ruble. 1966. Stepwise deletion of variables from a least square equation. State series description No. 7. Michigan Agricultural Experiment Station.

Rao, C. B. 1953. On the distribution of algae in a group of six small ponds. J. Ecol. 41: 62-71.

Ribbons, D. W., J. E. Harrison, and A. M. Wadzinski. 1970. Metabolism of single carbon compounds. Annu. Rev. Microbiol. 24: 135-158.

Rich, P. and R. G. Wetzel. 1969. A simple, sensitive underwater photometer. Limnol. Oceanogr. 14: 611-613.

________, ________, and N. V. Thuy. 1971. Distribution, production and role of aquatic macrophytes in a southern Michigan marl lake. Freshwat. Biol. 1: 3-21.

Romanova, A. P. 1957. Seasonal dynamics of the nitrogen cycle in water and bottoms of the littoral of Lake Baikal. In N. A. Akatova, E. N. Pavlovskii, and B. K. Shtegman (ed.) Transactions of the 6th Conference on the biology of inland waters. AEC-Natl. Sci. Found., Washington, D.C. (Engl. trans.).

Ruttner, F. 1960. Fundamentals of limnology, 3rd ed. University of Toronto Press, Toronto. (Engl. trans.).

Sakamoto, M. 1971. Chemical factors involved in the experimental lake area, northwestern Ontario. J. Fish. Res. Bd. Can. 28: 203-213.

Saunders, G. W. 1957. Interrelations of dissolved organic matter and phytoplankton. Bot. Rev. 23: 389-409.

________. 1963. The biological characteristics of fresh water. Great Lakes Res. Div., Univ. Michigan pub. No. 10: 245-267.

Schick, H. J. 1971a. Substrate and light dependency of molecular nitrogen fixation in Rhodospirillum rubrum. Arch. Mikrobiol. 75: 89-101.

Schick, H. J. 1971b. Interrelationship of nitrogen fixation, hydrogen evolution and photoreduction in *Rhodospirillum rubrum*. Arch. Mikrobiol. 75: 102-109.

________. 1971c. Regulation of photoreduction in *Rhodospirillum rubrum* by ammonia. Arch. Mikrobiol. 75: 110-120.

Schindler, D. W. and S. K. Holmgren. 1971. Primary production and phytoplankton in the experimental lakes area, northwestern Ontario, and other low-carbonate waters, and a liquid scintillation method for determining ^{14}C activity in photosynthesis. J. Fish. Res. Bd. Canada 28: 189-201.

Schneibner, R. A. 1958. The survey of the insect bottom fauna of a limited area of Wintergreen Lake, Kalamazoo County, Michigan. M.S. thesis, Michigan State University.

Schöllhorn, R. and R. H. Burris. 1966. Study of intermediates in nitrogen fixation. Fed. Proc. 24: 710.

________ and ________. 1967. Acetylene as a competitive inhibitor of N_2 fixation. Proc. Natl. Acad. Sci. U.S.A. 58: 213-216.

Shapiro, J., W. Chamberlain, and J. Barrett. 1969. Factors influencing phosphate use by algae. Int. Conf. Water Res., 4th, pp. 149-160.

Siebert, M. L. and W. H. J. Hattingh. 1967. Estimation of methane-producing bacterial numbers by the most probable number (MPN) technique. Water Res. 1: 13-19.

________, D. F. Toerien, and W. H. J. Hattingh. 1968. Enumeration studies on methanogenic bacteria. Water Res. 2: 545-554.

Singh, R. N. 1955. Limnological relation of Indian waters with special reference to waterblooms. Verh. int Ver. Limnol. 12: 831-836.

Sorger, G. J. 1969. Regulation of nitrogen fixation in *Azobacter vinelandii* OP: the role of nitrate reductase. J. Bacteriol. 98: 56-61.

Sorokin, Yu. I. 1961. Role of chemosynthesis in production of organic substances in water reservoirs. Investigation of chemosynthesis production in Kuibyshev Water reservoir in 1958-1959. Microbiology 30: 756-763. (Engl. trans.).

Stadtman, T. C. 1967. Methane fermentation. Annu. Rev. Microbiol. 21: 121-142.

Stanier, R. Y., M. Doudroff, R. Kunisawa, and R. Contopoulou. 1959. The role of organic substrates in bacterial photosynthesis. Proc. Natl. Acad. Sci. U.S.A. 45: 1246-1260.

Steemann, Nielsen E. 1951. Measurement of the production of organic matter in the sea by means of carbon-14. Nature (London) 167: 684-685.

________. 1962. Inactivation of the photochemical mechanism in photosynthesis as a means to protect the cells against too high light intensities. Physiol. Plant 15: 161-171.

________ and E. G. Jørgensen. 1962. The physiological background for using chlorophyll measurements in hydrobiology and a theory explaining daily variations in chlorophyll concentration. Arch. Hydrobiol. 58: 349-357.

________. 1963. Productivity, definition and measurement, pp. 129-164. In M. N. Hill (ed.) The Sea, vol. 2, Interscience, New York.

Stewart, W. D. P. 1966. Nitrogen fixation in plants. The Athlone Press, University of London, London.

________, G. P. Fitzgerald, and R. H. Burris. 1967. *In situ* studies on N_2 fixation using the acetylene reduction technique. Proc. Natl. Acad. Sci. U.S.A. 58: 2071-2078.

________, ________, and ________. 1968. Acetylene reduction by nitrogen-fixing algae. Arch. Mikrobiol. 62: 336-348.

________. 1969. Biological and ecological aspect of nitrogen fixation by free-living microorganisms. Proc. Roy. Soc. Ser. B. 172: 367-388.

________, A. Haystead, and H. W. Pearson. 1969. Nitrogenase activity in heterocysts of blue-green algae. Nature (London) 224: 226-228.

Stewart, W. D. P. 1970a. Nitrogen fixation by blue-green algae in Yellowstone thermal area. Phylologia 9: 261-268.

________. 1970b. Algal fixation of atmospheric nitrogen. Plant and Soil 32: 555-588.

________ and M. Lex. 1970. Nitrogenase activity in the blue-green alga *Plectonema boryanum* strain 594. Arch. Mikrobiol. 73: 250-260.

________ and H. W. Pearson. 1970. Effects of aerobic and anaerobic conditions on growth and metabolism of blue-green algae. Proc. Roy. Ser. B. 175: 293-311.

________, G. P. Fitzgerald, and R. H. Burris. 1970. Acetylene reduction assay for determination of phosphorous availability in Wisconsin lakes. Proc. Natl. Acad. Sci. U.S.A. 66: 1104-1111.

________, 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.

Stokes, J. E. and D. S. Hoare. 1969. Reductive pentose cycle and formate assimilation in *Rhodopseudomonas palustris*. J. Bacteriol. 100: 890-894.

Sylvester, R. O. and G. C. Anderson. 1964. A lake's response to its environment. J. San Eng. Div. ASCE. 90: 1-22.

Talling, J. F. 1957. Diurnal changes of stratification and photosynthesis in some tropical African waters. Proc. Roy. Soc. Sci. Ser. B. 147: 57-83.

Taniguchi, S., and M. D. Kamen. 1963. On the nitrate metabolism of facultative photoheterotrophs, pp. 465-484. In Studies on microalgae & photosynthetic bacteria. Japanese Society of Plant Physiologists, The University of Tokyo Press, Tokyo.

Taube, C. M. and E. H. Bacon. 1952. Gull Lake inventory summary-No. 2. Michigan Dept. Conserv. Inst. Fish. Res.

Thiel, P. G., D. F. Toerien, W. H. J. Hattingh, J. P. Kotzé, and M. L. Siebert. 1968. Interactions between biological and chemical characteristics in anaerobic digestion. Water Res. 2: 393-408.

Thiele, H. H. 1968. Die verwertung einfacher organicher substrate durch Thiorhodaceae. Arch. Mikrobiol. 60: 124-138.

Tolbert, N. E. and L. P. Zill. 1956. Excretion of glycolic acid by algae during photosynthesis. J. Biol. Chem. 222: 895-906.

Toerien, D. F. and M. L. Siebert. 1967. A method for the enumeration and cultivation of anaerobic "acid-forming" bacteria present in digesting sludge. Water Res. 1: 397-404.

________, ________, and W. H. J. Hattingh. 1967. The bacterial nature of the acid-forming phase of anaerobic digestion. Water Res. 1: 497-507.

________ and W. H. J. Hattingh. 1969. Anaerobic digestion-I. The microbiology of anaerobic digestion. Water Res. 3: 385-416.

________. 1970. Population description of the non-methanogenic phase of anaerobic digestion - I. Isolation, characterization, and identification of numerically important bacteria. Water Res. 4: 129-148.

Trentini, W. C. and M. P. Starr. 1967. Growth and ultrastructure of *Rhodomicrobium vannielii* as a function of light intensity. J. Bacteriol. 93: 1699-1704.

________. 1967. Defined medium allowing maximal growth of *Rhodomicrobium vannielii*. J. Bacteriol. 94: 1260-1261.

Uffen, R. L. and R. S. Wolfe. 1970. Anaerobic growth of purple nonsulfur bacteria under dark conditions. J. Bacteriol. 104: 462-472.

USCOMM-NOAA. 1971. Climatological data of Michigan, July 1971, Climatological data vol. 86, No. 7. US Gov. Print. Off., Washington, D.C.

van Niel, C. B. 1931. On the morphology and the physiology of the purple and green sulfur bacteria. Arch. Mikrobiol. 3: 1-112.

________. 1935. Photosynthesis in bacteria. Cold Spring Harbor Symposium, pp. 3-138.

van Niel, C. B. 1936. On the metabolism of the Thiorhodaceae. Arch. Mikrobiol. 7: 323-358.

________. 1944. The culture, general physiology, morphology, and classification of the non-sulfur purple and brown bacteria. Bacteriol. Rev. 8: 1-118.

________. 1971. Techniques for the enrichment, isolation and maintenance of the photosynthetic bacteria, pp. 3-28. In A. San Pietro (ed.) Methods in enzymology, vol. 23: Photosynthesis, part A. Academic Press, New York.

Verduin, J. 1956. Energy fixation and utilization by natural communities in Western Lake Erie. Ecology 37: 40-50.

________, E. E. Whitwer, and B. C. Cowell. 1959. Maximal photosynthesis rates in nature. Science 130: 268-269.

Vertebnaya, P. I. 1957. Photosynthesis of phytoplankton of the Klyaz'ma Reservoir, pp. 285-294. In N. A. Akatova, E. N. Pavlovskii, and B. K. Shtegman (ed.) Transactions of the 6th Conference on the biology of inland waters. AEC-Natl. Sci. Found., Washington, D.C. (Engl. trans.).

Vollenweider, R. A. and A. Nauwerck. 1961. Some observations on the C_{14} method for measuring primary production. Verh. int. Ver. Limnol. 14: 134-139.

Wall, J. S., A. C. Wagenknecht, J. W. Newton, and R. H. Burris. 1952. Comparison of the metabolism of ammonia and molecular nitrogen in photosynthesizing bacteria. J. Bacteriol. 63: 562-573.

Wallen, D. C. and G. H. Geen. 1971a. Light quality in relation to growth, photosynthetic rates and carbon metabolism in two species of marine plankton algae. Mar. Biol. 10: 34-43.

________ and ________. 1971b. Light quality and concentration of proteins, RNA, DNA and photosynthetic pigments in two species of marine plankton algae. Mar. Biol. 10: 44-51.

________ and ________. 1971c. The nature of the photosynthate in natural phytoplankton populations in relation to light quality. Mar. Biol. 10: 157-168.

Watanabe, A. 1967. The blue-green algae as the nitrogen fixators. Proc. ixth. Congr. Microbiol., mdscow (1966), p. 75-87.

Westlake, D. F. 1969. Estimating quantity and quality of biomass—Mactophytes, pp. 25-32. In R. A. Vollenweider (ed.) A manual on methods for measuring primary production in aquatic environments. Blackwell Scientific Publication, Oxford.

Wetzel, R. G. 1964. A comparative study of the primary productivity of higher aquatic plants, periphyton, and phytoplankton in a large, shallow lake. Int. Revue ges. Hydrobiol. 49: 1-61.

________. 1966. Productivity and nutrient relationships in marl lakes of northern Indiana. Verl. int. Ver. Limnol. 16: 321-332.

________. 1967. Dissolved organic compounds and their utilization in two marl lakes. Hidrol. Kozl. 47: 298-303.

________. 1968. Dissolved organic matter and phytoplanktonic productivity in marl lakes. Mitt. Internat. Verein. Limnol. 14: 261-270.

________. 1969. Factors influencing photosynthesis and excretion of dissolved organic matter by aquatic macrophytes in hard water lakes. Verh. int. Ver. Limnol. 17: 72-85.

________ and D. F. Westlake. 1969. Estimating quantity and quality of Biomass-Periphyton, pp. 33-40. In R. A. Vollenweider (ed.) A manual on methods for measuring primary production in aquatic environments. Blackwell Scientific Publication, Oxford.

________ and B. A. Manny. 1972. Secretion of dissolved organic carbon and nitrogen by aquatic macrophytes. Verh. int. Ver. Limnol. 18 (In press).

Whatley, F. R. 1971. Recycling through higher plants. Proc. Roy. Soc. Ser. B. 179: 193-200.

Wiessner, W. 1970. Photometabolism of organic substrates, pp. 95-133. In P. Halldal (ed.) Photobiology of microorganisms. John Wiley & Sons, New York.

Wilkinson, J. F. 1958. The extracellular polysaccharides of bacteria. Bacteriol. Rev. 22: 46-73.

Winter, H. C. and D. I. Arnon. 1970. The nitrogen fixation system of photosynthetic bacteria. I. Preparation and properties of cell-free extract from *Chromatium*. Biochim. Biophys. Acta 197: 170-179.

Whiteside, E. P., I. F. Schneider, and R. L. Cook. 1968. Soil of Michigan. Michigan State University Coop. Ext. Serv. Bull. E-630.

Wolk, C. P. 1970. Aspect of the development of a blue-green algae. Annu. Acad. Sci. 175: 641-647.

________ and E. Wojciuch. 1971. Photoreduction of acetylene by heterocysts. Planta 97: 126-134.

Wood, H. G. and R. L. Stjernholm. 1962. Assimilation of carbon dioxide by heterotrophic organisms, pp. 41-117. In I. C. Gunsalus and R. Y. Stanier (ed.) The Bacteria, vol. III: Biosynthesis. Academic Press, New York.

Wright, R. T. 1964. Dynamics of phytoplankton community in an ice-covered lake. Limnol. Oceanogr. 9: 163-178.

________ and J. E. Hobbie. 1965. The uptake of organic solutes in lake water. Limnol. Oceanogr. 10: 22-28.

________ and ________. 1966. Use of glucose and acetate by bacteria and algae in aquatic ecosystems.