A STUDY ON NITROGEN FIXATION BY BLUE-GREEN ALGAE USING THE ACETYLENE REDUCTION TECHNIQUE

BY

BYONG-IE, HAN

A STUDY ON NITROGEN FIXATION BY BLUE-GREEN ALGAE USING THE ACETYLENE REDUCTION TECHNIQUE

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Master of Science

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by

Byong-Ie, Han

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To the Graduate Council:

I am submitting herewith a Research Paper written by Byong-Ie, Han entitled "A Study on Nitrogen Fixation by Blue-Green Algae using the Acetylene Reduction Technique."

I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biology.

Major Professor

Accepted for the Council:

Dean of the Graduate School

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION

The whole process of this study was based on the fact that many blue-green algae, like certain bacteria such as Azotobacter, Clostridium, and Rhizobium sp., are able to fix elementary nitrogen (N_2) from the air and the significance of biological nitrogen fixation in lakes is slowly emerging.

Although nitrogen fixation does not appear to be a common characteristic of blue-green algae, many blue-green algae, over 50 strains and species of heterocystous blue-green algae are thought to be primarily responsible for this phenomenon (Fay et al., 1968; Stewart, 1973; Stewart et al., 1969).

There are also various reports in the literature of nitrogen fixation by non-heterocystous, filamentous blue-green algae and a few strains of unicellular blue-green algae.

So far, there is no good evidence for nitrogen fixation by algae belonging to groups other than blue-green algae (Fogg, 1962).

However, it is now generally accepted that the contribution by blue-green algae to the nitrogen status of natural ecosystems, particularly in aquatic ecosystems, is much more important than the contribution by heterotrophic micro-organisms (Stewart, 1963).

Thus, nitrogen fixation by blue-green algae might be one of the major source of combined nitrogen in aquatic environments.

In this study, nitrogen fixation by blue-green algae in aquatic habitats has been investigated with samples collected from Lake Barkley, Kentucky Lake, and Dunbar Lake in Tennessee from April through July, 1974, by the acetylene reduction technique which was first used by Stewart et al. (1967).

Some investigations on nitrogen fixation were also carried out with the pure colonies of the genus Nostoc.

Since nitrogen fixation by blue-green algae is the enzymic conversion of dinitrogen (N_2) from atmosphere to ammonia by the enzyme nitrogenase, it depends on enzyme nitrogenase activity.

So, by using the acetylene reduction technique, nitrogenase activity as well as total nitrogen fixed by blue-green algae can be measured to estimate potential nitrogen fixation by blue-green algae in the field and laboratory (Stewart et al., 1967).

The acetylene reduction technique is based on the fact that nitrogenase reduces acetylene to ethylene (Dilworth, 1966; Stewart et al., 1967).

Therefore, the rate of acetylene reduction can be used as an index of the rate of nitrogen fixation.

In this research paper, data obtained in experiments were also expressed in the term of acetylene reduction or ethylene production.

The objective of this study was to collect possible information about biological nitrogen fixation from aquatic environments of three lakes in Tennessee and colonies of the genus Nostoc growing on the surface of damp soil to observe the variations of nitrogen fixation by blue-green algae.

Also, the author's aim has been to compare his findings with those of other workers in nearby areas.

CHAPTER II

REVIEW OF RELATED LITERATURE

It is now generally accepted that blue-green algae fix much more nitrogen than do heterotrophic micro-organisms (Stewart, 1973; Wolk, 1973).

Probably, because of this importance of the contribution by blue-green algae to the nitrogen status of natural ecosystems, interest about the role of blue-green algae is increasing among phycologists, soil scientists, and other biologists, and numerous studies have been reported.

Particularly, blue-green algae have been economically considered as an important agent to provide nitrogen source for the rice plants in water logged rice paddies especially in India, China, Korea, Japan and other oriental countries (Stewart, 1973; Watanabe et al., 1951; Yoshida, 1973).

However, a careful literature survey of works relating to nitrogen fixation of blue-green algae revealed that within the last two decades, most of the important works in this area have been conducted in many countries of the world.

According to the report of Stewart (1973), Drewes (1928) was the first to demonstrate unequivocally that pure cultures of certain blue-green algae fixed nitrogen.

However, Drewes (1929), Allison and Morris (1930), and Fogg (1942) are considered to be earlier workers on nitro-

gen fixation by blue-green algae and considered that they first demonstrated fixation of elemental nitrogen by axenic cultures of blue-green algae, in species of Nostoc and Anabaena.

Subsequently, under aerobic conditions, Odintzova (1941) demonstrated nitrogen fixation in members of the order Chroococcales and Watanabe (1951) demonstrated nitrogen fixation in other members of the order Nostocales. Allen and Arnon (1955) also reported aerobic nitrogen fixation by Anabaena cylindrica Lemm. and Fogg (1951) demonstrated nitrogen fixation by Mastigocladus laminosus Cohn. of the order Stigonematales.

In 1969, Wyatt and Silvey confirmed and reported that nitrogen fixation by blue-green algae is not solely confined to filamentous genera with heterocysts. They demonstrated atmospheric nitrogen fixing ability in the genus Gloeocapsa of the order Chroococcales. They also pointed out that nitrogen fixation is not widespread among the Chroococcales. They found that another Gloeocapsa species did not fix nitrogen.

More recently, Stanier et al. (1971) and Rippka et al. (1971) demonstrated fixation of elemental nitrogen by members of the order Chroococcales.

Stewart and Lex (1970) demonstrated that among the filamentous blue-green algae which do not form heterocysts,

Plectonema is capable of fixing nitrogen. There are also reports of nitrogen fixation by non-heterocystous blue-green algae, Trichodesmium (Bunt et al., 1970).

It is now generally known that heterocysts have abundant nitrogenase and these are the most common sites of nitrogen fixation of blue-green algae (Fay et al., 1968; Ogawa and Carr, 1969; Stewart, 1970; Stewart et al., 1969). Jewell and Kulasooriya (1970) suggested that an estimate of the nitrogen fixation rates in blue-green algae may be made by "in situ" heterocyst counts and acetylene reduction measurements.

Nitrogen fixation in the surface water of lakes, oceans and other aquatic ecosystems is also well documented.

Before the acetylene reduction assay was introduced by Stewart et al. (1967), earlier works on nitrogen fixation by blue-green algae in aquatic ecosystems were usually performed by the heavy nitrogen (N^{15}) tracer method.

Schneider et al. (1960) first reported high but very variable fixation of N^{15}-labeled nitrogen gas by cell free extracts of blue-green algae (Wolk, 1973). However, according to the reports of Dugdale et al. (1959), N^{15} had been used successfully in the study of biological nitrogen fixation by Rittenberg et al., Burris and Miller and Burris et al. in early 1940s.

Numerous studies on heavy nitrogen (N^{15}) have reported

rates of nitrogen fixation in lakes and other aquatic ecosystems and have associated with the presence of certain blue-green algae, primarily in the order Nostocales.

Earlier works on nitrogen fixation in lakes and other aquatic ecosystems using N^{15} as a tracer are considered by Dugdale et al. (1959), Dugdale and Goering (1964), Dugdale and Dugdale (1962 and 1965), Goering and Neess (1964), Neess et al. (1962) and Williams and Burris (1952).

In 1966, Schöllhorn and Burris and Dilworth independently reported that the nitrogen fixing enzyme system (nitrogenase) can reduce acetylene to ethylene. Schöllhorn and Burris (1967) again demonstrated in vitro that acetylene completely inhibits nitrogen fixation at partial pressures at or over 0.1 atm through competition with nitrogen.

Subsequently, Stewart et al. (1967 and 1968) first used this acetylene reduction technique with blue-green algae and showed acetylene reduction by algae in lakes, in soil, in laboratory cultures and in cycad root nodules and advocated the use of the technique as a simple and inexpensive rapid-scan method for detecting potential nitrogen-fixing microorganisms in nature.

Hardy et al. (1968) also reported the successful application of acetylene reduction assay for measuring biological nitrogen fixation.

Since then, the acetylene reduction method has become more common than the N^{15} tracer method because the latter method is insufficiently sensitive and accurate and the N^{15} method is time consuming, expensive and requires a mass spectrometer (Stewart, 1967).

This basic technique used on water samples was described by Brezonik and Harper (1969), who measured nitrogen fixation at different depths in Lake Mary, Wisconsin, and Lake Mize, Florida by acetylene reduction assay, and on sediments by Brooks et al. (1971).

Using this acetylene reduction technique, Howard et al. (1970) determined fixation potential by blue-green algae "in situ" in water of Lake Erie from June through November, 1969. Rusness and Burris (1970) carried out experiment in representative oligotrophic and eutrophic lakes (Mendota and Madison) in Wisconsin from 1968 through 1969 by the acetylene reduction technique.

Granhall and Lundgren (1971) reported that the annual contribution of nitrogen fixation in the pelagial of a moderately eutrophic and unpolluted lake was of the order 0.5 gmN/m^2. Horne and Goldman (1972) investigated and concluded the annual contribution of nitrogen fixation to Clear Lake, California in 1970 was about 18 kg ha^{-1} which is 43% of the lake's yearly nitrogen flow. They reported that annual rates of nitrogen fixation were correlated with the proportion of

heterocysts to vegetative cells in Aphanizomenon and with total number of heterocysts in Anabaena.

Findley et al. (1973) investigated interrelation between plankton and surface nitrogen levels in Skaha Lake, British Columbia (Canada) and reported that the large increase in nitrogen in late July and August correlated with the Gloeotrichia echinulata J. E. Smith ex P. Richt. bloom and the increase in nitrogen fixation. They also reported that the cyanophyte bloom could have contributed a minimum of 2350 kg nitrogen to the lake in August, 1970 which is c. 62% of the nitrate-nitrogen occurring at any time.

Horne and Viner (1971) determined nitrogen fixation and its significance in tropical Lake George, Uganda. Bunt et al. (1970) and Stewart (1965) have determined nitrogen fixation by marine blue-green algae.

However, according to the author's own survey of the literature, so far no works on nitrogen fixation in Kentucky Lake, Lake Barkley and Dunbar Lake have been conducted.

CHAPTER III

METHODS AND MATERIALS

1) Collections

Lake samples were collected from Lake Barkley (at Gatlin Point, Bards Dam and Blue Creek), Kentucky Lake (at Boswell Landing and Rushing Creek), and Dunbar Lake (at five randomly selected places) from April 1 through July 16, 1974.

Also, the Genus Nostoc was obtained from Dr. Chester, Department of Biology, Austin Peay State University. On July 5, 1974, more Nostoc was collected at the garden of United Methodist Church, 5 Dotsonville Rd. Clarksville, Tennessee 37040, after a rainfall.

Surface waters, as samples of lake, were collected offshore with or without prior concentration of the algae, depending on the density of the algal population. Samples were concentrated by using a phytoplankton net (173 meshes per inch).

After collecting, samples were carefully placed in glass bottles with lake water for transport to the laboratory. All samples were brought directly to the laboratory without further delay. However, in the case of further delay in the field, the glass bottles containing lake samples were put in lake water to protect algal sam-

ples from any possible change of temperature.

The time for a returning tour of samples took a minimum time of 30 minutes but never exceeded 90 minutes.

2) Laboratory Studies

All samples were exposed under a constant daylight type fluorescent lighting for desired periods (See each Table in Chapter IV) at $70^{\circ}F$ ($21.1^{\circ}C$).

The lake samples were allowed to remain in the original bottles in which the samples were placed with lake water for desired period (See each Table, Chapter IV) to allow further growth of organisms.

In the case of Nostoc, it was transferred from the collecting containers into 75 millilitre capacity flasks containing 25 ml nutrient solution (Beijerinck) each and plugged with cotton plugs.

Before transferring the Nostoc samples into flasks containing nutrient solution, the colonies were washed by shaking them well with distilled water within a sterile flask and then, pouring off distilled water.

Each flask was inoculated with 3-5 colonies (about 25 cm^2 in area) and was placed under the above described laboratory conditions until the samples were used for the assay.

After the desired period of pretreatment (allowance

under above conditions), the acetylene reduction technique as described by Stewart et al. (1967) was used with some modifications.

Samples (lake sample; 1 ml each, Nostoc; 1 colony or 5 cm^2 in area with 0.5 ml of nutrient solution, or desired quantity of samples to measure) were carefully transferred into 7 ml capacity glass serum bottles fitted with rubber lined metal stoppers.

Then, acetylene (0.5 ml, ambient atmospheric pressure) was injected directly into 7 ml serum bottles containing algal suspensions or Nostoc without evacuation or flushing with any artificial gas mixture.

Schollhorn and Burris (1967) have shown in vitro that acetylene completely inhibits nitrogen fixation at partial pressures at or over 0.1 atm through competition with nitrogen. This was also applied to in vivo systems by Granhall and Lundgren (1971).

Basing on this theory and the results of their experiments, nitrogen did not have to be removed from the reaction bottles because measurement of ethylene produced represents the amount of nitrogen which would otherwise have been reduced during the incubation period.

Generally, each sample was incubated for 1 hour, but samples for longer period of incubation were also prepared to detect possible low rates of nitrogen fixation en-

countered.

During the period of incubation, the samples were subjected to a temperature of 70°F and constant light intensity, in addition the samples were frequently shaken.

Biological reactions were terminated by the injection of 0.2 ml of 50% TCA (Tri-Chloro Acetic acid) to each lake sample and 0.3 ml to Nostoc samples.

Then, samples were analyzed for ethylene production which represents the amount of nitrogen fixation.

All samples were observed under light microscope to determine dominant species.

3) Analysis

3---I. Gas Chromatography

3--II. Identification

3-III. Quantitative Analysis

3-I. Gas Chromatography

Ethylene formation was detected by Gas-Chromatography with a Varian-Aerograph Model 1400 gas chromatographic apparatus (H_2-flame ionization detector) fitted with a 9 ft. long, 1/8 inch in diameter column containing Porapak R, with N_2 serving as the carrier gas.

The operation of the gas-chromatography apparatus was as follows.

* Gas Flow Rate

a. Carrier gas (N_2)	25 ml per minute.
b. H_2	30 ml per minute.
c. Compressed Air	300 ml per minute.

* Temperature

a. Detector temp.	150°C
b. Injector temp.	120°C
c. Oven temp.	100°C

3-II. Identification

To identify the gases produced by the reduction of acetylene to ethylene, two chromatographic methods, the measurement of retention times and the addition of suspected substance were employed.

3-II-1. "Retention Times" method

The retention time is the time, in minute or seconds, elapsing between the time that a standard air peak emerges from a chromatographic column at a given temperature and gas flow and the time that a given substance emerges under the same conditions.

Thus, when operating variables are maintained constant, the retention time is characteristic of a compound. Therefore, a comparison of the retention times of known compounds (gases) with those of the unknown can identify

the unknown gases produced.

In this study, the time elapsing between the time of injection of samples and the time that sample gases emerge under the same conditions was compared with that of pure ethylene or acetylene (Matheson Gas Co.). This was because standard air peaks were not emerged due to the adjustment of the range switch and the attenuator of electrometer for keeping peaks of sample gases produced within the scale.

The elapsing times of pure ethylene and acetylene from the time of injection to the time of emergence of peaks were 1 (exactly one) minute and 1 minute and 9 seconds respectively in this study.

In obtaining these retention times, the identical operating conditions of the Gas-Chromatography were maintained as constant as possible.

3-II-2. "The Addition of Suspected Substance" method

To avoid some possible errors in performing above "retention times" method, and for the absolute identification, it is necessary to test sample gases produced by some other method.

The technique of "the addition of suspected subtance" method involves adding a small amount of a known material to a portion of the sample being investigated

followed by an examination of the subsequent chromatogram (Bobbitt et al., 1968; Nogare and Juvet, 1962).

If the peak of interest is enhanced, the identification is positive, if a new peak or shoulder results, the identification is negative.

Thus, identification of sample gases newly produced were carried out by this technique whenever needed in this study.

3-III. Quantitative Analysis

"Peak height method" was chosen to measure the amounts of ethylene produced or acetylene reduced in this study, since measuring the height of the peak is the simplest method of quantitative evaluation and it is primarily suitable for rapidly eluted components producing high and narrow peaks (Nogare and Juvet, 1962). Besides, by using this method, most of possible chromatographic apparatus variables in operation could be nullified, since both the internal standard (known amounts of ethylene or acetylene) and the sample gases were influenced to the same extent of instrumental variations.

Thus, calibration of peak heights with both the known amounts of ethylene and acetylene as internal standards and the unknown amounts of sample gases detected were performed.

Then, the direct comparison of the peak heights of the unknown ones with those of standard gases were carried out to yield the actual amounts of ethylene produced of acetylene reduced. All results were interpreted into the term of nanomoles.

CHAPTER IV

RESULTS

Various lake algae, including blue-green algae, were collected from Kentucky Lake, Lake Barkley, and Dunbar Lake in Tennessee and investigated initially or after certain periods of incubation for nitrogen fixation by the acetylene reduction technique in the laboratory.

The data in Table 1 show the entire series of collections of algal samples from lake waters including Nostoc sp. collected from damp soil and their general results during the period of this study from April through July.

The results were largely negative except those with pure colonies of Nostoc samples.

The values of acetylene reduction of lake samples were measurable only in concentrated samples collected from Dunbar Lake in mid-July, 1974 when there was a partial algal bloom, and in long-period-incubated samples such as Lake Barkley sample A and B.

Table 2 shows the dominant species and the blue-green algae which were frequently observed under the light microscope in each sample of collections.

Lake Barkley samples usually appeared thick and green, and abundant algae were collected each time.

The two areas of Kentucky Lake where the samples were

TABLE 1

Collections of Algal Samples and General Results

Date	Exposure Period in Lab.	Samples	Surface Water Temp.	Concentration	Nitrogen Fixation (Ethylene Production)
4/29/74	1½ months	Nostoc species			Measurable but, small amount
5/1/74	2 months	Dunbar Lake, A		Arbitrary, many times	Not detectable
5/1/74	2 months	Barkley, A (Near Blue Creek)		Arbitrary, many times	Measurable
5/1/74	2 months	Barkley, B (Near Dyers Creek)		Arbitrary, many times	Measurable
5/27/74	1 month	Dunbar Lake, B		Arbitrary, many times	Not detectable
6/15/74	Initially detected	Lake Barkley (Blue Creek)	77°F	Arbitrary, many times	Not detectable
6/15/74	Initially detected	Dunbar Lake, C	74°F	Without concentration	Not detectable
6/29/74	24 hours	Lake Barkley (Blue Creek)	80°F	1000 times	Not detectable
6/29/74	24 hours	Lake Barkley (Bards Dam)	78°F	1000 times	Not detectable

TABLE 1 (Continued)

6/29/74	24 hours	Lake Barkley (Gatlin Point)	80°F	1000 times	Not detectable
6/29/74	24 hours	Kentucky Lake (Boswell Landing)	81°F	1000 times	Not detectable
6/29/74	24 hours	Kentucky Lake (Rushing Creek)	78°F	1000 times	Not detectable
6/30/74	Initially detected	Dunbar Lake, A	75°F	1000 times	Not detectable
6/30/74	Initially detected	Dunbar Lake, B	74.5°F	1000 times	Not detectable
7/5/74	Variables	Nostoc species			Well produced
7/11/74	Initially detected	Dunbar Lake, D	76°F	W/O conc., 200X,& 500X	Detected, in small amount
7/16/74	Initially detected	Dunbar Lake, E	77°F	W/O conc., 200X,& 500X	Detected, in small amount

All lake samples were collected offshore between 12:00 Noon and 3:00 P.M. except Blue Creek on June 29 at 6:00 P.M. and both Kentucky Lake samples on June 29 between 4:30 P.M. and 5:30 P.M..

* Note; Data which were in doubt for any reason were not included in this Table.

TABLE 2

Dominant Species and Blue-green algae
which were observed in Each Lake Sample

Samples	Date	Dominant Species	Blue-green algae Observed frequently
Dunbar A	5/1/74	** Chrysosphaera sp. * Microspora floccosa * Spirogyra sp.	Oscillatoria sp.
Dunbar B	5/27/74	* Ankistrodesmum falcatus	
Barkley A (Near Blue Creek)	5/1/74	Cylindrospermum sp. Lyngbya sp. * Microspora sp.	Anabaena sp. Cylindrospermum sp. Lyngbya sp. Merismopedia elegans Nostoc sphaericum
Barkley B (Near Dyers Creek)	5/1/74	Anabaena sp. Merismopedia elegans * Scenedesmus sp. Trichodesmium laucustre * Ulothrix sp.	Anabaena sp. Cylindrospermum sp. Lyngbya sp. Merismopedia elegans Nostoc muscorum Tolypothrix sp. Trichodesmium laucustre
Dunbar Lake	6/15/74	* Gloeocystis sp. * Scenedesmus sp.	Chroococcus dispersus Gloeotrichia sp.

TABLE 2 (Continued)

Barkley (Blue Creek)	6/15/74	* *Ankistrodesmus falcatus* * *Dispora crucigenioides* ** *Epipyxis* sp. * *Scenedesmus* sp. * *Volvox* sp.	*Aphanocapsa delicatissima* *Oscillatoria* sp.
Lake Barkley (Blue Creek)	6/29/74	*Oscillatoria* sp. *Spirulina* sp.	*Oscillatoria* sp. *Spirulina* sp.
Lake Barkley (Bards Dam)	6/29/74	* *Microspora floccosa* *Oscillatoria subtilissima* * *Scenedesmus* sp. * *Volvox* sp.	*Anabaena* sp. *Chroococcus dispersus* *Oscillatoria* sp.
Lake Barkley (Gatlin Point)	6/29/74	*Ceratium* sp. * *Microspora* sp. *Synechococcus* sp. * *Volvox* sp.	*Merismopedia convoluta* *Oscillatoria amoena* *Phormidium angustissimum* *Synechococcus* sp. *Trichodesmium laucustre*
Kentucky Lake (Boswell Landing)	6/29/74	*Oscillatoria* sp. * *Spirogyra* sp. * *Volvox* sp. * *Xanthidium* sp.	*Anabaena* sp. *Chroococcus dispersus* *Oscillatoria subtilissima* *Schizothrix symplocoides*
Kentucky Lake (Rushing Creek)	6/29/74	*Lyngbya* sp. * *Microspora* sp. * *Scenedesmus* sp.	*Anabaena* sp. *Lyngbya* sp. *Trichodesmium* sp.
Dunbar Lake, A	6/30/74	* *Gloeocystis* sp.	* Algae were not abundant.

TABLE 2 (Continued)

Dunbar Lake, B	6/30/74	* Gloeocystis sp. * Spirogyra sp.	* Algae were not abundant
Dunbar Lake, D	7/11/74	Chroococcus sp. Gloeocapsa sp. (Colonial) Lyngbya sp. * Spirogyra sp. Tolypothrix sp.	Chroococcus sp. Gloeocapsa sp. Lyngbya sp. Merismopedia elegans Oscillatoria chlorina Tolypothrix sp.
Dunbar Lake, E	7/16/74	** Dinobryon sp. ** Epipyxis sp. Peridinium sp. (Dinophyceae)	Aphanocapsa sp. Gloeocapsa sp.

In this table, * denotes the class of Chlorophyceae, ** denotes the class of Chrysophyceae and those without specific sign are the class of Cyanophyceae (blue-green algae). Other abundant species such as Diatoms and Euglenas were omitted in this table.

collected was clear and less abundant algae were obtained in comparison with Lake Barkley.

Dunbar Lake was divided largely into two parts, Road (Dunbar Cave Road) side and Inner part, for the convenience of this study.

The road side where sample A, B and C were collected was clear to some degree and the algae were less abundant, while the shallow inner part where sample D and E were collected appeared to be filled with aquatic plants and was where large algal population developed during the period of this study.

However, the data in Table 2 show that blue-green algae, especially the family Nostocaceae were observed to be abundantly when acetylene reduction was detected, suggesting clearly that acetylene reduction is associated with blue -green algae.

Although the data in Table 3 do not represent "in situ" reduction rates at the time of collection since the samples were concentrated many times arbitrarily and allowed to grow for long periods of time, it does show that incubation was successful in producing acetylene reduction by blue-green algae.

Lyngbya sp. Trichodesmium sp. and Merismopedia elegans were found to be abundant when samples were observed with a light microscope (See Table 2).

TABLE 3

Variation in Ethylene Production by Lake Samples Cultivated in Laboratory

Sample	nMoles C_2H_4/ml/hr.
Lake Barkley A, 1	0.00
Lake Barkley A, 2	0.00
Lake Barkley A, 3	9.30
Lake Barkley A, 4	5.23
Lake Barkley A, 5	0.00
Lake Barkley B, 1	6.94
Lake Barkley B, 2	3.47
Lake Barkley B, 3	2.31
Lake Barkley B, 4	3.77
Lake Barkley B, 5	0.00

Samples were concentrated many times arbitrarily with a phytoplankton net offshore in Lake Barkley on May 1, 1974, between 2:00 P.M. and 3:00 P.M. and cultivated for 2 months in the laboratory.

Anabaena sp., Cylindrospermum sp. and Lyngbya sp. were commonly observed in both sample A and B (See Table 2).

Heterocysts which are thought to be primarily responsible for nitrogen fixation in filamentous blue-green algae were not found in Lyngbya sp..

As illustrated above, acetylene reduction "in situ" was detected mainly in concentrated samples which were collected from Dunbar Lake (D and E, Inner Part) on July 11, 1974, and July 16, 1974, between 12:00 Noon and 1:00 P.M. when the Lake was partially in algal bloom. Table 4 shows these results. Only a few samples collected without concentration showed acetylene reduction after long period of incubation.

However, to more closely approximate natural rates of fixation at the time of collection, the data in Table 4 show the results of acetylene reduction by samples without concentration.

On the other hand, with pure colonies of Nostoc which was identified as Nostoc commune Vaucher, abundant results were obtained.

Acetylene reduction rates by Nostoc sp. incubated in different media over short and long time periods are presented in Table 5 and Table 6. Each medium (nutrient solution, distilled water and pond water), demonstrated different effects on the acetylene reduction by Nostoc in the laboratory.

In the short periods of incubation, acetylene reduc-

TABLE 4

Acetylene Reduction "in situ" by Mid-July Lake Samples

Sample	Date	Concentration	Periods of Incubation	nMoles C_2H_2/ml
1	7/11/74	W/O	24 hrs.(1day)	0.000
2	7/11/74	"	48 " (2days)	0.000
3	7/11/74	"	72 " (3 ")	0.075
4	7/11/74	"	216 " (9 ")	0.818
5	7/16/74	"	48 " (2 ")	0.000
6	7/16/74	"	72 " (3 ")	0.000
7	7/16/74	"	216 " (9 ")	0.145
8	7/16/74	500X	216 hrs.(9days)	0.580
9	7/16/74	"	216 " (9 ")	1.02/2ml
10	7/16/74	"	216 " (9 ")	2.10/3ml

Samples of 1 ml and 2 ml with and without concentration were incubated with acetylene in the laboratory. All samples were collected from Dunbar Lake between 12:00 Noon and 1:00 P.M., when it was partially in algal bloom, and set up for incubation with acetylene initially.

Chroococcus sp. Gloeocapsa sp. and Tolypothrix sp. were observed frequently (See Table 2).

* Note; Periods of Incubation were prolonged to detect the positive values.

TABLE 5

Variation in Ethylene Production by
Nostoc commune Vaucher

Sample	Solution for Incubation	nMoles $C_2H_4/cm^2/hr.$
1	Nutrient Sol. (Beij.)	0.00 (Not detectable)
2	"	"
3	"	"
4	"	"
5	"	"
6	Pond Water	0.00 (Not detectable)
7	"	"
8	"	"
9	"	"
10	"	"
11	Distilled Water	0.69
12	"	1.39
13	"	1.04
14	"	1.03
15	"	0.69

Nostoc was collected from damp soil on July, 1974 after a rainfall and pretreated with above solutions for 24 hours in the laboratory before the test. The Nostoc colony whose size was 10 cm^2 in area was chosen for 1 hour incubation with C_2H_2.

TABLE 6

Effect of Medium on the Amount of Ethylene Produced by *Nostoc commune* Vaucher

Medium	Sample	Size (cm^2)	nMoles C_2H_4 Produced
* (8 hours of Incubation)			
Distilled Water (H_2O)	1	10	34.85
"	2	15	66.25
"	3	20	62.75
Pond Water	4	10	10.46
"	5	15	14.95
"	6	20	0.00
Nutrient Solution (Beij.)	7	10	9.42
"	8	15	0.00
"	9	20	9.46
* (80 hours of Incubation)			
Distilled Water (H_2O)	1	10	191.31
"	2	15	449.90
"	3	20	1,339.50
Pond Water	4	10	191.35
"	5	15	306.40
"	6	20	432.35
Nutrient Solution (Beij.)	7	10	415.10
"	8	15	453.40
"	9	20	854.65

All samples were pretreated with each solution for 48 hours. (See further illustrations on Page 30)

tion was measurable only in the samples treated with distilled water. The samples treated with nutrient solution produced measurable ethylene only after long period of incubation in comparison with samples treated with distilled water. The samples treated with distilled water showed the highest rate of acetylene reduction.

In Table 6, data obtained from experiments were compared directly one another without interpretation into "per units", since no exact mathematical linear relations were observed in the results of biological activities of Nostoc.

Table 7 shows that acetylene reduction by Nostoc was severely decreased when placed in Beijerinck solution for long time periods.

The rates of acetylene reduction in relation to the size of Nostoc colony are shown in Table 8 and data obtained on the effect of incubation time on the amount of ethylene produced by Nostoc is presented in Table 9.

No exact mathematical linear relations were observed between the size of colony and the amount of nitrogen fixation. The duration time of nitrogen fixation and the amount of nitrogen fixed were also found to have no exact mathematical linear relation to each other.

However, judging from the results of experiments shown in Table 5 through Table 9 it seemed that an incubation time of at least 1 hour is needed to detect nitrogen fixa-

TABLE 7

Effect of Beijerinck Solution
on the Amount of Acetylene Reduction
by Nostoc commune Vaucher
(In Comparison with other Incubation Media)

Sample	Incubation Media	nMoles C_2H_2/10cm^2/24 hrs.
1	Beij. (Soaked)	0.15
2	"	0.60
3	"	0.30
4	"	1.80
5	"	0.94
6	Beij. (Exposed)	91.60
7	"	28.26
8	"	23.20
9	Pond Water	31.38
10	"	6.93
11	"	25.37
12	Distilled Water	134.55
13	"	132.55
14	"	288.20

Samples 1 to 5 were placed in flasks containing Beijerinck solution for 1 month and were submerged into 1 ml of Beijerinck solution when incubated with acetylene while sample 6 to 8 were exposed to air (C_2H_2 gases) without soaking in solution. Samples 6 to 14 were pretreated with each solution for 48 hours.

TABLE 8

Acetylene Reduction in Relation to the Size of *Nostoc commune* Vaucher Colony per Unit Hour

Size of Sample	nMoles C_2H_2/hr.	Size of Sample	nMoles C_2H_2/hr.
1 cm^2	0.00	6 cm^2	3.67
2 "	0.00	7 "	0.00
3 "	0.00	8 "	4.03
4 "	1.74	9 "	3.95
5 "	3.47	10 "	5.67

TABLE 9

Effect of Incubation Time on the Amount of Ethylene Produced by *Nostoc commune* Vaucher

Incubation Time (hour)	nMoles C_2H_4/cm^2	Incubation Time (hour)	nMoles C_2H_4/cm^2
0.5 hour	0.00	4.0 hours	1.49
1.0 "	0.00	6.0 "	1.94
1.5 hours	0.69	12.0 "	4.36
2.0 "	0.69	24.0 "	9.16
2.5 "	0.74	48.0 "	15.62
3.0 "	1.39	80.0 "	57.44
3.5 "	0.95		

All samples in Table 8 and 9 were pretreated with nutrient solution for 1 day. Duplicate samples were set up and average values were taken for the results.

tion by Nostoc, especially when small samples are used.

The effect of light on acetylene reduction was checked using Nostoc samples in the laboratory. As Table 10 shows, among the 9 samples which were set up in the dark for different hours, 5 samples clearly showed acetylene reduction in small amounts. Evidently it is certain that nitrogen fixation occurs in the dark.

Sample 7-9 in Table 10 show that acetylene reduction rate of the samples incubated in the dark was less than 10% of that of the samples incubated in the light.

In addition, several other experiments such as 'Hourly variation in "in situ" C_2H_2 reduction in the surface waters of lake' were also conducted, but unfortunately since the rates of acetylene reduction in the lake was rarely measurable during the period of this study, the data denoted only the negative results.

TABLE 10

Variation in Ethylene Production by *Nostoc commune* Vaucher Incubated in the Dark and in the Light

Sample	Hour of Incubation	nMoles C_2H_4/cm^2 Dark	Light
1	0.5	0.00	0.00
2	1.0	0.00	0.00
3	1.5	0.21	0.69
4	2.0	0.02	0.69
5	2.5	0.00	0.74
6	3.0	0.00	1.39
7 (3 cm^2)	216.0 (9 days)	10.50	126.00
8 (5 ")	216.0 (9 ")	13.72	238.00
9 (7 ")	216.0 (9 ")	15.65	252.00

Nostoc (Sample 1 through Sample 6) which was pre-treated with nutrient solution (Beijerinck) for 24 hrs. in the laboratory was chosen for this test. Samples 7 to 9 were placed in a flask containing distilled water for 5 days and allowed to remain in the laboratory for an extended incubation period. All samples were wrapped with aluminum foil and incubated respectively.

CHAPTER V

DISCUSSION OF RESULTS

As illustrated in Chapter III, the rates of nitrogen fixation in three lakes (Barkley, Kentucky and Dunbar) in Tennessee, which were investigated with algal samples from each lake by acetylene reduction assay as first used by Stewsrt et al. (1967), were largely negative through the period of May to mid-July.

As shown in Table I and II, even though blue-green algae capable of fixing nitrogen were observed in almost all of lake samples under the microscope, positive acetylene reduction occurred only in the mid-July samples collected from Dunbar Lake when a partial algal bloom was visually apparent.

Investigations such as seasonal variations and the environmental variation of aquatic habitats in which nitrogen fixing blue-green algae live were not conducted concurrent with observations on nitrogen fixation, therefore it is impossible to state with certainty what is responsible for the negative results since nitrogen-fixing blue-green algae were present.

It is possible, however, that two major factors might be involved in these negative results. First, the biomass of blue-green algae capable of fixing nitrogen was not large enough during the period of this study. As shown in Table 2, the lake samples are characterized by the presence of

mainly Chlorophyta (green-algae) and Chrysophyta and the frequency of nitrogen-fixing blue-green algae were rare in comparison with the abundance of green algae.

More-over, it was found that heterocysts of filamentous blue-green algae which are thought to be primarily responsible for nitrogen fixation were slowly increasing in their total number only after mid-July. There are several reports supporting this phenomenon. Granhall and Lundgren (1971) have reported in their experiments conducted on Lake Erken, Sweden, that both the amount of acetylene reduction and the abundance of nitrogen-fixing blue-green algae were developed after July and the highest total reduction was found at the end of August.

Ogawa and Carr (1969), who investigated the relation between nitrogen fixation and heterocyst production in Lake Erie in 1967, reported that the number of heterocysts of Anabaena sp. and Aphanizomenon sp. increased only from mid-July and in mid-August, the heterocyst frequency of Anabaena sp. was in its maximum while the heterocyst frequency of Aphanizomenon sp. was in its maximum at the end of August.

This report coincides with the report of Howard et al. (1970), who also investigated biological nitrogen fixation in Lake Erie in 1969. They reported that nitrogen- fixing activity in the water was not detected in samples taken in June and July and appeared only in August when an algal

bloom was visually apparent.

Dugdale and Dugdale (1962) reported that the rates of nitrogen fixation in Sanctuary Lake, Pennsylvania, coincided with the Anabaena population and low fixation rates occurred until July and fixation rates rose suddenly at the time of mid-August. They pointed out that this minimum fixation rate until mid-summer is to be expected yearly and marked a quiescent period. They also reported that the presence of Anabaena does not necessarily imply active fixation and fixation was undetectable in their mid-October experiment although a substantial population of Anabaena was still present.

There is another report from Wisconsin Lake where Rusness and Burris (1970) reported that seasonal variation in acetylene reduction by surface samples show none or very little ethylene production until July 29 in both of oligotrophic and eutrophic lake samples but was vigorous in samples taken in late-August from eutrophicated lakes.

Although all of these reports came from more or less different temperate regions, the rates of nitrogen fixation of the three lakes in this study also coincide with these reports.

Secondly, the environmental conditions of these three lakes might be unfavorable for nitrogen fixation by blue -green algae as there are several unfavoralbe factors for

nitrogen fixation by blue-green algae.

Howard et al. (1970) postulated that light (total short wave radiation), temperature, nutrients and other factors affect nitrogen fixation by blue-green algae and these factors are significant over the extremes of seasonal variation.

Burris and Wilson (1946), Dugdale and Dugdale (1962), and Fogg (1956, 1962) reported that the presence of combined inorganic nitrogen acts to suppress the fixation of nitrogen.

Toetz (1972) reported the enrichment of $(NH_4)_2SO_4$ in two farm ponds at Oklahoma where the rate of nitrogen fixation was negative at the time of environmental investigation.

The concentration of combined nitrogen (NH_4-N and NO_3-N) in these three lakes as well as other unfavorable environmental conditions needs further investigation.

Nitrogen fixation by blue-green algae is known mainly in those filamentous groups with heterocysts (Fay et al. 1968; Stewart et al. 1969; Stewart 1973). The common nitrogen-fixing blue-green algae are the genera Anabaena, Anabaenopsis, Aulosira, Calothrix, Chlorogloea, Cylindrospermum, Fischerella, Gloeotrichia, Hapalosiphon, Mastigocladus, Nostoc, Scytonema, Stigonema, Tolypothrix, Westiellopsis, and Aphanizomenon flosaquae.

Among these genera, Anabaena and Nostoc are the most

well known nitrogen fixing blue-green algae.

There are also various reports in the literature of nitrogen fixation by non-heterocystous filamentous blue-green algae and unicellular algae.

Gleocapsa sp. and Chroococcus sp. are the well known nitrogen-fixing unicellular blue-green algae (Stanier et al. 1971; Stewart 1973; Wolk 1973; Wyatt and Silvey 1969).

As shown in Table 2, lake samples usually contained Oscillatoria sp. Lyngbya sp. and Chroococcus dispersus as the predominant forms of blue-green algae and the Nostoc and Anabaena as well as other nitrogen fixing forms were less evident.

The nitrogenase activities shown in Table 3 may be due to the presence of Cylindrospermum, Anabaena and Nostoc.

Although Table 3 presents significant values of acetylene reduction of lake samples, it is assumed that the values can not reflect the natural rate of nitrogen fixation at the time of collections since samples were allowed to grow in the laboratory for long periods of time.

However, data in Table 3 give information about the possibility and potentiality of nitrogen fixation in these three lakes in Tennessee.

The data in Table 4 presented the first positive evidence of acetylene reduction in these three lakes in Tennessee during the period of this study.

The nitrogen fixation rates were merely 0.0004%/24 hrs. on July 11, 1974 and 0.00007%/24 hrs. on July 16, 1974, in Dunbar Lake.

On the other hand, considerable acetylene reduction was obtained with Nostoc as shown in Table 5 through 10. These results are substantiated by the fact that Nostoc is the most well known nitrogen-fixer among the blue-green algae.

However, all samples in Table 5 do not show acetylene reduction. Only sample 11 to 15 which were treated with distilled water show acetylene reduction. None of samples treated with nutrient solution (Beijerinck) or pond water shows acetylene reduction within an hour.

As shown complementarily in Table 6 and 7, samples treated with Beijerinck solution or pond water always produced less abundant ethylene in comparison with samples treated with distilled water.

It is again impossible to state the reason of this phenomenon with certainty.

However, it is assumed that combined nitrogen in nutrient solution (Beijerinck) and pond water could inhibit the biological reactions involved in nitrogen fixation by Nostoc, since Beijerinck solution contains minerals including combined nitrogen as the form of NH_4NO_3.

However, data in Table 6 show that, as time passed, the

fixation rates of samples treated with Beijerinck or pond water increased while the fixation rates of samples treated with distilled water decreased, suggesting that eventually, Nostoc require certain minerals, possibly Mg^{++}, for their biological activities.

Smith and Evans (1970) and Stewart (1973) showed that ATP, Mg^{++} and $Na_2S_2O_4$ were required for active nitrogenase activity.

Table 7 shows that samples treated with Beijerinck solution for long period of time and submerged in Beijerinck solution during the period of incubation with C_2H_2 show only 0.76 nMoles C_2H_2 reduction per unit area (10 cm^2) while others show 20 to 280 nMoles C_2H_2 reduction per 10 cm^2 on an average within an equally given time of incubation. These results require further substantiation.

Data in Table 8 and 9 show acetylene reduction in relation to the size of Nostoc colony and incubation time respectively.

As mentioned previously in Chapter IV, no mathematical linear relations between size of colony or incubation time and the amounts of ethylene produced were observed. This could be due to some possible errors in measuring the size of Nostoc colony in the experiments were involved with the final calculations and/or it is perhaps because heterocysts of Nostoc are not distributed evenly within their colonial

gelatinous tegument.

However, the average productivity of ethylene was 0.332 nMoles per an hour and per unit size (cm^2).

Table 10 presents the acetylene reduction by blue -green algae in the dark. As shown in this table, it is clear that nitrogen fixation occurs in the dark but occurs at a lower rate than in the light.

Stewart (1973) has reported that glucose-6-phosphate and NADP system support and stimulate dark nitrogenase activity while direct photoreduction of nitrogen as a source of reductant occurs possibly in vivo in the light. The rate of acetylene reduction in the dark was less than 10% of that in the light as shown in table 10.

Generally, in reviewing all of the experiments of this study, it is worth while to discuss the relation of incubation time and the measurement of nitrogen fixation by acetylene reduction technique.

It is assumed that incubation time must be reduced to a minimum when the natural rate of fixation is being measured at the time of collection.

However, in this experiments, as shown in Table 4 through 10, no great change in rate occurred during the first 72 hours (possibly more than this) in lake samples and 6 hours in Nostoc samples.

Therefore, it is recommended that the incubation time

should be prolonged, especially with the April through July lake samples.

Otherwise, the use of possible larger bottles to accommodate larger samples might be necessary in order to detect the low rates encountered.

In conclusion, although the rates of acetylene reduction of these lake samples were largely negative, more frequent observations at more extended sites may reveal a greater nitrogen fixing potential in these three lakes.

CHAPTER VI

SUMMARY

Nitrogen fixation of blue-green algae has been detected by the acetylene reduction method with lake samples collected from Lake Barkley, Kentucky Lake and Dunbar Lake in Tennessee.

Even though blue-green algae capable of fixing nitrogen were present in these lakes, fixation rates were largely negative during the period of May through July. The positive values of acetylene reduction were detected only in mid-July lake samples collected from Dunbar Lake when a partial algal bloom was visually apparent. The fixation rates were 0.0004 %/24 hrs. on July 11, 1974 and 0.00007%/24 hrs. on July 16, 1974.

No fixation was observed in the Lake Barkley and Kentucky Lake during May to July period.

All evidence indicates that the beginning of the blue-green algal development is after July and presence of nitrogen fixing blue-green algae does not necessarily imply active fixation. Other factors may be of some importance in controlling fixation rates.

On the other hand, pure colonies of Nostoc commune Vaucher were well capable of nitrogen fixation. The average productivity of ethylene was 0.332 nMoles per an hour and per

unit size (cm^2) during the first 24 hours of incubation. The incubation media, Beijerinck, pond water and distilled water, showed different effects on acetylene reduction. It is assumed that the combined nitrogen in Beijerinck solution inhibits biological reactions of acetylene reduction by Nostoc. However, this needs further investigation.

The acetylene reduction rate by blue-green algae in the dark was less than 10% of that in the light.

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