BIOLOGICAL NITROGEN FIXATION IN A NITROGEN LIMITED TROPICAL LAKE, LAKE CHAPALA, MEXICO

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by
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Abstract

The objective of my study was to quantify the rate of biological nitrogen fixation in Lake Chapala, Mexico. Lake Chapala is the largest lake in Mexico. It is an ancient, tropical lake which is shaped in an east-west cul-de-sac with the inflow and outflow at the eastern end. Previous work showed low available nitrogen leading to nitrogen limitation of primary production. These measurements of available nitrogen ranged from undetectable to 1.8 mg l^{-1} on an east-west gradient of sampling stations throughout the year. Nitrogen levels were lowest at the western end of the lake. Biological nitrogen fixation rates were expected to increase as the ambient nitrogen decreased. However, biological nitrogen fixation, measured by the acetylene reduction technique, was negligible at all stations.

A reverse from the expected gradient of nitrogen fixation was found in the water from Lake Chapala when mixed with known nitrogen-fixing cyanobacteria. Greater nitrogen fixation by the added cyanobacteria was found with greater available nitrogen in the water. Also, the rate of nitrogen fixation by the introduced cyanobacteria increased when the water from Lake Chapala was filtered. This increased rate

of nitrogen fixation by the introduced cyanobacteria was attributed to the removal of an inhibitor within the water of Lake Chapala, Mexico.

The lack of nitrogen fixation in Lake Chapala was attributed to the lack of filamentous cyanobacteria. There are three factors which may have inhibited the growth or functioning of cyanobacteria: 1) The high winds mixing this shallow lake inhibited growth of chains and mat formation of cyanobacteria. 2) Wind-induced turbidity contributed to light inhibition of cyanobacteria in the water column and sediments. 3) Organism on the suspended particulates or chemical compounds adsorbed to the suspended particulates may have been an inhibiting factor.

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CHAPTER I

INTRODUCTION

Study Site

My study was conducted to quantify the rate of biological nitrogen fixation in Lake Chapala, Mexico. Previous studies by the Centro de Estudios de Limnológicos and Baylor University, 1983-1984, had determined that the ambient levels of biologically usable nitrogen in the water of Lake Chapala limited photosynthesis. Also, a one-year algal bioassay indicated that nitrogen was the limiting nutrient for algal growth in the water of Lake Chapala (Lind and Dávalos, unpublished).

During my study of Lake Chapala (summer, 1984), the levels of inorganic nitrogen were low. The ambient total inorganic nitrogen in the water column of Lake Chapala ranged from undetectable to 1.8 mg l^{-1} (Study of Lago de Chapala from 1982-1986, unpublished data, Centro de Estudios de Limnológicos). This low nitrogen content, combined with high phosphorus loading (Doyle, 1985) during the rainy season, created the conditions for a cyanobacterial bloom (which did not occur). If this bloom were present, the cyanobacteria would biologically fix nitrogen, thus enhanc-

ing the productivity in this tropical system. Therefore, I expected a peak of biological nitrogen fixation at the beginning of the rainy season.

I expected bacterial nitrogen fixation in the sediments to be important because of the shallowness of the lake. The top 5 cm of the sediments have been shown to support the majority of the biological nitrogen fixation (Keirn & Brezonik, 1971; Hanson, 1977). Bacteria on and in the top 5 cm of the sediments utilize penetrating light, the sediment nutrient sink, the substrate attachment and the lowered partial pressure of oxygen to facilitate nitrogen fixation (Chen et al, 1972; Dicker & Smith, 1980a; 1980b; 1980c).

To estimate the rate of nitrogen fixation, I initiated my study at the onset of the rainy season (June) and continued it throughout the summer. This period was expected to contain the highest nitrogen fixation rates. Rates of biological nitrogen fixation would decrease later in the rainy season due to nutrient inputs from rainfall and runoff. Logically, any inhibition of biological nitrogen fixation due to additions of nitrogen through precipitation runoff and increased river inflow would also be quantified during this period. An inverse relationship between the rate of biological nitrogen fixation by bacteria and the concentration of combined nitrogen in lake water has been established (Wetzel, 1983).

Answers to five questions were sought during my study:

1) Does the rate of nitrogen fixation change over a 24-h period? 2) Does the rate of nitrogen fixation differ spatially through the lake or over the time tested? 3) Does depth affect the rate of nitrogen fixation? 4) Does cell size of the organisms present relate to the rate of nitrogen fixation? 5) Does the rate of nitrogen fixation relate to ambient-inhibiting conditions? And if so, which conditions are more important, i.e. . . ammonia or total nitrogen (Stewart, 1971), substrate (sand preferred) (Moeller, 1978), turbulence (Horne & Galat, 1985), turbidity (fixation rates were higher with suspended clays) (Henriksson, 1981) or pH (Haystead & Stewart, 1972; Laane et al, 1980)?

Ecology of Biological Nitrogen Fixation

The concepts of productivity and limiting nutrients in aquatic systems are old ones. The most common limiting nutrients in aquatic systems are phosphorus and nitrogen (Wetzel, 1983). In highly studied temperate systems, where the drainage basin is wide and there is a low degree of slope, phosphorus is the most common limiting nutrient (Goldman & Horne, 1983). If phosphorus is supplied, then nitrogen limits productivity and also must be supplied to sustain increased primary productivity. Other factors determining productivity in temperate systems are the amount of light available (Finke & Seeley, 1978; Bergstein et al, 1981) and the water temperature (Benemann et al, 1971). For many years, this was the general model set forth for all

aquatic systems.

In recent years, primary productivity in tropical systems was found to be independent of light or temperature regulation. Primary productivity in tropical systems is frequently regulated by nitrogen limitation (Talling, 1965; Brezonik & Harper, 1969; Viner, 1973; Ganf & Horne, 1975; Gotterman, 1980; Levine & Lewis, 1984; Henry et al, 1984; Payne, 1986). Because temperature and light are usually not factors causing great variation in production of tropical aquatic systems (Ganf & Horne, 1975), the limiting nutrient has an even greater significance in regulation of productivity.

Plants can not use nitrogen in the atmospheric form (N_2). Atmospheric nitrogen is a triple-bonded molecule that is essentially inert. The usable form of nitrogen is ammonia (NH_3), with the oxidized nitrate (NO_3^-), being the most important form in primary productivity (Stewart, 1973).

Determination of nitrogen limitation in tropical systems has been made by many methods. The limiting nutrient has been identified by algal bioassays, determination of absolute concentration of ambient inorganic nitrogen or by relative concentrations of the ambient inorganic nitrogen. However, the ambient nitrogen concentrations can be very small or non-existent in highly productive tropical waters (Payne, 1986). In some tropical systems, cyanobacterial blooms persist almost permanently. These nitrogen-fixing bacteria

contribute to the nitrogen fertility of the tropical systems (Fay, 1983).

Two major sources of new, reduced nitrogen to the aquatic environment are precipitation, with its associated runoff, and nitrogen fixation (biological nitrogen fixation and industrial nitrogen fixation). Precipitation contains reduced nitrogen generated by lightning. Runoff leaches natural nitrogen compounds and industrially fixed nitrogen compounds from the soil and carries them into the aquatic system.

Biological nitrogen fixation is the direct reduction of elemental nitrogen (N_2) to ammonia (NH_3) by prokaryotes. Nitrogen fixation is an expensive process biologically. The energy of activation for nitrogenase is 30 Kj $mole^{-1}$ (1 Kcal $gram^{-1}$) between 20-30^{o}C (Anderson & Shanmugam, 1977). In legumes, bacteria use 40 Kcal $gram^{-1}$ of nitrogen converted into ammonia (10% efficiency). However, in free-living bacteria, the cost of nitrogen fixation is 400 Kcal $gram^{-1}$ nitrogen reduced (1% efficiency) (Odum, 1983). Biological nitrogen fixation occurs in normal pressure (1 atm.) with energy from excited electrons via the uncoupled Photosystem I. Photosystem I can operate without oxygen (Donze et al, 1972; Weare, 1973).

Although biological nitrogen fixation is an important process, the organisms capable of nitrogen fixation are facultative. Therefore, the presence of active nitrogen

fixation is heterogeneous. The rates of biological nitrogen fixation vary by area (Horne, 1977) and time (Finke & Seeley, 1978). Microhabitats develop patches of cyanobacteria with the formation of mats or scums. When the cyanobacteria are in the form of mats or scums, they carry out the majority of the nitrogen fixation. In nitrogen-limited tropical systems, biological nitrogen fixation can contribute almost 50% of the available nitrogen (1280 tones $year^{-1}$ of the total available nitrogen in Lake George, Africa) (Payne, 1986). The importance of biological nitrogen fixation in other tropical systems is based upon the suitability of the water for prokaryotes. Alkaline water enhances nitrogen fixation when the necessary nutrients and conditions are present (Haystead & Stewart, 1972; Horne, 1979; Laane et al, 1980).

An understanding of the methods available for detecting biological nitrogen fixation is essential to interpretation of the literature. Values in the literature often reflect the personal preference of each author. Without understanding the technique and methods used, errors in printed material can be accepted as fact. Also, due to the imperfect understanding of the nitrogenase system, background information on the structure, functioning and current research areas in biological nitrogen fixation enhance the understanding of this field.

CHAPTER II

BIOLOGICAL NITROGEN FIXATION: A REVIEW

Investigations and Techniques

Early Concepts of Nitrogen Uptake by Plants

Investigations into inorganic nitrogen (N_2) use by plants produced conflicting results for over 100 years. The theory of aerial nutrition for plants was assumed (Priestley, 1771 from Burns & Hardy, 1975 and Ingen-Housz, 1778 from Keeton, 1972) without testing shortly after the discovery that nitrogen was a major component (78%) of the atmosphere (Rutherford, 1772 from Burns & Hardy, 1975). This was based upon the ubiquity of nitrogen in living matter and the other atmospheric gases, oxygen (O_2) and carbon dioxide (CO_2) in living processes. If living organisms met their needs for oxygen (21% of the atmosphere) and carbon dioxide (0.03% of the atmosphere) by atmospheric content, surely the nitrogen requirements could be satisfied.

In 1806, Sausser tested this assumption with potted plants. He determined the source of plant nitrogen came from the soil (Sausser, 1806 from Burns & Hardy, 1975). Seven years later, Sir Humphrey Davy observed "peas and beans in all instances seem well adapted to prepare the ground. . . it seems that the azote which forms a constituent part

of (their) matter is derived from the atmosphere" (Azote was a name given to the triple bonded inorganic nitrogen, the most common form of nitrogen in the atmosphere, because it is unfit for respiration.) (Davy, 1813 from Burns & Hardy, 1975). For more than 70 years, from 1813 until 1886, results from studies on the source of plant nitrogen alternated between a mineral source (from the soil) and an atmospheric source. Although results continued to vary from one experiment to the other, the majority of the scientific community had now accepted a mineral source of nitrogen for plant nutrition.

Methods to Measure Changes in Total Nitrogen Content

The nitrogen-uptake experiments prior to 1883 used plant size and weight to quantify increased nutrition. These methods failed to show the actual amounts of nitrogen present within the tissue of the organism studied. Methods developed to quantify actual nitrogen amounts present in living tissues analyzed the changes in total nitrogen content of the test material. Changes in the total nitrogen content were measured by two oxidation methods, the Dumas analysis and the Kjeldahl procedure.

The first technique developed, the Dumas analysis, was a dry oxidative method (Dumas, 1834 from Bergersen, 1980). Organic material was oxidized in the presence of copper oxide and organic nitrogen was converted into inorganic nitrogen gas (N_2). The amount of organic nitrogen present was deter-

mined by measuring the volume of nitrogen gas produced with an analysis performed on the test material before and after the experiment. The two analyses often had very minute differences which increased the importance of experimental errors. Conflicting test results using the same organism were reported frequently (Aulie,1970).

In 1883, Boussingault made comparisons of results from the Dumas analysis for total nitrogen content. Boussingault's study supported the theory of an atmospheric source of nitrogen when he grew clover and peas in a greenhouse with nitrogen-poor, sandy soil. His results demonstrated increased nitrogen content by leguminous plants. However, his results did not define the nature of nitrogen from the atmosphere (elemental nitrogen, N_2, ammonia, NH_3, ionic ammonium, NH_4^+, or organic particulates) (Boussingault, 1883 from Aulie, 1970).

A wet oxidative method, the Kjeldahl procedure, was the second oxidative method. The Kjeldahl procedure used a digestion reagent, heat distillation and acid titrations for quantifying total nitrogen content (Kjeldahl, 1883 from Bergersen, 1980). Dried organic material was digested in a liquid phase of sulfuric acid and oxidized in the presence of a mercuric sulfate catalyst. Organic nitrogen, converted into ammonia, was collected by distillation and quantified by acid titration. The results, from analysis before and after experimentation, were compared to determine if the

nitrogen content increased in the test organism. Increases in legume plants' total nitrogen content, not found in a non-legume control, were presumed to be due to atmospheric nitrogen fixation (Fogg, 1942 from Fogg et al, 1973). Two advantages of the Kjeldahl procedure for total nitrogen content were: 1) The liquid end product was easier to determine. 2) The procedure did not require specialized equipment or training.

There were several disadvantages of the Kjeldahl procedure. These disadvantages were:1) The limit of sensitivity is 1 mg nitrogen l^{-1} which eliminated short term and small sample size experiments. 2) The experimental organism was destroyed. 3) The results made no distinction between the composition of nitrogen forms present in the test material, i.e. nitrite (NO_2), nitrate (NO_3), ammonia (NH_3^-), ammonium ions (NH_4^+), or elemental nitrogen (N_2). 4) The procedure required precision and patience. 5) The procedure failed to account for nitrogen in the form of azide, azine, azo, hydrazone, nitrile, nitro, nitroso, oxime and semi-carbazone.

A definitive study of an atmospheric nitrogen source was presented in 1886 which ended the controversy regarding the existence of the use of atmospheric nitrogen by plants. These findings were based upon comparisons of results of total nitrogen content as determined by the Kjeldahl procedure. The results of this study supported these three

facts: "1) Legumes were different from other plants; specifically, they could utilize atmospheric nitrogen (N_2), 2) This utilization, or fixation, depended on the active participation of certain microorganisms in a legume-microorganism symbiosis, and 3) The root nodules were the active centers of N_2 fixation" (Hellriegel & Welfarth, 1888 from Burns & Hardy, 1975).

With the identification of the root nodule microorganism, Rhizobium, as the symbiotic organism in legume plants (Beijerinck, 1888 from Aulie, 1970), several questions emerged. One question was whether or not root-nodule bacteria fixed atmospheric nitrogen when grown in a pure culture. Another question was whether or not there were other forms of bacteria which fixed nitrogen. These questions sparked a search for free-living nitrogen fixers (Burris & Miller, 1941). Biological nitrogen fixation by free-living organisms was found independently in soil and in water; however, the experiments failed to separate the cyanobacteria from the accompanying fungi and bacteria. Therefore, the organisms responsible for nitrogen fixation were not positively identified (soil - Frank, 1889; water - Prantl, 1889 from Fogg et al, 1973). Other researchers also found nitrogen fixation in impure cultures, but they were unable to isolate the bacteria in pure cultures, i.e. Clostridium (Winogradsky, 1893 from Burns & Hardy, 1975) and Azotobacter (Beijerinck and Van Delden, 1902 from Burris, 1972). Several

researchers obtained pure cultures of the symbiotic bacteria, Rhizobium, by 1914, but the cultures failed to fix nitrogen (Pringsheim, 1914; Glade, 1914; Maertens, 1914 from Fogg et al, 1973). The momentum for research on nitrogen fixation and performance of repeatable experimentation remained erratic due to these problems: 1) There were many problems with the pure culture techniques. 2) The nitrogenase inhibition was poorly understood. 3) The tests available were unable to detect small changes of nitrogen content (Burns & Hardy, 1975).

In 1928, results from the Kjeldahl procedure supported the fact that pure cultures could fix nitrogen. Five cyanobacteria cultures were identified as biological nitrogen fixers: Azotobacter, Clostridium, Anabaena, Nostoc, and the Rhizobium symbiont (Drews, 1928 from Burris, 1974). Previous researchers had assumed that a diazotroph (an organism which fixes elemental nitrogen) grew in the presence of elemental nitrogen and in the absence of fixed nitrogen (NO_2, NO_3, NH_3 and the ionic NH_4^+). This assumption did not allow for the many inhibiting conditions: repression by oxygen, hydrogen inhibition, light inhibition, presence of atmospheric ammonia, temperature regulation, micronutritional needs, pH tolerance range, inhibition by ammonia released by the bacteria itself, scavenging of fixed nitrogen by other microorganisms, other bacteria denitrifying the ammonia produced, impure reagents, as well as many other critical conditions (Hardy et al, 1973;

Stewart, 1973; Torrey & Lea, 1976; Horne, 1979).

Methods to Measure Changes in Volume of Nitrogen

Due to the inability of the Dumas and Kjeldahl results to determine the rate of nitrogen assimilation, manometry and the argon to nitrogen (Ar/N) ratio methods were developed. These methods measured changes in the atmospheric volume of a closed system. The first development was the Warburg manometer, which recorded changes in gas pressure (Warburg, 1923 from Burk & Lineweaver, 1930). Manometry had several advantages: 1) It was simple. 2) It was rapid. 3) It allowed for total control of the environment. 4) The test organism was not destroyed. However, there were also two disadvantages: 1) There was no measure of the rate of incorporation of fixed nitrogen for the organism. 2) There was no elucidation of the processes or intermediates in biological nitrogen fixation.

Manometry results indicated nitrogen uptake and nitrogenase inhibition in cultures of nitrogen-fixing organisms. Biological nitrogen-fixation studies, in 1928, reported the effects of partial pressures of oxygen. Partial pressure slowed the uptake of nitrogen, thus affecting the nitrogen-fixation process (Meyerhof & Burk, 1928 from Burris, 1974). Two years later, results from manometry identified the forms of reduced nitrogen which inhibited biological nitrogen fixation. These results showed that additions of nitrate, nitrite and especially ammonia inhibited the amount of

uptake of atmospheric nitrogen (Burk & Lineweaver, 1930). The age of the culture also slowed the rate of atmospheric nitrogen uptake (Cox, 1967).

Manometry was also used to clarify the role of oxygen and light in biological nitrogen fixation. These data indicated that the biological nitrogen-fixation process and the photosynthetic process were not the same. Results indicated that nitrogen uptake occurred in light conditions with photosynthesis and also continued in the dark when photosynthesis inhibition occurred. In the dark, only hydrogen was evolved by the cyanobacteria. These results supported the hypothesis that the evolution of oxygen (a waste product of photosynthesis) was not a part of biological nitrogen fixation (Cox, 1967).

After 1960, cell-free nitrogenase studies used micro-manometric techniques. The micro-manometric techniques quantified either the nitrogen uptake rates or the ammonia-production rates. Manometric results gave nitrogen-uptake rates for test material. The amount of ammonia, quantified by Kjeldahl acid titrations or the Nessler reaction with spectrophotometric determinations, gave ammonia-production rates (Burns & Hardy, 1975). The micro-manometric techniques provided an easy method for determinations of biological nitrogen fixation in small sample sizes.

The ratio of argon to atmospheric nitrogen (Ar/N) was the second method used to measure changes in atmospheric nitrogen. The test organism and atmosphere were placed in

an incubation chamber. The Ar/N ratio was immediately determined for the atmosphere within the incubation chamber. At the end of the experiment, the Ar/N ratio was again determined on a sample of the atmosphere within the incubation chamber. Any change in the ratio permitted calculations to obtain the amount of nitrogen uptake that occurred (Fay & Fogg, 1962; Stewart, 1973). Because it proved to be very insensitive, extensive use of the Ar/N ratio method did not occur (Burns & Hardy, 1975).

Indirect Methods to Identify the Role of the Heterocyst

Early research in biological nitrogen fixation used cultures of heterocystous cyanobacteria. Researchers using these cyanobacteria began to question why there were higher rates of nitrogen fixation in some cultures and lower rates of nitrogen fixation in other cultures. These researchers noted larger numbers of enlarged, hollow-looking cells (heterocysts) in cultures with higher rates of nitrogen fixation. It was assumed that these cells were responsible for biological nitrogen fixation and contained the active enzyme, nitrogenase. Colorimetry was used to test this hypothesis, by verifying the presence of a reducing environment within the heterocyst cells. Colorimetry tests used one of the following three compounds: 1) Triphenyl tetrazolium chloride (TTC) which was reduced into an insoluble, red formazan crystal (E' for TTC = +490 mV) (Stewart *et al*,

1969). 2) Nitroblue tetrazolium (NBT) which was reduced to an insoluble, blue formazan crystal (E' for NBT = +50 mV) (Fay & Kulasooriya, 1972). 3) Iodonitrotetrazolium (INT) which was reduced to an insoluble, brown formazan crystal (INT was not often used due to difficulty in detecting its small crystals). Tetrazolium salts had low redox potentials and were used extensively as artificial hydrogen acceptors to indicate general reducing activity. A microscope was necessary for detection of the formazan crystals which indicated a reducing environment within a cell (Drawert & Tischer, 1956 from Fay & Kulasooriya, 1972).

Tetrazolium salt experiments used both whole filaments and isolated heterocysts to verify a reducing environment. TTC formed red formazan deposits at the periphery of heterocyst cells (Drews, 1955 from Fay & Kulasooriya, 1972). TTC also formed red formazan crystals at bacterial attachments to heterocysts which indicated microzones of anaerobic reducing power in aerobic invironments (Paerl et al, 1981). Immature heterocysts reacted very slowly to TTC; however, the reaction was faster than in vegetative cells. NBT reacted with vegetative cells and immature heterocysts and showed granular blue deposits in 15-20 min. while mature heterocysts showed no reaction. Double-labeled experiments using TTC and NBT showed red heterocysts, blue immature heterocysts and blue vegetative cells which had a lighter blue color gradient going away from the heterocyst cell.

Vegetative cells adjacent to heterocysts formed more blue crystals (Stewart et al, 1969; Fay & Kulasooriya, 1972). Also the formation of red formazan crystals in heterocysts inhibited acetylene reduction by over 95%, however, photosynthesis, (carbon fixation as determined by radioactive ^{14}C) continued unchanged. After these studies, the heterocyst of cyanobacteria filaments were accepted as the site of nitrogen fixation (Fogg et al, 1973). The acceptance of biological nitrigen fixation in free-living organisms without heterocysts did not occur until after 1969. Gleocapsa was the first non-heterocystous organism identified as a nitrogen fixer (Wyatt & Silvey, 1969 from Carr & Whitton, 1973, 1982). Since that time, many free-living, non-heterocystous nitrogen fixers have been found (Carr & Whitton, 1982).

The disadvantages of using tetrazolium salts were: 1) Tetrazolium salts and oxygen competed for electrons. 2) The tetrazolium salts could not penetrate the multiple cell membranes in cyanobacteria heterocysts. 3) The researchers were unable to obtain functional cell-free extracts. 4) The crystal end product destroyed the test material. 5) Many nitrogen-fixing organisms were sensitive to oxygen and light, with no methods to eliminate these factors, at that time (Stewart et al, 1969; Fay & Kulasooriya, 1972).

Direct Methods to Measure Biological Nitrogen Fixation Rates

Methods that followed atmospheric nitrogen through the process of biological nitrogen fixation and incorporation into the plant biochemical pathways were developed. With the discovery of the nitrogen isotopes ^{15}N and ^{13}N, molecules of nitrogen could be traced into the chemicals and tissues of plants.

The stable isotope of nitrogen (^{15}N) was used for studies on biological nitrogen fixation by Burris and Miller in 1940 (Burris & Miller, 1941). Incorporated nitrogen was detected by an increase in the abundance of the isotope ^{15}N above the background value of 0.364% of the naturally occurring concentration of ^{15}N. The minimum accepted evidence of nitrogen fixation was an increase of 0.015 atom % of mass ^{15}N over unexposed controls (Burris & Wilson, 1946; Stewart, 1968). The ^{15}N methodology was based upon the facts that the isotope ^{15}N could not be incorporated into the cell by exchange of nitrogen through the membranes (Burris & Miller, 1941) and that plants could not discriminate between the abundant nitrogen (^{14}N) and the more rarely occurring isotope of nitrogen (^{15}N) (Burris & Wilson, 1946). The sensitivity limit of ^{15}N isotope detection by mass spectrometry was 1 μg of nitrogen l^{-1} (a 1,000-fold increase over the Kjeldahl procedure) (Stewart et al, 1969; Burns & Hardy, 1975). The ^{15}N method was a sensitive test for short-term, small-sample, and cell-free extract experiments.

Disadvantages of the ^{15}N method were many: 1) Test organisms were destroyed. 2) Crystalline derivatives of amino acids were purified from a hydrolysate (Burris & Miller, 1941). 3) Kjeldahl digestion equipment and chemicals were needed. 4) Mass spectrometry equipment was expensive and difficult to obtain (Burns & Hardy, 1975). 5) Special training for mass spectrometry was required. 6) The ^{15}N isotope was costly and difficult to obtain (Burris & Wilson, 1957).

Another ^{15}N method for following a nitrogen atom through biological nitrogen fixation, optical emission spectroscopy, has become widely used in laboratory studies of cell-free extracts. This procedure used the isotope shift in emission spectra of nitrogen when it was excited by microwave radiation. Different emission lines produced by $^{14}N_2$ (at 297.7 nm), $^{14}N^{15}N$ (at 298.3 nm) and $^{15}N_2$ (at 298.9 nm) were detected (Proksch, 1972 from Bergersen, 1980). The intensity of the bands corresponded to the concentration of the various nitrogen forms. The intensity of the bands measured by a photomultiplier was recorded as a mass spectrum. Optical-emission equipment was cheaper than most mass spectrometers, which encouraged the use of this method. The optical-emission method was useful when sample sizes of 100 µg of N_2 or larger could be used. The minimum detection level of this test was 0.10 atom % with a standard deviation of 3%. This test had two disadvantages: 1) The nitrogen

forms had to be converted into ammonia. 2) The range of sensitivity for this procedure was too wide for cell-free experiments (Bergersen, 1980).

Radioactive isotopes have not played an important role in determination of biological nitrogen fixation. The radioactive isotope of nitrogen (^{13}N) had been used as early as 1940 for biological nitrogen fixation studies. The 9.96 min. half-life restricted its use despite greater sensitivity (Campbell et al, 1967 from Bergersen, 1980). ^{13}N, a positron (B_{+max} $^{1.19}MeV$) emitter, was often incorporated as a double labeled experiment with radioactive carbon (^{14}C). Analysis on ^{13}N had to be completed within 1.5 h of freshly produced ^{13}N before the radioactivity was too low for accurate measurement (Bergersen, 1980). Analysis for photosynthesis, ^{14}C measurements, was completed at a later time. Incubation times for ^{13}N ranged from 2 to 30 min. with the same steps as the stable isotope method (Bergersen, 1980).

Disadvantages of radioactive isotopes were: 1) The researcher had to obtain state and federal licensing for handling radioactive material. 2) The work areas had to be inspected for safety in handling and disposal of radioactive material. 3) The researcher had to generate the radioactive ^{13}N (a minimal health hazard of exposure to radiation during handling of the radioactive isotope was calculated at 2 mrem per batch during preparation of the ^{13}N isotope and 4 mrem per batch for the test operation). 4) The researcher

had to obtain liquid scintillation or scanning equipment and chemicals. 5) The researcher needed training for operating liquid scintillation or scanning equipment.

The nitrogen-isotope results, which normally used ^{15}N, were the basis for increased understanding and advancements in bacterial and biochemical work. The work on biological nitrogen fixation for over 25 years (from 1940 to 1966) was based on the ^{15}N test (Burns & Hardy, 1975). Experiments using the labeled reactant $^{15}N_2$ identified rates in the process of nitrogen fixation, elucidated the nitrogenase inhibitors, identified biochemical pathways for fixed nitrogen and gave major advancements in the understanding of the nitrogenase enzyme. Confirmation of previously identified biological nitrogen fixers and identification of new organisms were made, i.e. . . Calothrix. Mastigigocladus, Westiellopsis, Chlorogloea (Burris, 1943 from Burris, 1974), Rhodospirillum, Anabaena, Nostoc (Kamen & Gest, 1949 from Bergersen, 1980), Bacillus (Lindstrom, 1949 from Bergersen, 1980), and Klebsiella (Lindstrom, 1950 from Burris, 1974). For 16 years, following the introduction of the isotopes of nitrogen, research in biological nitrogen fixation flourished followed by 20 years with sparse research.

Indirect Methods to Measure Biological Nitrogen Fixation Rates

The years from 1966 to the present represent a phenomenal resurgence of interest in the field of nitrogen fixation.

The catalyst for this renewed interest was the discovery that acetylene (C_2H_2) inhibited nitrogen fixation (a non-competitive inhibitor - Dilworth et al, 1966; a competitive inhibitor - Schodlhorn, 1965; Dilworth was later shown to be correct; from Burris, 1972). More importantly, acetylene was shown to be accepted by nitrogenase as a substrate and reduced to ethylene (C_2H_4) (Dilworth, 1966). It was demonstrated that the analytical possibilities of measuring substrate reduction by nitrogenase, with determinations made by flame ionization detection (FID) gas chromatography, were far superior to all previous methods (Hardy et al, 1968; Stewart et al, 1968; Stewart, 1971, 1973; Burris, 1974). The product from acetylene reduction by nitrogenase was found to be stereospecific (a cis addition of hydrogen) which supported the concept of an enzyme-catalyzed reaction (Dilworth, 1966).

Many other substrates capable of being reduced by nitrogenase were soon discovered. Substrates which nitrogenase reduced included: azide (Schollhord & Burris, 1966), N_2O, cyanide (Hardy & Knight, 1967), nitriles, isonitriles (Hardy & Knight, 1967), alkyne series up to CH_3DDH (CH_3CCCH_3 was not accepted by nitrogenase) (Burns & Hardy, 1975), protons added with ATP and a strong reductant were reduced to hydrogen (H_2) (Burris, 1972), nitric oxide, carbon monoxide (Dalton & Mortenson, 1972 from Burris, 1972), nitrous oxide (Mozen & Burris, 1972), and analogs of cyclo-

propene (Boothe et al, 1981). Difficulties were found with all substrates except acetylene. Most of the difficulties were due to toxicity to whole cells or difficulty in assay techniques (Sorokin et al, 1972; Martensson & Lundgren, 1984).

Acetylene-reduction tests were first advocated as a general, rapid scan technique in 1967 (Stewart et al, 1968). This technique is an indirect method with the necessity of verification via ^{15}N for quantitative results. However, for identification and comparative in situ purposes, the test is invaluable (Stewart, 1973; Martensson & Lundgren, 1984). Samples for testing of nitrogen fixation by the acetylene-reduction method use almost any type of enclosure provided the material is not porous or reactive with acetylene or ethylene. Most methods use glass or plastic vessels of fixed volumes ranging from 2 ml to 5 l. However, sealed plastic bags, metal cylinders driven in sediments and glass or plastic canopies for ecosystem studies have been used. Disposable syringes have been used successfully for assays of nodulated roots, soil cores, marine samples, freshwater samples and culture samples.

In the acetylene-reduction method, samples of cell suspensions or tissues are introduced into the test chamber. An atmosphere containing mixtures of gases and acetylene or pure acetylene is then introduced (0.2 atm of acetylene is minimum) into the test chamber. Controls for the acetylene-reduction method generally consisted of three

methods: 1) killed organisms; 2) chambers with no organisms; or 3) gas phase samples taken immediately after introduction of the acetylene. At the completion of the assay, the gas phase is removed. The gas phase from the sample is collected in other syringes or evacuated into tubes for storage before ethylene determination (Stewart et al, 1971; Hardy et al, 1973).

A FID gas chromatograph is used to quantify the gas phase from the acetylene-reduction method. The amount of ethylene in the sample is quantified (acetylene is reduced to ethylene by nitrogenase). A variety of column-packing material can be used to separate ethylene and acetylene. Some of these are: Porapak N, Q, R, and T (Hardy et al, 1973), and a 20% suspension of ethyl-N'N'-dimethyloxalamide on a 100-120 mesh acid-washed firebrick. Nitrogen is the most common FID carrier gas, but helium is also suitable.

Biological nitrogen fixation rates, in whole-cell cultures, increase with the length of incubation time. The increases continue up to 24 h and are credited to anoxic conditions within the incubation chamber itself. Therefore, short incubation times are necessary to test in aerobic environments to obtain a true representation of the biological nitrogen-fixation rates (Neess et al, 1962). Laboratory studies of cell-free extracts have found nitrogen-fixation rates increase in the presence of CO_2, specific light conditions (Saturation at 20 - 30 klux; Finke & Seeley,

1978) and added amounts of sucrose (Fay, 1965). Hydrogen evolution protects the nitrogenase system from oxygen. Several cyanobacteria cultures in aerobic conditions failed to fix nitrogen but evolved hydrogen gas. At pH 6.5, in cell-free extracts from cyanobacteria, hydrogen evolution is the only product of nitrogenase (Arnon et al, 1961; Fay, 1974).

The advantages of the acetylene-reduction technique are: 1) The sensitivity limit is 1×10^{-9} nmol of nitrogen l^{-1}. Ranges reported are from 10 nmol N mg protein^{-1} min^{-1}, the optimum rate reported in a laboratory (Stewart, 1973), to a minimum of 3×10^{-8} nmol of N l^{-1} h^{-1} (Bergersen, 1980). This sensitivity is three orders of magnitude increase of sensitivity over the ^{15}N method. 2) Standards are easily prepared and sample values easily calculated from the standards (Sorokin et al, 1972). 3) Simple gas handling techniques and equipment are used. 4) There is no need to flush existing nitrogen out of the incubation chamber if acetylene is above 0.17 atmospheres for 30 min. (Vanderhoef et al, 1975). 5) The test organism is not destroyed. 6) The test material may be sampled multiple times. 7) The test allows short incubations and rapid analysis. 8) The gas samples can be stored prior to analysis. 9) The apparatus is relatively inexpensive and mobile for in situ studies. 10) The product, C_2H_4, is quantified and identified easily by FID gas chromatography (Sorokin et al, 1972). 11) There is a built-in internal standard of C_2H_2 for ease in identify-

ing the ethylene peak. 12) The incubation and storage containers can be glass, metal or plastic (Hardy et al, 1973; Martensson & Lundgren, 1984). 13) Acetylene completely suppresses nitrogenase-catalyzed-hydrogen evolution (Anderson & Shanmugam, 1977; Peterson & Burris, 1978). 14) Reactions are stopped with many compounds: trichloroacetic acid (TCA) (Shah et al, 1972, 1977); $(NH_4)_2SO_4$ (Lannergren & Lundgren, 1974); 50% solution of mercuric chloride ($HgCl_2$) (Goreau et al, 1980); and Lugols iodine (Lannergren & Lundgren, 1974). 15) Reactions by acetylene in the gas phase are reduced because acetylene is 30 times more stable than oxygen (Picker & Smith, 1980).

Disadvantages and criticisms of the acetylene-reduction technique are: 1) Acetylene gas is very explosive. 2) Acetylene is an indirect substrate of nitrogenase (3 moles of acetylene reduced to ethylene equals 1 mole of nitrogen reduction, since acetylene needs 2 electrons and nitrogen needs 6 electrons) (Burns & Hardy, 1975). 3) Differences in the conversion factors between ^{15}N and acetylene reduction have occurred both in situ, in vitro, in vivo, and over time. The differences appear to be related to the log and lag culture growth phases (Hardy et al, 1973). Reported ratios of nitrogen reduction ^{15}N studies compared to acetylene reduction were: 1:1.4 - 1:1.8 (Stewart, 1973), 1:3 (Alimagno & Yoshida, 1977), 1:3.3 (Bergersen, 1970; Shah et al, 1972), 1:3 - 1:8 (Brezonik & Harper, 1969) and 1:6 - 1:8, due to

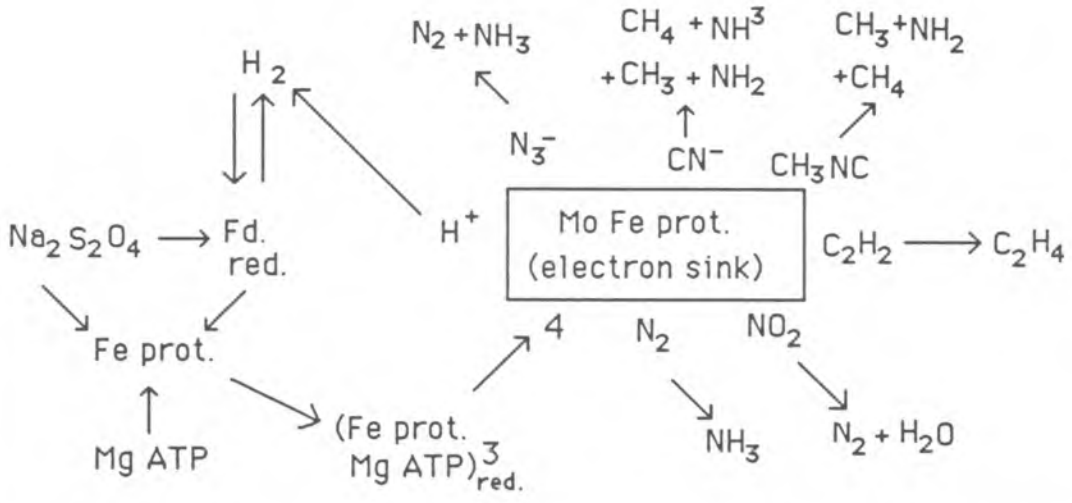
light reduction by turbidity (Flett et al, 1980; Brownlee & Murphey, 1983). 4) The measured fixation is not a true representation of actual rates due to the elimination of competitive inhibitors. The value was double the actual nitrogen-fixing rates as determined by ^{15}N methods and acetylene reduction (Flett et al, 1980). 5) There are many difficulties in comparing data from different acetylene-reduction studies. This is a result of non-standard reporting values (g l^{-1} h^{-1}, mol m^{2-1} h^{-1}, g l^{-1} hectare^{-1}, mol g sediment^{-1}, etc. (Flett et al, 1976). 6) The ethylene is unstable in some situations due to methane-oxidizing bacteria transforming ethylene into water-soluble compounds (Flett et al, 1975; Stewart, 1977). 7) The exact nature of the nitrogenase enzyme remains an enigma (Alexander, 1984; Stewart, 1985; Golden et al, 1985).

The Nature of Nitrogenase, The Enzyme

Due to variations in rates of fixation which can not be explained, an understanding of the nitrogenase enzyme is necessary. Nitrogenase has been studied extensively but still is not completely understood structurally (Alexander, 1984; Stewart, 1985) or functionally (Fay, 1983; Horne & Galat, 1985). Nitrogenase is highly conservative. The structure of nitrogenase appears to be identical in all the organisms possessing it (interchanging of the component parts of nitrogenase between different species still produces

an active enzyme). Nitrogenase is a nonspecific enzyme (almost any small molecule with a triple bond will be accepted and reduced, see Figure 1.). Nitrogenase has a tetrameric structure with two different-size components. These two components are paired iron-sulphur compounds which individually have no enzymatic activity (Mortenson, 1966; Burris, 1974). The larger component has a high molecular weight and exists as eight subunits. The smaller component has a low molecular weight and exists as four subunits. The two components of the protein are interchangeable between other genera and species of cyanobacteria and bacteria with retention of nitrogenase activity. (A modification has been found in three members of photosynthetic bacteria and also in the non-photosynthetic Azospirillum lipoferum. The combined components would not form an active nitrogenase enzyme.) (Boothe & Trebst, 1981).

The larger component of nitrogenase is known as (di)nitrogenase sensu stricto (Mo-Fe component). When purified, the Mo-Fe component is a brownish-yellow solution which is slightly sensitive to oxygen. The Mo-Fe component has a molecular mass of 360,000 daltons and contains two molybdenum atons, 30±2 iron atoms and an equal amount of acid-labile sulphur atoms. The Mo-Fe component binds the substrate and catalyzes the substrate reduction. The electron pair on the nitrogen atom retains any intermediate within the Mo-Fe component of nitrogenase (Dilworth, 1966).



1) Reduced ferredoxin ($Na_2S_2O_4$) reduces iron protein. 2) The reduced iron protein binds Mg ATP and the potential lowers to about -490mv. It also acquires the unique capacity to reduce the molybdenum iron protien of nitrogenase. The reduced molybdenum iron protein serves as an electron sink for the reduction of various substrates of nitrogenase. The molybdenum iron protein becomes reoxidized in the process. (The four sides of the box represent enzyme sites modified to give each special characteristic. N_2 and NO_2 compete for a common site; N_3^-, CN^- and CH_3NC compete for a common site: H_2 evolution via hydrogenase does not require ATP but is CO_2 sensitive, whereas H_2 evolution via nitrogenase requires ATP but is insensitive to CO_2).

Figure 1. Nitrogenase and its reactions (figure from Burris, 1974).

A separate binding site for inhibitors occurs on the Mo-Fe component of nitrogenase.

The Mo-Fe component exists as two clusters of paired subunits. Each cluster is composed of four paired subunits (two alpha and two beta). The alpha subunit has a molecular weight of about 40,000 daltons. Three tryptophan residues are in each of the alpha subunits (no tryptophan is found in the beta subunits). The beta subunit has a molecular weight of about 52,000 daltons. For the Mo-Fe component activation, an iron-molybdenum cofactor (FeMo-co) is needed. FeMo-co contains iron, sulfur and molybdenum (with $8Fe6S^{2-}$ atoms per Mo atom) (Shah et al, 1977). Six of the iron atoms are magnetic in a Mossbauer spectroscopy. FeMo-co binds reversibly with carbon monoxide (CO), thereby protecting the Mo-Fe component from this irreversible inhibitor. Many structural features of this larger component have not been elucidated to date (Fenchel & Blackburn, 1979; Bergersen, 1980; Alexander, 1984; Stewart, 1985; Golden et al. 1985). The smaller component of nitrogenase is known as (di)nitrogenase reductase (Fe component). When purified, the Fe component is a cold-labile, straw-yellow solution which is extremely sensitive to oxygen. The cell-free extracts of the Fe component lost activity when stored at 0^{o} K. Comparisons of the Fe component extracts from several different organisms showed similar physical and chemical properties with acid residues predominating over base residues (Bergersen, 1980).

The Fe component is a diamer with a moleçular mass of 72,000 daltons. Each of the four subunits is composed of 4Fe-4S clusters which have 73 amino acids and six cysteinyl residues interconnected by sulfur bridges. The cystein groups in this protein are not clustered, unlike ferredoxins. The Fe component exists as diamers which oscillate between the $(Fe_4\text{-}S_4)^{2-}$ and the $(Fe_4\text{-}S_4)^{3-}$ state (Emerich et al, 1978).

An activating compound is also needed to achieve catalytic activity in the Fe component. The activator removes an adenine-like molecule and ATP opens the site of the modifying group via a conformational change. Once activated, the nitrogenase remains active and no further action is required (Zumft, 1981). There are two non-competitive binding sites for Mg-ATP and a single binding site for Mg-ADP (see Figure 1). When the Mg-ADP site is filled, cooperation occurs in the Mg-ATP sites (McNary & Burris, 1962; Emerich et al, 1978). There is a stoichemical rate of two moles of ATP hydrolyzed per mole of electrons transferred to the larger Mo-Fe component (Boothe & Trebst, 1981; Carr & Whitton, 1982; Gottschalk, 1986). Both ATP binding sites must be filled before the Fe component undergoes conformational change. The two Mg-ATP sites have different affinities for Mg-ADP. When one Mg-ATP-binding site is filled with Mg-ATP, the electron transfer rate slows. If both binding sites are filled, inhibition of the substrate reduction occurs. The redox potential lowers approximately -100 to -200 mV from the normally active -295 mV (Zumft et

al, 1974 from Bergersen, 1980). Also, increased oxygen sensitivity occurs during inhibition. These findings are consistent with a gross-conformational change in the Fe component (Walker & Mortenson, 1973 from Bergersen, 1980).

Electrons are transferred from the Fe component to the Mo-Fe component. The transfer of electrons requires the formation of a complex which is coupled to ATP hydrolysis. The electrons are transferred at random from the Fe component to the pool of the Mo-Fe-component molecules during the transient complex formation. A lag occurs after every electron transfer, which indicates the nitrogenase proteins dissassociate (Hageman & Burris, 1979). Hydrogen evolution occurs during the lag phase and protects nitrogenase from oxygen. The 2 electrons transfer ratio was shown to be responsive to temperature and pH (Haystead & Stewart, 1972; Boothe et al, 1977; Peterson & Burris, 1978; Walker & Yates, 1978; Hageman & Burris, 1979; Stewart, 1985; Wang et al, 1985).

Based upon the assumption that the Mo-Fe component is the active nitrogen-reducing site, the specificity of molybdenum within nitrogenase is supported by previous research. A culture, deprived of molybdenum, was supplied with vanadium as a substitute (Chemically, vanadium is very similar to molybdenum in properties and size). The deprived organisms produced a vanadium-iron nitrogenase. This vanadium-iron protein is inhibitory to normal cultures.

In the nitrogen starved cultures, the vanadium-iron nitrogenase amplified the nitrogen inhibition problems of the organisms. The vanadium-iron nitrogenase is incapable of acetylene reduction and $^{15}N_2$ fixation (Fay & deVasconcelos, 1974).

Nitrogen Fixing Organisms

Only prokariotic organisms contain the enzyme nitrogenase and reduce nitrogen (N_2) to ammonia (NH_3). Diazotrophs are divided into two main groups, symbiotic and free-living. The free-living group can be subdivided into aerobes and anaerobes. The aerobic nitrogen-fixing organisms must live in an oxygen free environment. These organisms include: Clostridium, Desulfivibrio, and Desulfotomaculum. Facultative anaerobes can live in either an aerobic or anaerobic environment but they only fix nitrogen in an anaerobic environment. These organisms include: Bacillus, Klebsiella Enterobacter, Erwinia, Citrobacter, and Escherichia. The other organisms belong to the most important group of nitrogen fixers, the aerobic organisms (see Table 1). The aerobic organisms fix nitrogen into ammonia in the presence of oxygen. The most important aerobic organisms are the free-living cyanobacteria. These cyanobacteria are composed of three main groups: heterocystous-filament forms (*), non-heterocystous-filament forms (**), and unicellular forms (***) (Table 1).

Table 1. Organisms known to biologically fix nitrogen.

Order	Family	Genus	Species
Eubacteriales	Azotobacteraceae	Azotobacter	beijerinckii
			chroococcum
			paspali
			vinelandii
		Azomonas	insignis
			macrocytogenes
			agilis
		Beijerinckia	indica
			fluminensis
			derxii
		Derxia	gummosa
	Rhizobiaceae	Rhizobium	species
			aponicum
			leguminosarum
	Bacillaceae	Bacillus	macerans
			polymyxa
		Clostridium	butyricum
			pasteurianum
			saccharobutyricum
			acetobutyricum
			beijerinckia
			tyrobutyricum
			acetobutylicum
			felsineum
			kluyverii
			lactoacetophilum
			madisonii
			pectinovorum
			tetanomorphum
			butylicum
	Enterobacteriaceae	Klebsiella	pneumoniae
			aerogenes
		Enterobacter	aerogenes
			cloacae
			agglomerans
		Erwinia	herbicola
		Citrobacter	freundii
			intermedius
			Escherichia coli
Actinomycetale	Mycobacteriaceae	Mycobacterium	Flavum
			roseo-album
			azotabsorptum
	Corynebacteriaceae	Xanthobacter	autotrophicus

Table 1 Continued

Order	Family	Genus	Species
Hyphomicrobiales	Hyphomicrobiaceae	Rhodomicrobium	vannielii
Pseudomonadales	Athiorhodaceae	Rhodopseudomonas	palustris
			capsulata
			gelatinosa
			spheroides
			"x"
		Rhodospirillum	rubrum
	Thiorhodaceae	Chromatium	vinosum
			minutissimum
			sp.
	Chlorobacteriaceae	Chlorobium	thiosulfatophilum
	Ectothiorhodaceae	Ectothiorhodospira	shaposhnikovii
	Methanomonadaceae	Methylosinus	trichosporium
			sporium
		Methylococcus	capsulatus
	Thiobacteriaceae	Thiobacillus	ferro-oxidans
Pseudomonadales	Spirillaceae	Azospirillum	lipoferum
			brasilense
	Aquaspirillum	Peregrinum	fasciculus
		Desulfovibrio	desulfuricans
			vulgaris
			gigas
		Desulfotomaculum	orientis
			ruminis
Nostocales	Nostocaceae	Anabaena*	
		Anabaenopsis*	
		Aulosira*	
		Cylindrospermum*	
		Nostoc*	
	Rivulariaceae	Calothrix*	
	Scytonemataceae	Scytonema*	
		Tolypothrix*	
	Oscillatoriaceae	Trichodesmium **	
		Lyngbya **	
		Phormidium **	
		Plectonema **	
Stigonematales	Stigonemataceae	Fischerella*	
		Mastigocladus*	
		Stigonema*	
		Westiellopsis*	
Chroococcales		Gloeocapsa***	

The Ecological Importance of Biological Nitrogen Fixation

Nitrogen, an element essential to life, is biologically unusable in the abundant-atmospheric form (N_2). Sources of usable nitrogen are precipitation, runoff on the surface, groundwater drainage, and nitrogen fixation (Wetzel, 1983). Biological nitrogen fixation is the reduction of elemental nitrogen (N_2) to ammonia (NH_3) by prokariotic organisms. This is one of the fundamental processes for maintenance of the biosphere.

In undeveloped countries, which continue to have shortages of dietary protein, biological nitrogen fixation is a critical process. Protein shortages reflect a deficiency in available nitrogen. Nitrogen is essential for protein synthesis. The important role of nitrogen fixation is to continuously provide new reduced nitrogen to the environment. The free-living and symbiotic nitrogen-fixing organisms contribute significantly to the nitrogen fertility of both aquatic and terresterial habitats.

Reduced nitrogen is continuously lost by the process of denitrification. Denitrifying bacteria change reduced nitrogen (NH_3) into nitrite (NO_2). Other bacteria reduce the nitrite (NO_2) into elemental nitrogen (N_2) (Belser, 1979).

Due to the uneven distribution of nitrogen fixation, an understanding of factors in this process is beneficial. An understanding of the ecological distributions, the symbiotic and other relationships, and the physiological and

biochemical functions in biological nitrogen-fixing organisms is extremely important. It has been estimated that biological nitrogen fixation contributes approximately 175 million metric tons (80% of the new fixed nitrogen per year) to our biosphere. Lightning provides about 5 million metric tons (1% of the new fixed nitrogen per year) (Stewart, 1977). In developed countries where nitrogen fertilizer is available, biological nitrogen fixation is of lesser economic importance. The fertilizer industry provides about 60 million metric tons (20% of chemically fixed new nitrogen yr^{-1} via the Haber-Bosch process) to the biosphere. The Haber-Bosch process, costly both financially and environmentally, uses an iron-oxide catalyst and petroleum energy to compress (12 to 15 atm.) and then heat (450^{o} to 600^{o} C) dinitrogen (N_2) and liquid hydrogen (H_2). The exothermic reaction (-9 Kcal mol^{-1} ammonia) occurs and produces ammonia (NH_3) (Hardy et al, 1971; Alexander, 1984). Nitrogen fertilizer is ofter applied to soil as anhydrous ammonia.

Research in terrestrial nitrogen cycles has been extensive. The flow of nitrogen between atmospheric, terrestrial and aquatic systems has been quantified and illustrated (Figure 2). There are three processes which remove ammonia in terrestrial environments: 1) Volatilization removes 2-12% as nitrous oxides (nitrous oxides are toxic to living organisms and harmful to the ozone layer of the biosphere). 2) Denitrification removes about 20% of

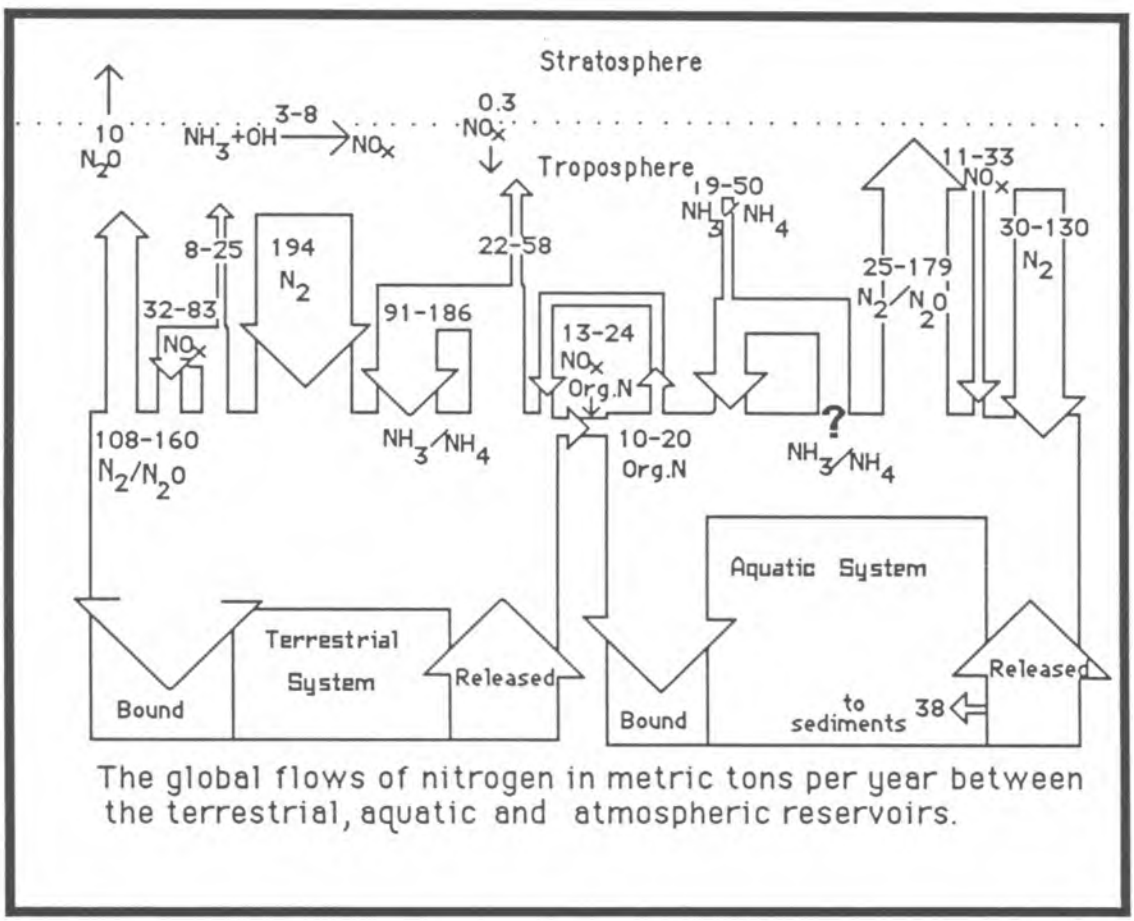


Figure 2. Global nitrogen flows (figure from Bolin & Arrhenius, 1977).

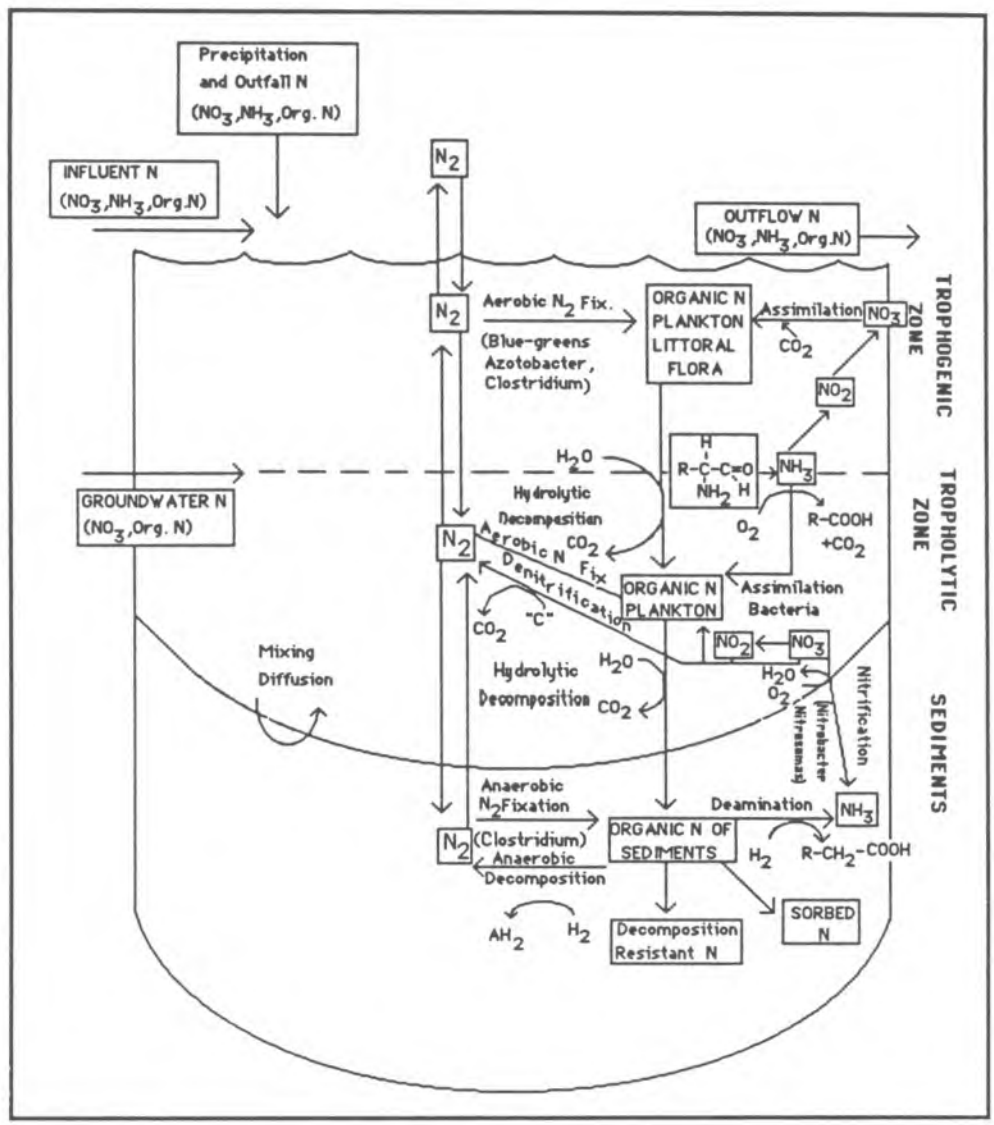


Figure 3. A generalized nitrogen cycle for fresh waters (from Wetzel, 1983).

the ammonia as gaseous N_2 and NO_2. 3) Leaching removes 43% of the ammonia into the aquatic system. The aqatic system ammonia (NH_3 and ionic NH_4^+) content losses and gains are an area of active research. Research in the nitrogen cycling for both marine and freshwater is critical for understanding of the nitrogen cycle of the biosphere. Nitrogen cycling in aquatic systems is complex and poorly understood. The sources and sinks of nitrogen in aquatic systems have been identified (Figure 3) but not quantified (Wetzel, 1983).

Knowledge of the process of biological nitrogen fixation has been slowly obtained over the last 200 years. Although research in aquatic environments began in 1889, many aspects of the organisms and the enzyme responsible for biological nitrogen fixation remain unsolved. Within the last 10 years, the blue-green algae have been renamed cyanobacteria and have been moved to be included with prokariotic organisms in the Kingdom Monera. Several bacteria have been identified as biological nitrogen-fixing organisms in aquatic and terrestrial systems, but cyanobacteria are the main nitrogen-fixing organisms in aquatic systems. Little is known about cyanobacteria in several important areas: 1) The homeostatic mechanisms which govern nitrogen fixation in aquatic habitats. 2) The relative importance between bacteria and cyanobacteria in aquatic systems. 3) The amount of nitrogen fixation for aquatic systems in warm and

tropical areas. 4) The amount of nitrogen fixation in new reservoirs. 5) The control methods specific for nitrogen fixation when this process becomes a polluting factor. 6) The exact nature of the enzyme responsible for biological nitrogen fixation. 7) Which organisms are capable of biological nitrogen fixation.

Acetylene-reduction tests advocated as a general, rapid-scan technique in 1967 (Stewart et al, 1968), have revealed the relationships between biological nitrogen fixation and productivity of the aquatic environment (one aspect of water quality is based upon productivity with oligotrophic denoting low productivity, mesotrophic denoting some productivity, and eutrophic denoting highly-productive water). Biological nitrogen-fixing organisms are rarely found in abundance in the sea. However, biological nitrogen-fixing organisms are abundant in freshwater. The role of biological nitrogen fixation in large rivers is unknown, but is not expected to be of importance except where the river flows into a nitrogen-depleted reservoir or lake. Reservoirs or lakes situated in volcanic areas are examples of such nitrogen deficient systems (Horne, 1977). Generally, lotic (lake) systems are better suited for cyanobacteria which require calm water for mat or scum formation (a zone where oxygen content is reduced) (Horne, 1977), but some cyanobacteria (Nostoc) thrive in fast-flowing streams and rivers (Horne, 1975). Although biological nitrogen-fixing organisms require

low ammonia-nitrogen content in the water, biological nitrogen fixation is most commonly associated with mesotrophic and eutrophic waters. The knowledge of how biological nitrogen fixation contributes to the ecosystem and how to regulate the process of biological nitrogen fixation is essential.

The factors that regulate biological nitrogen fixation are numerous and are not completely understood. The organisms that are capable of biological nitrogen fixation are ubiquitous. The process of biological nitrogen fixation does not occur until the organisms are stressed by lack of available nitrogen. Biological nitrogen-fixing organisms appear to modify the environment and facilitate their replacement by other organisms. The nitrogen-fixing organisms "bloom" (increase to very high numbers and fix nitrogen) after a stress period in the aquatic environment, i.e. . . at ice melt in the Arctic (Watanabe et al, 1979), at spring and fall overturn in temperate lakes (Horne & Goldman, 1972; Horne et al, 1972) and after the dry season in the tropics (Viner & Horne, 1971; Ganf & Horne, 1975). Some environments represent continuing stresses, i.e. . .hot springs (Watanabe & Yamomota, 1971) and oxygen deficiency in permanently stratified lakes, where these organisms are continuously found in abundance.

Testing for organisms capable of nitrogen fixation has been by the trial-and-error method because fixation varies

and is not correlated with bacterial presence or presence of heterocysts by depth or by time. In Clear Lake, two different fixation peaks were found to occur in a 24-h period. One peak of nitrogen fixation began at dawn and had a high at about noon. A second nitrogen-fixation peak began at dusk with a high at midnight. If cloudy conditions occur during the 24-h period prior to testing, then the nocturnal period of nitrogen fixation did not occur (Horne, 1979). Some other factors that affect the rate of nitrogen fixation are: 1) A small amount of molybdenum must be available for active enzyme formation. 2) Copper must be present in the water column (nitrogen fixation stops if copper remains below 10 $\mu g\ l^{-1}$). 3) The range of pH is from 5.0 to 10.5 (an optimum range is 6 to 9). 4) The amount of iron present is 10 $\mu g\ l^{-1}$ or more. 5) The amount of NH_3 is below 100 $\mu g\ l^{-1}$. 6) The amount of NO_3 is below 28 $\mu g\ l^{-1}$. 7) Turbulence in the water column, especially at the surface, must remain low. 8)Turbidity within the water column is low (light is critical for energy storage in free-living nitrogen fixers). Also, there are many micronutritional needs for biological nitrogen fixation. Because knowledge of the true nature of the nitrogen-fixing process is still elusive, research continues by adding and deleting the suspected requirements of nitrogen fixation.

Hourly rates of biological nitrogen fixation in areas around the world, as determined by the acetylene-reduction

method, have been calculated. The majority of funding for research in biological nitrogen fixation, and therefore the majority of tested areas, has been in developed countries. Rates of nitrogen fixation in aquatic environments all over the world are reported. Values for maximum rates of biological nitrogen fixation for the study site were used (Table 2).

Table 2. Rates of nitrogen fixation for different areas of the world by lattitude. Nitrogen fixation rates were determined by the acetylene reduction method. Values for biological nitrogen fixation are reported in nmol N_2 $l^{-1}hr^{-1}$.

Rate of Fixation	Location (Researchers)
Arctic (Above 50°)	
0.00016	Lake Erken, Sweden (Lannergren & Lundgren, 1974)
0. - 29.	Copenhagen, Denmark (Ahmad, 1981)
89	Prairie Lake, Canada (Brownlee & Murphy, 1983)
0. - 0.007	Shield Lake, Canada (Flett et al, 1980)
Temperate (Between 50° & 30°)	
0. - 0.303	Lake Mendota, WS (Goering & Neess, 1964)
4.6	Lake Mendota, WS (Stewart, 1971)
0.21 - 0.025	Lake Mendota, WS (Torrey & Lea, 1976)
0.01 - 6.6	Green Bay, WS (Stewart et al, 1971)
0.03 - 0.07	Green Bay, WS (Mague & Burris, 1973 from Burris, 1974)
0. - 29.3	Lake Superior, WS (Rusness *et al*, 1970 from Burris, 1974)
0.04 - 0.4	Sanctuary Lake, PN (Dugdale & Dugdale, 1962)
0.27 - 2.5	Clear Lake, CA (Horne & Goldman, 1971)
0.06 - 0.2	Clear Lake, CA (Horne, 1979)
0. - 0.11	Smith Lake, AK (Billand, 1966 from Bergerson, 1980)
Tropical (Between 30° N and 30° S)	
0.18 - 1.66	Lake Mary, FL (Brezonik & Harper, 1969)
2.96 - 10.99	Lake Mize, FL (Brezonik & Harper, 1969)
0.003 - 0.12	Lake Mize, FL (Keirn & Brezonik, 1971)
53.	Peel-Harvey Estuary, Australia (Huber,1986)
31. - 37.	Lake Valencia, Venezuela (Levine & Lewis,1984)
1.36 - 17.84	Lake George, Africa (Viner & Horne, 1971)
0.02 - 0.03	Lake George, Africa (Ganf & Viner, 1973)

CHAPTER III

MATERIALS AND METHODS

Site

Lake Chapala, the largest lake in Mexico, is located $102^{o}41'$ - $103^{o}25'$ W and $20^{o}06'$ - $20^{o}21'$ N (Figure 4). It is a massive graben in an ancient series of tertiary lakes in the Lerma drainage system (Serruya & Pollingher, 1983). Lake Chapala is a tectonic trench with an east-west cul-de-sac shape. The Rio Lerma inflow is located within 4 km of the Rio Grande de Santiago outflow on the eastern end of the lake (Figure 5). The lake is 1524 m above sea level. The surface area of Lake Chapala is approximately 1109 km^2. The volume is 7,962 x 10^6 m^3 (Serruya & Pollingher, 1983). The average depth of Lake Chapala is 5 m and the fetch (length) is 77 km. Lake Chapala is polymictic and well oxygenated. The temperature range of the water is 20^{o} - 24^{o} C (Serruya & Pollingher, 1983). The bottom fauna is mostly tubificids and particularly meager, however, fish production is rated at 1,000 tonnes $year^{-1}$ (Serruya & Pollingher, 1983). The soil of the region is volcanic ash and poor in nutrients (Anonymous, 1981).

Previous studies showed that Lake Chapala was nitrogen

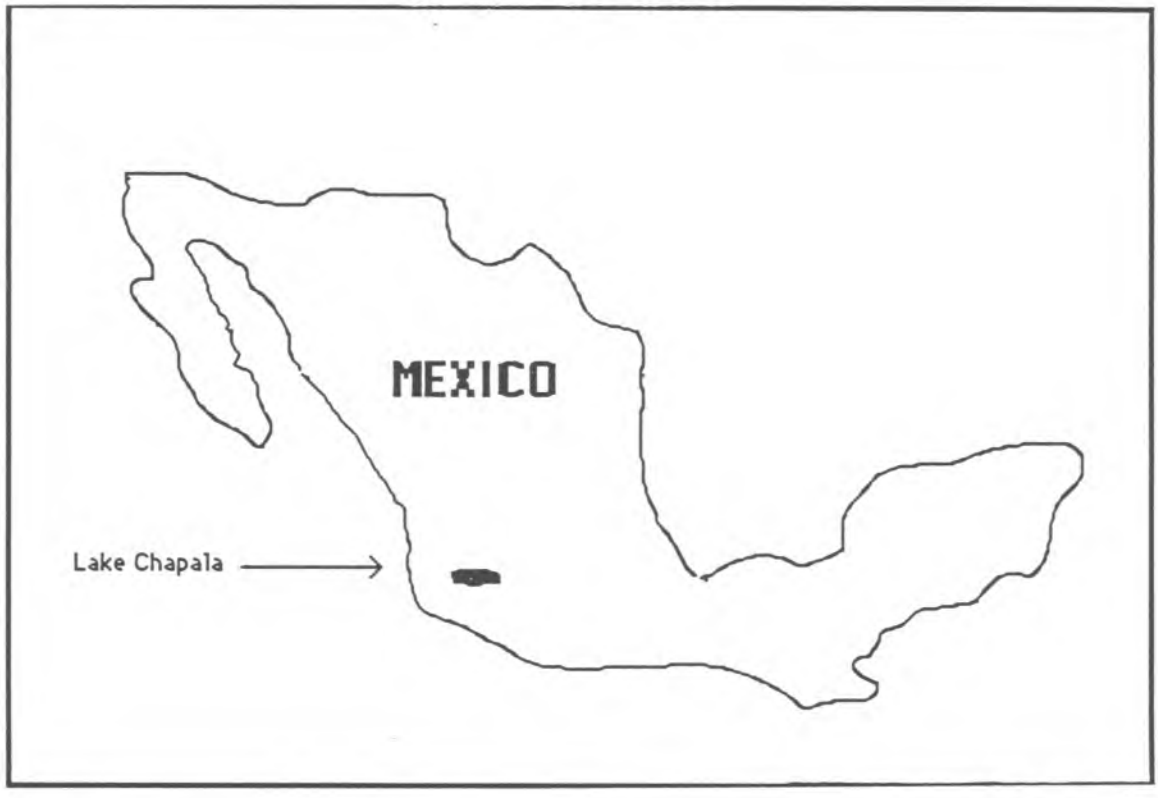


Figure 4. Lake Chapala is located in the southwestern region of Mexico, in the states of Jalisco and Michoacan.

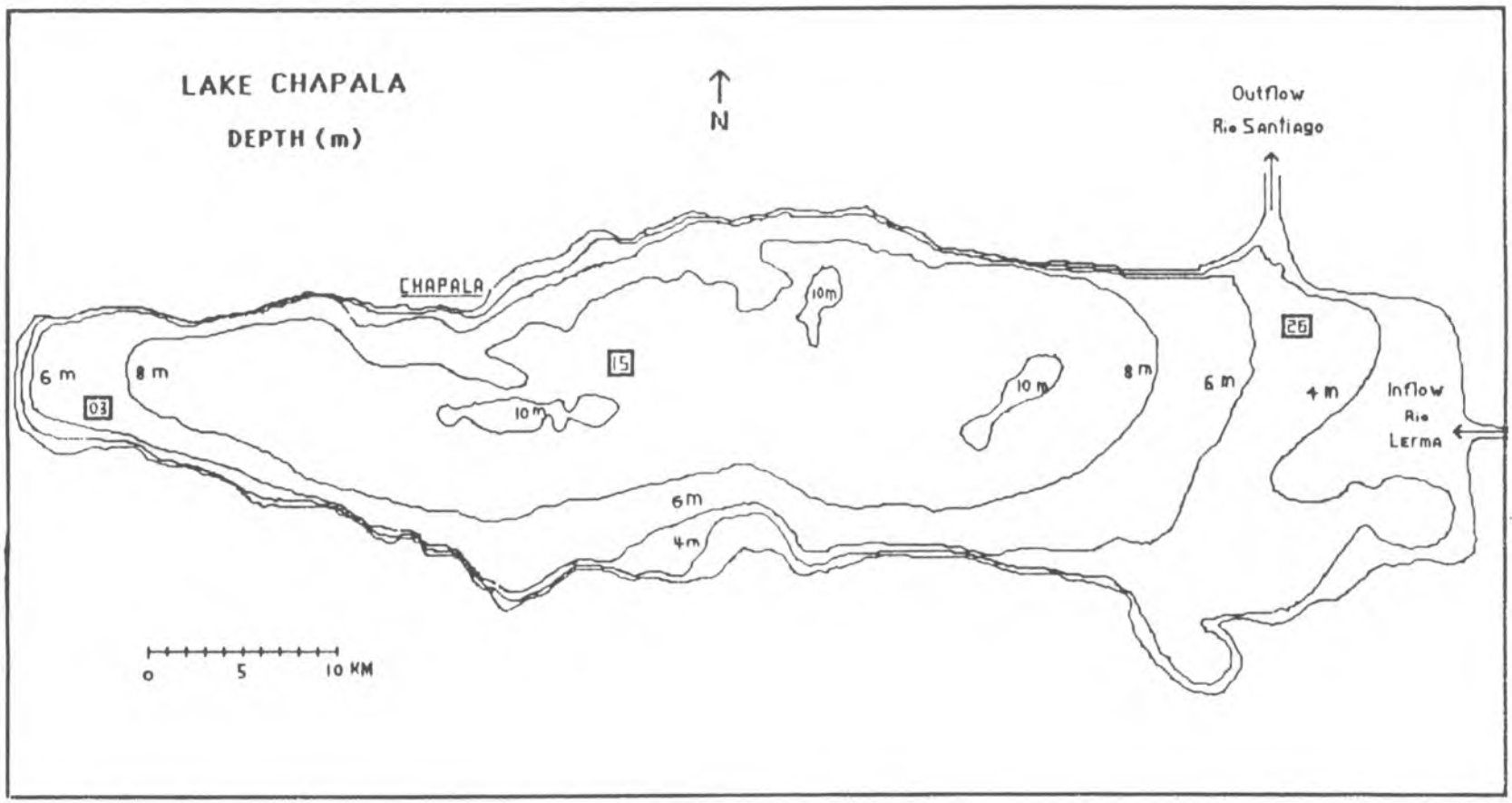


Figure 5. Lake Chapala, Mexico. Stations sampled represented by □. The inflow and outflow are on the eastern end of the lake. The launch site was the town of Chapala on the northern shore.

limited (Anonymous, 1981; Serruya & Pollingher, 1983; Limón, unpublished). The amount of ambient-inorganic nitrogen follows an east-west gradient with inorganic nitrogen often undetectable at the western end of the lake (Table 3). Average lake-wide values for nitrogen in 1984 were 80 $\mu g\ l^{-1}$ for ammonia-nitrogen, 240 $\mu g\ l^{-1}$ for nitrate-nitrogen and 750 $\mu g\ l^{-1}$ for organic-nitrogen (Limon, unpublished). The total-inorganic nitrogen (mg l^{-1}) to total-reactive phosphorus (mg l^{-1}) ratio needs to be above 12 for optimum algal growth (Smith, 1982). A ratio below 12 indicates a lack of available nutrients needed to support the metabolism of algae. This ratio remains less than 9 in Lake Chapala (Anonymous, 1981).

Methods

Stations

Three stations were chosen for sampling across Lake Chapala (Figure 5). Ambient levels of nitrogen at these stations represented the range of nitrogen limitation present in Lake Chapala. Station 26 was at the eastern end of the lake near the Rio Lerma inflow. Station 26 represented the high values of nitrogen due to the inflow of nitrogen from the river. The amount of nitrogen in water from Station 15 contained the medium level of ambient nitrogen found in the main body of the lake. Water from Station 03 was low in ambient nitrogen and represented the nutrient depleted western end of the lake. Samples were

Table 3. Mean values of inorganic nitrogen from 1972 to 1984 for three stations on Lake Chapala, Mexico. Nitrogen limitation is most extreme in the western end (Station 03) of this cul-de-sac lake. The inflow and outflow are located at the eastern end of the lake (Station 26). Values are in $\mu g\ l^{-1}$.

Nitrogen Form	Station 03	Station 15	Station 26
Ammonia-Nitrogen	164	112	219
Nitrate-Nitrogen	295	348	453
Kjeldahl-Nitrogen	682	658	874

(from Lind *et al*, 1987 manuscript)

collected bi-weekly, from June until August, 1984. A total of twelve samples were taken.

Sampling and Incubation

Water samples were obtained early in the morning. The water samples were collected between 6 and 9 am. Samples were taken at 2 m intervals at each station. The water was pumped on board into a 500 ml jar. For each sample, the 500 ml jar was allowed to overflow more than three times the jar capacity to remove any contamination by previous samples. A sediment sample was obtained with an Eckman dredge and placed in a polyethylene bucket. Each lake-water sample and the sediment sample was then prepared for incubation.

Incubation of water and sediment samples was achieved by preparing triplicate test chambers for each sample. The test chambers consisted of 50 ml disposable syringes with needles. Water from a sample was pulled into the syringe, and then immediately after,10 ml of acetylene was pulled into the syringe. The chambers were sealed by inserting the needle into a rubber stopper (Hardy et al, 1971). Test chambers (syringes) with the water or sediment sample were prepared to simulate the dark and light reactions of nitrogen fixation. Triplicate water or sediment samples were covered in aluminum foil to simulate the dark reactions. These samples were incubated in the boat in closed lake-water-filled ice chests painted black on the inside (Stahl et al, 1984; Henry et al, 1984; Huber, 1986). The remaining

water or sediment samples were incubated in the boat in open lake-water-filled ice chests. Samples were incubated 4 to 10 h. Incubation temperature of the ice chest was maintained at ambient lake temperature by adding lake water.

From the middle of July until the end of the study in August, all samples from Lake Chapala were returned to the laboratory in Guadalajara rather than incubated in the field. In the laboratory, I determined if the lake water could support artificially-induced biological nitrogen fixation. These lake-water samples were combined with a culture of known nitrogen-fixing cyanobacteria (Anabaena sp.). Incubation of the combined lake-water-with-cyanobacteria samples was carried out in an outdoor pond. The prepared lake-water-with-cyanobacteria samples were incubated at a depth of 1 m.

The Anabaena culture was maintained in the laboratory prior to use. The culture stock was obtained at a local pond. The Anabaena was present in many of the recently flooded ponds that had large populations of water hyacinths. Prior to mixing the Anabaena culture with the lake-water samples, the Anabaena was shaken and course filtered through a 1 mm screen to remove cyanobacteria clumps. Portions of the Anabaena culture were placed in a separate Erlenmyer flask for each type of sample being quantified. These portions contained 13 ml of the culture per sample to be prepared in a test. Water was then added to each Erlenmeyer flask and the flask was mixed by stirring with a clean glass

rod. When the sample was thoroughly mixed, the water-cyanobacteria sample was pulled into the test chambers. Water-cyanobacteria samples were incubated at a depth of 1 m for 4 to 10 h in an outdoor pond.

Tests were conducted for oxygen inhibition of biological nitrogen fixation. First, inhibition by the presence of oxygen was assessed. Oxygen was removed from three lake-water samples by bubbling helium through the water samples (Stahl et al, 1984) before pulling the water sample into the syringe. The samples were then incubated for 5 h. Another inhibition, the lack of sufficient oxygen, was assessed. Triplicate lake-water samples were prepared using 5 ml of acetylene and 5 ml of ambient air. These samples were then incubated for 5 h. Tests for inhibition were repeated in triplicate with lake-water samples and with lake-water-cyanobacteria samples. The ability of the cyanobacteria (Anabaena sp.) to fix nitrogen was verified by preparing test samples in triplicate prior to and along with each test.

I also researched possible inhibition of biological nitrogen fixation by particulates. These tests were divided into two parts. The two types of particulates were course particulates and fine particulates. The first type of particulates consisted of planktons capable of grazing on the nitrogen fixers (Lampert et al, 1986) and large algae which would compete for nutrients. Tests for course particulates included three treatments: 1) A test using

lake-water-cyanobacteria samples with no manipulation. 2) A test using course-filtered lake-water-cyanobacteria samples. 3) A test using lake-water-cyanobacteria samples with the course particulates doubled. Inhibition from grazing planktors and large algal forms was quantified by filtering water samples through a 64 μm plankton net (Lind, 1979) before assaying for nitrogen fixation by lake-water-cyanobacteria samples. The filtered plankton were then added to other samples to double the grazing planktons and large algal forms. The doubled plankton-lake-water samples were then combined with the Anabaena culture, prepared and incubated as described previously.

The second type of particulates was small organisms and suspended clay particulates. Inhibition related to competition by small organisms and any sorbed compounds or organisms on clay particulates suspended in the water was quantified. Tests for fine particulates included three treatments: 1) A test using lake-water-cyanobacteria samples without manipulation. 2) A test using filtered lake-water-cyanobacteria samples. 3) A test using lake-water-cyanobacteria samples with double the fine particulates. First, the lake water was gently filtered through a plankton net and then gravity filtered through a 0.2 μm Nucleopore filter (Lind, 1979). Lake water for twelve samples was filtered through the plankton net. The filtered water was then placed in the filtration column and when half of the water

had been filtered, filtration was stopped. The unfiltered water was shaken to resuspend the small organisms and fine particulates (thereby doubling the amount of small organisms and fine particulates). Water samples were prepared in triplicate with filtered water (small organisms and particulates removed) and unfiltered water (small organisms and particulates doubled). The water samples were assayed for nitrogen fixation by adding the Anabaena culture to the water samples after the filtration process.

Cyanobacteria (Anabaena sp.) were found on hyacinth mats in the ponds surrounding Lake Chapala. Therefore, water samples from within hyacinth mats in Lake Chapala were also sampled for biological nitrogen fixation. These samples were obtained within the floating hyacinth mats just below the surface of the lake. Water samples were returned to the laboratory and prepared without adding the cyanobacteria culture. Incubations for five replicates were carried out in the outdoor pond at a depth of 1 m.

The Acetylene Method for Analysis of Nitrogen Fixation

Nitrogen fixation was measured using a modification of the acetylene-reduction thchnique (Stewart et al, 1968). The acetylene-reduction technique was developed on the non-specificity of the nitrogenase enzyme. Active nitrogenase reduces acetylene into ethylene within

the gas phase of the test chamber. At the end of the incubation time, all biological activity is terminated and a sample of the gas phase is removed and tested for ethylene.

Modifications were made to facilitate the conditions of Lake Chapala, Mexico. The modifications used were: 1) in situ and laboratory incubations; 2) larger sample sizes (40 ml) (Horne & Goldman, 1972); 3) use of syringes for incubation chambers (Horne et al, 1979); and 4) only acetylene in the chamber atmosphere (Flett et al, 1976).

At the end of the incubation period, the gas phase containing the acetylene, and any ethylene produced, was removed from the incubated test chamber (syringe) and analyzed. This was accomplished by injecting the gas phase of the incubation chamber into 5 ml vacutainers (Hardy et al, 1973). On the following day, the gas phase was analyzed on a Schmadsu GC-4C flame ionization detection (FID) gas chromatograph. The nitrogen carrier gas flow was 30 ml min^{-1}. The injection port temperature was 110°C with the column temperature at 47°C. Column packing was Porapak N (Hardy et al, 1973) in a stainless steel column. FID gas chromatograph signals were recorded by a Perkin & Elmer 56, 10 inch chart recorder attached to a Varian CDSIII digital integrator. Calibration of the chromatograph was by ethylene gas standard (Scotty Gas) and a series of standards of ethylene diluted in acetylene.

The values of ethylene obtained from FID gas chromatography were then converted in nitrogen fixation potential. Ethylene production was equated with nitrogen fixation by using the chemical formula ratio of 3:1 (ethylene to ammonia produced). The 3:1 ratio was used since no verification by the isotope of nitrogen (^{15}N) could be obtained and precedent has been for the balanced chemical equation (three acetylene molecules reduced equivalent to one nitrogen molecule reduced to ammonia).

A control is used to quantify contamination. A control consists of the sample which has all biological activity stopped prior to introduction of the acetylene. A control was necessary due to ethylene contamination within tank acetylene. Tests were made on three possible methods for control. The methods were two common killing agents to stop biological activity (TCA and $HgCl_2$) and taking a sample of the gas phase before incubation. Actual nitrogen-fixation rates would then be a value above the background ethylene contamination found in the tank acetylene (mean control value for that sample).

When tank acetylene is used for incubations in the acetylene-reduction technique, a control is necessary. Because a control carried through all the steps of a process best replicates the true conditions of the test, controls using a killing agent would be treated the same as samples and would be preferred. The killing agents tested were

trichloroacetic acid (TCA) (Stewart et al, 1968; Brezonic & Harper, 1969; Flett et al, 1976; Stahl et al, 1984), mercuric chloride ($HgCl_2$) (Goreau, 1980). Also, a gas phase sample taken immediately after inoculation of the test chamber with acetylene was compared with a gas phase sample from the same chamber after incubation (Thake & Rawle, 1972; Horne & Carmiggelt, 1975).

Development and Validation of Methods

The acetylene-reduction technique uses a series of standards of ethylene to calibrate the FID gas chromatograph. These standards can also be used to verify the relationship of the concentration of ethylene in the water samples. The values from a series of dilutions of ethylene are used to prepare a standard curve. The ethylene diluted in acetylene was prepared in an open-ended vial submerged in a beaker to simulate the conditions in a test chamber. The standard curve included the ranges reported in the literature for nitrogen fixation (1% to 50% composition of ethylene) (Figure 6). The water samples from Lake Chapala, with the cyanobacteria culture added, were within the lower end of this range.

Data Analysis

Student's t tests were used to compare the mean values of nitrogen fixation for samples with the control for each test. Additional statistical analyses were

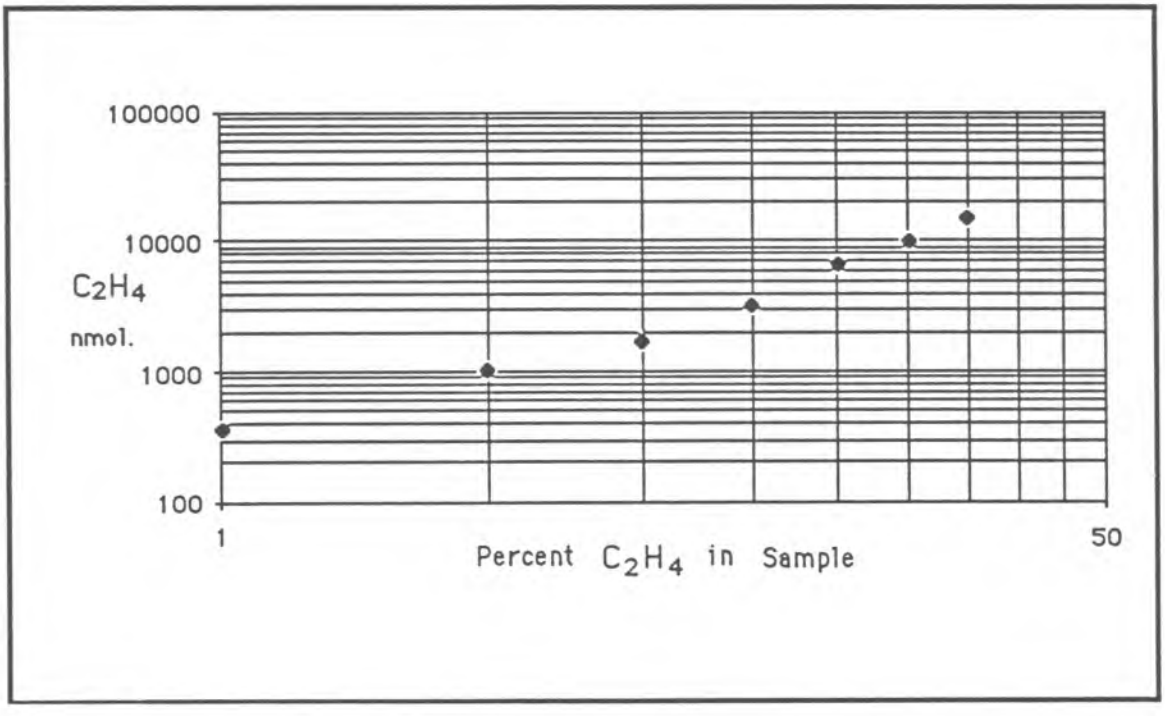


Figure 6. Standard curve of ethylene in acetylele. Ethylene concentrations ranged from 1% to 50% of the gas composition. Determination was by FID gas chromatography.

performed on the nitrogen-fixation results from lake-water-cyanobacterial culture samples. A 2-way analysis of variance (ANOVA) was used to compare means between depth and station in the tests for nitrogen fixation with the lake-water-cyanobacteria samples. Pairwise comparisons were made using Student-Newman-Keuls test to compare the mean nitrogen fixation values between depths from the lake-water-cyanobacteria samples. A 3-way ANOVA was used to test means of nitrogen fixation of the lake-water-cyanobacteria samples by station by depth. Statistical data analysis were done with the statistical programs of BMDP Statistical Software (Dixon, 1985) and the Statistical Package for the Social Sciences (SPSSx) (SPSS, Inc., 1986).

CHAPTER IV

RESULTS

Nitrogen Fixation in Lake Chapala

Only one lake water sample had nitrogen fixation during the entire summer sampling period ($p < 0.05$). The one sample was on August 6th in the surface waters of a recently flooded bay at the Chapala Town Dock (Table 4). Although testing was performed from June through August, no other Lake Chapala water samples had nitrogen fixation. There were no heterocystous cyanobacteria found in the lake water during the entire study. The common cyanobacteria found in Lake Chapala was a non-heterocystous, unicellular form.

Without biological nitrogen fixation, I was unable to answer the originally posed questions. During the diel study, there was no nitrogen fixation by any sample (the first question) (Table 5). Likewise, in the study of spatial variation (the second question) there was no biological nitrogen fixation at any station (Table 6).

Due to the lack of biological nitrogen fixation, I sought to determine if the water from Lake Chapala was capable of supporting nitrogen fixation. Lake-water-cyano-

Table 4. Mean (± 2 sd, N = 5) Rates of nitrogen fixation from hyacinth mats in Lake Chapala, Mexico. Estimation of nitrogen fixation was by acetylene-reduction technique. Values are reported in 10^{-5} nmol of N_2 l^{-1} h^{-1}.

Hyacinth mat in bay at Town Dock	11.47±1.06*
Hyacinth mat out from Town Dock	9.12±1.68
Hyacinth mat near Station 15	9.92±1.23
Hyacinth mat near Station 03	9.43±1.26
Hyacinth mat in front of Yacht Club	10.09±1.76
Control	9.11±1.24

* Significant $p < 0.05$

Table 5. Ambient rates of biological nitrogen fixation at Station 15 over a period of 14 hours on June 17, 1984 in Lake Chapala, Mexico. Values reported are means and two standard deviations for estimated nitrogen fixation in samples incubated in the light and samples incubated in the dark. Nitrogen fixation is estimated by the acetylene reduction method. Values are reported in 10^{-5} nmol N_2 l^{-1} hr^{-1}.

	6 am	9 am	12 pm	5 pm	8 pm
1 m:					
Light	3.71± 1.10	3.95±0.72	1.35±0.37	4.64±1.17	0.
Dark	2.83±0.84	3.20±0.25	2.07±0.81	2.85±0.18	3.74±2.66
Control	3.84±1.06	3.06±0.84	2.61±0.89	3.53±0.87	3.93±1.98
2 m:					
Light	2.63±0.44	5.27±0.30	2.42±0.93	0.	2.64±1.25
Dark	4.13±0.63	3.90±0.85	3.00±0.33	4.54±1.33	3.14±0.98
Control	3.76±0.58	4.56±0.59	3.84±2.04	2.73±0.83	3.67±1.37
4 m:					
Light	3.44±0.89	4.20±0.19	2.32±0.86	4.97±1.62	0
Dark	2.56±0.59	4.00±0.25	2.26±0.80	0	2.73+0.47
Control	3.73±0.89	4.83±0.67	2.10±0.28	4.72±2.10	3.09±0.62
Sediments:					
Light	2.10±0.36	4.80±0.19	3.95±2.11	0.	0.
Dark	1.95±0.42	3.20±0.19	3.56±1.48	1.07±0.29	5.00±1.65
Control	2.27±0.53	3.73±1.04	3.15±1.08	1.17±0.63	5.27±1.78

Table 6. Mean (± 2 sd, N=3) rates of biological nitrogen fixation from June till August, 1984 for three stations in Lake Chapala, Mexico. No statistical difference between experimental and control samples was found, except 24 July at 2 meters. Values are reported in 10^{-5} nmol N_2 L^{-1} hr^{-1}. E = Experimental: C = Control.

Date	Depth	Station 03	Depth	Station 15	Depth	Station 26
06 June						
	-		1m	E 0.	1m	E 0.
	-			C 1.33±1.05		C 1.18±1.28
	-		2m	E 1.51±0.73	2m	E 0.
	-			C 1.97±0.80		C 2.06±1.49
	-		4m	E 3.33±1.14		-
	-			C 3.88±1.63		-
	-		Sed.	E 2.54±1.02	Sed.	E 0.
	-			C 2.48±0.72		C 1.26±0.80
17 June						
	-		1m	E 3.71±1.10	1m	E 4.96±1.80
	-			C 3.58±1.07		C 4.23±1.81
	-		2m	E 2.62±0.44	2m	E 4.63±1.28
	-			C 2.91±176		C 4.73±1.70
	-		4m	E 2.55±0.59		-
	-			C 2.90±1.92		-
	-		Sed.	E 2.10±0.36	Sed.	E 0.
	-			C 2.90±1.92		C 0.
28 June						
	-		1m	E 0.	1m	E 2.54±0.60
	-			C 0.		C 1.67±0.42
	-		2m	E1.43±0.83	2m	E 1.04±0.22
	-			C 2.58±1.96		C 1.73±0.70
	-		4m	E1.46±0.47		-
	-			C 2.06±0.37		-
	-		Sed.	E 1.98±0.49	Sed.	E 2.27±0.31
	-			C 1.75±1.21		C 1.83±0.73
11 July						
	-		1m	E 3.91±0.80		-
	-			C 3.39±0.89		-
	-		Sed.	E 4.85±1.88		-
	-			C 4.03±1.42		-
13 July						
	1m	E 3.47±0.74		-		-
		C 2.21±0.72		-		-
	Sed.	E 2.85±0.88		-		-
		C 3.03±1.21		-		-

Table 6. continued

Date	Depth	Station 03	Depth	Station 15	Depth	Station 26
16 July						
		-		-	1m	E 2.47±0.90
		-		-		C 3.64±0.56
24 July						
	1m	E 1.11±0.46	1m	E 9.85±0.62		-
		C 1.06±0.94		C 8.89±0.37		-
	2m	E 1.10±1.08	2m	E 8.78±1.03		-
		C 1.89±1.21		C 3.91±1.06		-
27 July						
		-		-	1m	E 1.71±0.42
		-		-		C 0.96±0.95
31 July						
		-	1m	E 4.06±0.84		-
		-		C 4.85±1.10		-
02 August						
		-	1m	E 1.04±0.56		-
		-		C 0.91±0.48		-

bacteria samples (v/v 3:1 lake water to Anabaena sp. culture) were used. The values for biological nitrogen fixation by the lake-water-cyanobacteria samples were standardized by calculating the amount of biological nitrogen fixation per heterocyst in the culture added to the lake-water sample. Counts of heterocysts were made each time the Anabaena culture was used.

Lake water from Station 26 supported the highest rate of nitrogen fixation by the introduced culture. The lowest rate of nitrogen fixation by the introduced culture occurred in the lake water from Station 03. An intermediate value for the rate of nitrogen fixation was found in the lake water from Station 15 (Tables 7 and 8). Note that the rate of biological nitrogen fixation from water at Station 03 and Station 26 was not statistically different from the rate of biological nitrogen fixation in the lake water at Station 15. However, the rate of biological nitrogen fixation in the lake water from Station 26 and Station 03 were statistically different ($p < 0.05$).

A 2-way ANOVA compared the rate of biological nitrogen fixation by the introduced Anabaena sp. for the water samples taken from a depth of 1 m and 2 m at all three stations. The significant mean variations were due to station and not depth ($p < 0.05$) (Table 8).

The fourth question was to relate cell size to rates

Table 7. Mean (± 2sd, N=10) nitrogen fixation rates per heterocyst in water from a depth of 1 m at three stations of Lake Chapala, Mexico. Samples of water from the station were combined in a 3:1 ratio (v/v) with a culture (*Anabaena sp.*). Values are in 10^{-9} nmol of N_2 l^{-1} hr^{-1}. Station 03 and Station 26 are statistically different ($p<0.05$).

Station 03	Station 15	Station 26
72.31+15.70	81.87+28.82	126.01±31.07

Table 8. Mean (± 2 sd, N=5) biological nitrogen fixation values per heterocyst by a cyanobacterial culture mixed with water from Lake Chapala, Mexico. Samples contained a 3:1 v/v ratio of lake water and culture mixture. Values are in 10^{-9} nmol N_2 l^{-1} hr^{-1}. Note that at 1 m Station 03 and 15 are not different but at 2 m these stations are statistically different.

Treatment	Depth	Station 03	Station 15	Station 26
Lake Water with Cyano-bacteria added:				
	1m	48.50±8.72	66.31±23.82	71.32±17.59
	2m	59.67±5.80*	97.39±6.07*	No Sample
Filtered Lake Water with Cyanobacteria added:				
	1m	83.45±8.72	81.38±6.95	125.37±2.56
	2m	97.74±18.02	108.13±6.52	No Sample
Metazoans Removed:				
	1m	No Sample	83.56±33.82	43.18±9.82
Metazoans Doubled:				
	1m	No Sample	59.91±10.43	37.08±10.54
Fine Particulates Removed:				
	1m	No Sample	101.46±44.15	164.93±25.83
Fine Particulates Doubled:				
	1m	No Sample	25.52±6.45	21.77±2.87

* Significant $p < 0.05$

of nitrogen fixation. This question was originally based upon the idea that a certain fraction of the cyanobacteria community would be playing a dominant role as the biological nitrogen-fixing organism. No ambient nitrogen fixation was found in Lake Chapala, and therefore, this question could not be answered.

The fifth question addressed the possible biological nitrogen fixation controlling factors in the water of Lake Chapala. Due to the lack of biological nitrogen fixation in Lake Chapala, this question became the most important aspect of this study. Removing or adding the metazoans, filamentous green algae, diatoms and other large particulates had no effect on the rate of nitrogen fixation for the added cyanobacterial culture. However, the removal or addition of fine particulates (0.2 μm to 64 μm) had a significant effect on the rate of biological nitrogen fixation for the added cyanobacteria (Table 8). Removing the fine particulates increased the rate of nitrogen fixation by the introduced cyanobacteria at all stations in this study. The increase in the rate of nitrogen fixation was less for the water samples from Station 15 compared to the other two stations. Doubling the fine particulates greatly reduced biological nitrogen fixation for the cyanobacteria at all the stations (Table 8).

Results from Development and Validation of Methods

Due to the negligible values of nitrogen fixation obtained from the water from Lake Chapala, other tests were performed to verify appropriateness of the test materials, incubation chambers and detection methods used in the acetylene-reduction technique and analysis. These tests validated the experimental techniques and equipment used in this study.

A potential problem is the presence of methane bacteria which will convert the end product of acetylene reduction (ethylene) into a water-soluble compound. Ethylene is almost insoluble in water and is maintained in the gas phase of the incubation chamber. If the ethylene is converted to water soluble compounds, it will not be quantified. The ethylene spiked samples had a 95% recovery of the ethylene after incubation. Ethylene can also be produced abiotically by chemicals present in the water in the test chambers. This abiotic ethylene production would increase the baseline value of the control. Distilled water samples were used to test for this problem. The distilled water samples remained free of ethylene. Another problem was the appropriateness of the testing procedure. To check the equipment and test chambers, a culture of Anabaena was used. The cyano-bacteria samples had significant biological nitrogen fixation (Table 9). Note that the only values that were

Table 9. Mean rates (± 2 sd, N = 3) for nitrogen fixation to compare test chambers and materials used in the acetylene-reduction method. Values are reported in 10^{-5} nmol of N_2 l^{-1} h^{-1}.

Test condition	Value
Plastic chambers:	
Distilled water	0.
Lake water	0.
Cyanobacterial culture added	75.52±6.46*
Glass chambers:	
Distilled water	0.
Lake water	0.
Cyanobacterial culture added	72.47±7.54*
Ethylene added to lakewater sample:	
Immediate sample	139.07±38.42*
Incubated sample	132.12±36.98*
(This is a 95% recovery)	

* Samples significantly different from controls $p < 0.05$.

significantly different from the respective controls were the cyanobacteria samples.

The mean values of samples using two killing agents (TCA and $HgCl_2$) and also a 5 ml gas phase taken out immediately after injection of the acetylene (no killing agent involved) were compared to samples with no killing agents. All samples were incubated the same length of time (Table 10). The water samples with TCA added had significantly higher values (p 0.05) of nitrogen fixation than the samples with no killing agent. No differences were significant between mean values of nitrogen fixation in $HgCl_2$ killed samples and the samples with no killing agent and the immediate gas phase samples, taken after inoculation with acetylene.

Table 10. Mean rates (± 2 sd, N = 9) for biological nitrogen fixation for water samples with killing agents and water samples without killing agents to compare control methods. Values are reported in 10^{-5} nmol N_2 l^{-1} h^{-1}.

Killing Agent	Sample with Killing Agent	Sample without Killing Agent
TCA killed	11.56±7.47	3.26±1.40*
$HgCl_2$ killed	2.85±0.80	2.03±1.26

* Significant p 0.05

No Killing Agent	Before Incubation	After Incubation
Samples taken from same chambers	1.67±0.38	2.01±0.90

CHAPTER V

DISCUSSION

The relationship of biological nitrogen fixation to limited-available nitrogen in the environment is established (Wetzel, 1983). However, there are many factors which inhibit nitrogen fixation in a nitrogen-limited environment. The lack of nitrogen fixation in Lake Chapala can be attributed to the lack of heterocystous cyanobacteria. In open waters, biological nitrogen fixation has been strictly correlated with the presence of cyanobacteria (Fogg, 1971; 1973). The lack of cyanobacteria is attributed to wind-driven turbulence.

In Lake Chapala, the 77 km fetch and the 5 m mean depth combine to facilitate constant mixing of the water column and high turbulence. During the rainy season, which lasts the entire summer, thunderstorm-generated winds increased during the evening hours and continued throughout each night. Other researchers have noted that rates of nitrogen fixation are reduced or inhibited by wind turbulence. Wind action was noted to prevent scums by resuspension of the cyanobacteria in Lake Carl Blackwell, Oklahoma (Toetz, 1973). The loose aggregate shape of the

cyanobacterial mats were noted to be easily broken by turbulence in Clear Lake, California (Horne, 1979). Also mats were found only in wind-protected areas in many studies (Moeller & Roskoski, 1978; Horne, 1979; Horne & Galat, 1985). Even in the ocean the effect of turbulence has been noted ". . . a very calm bay preceding a cyanobacterial bloom" (Vanderhoef et al, 1972). During one ocean study on Oscillatoria, Carpenter noted ". . . in turbulent seas, the colonial habit of the cyanobacteria is lost and nitrogenase activity ceases" (Carpenter, 1976). Recently, the effect of turbulence on cyanobacteria and the decreased rate of nitrogen fixation has been studied. These researchers found that only when nutrients, light, and turbulence are within cyanobacterial limitations will heterocyst counts provide an adequate real-time estimate of nitrogen fixation (Horne & Galat, 1985).

Another factor related to turbulence is turbidity. Turbidity reduces sunlight penetration through the water column. Sunlight is the energy source for the biologically expensive process of nitrogen fixation. The rate of biological nitrogen fixation in situ has been established as a maximum rate just below the surface with a rapid decline with depth (Dugdale & Dugdale, 1962; Goering & Ness; 1964; Fogg, 1971; Horne, 1979). This relationship is due to light penetration similar to the rate of photosynthesis (Ward & Wetzel, 1980). Also associated with

turbidity is organic and inorganic molecules adsorbed on the clay particles. These adsorbed molecules can enhance or inhibit cellular metabolism. However, the least turbidity was at Station 03, with a Secchi depth < 1 m, but the highest rate of fixation was at Station 26, which sometimes becomes so turbid that the Secchi depth is 0.15 m. If the inhibiting substance was associated with the suspended clay particles, Station 26 should be severely inhibited, but it was not.

The relationship for increased biological nitrogen fixation with decreased concentration of ambient inorganic nitrogen was expected. This relationship was not found with the introduced cyanobacteria. The reverse of the expected trend was found when the cyanobacteria culture was added to water from Lake Chapala. The most nitrogen limited station had the lowest nitrogen-fixation rate. This appears to indicate a limiting nutrient not quantified in this study. Conversely, there could be present high concentrations of some chemical factor, inhibiting to nitrogen fixation, which builds up in the cul-de-sac lake. The idea of an additional inhibiting substance in the water column of Lake Chapala is supported. Lake-water samples, with suspended particulates filtered out and cyanobacteria added, had the highest rate of nitrogen fixation. The water samples, with added cyanobacteria culture, with the suspended particulates doubled had the

lowest rate of nitrogen fixation. Since adding or subtracting metazoans did not change the rate of nitrogen fixation in this lake, the concept of an inhibiting substance adsorbed to the suspended particulates appears possible. Further investigation into a limiting or inhibiting substance is needed.

Filamentous cyanobacteria are the most common biological nitrogen-fixing organisms. In Lake Chapala, filamentous cyanobacteria were not found in the lake during my study. Surrounding Lake Chapala are many low areas which fill with water during the summer rains. These transient ponds quickly develop massive water hyacinth mats over their entire surface. On the dying water hyacinths were found extensive colonies of Anabaena sp. Therefore, cyanobacteria are available to Lake Chapala and yet are not found in the water column or in the sediments.

Previously, researchers using the acetylene-reduction method have found abiologically generated ethylene in controls using TCA in carbonate aquatic systems (Thake & Rawle, 1972; Horne & Carmiggelt, 1975). The occurrence of abiological ethylene production by adding TCA has been associated with alkaline waters. Lake Chapala is an alkaline system and using TCA as a killing agent also abiologically produced ethylene.

Methane bacteria utilizing the ethylene produced in the acetylene-reduction method have been reported (Horne

& Carmiggelt, 1975; Flett, 1976). The samples of water from Lake Chapala, spiked with ethylene, did not lose ethylene. This verified that no methane bacteria capable of changing ethylene into water soluble compounds (which are not detected by the acetylene method) were present.

In conclusion, there was no ambient nitrogen fixation found in nitrogen-limited Lake Chapala during the summer of 1984. This was mainly attributed to the absence of heterocystous cyanobacteria. Based on the work of others, I believe this absence is partially due to wind-driven turbulence and its associated light inhibition due to turbidity. The nitrogen fixation by cyanobacteria introduced to the lake-water samples indicated inhibition beyond turbulence and light inhibition. Future studies are needed to test for inhibitory substances adsorbed to the suspended particulates in the water of Lake Chapala, Mexico.

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