NITROGEN FIXATION IN CANADIAN PRECAMBRIAN SHIELD LAKES

A Thesis

Submitted to

the Faculty of Graduate Studies

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

by

Robert John Flett

August, 1976

NITROGEN FIXATION IN CANADIAN PRECAMBRIAN SHIELD LAKES

BY

ROBERT JOHN FLETT

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

ABSTRACT

NITROGEN FIXATION IN CANADIAN

PRECAMBRIAN SHIELD LAKES

by

ROBERT JOHN FLETT

Rates of nitrogen fixation were measured in a number of lakes in the Experimental Lakes Area of northwestern Ontario, in the Canadian Precambrian Shield. An improved syringe technique was devised to make these acetylene reduction measurements of nitrogen fixation and a rapid and more accurate method of preparing and analysing N-15 containing samples by emission spectrometry was devised to calibrate the acetylene assay. Results from three years of study indicate that fixation can supply significant quantities of nitrogen to lakes that are receiving nutrient loads with total dissolved inorganic nitrogen: total dissolved phosphorus weight ratios of less than approximately 10.

TO BARBARA

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Dr. R.D. Hamilton and Dr. N.E.R. Campbell for their support as well as their contributions to my philosophical and practical education in science. John W.M. Rudd was unselfish with his time and advice and thereby contributed much in constructive criticism. Contact with Dr. D.W. Schindler was an important force in shaping the direction of this study. Barbara M. Graham was responsible for most of the 1975 data as well as giving freely of her own time for the thesis typing. Grace Decterow kindly provided administrative guidance and much secretarial aid. Many times Ron Reid was called upon for field help which he willingly gave. Dr. E.J. Fee was of considerable aid in computer modeling and analysis of my data, as were Dave Jacobs and Don Costin. Aid with incubator studies was often received from Hedy Kling, John Shearer and Doug Declercq, while the data processing and administrative problems were often solved by Russ Schmidt, Toos Reid, Thérèse Ruszczynski, Hugh Valiant and Akira Furutani. Dave Findlay often did phytoplankton analyses which were very important to the study. Jim Prokopowich, Sheila Michaelis and others of his staff periodically performed much needed chemical analyses and often supplied data as did Mike Capel, Ron Schade and Brian Hauser. Herman Schneider and Phil Michaelis, on numerous occasions, helped construct scientific equipment, as did Willy Burton, Cliff Jones and staff, Peter Harrop, Bert van der Veen and Ron Skaritko. Shelly Zettler, Connie Royal, Lynn Davies and Laurie Taite were responsible for most

of the graphics. Safe SCUBA diving procedures were ensured by Bruce Townsend and Ian Davies while Eric Matheson saw that sampling equipment and boats were functional. Marge and Diane Nevison unfailingly saw that excellent meals were available at any hour and on any day, forest fires included. Enlightening ideas were regularly received from Mike Stainton, Dr. Pat Healey, Ray Hesslein and Paul Quay. Library services supplied by Eric Marshall are gratefully acknowledged.

ABSTRACT

Rates of nitrogen fixation were measured in a number of lakes in the Experimental Lakes Area of northwestern Ontario, in the Canadian Precambrian Shield. An improved syringe technique was devised to make these acetylene reduction measurements of nitrogen fixation and a rapid and more accurate method of preparing and analysing N-15 containing samples by emission spectrometry was devised to calibrate the acetylene assay. Results from three years of study indicate that fixation can supply significant amounts of nitrogen to lakes that are receiving nutrient loads with total dissolved inorganic nitrogen: total dissolved phosphorus weight ratios of less than approximately 10.

TABLE OF CONTENTS

TABLE OF CONTENTS - continued

LIST OF FIGURES

Page

Figure

FIGURES - continued

FIGURE - continued

LIST OF TABLES

GENERAL INTRODUCTION

This study was conducted in an attempt to better understand the role of nitrogen fixation in the budgets of several well defined lakes. The logic behind the study was as follows: The increasing eutrophication of the Lower St. Lawrence Great Lakes had caused the International Joint Commission to investigate the problem and to try to determine how to combat it. It was thought that phosphorus removal from sewage would probably stop eutrophication, and in fact the Commission has already acted upon this assumption. Scientists believed that both carbon and nitrogen could be supplied to the lakes from the atmosphere by biological fixation and therefore these elements were considered essentially uncontrollable. It appeared that phosphorus was the only important nutrient supply that could be controlled by man.

The fact that atmospheric carbon dioxide could support algal production in lakes similar to the Lower Great Lakes was well illustrated by Schindler et al. (1973), Schindler and Fee (1973) and Emerson et al. (1973). In addition, the fixation of atmospheric nitrogen gas had been demonstrated by Horne and Goldman (1972) and Horne and Viner (1971) to be important in several extremely eutrophic lakes in California and Africa. There had been no thorough studies done, however, to determine if nitrogen fixation could supply significant amounts of nitrogen to the algae of the Lower Great Lakes or similar waters. It was our intention to perform such a study.

In addition, it was hoped that we might gain some insight into what causes the blooms of blue-green algae that are so often a nuisance in lakes and ponds. Such information would prove valuable in lake

management schemes or perhaps in projects concerning protein production by blue-green algae.

The Experimental Lakes Area of northwestern Ontario was the obvious location for this study. Extensive physical, chemical and biological background data were available for a series of lakes in different trophic states, the range encompassing Secchi depths of 0.25 to 10 meters (Cleugh and Hauser, 1971).

As Johnson and Vallentyne pointed out (1971), these lakes were ideal test subjects. They were isolated from the effects of man and had bed-rock controlled basins. In addition, a long term lease of the area permitted researchers to fertilize particular lakes with known amounts of nutrients and then record the results. There was no need to guess at nutrient inputs as is the case in most other culturally modified lakes studied elsewhere.

A previous study by Flett (1972) had shown nitrogen fixation to be occurring in two fertilized eutrophic lakes in the Experimental Lakes Area but the techniques employed in measuring the fixation were crude. This present work is much improved and is both a more intensive and extensive study of nitrogen fixation in these lakes than the previous one.

GENERAL HISTORICAL

Man has known at least since 1862 that biological fixation of atmospheric nitrogen occurs (Jodin, 1862). The most common measurement technique has been to do Kjeldahl analyses at successive moments in time and to look for increases in total nitrogen (Burris and Wilson, 1957). Unfortunately, such methods are insensitive and studies of the fixation process were greatly handicapped until the N-15 isotopic technique was developed in 1941 by Burris and Miller. Subsequently, accurate measurements of low rates of nitrogen fixation were made possible, an early example being the work of Dugdale et al. (1959). This date marks the beginning of productive studies upon nitrogen fixation in aquatic environments.

The first comprehensive attempts at determining the quantitative importance of nitrogen fixation (via the N-15 technique) in a lake were by Horne and Fogg (1970). They concluded that nitrogen fixation by planktonic algae contributes less than 1% of the total nitrogen income of the English Lakes Windermere and Esthwaite Water. A later N-15 study by Horne and Viner (1971) on tropical Lake George showed nitrogen fixation to supply a considerable portion of the total nitrogen input.

Many species of freshwater algae were examined for nitrogen fixing ability using the tracer technique and the list of capable species grew rapidly. Table I shows the maximum recorded rates of fixation for surface waters of various lakes in situ in the light (after Fogg, 1971).

In the marine environment, algal nitrogen fixation has been measured via the N-15 technique by Dugdale et al. (1961), Stewart (1964), Dugdale et al. (1964), Goering et al. (1966), and Stewart (1967).

Table I shows the maximum recorded rates of nitrogen fixation for surface waters of various lakes *in situ* in the light (after Fogg, 1971).

Lake and Date	Investigators	Principal spp. of Blue-green algae present	Total organic N mg/L	Nitrogen fixed ug/ L/ day
Sanctuary, Pennsylvania Aug. '59	Dugdale and Dugdale, 1962	Anabaena flos-aquae A. circulanis A. spiroides	3.6	125.
Mendota, Wisconsin Aug. '61	Goering and Neess, 1964	Gleotrichia echinulata	0.72	8.5
Wingra, Wisconsin July '61	Goering and Neess, 1964	Microcystis aeruginosa Anabaena sp.	2.1	12.
Smith, Alaska June, '64	Billaud, 1967	Anabaena flos-aquae	(0.41)*	2.88
Windermere (N), England June '66	Horne and Fogg, 1970	Anabaena flos-aquae Oscillatoria spp.	0.164	0.098
Windermere (S), England Oct. '65	Horne and Fogg, 1970	Anabaena solitaria A. flos-aquae Oscillatoria spp.	0.47	2.82
Esthwaite Water , England Aug. '65	Horne and Fogg, 1970	Aphanazomenon flos-aquae Anabaena flos-aquae Anabaena circulanis	0.255	0.244
Loch Leven Scotland, May, July, Sept. '68	Horne, unpublished	Synechococcus sp. Oscillatoria sp. Anabaena sp.	0.186	0
Tjeukemeer, Holland Sept. '66	Horne, unpublished	Aphanazomenon sp. Oscillatoria sp.	2.1	14.9
George, Uganda March '68	Horne and Viner, 1971	Anabaena sp. Anabaenopsis sp.	2.2	4.1
McIlwaine, Rhodesia March '68	Horne, unpublished	Microcystis sp.	1.275	0
Kariba, Rhodesia March '68	Horne, unpublished	Oscillatoria sp.	0.30	0

* Particulate N only

The insensitivity of the method and the few measurements performed have not yet permitted any good estimates to be made of total oceanic nitrogen fixation.

In 1966 the acetylene reduction technique was introduced as an indirect but very sensitive and inexpensive method for measuring nitrogen fixation (Dilworth, 1966; Schöllhorn and Burris, 1966). It was very quickly applied by Stewart (1967) to measure algal nitrogen fixation in cultures and lakes. It was from that time on that data began to rapidly accumulate as researchers made extensive use of this new and simple technique. The physiology of acetylene reduction was intensively investigated in the laboratory by Stewart et al. (1968), Hardy et al. (1968, 1973), Stewart and Lex (1970), Bergerson (1970), and Brouzes and Knowles (1971). Pelagic in situ lake acetylene reduction experiments were introduced by Rusness and Burris (1970) and by Howard et al. (1970). Attempts at lake nitrogen budgets were made by Stewart et al. (1971), Granhall and Lundgren (1971), Horne and Goldman (1972), Vanderhoef et al. (1974) and Lannergren et al. (1974). Contributions of fixed nitrogen were determined to be substantial in the last four studies and in several cases appeared to supply more than 40% of the total nitrogen income.

Florida lake sediments have been examined for nitrogen fixation by the acetylene reduction technique (Kiern et al. 1971) and in some cases have been found active in the fixation process. The same was true in Florida estuarine sediments (Brooks et al. 1971). Macgregor and Keeney (1973) also found evidence of fixation in Wisconsin lake sediments using acetylene reduction.

In marine measurements of nitrogen fixation, the acetylene reduction technique appears to have all but displaced the N-15 method. This can be seen in the work by Bunt et al. (1970), Carpenter (1972), Taylor et al. (1973) and Mague et al. (1974). Many more measurements are needed to determine the impact of the fixation process upon the nitrogen budget of the world ocean. Some excellent work by Wiebe et al. (1975) and Webb et al. (1975) has been done on nitrogen fixation (acetylene reduction) in coral reefs. It appears that atmospheric nitrogen is a major source of combined nitrogen in the Pacific reefs studied.

METHODS

GENERAL INTRODUCTION TO METHODS SECTIONS

The set purpose of this study was to establish what was the role of nitrogen fixation in some selected Canadian lakes. Initial investigations revealed that existing acetylene reduction techniques were too imprecise to be used in this work. A major effort was therefore made to improve the precision of the assay. It was also found that, using the equipment available to us, the amount of nitrogen required for mass spectrometric analysis of N-15 was so large (>1 mg N) that isotopic experiments were impractical; the samples would have had to be concentrated to such an extent that it would have been difficult to relate the results back to in situ conditions. Fortunately, as this study was just begun, a Statron NOI-5 N-15 Analyser became available to us. This emission spectrometer had the capability of analysing very small amounts of nitrogen and thus it was appealing because it would allow measurement of N-15 uptake on unconcentrated water samples. The preparation of samples for spectrometry, as described by the manufacturer, was tedious and difficult, however. Thus, a second major effort was made to devise a fast and convenient method of sample preparation for the spectrometer. In this way, the indirect acetylene reduction method could be calibrated with the more direct isotopic method.

Procedural development was of prime importance in this study and this is reflected in the large methods section of the dissertation. The two techniques have been handled separately because, while purporting to measure the same activity, they are so very different. Other ancillary procedures have been appended as a third portion of the methods section.

SECTION I OF METHODS

N-15 TECHNIQUES

INTRODUCTION

Isotope analysis of organically derived nitrogen has generally been an expensive and tedious procedure. The most widely used method of isotope analysis (Rittenberg) involves the conversion of the organic material to ammonia via Kjeldahl digestion, distillation of the ammonia into acid and then titration of the acid to determine the amount of ammonia present. The ammonia is then converted into nitrogen gas via chemical oxidation by alkaline hypobromite, and the resulting nitrogen gas, after drying in liquid nitrogen traps, is fed into a mass spectrometer where mass ratio analysis is carried out.

A much improved automated system has been described (Barsdate and Dugdale, 1965) wherein a Coleman[1] Nitrogen Analyser was connected directly to a mass spectrometer. The analyser operates on a Dumas principle and directly converts, in the presence of catalysts, organic nitrogen to nitrogen gas. The factory installed nitrometer, which measures the nitrogen gas volume, is bypassed. A liquid nitrogen trap is used to remove the analyser sweep gas before the effluent (containing the nitrogen) enters the mass spectrometer. Barsdate and Dugdale are not clear on how the amount of nitrogen produced is measured. They do claim precision of about $\pm$ 0.01 at %, a rapid analysis (5/hour) and the ability to handle small samples (10 µg N/sample). Unfortunately, the very high equipment costs will preclude its use for many researchers.

Nitrogen isotope analysis by emission spectroscopy has recently become more popular due to the introduction of commercially available

---

[1] Coleman Instruments - Div. of Perkin-Elmer, Maywood, Ill., U.S.A.

spectrometers especially designed for the purpose - Statron[2] NOI-5 and Jasco[3] NAI-1 Analysers. The precision of these machines is not as good as that provided by mass spectrometry but they are much less expensive and can handle very small sample quantities (<1 µg N) (Cook et al. 1967). Several sample preparation schemes have been described for these units (Keeney and Tedesco, 1973; Meyer et al. 1974). The manufacturers of the Statron suggest an in vacuo technique using hypobromite but it suffers from the inherent disadvantage of requiring previous Kjeldahl chemistry as well as titration to determine the ammonia concentration. In addition, a prescribed amount of the ammonium salt so produced must be weighed out so that the final pressure of nitrogen is the same in all samples. This can be very troublesome when the weight of sample is less than 50 µg. The specially shaped electrodeless discharge tubes are heat sealed after being filled with the sample and can only be used once, thereby considerably increasing the cost per analysis.

To speed up sample preparation, to reduce costs and to enable easy analysis of small samples (10 µg N/sample) a new preparation unit was devised. The unit combusts organic material in oxygen, measures the quantity of nitrogen so produced, and then directs the gas into the emission spectrometer for analysis. The quantity of nitrogen per sample is not critical (10 - 200 µg) and the electrodeless discharge tubes can be reused many times.

---

[2] P.G.H. Statron, Ehrehfried - Jopp - Str. 59, G.D.R.

[3] Jasco Incorporated, Easton, Maryland, U.S.A.

Methods and Materials

The preparation unit consists of two parts: the combustion tube (Fig. 1) and the vacuum rack (Fig. 2). The combustion tube consists of a stainless steel tube 0.87 cm (11/32 in) I.D., 1.0 cm (19/32 in) O.D. and 43.2 cm (17 in) long. A 0.79 cm (5/16 in) stainless steel rod is welded into the tube, closing one end. When the tube is in the furnace (Lindberg Hevi-Duty[1]), care must be taken to position the tube such that the weld junction is at least two inches out of the furnace; otherwise failure of the weld may occur after a number of heating cycles. Another 0.79 cm (5/16 in) stainless steel rod serves as the sample holder. Its end is threaded to accept a stainless steel cup into which the sample is placed before combustion.

The open end of the tube is machined to 0.95 cm. (3/8 in) O.D. for about 5 cm (3 in.) down the length to accommodate a Swagelok[2] 0.95 - 0.64 cm (3/8-1/4 in) reducing union (Fig. 1). The front ferrule of the 0.95 cm (3/8 in.) end has been replaced with a rubber 'O' ring and the back ferrule has been reversed, thereby producing a good 'finger tight' vacuum seal. The 0.64 cm (1/4 in) end of the union has a teflon front ferrule which accepts a 2 mm bore glass vacuum stopcock with a 2.54 cm (1 in) long capillary bore (0.24 cm. or 3/32 in) tubing extending from each side. To the other side of the stopcock a Swagelok 0.64-0.32 cm (1/4-1/8 in) reducer is fitted, also using teflon front ferrules. The combustion tube can be quickly connected to the

---

1 Lindberg Hevi-Duty - Div. Sola Basic Industries, Watertown, Wisc., U.S.A.

2 Swagelok - T.M. Crawford Fittings (Can.) Ltd., Niagara Falls, Ont.

Figure 1. A diagram of the combustion tube assembly.

2 - Reducing union 0.95 - 0.64 cm (3/8 to 1/4 in.)
3 - High vacuum glass stopcock valve.
4 - Reducing union 0.64 - 0.32 cm (1/4 to 1/8 in.)
40 - Outer stainless steel shell of combustion tube
41 - Inner stainless steel filler rod and combustion tube end
42 - Weld making vacuum tight union between 40 and 41
43 - Stainless steel sample cup
44 - Vent hole in stainless steel sample cup
45 - Stainless steel filler and sample holder rod
46 - Brass back ferrule, reversed
47 - Rubber 'O' ring
48 - Brass hex nut for finger tightening 'O' ring.

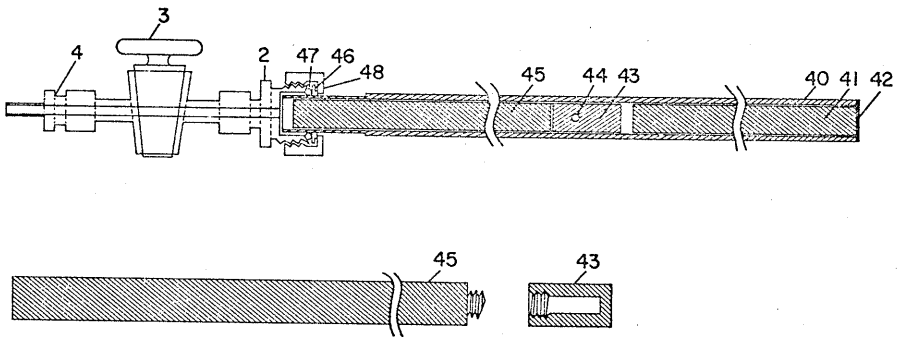
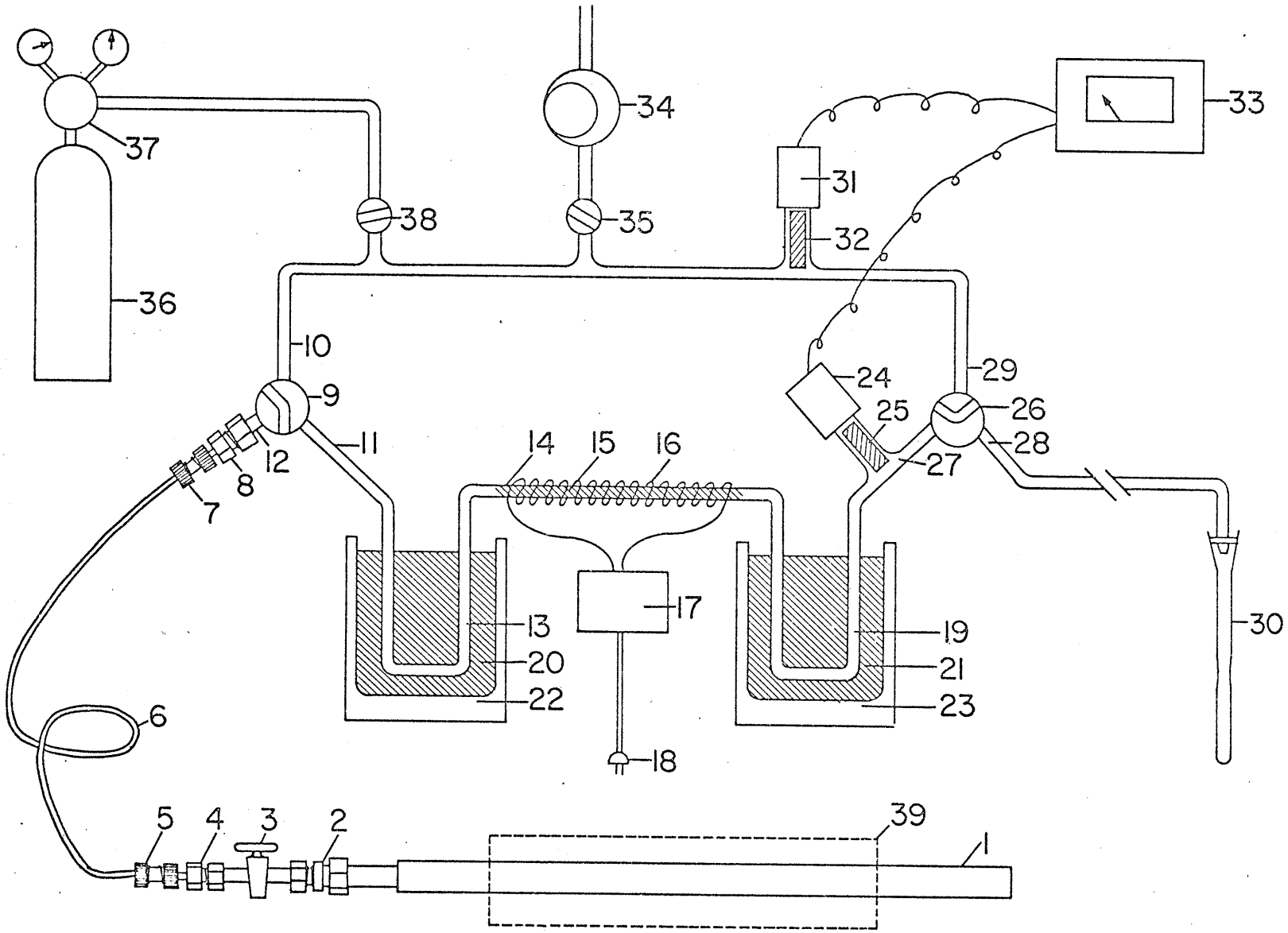

3
4
2
47
46
48
45
44
43
40
41
42
45
43

Figure 2. A diagram of the preparation unit.

1 - Stainless steel combustion tube (whole assembly)
2 - Reducing union 0.95 - 0.64 cm (3/8 - 1/4 in)
3 - High vacuum glass stopcock valve
4 - Reducing union 0.64 - 0.32 cm (1/4 - 1/8 in)
5 - High vacuum finger tight union 0.32 cm (1/8 in)
6 - Copper tubing, flexible 0.32 cm (1/8 in) O.D.
7 - High vacuum finger tight union 1/8 in.
8 - Reducing union 0.64 - 0.32 cm (1/4 - 1/8 in.)
9 - High vacuum glass 3-way stopcock valve
10 - Valve leg
11 - Valve leg
12 - Valve leg
13 - Glass U-shaped cold trap (Pyrex)
14 - Glass reduction column (Pyrex)
15 - Reduced copper turnings, fine
16 - Nichrome heater wire
17 - Variable voltage transformer
18 - A.C. line plug
19 - Glass U-shaped cold trap (Pyrex)
20 - Liquid nitrogen
21 - Liquid nitrogen
22 - Dewar flask
23 - Dewar flask
24 - Pirani guage sending unit
25 - Glass filler rod in sending unit stem
26 - High vacuum glass 3-way stopcock valve
27 - Valve leg
28 - Valve leg
29 - Valve leg
30 - Glass electrodeless discharge tube installed in analyser
31 - Pirani guage sending unit
32 - Glass filler rod in sending unit stem
33 - Dual station Pirani guage readout
34 - Mechanical vacuum pump
35 - High vacuum glass stopcock valve
36 - Tank containing ultra high purity oxygen
37 - Two stage gas regulator
38 - High vacuum glass stopcock valve
39 - Tubular furnace for stainless steel combustion tube.


37
36
38
34
35
31
32
33
10
9
11
12
8
7
6
14
15
16
24
25
26
27
28
29
30
13
20
22
17
18
19
21
23
5
4
3
2
39
1

vacuum system by inserting the 0.32 cm (1/8 in) male end of the reducer into a 0.32 cm (1/8 in) Ultra-Torr[1] union installed on a copper tube leading to the vacuum rack, and making it finger tight. The total volume of the combustion tube is about 3 ml up to the closed stopcock, when the sample holder is in place.

The internal volume of the preparation unit (discharge tube, vacuum rack between legs 11 and 27, and combustion tube) used in this study is approximately 20 ml. It is important that the internal volume of the preparation unit be small if the samples to be analysed contain little nitrogen. This is because the pressure of nitrogen is inversely proportional to the volume of the preparation unit. The smaller the preparation unit volume, the less sample needed to attain at least the absolute minimum of 0.2 Torr pressure in the discharge tube.

The preparation unit is constructed of 3 mm bore, 1.5 mm wall pyrex tubing and the valves are 2 mm bore spring loaded vacuum stopcocks lubricated with silicone high vacuum grease. The copper reduction column is wound with approximately 100 turns of about 18 guage nichrome wire across which 65 volts A.C. is applied from a variable transformer. The temperature of the column could not be accurately measured but the temperature was such that the heater wire glowed faintly when viewed in a darkened room. Vacuum is attained using a Welch[2] Duo-Seal 1405 mechanical pump capable of obtaining pressures <1 mTorr without the use of cold traps. Pressure is monitored with a

---

1 Ultra-Torr - T.M. Cajon Co., Crawford Fittings (Can.) Ltd., Niagara Falls, Ont.

2 Welch Duo-Seal - T.M. Sargent-Welch Scientifc Co., Skokie, Ill., U.S.A.

dual range (0 - 100 mTorr, 0-5 Torr) two station Pirani guage (Kinney[3]). Ultra high purity oxygen (Linde[4]) is supplied via copper tubing to the vacuum rack at approximately 2 lb./in.^{2}. The combustion tube is connected to the rack via 0.32 cm (1/8 in.) O.D. copper tubing, Swagelok and Ultra-Torr fittings being used to make the reductions and unions. The electrodeless discharge tube, which remains permanently fixed in the emission spectrometer (Meyer et al. 1974), is linked to the preparation unit by 0.32 cm (1/8 in.) copper tubing also. The discharge tubes as supplied with the Statron NOI-5 analyser are designed to roughly fit on a taper joint into which molten picein wax is then poured to effect a vacuum tight seal. This messy procedure is circumvented by employing an 'O' ring around a 0.64 cm (1/4 in.) tapering glass tube; it yields an effective yet easily made seal (Fig. 2).

Theory of the Preparation Unit Operation

A dry particulate sample, cellular material for example, is put into the combustion tube and combusted in a pure oxygen atmosphere. The combustion products, assumed to be mostly H_2O, CO_2, N_2 and various nitrogen oxides, then pass through a liquid nitrogen trap where the H_2O, CO_2 and some of the unconsumed oxygen condense. The nitrogen, nitrogen oxides and remaining oxygen next pass through a column of hot reduced copper that removes the oxygen and reduces any nitrogen oxides

---

3 Kinney Vacuum Co., Boston, Mass. U.S.A.

4 Linde - Union Carbide Canada Limited, Toronto, Ont.

to nitrogen gas. The nitrogen gas is finally passed through another liquid nitrogen cold trap wherein any remaining condensables are removed before the gas enters the electrodeless discharge tube of the emission spectrometer.

Practical Operation of the Preparation Unit

It is difficult to describe in an easily readable form the sequential steps that must be followed for the proper operation of the sample preparation unit. It is suggested that the text be read with continual reference to Fig. 2. The meaning of the word 'straddle' as used here is seen in Fig. 2: valve 9 is shown straddling leg 12.

With valve 38 closed, 35 open, 9 joining 10 to 11, and 26 joining 27 to 29, the vacuum is applied. When the pressure drops below 20 mTorr the heater for the copper reduction column is turned on. After several days initial running, typical pressure readings are <1 Torr and <1m Torr stations 31 and 24 respectively. It takes this long a period of time to remove water and contaminating gases from the unit when it is turned on for the first time. Subsequent pumpdowns between samples require only about 15 minutes. Liquid nitrogen is then added to the cold traps and pressures of <1m Torr and 0 mTorr should appear at stations 31 and 24 respectively.

The cold traps and copper reduction column are then closed off by turning 9 to a position straddling 10 and turning 26 to a position joining 29 to 28. This will evacuate the electrodeless discharge tube. It has been previously (Meyer et al., 1974) demonstrated that

a 15 minute evacuation period will remove memory of previous samples from the discharge tubes provided the tube is excited during pumpdown. For this reason, the 15 minute pumpdown and excitation period has been adopted as standard.

A dry particulate sample containing 10-200 μg nitrogen is placed upon a 4 cm^2 piece of clean aluminum foil[1]. The foil is folded over several times and then dropped into the sample cup which is next screwed onto the sample holder rod. The whole assembly is then inserted into the combustion tube which is then sealed with the 'O' ring closure and connected to the preparation unit. Clean pliers and forceps are used for all these manipulations so that nitrogen contamination from the hands is avoided. Valve 26 is closed while valve 9 is turned so as to join 10 to 12, and then 26 is reopened thereby preventing a surge of atmospheric nitrogen into the partially cleaned up discharge tube. Pumpdown time for the combustion tube varies and depends upon the dryness of the sample. The time is shortened by gentle heating of the combustion tube, something easily accomplished by laying the tube on the exterior surface of the tube furnace. A final pressure of <1 mTorr is usually seen at station 31 after 5-10 minutes pumping.

When the combustion tube evacuation is complete, valve 9 is kept in the position joining 10 to 12, valve 26 is made to straddle leg 29, and oxygen is allowed into the unit via 38, for about 3 seconds. This oxygen serves as a rinse and is quickly removed by the pump.

---

[1] Pieces of foil were combusted as though they contained samples and they produced no appreciable nitrogen gas pressure, thus indicating that they were free of contaminating nitrogen.

Once the pressure again approaches 10 mTorr, valve 35 is closed and 38 is opened for about 5 seconds after which time the oxygen pressure in the combustion tube is approximately 2 lb./in.^{2}. Valve 3 is closed, 9 is made to straddle 10, 38 is closed and 35 opened so that the unit evacuates. Union 5 is loosened and the combustion tube disconnected from tube 6.

The combustion tube is placed into the tube furnace (890°C) and is reconnected to the preparation unit by union 5, valve 9 is turned to join 10 and 12 and the system is evacuated up to valve 3. Now the vacuum in the cold traps and reduction column as well as in the discharge tube is checked and if necessary, reestablished. After 5 minutes of combustion, valve 3 is opened and the combustion products are routed via 9 through the cold traps and reduction column, past Pirani guage 24 and up to closed valve 26 (straddling 29). Cold trap 13 removes condensables such as CO_2 and H_2O, the hot copper column 15 removes any unconsumed oxygen as well as reducing nitrogen oxides, and cold trap 19 removes residual condensables. The pressure reading at station 24 is therefore due to nitrogen gas only and one can easily calculate the weight of nitrogen originally present in a sample, with the aid of a standard curve. If the nitrogen pressure is above that desired for accurate analysis (in our case, above 2 Torr), excess gas can be evacuated via 26 in a position joining 27 to 29. When a suitable (0.4 - 2.0 Torr) pressure is obtained, 26 is turned to the position joining 27 to 28 and the nitrogen is routed to the discharge tube. The pressure reading on guage 24 should drop considerably (in this case, about 50%) because of the sudden increase in volume of the system. An effort shcuild be made to keep discharge tube pressure approximately

between 0.2 and 0.8 Torr for acceptable accuracy in at % N-15 measurements, the reasons for this being discussed later. This means that enough sample must be analysed to produce at least a minimum pressure of 0.2 Torr; pressures in excess of 1.0 Torr can easily be evacuated.

After approximately 30 seconds, the optical emission stabilizes and a series of scans (eg. 6) can be made of the mass 28, 29, and 30 nitrogen emission bandheads. The peak heights are then measured and the average N-15 enrichment is calculated (Ferraris and Proksch, 1972). If sample drying and combustion are done during discharge tube cleanup, it is possible to process a sample every 20 minutes.

Samples which are already in the gaseous form are handled differently than particulate samples. Basically, it is only required that the gas pass through the first liquid nitrogen trap, the hot copper column, then the other liquid nitrogen trap and finally form a pressure of at least 0.2 Torr in the discharge tube. Specifically the operation is as follows: The combustion tube is separated from the preparation unit by removing the flexible tubing from union 7. Into the open end of this union is fitted a rubber septum which is held secure under the knurled nut. With the unit in normal operating order and everywhere evacuated, valves 38 and 35 closed, valve 9 straddling leg 12 and valve 26 straddling leg 28, the gaseous sample is introduced through the septum by a syringe bearing a 26 G hypodermic needle. The quantity of gas is not critical; it is only necessary that there be an excess above that required by the analyser. The syringe is left in place so that no leakage can occur through the puncture and valve 9 is made to

momentarily join legs 12 and 10 and next made to straddle leg 10. The gas thereby distributes itself between legs 10 and 29 of the unit. By this procedure the septum is isolated from the unit and the syringe can be safely removed without fearing inward leakage of atmospheric nitrogen.

The portion of the unit between legs 10 and 29 serves as a sample gas reservoir. In order to facilitate easy subsampling of the gas, the pressure is reduced (if necessary) to approximately 1 Torr in the reservoir by evacuating through valve 35 until the correct pressure is reached. Next, a subsample is taken by turning valve 9 to join legs 10 and 11 for a period sufficient to obtain pressure equilibrium at sender 24 (approximately 30 seconds) and then turning 9 so as to straddle leg 11. Because the volume of the gas reservoir is substantially greater than the rest of the unit between legs 11 and 27, the pressure as read on sender 24 is only slightly less than that in the reservoir i.e. 1 Torr. Valve 26 is next made to join legs 27 and 28 and the sample is introduced to the spectrometer for analysis in the normal way.

Preparation Unit Maintenance

The combustion tube will burn out in time and start to leak. It is not feasible to repair the tubes and therefore when such leaks occur the tubes are discarded. The 'O' rings on the tubes are subject to wear and should be regularly checked and replaced if necessary. High vacuum silicone grease very lightly applied to the 'O' rings is helpful

in maintaining the integrity of the system.

The stopcocks of the preparation unit may in time become plugged or difficult to turn and therefore should be regularly cleaned and lubricated with silicone high vacuum grease. In our experience, the copper reduction column becomes mostly oxidized after perhaps 50 samples have been processed. The copper distinctly changes colour from red to black when it is oxidized and this is plainly visible through the glass walls of the column. When this occurs the heater is turned off so that the copper may cool and the liquid nitrogen dewars are removed so that the traps will reach ambient temperature. Valve 35 is closed and hydrogen gas is introduced into the assembly at valve 26. The gas passes at approximately 100 ml/min through the copper turnings and out of the other valve 9 through leg 12. This hydrogen must either be burned or properly vented from the room. Once all the air has been purged from the system by the hydrogen, the heater is again turned on and the copper is rapidly reduced. The water vapour produced condenses and form droplets which mostly pass out of the unit with the unused hydrogen. When all of the copper is reduced, the heater is turned off and the column allowed to cool. The hydrogen flow can then be halted and the preparation unit evacuated in the normal fashion. Several hours may be required to pump off the water from the system, a process that is considerably accelerated when heat is applied. Once the pressure is below 10 Torr the reduction column heater can again be turned on. Pressures below 1 mTorr are obtained after 8 hours of continuous pumping.

The liquid nitrogen dewars are removed at the end of each operating day so that any solidified or condensed gases and vapours will evaporate

and be pumped away from the unit. The dewars are replaced and filled with liquid nitrogen at the beginning of each operating day.

The electrodeless discharge tube is subject to discoloration after continued use. The discoloration has no apparent effect upon the accuracy of the N-15 measurements, but as a precaution, the tube is replaced with a new one after approximately every 100 sample runs.

Results and Discussion

A typical recorder tracing of the emission spectrometer output is given in Fig. 3. In this case, the sample consisted of a pre-combusted (550°C) glass fibre filter through which had passed 25 ml of eutrophic lake water, containing 10 µg particulate nitrogen of natural abundance. The baselines utilized for calculating peak height (see Fig. 3) are not those recommended by the manufacturer (Statron) but were found to yield the most accurate results over the largest range of N-15 enrichments encountered (0.37 - 50.0 at %). Figure 4 shows the relationship between actual at % in standards of 49.25 to 1.36 at % versus the observed at % determined by the emission spectrometer. The vertical bars represent 95% confidence limits for the observed at % reading of any single sample. The Student's t distribution was employed for this determination and the sample size is printed beneath each bar. Figure 5 is a similar plot for standards of 7.01 to 0.37 at %. The observed readings are very close to the actual values of the standards and for many purposes a standard curve may not be required because the observed readings will be sufficiently accurate without correction. If maximum accuracy is required, a standard curve that joins

Figure 3. The N-14/14 baseline consists of a line that is at one end tangential to the tail of the N-14/14 peak and at the other end intercepts the abscissa immediately below the inflection point between the N-14/15 and N-14/14 peaks. If a baseline, drawn tangential to both the head and tail of the N-14/14 peak, intercepts the abscissa further to the right than the previous baseline, then the second baseline is used instead. The N-14/15 baseline is as indicated in the figure.

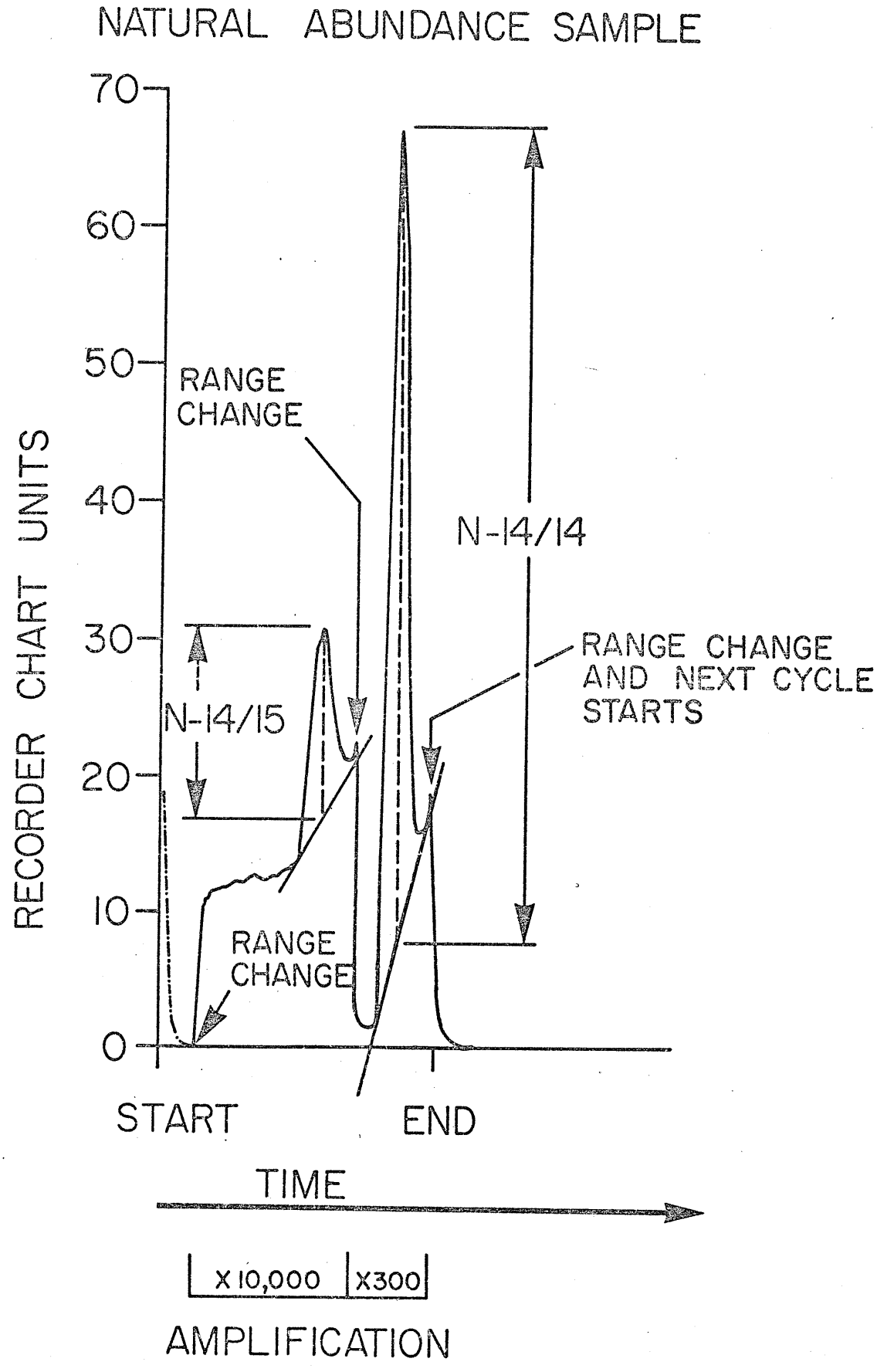

NATURAL ABUNDANCE SAMPLE
70
60
50
40
30
20
10
0
RECORDER CHART UNITS
RANGE CHANGE
N-14/14
N-14/15
RANGE CHANGE AND NEXT CYCLE STARTS
RANGE CHANGE
START
END
TIME
X10,000
X300
AMPLIFICATION

Figure 4. A plot of observed at % N-15 as a function of actual at % from 1.36 to 49.25 at % N-15. The numerals under each 95% confidence bar give the number of samples analysed.

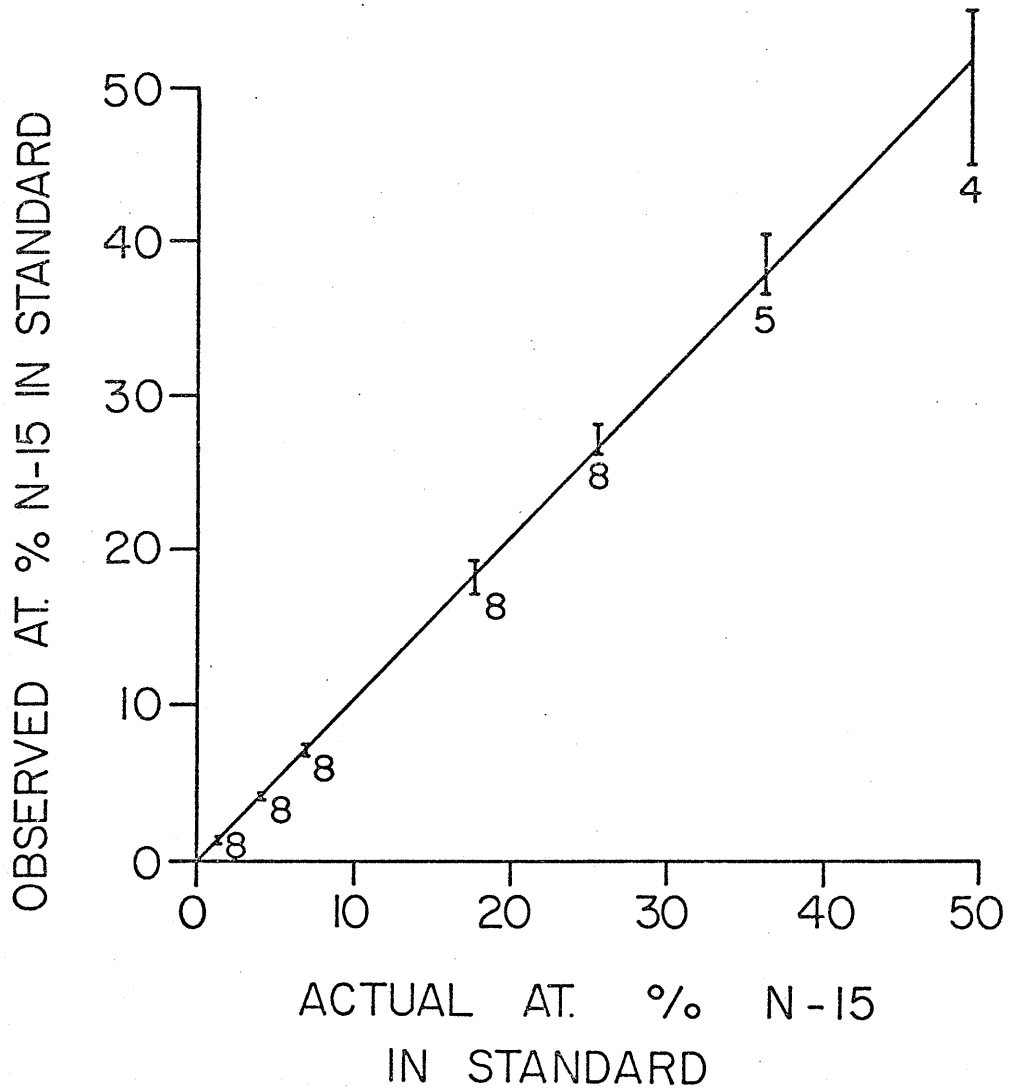
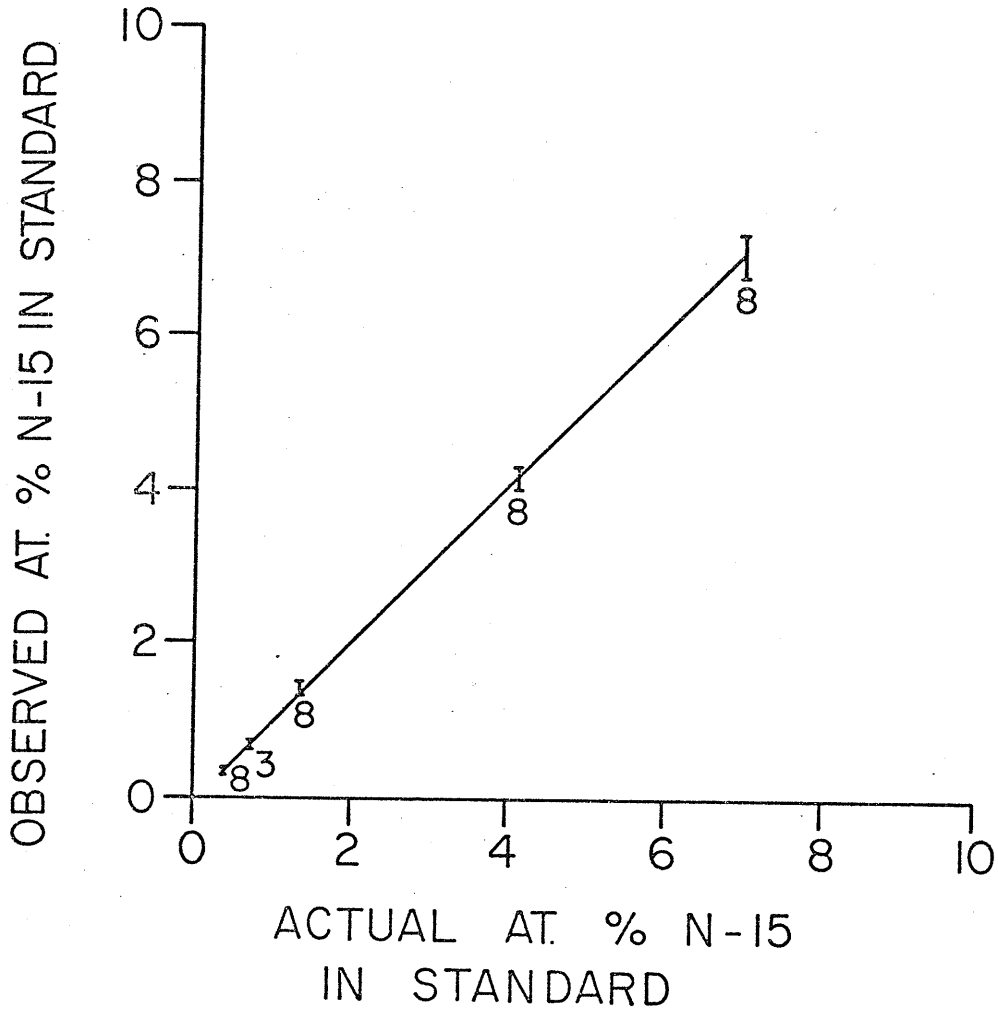

OBSERVED AT. % N-15 IN STANDARD
50
40
30
20
10
0
0
10
20
30
40
50
ACTUAL AT. % N-15
IN STANDARD
8
8
8
8
8
5
4

Figure 5. A plot of observed at % N-15 as a function of actual at % from 0.37 to 7.01 at % N-15. The numeral under each 95% confidence bar gives the number of samples analysed.


OBSERVED AT. % N-15 IN STANDARD
ACTUAL AT. % N-15 IN STANDARD
0
2
4
6
8
10
8
8
8
8
3

averages of observed measurements should be used because such a curve employs actual observations and does not assume that the relationship between real and observed measurements is linear. At the natural abundance level of 0.37 at %, the machine used in this study gave a mean reading of 0.337 at % for 8 samples with a 95% confidence interval of 0.321 to 0.353 at %.

The amount of nitrogen present in particulate samples is determined from a standard curve relating known weights of nitrogen to observed gaseous pressure in the preparation unit. The curve is constructed by pipetting 100 µl volumes of appropriate concentrations of NH_4Cl solutions onto precombusted Reeve Angel[1] 984-H glass fibre filters, allowing them to dry and then analysing them in the same fashion as for particulates. Figure 6 shows the results. The vertical bars are 95% confidence intervals for pressure readings of single samples obtained by the Pirani guage. The numbers under the bars represent the sample size employed to determine the confidence intervals using the Student's t distribution. The pressure-weight relationship appears to be linear.

When nitrogen gas from the combustion tube is permitted into the evacuated preparation unit, the pressure reaches a maximum value in about 30 seconds and from that instant on it declines slowly. Figure 7 illustrates the drop in pressure as a function of time. The pressure appears to reach a nearly stable end-point after about ten minutes. The pressure-weight standard curve given in Fig. 6 is constructed from maximum pressure values obtained about 30 seconds after opening the combustion tube to the preparation unit. The reason for this slow drop in

---

[1] Reeve Angel - Clifton, New Jersey, U.S.A.

Figure 6. A plot of gas pressure (Torr) in the sample preparation unit as a function of the weight of nitrogen (μg) combusted in the unit. The vertical bars (95% confidence limits) have the number of samples analysed given under them

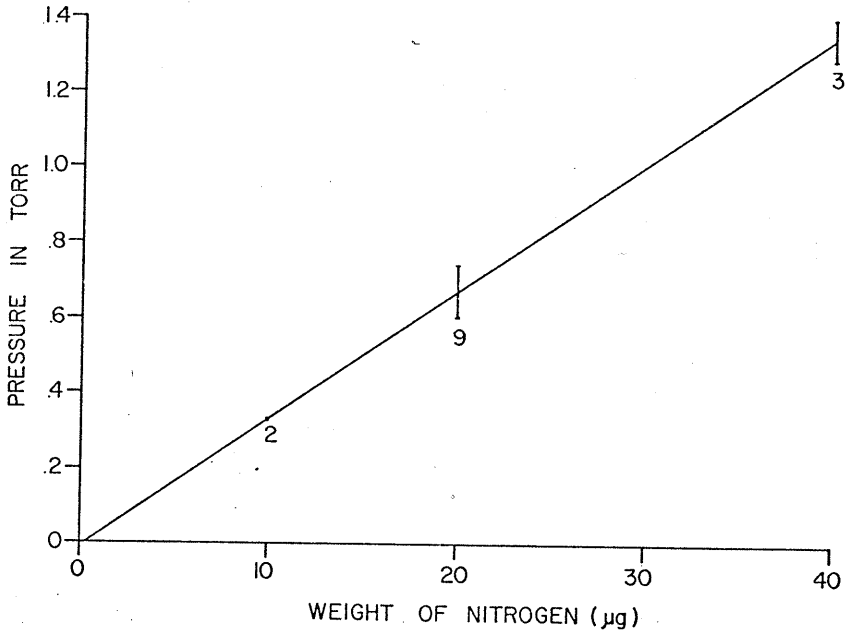
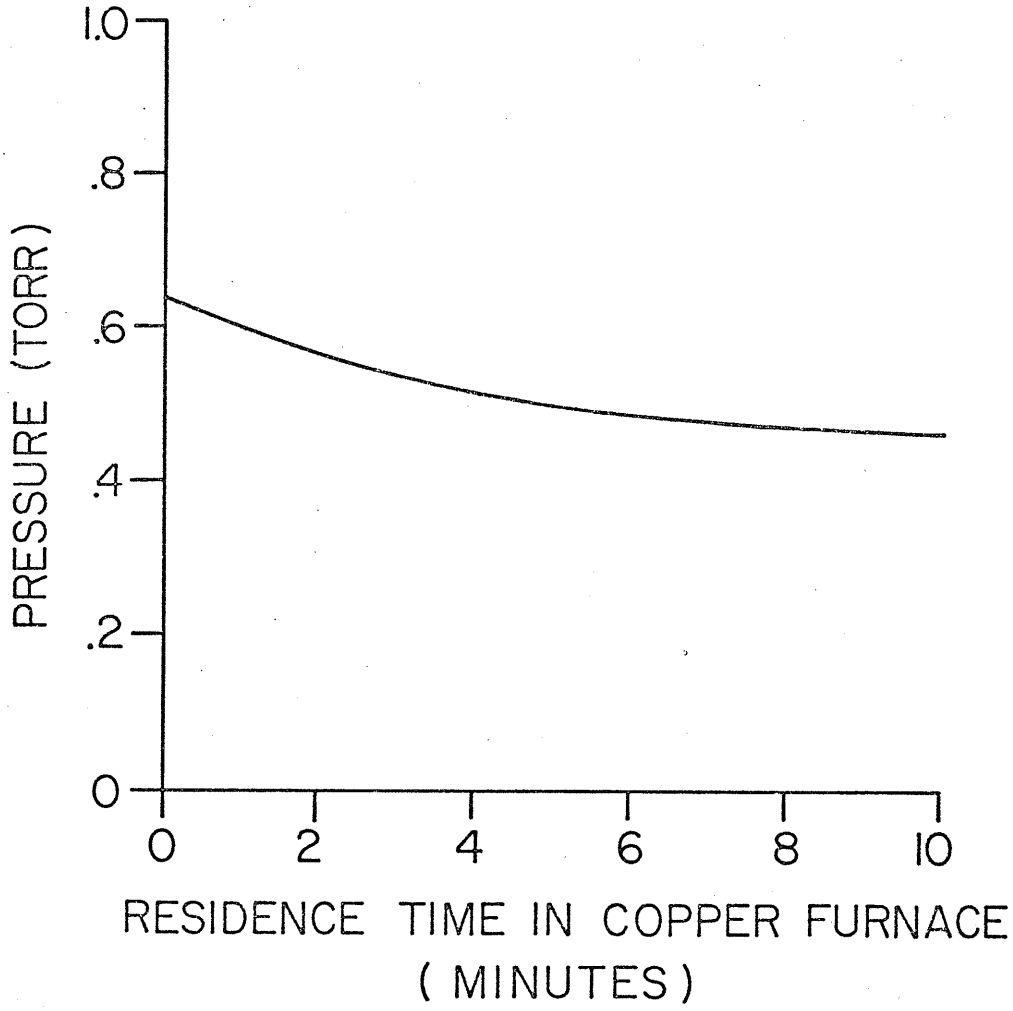

1.4
1.2
1.0
.8
.6
.4
.2
0
PRESSURE IN TORR
0
10
20
30
40
WEIGHT OF NITROGEN (µg)
2
9
3

Figure 7. A plot of gaseous pressure (Torr) in the sample preparation unit as a function of time (min). The first measurement actually was taken about 30 seconds after the sample had been allowed into the furnace, when the pressure had already reached a maximum and had begun its slow decline.

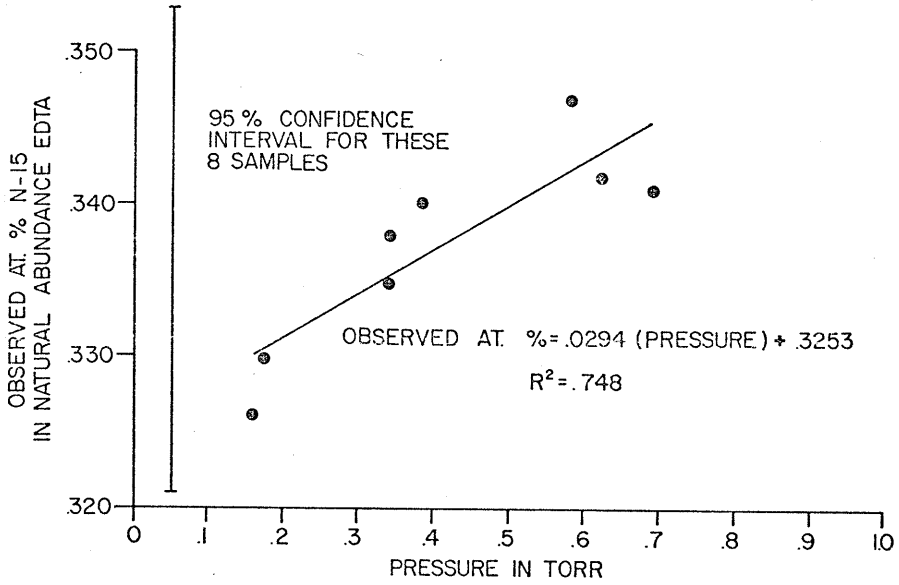

1.0
.8
.6
.4
.2
0
PRESSURE (TORR)
0
2
4
6
8
10
RESIDENCE TIME IN COPPER FURNACE
(MINUTES)

pressure is not known; it may be that the copper turnings are still reacting with unconsumed oxygen in the system or that carbon dioxide and water vapour are still being solidified in the cold traps.

Previous workers (Broida and Chapman, 1958; Keeney and Tedesco, 1973; Meyer et al. 1974) have shown that the observed at % readings are slightly dependent upon pressure of nitrogen in the discharge tube The same effect was noted in this study. Figure 8 illustrates how a natural abundance sample varies as a function of pressure in the discharge tube. The relationship between % N-15 and pressure is statistically significant as is revealed by the value of R^2=0.748 for the regression line drawn. It appears that a substantial portion of the variation in at % readings is in fact due to this pressure effect. The most precise measurements require that a constant pressure be adhered to for all analyses; for many less exacting purposes the pressure effect can probably be disregarded, at least within the pressure ranges shown here (0.2 - 0.8 Torr).

It has been a common practice to employ inert gases such as helium or argon to sustain light emission by nitrogen in electrodeless discharge tubes (Cook et al. 1967; Keeney and Tedesco, 1973; Meyer et al. 1974). These gases are employed in situations where the amount of nitrogen is low and therefore the pressure insufficient to permit ignition. Meyer et al. (1974) state that ignition can be attained with as little as 15 mTorr nitrogen pressure, provided that total pressure is brought up to 1 Torr with argon. They also state, however, that accurate results were obtained only when the nitrogen pressure was at least 200 mTorr, with an additional 900 mTorr of argon pressure as a sustainer.

Figure 8. A plot of observed at % N-15 of natural abundance samples as a function of gas pressure (Torr) in the discharge tube of the NOI-5 emission spectrometer.



Even when sufficient nitrogen is present to sustain light emission in the tube, the previous three groups of workers still recommend the use of sustainer gases.

We have found that bright emissions are easily obtained on pressures from 0.2 to 3.0 Torr without the addition of a sustainer gas. The emissions are long lived (>0.5 hours) even at the lowest pressures and the accuracy appears to be generally better than those claimed by other workers (Fig. 5). The coefficients of variation of 0.37 and 4.15 at % standards were 2.0% and 1.3% respectively (n=8 in both cases). These variations are significantly lower than those reported by Meyer et al. (1974). Of more importance is the fact that it is considerably easier to process samples if the sustainer gas is not required.

The gain setting for the photomultiplier tube appears to have no effect upon the at % reading within the range of 1000 to 1400 volts. This is at least in partial agreement with Meyer et al. (1974) and in contrast to the results of Keeney and Tedesco (1973). In our experience it is not necessary to exceed these voltage bounds.

SECTION II OF METHODS

ACETYLENE REDUCTION TECHNIQUES

INTRODUCTION

The acetylene reduction technique rapidly became popular as a method of measuring biological nitrogen fixation because of its high sensitivity and simplicity. It has been applied to a range of organisms broad enough to include termites (Benemann, 1973) and lichens (Hitch and Stewart, 1973). The number of variations on the basic assay procedure have increased almost proportionally to the increasing number of investigators interested in N_2 fixation. Unfortunately not all these variations can be considered useful and in particular, most methods used in aquatic environments seem unacceptable.

There are several factors affecting the assay that have been disregarded in past aquatic applications and results of such work are suspect. The refinements to the acetylene reduction assay that are essential to this study are described below and have been published earlier (Flett et al. 1976).

PROCEDURES

Typical Previous Procedure - "Method I"

Most aquatic acetylene reduction assays have been performed on water samples using variants of the following basic technique. A small serum bottle (5-100 ml) is partially filled with the sample, capped with a serum bottle stopper, and the vapour phase replaced with a synthetic atmosphere containing argon, oxygen, carbon dioxide and acetylene (Rusness and Burris, 1970). In some aerobic assays

(Stewart et al., 1971) an air vapour phase, to which acetylene has been added, is employed. Anaerobic assays usually have a synthetic vapour phase in which oxygen is replaced by argon. After a suitable incubation period, which ranges from several minutes to many hours (Taylor et al., 1973; Brezonik and Harper, 1969) a known volume of the vapour phase is removed by syringe and its ethylene content assayed by hydrogen flame ionization detector gas chromatography. A simple calculation is employed to relate this value to the amount of di-nitrogen fixed:

$$\text{moles } N_2 \text{ fixed} = .33 \times \text{moles } C_2H_4 \text{ produced}$$

Such methods appear to be based upon a number of untested assumptions involving the degree and velocity of gas exchange between aqueous and vapour phases. The methods also appear to be performed under suboptimal conditions due to this neglect of the basic physical chemistry of the assay. In aqueous systems many of these difficulties can be overcome by the following technique.

Modified Procedure - "Method II"

The water sample is obtained by any suitable sampler (in our case, Van Dorn) that has a female London Luer fitting installed in the end of the exhaust tube. Water is allowed to exhaust for several seconds before a 50 ml glass syringe (interchangeable London Luer) is connected to the Luer outlet of the sampler and partially filled with sample. Plastic syringes are unsuitable due to gas permeability and pronounced 'memory'. The syringe is then disconnected from the sampler and shaken sufficiently to rinse and completely wet the barrel and plunger. This

water is expelled and the syringe reconnected to the sampler from which ∿40 ml of sample [1] is gently drawn into the syringe. After disconnection from the sampler, the syringe is held point upward and the sample volume reduced to ∿31 ml, thus expelling any air bubbles. A 26 G, 1.3 cm (1/2 in) hypodermic needle is fitted to the syringe and the volume reduced to exactly 30 ml. A second method of obtaining water samples is described in Section III of Methods.

Acetylene is obtained directly from a small MC cylinder (welding grade) equipped with a regulator that supplies gas via a short length of surgical tubing that is end-plugged with a serum stopper. The tube is flushed with acetylene prior to being sealed by the serum stopper.

With the syringe pointing upward, the needle is forced through the acetylene tube serum stopper and ∿7 ml of acetylene is allowed into the syringe over the water sample. It is important that the gas does not bubble through the water because it is very rapidly dissolved and thus an unknown excess of acetylene may accidentally be added. While avoiding agitation, which would cause acetylene to dissolve, the needle is withdrawn from the serum stopper, the gas volume adjusted to exactly 5.0 ml and the needle is then sealed by forcing it into a solid rubber stopper. The sample is vigorously shaken for 10 seconds or until no more acetylene will dissolve, at which time it is our experience that the vapour phase has a volume of ∿0.25 ml. This moment is recorded as the beginning of the incubation period.

---

[1] Note all stated volumes are appropriate to our experiments and may vary according to individual requirements; see later.

After a suitable period of incubation, the assay can be terminated by the addition of trichloracetic acid (Stewart et al., 1967) or the equilibrated vapour phase can simply be removed immediately (see following).

Gas samples are obtained as follows: A gentle tension is exerted on the syringe plunger and the stopper is simultaneously removed. This procedure reduces the probability of losing gas when the stopper is removed. Room air is drawn into the syringe such that the vapour phase volume is 15 ml and then it is resealed with the stopper. The syringe is vigorously shaken for 30 seconds to equilibrate the vapour and aqueous phases. The final vapour phase volume is carefully noted because it will exceed the expected 15 ml, a consequence of acetylene degassing from the water.

Small serum bottles (7-13 ml) are used as storage vessels for the gas samples. They are prepared by completely filling them with distilled water and then capping them with serum stoppers. The water that is displaced by the stoppers is vented through a 26 G hypodermic needle. They must be free of entrained air.

The equilibrated gas phase from the syringe is introduced into the serum bottle as follows: a 26 G hypodermic needle is pushed just through the stopper of an upright serum bottle. The syringe needle is unsealed by removing the solid stopper, the syringe pointing downward while being held by the barrel. This may allow a very small amount of water to leak out but ensures that no gas contamination occurs. Next, the syringe needle is forced all the way into the serum bottle stopper and the whole assembly inverted so that the syringe points up. The gas is forced into the serum bottle and the water displaced through

the vent needle. After most of the gas has been expelled from the syringe and all of the water forced from the serum bottle, both needles are removed. The vent needle should not be left in place any longer than necessary because air may enter through it and contaminate the gas sample.

Gas chromatographic analysis of a sample can be accomplished by hydrogen flame ionization detector equipped G.C. using a variety of packings (Hardy et al., 1973). We have found phenylisocyanate/Porasil C[1] @ 45°C to be very effective because the large amounts of methane often found in lake bottom waters do not interfere with the ethylene peak nor do large quantities of acetylene interfere because the acetylene peak follows that of ethylene. Normally, 0.1 ml of sample gives adequate sensitivity yet prevents extensive loading of the column with acetylene. The serum bottles prove convenient because they may be repeatedly sampled with little loss of accuracy if only 0.1 ml is withdrawn per analysis.

Once the concentration of ethylene in the vapour has been determined, the actual quantity of ethylene produced in a given volume of water sample can be calculated using the relationship outlined later in this section of the dissertation.

---

[1] Waters Associates, Framingham, Mass.

RESULTS AND DISCUSSION

Ethylene Solubility

Procedures analogous to Method I depend heavily upon the assumption that ethylene is insoluble in water and therefore that all the ethylene produced is quickly transferred to the vapour phase. This assumption is incorrect.

Ethylene is quite soluble in water. Under 1.0 atmosphere pressure of pure ethylene at 20°C, 0.122 volumes of the gas will dissolve in 1.0 volume of water. Compared to methane, oxygen, and nitrogen, it is respectively 3.7, 3.9 and 8.1 times more soluble than these other gases (Loomis, 1928).

The amount of ethylene that passes from solution into the vapour phase in an equilibrated closed system depends upon the relative amounts of aqueous and vapour phase present. The actual quantity of the ethylene transferred can be calculated by the following variation of Henry's Law:

$$X = \frac{M}{1 + \frac{\alpha A}{B}}$$ where:

X = volume of the gas transferred from aqueous to vapour phase (ml), M = total volume of the gas dissolved in the aqueous phase (ml), α = Bunsen absorption coefficient = volume of gas that will dissolve in 1.0 ml of water at a given temperature and under 1.0 atmosphere partial pressure of the gas, A = volume of the aqueous phase (ml), B = volume of the vapour phase (ml). All of the gas in the system is assumed to be initially dissolved in the aqueous phase.

Application of this relationship is intrinsic to the success of Method II. It must be stressed that α depends upon the temperature of the aqueous phase when equilibrated, not that occurring during incubation. It should also be noted, that the value B is the volume of the vapour phase after equilibration; this value will be slightly larger than the volume of air initially employed for equilibration.

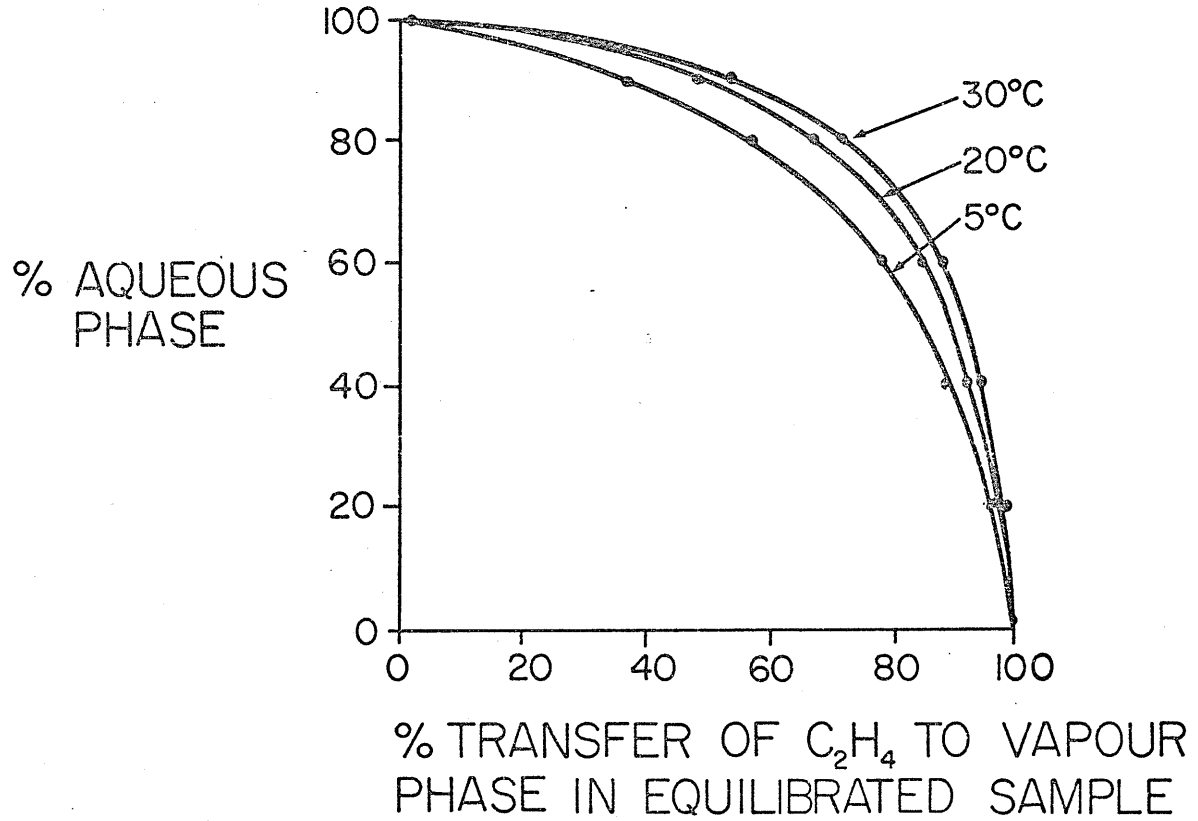
The percentage of ethylene transferred from aqueous to vapour phase for a given temperature can also be calculated:

$$\text{Percent transfer of ethylene} = \frac{X(100)}{M} = \frac{100}{1 + \frac{\alpha A}{B}}$$

From this formula, a plot of percentage transfer of ethylene versus aqueous phase as a percentage of total volume can be made for various temperatures (Fig. 9).

From this plot it is evident that the percentage transfer of ethylene is inversely proportional to the percentage of aqueous phase and is directly proportional to temperature. For example, at a temperature of 5°C only 57% of the ethylene would be transferred to the vapour phase from an aqueous phase that was 80% of the total volume of the sample bottle. Therefore, under conditions where the aqueous phase comprises a significant fraction of the total volume, a significant error can made if one assumes that ethylene is insoluble. In view of the fact that the curves shown in Fig. 9 are easily generated and applied, it would seem unreasonable not to use them when calculating ethylene concentrations

Figure 9. A thoeretical plot of percentage transfer of ethylene to the vapour phase versus aqueous phase as a percentage of total volume of the vessel for 5, 20 and 30°C.


100
80
60
40
20
0
0
20
40
60
80
100
30°C
20°C
5°C
% AQUEOUS PHASE
% TRANSFER OF C_2H_4 TO VAPOUR PHASE IN EQUILIBRATED SAMPLE

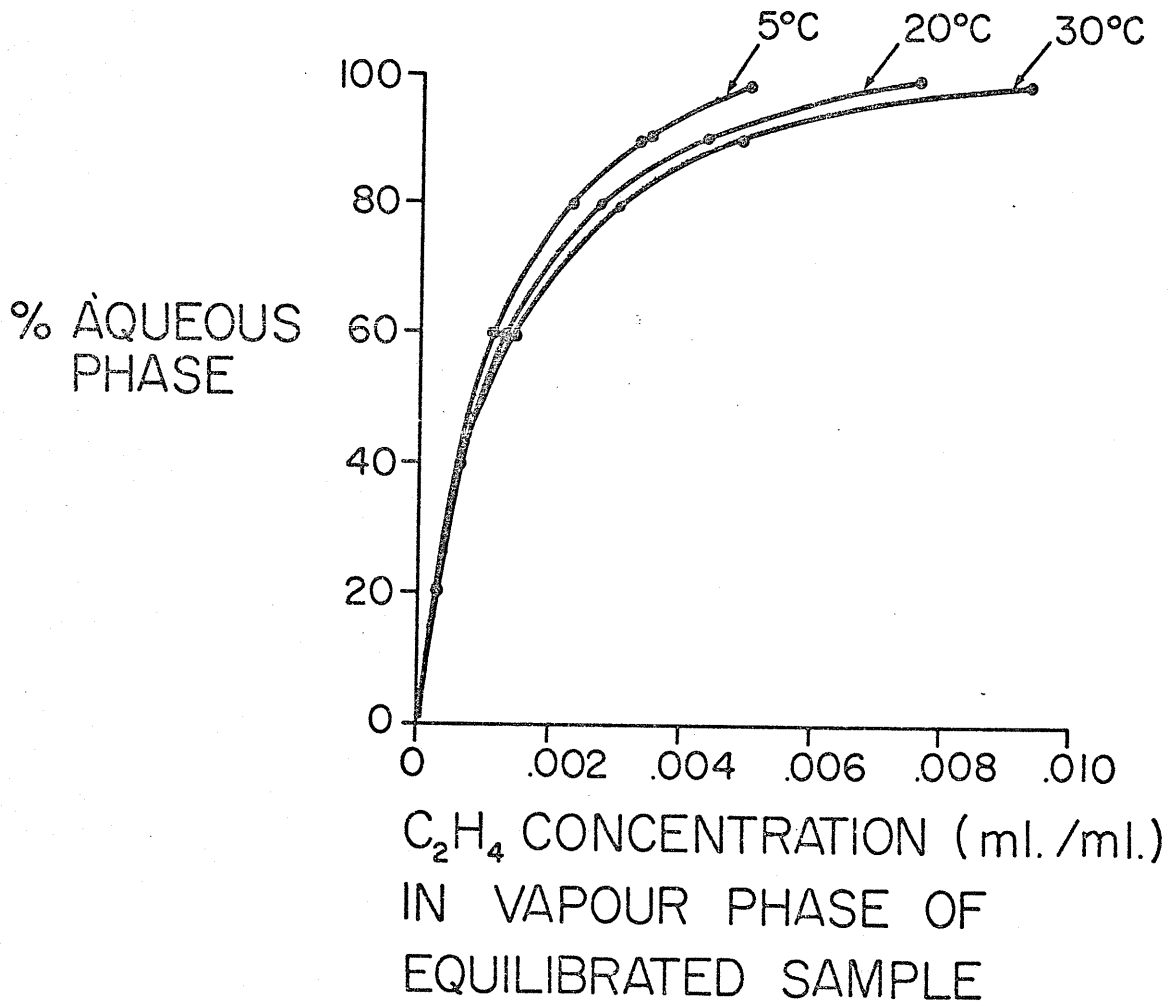
Assay Sensitivity

In order to increase the sensitivity of the acetylene reduction method, some workers have concentrated organisms in water samples to increase the amount of ethylene produced per unit volume of sample (Granhall and Lundgren, 1971). Concentration of natural samples is widely recognized in microbial ecology as a procedure best left undone if there are other methods available for increasing the sensitivity of an assay.

Figure 10 is a plot of theoretical calculations of percentage aqueous volume versus equilibrium ethylene concentration for a water sample at various temperatures and originally containing 1.0×10^{-3} ml C_2H_4/ml H_2O. It should be noted that the concentration of ethylene in the vapour phase is directly proportional to the percentage aqueous phase. For example, at 20°C the concentration of ethylene in a system with 80% aqueous phase is 11.2 times greater than in one having a 20% aqueous phase. This illustrates that assay sensitivity can be significantly enhanced simply by increasing the relative proportion of aqueous phase in the incubation bottle rather than by concentrating the sample. The limiting factor in this method of increasing sensitivity is the volume of the vapour phase as it is necessary that the vapour phase is kept large enough for accurate subsampling. If a vapour phase of ∿10 ml were required for accuracy, a 10:1 aqueous: vapour volume ratio could be employed on a 100 ml sample. Should still more sensitivity be required a 100:1 ratio could be used on a 1.0 ℓ sample while still maintaining the 10 ml vapour phase.

This method of increasing sensitivity has evidently been used in methods of type I but it appears (Stewart, 1968) that no correction has

Figure 10. A theoretical plot of ethylene concentration in the vapour phase versus aqueous phase as a percentage of total volume of the vessel for 5, 20, and 30°C. The original ethylene concentration was 1.0×10^{-3} ml C_2H_4/ml H_2O.


5°C
20°C
30°C
100
80
60
40
20
0
% AQUEOUS PHASE
0
.002
.004
.006
.008
.010
C_2H_4 CONCENTRATION (ml./ml.)
IN VAPOUR PHASE OF
EQUILIBRATED SAMPLE

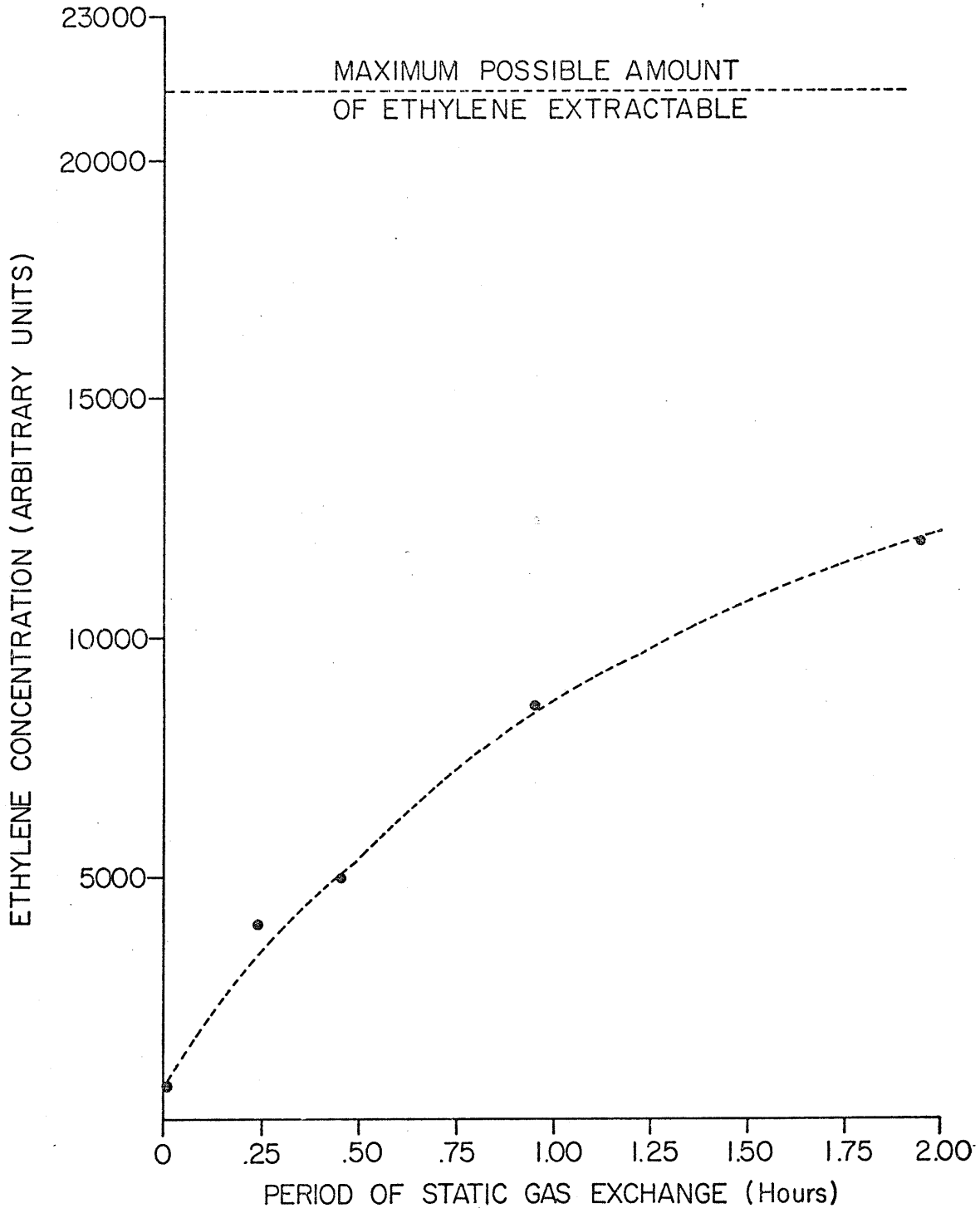
been made for ethylene solubility, an important factor in assays having proportionately large aqueous phases. Method II accommodates the problem of ethylene solubility and also provides flexibility in determining the final aqueous:vapour phase ratio. If this means of increasing the sensitivity proves inadequate in a given situation perhaps one should ask whether the rate of ethylene production (nitrogen fixation) is of any significance in the environment being examined.

Agitation and Gas Exchange

Many workers using Method I seem to have assumed that ethylene, by reason of its supposed insolubility was totally transferred to the vapour phase even though the samples were not agitated during or after incubation. To test this assumption a 50 ml serum bottle was half filled with water than contained a nonsaturating amount of dissolved ethylene. The vessel was sealed and left at rest on a table top. The vapour phase was subsampled for ethylene concentration at regular intervals, the results being seen in Figure 11.

Of the maximum amount of ethylene theoretically transferrable to the vapour phase, only 57% appeared there in 2 hours. While this experiment represents the extreme case of abolutely no agitation, it illustrates how underestimations of ethylene production can be obtained if one does not ensure complete equilibration. In an actual assay, such an underestimate would be further exaggerated because ethylene is constantly being produced, not as in this experiment where the total quantity of ethylene was present at time zero.

Figure 11. An experimental plot illustrating the transfer of ethylene from aqueous to vapour phase as a function of time. The vessel was static, had a 50 ml volume, and contained 25 ml of water in which was dissolved a subsaturating amount of ethylene.

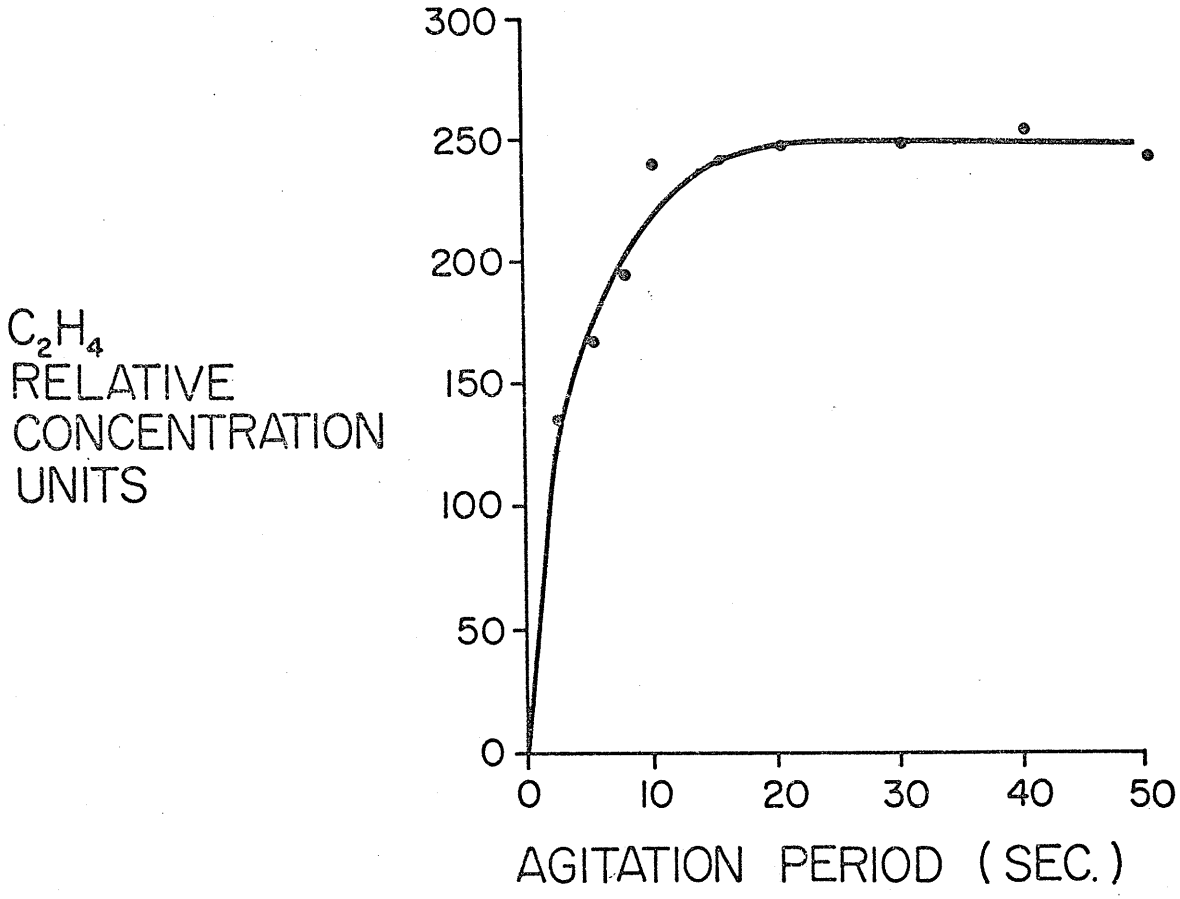

23000
20000
15000
10000
5000
MAXIMUM POSSIBLE AMOUNT
OF ETHYLENE EXTRACTABLE
ETHYLENE CONCENTRATION (ARBITRARY UNITS)
0
.25
.50
.75
1.00
1.25
1.50
1.75
2.00
PERIOD OF STATIC GAS EXCHANGE (Hours)

It being clear that equilibration of the ethylene between the aqueous and vapour phases was not immediate under static conditions, an experiment was undertaken to determine how quickly equilibrium could be obtained. The method was the same as in the previous experiment except that the vessels were vigorously hand shaken for various time periods.

The results (Fig. 12) reveal that equilibration occurred in about 20 seconds in this specific case, where the aqueous and vapour phases were equal in volume (25 ml). However, it is essential that each experimenter using a procedure like Method I determine the equilibration time for his own vessels and sample volumes if he wishes to obtain accurate results. For example, in cases where a 1.0 ml gas phase were utilized, equilibration periods of the order of 5 - 10 minutes might be required. Note that Method II stresses precise, standardized equilibration times and agitation procedures.

A final caution should be given about data obtained from experiments not employing agitation steps. As illustrated in Fig. 12, initially, very slightly different periods of agitation cause greatly different amounts of ethylene to be transferred. It is quite conceivable that duplicate samples could vary greatly simply because one sample was inadvertently handled (agitated) more than the other. Unfortunately, data obtained from such experiments is not amenable to later correction because each sample may have undergone a different and unknown degree of agitation. Due to this, some previously published data may be of litte real value.

Figure 12. An experimental plot showing the transfer of ethylene from aqueous to vapour phase as a function of time. The vessel was vigorously shaken by hand, had a 50 ml volume, and contained 25 ml of water in which was dissolved a subsaturating amount of ethylene.


C_2H_4
RELATIVE
CONCENTRATION
UNITS
300
250
200
150
100
50
0
0
10
20
30
40
50
AGITATION PERIOD (SEC.)

True Substrate Concentrations

The concentration of acetylene or nitrogen (expressed in atmospheres) in a sample can be determined by a further variation of Henry's Law:

$$\frac{M - X}{A} = \text{Concentration of dissolved gas in the sample (ml gas/ml } H_2O)$$

Therefore: $$\frac{M-X}{A} = \frac{M - \frac{M}{1 + \frac{\alpha A}{B}}}{A} \qquad \text{where } x = \frac{M}{1 + \frac{\alpha A}{B}}$$

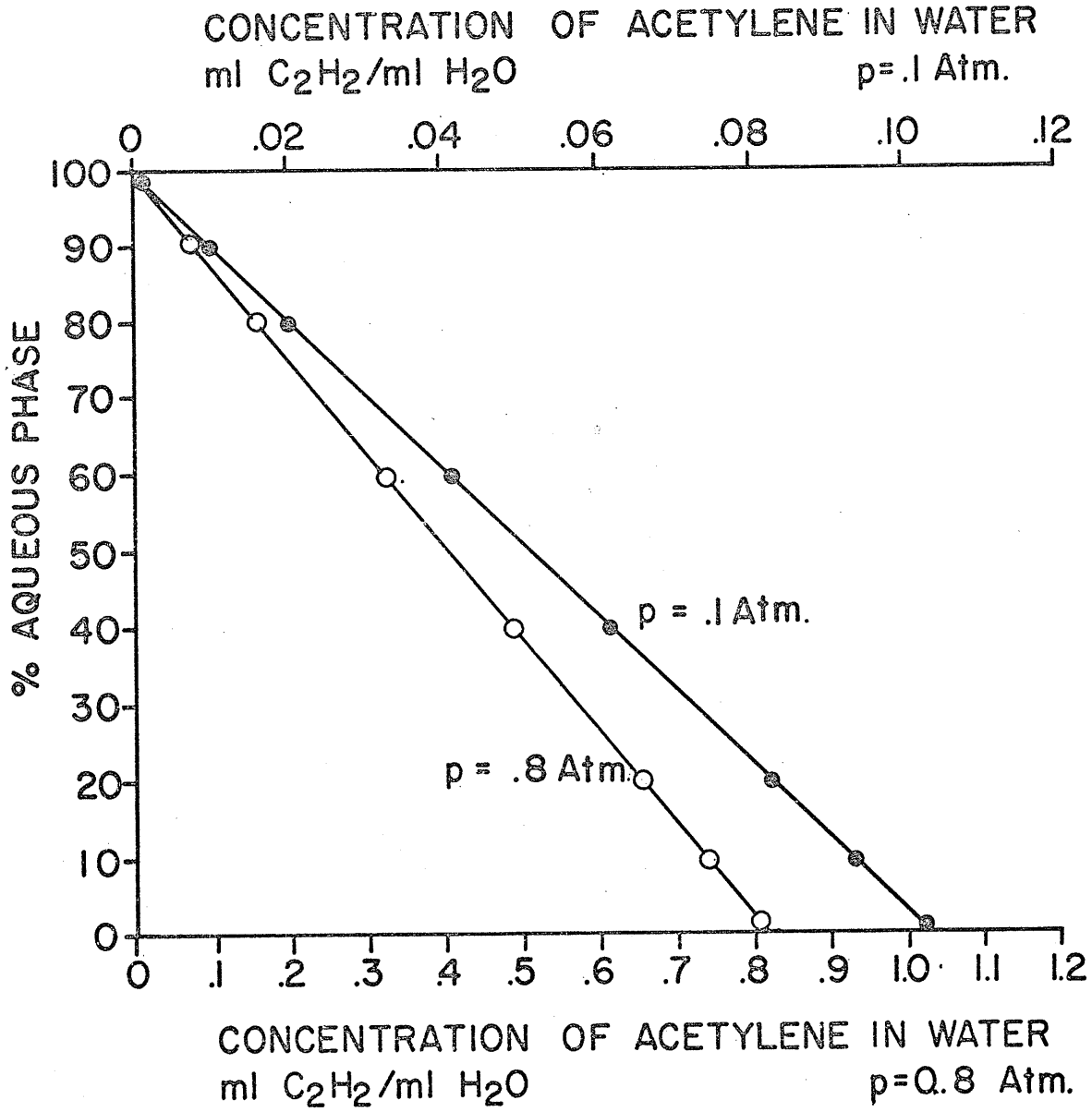
p is defined as the initial partial pressure of the gas in atmospheres. Therefore pB = M

$$\frac{M-X}{A} = \frac{pB - \frac{pB}{1 + \frac{\alpha A}{B}}}{A}$$

From this one is able to calculate the concentration of dissolved gas in samples of any aqueous:vapour phase volume ratio and under any partial pressure of the gas.

Figure 13 is a theoretical plot of dissolved C_2H_2 concentration in a series of samples having various % aqueous phases initially under 0.1 and 0.8 atmosphere C_2H_2 vapour phases. It is obvious that the % aqueous phase, as well as the partial pressure, has a marked effect upon the concentration of dissolved acetylene.

Figure 13. A theoretical plot of dissolved acetylene concentrations versus % aqueous phase for vapour phases initially containing 0.1 and 0.8 atmospheres of acetylene.

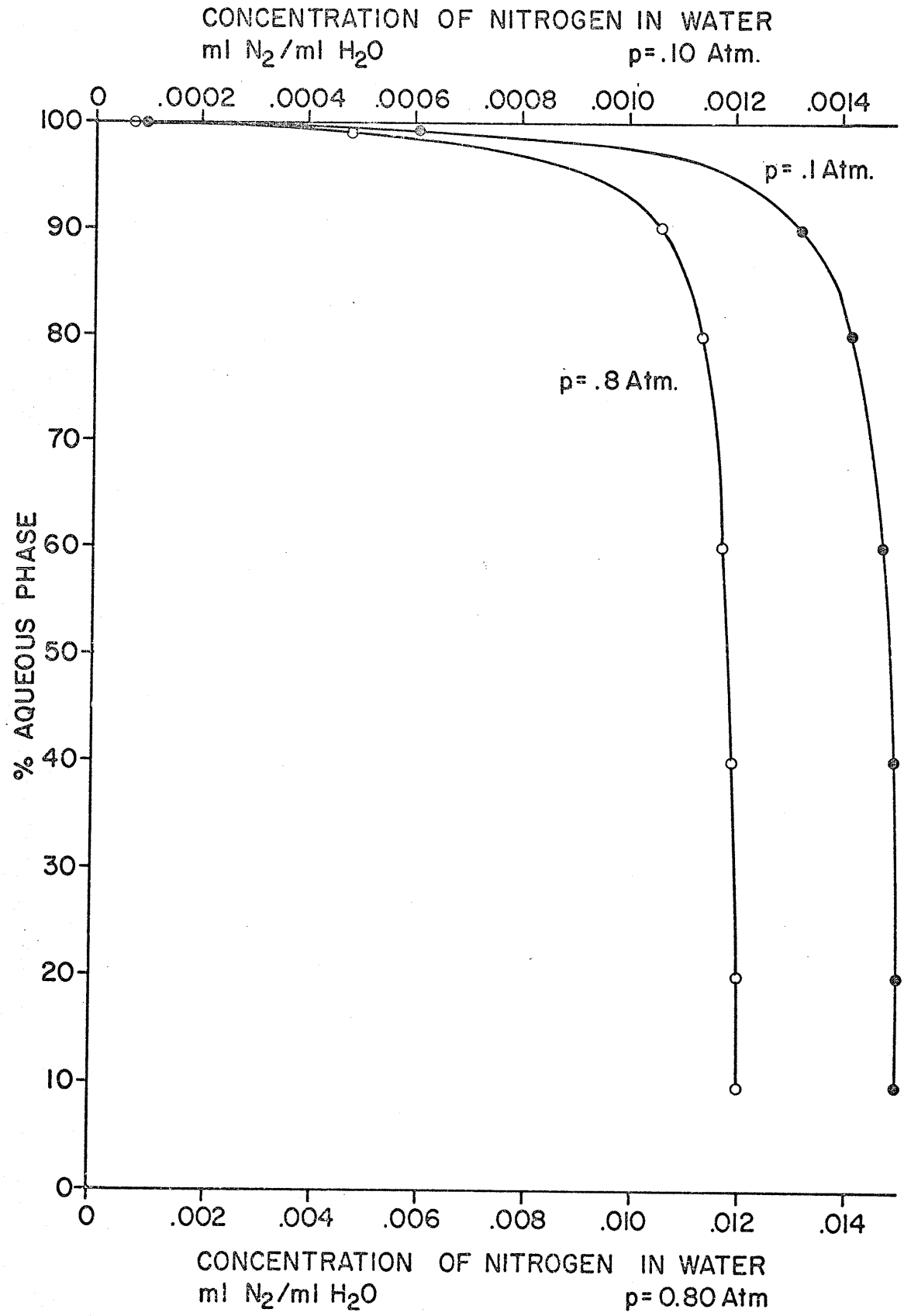

CONCENTRATION OF ACETYLENE IN WATER
ml C_2H_2/ml H_2O
p=.1 Atm.
0
.02
.04
.06
.08
.10
.12
100
90
80
70
60
50
40
30
20
10
0
% AQUEOUS PHASE
p = .1 Atm.
p = .8 Atm.
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
1.1
1.2
CONCENTRATION OF ACETYLENE IN WATER
ml C_2H_2/ml H_2O
p=0.8 Atm.

Conceivably, the nitrogenase of one sample could be saturated with acetylene while that of a second sample might not be, even though both samples were exposed to the same partial pressures of acetylene. The samples only need to have different % aqueous phases for this difference to occur. For example, Stewart (1968) has determined that Anabaena flos-aqua are just saturated at 0.1 atm. C_2H_2 in a chamber having a 4.0 ml vapour phase and a 1.0 ml aqueous phase (C_2H_2 concentration = 0.082 ml C_2H_2/ml H_2O). If the same assay were performed in a chamber with 0.1 atm C_2H_2, a 4.0 ml vapour phase and a 4.0 ml aqueous phase, the ethylene production/ml of culture might be only 60% of that in the previous test, due solely to subsaturation of the nitrogenase (0.051 ml C_2H_2/ml culture).

Moreover, if the half-saturation constant (K_m) values are expressed in atmospheres C_2H_2, values obtained in one experiment are not directly comparable to K_m values obtained from other experiments; the % aqueous phase must be included in the data for such comparisons to be possible. This could be avoided by expressing K_m values in terms of concentration units and then direct comparisons between K_m values could be made without having to recalculate past data.

Figure 14 is a plot of dissolved N_2 concentration in a series of samples having various % aqueous phases initially under 0.1 and 0.8 atmosphere N_2 vapour phases. As was the case with acetylene, both partial pressure and the % aqueous phase can be seen to have effects upon the concentration of dissolved N_2 in the water. The change in dissolved N_2 in the water, as a function of % aqueous phase, is not very pronounced for values below 80%. Therefore, all data concerned with nitrogen gas concentrations and obtained from samples with less

Figure 14. A theoretical plot of dissolved nitrogen gas concentrations versus % aqueous phase for vapour phases initially containing 0.1 and 0.8 atmospheres of nitrogen.


CONCENTRATION OF NITROGEN IN WATER
ml N_2/ml H_2O
p=.10 Atm.
0
.0002
.0004
.0006
.0008
.0010
.0012
.0014
100
90
80
70
60
50
40
30
20
10
0
p=.1 Atm.
p=.8 Atm.
% AQUEOUS PHASE
0
.002
.004
.006
.008
.010
.012
.014
CONCENTRATION OF NITROGEN IN WATER
ml N_2/ml H_2O
p=0.80 Atm

than 80% aqueous phases are probably intercomparable even though they are expressed in atmospheres. This means that most nitrogen gas K_m values which are expressed in atmospheres of nitrogen can probably be validly intercompared. Nonetheless, nitrogen gas concentrations would be best described in concentration units so that no ambiguity exists.

A convention should be adopted with respect to the expression of dissolved gas concentration. Oxygen and carbon dioxide are usually expressed in terms of concentration units eg. μ moles/l or mg/l; so it would seem should nitrogen and acetylene.

Acetylene Additions

It has been suggested previously (Hardy et al., 1973) that any nitrogen gas originally present in a sample should be removed and then replaced by a quantity of acetylene that will "saturate N_2-ase to the same degree that it is saturated by ambient N_2". Such an approach, though perhaps correct in theory, is not desirable in aquatic field studies because other gases (CO_2, O_2, CH_4 etc.) will be removed with the nitrogen and subsequently should be replaced (see later). This presents an almost insurmountable problem for field biologists.

Another technique has been developed (Granhall and Lundgren, 1971) wherein acetylene is applied to a sample in such high concentrations that fixation of ambient nitrogen is completely inhibited. This is an especially attractive technique because the gases originally dissolved in the sample can be retained provided a sufficiently small vapour phase is employed as in Method II. The main consideration is to ensure

that saturation of the nitrogenase has substantially occurred; this should be verified experimentally for the particular ecosystem being assayed. However, it must also be demonstrated that inhibition of acetylene reduction is not taking place (Hardy et al., 1968; Brouzes and Knowles, 1971) due to an excess addition of acetylene. A typical addition (Stewart, 1968) in methods of type I, has been 0.1 atmospheres acetylene (4.0 ml vapour phase/1.0 ml aqueous phase) and the concentration can be calculated, by methods previously described in this paper, to be 0.082 ml C_2H_2/ml H_2O. The acetylene concentration, as given in method II, is 0.158 ml C_2H_2/ml H_2O when 5.0 ml of acetylene are added. Both have been found satisfactory.

Though very soluble in water (1.032 ml C_2H_2/ml H_2O @ 20°C and 1.0 atmosphere pressure C_2H_2), in our experience acetylene in the vapour phase will not quickly reach equilibrium with the aqueous phase unless the container is agitated. Therefore, samples should be shaken immediately upon injection of the acetylene, as in Method II; this will ensure that the nitrogenase is saturated as the start of the incubation period and not at some later time.

Blank Determinations

The use of blanks in the acetylene reduction technique is imperative. From them one determines the ambient ethylene concentration of the water and the quantity of ethylene added to the sample via impure[1] acetylene. The estimation of ethylene can be performed by

[1] Welding grade acetylene is often found to contain as little ethylene as high purity acetylene and therefore the welding grade gas is used because of its lower cost. The purity of gas improves as the cylinder empties and therefore the first half of the gas is often vented off so as to obtain acetylene containing little ethylene (Brooks et al. 1971).

adding the acetylene (and contaminating ethylene) to a sample, equilibrating it by agitation and then immediately subsampling the gas phase for gas chromatographic analysis of ethylene. Provided that water and gas phase volumes are the same in both blank and sample, the ethylene determined in the blank may be directly subtracted from that determined in the sample vessel that has been incubated and then equilibrated. Failure to equilibrate the contaminating ethylene may result in high blank values which in turn will lower estimates of total ethylene produced during incubations. A search of the literature indicates such errors may be common because there is no evidence that they have been deliberately avoided.

Vapour Phase Effects

Perhaps the least discussed yet most difficult problem in the application of the acetylene reduction assay to aquatic environments has been the presence of the vapour phase in the assay bottle, such as seen in Method I. Unless the gaseous constituents of the vapour phase are in exactly the right concentrations, a nonequilibrium situation will exist between the vapour and aqueous phases. This will cause the concentrations of dissolved gases in the water (O_2, CO_2, CH_4, N_2 etc.) to change from those originally occurring *in situ*.

A general statement about the biological effects of altered dissolved gas concentrations upon acetylene reduction cannot be made; there probably are many. For example, Stewart and Pearson (1970) found that changes in dissolved oxygen concentrations can affect rates of

acetylene reduction in algae. These workers have shown that acetylene reduction was enhanced at oxygen levels below or equal to atmospheric equilibrium and that it was inhibited at higher concentrations. Such findings have implications for the measurement of nitrogen fixation in the field. An actively photosynthesizing sample, one taken from the surface of a lake for example, may well contain higher than equilibrium concentrations of dissolved oxygen. Acetylene reduction rates should be depressed in this condition. Unfortunately, if most current field assay techniques were applied to such a sample, the dissolved oxygen concentration would drop due to equilibration with a relatively large vapour phase usually containing 20% oxygen. The probable result of a lower dissolved oxygen concentration would be a higher than in situ rate of acetylene reduction, and hence, an overestimate of nitrogen fixation.

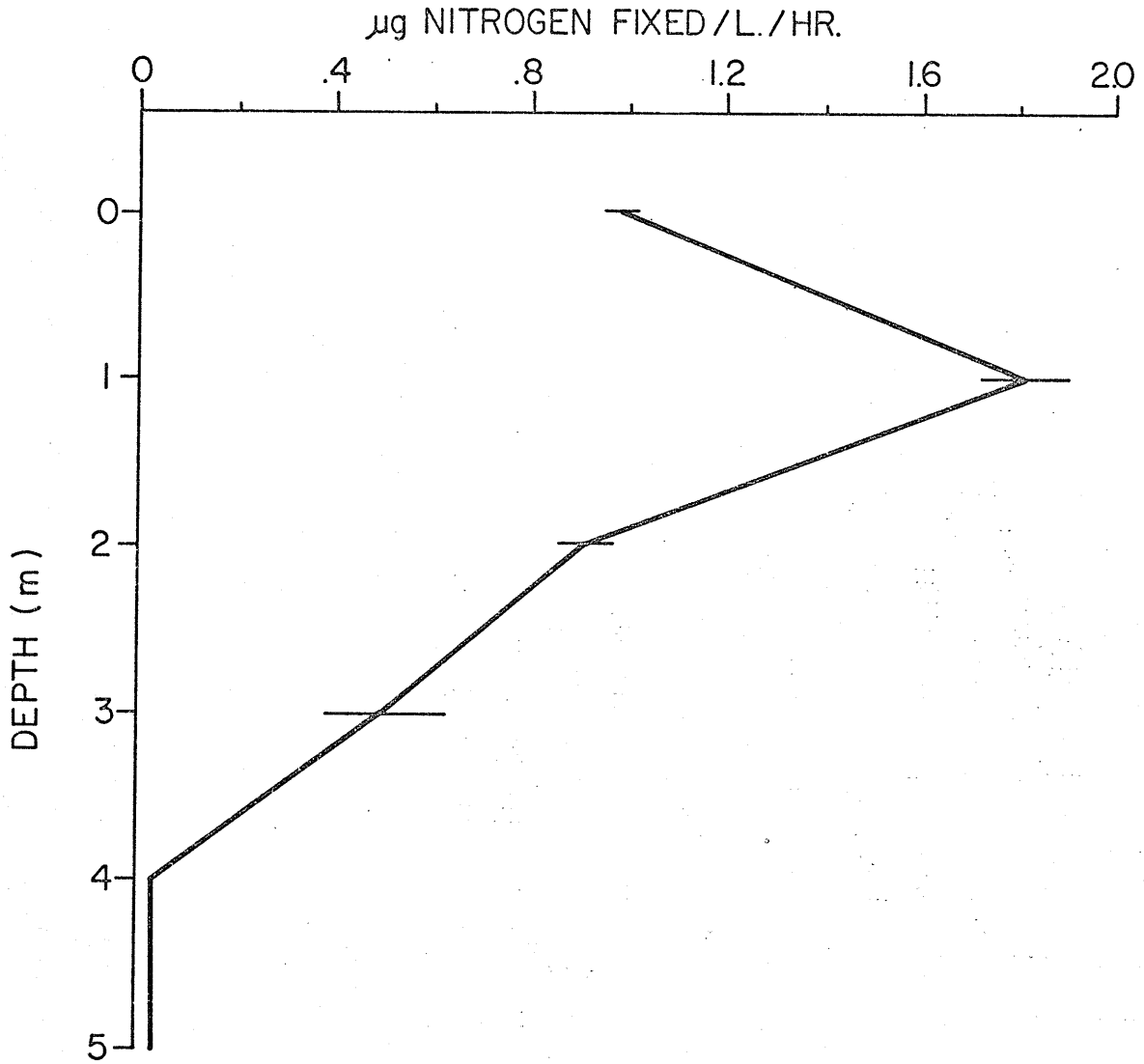
It has also been demonstrated by Stewart and Pearson (1970) that, after several hours acclimatization to lower than equilibrium oxygen concentrations, algae reduce acetylene more rapidly than at the higher atmospheric equilibrium concentrations to which they were previously exposed. Therefore, samples containing reduced amounts of oxygen when collected would probably yield underestimates of in situ nitrogen fixation when incubated under a 0.2 atmosphere oxygen vapour phase.

Several species of the aerobic nitrogen fixing bacterium Azotobacter have been observed to be affected by changes in dissolved oxygen concentrations by Drozd and Postgate (1970). These workers have shown that Azotobacter grown under vapour phases containing less than than 0.2 atmospheres oxygen exhibit lower acetylene reduction rates when

exposed to 0.2 atmosphere oxygen vapour phases. Since large numbers of lakes contain less oxygen than that expected for atmospheric equilibrium, it is quite possible that acetylene reduction assays performed under a 20% oxygen atmosphere would yield underestimates of Azotobacter nitrogenase activity. This effect may be especially troublesome when sampling from stratified lakes where oxygen concentrations can range with depth from levels equivalent to >0.4 atmospheres to 0 atmospheres. In addition, the effects of increased oxygen concentration upon acetylene reduction appear to be much more pronounced under conditions of carbon and phosphorus limitation (Drozd and Postgate, 1970). Such conditions are not uncommon in lakes (Schindler and Holmgren, 1971) and therefore oxygen inhibition must be carefully guarded against when assaying these water bodies by acetylene reduction.

The various problems associated with a vapour phase are largely avoided by Method II because of the very small vapour phase employed. Figure 15 is a typical example of many profiles of acetylene reduction, obtained by this method from the northeast basin of Lake 226, the Experimental Lakes Area of northwestern Ontario (Johnson and Vallentyne, 1971). This lake has two basins which are separated by a plastic curtain; the northeast basin receives carbon, nitrogen and phosphorus while the southeast basin receives only carbon and nitrogen. In the first and second years of fertilization (1973, 1974) extensive blooms of blue-green algae (Anabaena spiroides and Anabaena planktonica) occurred in the northeast basin (Schindler, 1974). The results of Fig. 15 were obtained on August 13, 1974 when the bloom was quite active. A C_2H_4 : N_2 ratio of 3 : 1 was assumed in order to estimate

Figure 15. An experimental plot of nitrogen fixation rates versus depth, for Lake 226 NE, the Experimental Lakes Area. It was performed according to Method II of the text and a C_2H_4 : N_2 ratio of 3:1 was assumed.


µg NITROGEN FIXED/L./HR.
0
.4
.8
1.2
1.6
2.0
DEPTH (m)
0
1
2
3
4
5

nitrogen fixation. It can be seen that duplicate measurements (ends of the horizontal bars) gave quite similar results, this being the case in most profiles employing the syringe technique.

Metabolism of the Gaseous Hydrocarbons

It had been previously determined that ethylene was rapidly metabolized by methane oxidizing bacteria (Flett et al., 1975) which were present in the lake at the aerobic/anaerobic interface, where oxygen concentrations were between 1.0 and 0.1 mg/l (Rudd and Hamilton, 1975). It was subsequently shown that a mixed culture containing these same methane oxidizers fixed nitrogen gas (Rudd et al., 1976) when no other nitrogen sources were present. From this it appeared that the acetylene reduction assay could not be used to measure nitrogen fixation in the presence of these organisms. However, de Bont and Mulder (1976) have found that the ethylene is not metabolized when acetylene is present in quantities normally used for acetylene reduction assays. This newer information indicated that the acetylene reduction assay could be used even in the presence of the methane oxidizers. It is unlikely, however, that nitrogen fixation by the methane oxidizers themselves can be measured, using the acetylene reduction technique, because acetylene interferes with the oxidative metabolism of methane (de Bont and Mulder, 1974; Whittenbury et al., 1975). The overall effect is that the acetylene reduction technique probably can be used to measure nitrogen fixation on all aerobic or microaerophyllic organisms except the methane oxidizers.

SECTION III OF METHODS

OTHER TECHNIQUES USED IN MEASURING NITROGEN FIXATION

Water Sampling Techniques

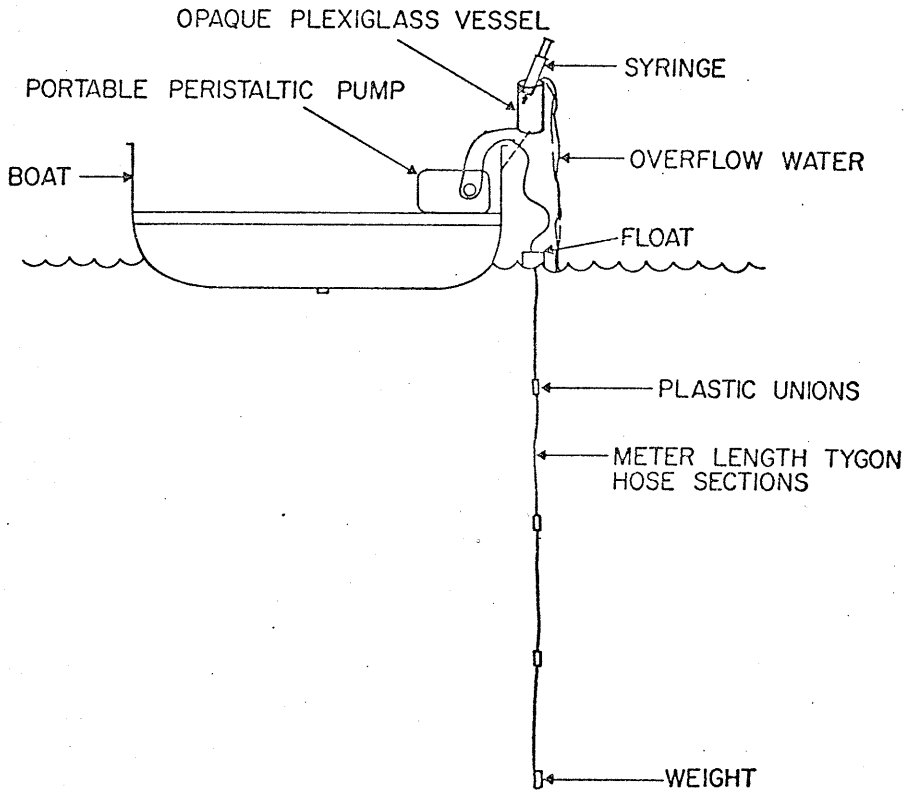
Water samples were obtained by boat in several ways. In 1972-74 most samples were taken with a Van Dorn sampler. This sampler was 0.45 meters long and 3 liters in volume. Thus, the sample was a composite of the water over the 0.45 meter depth interval that the device encompassed. The outlet hose of the Van Dorn was equipped with a female Luer fitting that could be used to fill syringes or bottles. In 1975 the samples were obtained by a portable peristaltic Cole-Parmer[1] pump because it allowed water to be taken at closely spaced depth intervals, an important advantage when one is examining gradients of microbial activity over small depth increments. Meter length sections of 0.95 cm (3/8 in) O.D. Tygon[2] tubing were connected together by plastic unions until the hose was the correct length for sampling the desired depth. This assured that the hose length was as short as possible, an important factor if degassing of methane, nitrogen, oxygen, etc. from the water because of pressure and temperature changes, is to be avoided (Rudd and Hamilton, 1975). The sampling hose was suspended from a plastic float so that up and down movements of the boat were not transmitted to the hose. The pump fed the sample water either directly into bottles (overflow 3 times) or into the bottom of an opaque plexiglass vessel of 500 ml volume which overflowed from the open top (see Fig. 16). This opaque vessel partially protected the water from bright surface light and yet permitted easy sub-

---

1 Cole-Parmer - Chicago, Illinois, U.S.A.

2 Tygon - T.M. of Norton Plastics and Synthetics Div., Akron, Ohio, U.S.A.

Figure 16. A diagram of the water sampling apparatus used in 1975.


OPAQUE PLEXIGLASS VESSEL
SYRINGE
PORTABLE PERISTALTIC PUMP
BOAT
OVERFLOW WATER
FLOAT
PLASTIC UNIONS
METER LENGTH TYGON
HOSE SECTIONS
WEIGHT

sampling of the water, by syringe, through the top. Sufficient pumping time (30 seconds) was allowed at each new depth for the vessel to be theoretically filled 3 times. This appeared to effectively eliminate 'memory' of previous samples. Gas exchange with the atmosphere was judged to be minimal because Winkler oxygen determinations showed anaerobic bottom water samples could be obtained in the syringes.

Additions of Acetylene Gas

Acetylene gas was added to samples as described in the 'Acetylene Reduction Assay' section of Methods.

Addition of Nitrogen - 15 Gas

Nitrogen-15 gas (Onia)[1] of 57 to 95 at % was obtained in 500 ml ampoules that were suitable for connection to vacuum racks. The vacuum fitting was carefully broken off about 3 cm above the break seal and a 2 cm metal rod was gently placed in the open end. A #57 Suba Seal[2] was forced over the end of the tube to form a tight seal, the tube being inside the Suba Seal. The dead space between the Suba Seal and the break seal was next flushed for several minutes with commercial grade tank nitrogen, it being introduced by one 26 G hypodermic needle and vented through a second 26 G needle. The needles were removed and the ampoule vigorously shaken until the break seal was visibly broken. Next, about 20 ml of boiled distilled water was injected into the ampoule by syringe with a 26 G needle. This water

---

1 Onia - Azote et Produits Chimiques S.A., Paris, France

2 Suba Seal - Griffin and George Ltd., Toronto, Canada

raised the pressure of the nitrogen in the ampoule slightly above that in the surrounding air and ensured that any leaks would be in a direction out of the ampoule. The water was previously boiled to partially remove dissolved oxygen which would contaminate the N-15.

Nitrogen-15 gas was obtained from the ampoule as follows: a syringe, of sufficient volume to contain the desired amount of nitrogen was filled with boiling distilled water which was then injected through a 26 G needle into the ampoule. After the water was injected, an equal amount of nitrogen-15 was pulled into the same syringe which was next removed from the Suba Seal. The syringe was immediately sealed by inserting the needle into a solid rubber stopper (Fig. 17A).

If N-15 gas was to be added to samples in serum bottles (4 ml N_2 + 116 ml H_2O), the bottles were sealed with serum stoppers in such a way as to exclude air bubbles (see 'Acetylene Reduction Assay' section in Methods). The N-15 gas was injected into the bottle through the serum stopper, the bottle being inverted and the excess water being released through a second 26 G needle (Fig. 17B).

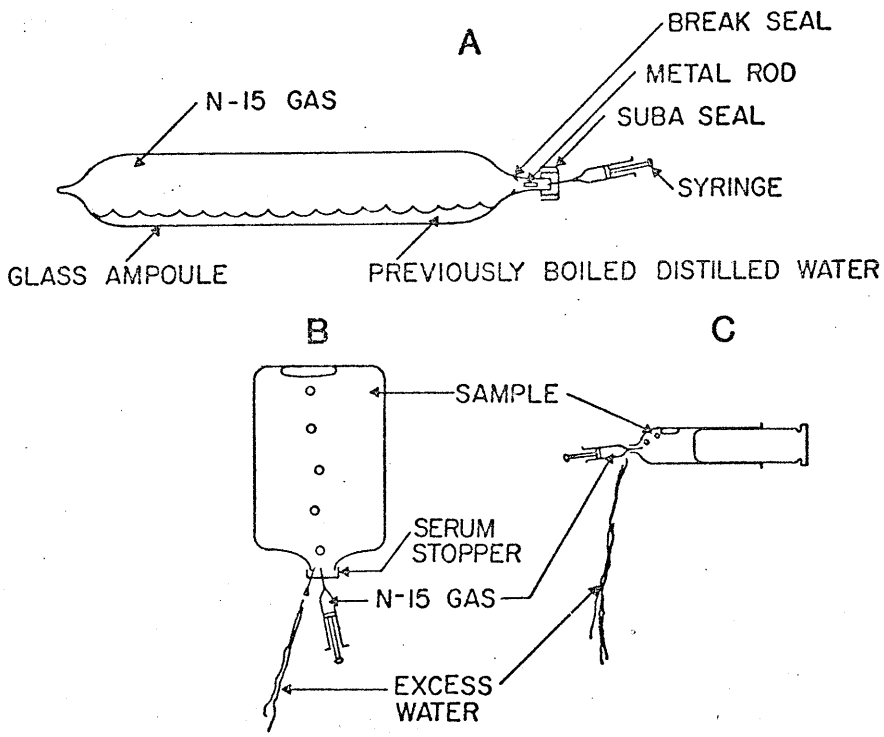
If N-15 gas was to be added to samples in syringes (1 ml N_2 + 29 ml H_2O), the gas was simply added through the male Luer fitting (the needle off the syringe - Fig. 17C) with the syringe lying on its side. The syringe was then sealed with a needle that was forced into a solid rubber stopper.

Vigorous agitation of the samples was required to dissolve and equilibrate the N-15 gas. The time period required for equilibration was variable, depending upon the aqueous : gaseous volume ratios, temperature and degree of agitation. For example, equilibration of

Figure 17 (A) A diagram showing how N-15 gas was obtained from the gas ampoule.

(B) A diagram showing how N-15 gas was added to a water sample contained in a serum bottle.

(C) A diagram showing how N-15 gas was added to a water sample contained in a glass syringe.


A
BREAK SEAL
METAL ROD
SUBA SEAL
N-15 GAS
SYRINGE
GLASS AMPOULE
PREVIOUSLY BOILED DISTILLED WATER
B
C
SAMPLE
SERUM STOPPER
N-15 GAS
EXCESS WATER

1 ml of N-15 in 29 ml of water at 20 C required about 5 minutes of vigorous hand shaking.

The sample was incubated either in situ or in an incubator (see Incubator Procedures in Methods) for a period generally shorter than 4 hours.

Determinations of Specific Activities of N_2 - 15 in Samples

The actual concentration of N_2 - 15 in a sample was estimated theoretically:

$$S = \frac{(V_E \times P_E) + V_N \times P_N)}{V_E + V_N} \quad \text{where}$$

V_E = volume of enriched N_2 - 15 gas added (known)

P_E = at % N - 15 in enriched gas (known approximately)

V_N = volume of originally dissolved natural abundance nitrogen (calculated)

P_N = at % N - 15 in natural abundance nitrogen (known)

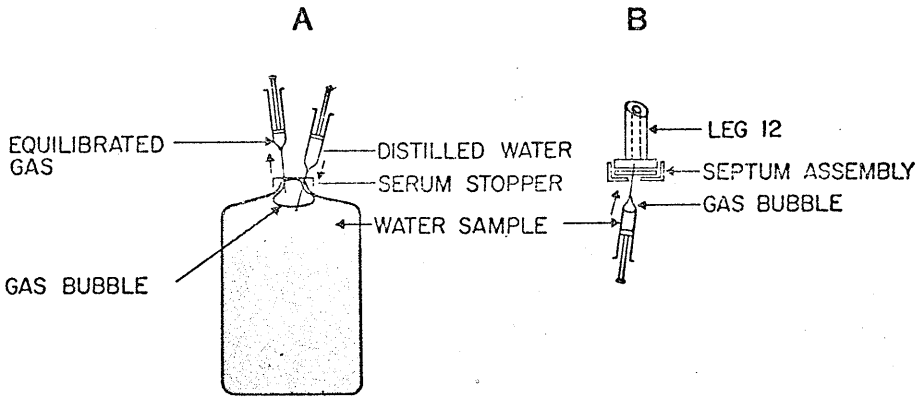
S = specific activity (at %) of equilibrated N_2 gas in sample

In most cases the estimated specific activity was found to be accurate enough. Where a better accuracy was demanded, or where S could not be calculated, the actual at % was measured directly:

For bottled samples, at the end of an incubation the equilibrated gas bubble was forced into a syringe by adding distilled water to the sample bottle (Fig. 18A). The gas so collected was injected directly into the sample preparation unit (Fig. 18B) of the N-15 analyser, through a septum installed on leg 12. For syringe-enclosed samples, the gas

Figure 18. (A) A diagram showing how to obtain gas sample of the equilibrated gas phase in a serum bottle so that N-15 specific activity can be determined.

(B) A diagram showing how the gas samples (from serum bottles or syringes) are injected into the preparation unit for N-15 analysis.


A
B
EQUILIBRATED GAS
DISTILLED WATER
SERUM STOPPER
WATER SAMPLE
GAS BUBBLE
LEG 12
SEPTUM ASSEMBLY
GAS BUBBLE

bubble was injected directly from the syringe into the preparation unit (Fig. 18B). The amount of nitrogen in the sample was far in excess of that required for analysis and therefore most of the gas was pumped away from the preparation unit before it was passed through the traps and column on its way to the analyser (see 'N - 15 Techniques' section in Methods).

Incubation Procedures

I. In situ incubations

A. Profiles of depth

Sample vessels were suspended in the water column at one meter intervals from a 0.64 cm (1/4 in) diameter polypropylene rope which was attached to a 1 gallon glass bottle (Fig. 19). The meter increments of depth were marked with chrome-plated hose pinch clamps, to which the samples (bottles or syringes), could be easily fastened. Spring 'dog leash' clips were secured to the vessels with wire or suitable clamps thus allowing quick connection or disconnection of the vessels from the incubation rope. Duplicate samples could also be conveniently placed at each depth. Depth of incubation was generally 0.2, 1.2, 2.2, 3.2, 4.2, 5.2, 7.2 and 9.2 meters although the deepest depths were not always sampled.

B. Subsurface replicates

If a number of samples were to be incubated at the same in situ light intensity, a horizontal, clear plexiglass rod was suspended between two floats and the samples in turn suspended from the rod (Fig. 20). This incubation technique worked well for both syringes and bottles and ensured that no shading of samples occurred.

Figure 19. A diagram of the in situ incubation apparatus for depth profiles of nitrogen fixation.

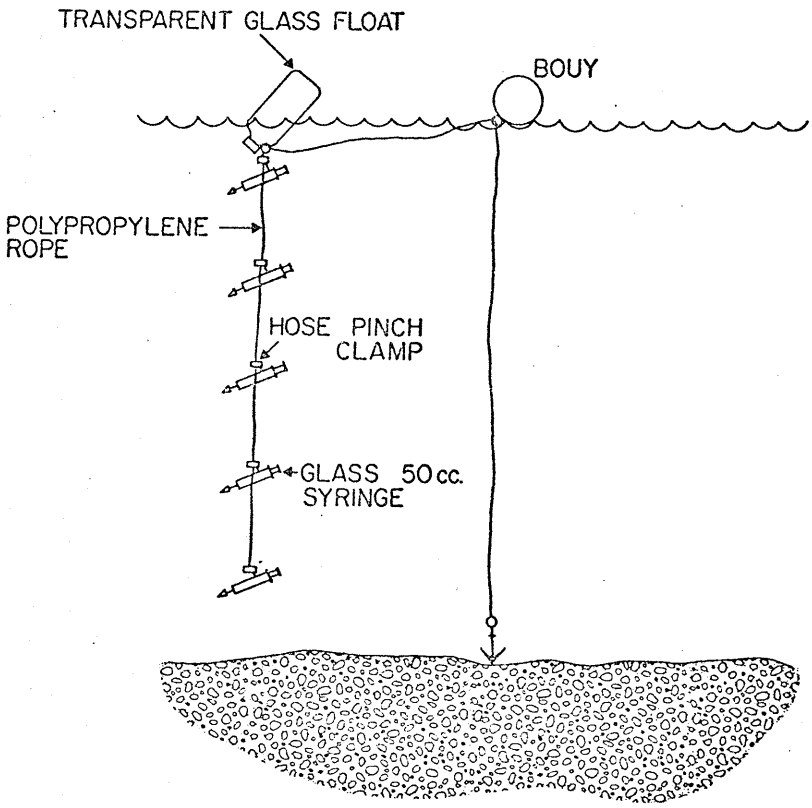
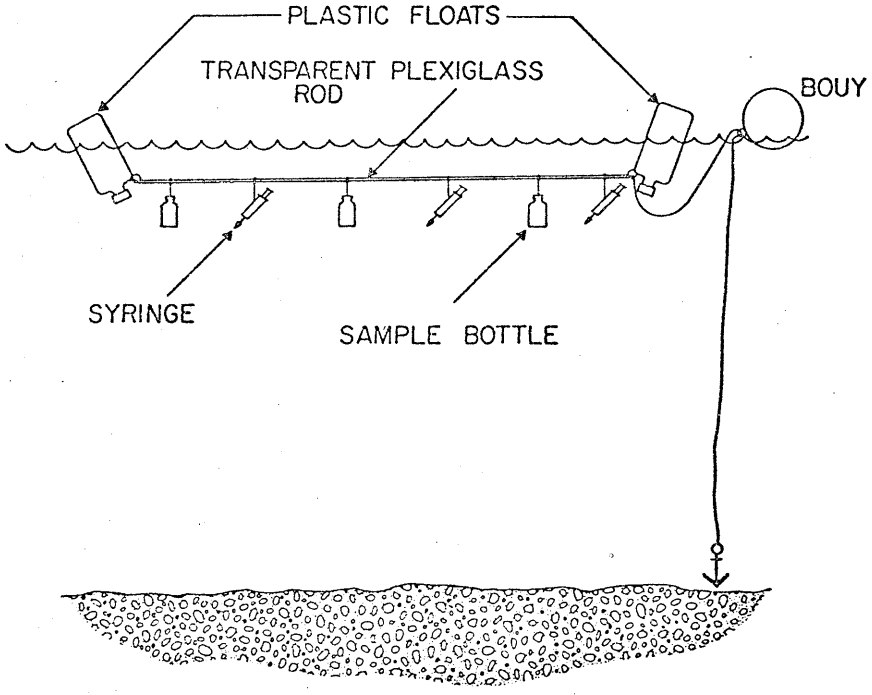

TRANSPARENT GLASS FLOAT
BOUY
POLYPROPYLENE ROPE
HOSE PINCH CLAMP
GLASS 50cc. SYRINGE

Figure 20. A diagram of the in situ incubation apparatus used for comparing rates of nitrogen fixation between a number of samples at the same subsurface light intensity.


PLASTIC FLOATS
TRANSPARENT PLEXIGLASS ROD
BOUY
SYRINGE
SAMPLE BOTTLE

II. Variable light intensity incubator

The rates of nitrogen fixation and acetylene reduction were determined as functions of light in a variable light intensity incubator (Fig. 21). The incubator was designed by Fee (1973) for studies of primary production of phytoplankton in lakes. It features 4 rotating wheels, each at a different light intensity, to which are fastened clear glass vessels containing the samples to be studied (Fig. 22). The rotation of the wheels ensured that all samples received equal incident light and agitation. The illumination was supplied by a bank of tungsten-iodine and mercury vapour light bulbs. The lamp intensity could be adjusted with variable transformers as required to simulate seasonal variances in the incident solar radiation. The spectral quality of the light was similar to that of sunlight (Fig. 23). The light intensity at each wheel was determined with a Lambda[1] quantum meter and therefore nitrogen fixation or acetylene reduction were expressed as functions of light in μEinsteins (1 Einstein = 6.02×10^{23} quanta ≡ 1 mole of quanta).

Sediment Core Methods

A technique was developed that allowed us to isolate and manipulate 7.0 cm diameter sediment cores in very shallow waters (<0.5 meters). A suitable shallow area was first identified (Lake 239, S.E. sub-basin) and then a working platform about 1 x 2 m was constructed 0.3 m above the water level. Pre-existing fallen trees served as foundations for

---

[1] Lambda - Lambda Instruments Corporation, Lincoln, Nebraska, U.S.A.

Figure 21. A diagram of the variable light intensity incubator of Fee, shown here with primary production bottles in place.

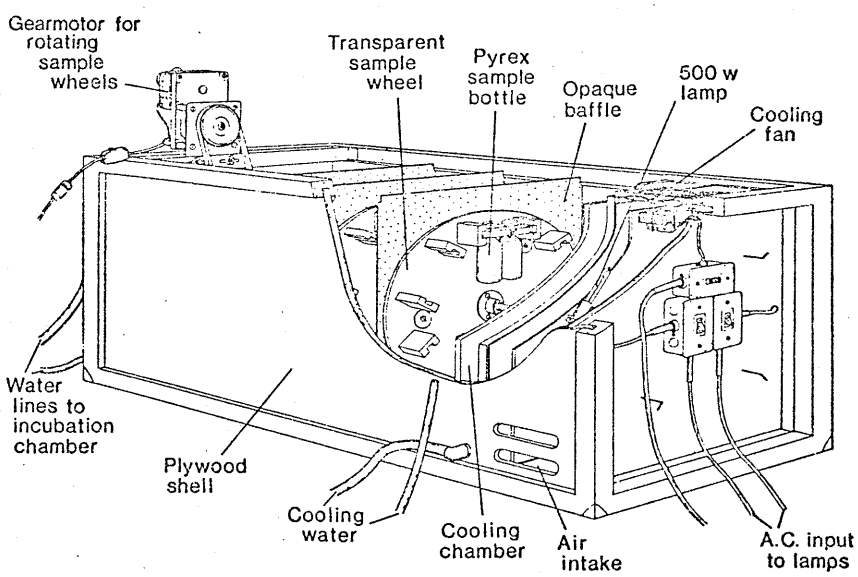

Gearmotor for rotating sample wheels
Transparent sample wheel
Pyrex sample bottle
Opaque baffle
500 w lamp
Cooling fan
Water lines to incubation chamber
Plywood shell
Cooling water
Cooling chamber
Air intake
A.C. input to lamps

Figure 22. A diagram of one of the rotating wheels of the Fee incubator, showing syringes in position for a nitrogen fixation assay.

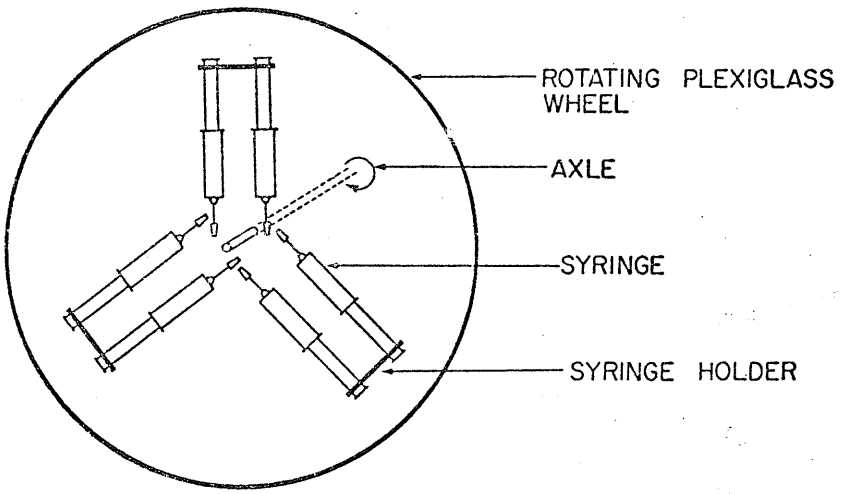
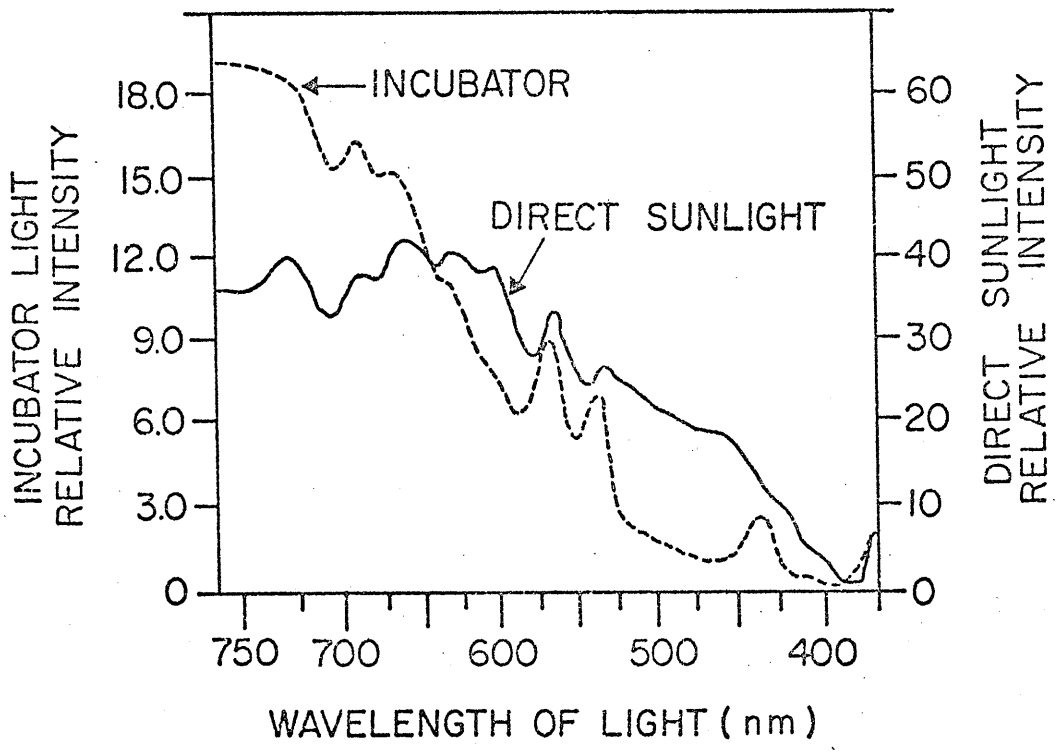

ROTATING PLEXIGLASS WHEEL
AXLE
SYRINGE
SYRINGE HOLDER

Figure 23. A plot of light relative intensity vs. wavelength for direct sunlight on June 15, 1976 at 2:00 pm at E.L.A. compared to a spectrum obtained from the front of the brightest chamber in the Fee incubator on the same day. Although the sunlight is much brighter (∿3X), the relative distribution of light at the different wavelengths is quite similar to incubator light until the wavelength becomes greater than 650 nm, at which point the incubator light is considerably richer in the red.


INCUBATOR
DIRECT SUNLIGHT
INCUBATOR LIGHT
RELATIVE INTENSITY
DIRECT SUNLIGHT
RELATIVE INTENSITY
18.0
15.0
12.0
9.0
6.0
3.0
0
60
50
40
30
20
10
0
750
700
600
500
400
WAVELENGTH OF LIGHT (nm)

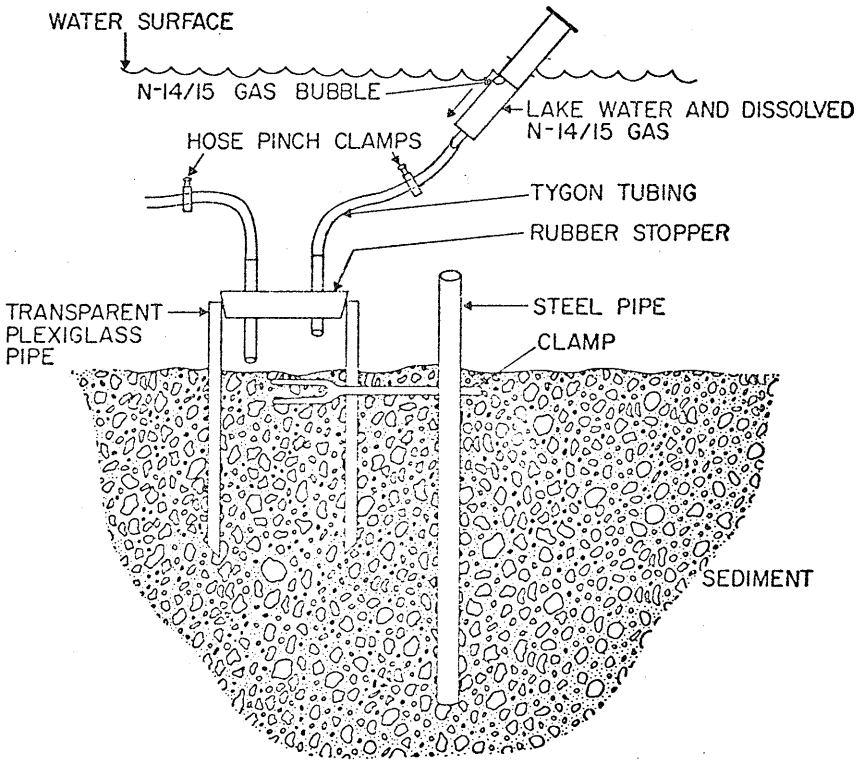
the platform. Next a steel pole of 2 cm diameter and 1 m length was driven about 80 cm into the sediment. Onto this was fitted a clamp capable of holding a vertical pipe of 7.0 cm diameter. A 20 x 7.0 cm diameter plexiglass pipe, with a beveled leading edge, was forced very gently into the sediment, through the open arms of the clamp. Once the leading edge of the pipe was to a sediment depth of 15 cm, the clamp was closed so that the pipe was firmly held in position. The pipe was allowed to stand for at least 2 weeks before any further manipulations were performed, in an attempt to allow the sediments to reassume their undisturbed state.

After this waiting period the pipe was very gently capped with a large stopper that had 2 glass tubes through it (Fig. 24). About 20 cm of Tygon tubing had previously attached to each of the glass tubes, and on each piece of Tygon was an open hose pinch clamp.

Into a 50 cc glass syringe was drawn 50 ml of water from the lake immediately adjacent the pipe. To this syringe was added 2.0 ml of 95 at % N - 15 gas from a second 5 cc syringe (see 'Addition of Nitrogen - 15 Gas in Methods). The syringe was sealed with a needle forced into a stopper and shaken vigorously for 5 minutes by hand. The same process was repeated for a second 50 cc syringe.

The needle was next removed from one of the 50 cc syringes and the N - 15 containing lake water was gently forced into the stoppered pipe through one of the Tygon tubes, as was the equilibrated N - 14/15 bubble. The gas bubble also contained, in addition to the nitrogen, some CH_4, O_2 and CO_2 removed from the water during equilibration; by reintroducing the gases they were again available to the organisms in the tube if the existing dissolved CH_4, O_2 and CO_2 were consumed. This

Figure 24. A diagram of the apparatus used to isolate sediment cores and then enrich them with N-15 gas.


WATER SURFACE
N-14/15 GAS BUBBLE
LAKE WATER AND DISSOLVED N-14/15 GAS
HOSE PINCH CLAMPS
TYGON TUBING
RUBBER STOPPER
TRANSPARENT PLEXIGLASS PIPE
STEEL PIPE
CLAMP
SEDIMENT

was repeated for the second syringe so that the desired 100 ml of N-15 containing water was added and then both hose pinch clamps were closed. The system was allowed to incubate for a period of 1 to 3 weeks.

At the end of the incubation period, the hose pinch clamps were opened and the stopper gently removed from the plexiglass pipe. A tiny piston corer, constructed from a 10 cc plastic syringe, was used to obtain a 1.5 cm diameter by 5 cm long core (Fig. 25). The piston could be firmly held by one hand that rested on the pipe edge while the other hand was used to force the barrel of the syringe down into the sediments. Once the desired depth was obtained, the entire syringe was pulled slowly out of the sediment and a rubber stopper was forced into the open end of the syringe to seal it. Cores appeared to retain their integrity when obtained in this fashion, even after being transported back to the laboratory where they were immediately frozen by placing them vertically upright in a -40°C freezer for 2 hours.

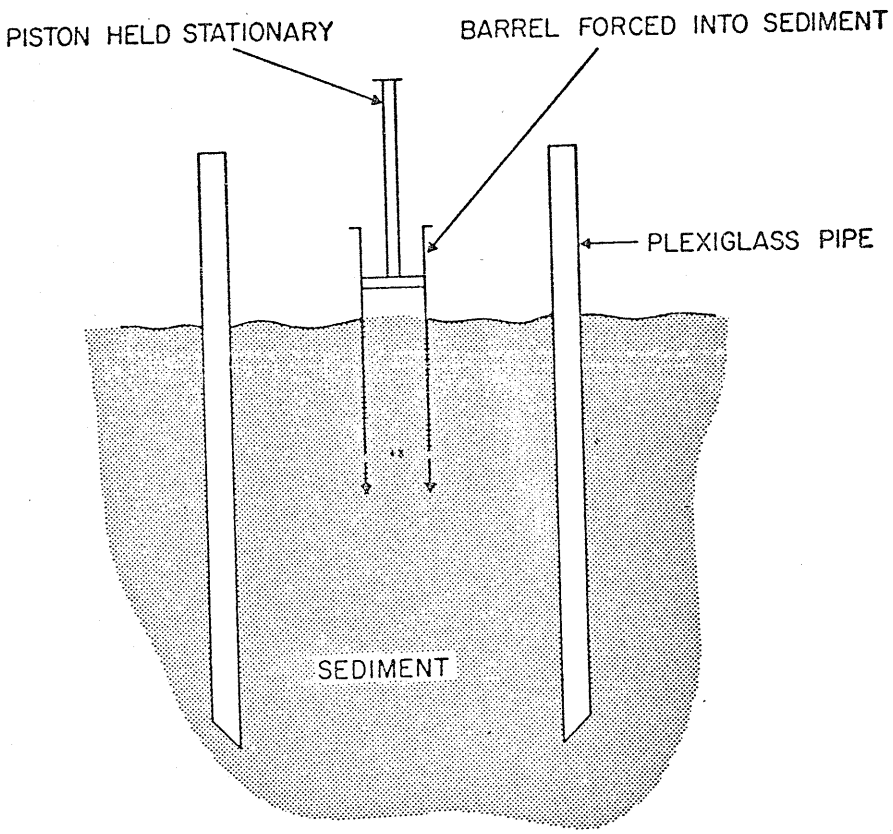
Once frozen, the core was extruded from the syringe in 0.3 cm increments, the core being forced from the syringe by the plunger. The 0.3 cm sections were carefully cut off by a clean jeweler's saw and deposited onto prefired (550°C for 12 hours) Reeve Angel glass fibre filters of 2.5 cm diameter. The filters were then held by clean forceps in front of a air stream from a Heat gun[1] until the samples were dry (approximately 1 minute). The samples, on the filters, were next placed in 5 cm diameter Millipore[2] disposable petri plates for room temperature

---

1 Heat Gun - Master Appliance Corp. Racine, Wisconsin, U.S.A.

2 Millipore - Millipore Corporation, Bedford, Mass., U.S.A.

Figure 25. A diagram showing the procedure used to obtain cores of sediment for later analysis of N-15 content.


PISTON HELD STATIONARY
BARREL FORCED INTO SEDIMENT
PLEXIGLASS PIPE
SEDIMENT

storage until analysis which was accomplished within several weeks.

For nitrogen isotope analysis, several cubic millimeters of the dry sediment was placed on another prefired Reeve Angel 984 H glass fibre filter and treated as was described in the 'N-15 Techniques' section of Methods.

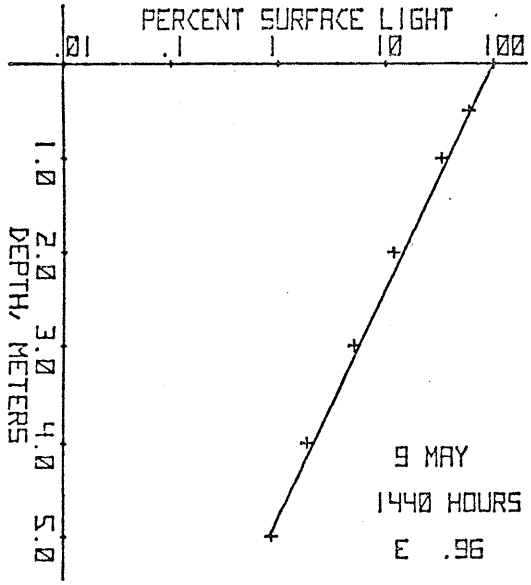
Computer Modeling

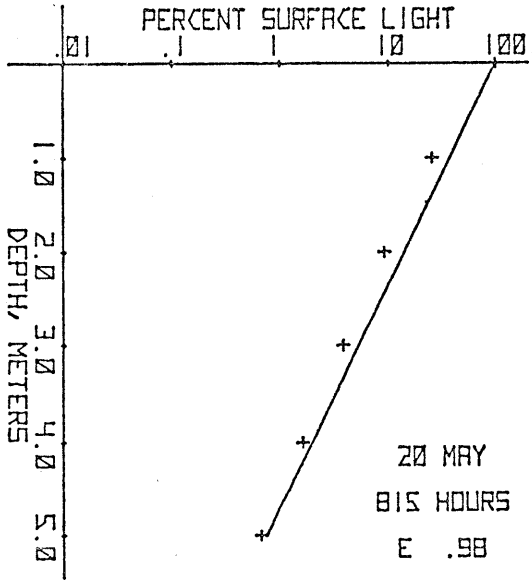
Once weekly measurement of acetylene reduction gave an estimate of the annual rate of nitrogen fixation although considerable interpolation between measurements was required. The organisms that fixed nitrogen in these lakes were photosynthetic and therefore were strongly under the influence of incident solar radiation. For this reason it was considered important to relate acetylene reduction directly to incident light in order to account for the many variations in light due to cloud cover and day length (Schindler and Fee, 1975). Incident light has been measured at the E.L.A. meteorological station for the whole period of this study. Light extinction coefficients, based upon biweekly measurements, were available for each lake being studied (Shearer, 1976; Shearer and Declercq, 1976); see example Fig. 26. These allowed calculation of the light available for fixation at any depth, as a function of incident solar radiation. The relationship between light intensity and rate of acetylene reduction was the other information required. Such data were obtained using the variable light intensity incubator of Fee (1973) - see Incubation Procedures, II of Methods section.

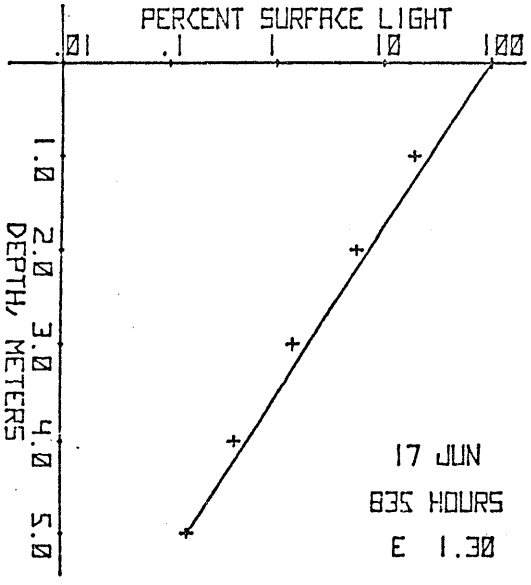
Figure 27 is a plot of average nitrogen fixation rates as functions of light intensity for 1.5 m samples obtained from Lake 226 NE on

Figure 26. Examples of plots showing % light transmission as a function of depth for various dates in 1975 (Lake 227). Courtesy of J. Shearer and D. Declercq (in press). The extinction coefficient, E, is given for each plot.

LAKE 227


PERCENT SURFACE LIGHT
.01
.1
1
10
100
DEPTH, METERS
0.1
0.2
0.3
0.4
0.5
9 MAY
1440 HOURS
E .96


PERCENT SURFACE LIGHT
.01
.1
1
10
100
DEPTH, METERS
0.1
0.2
0.3
0.4
0.5
20 MAY
815 HOURS
E .98


PERCENT SURFACE LIGHT
.01
.1
1
10
100
DEPTH, METERS
0.1
0.2
0.3
0.4
0.5
17 JUN
835 HOURS
E 1.30

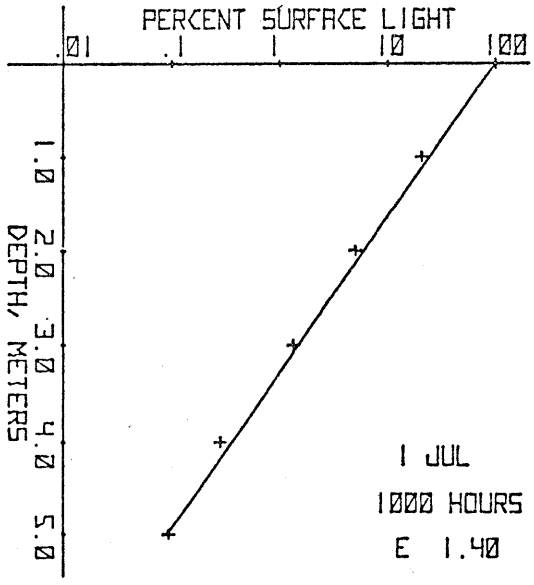
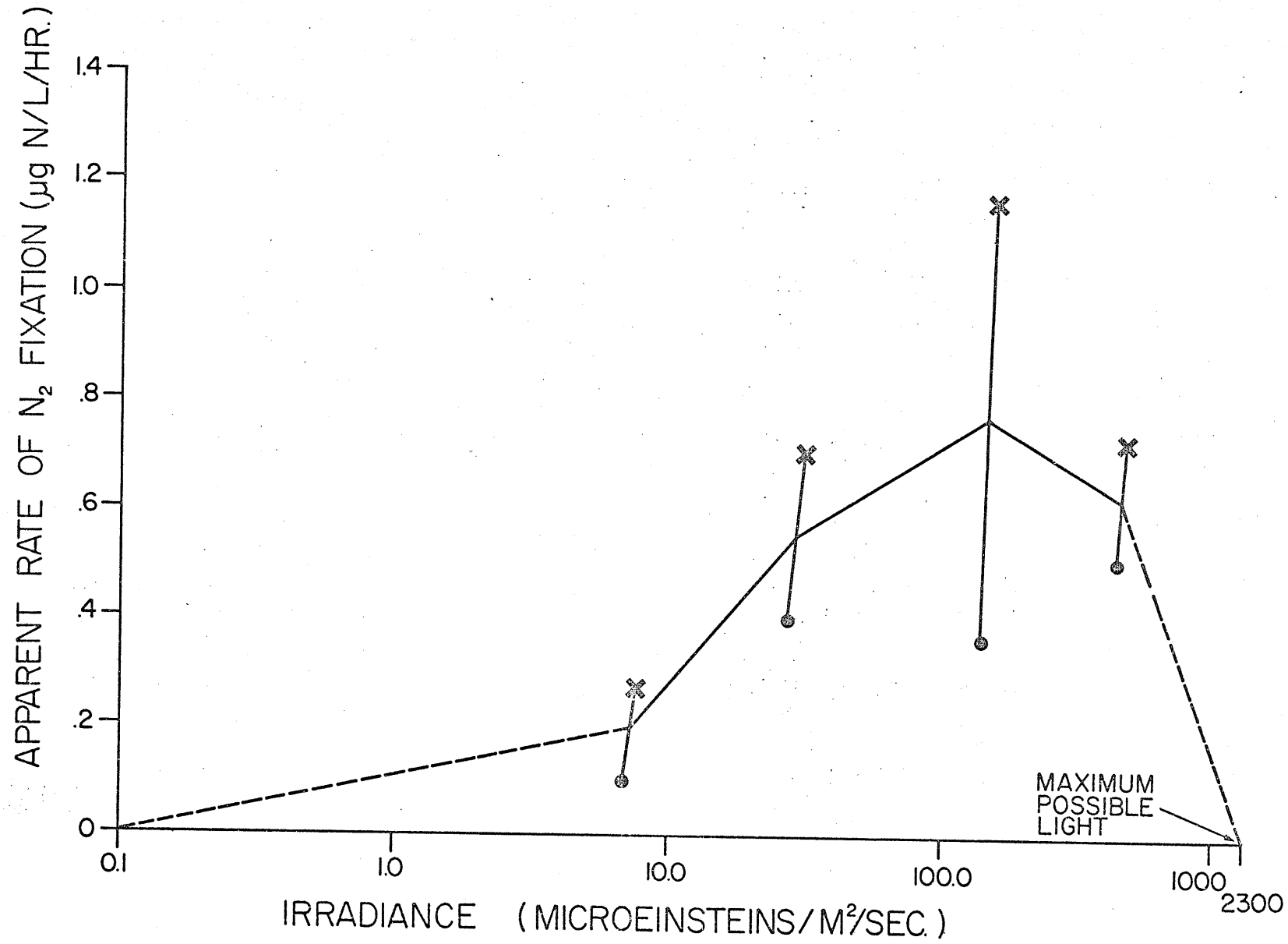

PERCENT SURFACE LIGHT
.01
.1
1
10
100
DEPTH, METERS
0.1
0.2
0.3
0.4
0.5
1 JUL
1000 HOURS
E 1.40

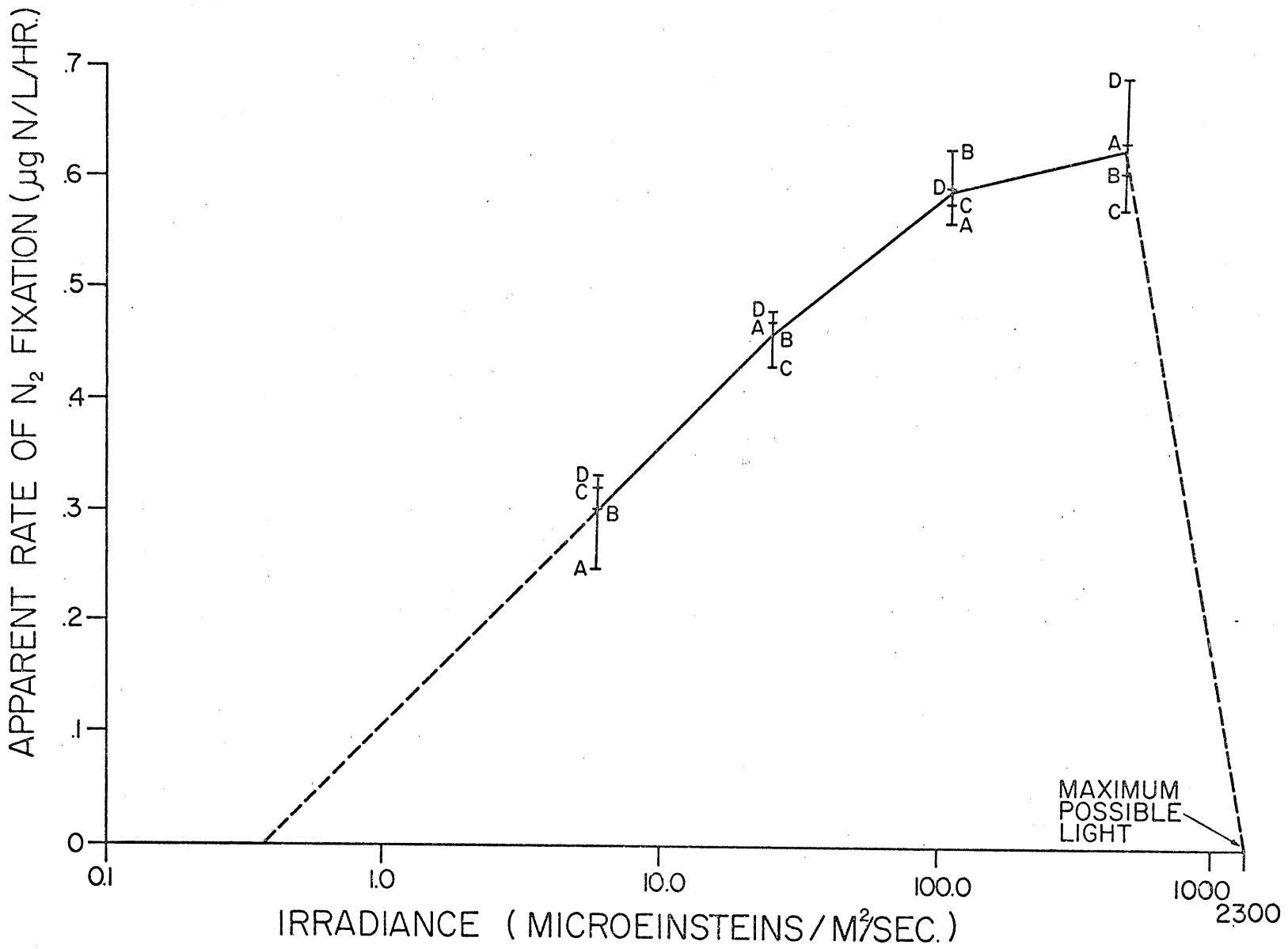
Figure 27. A plot of average (for 2 dates) nitrogen fixation rates as functions of light intensity from Lake 226 NE on August 10 and 21, 1974 (solid lines). The incubation was done in the Fee incubator for 2 hours, the samples having been taken at about 10 a.m. Dashed lines are explained in text.


APPARENT RATE OF N_2 FIXATION (µg N/L/HR.)
1.4
1.2
1.0
.8
.6
.4
.2
0
0.1
1.0
10.0
100.0
1000
2300
IRRADIANCE (MICROEINSTEINS/M²/SEC.)
MAXIMUM POSSIBLE LIGHT

August 10 and 21, 1974. Figure 28 gives a similar plot for Lake 226 NE, 1975. In this case, however, the line represents an average of four separate measurements on the same day over different time periods. A ratio of one nitrogen molecule reduced per three ethylene molecules produced was assumed. The dashed lines were arbitrarily chosen to complete the fixation:light relationship for low and high light intensities, where actual measurements could not be made. For 1974, the decision to make fixation rates equal zero at 0.1 $\mu E/m^2/sec$ or lower light intensity was based on *in situ* results which showed fixation to be just detectable at light levels of $\sim$0.1 $\mu E/m^2/sec$ on August 17, 1974, a date intermediate to the two incubator studies. The dashed line at the high light intensities was plotted so that fixation would equal zero if the fixing organisms experienced maximum possible irradiance from the sun (2300 $\mu E/m^2/sec$) at the very surface of the lake. This presumed that the organism would exhibit 100% light inhibition under such circumstances, a rather unlikely presumption. Both of these arbitrarily positioned dashed lines were assumed to underestimate nitrogen fixation rates. For 1975, the assumptions were the same except that fixation was assumed to equal zero when light was $<0.38\ \mu E/m^2/sec$. In all cases, it was assumed that the relationship between incident light and nitrogen fixation, as seen in the curves of Fig. 27 and 28, were valid over the entire period that fixation was measured.

The program functioned as follows: For each day on which *in situ* acetylene reduction results were positive, predictions of nitrogen fixation were obtained from the model for the same time periods over

Figure 28. A plot of average nitrogen fixation rates as functions of light intensity, for integrated samples (0-5 m depth) on Sept. 12, 1975 (solid lines). Samples were taken at 6:00 a.m., 10:00 a.m., 2:00 p.m. and 5:00 p.m. and incubated for 2.0 hours in the Fee incubator. The results of all the different sampling periods were averaged to give the plot illustrated here. Dashed lines are explained in text. A = 6:00 a.m., B = 10:00 a.m., C = 2:00 p.m. and D = 5:00 p.m.


APPARENT RATE OF N_2 FIXATION (µg N/L/HR.)
.7
.6
.5
.4
.3
.2
.1
0
0.1
1.0
10.0
100.0
1000
2300
IRRADIANCE (MICROEINSTEINS / M²/SEC.)
MAXIMUM POSSIBLE LIGHT
A
B
C
D

which the in situ assay was performed. The predicted fixations at 0.2, 1.2, 2.2, 3.2, 4.2 and 5.2 m depths were compared with the observed fixations at the same depths and a factor were calculated for each depth that expressed the observed value as a fraction of the predicted value. Discrepancies between observed and predicted values were attributed to changes in population activity and density of the nitrogen fixing organisms. Next, the model was asked to predict the fixation rates at discrete depths over the whole lighted day, at 0.2 meter intervals. The predicted value at each depth was multiplied by the previously obtained factor from the same or closest depth. For example, the factor obtained from the in situ depth of 1.2 meters would be used to multiply the model predictions at 0.8, 1.0, 1.2, 1.4 and 1.6 meter depths. Similarly, the factor obtained from the 2.2 meter depth in situ incubation was used to multiply the model predictions from 1.8, 2.0, 2.2, 2.4 and 2.6 meter depths etc.

The rates of adjacent 0.2 meter depths were then averaged and this value was used to multiply the total lake volume enclosed between the two depths. This was performed for depths from 0.0 to 5.0 meters, the only depths for which significant activity was noted. Twenty-five predictions were summed and the resultant value represented the total day's fixation of nitrogen into the lake.

The model was then asked to calculate fixation for days other than those when in situ fixation data were available. The assumption was made that the factors remained constant with time and therefore the only new input required for the program to run was the light data for each day examined. In situ fixation measurements were normally done once weekly; the factors derived from these measurements were applied to the three

days preceding and the three days following the in situ measurements.

The program produced daily totals of fixation and the sum of these totals represented the estimated yearly total input of fixed atmospheric nitrogen to the lake.

The actual program listing and an explanation of it is given in the Appendix of this thesis.

RESULTS

Acetylene Reduction Assays for Blue-Green Algal Nitrogen Fixation in the Watercolumn

Beginning in 1973, measurements of acetylene reduction were carried out in a number of lakes of the Experimental Lakes Area, using the syringe technique previously described in Methods. Three lakes, 227, 302 North and 261 were monitored beginning in May and Lake 226 Northeast was added to the list in late August of the same year.

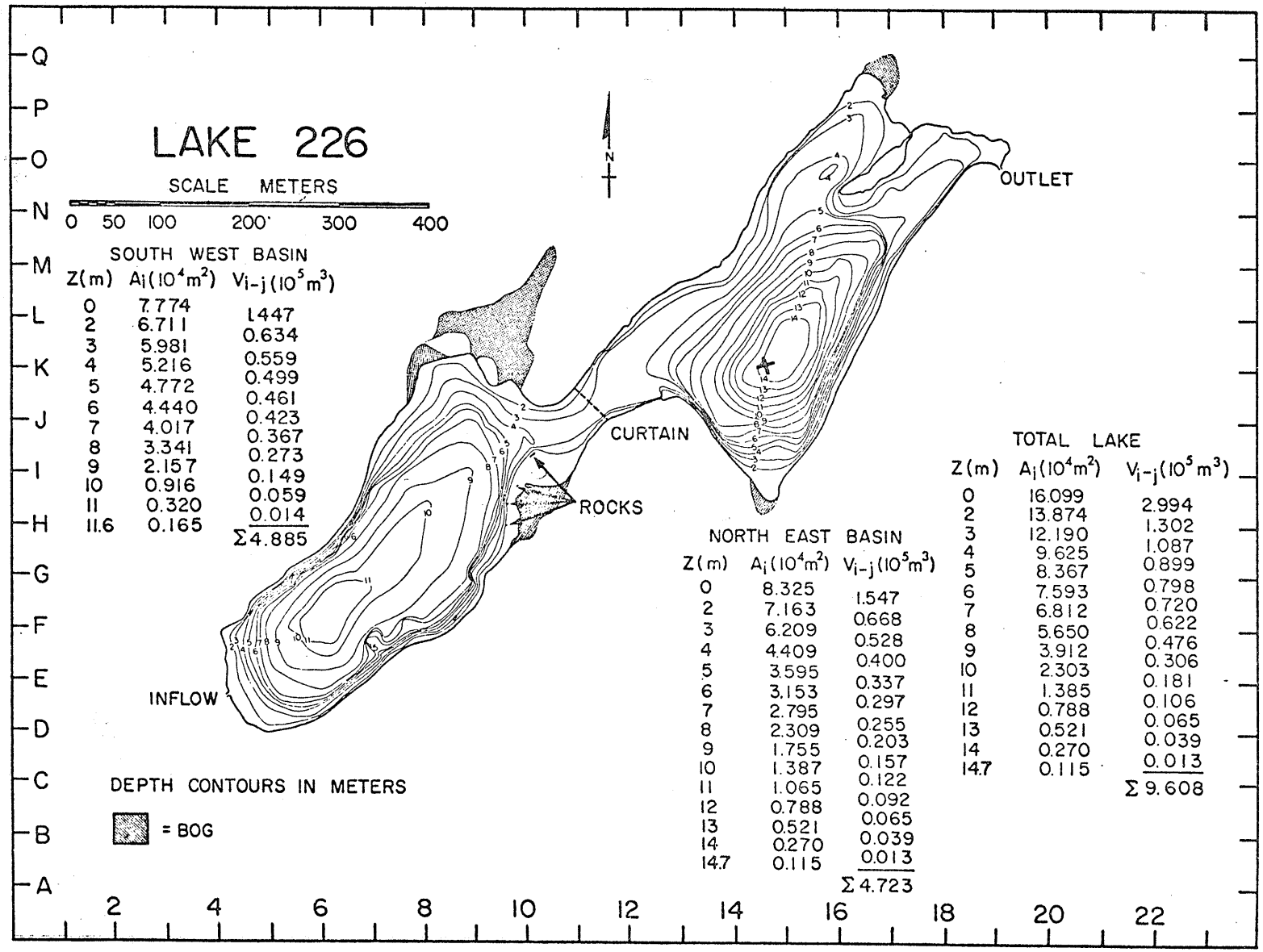
Lake 226 is a well stratified double basin lake of 16.1 hectares total area, the southwest basin being 7.8 hectares and northeast basin being 8.3 hectares (Fig. 29). The lake was classified as oligomesotrophic prior to 1973 when a fertilization program was initiated. In the spring of that year the two lake basins were separated by positioning a plastic Sea Curtain[1] across the lake at the narrow neck connecting the two basins. This effectively created two separate lakes.

The southwest basin was continuously fertilized over a 21 week period during the open water season with 5.89 g $C/m^2/yr$ as sucrose and 3.08 g $N/m^2/yr$ as $NaNO_3$. The northeast basin was similarly fertilized except that in addition to the C and N, a further 0.58 g $P/m^2/yr$ was added as phosphoric acid. The northeast basin rapidly responded to the nutrient additions and algal biomass increased above that in the other basin (Schindler, 1974). Toward the end of August 1973 a thick epilimnetic bloom of Anabaena spiroides appeared.

---

[1] Sea Curtain - Kepner Plastics, Torrance, California, U.S.A.

Figure 29. A bathymetric map of Lake 226 (Courtesy E.L.A. Staff). The X marks the sampling location.



SOUTH WEST BASIN

Z(m)	$A_i(10^4 m^2)$	$V_{i-j}(10^5 m^3)$
0	7.774	1.447
2	6.711	0.634
3	5.981	0.559
4	5.216	0.499
5	4.772	0.461
6	4.440	0.423
7	4.017	0.367
8	3.341	0.273
9	2.157	0.149
10	0.916	0.059
11	0.320	0.014
11.6	0.165	Σ 4.885

NORTH EAST BASIN

Z(m)	$A_i(10^4 m^2)$	$V_{i-j}(10^5 m^3)$
0	8.325	1.547
2	7.163	0.668
3	6.209	0.528
4	4.409	0.400
5	3.595	0.337
6	3.153	0.297
7	2.795	0.255
8	2.309	0.203
9	1.755	0.157
10	1.387	0.122
11	1.065	0.092
12	0.788	0.065
13	0.521	0.039
14	0.270	0.013
14.7	0.115	Σ 4.723

TOTAL LAKE

Z(m)	$A_i(10^4 m^2)$	$V_{i-j}(10^5 m^3)$
0	16.099	2.994
2	13.874	1.302
3	12.190	1.087
4	9.625	0.899
5	8.367	0.798
6	7.593	0.720
7	6.812	0.622
8	5.650	0.476
9	3.912	0.306
10	2.303	0.181
11	1.385	0.106
12	0.788	0.065
13	0.521	0.039
14	0.270	0.013
14.7	0.115	Σ 9.608

Acetylene reduction depth profiles were obtained on September 4 and 11 of that year. Total inputs of nitrogen to the lake were calculated in the following way: For each profile, rates of adjacent 1.0 m depths were averaged together and the average was used to multiply the volume of the lake water between the two adjacent depths. This result was the amount of nitrogen being fixed in the lake per hour in the layer between the adjacent depths. The total nitrogen being fixed per hour in the entire lake was next obtained by summing the results for all the layers examined in the profile. The hourly whole lake fixation rate was assumed to continue for approximately 10 hours[1] and this yielded a rate of daily fixation. The two rates so obtained are graphically expressed as '*' in the top panel of Fig. 30. The dashed lines represent biomass of nitrogen fixing blue-green algae in the epilimnion of the lake (data of D. Findlay, E.L.A. project).

The assumption was made that fixation remained constant for 3 days prior to and 3 days after the in situ incubations, and that fixation did not occur on other dates. The total nitrogen fixation input to the lake was estimated to be 26 Kg N, based on these assumptions.

In 1974 a bloom of Anabaena solitaria f. planktonica formed in Lake 226 NE; nitrogen fixation was initially detected on July 26 and persisted up to September 9. The data was complete enough for an attempt

---

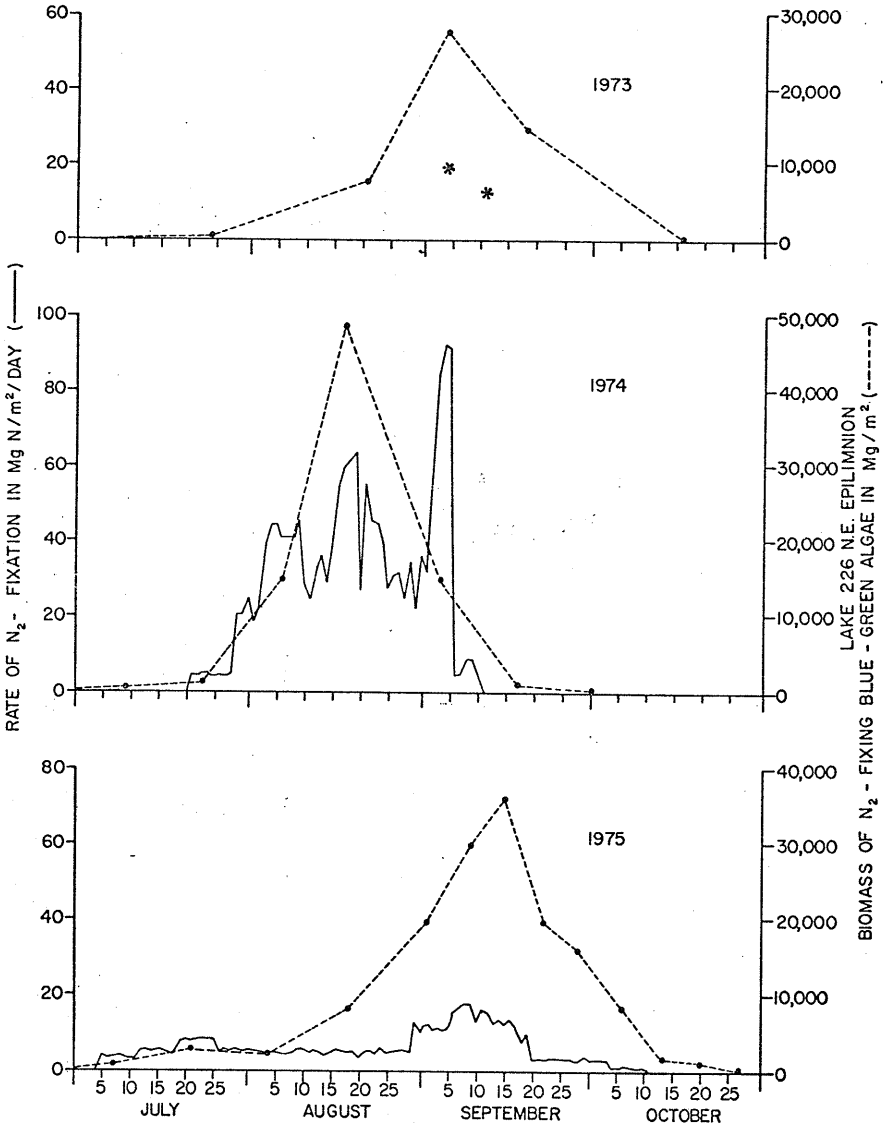
[1] Diurnal experiments on Lake 226 NE (Aug. 17, 1974) had later shown that for a day with about 13 hours of sunlight, one could assume the 10:00 a.m. to 2:00 p.m. rate of fixation x 0.77 to be the whole day rate. This meant that one could assume 0.77 x 13 = 10 hours of fixation at the 'noon' rate. The figure is obviously approximate and mass calculations based upon it should be treated with reservation.

Figure 30. The wet weight biomasses of the nitrogen fixing blue-green algae are given by the dashed lines while the asterisks or solid lines represent nitrogen fixation rates. In all cases the fixation was estimated using the acetylene reduction technique, assuming that the C_2H_4: N_2 ratio was 3:1.

Top Panel. A plot of nitrogen fixation rates and N_2 fixing blue-green algal biomass as a function of time in Lake 226 NE, 1973. Only data from Sept. 4 and 11 are shown here and no modeling was attempted.

Middle Panel. A plot of nitrogen fixation rates and N_2 fixing blue-green algal biomass as a function of time in Lake 226 NE, 1974. The fixation rates shown are due to computer modeling described in the text.

Lower Panel. A plot of nitrogen fixation rates and N_2 fixing blue-green algal biomass as a function of time in Lake 226 NE, 1975. The fixation rates shown are due to computer modeling described in the text.


1973
1974
1975
RATE OF N_2 - FIXATION IN Mg N/m²/DAY (——)
BIOMASS OF N_2 - FIXING BLUE - GREEN ALGAE IN Mg/m² (------)
LAKE 226 N.E. EPILIMNION
JULY
AUGUST
SEPTEMBER
OCTOBER

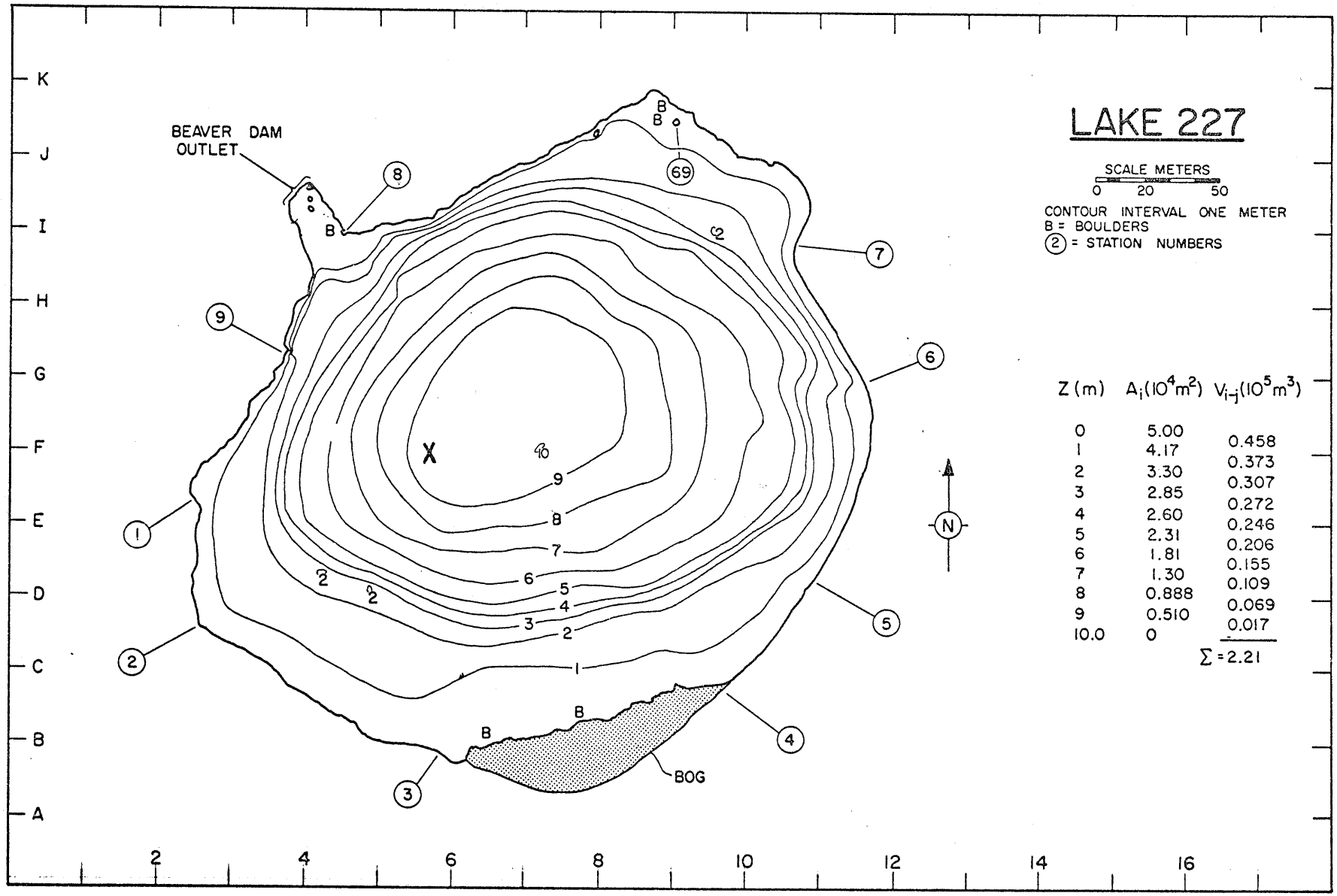
to be made at modeling nitrogen fixation as a function of incident solar radiation to the lake. This is described in the Computer Modeling section of Methods.

The middle panel of Fig. 30 shows the computer estimated daily fixation rates to Lake 226 NE, 1974. The dashed lines are measures of biomass of nitrogen fixing blue-green algae (data of D. Findlay, E.L.A. project).

In 1975 a bloom of Anabaena solitaria f. planktonica appeared in Lake 226 NE with nitrogen fixation first being detected on July 8 and last detected on October 7. Nitrogen fixation as a function of light was modeled in a similar way to that of Lake 226 NE (1974). The results are seen in the lower panel of Fig. 30. The biomass of the nitrogen fixing blue-green algae in the epilimnion is given by the dashed lines (data of D. Findlay, E.L.A. project).

Lake 227 is a small well stratified lake of 5.0 hectares in area with a maximum depth of 10 m (Fig. 31). In its natural state it was oligomesotrophic (Schindler et al. 1971) with averaged analyses from the euphotic zone showing total dissolved nitrogen = 160 µg/L, total dissolved phosphorus = 7 µg/L and chlorophyll a = 2-5 µg/L (Armstrong and Schindler, 1971). The Chrysophyceae were the dominant phytoplankton. In 1969 a program of fertilization was started (Schindler et al. 1971, 1973) wherein additions of 0.48 g $P/m^2/yr$ (as H_3PO_4) and 6.29 g $N/m^2/yr$ (as $NaNO_3$) were added in 21 equal increments from mid May to mid October. These additions were designed to match the N : P loading ratios observed in the St. Lawrence Great Lakes. The lake responded immediately to the increased nutrient loading and exhibited a higher algal standing crop, an elevated chlorophyll a concentration and a shift in population to

Figure 31. A bathymetric map of Lake 227 (Courtesy of E.L.A. staff). The X marks the sampling location.



Z (m)	$A_i (10^4 m^2)$	$V_{i-j} (10^5 m^3)$
0	5.00	
		0.458
1	4.17	
		0.373
2	3.30	
		0.307
3	2.85	
		0.272
4	2.60	
		0.246
5	2.31	
		0.206
6	1.81	
		0.155
7	1.30	
		0.109
8	0.888	
		0.069
9	0.510	
		0.017
10.0	0	

$\Sigma = 2.21$

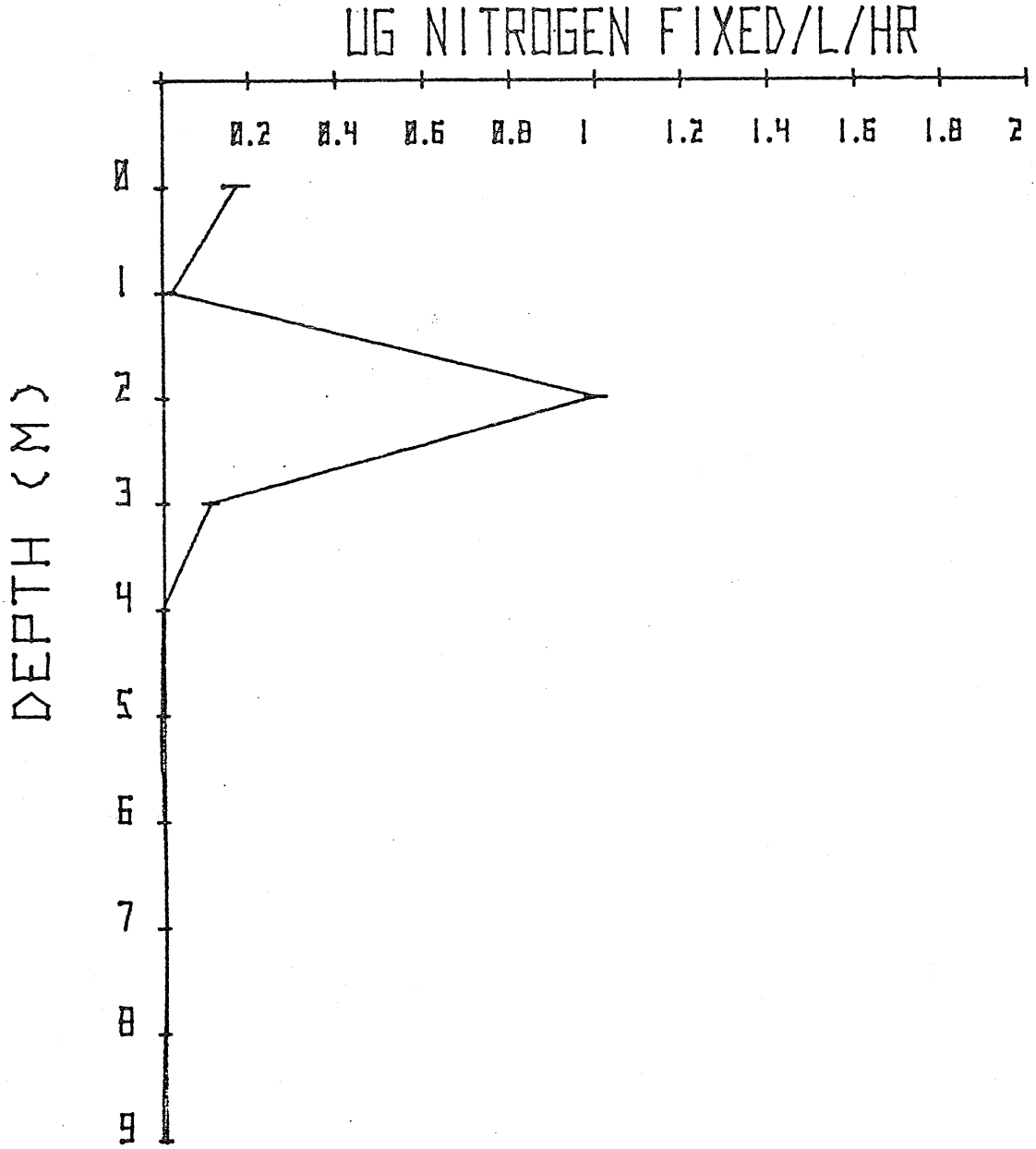
Chlorophytes. Cyanophytes were abundant but of the non-nitrogen fixing varieties such as Oscillatoria redekei (Schindler et al. 1973). In the years 1973 and 1974 acetylene reduction assays were negative throughout the water column.

It has been previously observed (Schindler, 1974) that Lake 226 NE in the E.L.A. produced blooms of nitrogen fixing algae when fertilized with 5.89 g $C/m^2/yr$, 0.58 g $P/m^2/yr$ and 3.08 g $N/m^2/yr$. It was therefore decided in 1975 to fertilize Lake 227 at a similar rate (P = 0.46 $g/m^2/yr$ and N = 2.25 $g/m^2/yr$) in an attempt to cause blooms of nitrogen fixing algae (D.W. Schindler, personal communication). The attempt was successful, for in early July of 1975 nitrogen fixation was noted to begin and it continued through until mid October. A concurrent bloom of Aphanazomenon gracilis (data of D. Findlay, E.L.A. project) was believed responsible for the fixation. Fig. 32, A-D, shows typical examples of acetylene reduction depth profiles. These measurements were performed once weekly over the entire bloom period. Total yearly nitrogen fixation was estimated using a computer model similar to that employed in the Lake 226 NE estimate. The only important variations were that light extinction coefficients for Lake 227 were used as were the in situ Lake 227 acetylene reduction data. Unfortunately, no variable light intensity incubator studies were performed on this Lake 227 population of Aphanazomenon gracilis. In lieu of such data relating nitrogen fixation to light intensity, the curves obtained for Lake 226 NE (1975) during the Anabaena solitaria bloom were used instead. A plot of the day to day estimated nitrogen fixation rates are given in Fig. 33. Also included on this plot are epilimnetic biomass concentrations of Anabaena gracilis, the only nitrogen fixing blue-green algae

Figure 32. A-D. Examples of nitrogen fixation depth profiles in Lake 227, 1975. The acetylene reduction technique was employed and a C_2H_4 : N_2 ratio of 3 : 1 was assumed.

LAKE 227
DAY 202
DATE 21 JUL 75

ACETYLENE
REDUCTION

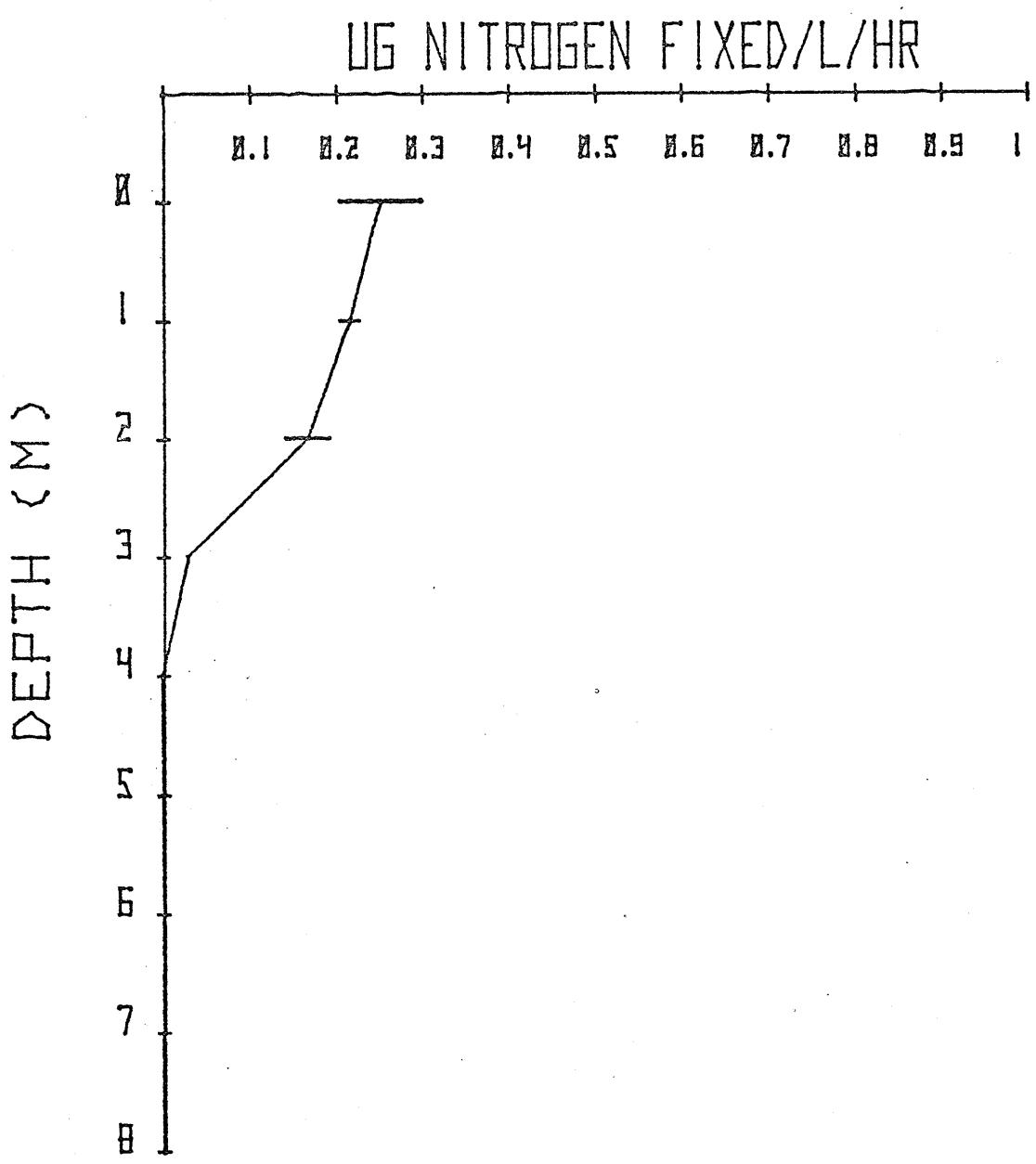


LAKE 227

DAY 244

DATE 1 SEP 75

ACETYLENE REDUCTION

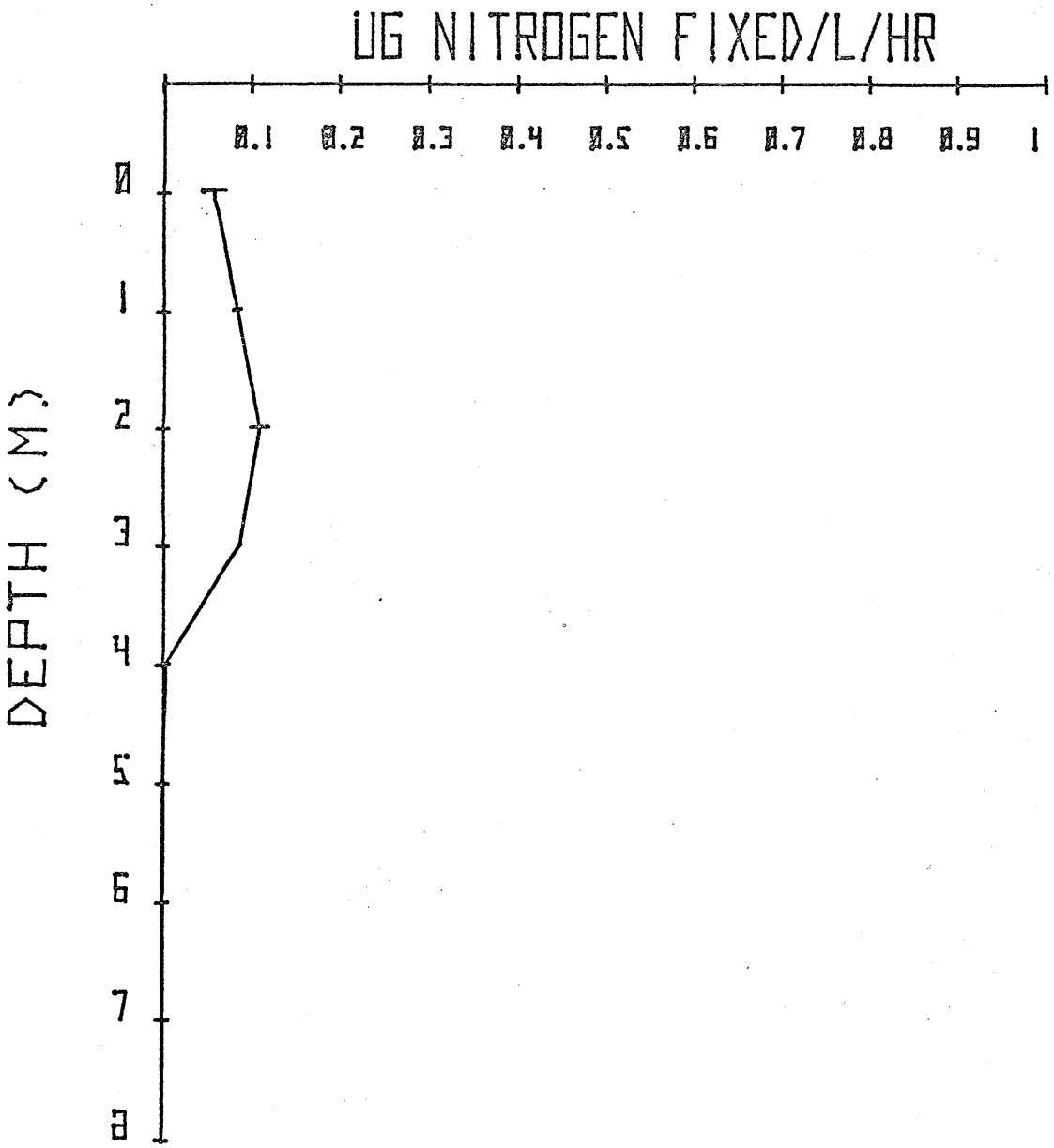


LAKE 227

DAY 272

DATE 29 SEP 75

ACETYLENE REDUCTION



LAKE 227

DAY 279

DATE 6 OCT 75

ACETYLENE REDUCTION

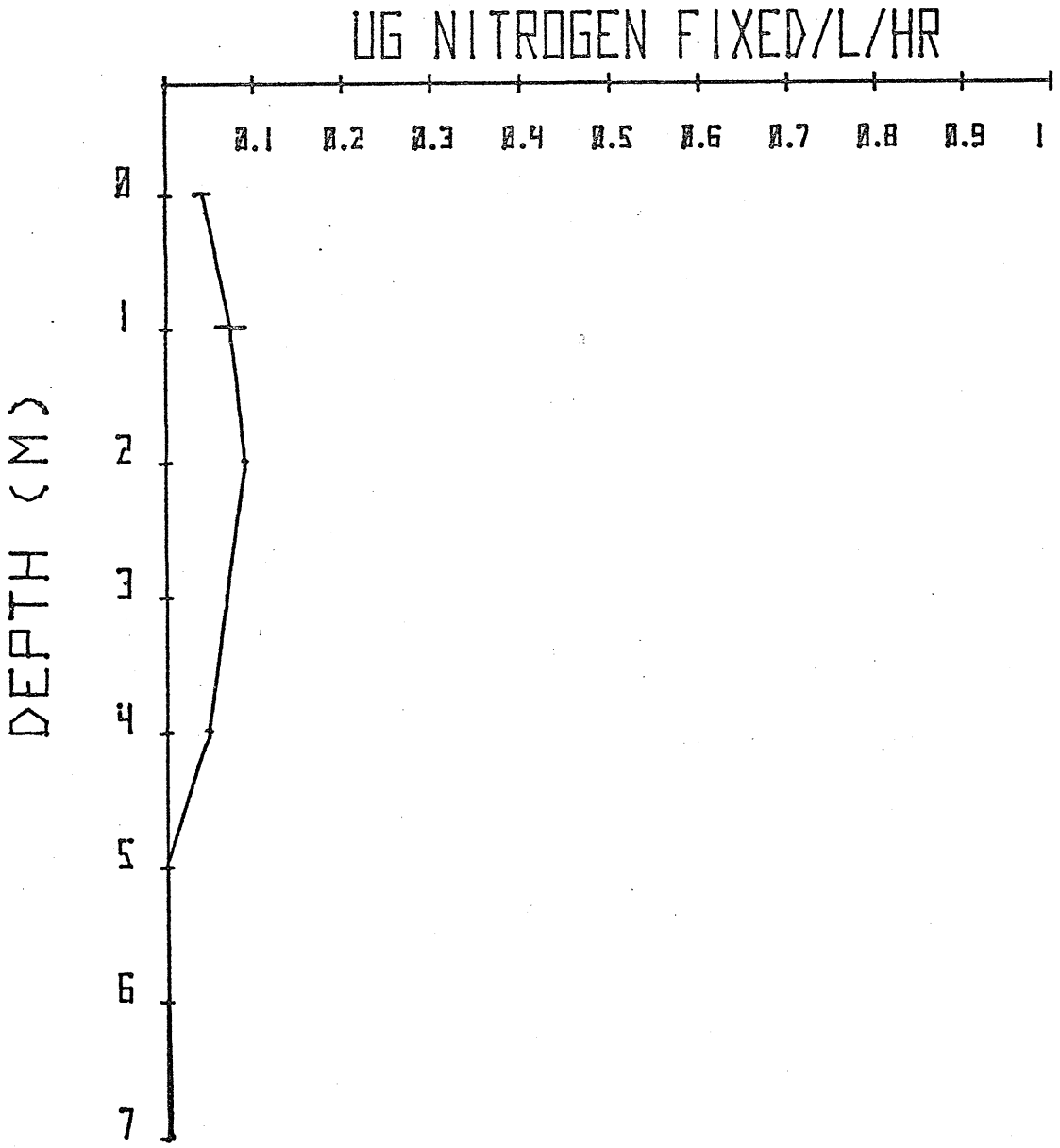
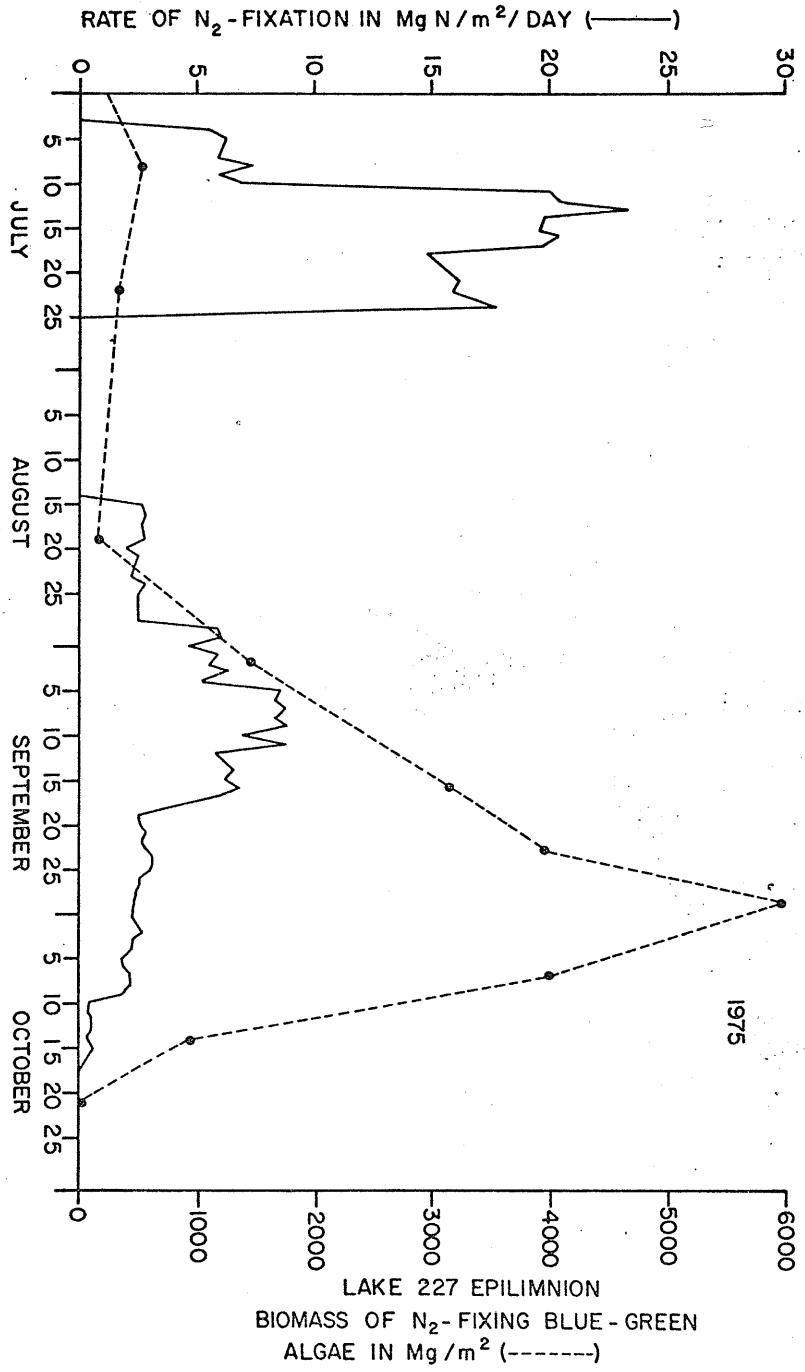


Figure 33. A plot of nitrogen fixation rates (solid lines) and nitrogen fixing blue-green algal biomass (dashed lines) as a function of time in Lake 227, 1975. The fixation rates were predicted using a computer model described in the text. The model estimated the amount of nitrogen fixed, in between the once weekly in situ measurements, as a function of incident solar radiation. The acetylene reduction technique was used and a C_2H_4 : N_2 ratio of 3:1 was assumed.


RATE OF N_2-FIXATION IN Mg N/m²/DAY (———)
0
5
10
15
20
25
30
JULY
AUGUST
SEPTEMBER
OCTOBER
1975
0
1000
2000
3000
4000
5000
6000
LAKE 227 EPILIMNION
BIOMASS OF N_2-FIXING BLUE-GREEN ALGAE IN Mg/m² (-------)

present in abundance in the lake (data of D. Findlay, E.L.A. project).

Lake 302 is a well stratified dimictic double basin lake of 23.7 hectares area, the north basin being 12.8 hectares and the south basin 10.9 hectares (Fig. 34). The two basins are separated by plastic Sea Curtains in a manner similar to Lake 226. The south basin received no fertilizer and remained oligotrophic; the north basin received additions to the hypolimnion of 3.73 g $C/m^2/yr$ (as sucrose), 0.54 g $P/m^2/yr$ (as H_3PO_4) and 2.79 g $N/m^2/yr$ (as NH_4Cl) (Schindler, 1975). Acetylene reduction measurements were performed approximately monthly in the south basin in 1973 and 1974. The results were always negative; significant numbers of nitrogen fixing blue-green algae were never observed (data of D. Findlay, E.L.A. project) in biweekly samples.

Lake 261 is well stratified, of 5.57 hectares in area (Fig. 35) and has been fertilized with H_3PO_4 at a rate of 0.246 g $P/m^2/yr$ from 1973 onward. The lake was classed as oligotrophic prior to fertilization and continues to be so classed. No nitrogen fixation was ever detected in the water column of this lake during the approximately once monthly sampling schedule of 1973 and 1974. No significant populations of planktonic nitrogen fixing blue-green algae were observed in monthly samples (data of D. Findlay, E.L.A. project).

Figure 34. A bathymetric map of Lake 302. (Courtesy of E.L.A. staff). The X's mark the sampling locations.

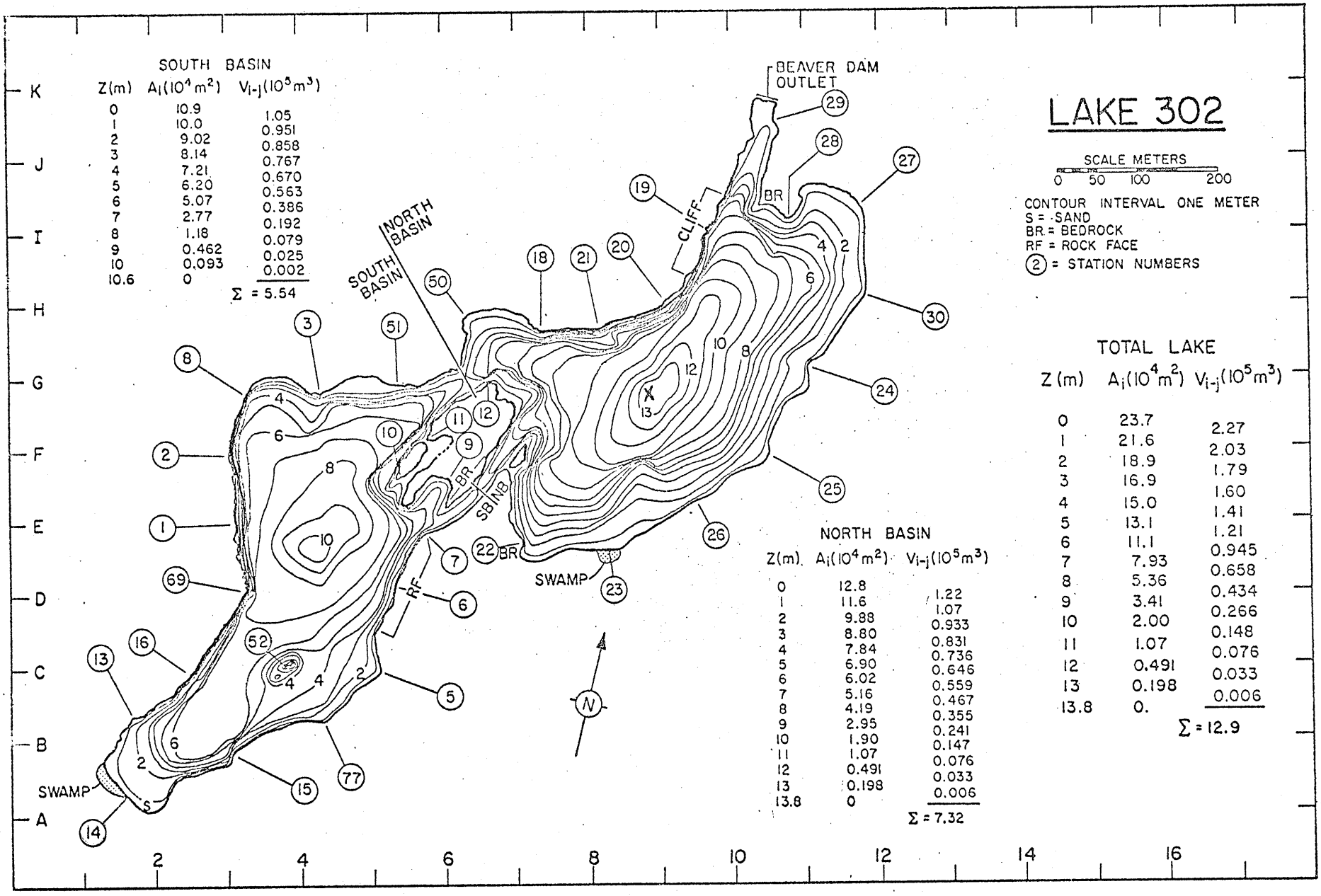
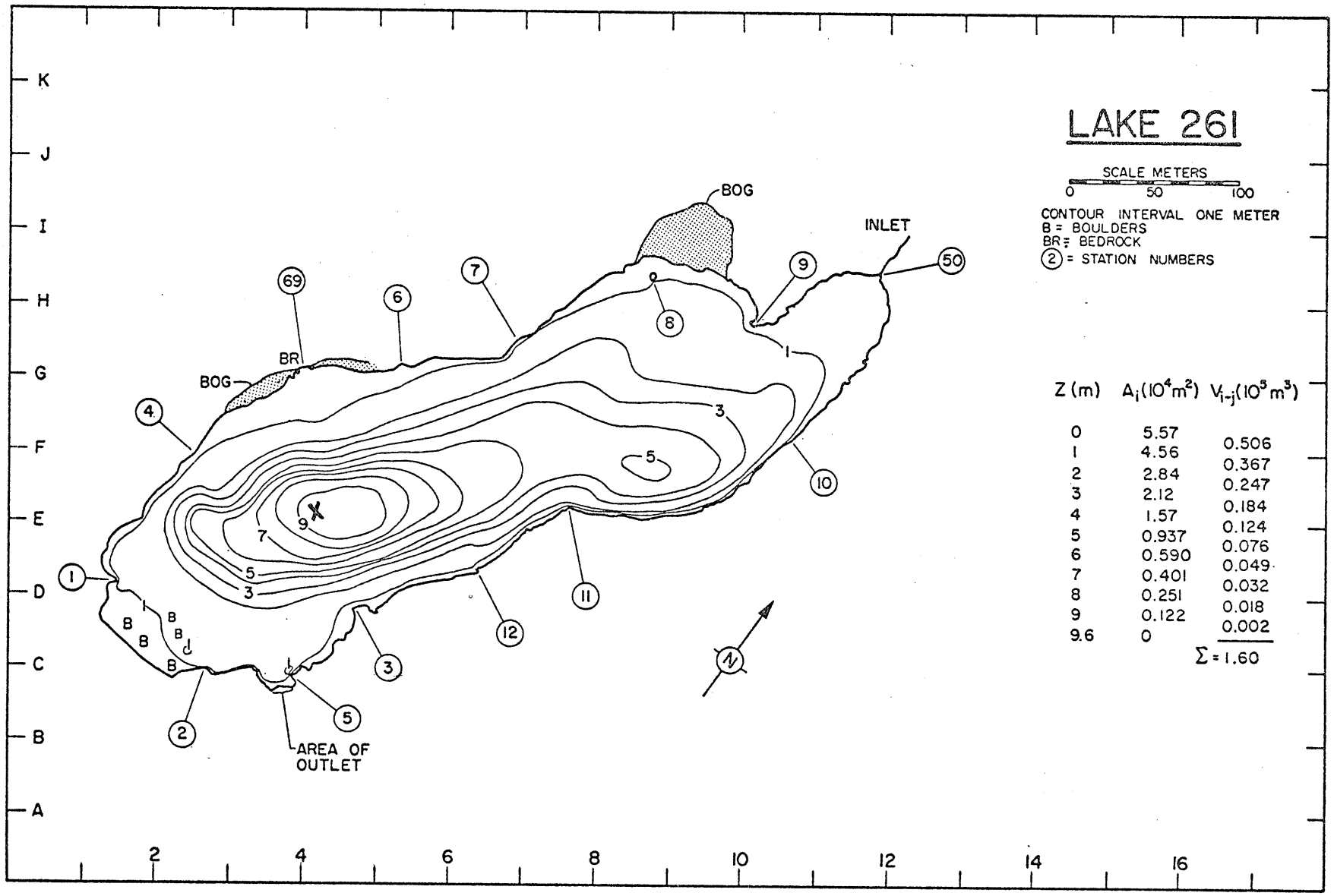

LAKE 302
SCALE METERS
0 50 100 200
CONTOUR INTERVAL ONE METER
S = SAND
BR = BEDROCK
RF = ROCK FACE
(2) = STATION NUMBERS
BEAVER DAM OUTLET
NORTH BASIN
SOUTH BASIN
CLIFF
SWAMP
SWAMP
BR
RF
SB
NB
N
SOUTH BASIN
Z(m) Ai(10⁴m²) Vi-j(10⁵m³)
0 10.9 1.05
1 10.0 0.951
2 9.02 0.858
3 8.14 0.767
4 7.21 0.670
5 6.20 0.563
6 5.07 0.386
7 2.77 0.192
8 1.18 0.079
9 0.462 0.025
10 0.093 0.002
10.6 0
Σ = 5.54
NORTH BASIN
Z(m) Ai(10⁴m²) Vi-j(10⁵m³)
0 12.8 1.22
1 11.6 1.07
2 9.88 0.933
3 8.80 0.831
4 7.84 0.736
5 6.90 0.646
6 6.02 0.559
7 5.16 0.467
8 4.19 0.355
9 2.95 0.241
10 1.90 0.147
11 1.07 0.076
12 0.491 0.033
13 0.198 0.006
13.8 0
Σ = 7.32
TOTAL LAKE
Z (m) Ai(10⁴m²) Vi-j(10⁵m³)
0 23.7 2.27
1 21.6 2.03
2 18.9 1.79
3 16.9 1.60
4 15.0 1.41
5 13.1 1.21
6 11.1 0.945
7 7.93 0.658
8 5.36 0.434
9 3.41 0.266
10 2.00 0.148
11 1.07 0.076
12 0.491 0.033
13 0.198 0.006
13.8 0. 0.006
Σ = 12.9

Figure 35. A bathymetric map of Lake 261 (Courtesy of E.L.A. staff). The X marks the sampling location.



Z (m)	A_i ($10^4 m^2$)	V_{i-j} ($10^5 m^3$)
0	5.57	
		0.506
1	4.56	
		0.367
2	2.84	
		0.247
3	2.12	
		0.184
4	1.57	
		0.124
5	0.937	
		0.076
6	0.590	
		0.049
7	0.401	
		0.032
8	0.251	
		0.018
9	0.122	
		0.002
9.6	0	

$\Sigma = 1.60$

N-15 Assays for Nitrogen Fixation by Methane Oxidizing Bacteria in the Watercolumn

It had been shown that methane oxidizing bacteria were active in some lakes of the Experimental Lakes Area at depths where the oxygen concentrations were between 1.0 and 0.1 mg O_2/l (Rudd et al. 1974). Some methane oxidizing bacteria had previously been observed to fix nitrogen (Davis et al. 1964) and therefore it was decided to investigate the possibility that the methane oxidizing bacteria present in Lake 227 were nitrogen fixers also. The N-15 technique was chosen to assay for nitrogen fixation because it was suspected that the acetylene reduction technique was not applicable to the methane oxidizers (Flett et al. 1975).

An attempt was made to demonstrate in situ N-15 fixation in Lake 227 on September 26, 1974. A sample of 120 ml volume, taken from 4.75 m depth and containing 1.02 mg O_2/l was equilibrated with 5 ml of 57 at % N-15 gas and then incubated in situ for 6 hours (see Section III of Methods). No fixation was detected.

A second attempt was made to show nitrogen fixation by the methane oxidizers, this time in Lake 302 N. The hypolimnion of this lake basin was receiving fertilizer additions (described previously in Results) and was anoxic during summer stratification. On July 31, 1975, samples of 120 ml volume were taken at different depths in the low oxygen zone: each sample was equilibrated with 4 ml of 97 at % N-15 gas and then incubated in situ for 18 hours. Fixation was noted to occur (Table II).

Table II shows nitrogen fixation rates, particulate nitrogen concentrations and oxygen concentrations as a function of depth in Lake 302 N, July 31, 1975.

Depth (m)	O_2 Concentration (mg/L)	Particulate N (µg/L)	N_2 Fixation (µg N/L/hr)
7.2	0.2	295	0.011
7.4	0.1	371	0.019
7.6	0.1	475	0.019
8.0	0.0	340	0.006

It is assumed that the nitrogen fixation was due to methane oxidizers because they are known to fix only at low oxygen levels (usually <1.0 mg O_2/L - Rudd et al. 1976) and because no nitrogen fixing blue-green algae were present in the samples. The fixation was occurring in a hypolimnetic bloom of algae, concentrated in a narrow band around the 7.5 m depth, this concentration being easily noted in the particulate nitrogen data of Table II. The dominant algae genera were, in order of importance, Chlamydomonas, Cryptomonas and Mallomonas (unpublished data of E.J. Fee of E.L.A.).

Nitrogen Fixation in the Sediments

In Lake 302 S and Lake 227 several sediment slurries were obtained in summer 1973 by allowing a 3 litre Van Dorn sampler to partially penetrate the soft bottom sediments of the hypolimnia before being triggered. The slurries were assayed for nitrogen fixation by the acetylene reduction syringe technique in the same way as for water samples. The samples were incubated in situ just above the lake bottoms. The hypolimnion water of Lake 302 S was nearly saturated with oxygen while that of Lake 227 was anoxic. No nitrogen fixation was detected in either slurry.

In Lake 239 (1975) several studies were done to determine if N-15 gas would be fixed by in situ sediment cores located in the organically rich, shallow water littoral sediments of the south-east sub-basin. These techniques are described in the Methods section under 'Sediment Core Methods'. An 8 day incubation (August 21 - 28) and a 21 day incubation (September 2 -23) with equilibrated N-15 concentrations of 70

at % in aqueous phases of approximately 100 ml over the cores proved to be negative with respect to fixation in the sediment cores. Methane bubbles were noted coming from the sediments when they were disturbed.

Nitrogen Fixation by the Periphyton

Periphyton was not examined for nitrogen fixation in this study. However, very preliminary results by M. Turner (personal communication) of the E.L.A. project indicated that nitrogen fixation was being carried out by the periphyton of Lake 261 in 1975, the only year in which measurements were made. The fixation was light dependent and appeared to be due to a blue-green Anabaena sp.

Comparison of Nitrogen Fixation Rates Determined by N-15 and Acetylene Reduction Techniques

Nitrogen fixation rate estimates obtained by acetylene reduction were compared to rates simultaneously obtained by N-15 gas uptake. The comparisons were performed on subsamples of larger samples taken from Lake 227 and 226 NE at various dates. The acetylene reductions were always done in syringes with 30 ml of water as previously described by 'Acetylene Reduction Assays' in Methods. The N-15 uptake was done either in syringes with 30 ml of water and 1 ml of N-15 gas, or in 120 ml bottles with 4 ml of N-15 gas as was described by 'Additions of Nitrogen-15 Gas' in Methods. During in situ comparisons, duplicates of acetylene reduction and quadruplicates of N-15 were normally done and incubations were carried out at 0.3 m depth on a horizontal plexiglas rod described

in 'Incubation Procedures'. In the incubator comparison, both N-15 and acetylene reduction techniques were performed in syringes in duplicate. The incubation periods were between 2 and 5 hours and they were the same for both assays in any given comparison.

The results are given in Table III. It is obvious that the acetylene reduction technique, using the ratio of 3 C_2H_4 produced : 1 N_2 fixed, usually gave much higher estimates of nitrogen fixation.

Table III shows the rates of nitrogen fixation as determined by the acetylene redcution and isotopic methods when applied to subsamples of the same water. The data marked with '*' are excluded from the average because it is believed that the activity was too low to be measured accurately.

Date	Incubator Light Intensity µEinsteins/m^2/sec	Rate by C_2H_4 µg N/L/hr	Rate by N-15 µg N/L/hr	Ratio of Rates C_2H_4/N-15
LAKE 226 NE				
Sept 4/74	-	2.02	0.51	3.96
Sept 4/75	-	0.261	0.123	2.12
Sept 12/75	462	0.574	0.193	2.97
	102	0.578	0.165	3.50
	25	0.433	0.143	3.03
	6	0.322	0.036	8.94*
Sept 18/75	-	0.247	0.108	2.29
Sept 24/75	-	0.116	0.047	2.47
			Average =	2.90
			Excluding *	
Lake 227				
July 21/75	-	0.99	1.40	0.71
Sept 18/75	-	0.24	0.088	2.73
Sept 25/75	-	0.095	0.046	2.05
			Average =	1.83

DISCUSSION

Nitrogen Fixation by Blue-Green Algae in the Watercolumn

In Lake 226 NE, nitrogen fixation obviously had some importance. Table IV shows the estimated masses of nitrogen fixed in the lake for the years of 1973-75. Also given in the table are other inputs of nitrogen as determined by Schindler and his co-workers at the Experimental Lakes Area (personal communications). The shape of the fixation curve for 1974 and 1975 generally corresponds with the biomass of the nitrogen fixing blue-green algae.

The Lake 227 results are summed up by Table V. Only in 1975 was nitrogen fixation detectable. Again, the other nitrogen input data are due to Schindler and his co-workers (personal communications). The relative contribution of fixed nitrogen to Lake 227 was less than than observed for Lake 226 NE but it was still significant.

The bimodal shape of the Lake 227 fixation curve seen in Fig. 33 is not easily explained; it is possible that organically derived NH_3 nitrogen was being returned to the watercolumn from the sediments during the period from July 25 to August 13, 1975 and that this nitrogen was repressing fixation. An analysis of the allochthonous nutrient inputs to the lake (total N and total P) did not reveal any significant differences between the N:P ratio of the nutrients during the summer periods when fixation was and was not occurring.

Large periodic changes can be noted in the rates of nitrogen fixation of both lakes (Fig. 30 and 33). These very abrupt changes only occur when new in situ nitrogen fixation data, very different from the previous data, are encountered by the computer model. The 1975 fixation

Table IV shows the contribution of nitrogen fixation to Lake 226 NE for 1973-75.

LAKE 226 NE

Year	N_2 Fixation Input (KG) A	Fertilizer N Input (KG) B	Other Sources N Input (KG) C	%N Contributed by N_2 Fixation A/(A + B + C)
1973	17.8	149.7	72.5	7.4%
1974	139.6	149.7	76.2	38.2%
1975	51.4	149.7	∿70	∿19%

Table V shows the contribution of nitrogen fixation to Lake 227 for 1972-75.

LAKE 227

Year	N_2 Fixation Input (KG) A	Fertilizer N Input (KG) B	Other Sources N Input (KG) C	%N Contributed by N_2 Fixation A/(A + B + C)
1972	0	314.2	61.9	0%
1973	0	314.2	47.6	0%
1974	0	314.2	66.8	0%
1975	26	∿105	∿60	∿14%

data from Lake 227 and 226 NE often show weekly stepwise changes in algal nitrogen fixation activity per unit volume of water. The smaller variations on top of each step are due to changes in light. In Lake 226 NE, 1974, the steps occur at irregular intervals because in situ measurements were not made on a rigid once-a-week schedule. In both lakes it appears that the fluctuations in nitrogen fixation rates are due mostly to changes in activity of the algae per unit volume of water and not to changes in incident light.

In none of the other lakes examined was planktonic blue-green algal nitrogen fixation detected.

Nitrogen Fixation by Methane Oxidizing Bacteria in the Watercolumn

It was suspected that nitrogen fixing methane oxidizing bacteria were responsible for the observed nitrogen fixation in Lake 302 N and therefore an attempt was made to estimate the possible magnitude of such a nitrogen input to the lake.

As a first estimate, if one assumes that a maximum rate of methane oxidation of 0.1 μM CH_4/L/hr (upper estimate - J.W.M. Rudd - personal communication) occurred steadily for a period of 3 months and if one mole of nitrogen was fixed for every 20 moles of methane oxidized, then approximately 300 μg of nitrogen would be fixed per litre of water. If one further assumed that the activity took place through a one meter depth, then the areal contribution of nitrogen would have been approximately 0.13 g N/m^2. As a second estimate, if one took 0.015 μg N/L/hr as an average fixation rate (from Table II) the areal contribution of nitrogen to this lake would have been 0.014 g N/m^2. The natural allochthonous

input of nitrogen to this lake is approximately 0.8 g $N/m^2/yr$ (D.W. Schindler, unpublished). There exists, therefore, only a slight possibility that this fixation might supply a significant fraction of natural nitrogen input to the lake. If one includes the fertilizer nitrogen input in these calculations (2.79 g N/m^2) the possible relative significance of nitrogen fixation is further reduced. The quantity of nitrogen fixed may be significant to the methane oxidizers themselves but it is unlikely that it is very important to the nitrogen budget of the entire lake.

The Lake 227 N-15 uptake experiment, designed to show fixation by the methane oxidizers, proved negative. The oxygen concentrations in this experiment may have been too high, however, and significant fixation cannot be definitely ruled out until the studies are repeated.

Nitrogen Fixation in the Sediments

Nitrogen fixation could not be detected by acetylene reduction in the sediments of aerobic Lake 302 S or anaerobic Lake 227. Unfortunately, this does not allow one to draw definite conclusions regarding sediment nitrogen fixation. The performance of the acetylene assay is questionable in anaerobic (Brouzes and Knowles, 1971; Oremland and Taylor, 1975) and near anaerobic (Acetylene Redution Assay in Methods) regions. The more reliable N-15 technique, applied to sediments in Lake 239, indicated that if fixation was occurring, rates were below that detectable by the method. The following calculation was performed to determine the maximum amount of nitrogen that could be fixed in the sediments of Lake 239 and still be undetected by the N-15 techniques:

Given: Sediment core length examined = 5.4 cm

Average density of examined core length = 0.6875 g/cm^3 @ 105°C

Highest nitrogen content of sediments noted in E.L.A. <4% (Brunskill et al. 1971).

Minimum increase in N-15 required to be certain that fixation is occurring = .04 at % when using Statron emission spectrometer.

Incubation period = 3 weeks (21 days)

Then: Maximum nitrogen content = .04 x .6875 = 2.75 mg N/cm^3

Maximum nitrogen in core to 5.4 cm depth = 5.4 x 2.75 = 14.85 mg N/cm^2

Best sensitivity of N-15 method = minimum .04 at % increase in N

$\therefore$ Amount of fixation that could go undetected = 0.0004 x 14.85 = 5.9 µg N/cm^2 = 59.0 mg N/m^2

Fixation rate that could go undetected = 59/21 = 2.8 mg N/m^2/day.

IF fixation was active for 6 warmer months of the year, 182 x 2.81 = 0.51 g N/m^2 could have been fixed and still have been undetected.

There is a possibility that the N-15 gas diffused so slowly into the sediments that insufficient N-15 gas was present to be fixed and later detected. It may be that the labelled nitrogen gas never diffused down into the deeper anaerobic sections of the core and therefore that anaerobic nitrogen fixation was not actually assayed. It is suspected that N-15 gas did diffuse at least to 2 cm depth in the core because

oxygen has been noted to penetrate to this depth from overlying oxygenated waters (R. Hesslein, personal communication). If N-15 gas did penetrate as far as oxygen into the core, nitrogen fixation by methane oxidizing bacteria or other aerobic bacteria should have been observed, provided the rates were high enough to be detected.

The loading rate of Lake 239 has been approximately 1.2 g $N/m^2/yr$ (average 1972-1974) (D.W. Schindler, unpublished E.L.A. data). If the sediment nitrogen fixation were actually occurring and at a rate just below detectability (<0.5 g $N/m^2/yr$), sediment nitrogen fixation could have supplied significant portions of the total nitrogen input to the lake and still have escaped detection.

The determination of the amount of nitrogen fixation that could go undetected is obviously a rough approximation and much confidence should not be placed in it. It does illustrate that a more sensitive means of measuring nitrogen fixation than was applied here is needed for the sediments before good estimates of rates can be obtained. Macgregor and Keeney (1973) have previously stated much the same opinion with respect to some Wisconsin lakes.

Nitrogen Fixation by the Periphyton

The positive nitrogen fixation results in Lake 261 were most interesting but far too preliminary to allow estimates of its importance to be made. The Σ N : Σ P (Σ = fertilizer and natural inputs) loading ratio of this lake is <5:1 and it was initially suspected that such a loading ratio would encourage a planktonic bloom of nitrogen fixing organisms (D.W. Schindler, personal communication). It seemed logical that nitrogen fixers would become dominant in a relatively nitrogen poor

environment. Such was the case for Lake 226 NE in 1973-75 and later for Lake 227 in 1975 when the N:P loading ratios were approximately 5:1. Lake 261 appeared aberant, when only planktonic nitrogen fixation was considered, because it did not fit the predicted pattern. If it is revealed that periphyton fixation supplies significant quantities of nitrogen to the lake (measurements are presently being made by E.L.A. staff), Lake 261 may still be performing as predicted i.e. making up its nitrogen shortage.

Comparison of Acetylene Reduction to N-15 Gas Uptake

The results given in Table III show that the acetylene reduction technique consistently gave higher estimates of nitrogen fixation than the N-15 uptake technique. Why was this so?

The N-15 uptake method as used here measured the increased N-15 content of the particulate material only. If rapid extracellular excretions of fixed N-15 occurred, underestimates of nitrogen fixation would be made. This is a plausible explanation for the discrepancy between the two methods. Although extracellular excretion was not investigated in this study, it has been shown to be an active and widespread process in the green and blue-green algae (Stewart, 1963; Fogg, 1966; Jones and Stewart, 1969 (A)). Excretion of numerous amino acids and peptides has been demonstrated. Compounds such as ammonia and amino acids are also actively taken up by microbes (Jones and Stewart, 1969 (B); Gardner and Lee, 1975) and therefore the net loss of fixed nitrogen from the particulate would depend upon its re-uptake. If all the extracellular products were taken back up immediately by

any of the particulate fractions, the N-15 technique as described here should have given results similar to the acetylene reduction technique.

A second explanation may be that the algal cells were partially broken when filtered through the Reeve Angel 984 H glass fibre filters used in this work. If this occurred, the probability is high that labelled cell contents would have passed by the filter into the filtrate where they would not have been measured.

Another possibility is that the N-15 technique was giving accurate results and that the 3:1 ratio assumed for ethylene production: nitrogen fixation is incorrect. The results from Table III indicate that acetylene reduction is overestimating nitrogen fixation by more than a factor of two. If this were so, all of the budget figures given in this work would have to be reduced by more than fifty percent. Although the significance of nitrogen fixation would be substantially reduced, the general conclusions to this study would not be greatly altered.

Further work concerning the ethylene:nitrogen ratio is being carried out by B.M. Graham at the E.L.A.

CONCLUSIONS

Conclusions Concerning the Methods

N-15 Techniques

The emission spectrometric techniques developed here possess some substantial advantages over previous methods. The very time consuming and tedious wet chemical sample preparation procedures are avoided by the preparation unit earlier described. The unit is relatively inexpensive, easily constructed and processes samples containing as little as 5 µg N, a considerable improvement over other methods (Meyer et al. 1974; Keeney and Tedesco, 1973). An important improvement of this preparation unit is that it gives an estimate of the quantity of nitrogen in each sample and thus avoids the titrations and delicate weighing procedures required by other methods. Another feature of the unit is its low operating cost : the electrodeless discharge tubes are reused many times instead of being discarded after each use.

The Statron emission spectrometer, when operated in the modified fashion described in this thesis, gives better precision than has been previously noted by others. The rate of sample analysis is not as rapid as that of the automated mass spectrometric procedure of Barsdate and Dugdale (1965) but equipment costs are considerably lower and only one operator is required versus the two needed to operate the automated unit.

Acetylene Reduction Techniques

The acetylene reduction assay, as used in this study, avoids as many of the obvious errors as possible. The solubility of ethylene is acknowledged and simply dealt with and the use of different gas: aqueous volumes to increase assay sensitivity is handled in a practical way. Problems associated with ethylene and acetylene gas exchange are also identified and solutions to these problems are given. The use of syringes for incubating aqueous samples is a major improvement in the assay procedure because it essentially removes the need for special gas mixtures in the headspaces of incubation vessels. With the syringe technique, the concentrations of dissolved gases in the samples remain basically unchanged and therefore the sample should yield more accurate results.

It is concluded that the physical problems associated with the assay have been mostly removed. Some biological problems such as acetylene inhibition or toxicity and uncertain C_2H_4 : N_2 ratios still remain to be solved.

Conclusions drawn from the Results

From the data presented in Tables IV and V, it may be concluded that nitrogen fixation can be a significant source of nitrogen to Canadian Shield Lakes. This would tend to support the view put forth by the International Joint Commission (1969), Schindler (1974) and others that phosphorus was the logical element to remove from effluents entering the Great Lakes. Both carbon and nitrogen can enter a water body directly from the atmosphere in gaseous form and then be fixed by algae. Control of these carbon and nitrogen inputs is impossible.

The data for Lake 227 in Table VI, relating the appearance of nitrogen fixing blue-green algae to the total nitrogen: total phosphorus loading ratio, leads one to further conclusions. It appears that the lower N:P loading ratios may encourage development of blue-green algal blooms while higher N:P loading ratios may discourage them. A problem arises here with respect to the terms total N and total P. In Lake 227 more than 80% of the total nitrogen loading and more than 89% of the total phosphorus loading was supplied in the readily assimilated soluble inorganic form via the fertilization program. In other, non-experimental lakes, however, the chemical composition of the nutrient loading is not as closely defined. It is probably not accurate to use total phosphorus and total nitrogen inputs to estimate effective nutrient loading to many lakes because not all of the total can be immediately assimilated. Particulate phosphorus, in the form of mineral dust, would certainly require time to be dissolved and particulate phosphorus held in living tissue would mostly not be available until cellular lysis at time of death.

Furthermore, it is well known that total dissolved phosphorus concentrations are very low in most lakes, even in those subjected to high phosphorus loading rates (e.g. Lake Erie) and this indicates that nearly all of the total dissolved phosphorus is available to algae. For this reason it was decided to use total dissolved phosphorus concentrations, measured in nutrient inputs to a lake, when trying to estimate the phosphorus loading rate. Similarly, it is well known that inorganic nitrogen concentrations (NO_3^-, NO_2^-, NH_3) are often very low in lakes subject to high nitrogen loading rates (e.g. Lake Erie) and it

Table VI shows the relationship of N:P loading ratio to occurrence of blue-green fixers in Lake 227 (Nutrient loadings are those of D.W. Schindler, E.L.A. data).

LAKE 227

Year	Total N Loading KG	Total P Loading KG	N:P Ratio Loading	Presence of Blue-Green Fixers
1972	376.1	26.1	14.4	-
1973	361.7	25.9	14.0	-
1974	381.0	26.9	14.2	-
1975	∿165	∿26	∿6.3	+

is obvious that inorganic nitrogen is rapidly assimilated. The roles of the other fractions of the total nitrogen pool (the dissolved organic nitrogen and the particulate nitrogen) are not so obvious. In the Experimental Lakes Area, for example, between 100 and 400 µg/L of dissolved organic nitrogen has normally been present in all the lakes studied, regardless of trophic state (Schindler, unpublished data of the E.L.A.). This suggested that the dissolved organic nitrogen was not readily assimilated by the algae and therefore it was decided not to consider this nitrogen when estimating effective nitrogen loading rates. Particulate nitrogen was considered an unimportant input when estimating effective nitrogen loading for the same reasons previously stated concerning particulate phosphorus. In order to estimate the effective nitrogen loading rates to lakes, therefore, it was decided to use only the dissolved inorganic nitrogen concentrations observed in the nutrient inputs.

To further investigate the possible relationship between the ratio of dissolved inorganic N to total dissolved P in nutrient loading and the presence of nitrogen fixing algae, data from a number of other lakes were examined to see if the relationship held for them also. The data are seen in Table VII. In addition, the ratios of dissolved inorganic nitrogen to total dissolved phosphorus already in the lakes were also calculated (Table VII) to determine if they were correlated to the occurrence of the nitrogen fixing blue-green algae.

In order to simplify the analysis, no attempt was made to assess the effects of sediment feedback of nutrients upon the N:P loading ratios.

Table VII shows the average ratios of inorganic nitrogen concentrations: total dissolved phosphorus concentrations in both nutrient loadings and lake waters and relates these ratios to the occurrence of blue-green nitrogen fixing algae for a number of lakes on various dates.

* These results are based upon Kjeldahl -N + NO_3^- - N rather than NO_3^- -N + NO_2^- -N + NH_3 -N.

Lake	Date	Average Ratio of $(NO_3^- + NO_2^- + NH_3)$-N : Tot. Dis. P in Summer (Wt/Wt)		Presence of Blue-Green N_2 Fixers
		In Loading	In Epilimnion	
226 NE	1973	6.8	2.8	+
226 NE	1974	7.1	3.1	+
226 NE	1975	∿7	1.3	+
226 SW	1973	72.4	4.4	-
226 SW	1974	68.4	32.8	-
226 SW	1975	∿70	21.6	-
227	1972	14.4	1.2	-
227	1973	14.0	1.6	-
227	1974	14.2	2.1	-
227	1975	6.3	0.4	+
239	1973	21	3.7	-
239	1974	23	5.0	-
239	1975	∿22	2.3	-
Gravenhurst Bay	1969-71	3.4*	1.6	+
"	1971-73	15.5*	4.3	+
Green Bay	1971-72	∿10	1.8	+
Little Otter	1971	8.8	0.5	+
" "	1972	∿106	2.9	-
Pasqua	1965-66	0.2	0.2	+
L. Wpg/ Red River	1968-69	6.9	4.0	+
L. Wpg./ Sask. River	1968-69	6.2	0.9	+

The data for the numbered lakes are all from Schindler (unpublished data of the E.L.A.). Algal species identifications are by D. Findlay (unpublished data of the E.L.A.). Gravenhurst Bay data are calculated from work by Michalski et al. (1973, 1975). The main nutrient input to this bay was from a sewage outfall and the concentrations of total dissolved P, Kjeldahl-N and NO_3^--N in the sewage were regularly determined by those workers. Unfortunately, NH_3-N concentrations were not measured and therefore direct comparisons of loading rates between this and the other lakes are not possible. In order to use the data, however, the Kjeldahl-N concentrations were substituted for the NH_3-N concentrations, it being realized that the Kjeldahl-N concentrations would be higher than the NH_3-N because Kjeldahl-N measured NH_3-N plus organic nitrogen. The result is that the N:P loading ratios shown in Table VII should probably be lower than are indicated for Gravenhurst Bay, 1969-73. The methods of determination of dissolved nutrient concentrations in the lake itself were similar to those used on the other lakes given in the Table. The 1969-71 results represent N:P ratios before tertiary treatment was installed on the sewage outfall and the 1971-73 results represent post-tertiary treatment ratios.

The Green Bay results are calculated from data of Vanderhoef et al. (1974); the loading ratio was assumed to be the ratio of the sum of the dissolved NH_3-N and NO_3^--N concentrations to the total dissolved PO_4-P concentration observed at Station 2, a site in the Fox River which is the main nutrient source of the Bay. The mean of measurements from Stations 5-14 in the Bay proper was used to estimate the average ratio of dissolved inorganic N : total dissolved P during summer in the epilimnion. The Little Otter Lake results are calculated from data of

Michalski and Conroy (1973). The nitrogen loadings were determined using sums of average NH_3-N and NO_3^--N concentrations and mean daily water inflows to Little Otter Lake. The phorphorus loadings were given by Michalski and Conroy and required no calculations. The 1971 results were obtained during a period when extremely high phosphorus inputs occurred from an industrial site. In 1972 that phosphorus input had been arrested. The Lake Pasqua results are calculated from the data of Hammer (1969) and Warwick (1967). The average yearly concentrations of NO_3^- -N and NO_2^- -N and NH_3-N and of dissolved $PO_4^{\equiv}$ -P at Station 9, in the Qu'Appelle River just before entering Pasqua Lake, were used to calculate the loading ratio. Similar measurements in Lake Pasqua, averaged over the summer, gave the ratio of dissolved inorganic N : total dissolved P in the lake water. For Lake Winnipeg, chemical data and station numbers are those of Brunskill et al. (unpublished) while the phytoplankton data are those of Holmgren and Kling (unpublished). The loading ratio was assumed to be equal to the sum of dissolved NO_3^- -N and NO_2^- -N and NH_3 -N versus the total dissolved phosphorus concentration periodically observed in the Red River at Station 0 and the Saskatchewan River at Station 29. Both of these river stations are close to the mouths of the respective rivers where they empty into Lake Winnipeg. Similar periodic measurements performed at Station 25 (approximately 25 Km into the lake from the Saskatchewan River mouth) and station 2 (3.2 Km into the Lake from the Red River mouth) permitted calculation of the dissolved inorganic N: total dissolved P ratios in the lake.

Table VII reveals that the nitrogen fixing blue-green algae generally appear to be associated with waters having N : P loading

ratios of less than 10. Such blue-greens are mostly absent from waters where the loading ratios are above 10. Figure 36 is a plot of Table VII which clearly demonstrates this trend. The + signs indicate the presence of nitrogen fixing blue-greens while the - signs indicate the absence of these algae. Gravenhurst Bay is an exception, and as was discussed earlier, the loading ratios assigned to that water body may be too high. The arrows indicate the directions in which the points should probably be shifted if they are to more truly represent the loading experienced by the Bay. The abscissa of this plot is expressed as the logarithm of total dissolved inorganic nitrogen concentration. It was hypothesized that, given a sufficiently high dissolved inorganic nitrogen concentration, nitrogen fixation would no longer be a competitive advantage for the blue-green algae. Stripped of their competitive advantage, it was suspected that the blue-greens would disappear in favour of other algae with faster growth rates. This hypothesis does not appear valid, at least for the inorganic nitrogen concentrations encountered here.

No clear relationships between the appearance of nitrogen fixing blue-greens and the ratio of dissolved $NO_3^- + NO_2^- + NH_3$: dissolved total P could be found. When the ratios were plotted (Fig. 37) the scatter of points appeared random. This tends to confirm the belief (Schindler and Fee, 1975) that measurements of dissolved P and N concentrations often do not yield data indicative of loading rates. If the relationship between N : P loading ratios and occurrence of blue-green N_2 fixers is valid, then it follows that measurement of dissolved summer epilimnetic N and P concentrations may be of little use for prediction of such blue-green blooms.

Figure 36. A plot of + and - occurrences of blue-green fixers as functions of N : P loading ratios and average dissolved inorganic nitrogen concentrations in the summer epilimnia of a number of lakes.

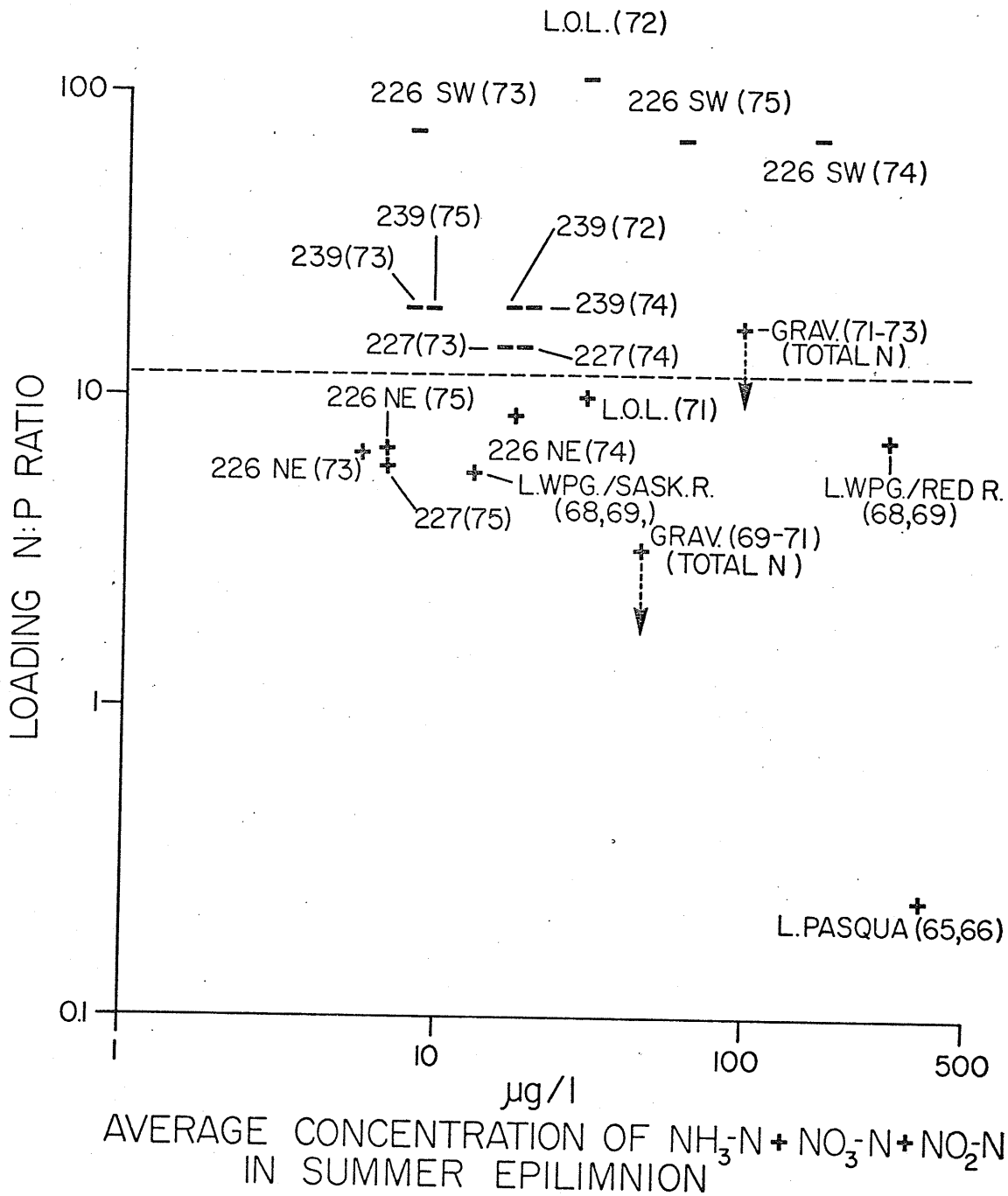
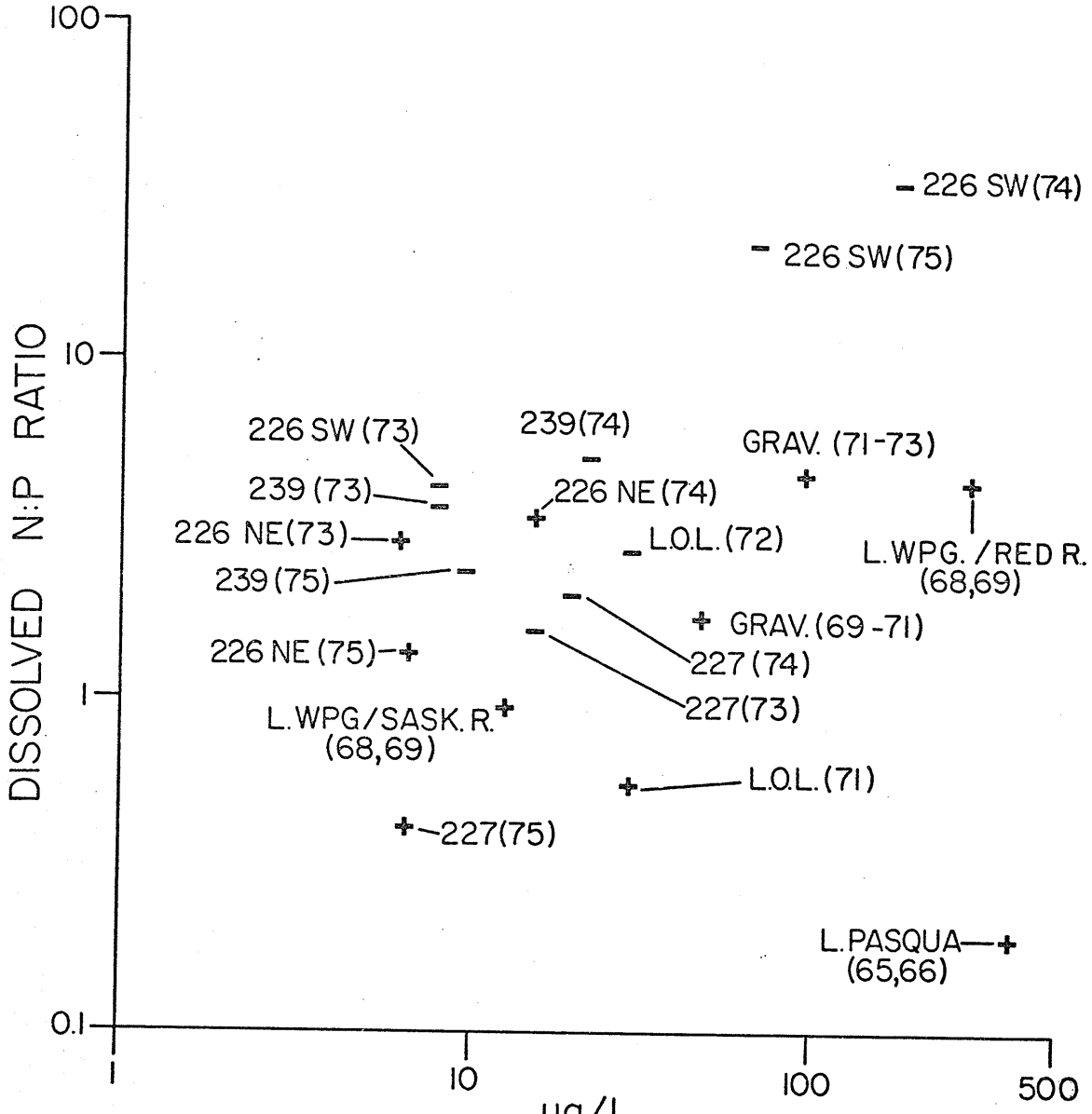

L.O.L.(72)
100
226 SW(73)
226 SW(75)
226 SW(74)
239(75)
239(72)
239(73)
239(74)
227(73)
227(74)
GRAV.(71-73)
(TOTAL N)
10
226 NE(75)
L.O.L.(71)
226 NE(74)
226 NE(73)
L.WPG./SASK.R.
(68,69,)
L.WPG./RED R.
(68,69)
227(75)
GRAV.(69-71)
(TOTAL N)
1
L.PASQUA(65,66)
0.1
1
10
100
500
µg/l
LOADING N:P RATIO
AVERAGE CONCENTRATION OF NH_3-N + NO_3-N + NO_2-N
IN SUMMER EPILIMNION

Figure 37. A plot of + and - occurrences of blue-green fixers as functions of dissolved inorganic N: total dissolved P ratios (summer epilimnion averages) and dissolved inorganic nitrogen concentrations in the summer epilimnia of a number of lakes.


100
10
1
0.1
DISSOLVED N:P RATIO
226 SW(74)
226 SW(75)
226 SW(73)
239(74)
GRAV. (71-73)
239 (73)
226 NE(74)
226 NE(73)
L.O.L.(72)
L.WPG./RED R.
(68,69)
239 (75)
GRAV.(69-71)
226 NE (75)
227 (74)
227(73)
L.WPG/SASK.R.
(68,69)
L.O.L.(71)
227(75)
L.PASQUA
(65,66)
1
10
100
500
µg/L
AVERAGE CONCENTRATION OF NH_3-N+NO_3-N+NO_2-N
IN SUMMER EPILIMNION

Of what practical use are these findings in water management techniques? If it is valid that blue-green nitrogen fixing algae occur only when the N : P loading ratio is less than 10, then a water manager would know, before starting tertiary treatments, the approximate quantity of phosphorus he must remove from an effluent in order to avoid these blue-green nuisance blooms in his receiving water. Such information would obviously be of primary importance when designing and operating treatment facilities.

In some cases, it may not be possible to remove enough phosphorus to raise the N : P ratio above 10. Under such circumstances it may be possible to raise the loading ratio by adding nitrogen instead of (or as well as) removing phosphorus. The addition of inorganic nitrogen may actually increase the areal primary production in a receiving water but the production would be of non-bloom forming algae which are generally distributed over much greater depths in the water columns. The surface concentrations of these algae would not be as high as might have been experienced if nitrogen fixing blue-greens had been dominant, and the water would be clearer at the surface. For example, in 1974 Lake 226 NE exhibited a very thick and highly visual bloom of nitrogen fixing blue-green algae. From appearances alone, it seemed that this lake was much more productive than Lake 227 in the same year. However, epilimnetic primary production figures (E.J. Fee, unpublished) for Lake 226 NE (82 g $C/m^2/yr$) and Lake 227 (158 g $C/m^2/yr$) show that Lake 227 was actually the more productive of the two even though it was visually less obvious. In short, the kind of primary production can be more important than the quantity when one is considering the recreational

potential and aesthetic appearance of a surface water.

The ability to inhibit nuisance blooms of nitrogen fixing blue-green algae would be valuable in some fish culturing operations. In numerous lakes (Barica, 1975) these blooms collapse so suddenly at their conclusion that their degradation can completely consume the dissolved oxygen in the water column and thereby cause massive fish kills. If one raised the N : P ratio by augmenting the inorganic nitrogen supply to such waters, it might be possible to encourage populations of other more stable (less subject to sudden collapse) algal families. The risk of 'summer kills' would be much reduced with these stable algal communities.

BIBLIOGRAPHY

Armstrong, F.A.J. and D.W. Schindler. 1971. Preliminary chemical characterization of waters in the Experimental Lakes Area, northwestern Ontario. J. Fish. Res. Bd. Canada 28: 171-187.

Barica, J. 1975. Collapses of algal blooms in prairie pothole lakes: their mechanism and ecological impact. Verh. Internat. Verein. Limnol. 19: 606-615.

Barsdate, R.J. and R.C. Dugdale. 1965. Rapid conversion of organic nitrogen to N_2 for mass spectrometry: an automated Dumas procedure. Anal. Biochem. 13: 1-5.

Benemann, J.R. 1973. Nitrogen fixation in termites. Science 181: 164-165.

Bergersen, F.J. 1970. The quantitative relationship between nitrogen fixation and the acetylene-reduction assay. Aust. J. biol. Sci. 23: 1015-1025.

Billaud, V.A. (Dugdale). 1967. Aspects of the nitrogen nutrition of some naturally occurring populations of blue-green algae. In Environmental requirements of blue-green algae. Proc. Symp. Sept. 23-24, 1966. U.S. Dept. Interior Federation Water Pollution Control Admin.

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

Broida, H.P. and M.W. Chapman. 1958. Stable nitrogen isotope analysis by optical spectroscopy. Anal. Chem. 30: 2049-2055.

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

Brouzes, R. and R. Knowles. 1971. Inhibition of growth of Clostridium pasteurianum by acetylene: implications for nitrogen fixation assay. Can. J. Microbiol. 17: 1483-1489.

Brunskill, G.J., D. Povoledo, B.W. Graham and M.P. Stainton. 1971. Chemistry of surface sediments of sixteen lakes in the Experimental Lakes Area, northwestern Ontario. J. Fish. Res. Bd. Canada 28: 277-294.

Bunt, J.S., K.E. Cooksey, M.A. Heeb, C.C. Lee and B.F. Taylor. 1970. Assay of algal nitrogen fixation in the marine subtropics by acetylene reduction. Nature 227: 1163-1164.

Burris, R.H. and C.E. Miller. 1941. Application of N^{15} to the study of biological nitrogen fixation. Science 93: 114-115.

Burris, R.H. and P.W. Wilson. 1957. Methods for measurement of nitrogen fixation. In Methods in Enzymology. S.P. Colowick and N.O. Kaplan, Eds. 4: 355-367. Academic Press, New York.

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

Cleugh, T.R., and B.W. Hauser. 1971. Results of the initial survey of the Experimental Lakes Area, northwestern Ontario. J. Fish. Res. Board Canada 28: 129-137.

Cook, G.B., J.A. Goleb and V. Middelboe. 1967. Optical nitrogen-15 analysis of small nitrogen samples using a noble gas to sustain the discharge. Nature 216: 475-476.

Davis, J.B., V.F. Coty and J.P. Stanley. 1964. Atmospheric nitrogen fixation by methane - oxidizing bacteria. J. Bacteriol. 88: 468-472.

de Bont, J.A.M. and E.G. Mulder. 1974. Nitrogen fixation and co-oxidation of ethylene by a methane-utilizing bacterium. J. gen. Microbiol. 83: 113-121.

de Bont, J.A.M. and E.G. Mulder. 1976. Invalidity of the acetylene reduction assay in alkane-utilizing nitrogen-fixing bacteria. Appl. Environ. Microbiol. 31: 640-647.

Dilworth, M.J. 1966. Acetylene reduction by nitrogen-fixing preparations from Clostridium pasteurianum. Biochim. biophys. Acta. 127: 285-294.

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

Dugdale, R.C., D.W. Menzel and J.H. Ryther. 1961. Nitrogen fixation in the Sargasso Sea. Deep-Sea Res. 7: 297-300.

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

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

Drozd, J. and J.R. Postgate. 1970. Effects of oxygen on acetylene reduction, cytochrome content and respiratory activity of Azotobacter chroococcum. J. gen. Microbiol. 63: 63-73.

Emerson, S., W. Broecker and D.W. Schindler. 1973. Gas-exchange rates in a small lake as determined by the radon method. J. Fish. Res. Board Can. 30: 1475-1484.

Fee, E.J. 1973. A numerical model for determining integral primary production and its application to Lake Michigan. J. Fish. Res. Board Can. 30: 1447-1468.

Flett, R.J. 1972. Measurement of nitrogen fixation rates by acetylene reduction and estimation of seasonal inputs of biologically fixed nitrogen to several artificially fertilized lakes in the Experimental Lakes Area of northwestern Ontario. M.Sc. Thesis, U. of Manitoba.

Flett, R.J., J.W.M. Rudd and R.D. Hamilton. 1975. Acetylene reduction assays for nitrogen fixation: a note of caution. Appl. Microbiol. 29: 580-583.

Flett, R.J., R.D. Hamilton and N.E.R. Campbell. 1976. Aquatic acetylene-reduction techniques: solutions to several problems. Can. J. Microbiol. 22: 43-51.

Fogg, G.E. 1966. The extracellular products of algae. Oceanogr. Mar. Biol. Ann. Rev. 4: 195-212.

Fogg, G.E. 1971. Nitrogen fixation in lakes. Pl. Soil, Special Volume: Biological nitrogen fixation in natural and agricultural habitats. pp. 393-401.

Gardner, W.S. and G.F. Lee. 1975. The role of amino acids in the nitrogen cycle of Lake Mendota. Limnol. Oceanogr. 20: 379-388.

Goering, J.J. and J.C. Neess. 1964. Nitrogen fixation in two Wisconsin lakes. Limnol. Oceanogr. 9: 530-539.

Goering, J.J., R.C. Dugdale and D.W. Menzel. 1966. Estimates of *in situ* rates of nitrogen uptake by *Trichodesmium* sp. in the tropical Atlantic Ocean. Limnol. Oceanogr. 11: 614-620.

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

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

Hardy, R.W.F., R.C. Burns and R.D. Holsten. 1973. Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. Soil. Biol. Biochem. 5: 47-81.

Hitch, C.J.B. and W.D.P. Stewart. 1973. Nitrogen fixation by lichens in Scotland. New Phytol. 72: 509-524.

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

Horne, A.J. and A.B. Viner. 1971. Nitrogen fixation and its significance in tropical Lake George, Uganda. Nature 232: 417-418.

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

Howard, D.L., J.I. Frea, R.M. Pfister and P.R. Dugan. 1970. Biological nitrogen fixation in Lake Erie. Science 169: 61-62.

International Joint Commission. 1969. Pollution of Lake Ontario and the International Section of the St. Lawrence River. Vol 3.

Jodin, O. 1862. Du rôle physiologique de l'azote. Comp. Rend. Acad. Sci. (Paris), 55: 612. (Cited from McKee, 1962).

Johnson, W.E. and J.R. Vallentyne. 1971. Rationale, background and development of experimental lake studies in northwestern Ontario. J. Fish. Res. Bd. Canada: 28: 123-128.

Jones, K. and W.D.P. Stewart. 1969a. Nitrogen turnover in marine and brackish habitats. III. The production of extracellular nitrogen by Calothrix scopulorum. J. mar. biol. Ass. U.K. 49: 475-488.

Jones, K. and W.D.P. Stewart. 1969b. Nitrogen turnover in marine and brackish habitats. IV. Uptake of the extracellular products of the nitrogen fixing alga Calothrix scopulorum. J. mar. biol. Ass. U.K. 49: 701-716.

Keeney, D.R. and M.J. Tedesco. 1973. Sample preparation for and nitrogen isotope analysis by the NOI-4 emission spectroscope. Anal. Chim. Acta 65: 19-34.

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

Lännergren, C., A. Lundgren and U. Granhall. 1974. Acetylene reduction and primary production in Lake Erken. Oikos 25: 365-369.

Loomis, A.G. 1928. In International critical tables of numerical data, physics, chemistry and technology. Vol. 3. Edited by E.W. Washburn. McGraw-Hill, New York. p. 260.

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

Mague, T.H., N.M. Weare and O. Holm-Hansen. 1974. Nitrogen fixation in the north Pacific Ocean. Mar. Biol. 24: 109-119.

Meyer, G.W., B.D. McCaslin and R.G. Gast. 1974. Sample preparation and 15-N analysis using a Statron NOI-5 optical analyser. Soil Science. 117: 378-385.

Michalski, M.F.P. and N. Conroy. 1973. The "oligotrophication" of Little Otter Lake, Parry Sound District - 1971-1972. Report from the Biology Section, Water Quality Branch, Ministry of Environment, Ontario, Canada.

Michalski, M.F.P., M.G. Johnson and D.M. Veal. 1973. Muskoka Lakes water quality evaluation. Rpt. No. 3, Eutrophication of the Muskoka Lakes. - Ontario Ministry of the Environment, Water Resources Branch, Toronto, Ontario.

Rudd, J.W.M., R.D. Hamilton and N.E.R. Campbell. 1974. Measurement of microbial oxidation of methane in lake water. Limnol. Oceanogr. 19: 519-524.

Rudd, J.W.M. and R.D. Hamilton. 1975. Factors controlling rates of methane oxidation and the distribution of the methane oxidizers in a small stratified lake. Arch. Hydrobiol. 75: 522-538.

Rudd, J.W.M., A. Furutani, R.J. Flett and R.D. Hamilton. 1976. Factors controlling methane oxidation in shield lakes: The role of nitrogen fixation and oxygen concentration. Limnol. Oceanogr. 21: 357-364.

Rusness, D. and R.H. Burris. 1970. Acetylene reduction (nitrogen fixation) in Wisconsin lakes. Limnol. Oceanogr. 5: 808-813.

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

Schindler, D.W., F.A.J. Armstrong, S.K. Holmgren and G.J. Brunskill. 1971. Eutrophication of Lake 227, Experimental Lakes Area, northwestern Ontario, by addition of phosphate and nitrate. J. Fish. Res. Bd. Canada 28: 1763-1782.

Schindler, D.W. and E.J. Fee. 1973. Diurnal variation of dissolved inorganic carbon and its use in estimating primary production and CO_2 invasion in lake 227. J. Fish. Res. Board Can. 30: 1501-1510.

Schindler, D.W., H. Kling, R.V. Schmidt, J. Prokopowich, V.E. Frost, R.A. Reid and M. Capel. 1973. Eutrophication of Lake 227 by addition of phosphate and nitrate: the second, third and fourth years of enrichment, 1970, 1971 and 1972. J. Fish. Res. Board Can. 30: 1415-1440.

Schindler, D.W. 1974. Eutrophication and recovery in experimental lakes: implications for lake management. Science 184: 897-899.

Schindler, D.W. and E.J. Fee. 1975. The roles of nutrient cycling and radiant energy in aquatic communities. In Photosynthesis and productivity in different environments. International Biological Programme. Vol 3. Cambridge University Press.

Schindler, D.W. 1975. Whole-lake eutrophication experiments with phosphorus, nitrogen and carbon. Verh. Internat. Verein. Limnol. 19: 3221-3231.

Schöllhorn, R. and R.H. Burris. 1966. Study of intermediates in nitrogen fixation. Fed. Proc. Fedn. Am. Socs. exp. Biol. 25: 710.

Shearer, J.A. 1976. Light extinction measurements in the Experimental Lakes Area - 1974 Data. Fish. Mar. Serv. Dev. Tech. Rep. 615: 96 pp.

Shearer, J.A. and D. Declercq. 1976. Light extinction measurements in the Exprimental Lakes Area - 1975. Data Fish. Mar. Serv. Dev. Tech. Rep. XXX. In Press.

Stewart, W.D.P. 1963. Liberation of extracellular nitrogen by two nitrogen-fixing blue-green algae. Nature 200: 1020-1021.

Stewart, W.D.P. 1964. Nitrogen fixation by Myxophyceae from marine environments. J. gen. Microbiol. 36: 415-422.

Stewart, W.D.P. 1967. Nitrogen turnover in marine and brackish habitats II. Use of ^{15}N in measuring nitrogen fixation in the field. Annals of Botany. N.S. 31: 385-407.

Stewart, W.D.P., G.P. Fitzgerald and R.H. Burris. 1967. In situ studies on N_2 fixation using the acetylene reduction technique. Proc. Nat. Acad. Sci. 58: 2071-2078.

Stewart, W.D.P., G.P. Fitzgerald and R.H. Burris. 1968. Acetylene reduction by nitrogen-fixing blue-green algae. Arch. Mikrobiol. 62: 336-348.

Stewart, W.D.P. 1968. Nitrogen input into aquatic ecosystems. In Algae, man and the environment. Edited by D.F. Jackson. Syracuse University Press, Syracuse, New York. pp. 53-72.

Stewart, W.D.P. and M. Lex. 1970. Nitrogenase activity in the blue-green alga Plectonema boryanum strain 594. Arch. Mikrobiol. 73: 250-260.

Stewart, W.D.P. and H.W. Pearson. 1970. Effects of aerobic and anaerobic conditions on growth and metabolism of blue-green algae. Proc. Roy. Soc. London, Ser. B. 175: 293-311.

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

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

Vanderhoef, L.N., C-Y Huang, R. Musil and J. Williams. 1974. Nitrogen fixation (acetylene-reduction) by phytoplankton in Green Bay, Lake Michigan, in relation to nutrient concentrations. Limnol. Oceanogr. 19: 119-125.

Webb, K.L., W.D. DuPaul, W. Wiebe, W. Sottile and R.E. Johannes. 1975. Enewetak (Eniwetok) Atoll: Aspects of the nitrogen cycle on a coral reef. Limnol. Oceanogr. 20: 198-210.

Whittenbury, R., H. Dalton, M. Eccleston and H.L. Reed. 1975. The different types of methane oxidizing bacteria and some of their more unusual properties. In Microbiol growth on C-1 Compounds. pp. 1-9. Proc. Symp. Sept. 5, 1974. Tokyo, Japan.

Wiebe, W.J., R.E. Johannes and K.L. Webb. 1975. Nitrogen fixation in a coral reef community. Science 188: 257-259.

APPENDIX

Computer program used for predicting nitrogen fixation input into a lake as a function of incident solar radiation.

University of Manitoba IBM-370 Computer. Language: Fortran - WATFIV Compiler.

```
      $JOB  WATFIV
    1       DIMENSION ALITE(140,230),ARVAL(120),AVOL(30),E(30),B(30),G(30)
    2       DIMENSION  IYINC(230),WVAL(100),ITONE(17),ITTWO(17),A(100)
    3       DIMENSION DAYNO(120)
    4       N=1
    5       DO 4 J=1,5
    6       M=N+4
    7       READ(5,5)(AVOL(I),I=N,M)
    8     4 M=N+5
    9     5 FORMAT(5F15.1)
   10       IY=1
   11       DO 20 I=1,15
   12       READ(5,10)ITONE(IY),ITTWO(IY)
   13    10 FORMAT(2I5)
   14    20 IY=IY+1
   15       N=1
   16       DO 24 J=1,15
   17       M=N+5
   18       READ(5,23)(WVAL(K),K=N,M)
   19    23 FORMAT(6F12.6)
   20    24 N=N+6
   21       X=1
   22       ISET=0
   23       IDATOT=105
   24    25 READ(5,30)DAYNO(X)
   25    30 FORMAT(F3.0)
   26       READ(5,40)IYINC(X)
   27    40 FORMAT(70X,I10)
   28       ISET=ISET+1
   29       ISTART=-12
   30    50 ISTART=ISTART+13
   31       IEND=ISTART+12
   32       READ(5,60)(ALITE(ISET,J),J=ISTART,IEND)
   33    60 FORMAT(13F6.0)
   34       IF(IEND .LT.IYINC(X))GO TO 50
   35       X=X+1
   36       IF(X .LE. IDATOT)GO TO 25
   37    12 J=0
   38       X=4
   39       IY=1
   40       ISET=4
   41       IJ=ISET+98
   42       C=0
   43       K=0
   44       DO 330 M=ISET,IJ,7
   45       IONE=ITONE(IY)
   46       ITWO=ITTWO(IY)
      C-THESE LIGHT ATTENUATION FACTORS(FITK) ARE FOR L.227-1975
   47       IF(DAYNO(X) .GT. 128 .AND. DAYNO(X) .LT. 136)FITK=0.96
   48       IF(DAYNO(X) .GT. 135 .AND. DAYNO(X) .LT. 148)FITK=0.98
   49       IF(DAYNO(X) .GT. 147 .AND. DAYNO(X) .LT. 162)FITK=1.13
   50       IF(DAYNO(X) .GT. 161 .AND. DAYNO(X) .LT. 176)FITK=1.30
   51       IF(DAYNO(X) .GT. 175 .AND. DAYNO(X) .LT. 190)FITK=1.40
```

```
      IF(DAYNO(X) .GT. 189 .AND. DAYNO(X) .LT. 204)FITK=1.69
      IF(DAYNO(X) .GT. 203 .AND. DAYNO(X) .LT. 218)FITK=1.45
      IF(DAYNO(X) .GT. 217 .AND. DAYNO(X) .LT. 232)FITK=1.15
      IF(DAYNO(X) .GT. 231 .AND. DAYNO(X) .LT. 246)FITK=1.20
      IF(DAYNO(X) .GT. 245 .AND. DAYNO(X) .LT. 260)FITK=1.05
      IF(DAYNO(X) .GT. 259 .AND. DAYNO(X) .LT. 274)FITK=1.32
      IF(DAYNO(X) .GT. 273 .AND. DAYNO(X) .LT. 288)FITK=1.08
      IF(DAYNO(X) .GT. 287 .AND. DAYNO(X) .LT. 302)FITK=0.98
      IF(DAYNO(X) .GT. 301 .AND. DAYNO(X) .LT. 309)FITK=1.25
      Z=0
      DO 300 I=1,27
      SUMFIX=0
      DO 280 J=IONE,ITWO
      QLITE=ALITE(ISET,J)*2.7183**(-FITK*Z)
      IF(QLITE .GT. 461.71)GO TO 190
      IF(QLITE .GT. 101.78)GO TO 210
      IF(QLITE .GT.  24.88)GO TO 230
      IF(QLITE .GT.   0.38)GO TO 260
      F=0
      GO TO 280
190   F=(ALOG10(QLITE)-(3.3424))/(-1.0815)
      GO TO 280
210   F=(ALOG10(QLITE)+8.083)/ 17.24
      GO TO 280
230   F=(ALOG10(QLITE)+0.082)/ 4.7723
      GO TO 280
260   F=(ALOG10(QLITE)+0.4202)/ 3.97
      GO TO 280
280   SUMFIX=SUMFIX+F
      Z=Z+0.2
300   A(I)=SUMFIX/(ITTWO(IY)-ITONE(IY))
      WRITE(6,308)DAYNO(X)
308   FORMAT(' DAYNO=',F4.0)
      WRITE(6,310)(A(I),I=2,27,5)
310   FORMAT(1X,F25.10)
      DO 315 I=2,27,5
      IF(A(I) .EQ. 0)A(I)=1
      C=C+1
      K=K+1
      ARVAL(C)=WVAL(K)/A(I)
315   WRITE(6,316)ARVAL(C)
316   FORMAT(' ARVAL=',F15.12)
      X=X+7
330   IY=IY+1
      X=1
      ISET=0
      GNDTOT=0
      DO 715 K=1,IDATOT
C-THESE LIGHT ATTENUATION FACTORS(FITK) ARE FOR L.227-1975
      IF(DAYNO(X) .GT. 128 .AND. DAYNO(X) .LT. 136)FITK=0.96
      IF(DAYNO(X) .GT. 135 .AND. DAYNO(X) .LT. 148)FITK=0.98
      IF(DAYNO(X) .GT. 147 .AND. DAYNO(X) .LT. 162)FITK=1.13
```

```
103             IF(DAYNO(X) .GT. 161 .AND. DAYNO(X) .LT. 176)FITK=1.30
104             IF(DAYNO(X) .GT. 175 .AND. DAYNO(X) .LT. 190)FITK=1.40
105             IF(DAYNO(X) .GT. 189 .AND. DAYNO(X) .LT. 204)FITK=1.69
106             IF(DAYNO(X) .GT. 203 .AND. DAYNO(X) .LT. 218)FITK=1.45
107             IF(DAYNO(X) .GT. 217 .AND. DAYNO(X) .LT. 232)FITK=1.15
108             IF(DAYNO(X) .GT. 231 .AND. DAYNO(X) .LT. 246)FITK=1.20
109             IF(DAYNO(X) .GT. 245 .AND. DAYNO(X) .LT. 260)FITK=1.05
110             IF(DAYNO(X) .GT. 259 .AND. DAYNO(X) .LT. 274)FITK=1.32
111             IF(DAYNO(X) .GT. 273 .AND. DAYNO(X) .LT. 288)FITK=1.08
112             IF(DAYNO(X) .GT. 287 .AND. DAYNO(X) .LT. 302)FITK=0.98
113             IF(DAYNO(X) .GT. 301 .AND. DAYNO(X) .LT. 309)FITK=1.25
        C-THESE ARE COUNTERS FOR ARVAL IN L.227-1975
114             IF(DAYNO(X) .GT. 184 .AND. DAYNO(X) .LT. 192)C=1
115             IF(DAYNO(X) .GT. 191 .AND. DAYNO(X) .LT. 199)C=7
116             IF(DAYNO(X) .GT. 198 .AND. DAYNO(X) .LT. 206)C=13
117             IF(DAYNO(X) .GT. 205 .AND. DAYNO(X) .LT. 213)C=19
118             IF(DAYNO(X) .GT. 212 .AND. DAYNO(X) .LT. 220)C=25
119             IF(DAYNO(X) .GT. 219 .AND. DAYNO(X) .LT. 227)C=31
120             IF(DAYNO(X) .GT. 226 .AND. DAYNO(X) .LT. 234)C=37
121             IF(DAYNO(X) .GT. 233 .AND. DAYNO(X) .LT. 241)C=43
122             IF(DAYNO(X) .GT. 240 .AND. DAYNO(X) .LT. 248)C=49
123             IF(DAYNO(X) .GT. 247 .AND. DAYNO(X) .LT. 255)C=55
124             IF(DAYNO(X) .GT. 254 .AND. DAYNO(X) .LT. 262)C=61
125             IF(DAYNO(X) .GT. 261 .AND. DAYNO(X) .LT. 269)C=67
126             IF(DAYNO(X) .GT. 268 .AND. DAYNO(X) .LT. 276)C=73
127             IF(DAYNO(X) .GT. 275 .AND. DAYNO(X) .LT. 283)C=79
128             IF(DAYNO(X) .GT. 282 .AND. DAYNO(X) .LT. 290)C=85
129             ISET=ISET+1
130             Z=0
131             DO 3000 I=1,26
132             SUMFIX=0
133             INC=IYINC(X)
134             J=0
135             DO 2800 N=1,INC
136             J=J+1
137             QLITE=ALITE(ISET,J)*2.7183**(-FITK*Z)
138             IF(QLITE .GT. 461.71)GO TO 1900
139             IF(QLITE .GT. 101.78)GO TO 2100
140             IF(QLITE .GT.  24.88)GO TO 2300
141             IF(QLITE .GT.   0.38)GO TO 2600
142             F=0
143             GO TO 2800
144     1900    F=(ALOG10(QLITE)-(3.3424))/(-1.0815)
145             GO TO 2800
146     2100    F=(ALOG10(QLITE)+8.083)/ 17.24
147             GO TO 2800
148     2300    F=(ALOG10(QLITE)+0.082)/ 4.7723
149             GO TO 2800
150     2600    F=(ALOG10(QLITE)+0.4202)/ 3.97
151     2800    SUMFIX=SUMFIX+F
152             Z=Z+0.2
153     3000    A(I)=SUMFIX/12.0
```

```
154             DO 500 I=1,3
155     500     E(I)=A(I)*ARVAL(C)
156             DO 510 I=4,8
157             AB=C+1
158     510     E(I)=A(I)*ARVAL(AB)
159             DO 520 I=9,13
160             AC=C+2
161     520     E(I)=A(I)*ARVAL(AC)
162             DO 530 I=14,18
163             AD=C+3
164     530     E(I)=A(I)*ARVAL(AD)
165             DO 540 I=19,23
166             AE=C+4
167     540     E(I)=A(I)*ARVAL(AE)
168             DO 550 I=24,26
169             AF=C+5
170     550     E(I)=A(I)*ARVAL(AF)
171             DO 600 I=1,25
172             AG=I+1
173     600     B(I)=(E(I)+E(AG))/2
174             DO 650 I=1,25
175     650     G(I)=B(I)*AVOL(I)
176             WRITE(6,670)(G(I),I=1,25)
177     670     FORMAT('0',5F15.1)
178             H=0
179             DO 700 I=1,25
180     700     H=H+G(I)
181             WRITE(6,720)DAYNO(X),H
182             X=X+1
183     715     GNDTOT=GNDTOT+H
184     720     FORMAT(' TOTAL FIXATION IN LAKE ON DAY',F4.0,'IS',
                GF15.1,'MICROGRAMS NITROGEN')
185     730     WRITE(6,740)GNDTOT
186     740     FORMAT(' GRAND TOTAL OF ALL N FIXED FOR YEAR IS',
                GF15.1,'MICROGRAMS NITROGEN')
187             STOP
188             END
```

Examples of the first and second sections of computer output. The first section gives day number, the 5 predicted fixation rates for 0.2, 1.2, 2.2, 3.2, 4.2 and 5.2 meter depths, and the 5 values of ARVAL. The second section gives the weight of nitrogen fixed on the indicated day into the whole lake in 0.2 meter thick layers from 0.0 to 5.0 meter depth.

An example of the first section of computer output

```
 DAYNO=188.
                0.3083612000
                0.6201755000
                0.4017630000
                0.4136653000
                0.2575891000
                0.1014914000
ARVAL= 1.286801000000
ARVAL= 0.143830200000
ARVAL= 0.175476600000
ARVAL= 0.030941380000
ARVAL= 0.014752170000
ARVAL= 0.000000000000
```

An example of the second section of computer output

```
    61104560.0    73598750.0    43913080.0    10258240.0    10675060.0
    10446340.0     9794063.0    10026030.0    10050160.0     8889287.0
     7535270.0     6555575.0     3613907.0      989355.1      941999.3
      947694.1      920182.9      619835.4      349068.3      300390.1
      266100.1      227628.4      103401.1           0.0           0.0
TOTAL FIXATION IN LAKE ON DAY185.IS    272124900.0MICROGRAMS NITROGEN
```

Table VIII shows the way in which predicted daily rates of nitrogen fixation were corrected by weekly observed in situ rates.

Depth Meters	Observed Rate 10:00 a.m.- 2:00 p.m. µg N/L/Hr	Predicted Rate 10:00 a.m. 2:00 a.m. µg N/L/Hr	Observed/ Predicted = Arval	Predicted Rate Entire Day µg N/L/Hr	Predicted Rate (corrected) Entire day µg N/L/Hr
0.0	-	0.6		0.4	0.4 x 2.0 = 0.8
0.2	1.6	0.8	2.0	0.5	0.5 x 2.0 = 1.0
0.4	-	1.0		0.7	0.7 x 2.0 = 1.4
0.6	-	1.2		1.0	1.0 x 1.0 = 1.0
0.8	-	1.2		1.1	1.1 x 1.0 = 1.1
1.0	-	1.1		0.8	0.8 x 1.0 = 0.8
1.2	1.0	1.0	1.0	0.8	0.8 x 1.0 = 0.8
1.4	-	0.9		0.6	0.6 x 1.0 = 0.6
1.6	-	0.8		0.6	0.6 x 0.5 = 0.30
1.8	-	0.7		0.5	0.5 x 0.5 = 0.25
2.0	-	0.7		0.5	0.5 x 0.5 = 0.25
2.2	0.3	0.6	0.5	0.4	0.4 x 0.5 = 0.20
2.4	-	0.6		0.3	0.3 x 0.5 = 0.15
2.6	-	0.5	0.3	0.3	0.3 x 0.5 = 0.15
2.8	-	0.4		0.2	0.2 x 0.67= 0.13
3.0	-	0.4		0.2	0.2 x 0.67= 0.13
3.3	0.2	0.3	0.67	0.2	0.2 x 0.67= 0.13
3.4	-	0.3		0.2	0.2 x 0.67==0.13
3.6 ↓ 5.2	-	0.3		0.1	0.1 x 0.67= 0.07

EXPLANATION OF PROGRAM

Lines 1-3, Dimension statements

Lines 4-9, Reading in 25 values of AVOL where AVOL = volume of the lake between adjacent 0.2 meter depths for 0.0 to 5.0 meters.

Lines 10-14, Reading in 15 values of ITONE and 15 values of ITTWO where ITONE is the n^{th} 5 minute interval of the lighted day and corresponds to the beginning of an in situ acetylene reduction measurement.
where ITTWO is the m^{th} 5 minute interval of the lighted day and corresponds to the end of an in situ acetylene reduction measurement.

Lines 15-20, Reading in 90 values of WVAL where WVAL = the rate of nitrogen fixation observed in situ in the lake. There are values for 0.2, 1.2, 2.2, 3.2, 4.2 and 5.2 meter depths for a period of 15 weeks ie. 6 x 15 = 90 values.

Lines 21-22, Internal counters.

Line 23, IDATOT tells the number of days to be examined and thereby instructs computer to read only that amount of light data.

Lines 24-25, Reading light data in. First number read is DAYNO(X) and it gives the day of the year.

Lines 26-27, Reading light data in. Second number read is IYINC(X) and it gives the number of 5 minute increments in the lighted day.

Lines 28-36, Reading light data in. Instructs computer to read the correct number of cards for each day's light data and also the number of days to read ie. IDATOT.

Line 37, Initializing counter.

Line 38, Defining first value of X for DAYNO(X).

Line 39, Initialization.

Line 40, ISET defines the rank of the day for which the first in situ data are available ie. on the 4th day of light data read in, in situ observations were obtained in Lake 227, 1975.

Line 41, IJ defines the rank of the last day for which in situ data are available.

Lines 42-43, Initialization.

Lines 44-95, Routine to estimate fixation at 0.2, 1.2, 2.2, 3.2, 4.2 and 5.2 meter depths for the same time interval as the in situ measurements. These estimates, A(I), are then compared with the actual in situ observations, WVAL, and the ratio of WVAL/A(I) = ARVAL. New values of ARVAL are generated for each week in which in situ data are available. In this case, there are 6 depths and 15 weeks for a total of 6 x 15 = 90 values of ARVAL.

Line 44, Defines which days are to be examined.

Lines 45-46, Redefinitions.

Lines 47-60, Light attenuation factors are given for a range of dates for the lake in question.

Line 61, Defines initial depth Z = 0.

Lines 62-82, Routine to predict rates of fixation at 27 depths, from 0.0 to 5.2 meters in 0.2 meter intervals, for the same time interval as the in situ measurement.

Line 63, Initializing value of SUMFIX = 0, where SUMFIX is the sum of the nitrogen fixation rates at a single depth that are determined every 5 minutes from the average incident solar radiation during each 5 minute period (summation occurring over the period of in situ incubation.

Lines 64-80, Routine for summing all the values of F, where F is the predicted rate of fixation over one 5 minute interval at one depth.

Line 65, Gives the light attenuation equation, where
QLITE = actual light intensity reaching the depth
ALITE = incident light intensity
2.7183 = e
FITK = extinction co-efficient
Z = depth in meters.

Lines 66-79, The correct equation for expressing light intensity: nitrogen fixation is chosen according to the light intensity and then the average rate of fixation, F, is calculated for that 5 minute interval.

Line 80, All of the values of F that were calculated for the period of the in situ incubation are summed.

Line 81, Depth is incremented by 0.2 meters.

Line 82, The average rate of fixation predicted for the in situ incubation period is determined at each depth.

Lines 83-86, Instructions to write DAYNO and the average fixation rates on that day number at 0.2, 1.2, 2.2, 3.2, 4.2 and 5.2 meter depths.

Lines 87-93, The values of ARVAL are calculated for each depth.

Lines 94-95, Incrementation.

Lines 96-98, Initialization.

Lines 99-183, Routine to predict total nitrogen fixation input to the lake, on a daily basis and as a total.

Line 99, Defines number of days to be examined.

Lines 100-113, Give required light attenuation factors,

Lines 114-128, Internal counters.

Lines 129-130, Initialization.

Lines 131-153, Routine predicts average rate of fixation over entire day at 0.2 meter intervals from 0.2 to 5.0 meters depth.

Lines 154-170, Series of statements that cause each average daily rate of fixation, at each 0.2 meter depth, to be multiplied by the appropriate ARVAL. For example assume that on day 74 an in situ incubation were performed and rates of fixation were determined at 0.2, 1.2, 2.2, 3.2, 4.2 and 5.2 meters between the hours of 10:00 a.m. and 2:00 p.m. Assume also the rates of fixation at the same time and depths were predicted from light values input into the program and that ARVAL were calculated from the rates at the six depths. Assume next that the entire days light data were used to predict the average rate of nitrogen fixation over the lighted day at 0.2 meter intervals from 0.0 to 5.0 meters depth. It is further assumed that if the nitrogen fixation rate determined in an in situ observation is different from that predicted by the light values, then the predicted rates are wrong and should be made the same as the in situ rates by multipylying the predictions by the ARVAL of the nearest or equivalent depth. Table VIII gives an example of how the ARVAL values are applied. The next assumption made is that the values of ARVAL are

Lines 154-170 - continued

valid for the 3 day periods immediately preceding and following the in situ measurements. The corrected predicted rates are then obtained for these other 6 days of the week by use of the appropriate solar radiation values. The procedure is repeated until all of the weeks in the experimental period have been examined.

Lines 171-173, Averages corrected predicted daily rates of nitrogen fixation from adjacent 0.2 meter depths.

Lines 174-177, Calculates and writes out predicted daily weight of nitrogen fixed in each 0.2 meter depth layer of the entire lake.

Lines 178-183, Calculates and writes out predicted daily weight of nitrogen fixed in entire lake.

Lines 184-186, Writes out predicted total weight of nitrogen fixed in entire lake over complete experimental period.

Lines 187-188, End statements.