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

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ABSTRACT

Nitrogen fixation rates were measured by the acetylene reduction technique in two artificially fertilized lakes. Total nitrogen fixed was found to range from 3.26 to 5.55 g - N/m^2 over a sampling period of about 4 months. This amount of nitrogen, fixed mainly by bacteria, represents a large proportion of the total nitrogen input to these lakes. Most fixation activity occurs in fairly defined but evenly distributed depths in the water column.

TO PATRICIA AND DAVID

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INTRODUCTION

Introduction

The purpose of this study was to measure the total rate of nitrogen fixation in several artificially fertilized lakes whose nitrogen input from all other sources was known. In this way, the relative importance of the fixation process, with respect to other forms of nitrogen input, could be determined.

Estimations of the nitrogen fixation rates were based solely upon results obtained from the acetylene reduction technique. No calibration of the results was attempted using $^{15}N_2$, therefore rates cannot be known with absolute precision. The relative rates, over time and with depth, however, most probably are correct.

Although other studies of this type have been done by a number of other workers, their fixation measurements for the most part were too infrequent and the amounts of nitrogen contributed by other sources were ill defined. This study is an attempt to correct these faults.

HISTORICAL

Historical

The ability of certain organisms to fix atmospheric molecular nitrogen has been recognized for over 100 years (1) and is now well established (2,3,4,5). Over this period of time many of the participating organisms have been identified and studied. Concomitantly, methods for measurement of fixation have been much improved.

Until 1940 nitrogen fixation was quantitively measured by the Kjeldahl technique. This method was tedious and often inaccurate and quickly gave way to the newer more sensitive $^{15}N_2$ tracer technique proposed by Burris and Miller (6,7). The $^{13}N_2$ isotope was employed by Campbell *et al* (8) to study nitrogen fixation of soil-borne microorganisms and, except for its short half life, was also found to be satisfactory. Both of these techniques, however, require expensive and complicated equipment and thus are not well suited to *in situ* field studies on nitrogen fixation (9).

After Dilworth's (10) discovery in 1966 that acetylene acted as a reducible analogue of nitrogen in the fixation reaction, Hardy and Knight (11) applied hydrogen flame ionization gas chromatography to the detection of ethylene formed from acetylene by nitrogenase. They proposed that the reduction of acetylene to ethylene, coupled with the detection and separation method, was a sensitive and rapid, although indirect, assay for nitrogen fixation activity.

Though relatively simple and inexpensive, the acetylene reduction technique does not appear to have been used intensively in studies upon aquatic environments. Perhaps this is partly due to uncertainties concerning the accuracy of the assay that were ably expressed by Bergersen (12). Estimations of aquatic nitrogen fixation rates that have been done, using the acetylene reduction technique, were usually poor due to completely inadequate sampling procedures (13,14,15,16). The best attempts to date at determining total nitrogen input into lakes by fixation are those of Horne and Fogg (17). They applied the $^{15}N_2$ technique over a 1.5 year period with a sampling frequency of about 1.5 months and they consistently sampled at several depths. Their data, which must be considered the best presently available, shows fixation to be from 0.037 to 0.287 g - N/m^2/yr and this is considered less than 1% of the total nitrogen income. Algae were held to be mainly responsible for the fixation.

Very recently Keirn and Brezonik (18) have applied the acetylene reduction technique to Lake Mize in Florida and they determined bacterial fixation to supply as much as 1.14 g - N/m^3/yr. According to their estimates, this amount of nitrogen represents 56% of the total nitrogen input to the lake and thus is very important. Granhall and Lundgren (19) have estimated algal nitrogen fixation by the same technique and have found it to contribute 0.5 g - N/m^2/yr in Lake Erken, Sweden. This evidently increases annual loading of combined nitrogen by 40%.

Wilkinson (20) has proposed much higher nitrogen fixation rates for a New Zealand river estuary that he studied. He calculated the nitrogen inputs and outputs of the estuary and constructed a fairly sound budget that required high rates of bacterial nitrogen fixation to make it balance. Analysis of his data for this highly polluted estuary appear to yield summer fixation rates greater than 0.1 g - $N/m^2/day$.

An extensive search of the literature has not revealed any full scale attempts at estimating total nitrogen fixation input into a lacustrine environment, using the acetylene reduction technique or any other. It is also evident that fixation input has not been measured in bodies of water where nitrogen inputs from all other sources are known with certainty. This set of circumstances would tend to indicate that the importance of nitrogen fixation as a nitrogen input has not yet to this date been determined with any degree of accuracy.

The Experimental Lakes

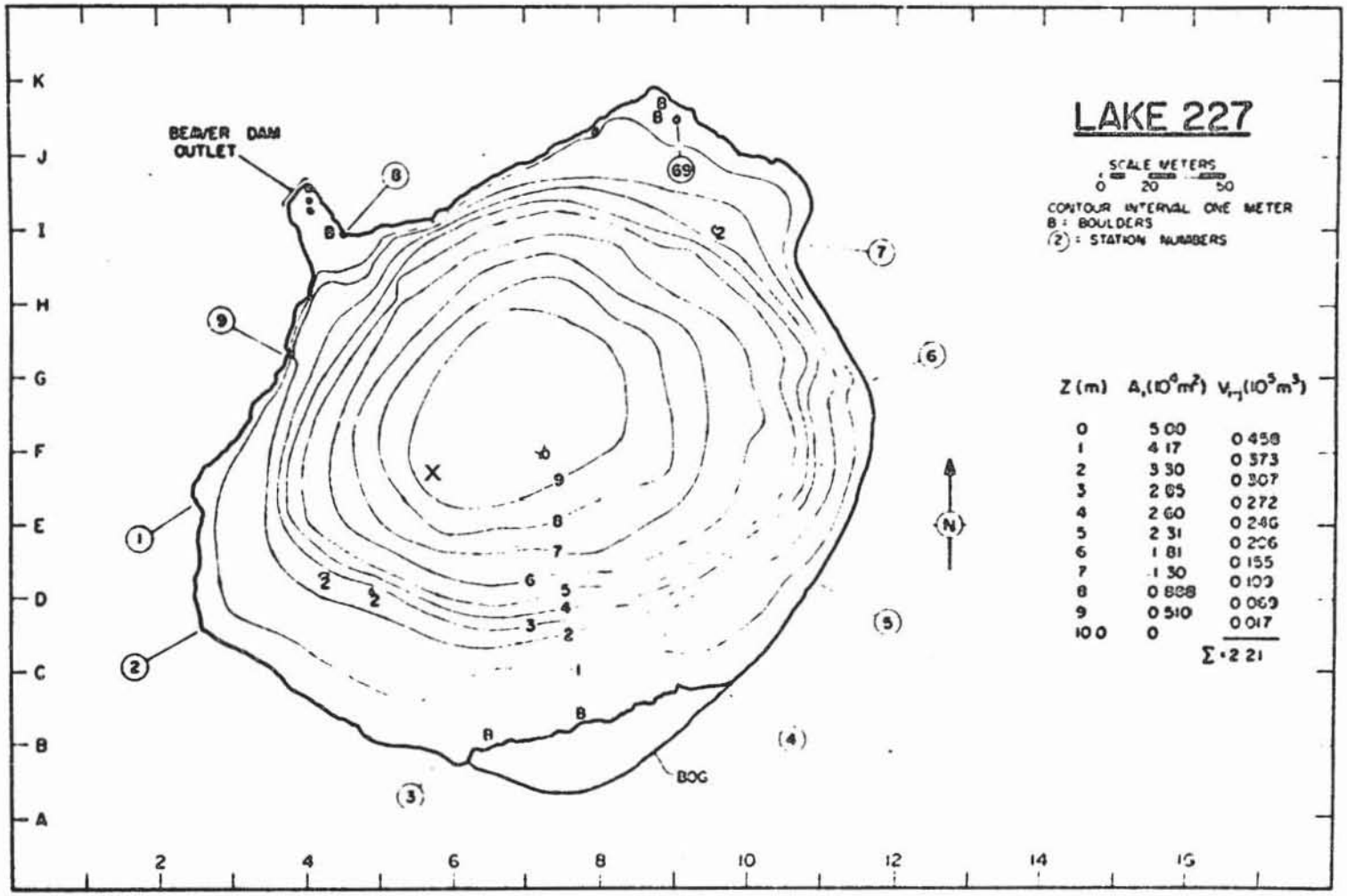
Situated in North-western Ontario in the Pre-Cambrian Shield, the lakes under study comprise a part of the Experimental Lakes Area (E.L.A.) of the Fisheries Research Board of Canada (21a). In common with most other lakes of the region, they were oligotrophic in nature (21b).

Two lakes, both of which are being artificially fertilized, were chosen for our experiments: Lake 227 has an area of 5 hectares with a maximum depth of 10 m (Fig 1) and was fertilized in 1969, 1970 and again this year; Lake 304 with an area of 3.62 hectares and a maximum depth of 6 m (Fig 2) was fertilized for the first time in 1971. Location of the lakes within the E.L.A. is given in Fig 3.

Fertilizer applications in both cases were on a weekly basis beginning in the first week in June and continuing for a 21 week period. Total loadings for Lake 227 were as follows: 0.48 g $P/m^2/yr$ as H_3PO_4 and 6.29 g $N/m^2/yr$ as $NaNO_3$. For Lake 304, total loading was 0.4 g $P/m^2/yr$ as H_3PO_4, 5.2 g $N/m^2/yr$ as NH_4Cl and 5.54 g $C/m^2/yr$ as sucrose.

A number of considerations determined the choice of these lakes for study. First, they are morphometrically alike and of approximately the same size. Second, the water chemistry of the lakes are similar and each displays a definite thermocline along with concurrent oxygen stratification (21c). Third,

Fig. 1. Bathymetric chart of Lake 227.



Z (m)	$A_z (10^6 m^2)$	$V_{z-1} (10^5 m^3)$
0	5 00	
1	4 17	0 458
2	3 30	0 373
3	2 85	0 307
4	2 60	0 272
5	2 31	0 246
6	1 81	0 206
7	1 30	0 155
8	0 888	0 109
9	0 510	0 069
10 0	0	0 017
		Σ 2 21

Fig. 2. Bathymetric chart of Lake 304.

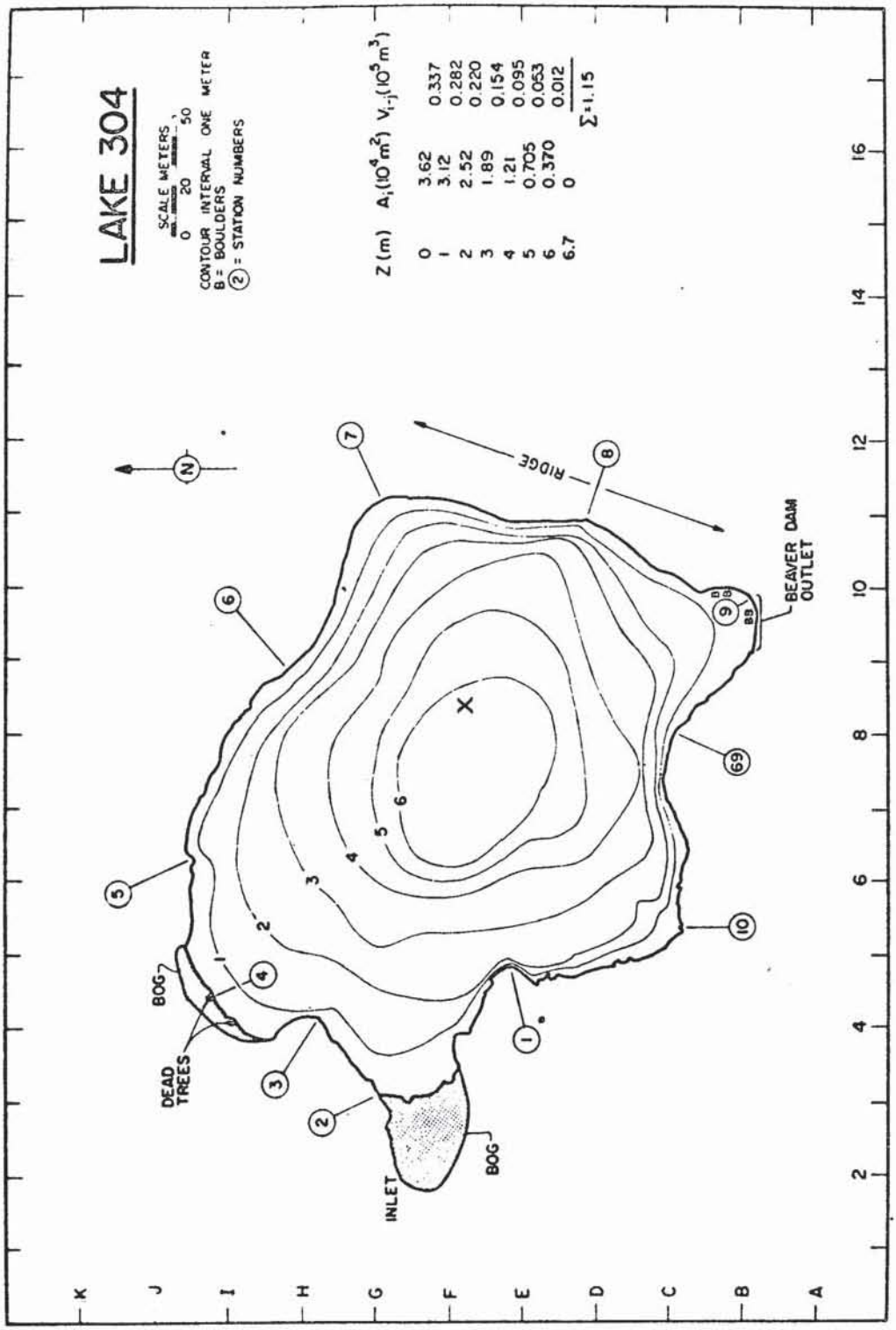
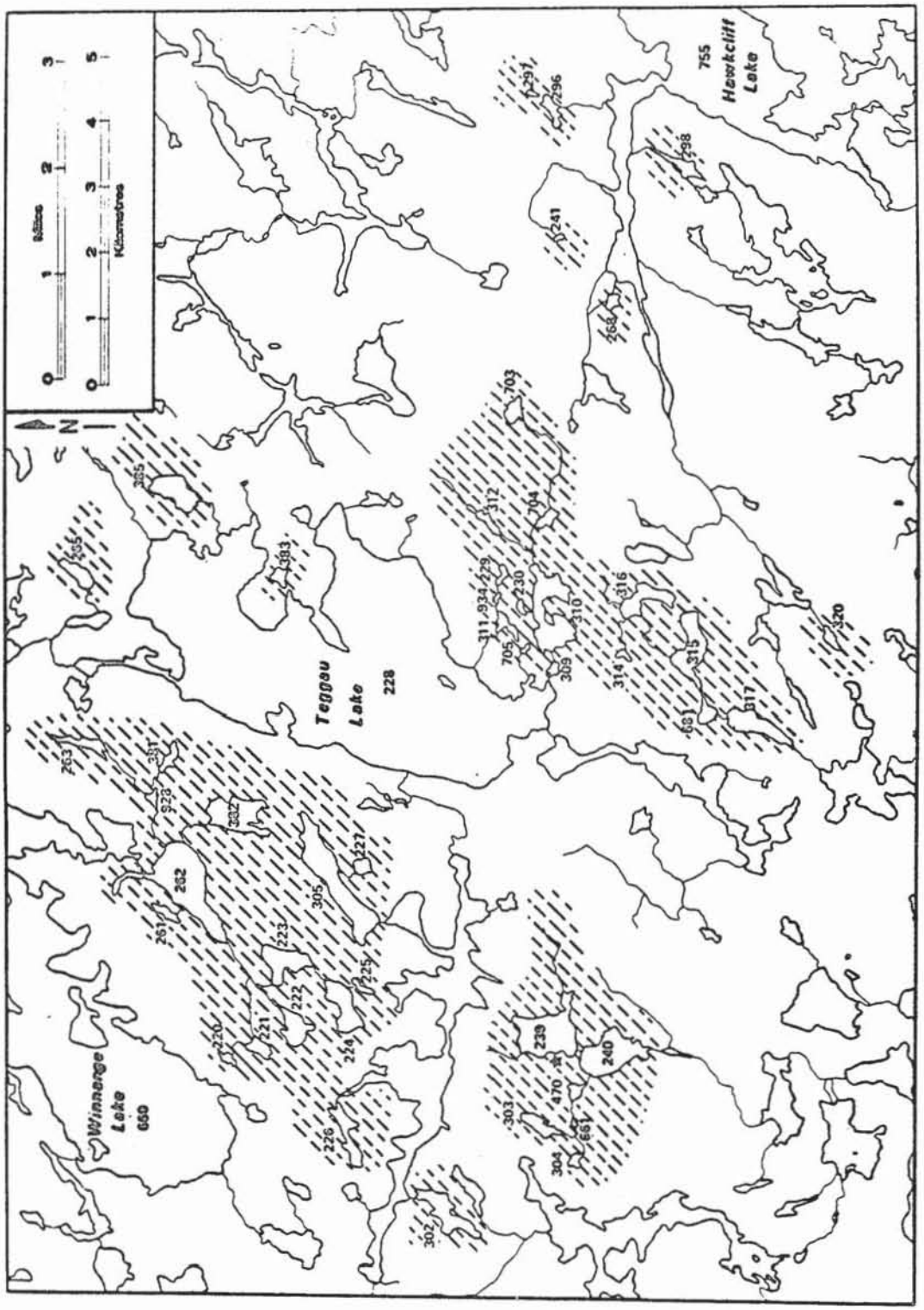

LAKE 304
SCALE METERS
0 20 50
CONTOUR INTERVAL ONE METER
B = BOULDERS
(2) = STATION NUMBERS
Z (m) $A_i(10^4 m^2)$ $V_{i-j}(10^5 m^3)$
0 3.62 0.337
1 3.12 0.282
2 2.52 0.220
3 1.89 0.154
4 1.21 0.095
5 0.705 0.053
6 0.370 0.012
6.7 0 Σ=1.15
N
RIDGE
BEAVER DAM OUTLET
BOG
DEAD TREES
INLET
BOG
A B C D E F G H I J K
2 4 6 8 10 12 14 16

Fig. 3. The central part of the E.L.A. region showing the 17 drainage basins (hatched areas) including 46 small lakes that have been set aside for research on eutrophication. A star denotes the site of the E.L.A. field station.


Miles
Kilometres
N
Winnange Lake
650
Teggau Lake
228
Hawkcliff Lake
755
263
265
261
262
331
323
332
305
227
223
225
222
221
226
224
226
302
303
239
470
304
661
240
311
934
229
230
705
309
310
316
314
315
317
320
703
312
704
1383
1241
1291
1296
1298
268

they are readily accessible with Lake 304 being about 1.25 miles by trail from the laboratory site and Lake 227 is about three miles distant. Travel time to either lake is seldom more than 40 minutes.

A substantial though indirect benefit arising from the choice of these lakes pertains in that F.R.B. personnel are carrying out intensive physical, chemical and biological studies conjointly with their fertilization program. Data derived from these investigations will be correlated with those obtained from our experiments.

Chemical tests for phosphates and nitrogen present in a lake at any given time do not necessarily give a history of its nutrient input. Rather, they reveal only the amount of nutrient available to the particular test employed (22). It is entirely possible that nitrogen fixation rates relate not to such transient nutrient conditions but to total loading conditions of the system in question. In respect to Lakes 227 and 304, which represent natural systems where loading rates and total loading are known with precision, an unparalleled opportunity to study aquatic nitrogen fixation under controlled nutrient conditions is provided.

METHODS AND MATERIALS

General Experimental Outline

The acetylene reduction technique was used to monitor the lakes for *in situ* nitrogen fixation. Unconcentrated water samples (2.0 litre) from various depths were introduced into 2.5 litre light and dark glass bottles and incubated under 1.0 atmosphere of acetylene for four hours.

After incubation at depths corresponding to those from which the various samples were taken, the bottles were raised and, from each, atmosphere samples (20.0 ml) were removed by syringe to evacuated stoppered glass vials for transport to the laboratory where assays for ethylene produced during incubation were carried out.

As well, water samples representative of each depth examined were analysed for ortho-phosphate, soluble nitrogen, particulate organic nitrogen, nitrate and nitrite. Primary productivity was measured occasionally under the acetylene gas phase to determine if photosynthesis was inhibited by the experimental conditions pertaining.

Acetylene Reduction Technique

It has been well established that the nitrogenase complex of nitrogen fixing organisms will reduce nitrogen to ammonia (2,3,4,5,6,7). It has also been shown that several other triply bonded nitrogen and carbon compounds are reduced by the same complex (23). Acetylene is one such compound, yielding ethylene upon reduction (10,24,25). Each NH_3 molecule

produced requires a 3 $\bar{e}$ reduction while each C_2H_4 molecule produced requires a 2 $\bar{e}$ reduction. Theoretically, a yield of 1.5 moles of ethylene should represent a yield of 1.0 moles of NH_3, provided each substrate is applied mutually exclusive of the other. In practice, the NH_3 to C_2H_4 ratio has been noted to be between 1:1 and 4:2 (12). Such ratios were obtained by direct comparison of $^{15}N_2$and acetylene reduction techniques.

Determination of nitrogen fixation rates on the scale used for this investigation would not have been practical by any method other than acetylene reduction. The very great costs involved when using $^{15}N_2$techniques in terms of the number and size of samples examined made it absolutely prohibitive. Direct measurements of fixation rates by Kjeldahl methods were also deemed unfeasible due to the short incubation times employed and the relative insensitivity of this assay.

On the other hand, the acetylene reduction technique possessed many advantages that favoured its use, especially in studies of aquatic fixation. Acetylene is 65 times more soluble in water than nitrogen (at 1 atm. and 25°C, acetylene in solution is 41.9 mM and nitrogen in solution is 0.64 mM) (12). The high solubility of acetylene permits rapid diffusion of the gas throughout the sample. This is most important when large volumes of sample are being incubated for short periods of time. Acetylene is also preferentially reduced as compared with nitrogen, and at a partial pressure of 10% has been noted to completely saturate the nitrogenase of Anabaena (9).

Applying a high p C_2H_2 removes the need to strip samples of dissolved nitrogen that competes for the nitrogenase complex. Stripping would remove the CO_2 and O_2 from the water, a situation that is best avoided. It was thought more practical to apply a pure C_2H_2 gas phase and to monitor its effect upon the phytoplankton population by C^{14} uptake.

The ethylene produced in the reduction process is insoluble in water, thus the compound concentrates in the gas phase of the sample bottle.

Incubation Vessels

On the assumption that concentrated water samples (volume reduced by filtration) may introduce anomolies in terms of interpretation of results of in situ incubation studies, it was decided to use as large a vessel as was practical to handle and fill under field conditions. Since our incubation periods were to be as short as four hours duration, the larger the sample taken the more likely that low levels of nitrogen fixation could be detected.

Glass bottles (2.5 litre) of the kind in which acid is sold commercially were selected. The bottle opening was stoppered either with a #49 or a #57 'Suba Seal'.* The glass handles proved most convenient for securing the harness assembly which positioned the vessels at appropriate depths along the anchoring cable. When filled with 2.0 litre samples

* Griffin and George Limited, London, England.

the bottles possessed a slight negative buoyancy which kept them hanging directly down from the surface marker float when secured by clips to the harness and anchor cable.

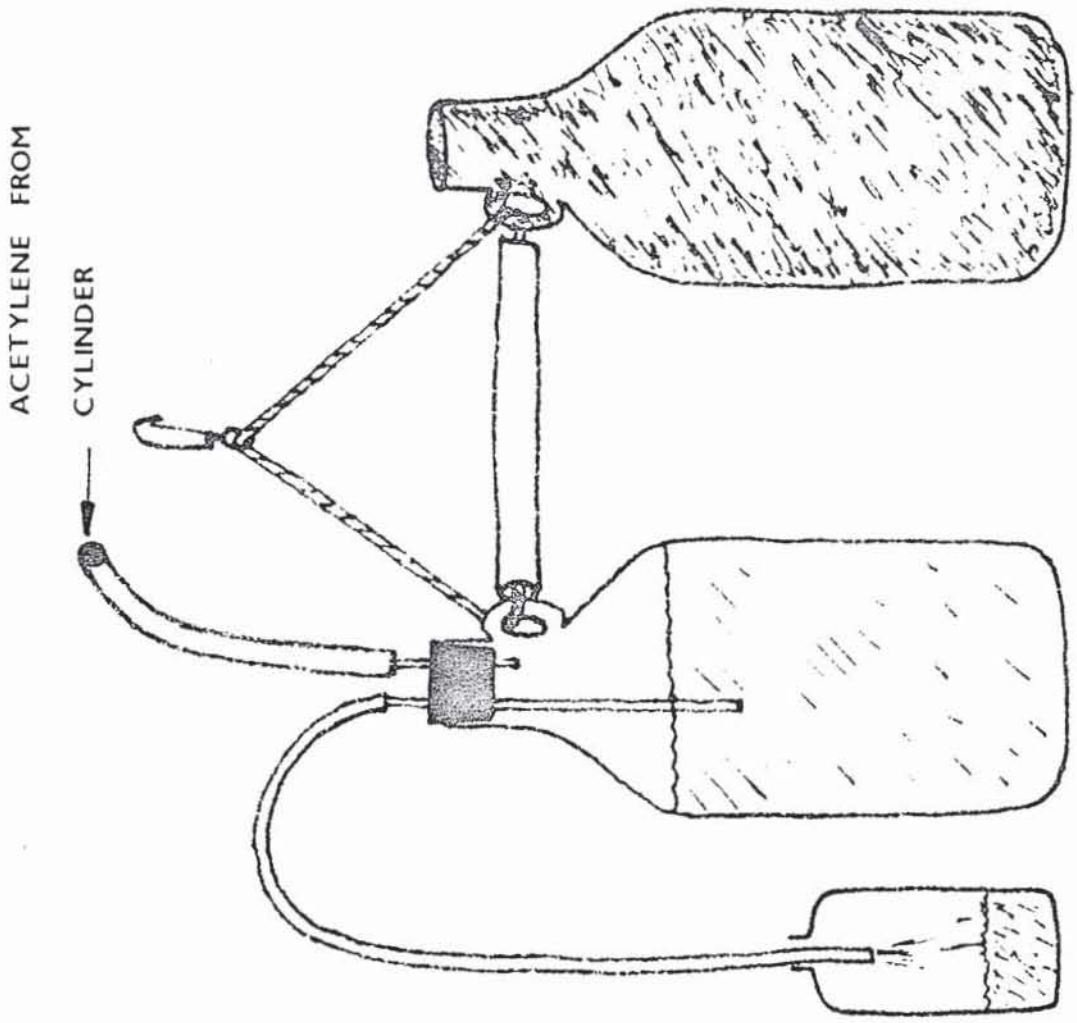
To provide a measurement of heterotrophic N-fixation as well as that proceeding under light conditions, several of the vessels in each experimental series were darkened by covering with aluminium foil over which black P.V.C. adhesive tape was tightly wrapped to completely cover the vessel. With the 'Suba Seal' in place with its apron folded down over the bottle neck an effective light tight condition resulted.

One and Two Bottle Harness

If only one bottle was to be incubated at a given depth, it was secured by a nine inch length of 1/4" diameter polypropylene rope tied to the bottle handle and attached at the other end by a spring clip to a ring on the anchor line.

Where both light and dark vessels were required at the same depth, a special harness was devised. This consisted of a spacer assembly to separate the bottles at one foot centres on either side of the anchor line and a bridle to keep the bottles level with one another. This spacing was necessary to ensure that no shading of the light vessel occurred. The spacer consisted of an 11 inch length of one inch rigid P.V.C. pipe. A clip was installed on the upper end of the bridle to facilitate easy attachment of the harness assembly to the anchor line. A schematic drawing of single and double harness assemblies is given in Fig 4.

Fig. 4. Diagram of the two bottle harness and the acetylene filling apparatus. Single bottles are supported by a short length of rope secured to the handle with a clip on the other end.


ACETYLENE FROM
CYLINDER

Floats, Anchors and Anchor Lines

The sampling position in each lake was marked by anchoring one gallon plastic bottle floats, Figs 1,2; these remained in position throughout the experimental season. From the floats, supporting lines were secured to heavy stone anchors. Brass rings tied at the surface, at 0.5 m, at 1.0 m and thereafter at metre intervals to the bottom provided the means for easily attaching the vessel harness clips as required.

Sampling

A plexiglas three litre Van Dorn sampler* was used to secure the water samples. Samples taken by this device represent a vertical cylinder of water about 0.5 m in length with the nominal sample depth being at about the middle of the column. The sampler, when full, contained enough water to completely fill one incubation vessel. Sampling always proceeded from surface to bottom to ensure that the thermocline was disturbed as little as possible.

The filling procedure was as follows: either one or two incubation vessels, depending upon the number to be incubated at the same depth were completely filled with water taken from that depth by the Van Dorn sampler. The vessels were covered during filling with a heavy light-proof cloth so that inhibition or enhancement of photosynthesis would not occur. The vessels were gassed, stoppered and immediately lowered to the prescribed incubation depth. Time of submergence was recorded. The next depth was then sampled and the filling procedure was repeated.

* Kahlsico Co., San Diego, California, U.S.A.

It was found that the whole process for either lake required about an hour. Since the sequential retrieval of the vessels after incubation and the gas sampling procedure that followed also took one hour to complete, the incubation period for surface vessels was the same as that for bottom vessels although later by one hour. The incubation period was four hours, usually from 10:00 A.M. to 2:00 P.M.

Gassing Samples with Acetylene

It had been decided that a 0.5 litre gas phase of pure acetylene would be used in the incubation vessels. Pressurized acetylene* was used to displace 0.5 litre of water sample from a full incubation vessel, see Fig 4. Immediately after displacement the sample vessel was stoppered with the appropriate sized 'Suba Seal'. The displaced water was collected by rubber tube to a Nalgene sample bottle graduated at the 500 ml level. The water so collected was transferred to two acid washed 250 ml Nalgene sample bottles and capped. These sub-samples were transported back to the field laboratory for processing.

Gas Sampling of the Incubation Vessels

Samples of the gas phase from vessels, after incubation, were removed using a 50 ml glass syringe fitted with a 26 gauge 1/2" needle. Since a partial vacuum developed in the incubation vessels due to the solubility of the acetylene added, the following procedure was adopted.

* A 2.0 litre steel cylinder, 300 psi controlled by a single stage regulator provided by Welders Supplies, Wpg., Manitoba. In the commercial gas, a trace amount of ethylene was always present but was accounted for by gas sample analysis for each experiment in the series.

The glass plunger was removed, moistened thoroughly with lake water, replaced in the barrel of the syringe and then pumped several times. This assured a gas tight seal as well as removing any traces of previous gas samples. A needle was then fitted to the syringe and forced through the 'Suba - Seal' of the incubation bottle. The plunger was pulled out to the 35 ml position and retained there for approximately 20 seconds. On release, the plunger would spring into the syringe to approximately the 25 ml mark. The syringe was removed and in this way a 25 ml sample, nominally at atmospheric pressure, was obtained.

Serum bottles of 7 ml volume were used to store gas samples until analysis. The preparation and use of these bottles was as follows.

Each bottle was completely filled with distilled water and then sealed with a serum stopper that had a 1/2" - 26 gauge hypodermic needle just passing through its septum from top to bottom. This needle acted as a vent to release any air that was entrapped under the serum stopper and to release any water that was displaced by the stopper during its insertion. The needle was then removed.

The transfer of gas from syringe to serum bottle was accomplished by displacing the water from the bottle with gas sample. A 1/2" - 26 gauge needle was passed just through the septum and the serum bottle was inverted. The needle on the syringe containing the sample was pushed all the

way through the septum into the bottle and gas injected until all the water was displaced through the other needle. Both needles were then removed. None of the serum stoppers was used more than twice and no gas leakage was evident even after storage periods of up to seven days.

Gas Analysis

Ethylene production was measured with a model 104 Pye vapour phase F.I.D. chromatograph and recorded on a Coleman model 165 millivolt recorder. The column[1] was 6' x 1/8" stainless steel packed with Poracil C/Phenylisocyanate.

Using ethylene and acetylene gas mixtures of known concentrations, standard curves were constructed to provide for the conversion of recorded peak heights to concentrations of ethylene (in n moles) and acetylene (in m moles) (Figs 5,6).

Determination of Partial Vacuum in Incubation Bottles

Acetylene is quite soluble in water while ethylene is relatively insoluble in water. A reduction in gas phase pressure in the incubation bottle occurs due to the solution of acetylene in the aqueous phase. Because gas samples were taken at atmospheric pressure (see Taking of Gas Samples p.22), both the concentration of acetylene

1. Column temperature was 23°C (ambient); carrier gas as N_2 at a flow rate of 20 ml/minute.

Fig. 5. Ethylene standard curve for chromatograph.

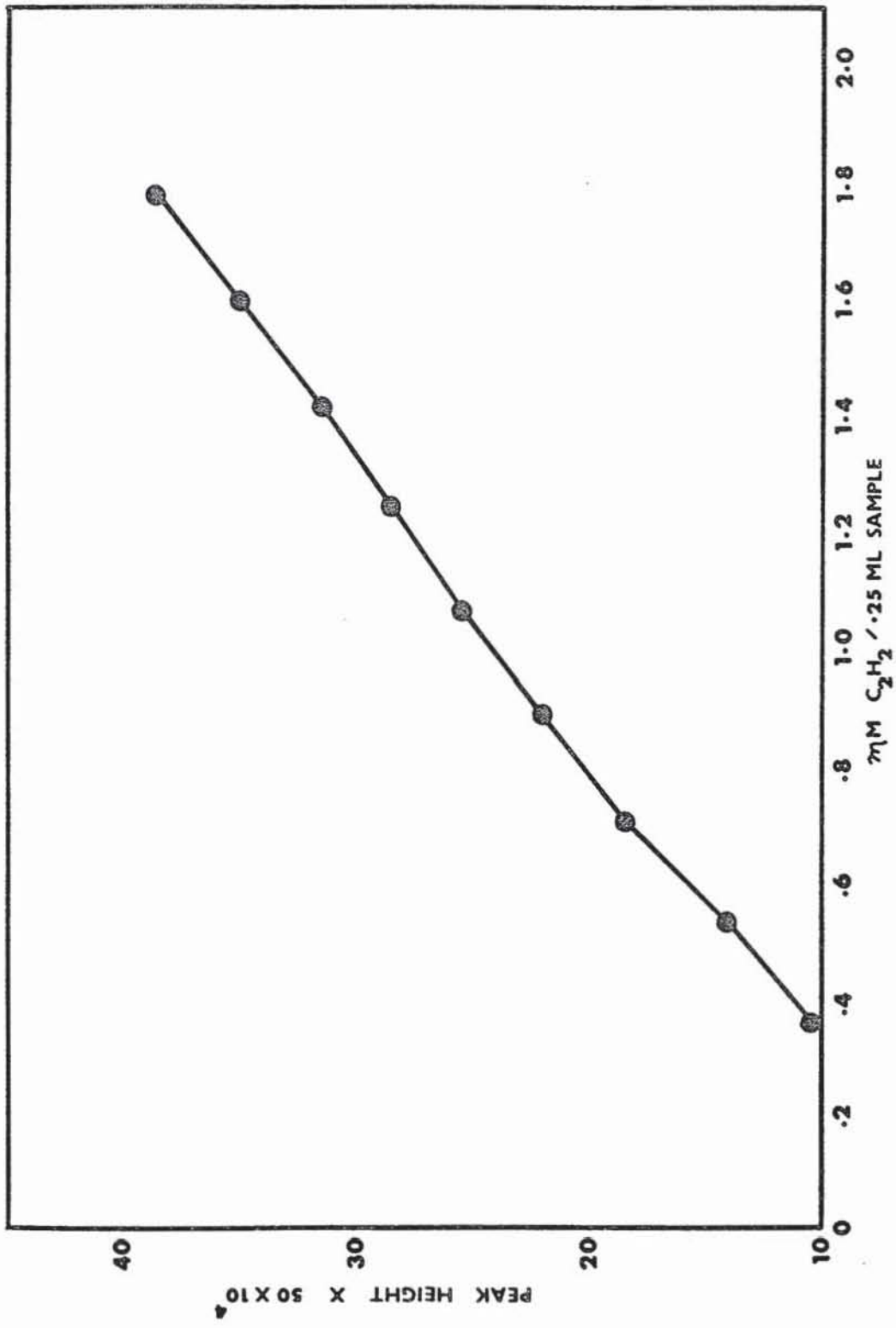
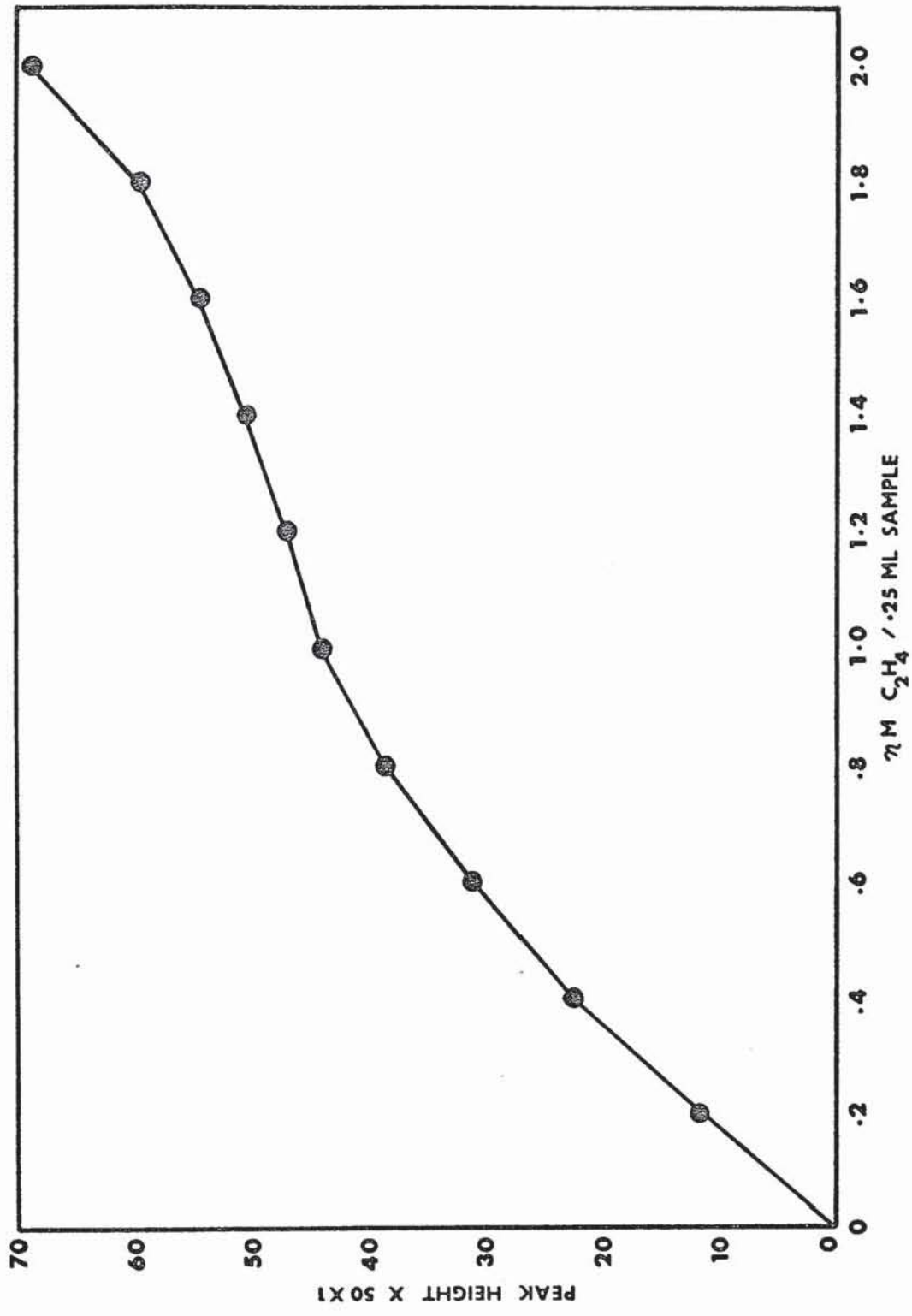

PEAK HEIGHT X 50 X 10
40
30
20
10
0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
mM C_2H_2 / .25 ML SAMPLE

Fig. 6. Acetylene standard curve for chromatograph.


PEAK HEIGHT X 50 X1
0
10
20
30
40
50
60
70
0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
nM C_2H_4 / .25 ML SAMPLE

and ethylene was increased by the sampling technique. The acetylene concentration was effectively increased to its original value that it had before any of the gas dissolved in the aqueous phase. The ethylene concentration, however, was increased above its original value. This was because none of it had gone into solution, and thus, when the volume was reduced by the sampling technique, the effective concentration was increased in the sample. In order to estimate the factor by which the ethylene was increased in the syringe, the partial vacuum of the incubation bottles was measured in the following manner.

The incubation bottles were filled, gassed and incubated in the usual way. Just prior to taking the gas samples, a diaphram vacuum gauge was connected to the incubation bottle by means of a rubber tube with a hypodermic needle sealed in one end. The needle was pushed through the 'Suba - Seal' into the bottle and the partial vacuum read directly off the gauge in mm of mercury. Dead volume of the vacuum apparatus was 5 ml. Replicate measurements provided the basis for statistical treatment and the calculation of a standard deviation value.

Dissolved Oxygen in Samples

It was assumed that the dissolved oxygen in samples might decrease in concentration during incubation under the acetylene gas phase. Accordingly, dissolved oxygen was measured before and after incubation in a number of acetylene reduction experiments using the Winkler procedure* as modified by Alsterberg (26).

Concurrently with oxygen measurements, $^{14}CO_2$ uptake measurements were made on the same samples. For details, see $^{14}CO_2$ uptake procedure, p. 31. Changes in rate of $^{14}CO_2$ uptake might be attributed to oxygen depletion. To determine if this condition produced any effect on primary production by the sample biomass, a control experiment was included which differed only from the routine acetylene reduction experiments in that helium gas was used in place of acetylene. Otherwise, all experimental treatments and conditions were the same as described in respect to $^{14}CO_2$ additions and dissolved oxygen measurements.

In this experiment, four sample bottles were incubated at each depth. Bottle #1 was a dark bottle with an acetylene gas phase; bottle #2, a light bottle, otherwise identical to #1. Sample bottle #3 contained a helium gas phase while #4

* The reagents, manganous sulphate, alkaline iodide - azide, sulfamic acid and P.A.O. were supplied by Hach Chemical Co., Ames, Iowa, U.S.A.

contained air or Argon 80%/oxygen 20% as the gas phase. Bicarbonate, ^{14}C labelled, was uniformly provided in all sample bottles. Before incubation, dissolved oxygen was measured in subsamples from each depth. After incubation, primary production was measured on all samples and dissolved oxygen was determined on the three light bottles only.

Estimation of Acetylene Toxicity by ^{14}C Uptake

The use of a pure acetylene gas phase made it necessary to determine whether or not the gas was adversely affecting the phytoplankton and bacteria. It was hypothesized that if $^{14}CO_2$ uptake by phytoplankton decreased in the presence of acetylene, the gas might be toxic to them and to other aquatic microorganisms. If the gas was found to be toxic, it might further be assumed that nitrogen fixation rates obtained under such a gas phase were not representative of actual fixation rates *in situ*. On the other hand, if CO_2 uptake was the same under an acetylene gas phase as under an air gas phase, it might be presumed that the acetylene assay system was not adversely affecting the plankton and that fixation results were reliable.

Several primary production ($^{14}CO_2$ uptake) measurements were made during the course of acetylene reduction experiments. The $^{14}CO_2$ uptake method of Schindler and Holmgren (22), modified by acidifying, bubbling and counting by liquid scintillation, was used (30). This method has been

used routinely to measure phytoplankton production in the lakes at the E.L.A.

Primary production in the presence and absence of acetylene was measured in the following manner. A dark and light bottle (one for each depth) was filled with water and gassed as described in previous sections. Another light bottle (one for each depth) was completely filled with water, and from this full bottle, 0.5 litre of water was poured out. This left an air gas phase of 0.5 litre over the water. The bottle was then sealed with a 'Suba - Seal'. To each of the bottles (3 at each depth) 1.6 ml of $NaH^{14}CO_3$ (10 uc/ml) was added by Hamilton* syringe. Incubation was as in routine acetylene reduction experiments. At the end of incubation, 250 ml of water from each bottle was poured into separate Nalgene bottles of 250 ml volume and immediately put into a light tight container. These subsamples were then transported back to the E.L.A. laboratory and processed for counting within several hours.

* The Hamilton Company Inc., Whittier, California.

Water Chemistry

It was thought that, in addition to the nitrogen fixation assay, certain chemical analyses should be carried out concurrently on the lake water. A brief outline of the analyses is given here. They are all based upon procedures found in "A Practical Handbook of Seawater Analysis" (31). Methods which were modified are included in the appendices of this work.

Preparation of Samples for Analysis

Five hundred ml samples from each depth, in two 250 ml Nalgene bottles, were brought back to the E.L.A. laboratory within 2 hr of sampling. One bottle was filtered through acid washed (50% HCl) 5.5 cm Whatman GF/C* filters under 250 - 275 mm Hg vacuum. The filtrate was returned to the original bottle from which it was poured. Both the filtered and unfiltered samples were frozen in the bottles at -20°C for storage until they were transported back to the Winnipeg laboratory for analysis.

Reactive Phosphorus

The method of Murphy and Riley (32) was used for this analysis. It is free from interference by arsenic and silicates at concentrations present in the samples.

* W & R Balston Ltd., England

The range of the test is .03 - 5.0 ug - at/l. Precision at the 3.0 ug - at/l level lies in the range $\pm$ .03/$n^{1/2}$ ug - at/l where n = number of determinations. Precision at the 0.3 ug - at/l level lies in the range $\pm$ 0.02/$n^{1/2}$ ug - at/l. Duplicate determinations were carried out. Although more sensitive adaptations of this analysis are available, it was thought to be sufficiently accurate for these purposes (31).

Chemical measurements of orthophosphate have been much criticized of late for their lack of specificity (33). It is not known what percentage of labile phosphate compounds are hydrolyzed during the test and therefore it is impossible to meaningfully estimate the amount of orthophosphate present in the samples. It is sufficient to say that the analysis used was sensitive enough to give rough estimates of biologically available phosphorus in the lakes.

Nitrates

The cadmium reduction method (34) as outlined in "Waste and Wastewater Analysis Procedures", Cat. No. 10 (Hach Chemical Co.) was used to determine nitrate concentration. The effective range of the analysis was 10 - 2000 ug/l NO_3^- - N when using 19 mm path length rounded cuvettes. Precision of the test was within $\pm$ 5% at the 50 ug/l NO_3^- - N level when the mean of duplicate determinations was used. It was possible to increase the sensitivity of the measurement if 10 cm cuvettes were used instead of the rounded cuvettes.

Nitrites

The measurement of nitrite concentration was the only procedure not also carried out by the Fisheries Board. It was omitted by them because their preliminary surveys indicated concentrations to be so low as to be inconsequential (23). For this study, however, due to its intimate relation with nitrogen in all forms, it was felt wiser to carry out the determination. In this way, the quantitative importance of nitrite upon fixation could be definitely discounted, if, in fact, the concentration proved to be negligible.

The Hach method (34) for nitrite determination is basically the same as the nitrate determination except that the cadmium reductor is omitted. The precision was within $\pm$ 5% at the 10 ug/l NO_2^- - N level when the mean of duplicate determinations was used. The range of the analysis was 1.0 - 200 ug/l NO_2^- - N using 19 mm rounded cuvettes.

Particulate and Soluble Organic Nitrogen

Determination of particulate nitrogen was done using a substantially modified form of the method of Strickland and Parsons (31) designed to measure 'soluble' organic nitrogen. Kjeldahl digestion of 25 ml of unfiltered sample is followed by a colorimetric ammonia determination with ninhydrin - hydrindantin solution (35). This is a measure of total organic nitrogen, both 'soluble' and 'particulate'.

An identical analysis is then done on a filtered portion of the same sample. This gives an estimate of 'soluble' organic nitrogen. The concentration of soluble organic nitrogen is then subtracted from the total organic nitrogen concentration to yield particulate organic nitrogen concentration. All analyses were done in duplicate and the mean of the two was used to determine concentrations of NH_3 - N. The range of the analysis was from 0.25 - 2000 ug/l NH_3 - N. Precision at the 6.0 ug/l level was $\pm$ $.6/n^{1/2}$ ug/l N where n = number of determinations [$(NH_4)_3 SO_4$ standard].

RESULTS AND DISCUSSION

Results of Water Chemistry

Chemical analyses of the water samples were carried out at biweekly intervals on Lakes 304 and 227. Results of our analyses and those of the Fisheries Research Board were compared and, in general, both sets of data were in agreement. Specific values for the chemical concentrations will not be given in this work. No apparent correlation existed between nitrogen fixation rates and the concentrations of dissolved or suspended chemicals that were measured.

Reactive Phosphorus

Although dissolved concentrations as high as 10 ug $PO_4^{=}$ - P / litre were encountered in isolated cases, most samples yielded concentrations below 5 ug and the majority were close to the 0 ug level in both lakes. The dissolved phosphate appeared to be quite evenly distributed throughout the water column. Concentrations did not appear to change significantly with time over the 4 month sampling period.

Nitrate

In Lake 304, no definite pattern of nitrate distribution could be noted with certainty. The only trend that may be occurring is a slight lowering of nitrate levels with depth. The concentration of nitrate was usually less than 100 ug NO_3^- - N/l, the mean being fairly close

to 50 ug and the range being from 5 to 300 ug NO_3^- - N/l.

In Lake 227, an uneven distribution of nitrate was more evident. Highest concentrations were most often noted to be just above the thermocline. There was often a rapid drop in concentration as sampling continued through and below the thermocline. Typical results show 10 - 50 ug NO_3^- - N/l in the surface few meters, then a rapid rise to the 100 - 150 ug level at the upper edge of the thermocline, and finally, a rapid drop to the 10 - 20 ug level in the hypolimnion.

In neither lake could correlations be made between nitrate concentrations and fixation rates. It is generally believed that high levels of nitrate or ammonia shut down the fixation process. It is a possibility that the levels of nitrate encountered here are too low to inhibit fixation, and thus no correlation would exist.

Nitrite

Concentration of nitrite was measured several times in both Lake 227 and Lake 304. The nitrite concentration was barely detectable (below 1.0 ug NO_2^- - N/l). The analyses were discontinued at the end of July and no correlation between nitrite concentration and fixation was attempted.

Soluble Organic Nitrogen and Ammonia

The nitrogen detected in this analysis was probably present mainly as ammonia. Comparison of our results with

those of the Fisheries Research Board revealed fairly substantial discrepancies. Ammonia concentrations detected by our method gave consistently lower results, an occurrence that was thought to be due to the freezing and storage of our samples. The analytical procedure outlined in the appendix of this work is therefore not recommended for any but unfrozen samples not more than several hours old.

Data obtained from the Fisheries Research Board from Lakes 304 and 227 showed ammonia concentrations to be in the 20 - 100 ug NH_3 - N/l level in the epilimnions. From the top of the thermocline downward, ammonia concentration increased almost exponentially to the lake bottoms. In Lake 304, values as high as 2000 ug NH_3 - N/l were observed at the bottom, while in Lake 227, a value of 4000 ug NH_3 - N/l was obtained at the bottom. Such figures represent maximum ammonia accumulation observed in late summer after five months of stratification. In Spring, concentrations of ammonia near the bottom were similar to those in the epilimnion.

There did not appear to be any consistant correlation between ammonia concentration and nitrogen fixation rates. While ammonia was in low concentrations in the epilimnion and fixation (in Lake 304) was highest there, secondary peaks in fixation were noted at the 4 or 5 meter depths

where ammonia concentration were quite high (above 500 ug NH_3 - N/l) e.g. Aug. 3,10,18 and Sept. 15. Similarly, Lake 227 showed fixation peaks in the 3 to 7 meter depths, the fixation often being greater at these depths than at the surface e.g. July 13, Aug. 10, 20. It would seem more logical for fixation to be active in depths where dissolved nitrogen as NH_3 was low in concentration i.e. high fixation rates at the surface of Lake 227. This, however, was not usually the case. This might be explained if it is assumed, as with nitrate, that the dissolved ammonia was below that amount required to shut down the fixation process.

Particulate Organic Nitrogen

The results of these analyses are open to question because the samples had been frozen and stored, a procedure that had been found to be unsatisfactory for the soluble organic nitrogen measurements. Results from similar analyses by the Fisheries Research Board were not yet compiled up to this date and so no comparison is possible between the two methods.

Our data for Lake 304 indicates a gradual build-up of particulate organic nitrogen in the hypolimnion as the summer progressed, with levels rising from 200 to 1400 ug N/l. The distribution pattern throughout the water column appeared to be similar to that for ammonia. There was no apparent correlation, negative or positive, between nitrogen fixation rates and particulate nitrogen

accumulations.

Data from Lake 227 was for only the upper 6 meters and it was deemed too incomplete to be useful.

Incubation Bottle Partial Vacuum

Internal pressure was measured on one set of 14 incubation bottles from Lake 304 and one set of 13 bottles from Lake 227. In this way, measurements were made at all depths on both lakes. The mean pressure of the bottles was calculated to be .73 atmospheres after 4 hours of incubation. The standard deviation was $\mp$ 11%.

Changes in Dissolved Oxygen

Results obtained from measurements of dissolved oxygen in light samples before and after incubation are given in Figs 7 and 8. On September 21 the average changes, after incubation, of oxygen concentrations in the upper 4 meters of L. 304 were: -17% under helium, -10% under acetylene, +4% under argon 80%/oxygen 20%. Similarly, on September 22, the average changes after incubation were: -14% under helium, -10% under acetylene, +4% under air. Since the results were comparable in both experiments, they will not be discussed separately.

An increase in oxygen concentration under the argon/oxygen and air gas phases might well be expected. Sept. 21 and 22 were bright sunny days and thus net evolution and accumulation of oxygen would be expected in the samples during algal photosynthesis. The saturation level from 0 to 4 meters was approximately 10.5 mg oxygen/l of water on these dates (36). Because oxygen concentrations in the samples were well below this saturation level (Figs 7,8),

Fig. 7. Profile of dissolved oxygen in samples from Lake 304 under various gas phases on Sept. 21/1971. Solid circles joined by evenly dashed lines are preincubation dissolved oxygen concentrations; solid circles joined by solid lines are dissolved oxygen concentrations under oxygen 20%/argon 80% after 4 hours; solid circles joined by unevenly dashed lines are dissolved oxygen concentrations under helium after 4 hours; open circles joined by evenly dashed lines are dissolved oxygen concentrations under acetylene after 4 hours.

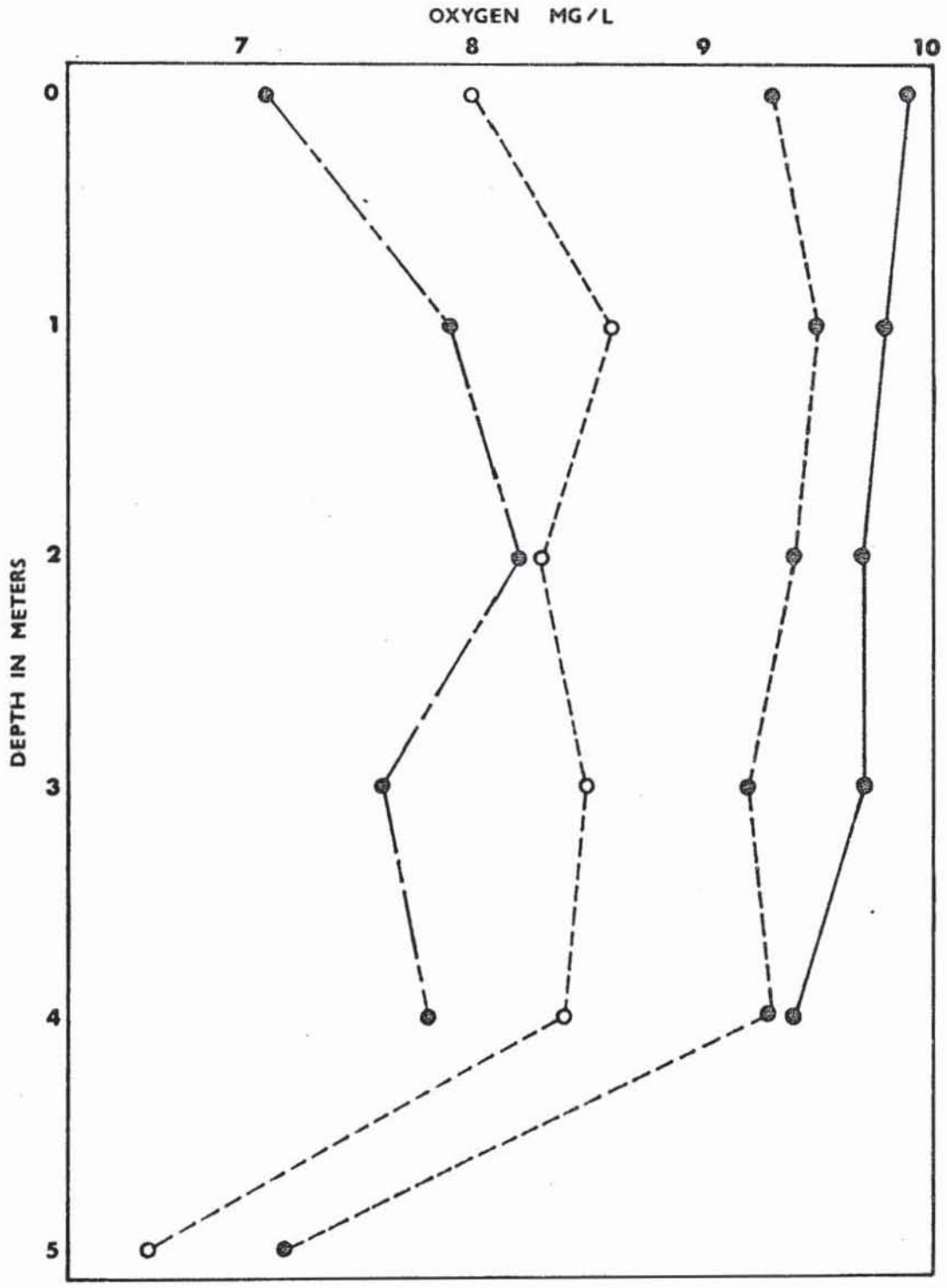
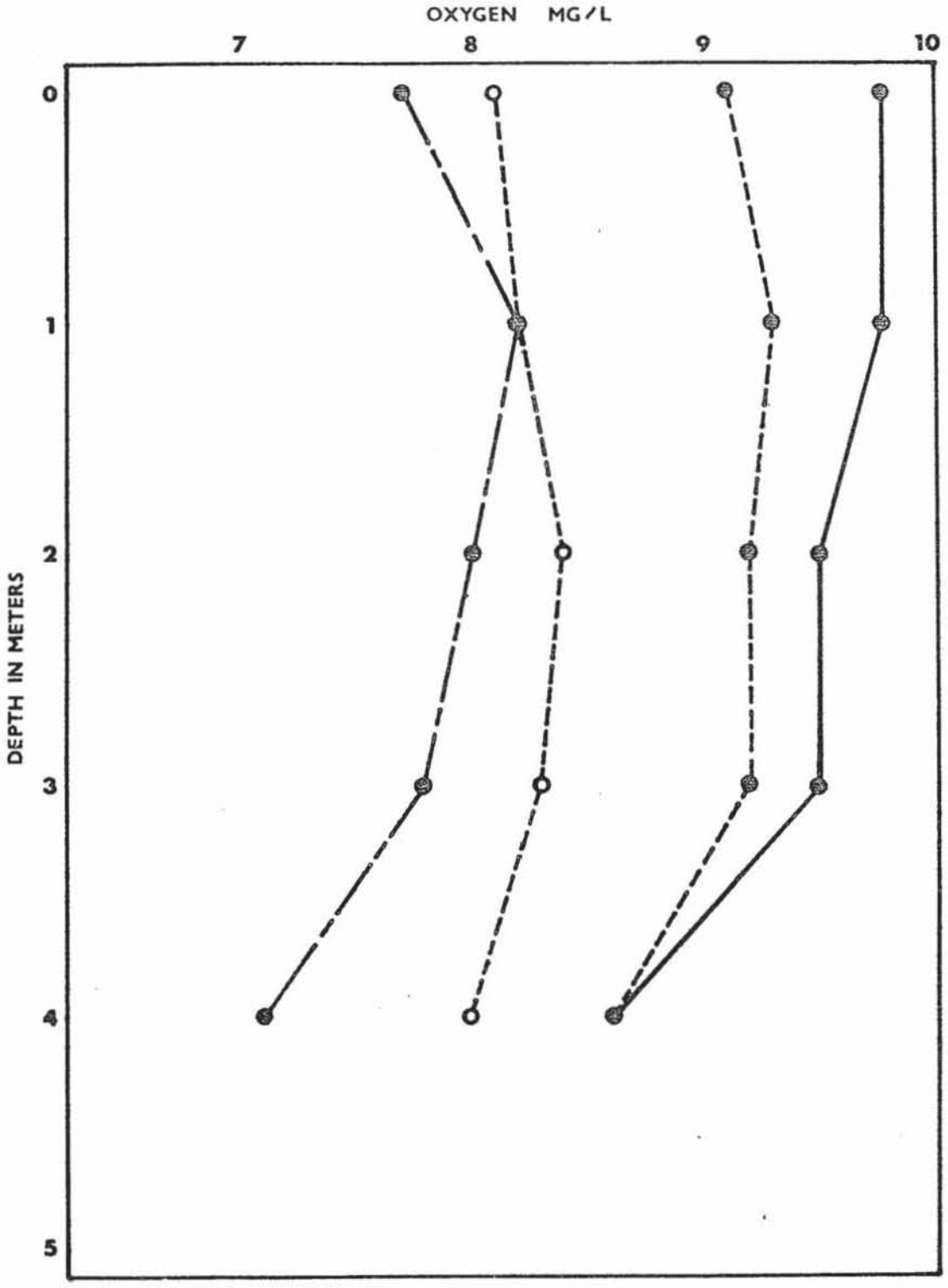

OXYGEN MG/L
7
8
9
10
0
1
2
3
4
5
DEPTH IN METERS

Fig. 8. Profile of dissolved oxygen in samples from Lake 304 under various gas phases on Sept. 22/1971. Solid circles joined by evenly dashed lines are preincubation dissolved oxygen concentrations; solid circles joined by solid lines are dissolved oxygen concentrations under air after 4 hours; solid circles joined by unevenly dashed lines are dissolved oxygen concentrations under helium after 4 hours; open circles joined by evenly dashed lines are dissolved oxygen concentrations under acetylene after 4 hours.


OXYGEN MG/L
7
8
9
10
0
1
2
3
4
5
DEPTH IN METERS

oxygen from the gas phases of the bottles may have also dissolved in the water and account for part of the increases.

Decreases in dissolved oxygen observed in samples incubated under acetylene may be due to loss of oxygen from the water to the gas phase. The same may be true with respect to decreases in oxygen observed under the helium gas phases.

The degree of oxygen depletion was greater under helium than under acetylene. It is postulated that passage of oxygen from aqueous to gas phase occurs at a higher rate under helium because of the low mass of the helium atom compared to that of the acetylene molecule. The oxygen molecule would tend to pass well out of the liquid-gas interface before its direction of travel was greatly influenced by collisions with the much lighter helium atoms. This would tend to keep the oxygen concentration relatively low at the boundary. When acetylene is the gas phase, oxygen colliding with this heavier particle is often reflected back to the interface. A concentration of oxygen builds up at the interface and causes the rate of diffusion to slow because concentrations of oxygen in the water and gas at the boundary are almost at equilibrium.

Reasons for the differences in dissolved oxygen observed under the various gas phases have been postulated. Primary production remained relatively constant at the

oxygen concentrations encountered. From this, it is concluded that dissolved oxygen changes of +4% to -17% had no noticeable effect upon the sample biomass, and specifically, that the use of a pure acetylene gas phase containing no oxygen was not adversely affecting the fixation assay. This appears to be supported to an extent by the work of Drozd and Postgate (37,38). Their studies indicate that changes of $\bar{+}$ 10% from original *in situ* oxygen concentrations have little effect upon acetylene reduction activity noted in cultures of nitrogen fixing bacteria.

Acetylene Toxicity

The uptake of ^{14}C by the phytoplankton did not appear to be adversely affected by the dissolved acetylene, in the series of samples tested (Figs 9,10). Results of Sept. 21, L.304, show uptake rates to be very similar under acetylene and under 80% argon/ 20% oxygen. Results of Sept. 22, L.304, also show uptake rates to be fairly similar under the two gas phases. Differences that do exist may well be due to experimental error.

It is well known that zooplankters are important grazers of phytoplankton and bacteria. If acetylene was toxic to these animals and decreased their grazing rates, a higher standing crop of nitrogen fixing organisms, among others, could be expected. Such circumstances might well lead to erroneous fixation results.

Fig. 9. Profile of primary production in samples from Lake 304 under various gas phases on Sept. 21/1971. The incubation period was 4 hours. Solid circles joined by evenly dashed lines are dark bottles under acetylene; solid circles joined by unevenly dashed lines are light bottles under helium; solid circles joined by solid lines are light bottles under oxygen 20%/argon 80%; open circles joined by evenly dashed lines are light bottles under acetylene.

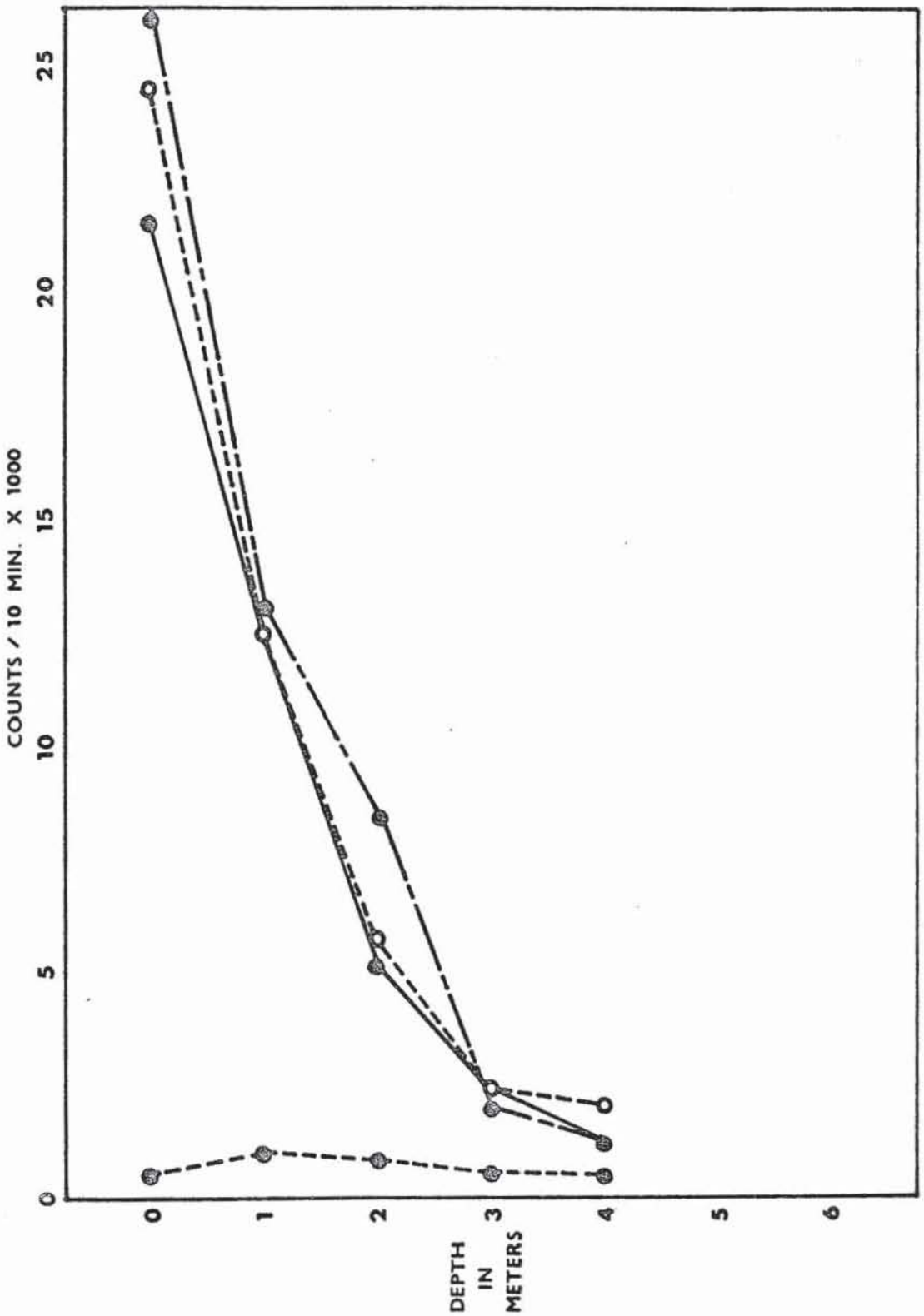
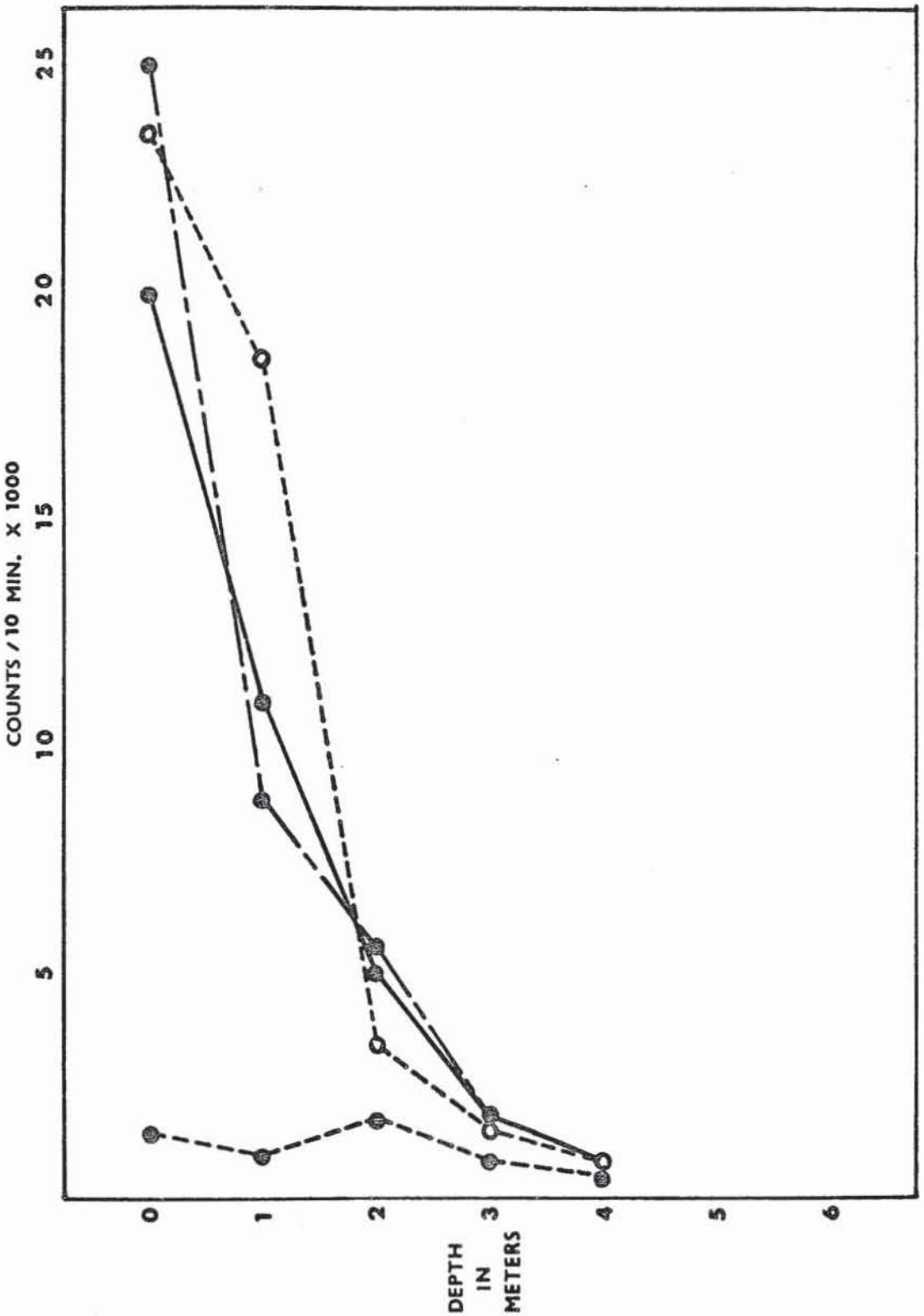

COUNTS / 10 MIN. X 1000
0
5
10
15
20
25
DEPTH IN METERS
0
1
2
3
4
5
6

Fig. 10. Profile of primary production in samples from Lake 304 under various gas phases on Sept. 22/1971. The incubation period was 4 hours. Solid circles joined by evenly dashed lines are dark bottles under acetylene; solid circles joined by unevenly dashed lines are light bottles under helium; solid circles joined by solid lines are light bottles under air; open circles joined by evenly dashed lines are light bottles under acetylene.


COUNTS / 10 MIN. X 1000
5
10
15
20
25
0
1
2
3
4
5
6
DEPTH IN METERS

Observations were made upon zooplankton that were visible to the naked eye. After the routine 4 hour incubation period, zooplankton were actively moving and appeared to be healthy. In several samples that had been incubated 24 hours, the visible zooplankters still appeared healthy showing no obvious harmful effects due to the dissolved acetylene.

Treatment of Acetylene Reduction Results

Ethylene peak heights observed on the chromatograms were converted to ethylene concentrations per 0.25 ml sample (atm. pressure) calculated on the basis of known concentrations using a standard curve (Fig 5). Acetylene concentrations were also measured using another standard curve (Fig 6). The acetylene measurements served as internal standards for the chromatography because the concentration of the gas should have been essentially the same in all samples analysed. If an increase occurred in a sample's acetylene concentration relative to the blank, the ethylene concentration noted in the sample could be adjusted downward. Such an adjustment would be inversely proportional to the excess acetylene noted in the sample. The opposite is true for samples containing less acetylene than that present in the blank. Mathematically, the relationship can be stated thus:

$$\text{ethylene (adjusted)} = \text{ethylene (sample)} \times \frac{\text{acetylene (blank)}}{\text{acetylene (sample)}}$$

This procedure of adjusting the ethylene concentration appears more logical than leaving it unadjusted. Dilution of gas samples by contaminating air is not nearly so important if this correction is used. Differences in sample volumes and pressures are also accounted for if one assumes that the acetylene concentration should be the same in samples and blanks.

Acetylene is quite soluble in water and a partial vacuum is formed in the incubation bottles as a result of this property. The syringe sampling technique employed caused a reduction in volume of the gas samples so that they were nominally at atmospheric pressure. The acetylene would essentially be at original pre-incubation concentration but the ethylene, because of its insolubility, would be effectively increased in concentration due to the sample volume reduction. It is for this reason that the partial vacuums of the incubation bottles were measured. Taking the average internal pressure of the bottles to be 0.73 atmospheres (see Incubation Bottle Vacuum p. 43) one can apply this factor to the relationship previously developed and obtain a true ethylene concentration.

$$0.73 \left(\text{eth. (sample)} \times \frac{\text{acet. (blank)}}{\text{acet. (sample)}} \right) = \text{actual ethylene concentration } /.25 \text{ ml}$$

It is from this corrected actual ethylene value that the ethylene concentration of the blank is subtracted.

The difference between these two values represents the net evolution of ethylene in the incubation bottle /.25 gas sample.

$$.73 \text{ (eth. (sample) x } \frac{\text{acet. (blank)}}{\text{acet. (sample)}} \text{ - eth. (blank)} =$$

net ethylene evolution /.25 ml gas sample

A further series of factors are then applied to the net ethylene evolution results. Ethylene production is converted to NH_3 production by dividing the ethylene figure by 1.5, the theoretical molar equivalence factor. The molar value of NH_3 is further multiplied by 17 to give its weight in grams. The weight of nitrogen is then obtained by multiplying 14/17 times the last result calculated. This weight of nitrogen represents fixation over a period of 4 hours in a water volume of 2.0 litres. Fixation results in terms of g-N/l/hr are obtained by multiplying the previous result times 0.5 and 0.25. Total ethylene production in the 500 ml gas phase of the incubation bottle (as opposed to that in a .25 ml chromatograph gas sample) is calculated by multiplying $\frac{500}{.25}$ times the last result.

If all these factors are reduced to simplest terms, a constant is obtained:

$$\frac{14}{17} \times 17 \times \frac{1}{1.5} \times .5 \times .25 \times \frac{500}{.25} = 2333$$

The final equation used to estimate nitrogen fixation rates in g/l/hour is given by:

$$\left(.73 \left(\text{eth. (sample)} \times \frac{\text{acet. (blank)}}{\text{acet. (sample)}}\right) - \text{eth. (blank)} \right) \times 2333 =$$

ηg - N fixed/l/hour

It is well to remember that the ethylene: ammonia ratio of 1.5 : 1.0 is an assumed value. Values as low as 1.0 : 1.0 and as high as 4.2 : 1.0 have been reported. Average values for many workers, however, tend toward the theoretical ratio of 1.5 : 1.0. Also, standard deviation for the partial vacuum correction of .73 has not been included in the final equation. Estimates on the accuracy of the assay system must take these facts into consideration.

Interpretation of Fixation Results

Lake 304

It is evident that considerable variation in fixation rates existed from week to week and from meter to meter in the water column (Figs 11,12,13,14). Fixation rates were usually highest at the surface and decreased with depth. Secondary peaks at deeper depths were quite pronounced on several dates i.e. Aug. 3, 10, 18 and Sept. 15. On all dates but July 7, fixation appeared to be nearly equal in both dark and light bottles. Such a finding would tend to implicate bacteria as the organisms mainly responsible for the nitrogen fixation. If algae were actually fixing, it was thought that the rate would have been much reduced when they were in darkness. On the day of July 7, a blue-green algal bloom may have been present and thus explain the large difference between dark and light fixation rates noted in that instance.

The fact that fixation rates were higher or highest at the surface may be attributable to several causes. Photosynthetic generation of ATP is probably greater near the surface than at lower depths and thus accumulation of surpluses could supply epiphytic bacterial nitrogen fixers with an increased source of energy. It is also possible that bacterial fixers concentrated in the surface layers because of preferred physical conditions existing there.

Fig. 11. Profiles of nitrogen fixation rates in samples from Lake 304 on July 7, 14, 21 and 26/1971. Solid lines are dark bottles and dashed lines are light bottles.

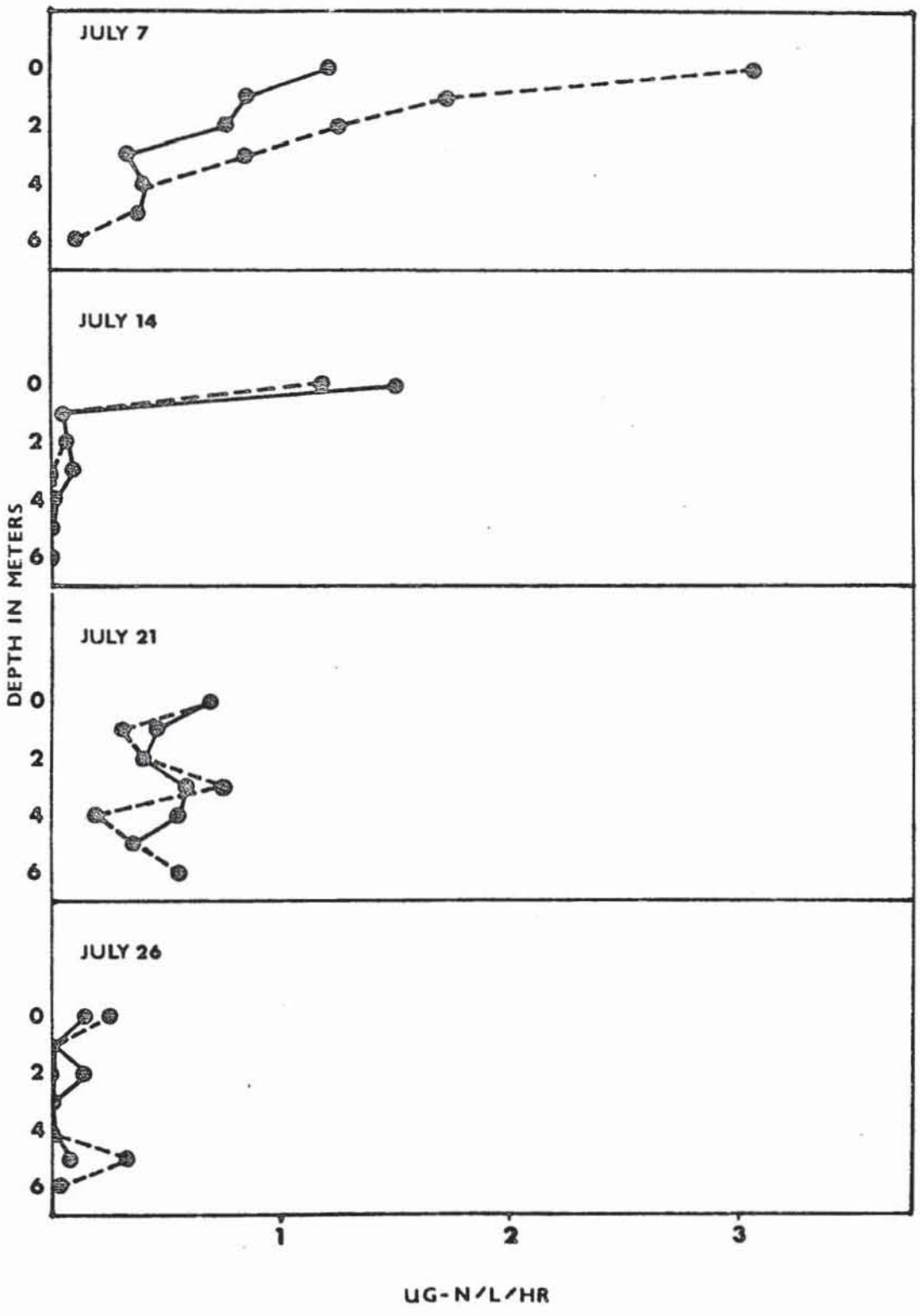

JULY 7
JULY 14
JULY 21
JULY 26
0
2
4
6
DEPTH IN METERS
1
2
3
UG-N/L/HR

Fig. 12. Profiles of nitrogen fixation rates in samples from Lake 304 on Aug. 3, 10, 18 and 25/1971. Solid lines are dark bottles and dashed lines are light bottles.

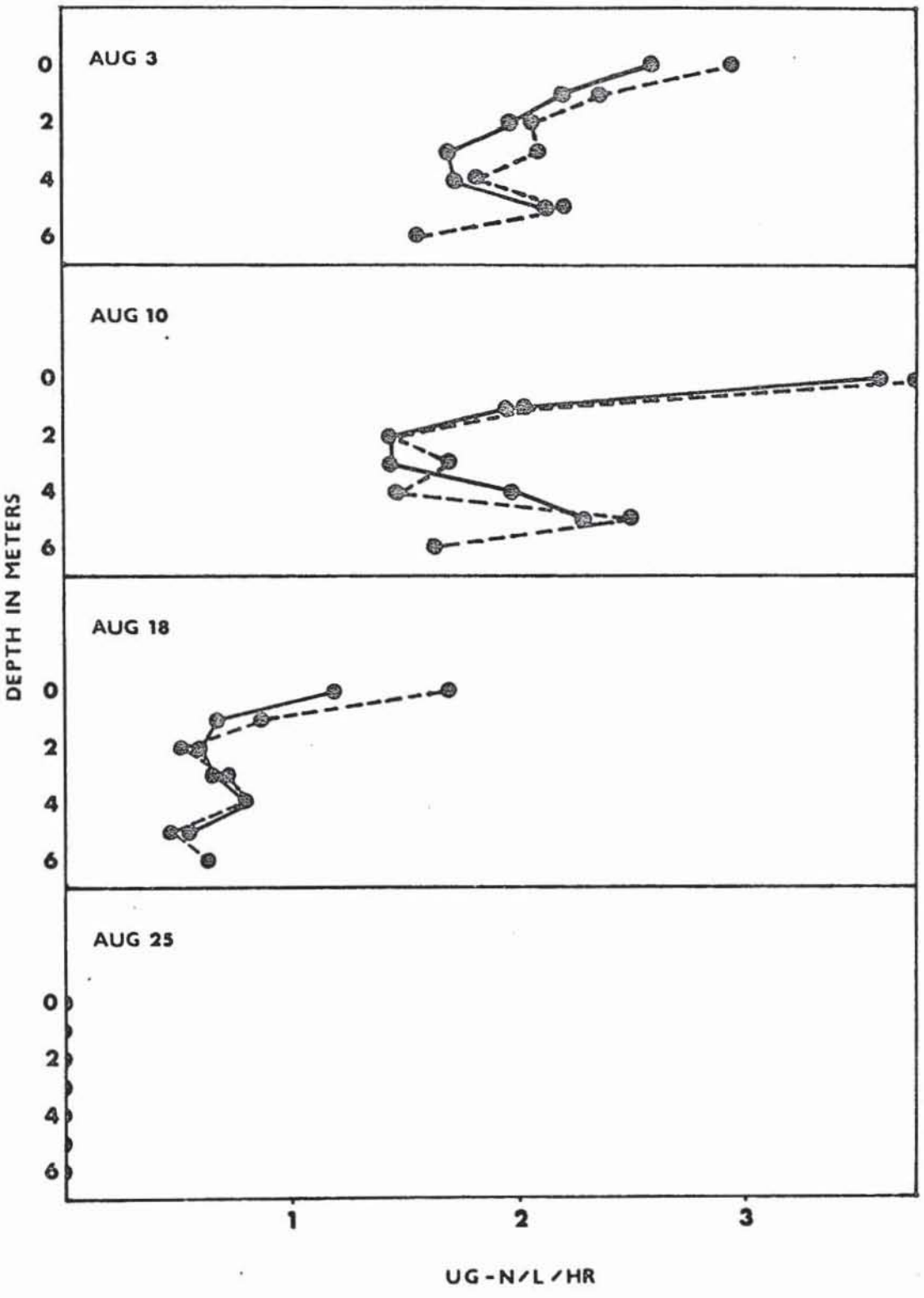

AUG 3
AUG 10
AUG 18
AUG 25
0
2
4
6
DEPTH IN METERS
1
2
3
UG-N/L/HR

Fig. 13. Profiles of nitrogen fixation rates in samples from Lake 304 on Sept. 1, 15 and 22 and Oct. 13/1971. Solid lines are dark bottles and dashed lines are light bottles.

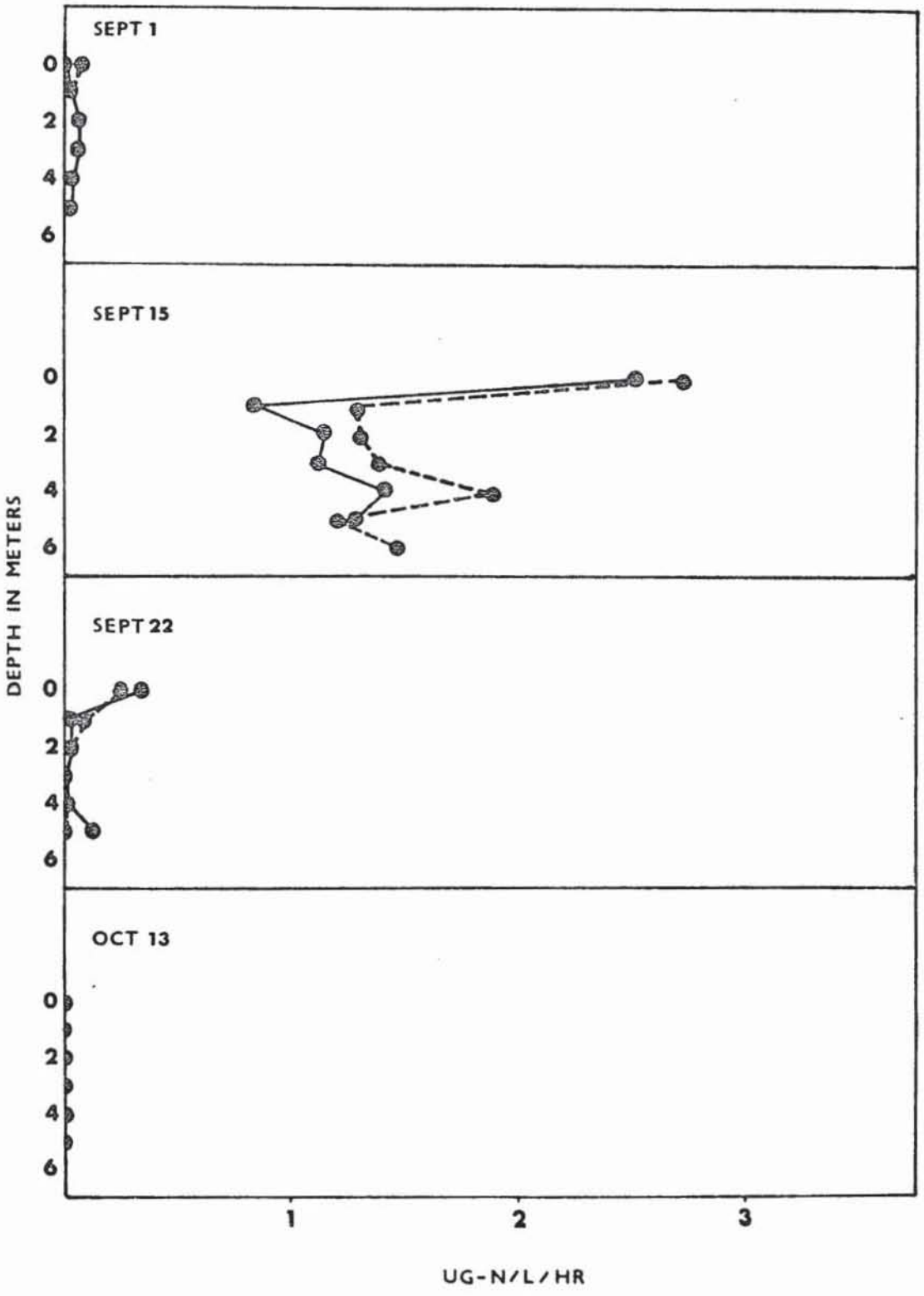
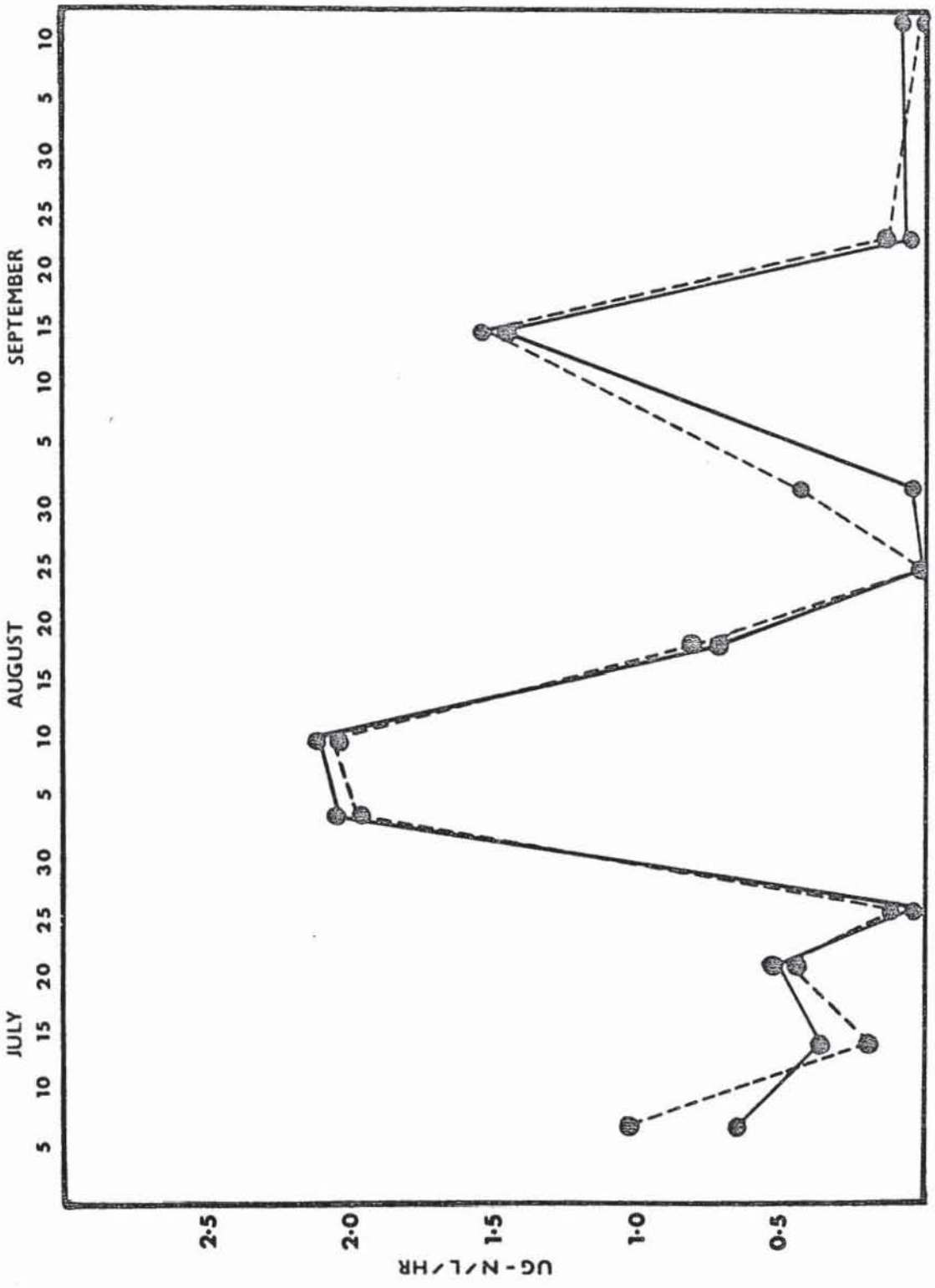

SEPT 1
SEPT 15
SEPT 22
OCT 13
0
2
4
6
DEPTH IN METERS
1
2
3
UG-N/L/HR

Fig. 14. Average rate of nitrogen fixation in the whole water column of Lake 304 as a function of time. These results are uncorrected with respect to lake volume development. Solid lines are dark bottles and dashed lines are light bottles.


UG - N/L/HR
2·5
2·0
1·5
1·0
0·5
JULY
AUGUST
SEPTEMBER
5 10 15 20 25 30 5 10 15 20 25 30 5 10 15 20 25 30 5 10

Similarly, certain species of algae may concentrate at the surface and have associated with them bacteria that are capable of fixation. Such an accumulation of algae might have excreted extracellular products (soluble organic matter) which bacteria found suitable as an energy and carbon substrate to sustain fixation.

The pronounced secondary peaks previously identified at depth are more difficult to interpret. It would appear that a set of conditions suitable for bacterial fixation existed at those times at the 4 and 5 meter depths. What parameters made up the set of conditions can only be surmised. Algal concentrations may have occurred at those depths and thus may have provided the carbon and energy substrates for bacterial nitrogen fixation. The substrates might have been extracellular excretions or breakdown products of dead and decaying algae.

The larger variations observed in fixation rates from week to week were in agreement with observations by other workers (14,17,18). In Lake 304, maximum rates appeared to decrease from July 7 to July 26, although total fixation in the water column did not e.g. July 21. Fixation began to increase Aug. 3 and reached the seasonal high on Aug. 10, then gradually fell to 0 by Aug. 25. Rates remained low until the end of the sampling period (Oct. 13) except for a brief but substantial increase around Sept. 15. It would appear that fixation occurred

in three distinct seasonal periods (Fig 14). Why these peaks occurred when they did is not known.

It is quite evident that the total amount of nitrogen fixed could not be estimated with a sampling frequency less than the one employed. Sampling once a month, for instance, would have been less than adequate because it might have yielded results for total fixation that were in error by an order of magnitude.

Lake 227

The fixation rates observed in this lake were generally lower than those of Lake 304. Variations in rates, on a week to week basis and from meter to meter in the water column, were smaller than in Lake 304 (Figs 15,16,17,18,19).

Dark incubation bottles were used in the 0, 1 and 2 meter depths only, therefore bacterial and algal fixation cannot be differentiated with the same degree of confidence as in Lake 304. It does appear, however, that dark and light fixation rates are similar, thus tending to implicate bacteria as the organisms usually responsible for the fixation observed. Only on July 13 do the results indicate fixation to be algal. On this date, the highest fixation rate of the season was observed at the surface. The fixation was high even in the dark, though still less than one half of that seen in the light. It is possible that reserve energy sources were utilized to accommodate the fixation process

Fig. 15. Profiles of nitrogen fixation rates in samples from Lake 227 on July 9, 13 and 23/1971. Solid lines are dark bottles and dashed lines are light bottles.

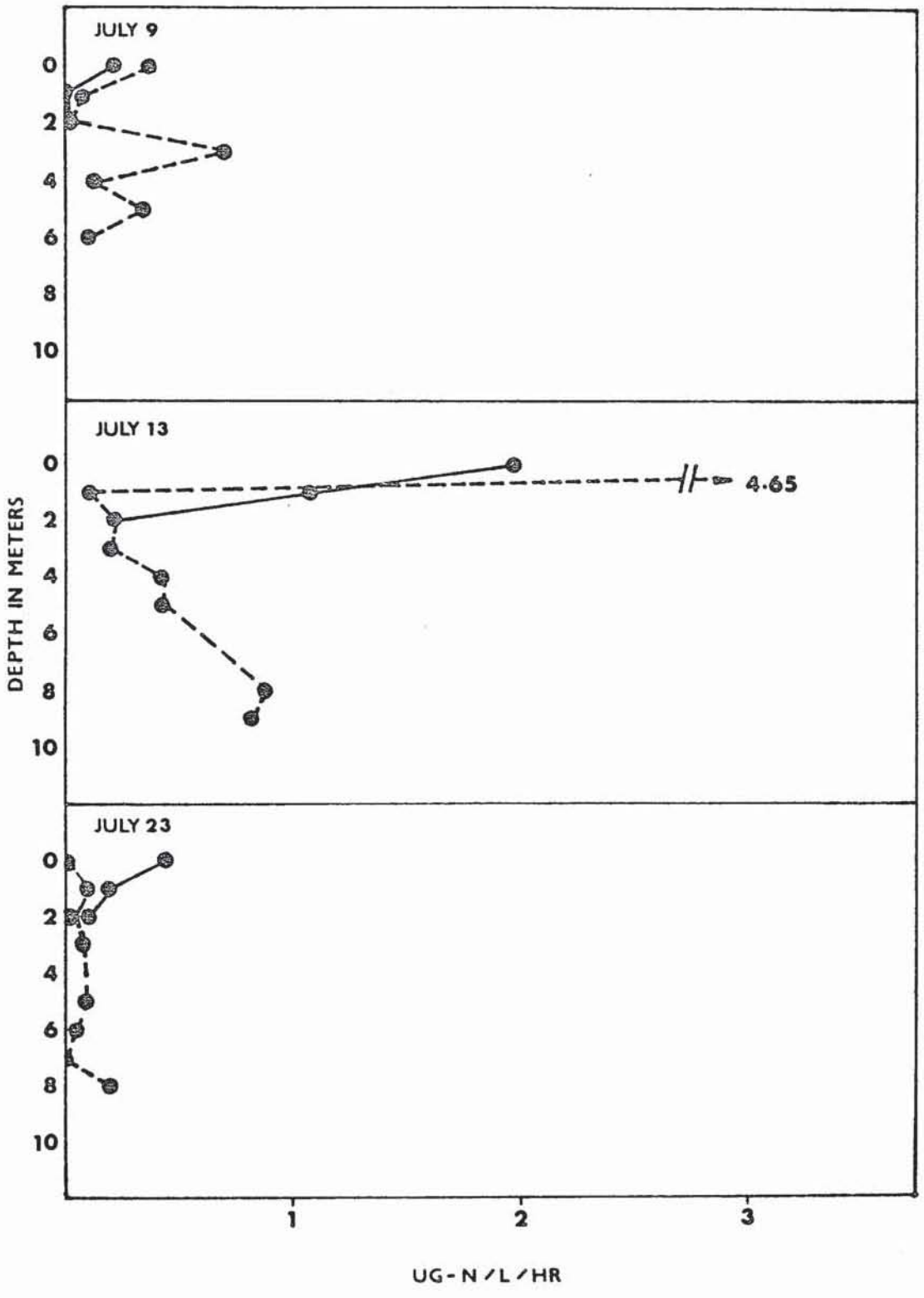

JULY 9
JULY 13
JULY 23
4.65
DEPTH IN METERS
0
2
4
6
8
10
1
2
3
UG-N/L/HR

Fig. 16. Profiles of nitrogen fixation rates in samples from Lake 227 on Aug. 3, 10 and 20/1971. Solid lines are dark bottles and dashed lines are light bottles.

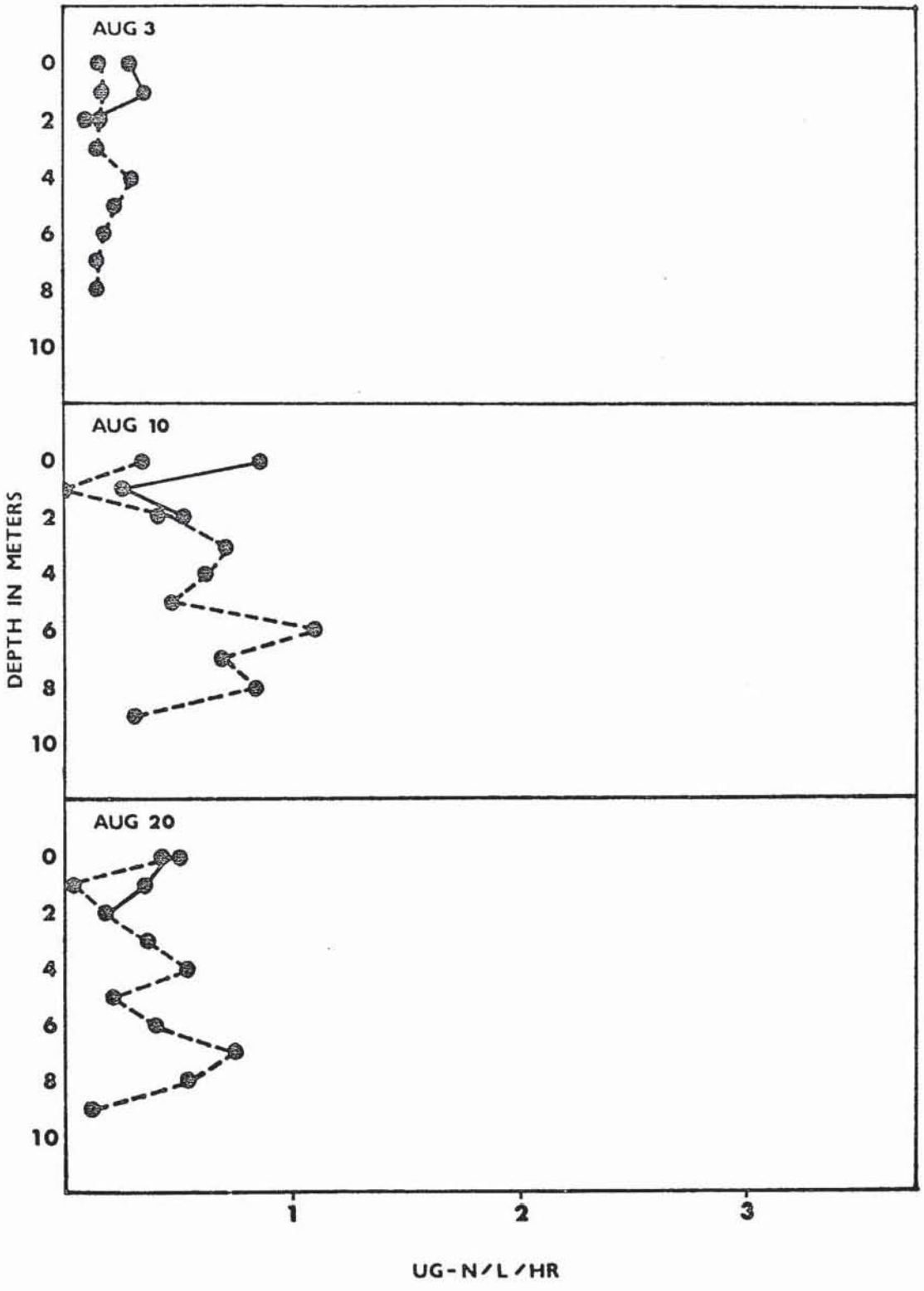

AUG 3
AUG 10
AUG 20
DEPTH IN METERS
0
2
4
6
8
10
1
2
3
UG-N/L/HR

Fig. 17. Profiles of nitrogen fixation rates in samples from Lake 227 on Sept. 2, 7 and 14/1971. Solid lines are dark bottles and dashed lines are light bottles.

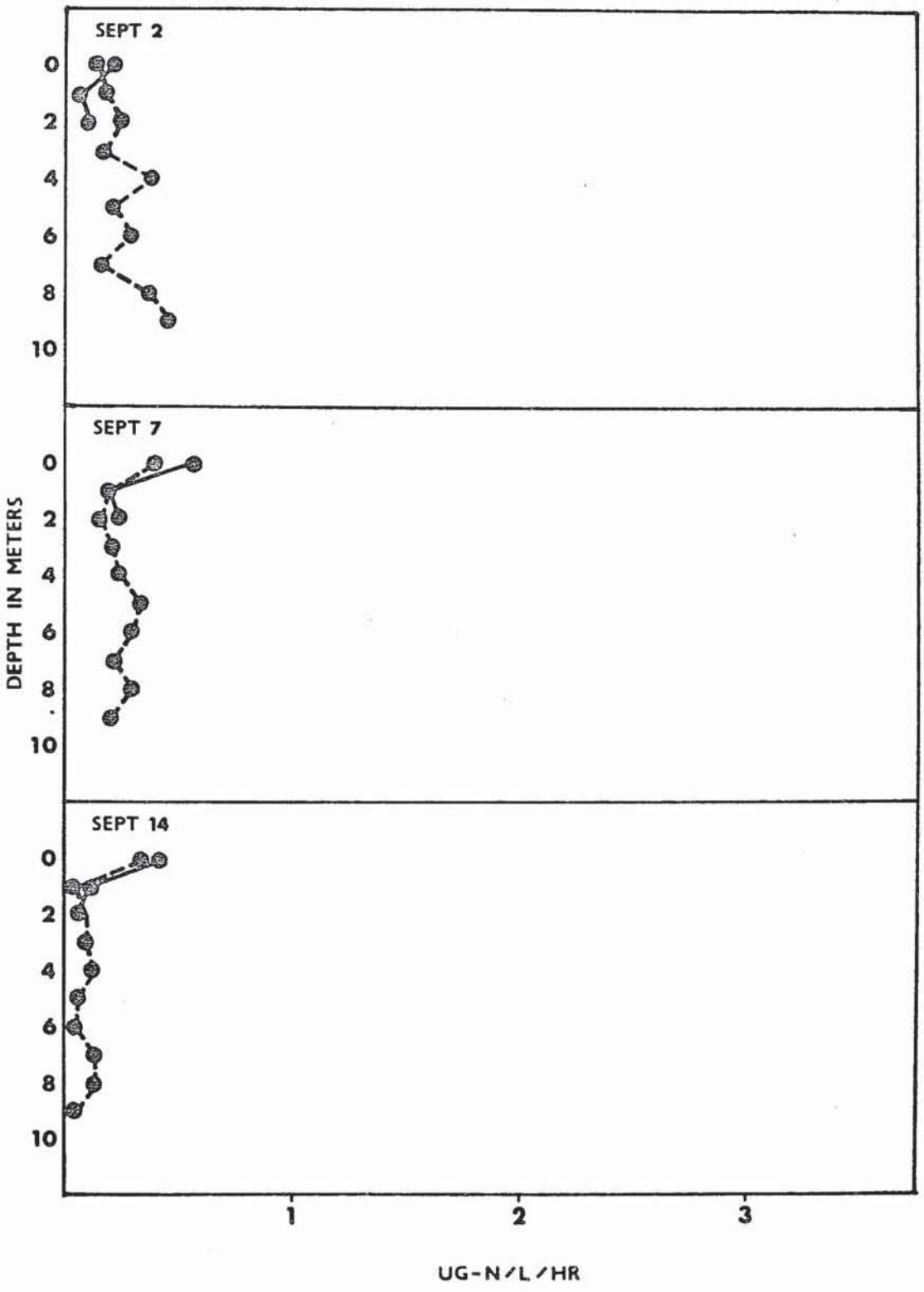

SEPT 2
SEPT 7
SEPT 14
0
2
4
6
8
10
DEPTH IN METERS
1
2
3
UG-N/L/HR

Fig. 18. Profiles of nitrogen fixation rates in samples from Lake 227 on Oct. 12/1971. Solid lines are dark bottles and dashed lines are light bottles.

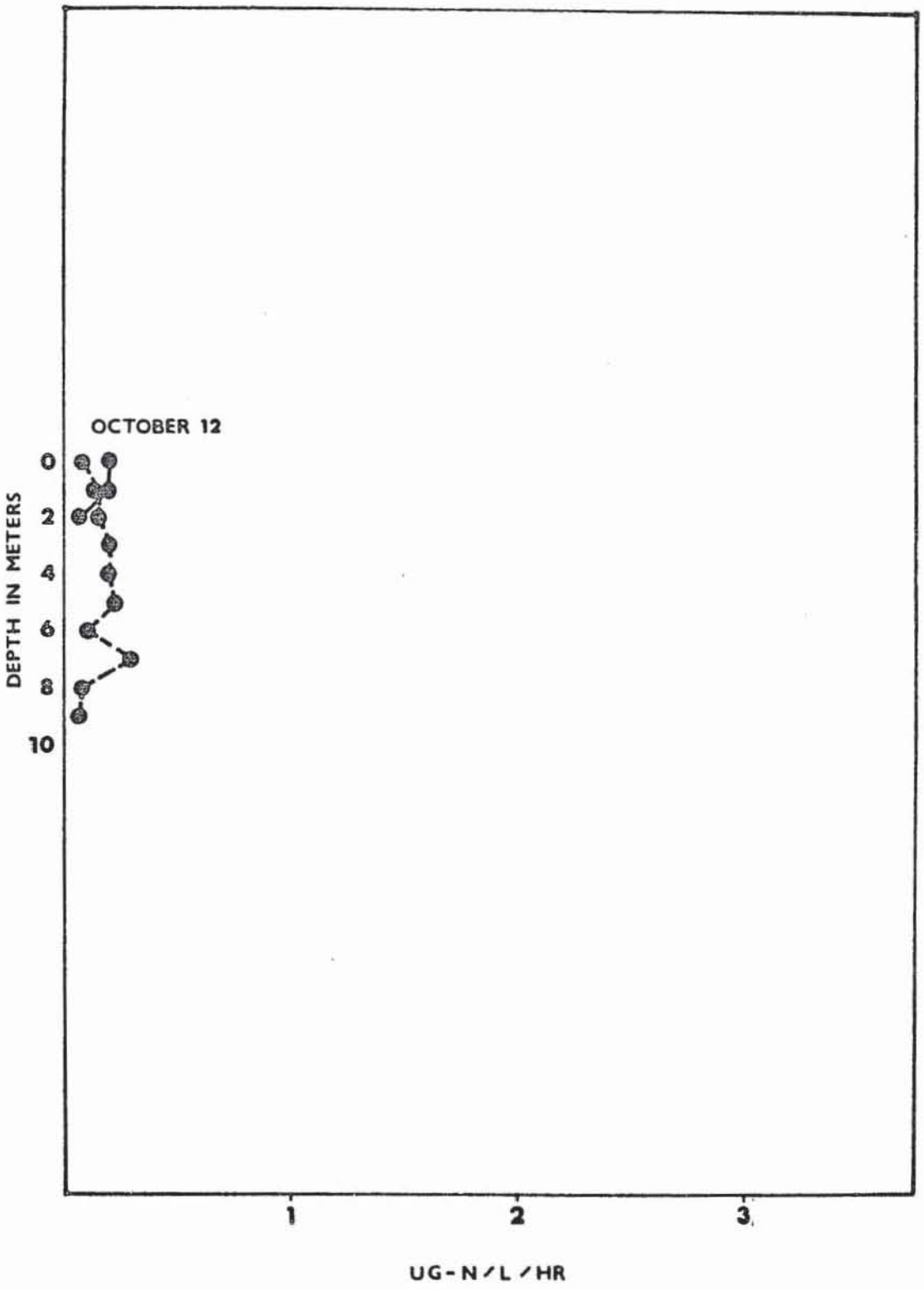
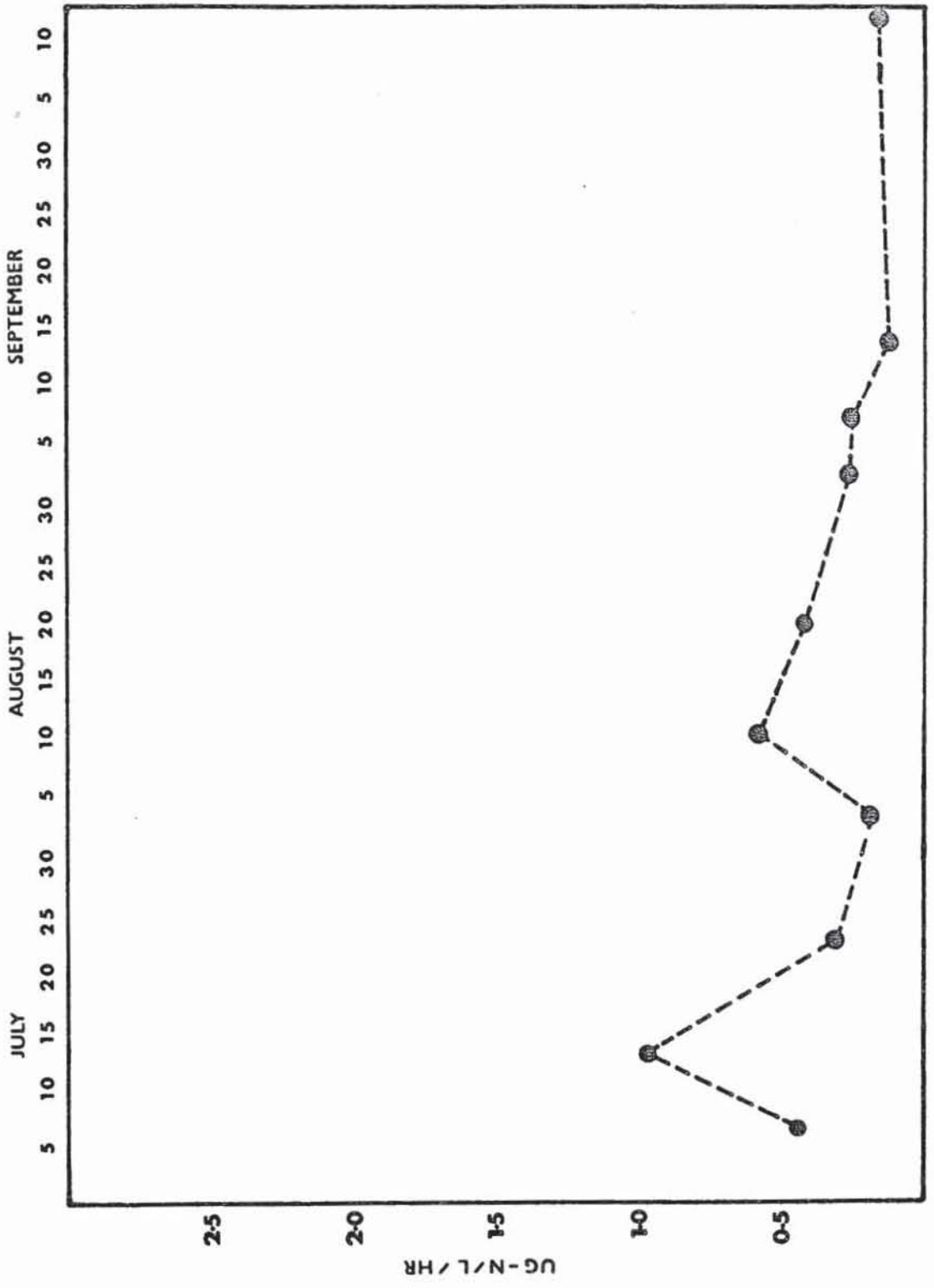

OCTOBER 12
DEPTH IN METERS
0
2
4
6
8
10
1
2
3
UG-N/L/HR

Fig. 19. Average rate of nitrogen fixation in the whole water column of Lake 227 as a function of time. The results are uncorrected with respect to lake volume development. Dashed lines are light bottles.


JULY
AUGUST
SEPTEMBER
5 10 15 20 25 30 5 10 15 20 25 30 5 10 15 20 25 30 5 10
UG-N/L/HR
0·5
1·0
1·5
2·0
2·5

in the dark and that before the four hour incubation period was over, the reserve was consumed. Fixation in the light was greater, perhaps, due to a constant supply of energy via photosynthesis.

The presence of secondary and tertiary fixation peaks in the water column on various dates (e.g. July 9, Aug. 10, 20) might be explained as for those observed in Lake 304. The seasonal distribution of fixation appears to follow that of Lake 304 in a general way but the average rates are much lower and less pronounced (19).

Estimation of Total Nitrogen Fixed

The seasonal averages of nitrogen fixation rates were determined for each depth. This was accomplished by adding rates of the first and second dates and dividing by two; adding rates of the second and third dates and dividing by two etc. These averages were weighted according to the number of days between sampling periods. For example, in the case of Lake 304, the first sampling was on July 7 and the second on July 14; the number of days between samplings was seven. The total number of days between first and last samplings was 98. The weighted average was therefore expressed as

$$\frac{7}{98} \times \frac{\text{July 7 rate} + \text{July 14 rate}}{2}$$

The procedure was then repeated for the second and third sampling dates, the third and fourth etc. All of the weighted averages were then totalled and the resulting sum represented the average seasonal fixation rate at the particular depth in question.

The sum was then multiplied by the volume of water (in liters) that was known to exist in the lake at the depth being studied. For example, the average rate of fixation obtained for the two meter level would be multiplied by the volume of the lake between the two and three meter depths. Mathematically, this treatment of data is not as precise as it might be; the volume of water between two adjacent depths should be multiplied by the average of rates from the two adjacent depths, not by the rate obtained in the upper depth. In practice, however, this apparently incorrect calculation does appear justified. Because of the sampling technique employed, samples taken from any given depth represent a column of water extending from the given depth to a depth one half meter deeper. Such a sample, though not in the strictest sense a composite of the meter interval, probably yields fixation results that are fairly representitive of fixation activity in any given interval.

Volumes and areas for Lake 304 and Lake 227 are given in Figs 1 and 2.

Having calculated the weights of nitrogen fixed/hr in each meter increment of the lake, these weights are totalled and the resulting sum is the weight of nitrogen fixed/hr in the entire lake over the sampling period. This weight can then be divided by the lake surface area (in meters^2) to yield an average fixation rate in g - N/m^2/hr.

Lake 304

Samples that were incubated from 10:00 A.M. to 2:00 P.M. in the light yielded fixation rates of 88.74 g - N/hr for the whole lake. If one assumes a daily 16 hr light exposure over the 98 day sampling period, total nitrogen fixed is 88.74 x 16 x 98 = 139.14 Kg. Dividing this figure by the lake area of 3.62 x 10^4 m^2 yields a fixation rate of 3.84 g - N/m^2/98 days in the light.

Samples that were incubated in dark bottles from 10:00 A.M. to 2:00 P.M. yielded fixation rates of 79.11 g - N/hr for the whole lake. If one assumes an eight hour dark period over the 98 days, total nitrogen fixed is 79.11 x 8 x 98 = 62.02 Kg. When this is divided by the lake area of 3.62 x 10^4 m^2 , dark fixation is noted to be 1.71 g - N/m^2/98 days.

Total nitrogen fixed over the 98 days would be the sum of the dark and light fixations i.e. 3.84 + 1.71 = 5.55 g - N/m^2/98 days.

Artificial fertilization of Lake 304 provides a nitrogen input of 5.2 g - $N/m^2/yr$ (see The Experimental Lakes p. 7) and it had been estimated that this accounted for about 75% of the total nitrogen input (23). If the calculated nitrogen fixation input is at all close to being correct, nitrogen addition to the lake would be almost double that of previous estimates that assumed little or no fixation.

A nitrogen budget is presently being constructed for this lake by F.R.B. personnel. Their estimate of how much nitrogen has gone to outflow and sediments or remained dissolved or suspended in the water column should help to clarify the situation and determine how plausible these high fixation rates are.

Lake 227

Samples that were incubated from 10:00 A.M. to 2:00 P.M. in the light gave fixation results of 71.07 g - N/hr for the whole lake. If one assumes a daily 16 hr light exposure over the 95 day sampling period, total nitrogen fixed is 71.07 x 16 x 95 = 108.03 Kg. Dividing this figure by the lake area of 5.00×10^4 m^2 yields a fixation result of 2.16 g - $N/m^2/95$ days in the light.

Estimation of fixation in the dark is slightly more difficult due to the fact that dark bottles were used only for 0, 1 and 2 meter depths. It is evident, however, that

fixation below these depths is nearly equal in dark and light samples (Figs 15,16,17,18). It was therefore assumed that if fixation data from the three uppermost dark bottles were combined with data from the light bottles of depths 3 - 9 meters, a fairly good approximation of dark fixation could be obtained. Using this method it was determined that nitrogen was fixed at the rate of 72.36 g/hr for the whole lake in the dark. For a daily 8 hr dark period over 95 days, total nitrogen fixed was 72.36 x 8 x 95 = 54.99 Kg. This represents a nitrogen input of 1.10 $g/m^2/95$ days.

Total nitrogen fixed in the lake over the 95 days would be the sum of the light and dark fixations i.e. 2.16 + 1.10 = 3.26 g - $N/m^2/95$ days.

Artificial fertilization is known to account for 6.3 g - $N/m^2/yr$ (see The Experimental Lakes, p. 7) and this and other data have been used to construct several nitrogen budgets for the lake. The following is the most recent budget prepared by F.R.B. personnel (23) and covers the period of April 9/1970 to March 31/1971.

Input	
Fertilization	6.3
Precipitation	0.6
Runoff	1.5
Total input	8.4 g - $N/m^2/yr$

Output

Sedimentation	3.6
Outflow	1.8
Dissolved	2.5
Suspended	0.5
Total output	8.4 g - $N/m^2/yr$

Examination of the various components and their techniques of measurement reveals that sedimentation and runoff are the only quantities that are not known with certainty. Of these two, sedimentation is probably most in doubt because it is not directly measured but is estimated upon the basis of the other nitrogen analyses and because it represents the larger amount of nitrogen.

It will be noted that this 1970 budget makes no allowance for nitrogen input by fixation. If it is assumed that such an input did exist and that it was of the same magnitude as encountered in our 1971 fixation studies, then one must adjust the budget to accommodate this extra input. A reasonable approach might be to hypothesize a much higher sedimentation rate, perhaps greater by a factor of two.

Another possibility may be that denitrification is actively proceeding and that it is responsible for losses of nitrogen equal in magnitude to the input by fixation. If such were the case, a new budget would have to be constructed and it might appear as follows:

Input

Fertilization	6.3	
Precipitation	0.6	
Runoff	1.5	
Fixation	3.0	(assumed)
Total input	11.4	g - $N/m^2/hr$

Output

Sedimentation	3.6	
Outflow	1.8	
Dissolved	2.5	
Suspended	0.5	
Denitrification	3.0	(assumed)
Total output	11.4	g - $N/m^2/yr$

A budget such as this is plausible if one considers the extreme oxygen stratification in the lake and correlates it to the physiological activities of nitrifying and denitrifying bacteria in similar environments.

General Conclusions

Fixation rates observed in these two lakes are quite high (17,18) and evidently account for a substantial portion of total nitrogen inputs. Bacteria appear to be the major organisms responsible for fixation, a role that is usually assigned to certain blue-green algae. Should these fixation rates be substantiated by the $^{15}N_2$ technique, the role of bacterial nitrogen fixation in lakes must be regarded to be much more important than previously thought. It is thus imperative that a comparison between the $^{15}N_2$ and acetylene reduction techniques be done *in situ* so that actual rates of fixation can be determined.

Apart from the difficulty of calibrating the acetylene reduction method, it must be considered a very attractive means of measuring nitrogen fixation (13). It was noted that acetylene appeared to have no toxic effects upon the phytoplankton even at the high concentrations of gas used. The gas is also rapidly dissolved in the aqueous phase and is preferentially reduced instead of nitrogen. All of the above factors, combined with the ease of gas chromatographic analysis, make this method a very valuable tool for aquatic fixation studies.

The possibility of experimental error cannot be ignored in this work. The most likely cause of error would stem from incomplete solution of acetylene in the aqueous phase.

If this occurred, the nitrogenase system would not be completely saturated with acetylene and would yield lower than actual fixation rates. Should oxygen or acetylene be toxic to the fixers, rates would again appear to be lower than they actually were. Small amounts of ethylene that were produced in the assay system, but remained dissolved in the water, would also tend to give low results. All of these most obvious errors would tend to yield fixation rates that were underestimates. Though the possibility of an overestimate still remains, it appears much less likely than an underestimate.

The extreme variations in fixation rates encountered, over the season and throughout the water column, make it obvious that sampling frequency and intensity must be high for this assay if one is to estimate the total nitrogen input. Estimates made by other workers (14,15,16,18) have little to substantiate them because of the small number of samples taken. Often, just surface samples were used. Making any estimates from such data must have a high degree of risk and probably, error.

Finally, one cannot disregard the possible role of denitrification in these aquatic environments. High fixation rates may well be countered in the sense of a nitrogen budget by high denitrification rates. The quantities of dissolved nitrogen gas might be expected to decrease if fixation was quite active, but at the same time, it might be regenerated

by denitrification. Further work using gas chromatography and $^{15}N_2$ might shed some light upon this area and further substantiate or refute the results obtained in this study.

APPENDIX

Nitrate & Nitrite Analysis

Precision: At the 50 ug/l NO_3^- - N level, precision was $\pm 5\%$.

Range: 10 - 2000 ug/l NO_3^- - N

Equipment:
- 25 ml volumetric flask
- 28 ml glass bottles with rubber lined screw caps
- stopwatch
- spectrophotometer (Coleman Junior II A, Maywood, Illinois, U.S.A.)
- rounded cuvettes, 19 mm x 105 mm (for above, Coleman)
- Nitra Ver IV powder pillows (Hach Chemical Co., Ames, Iowa, U.S.A.)

Method: A 25 ml filtered water sample at room temperature (see Preparation of Samples for Analysis, p. 32) was dispensed into a 28 ml bottle by volumetric flask. A powder pillow of Nitra Ver IV was added to the sample and the bottle capped. The bottle was shaken for exactly one minute and then allowed to sit for 2 minutes to facilitate settling of the cadmium filings. Approximately 20 ml of sample were decanted from the bottle into a cuvette which was then put into the spectrophotometer to allow air bubbles to come off. After a 4 minute period in the cuvette, the absorbance was read at 520 mu.

A standard curve was constructed from samples of known concentrations of NO_3^-. Each day that samples were analysed, a NO_3^- standard was included to check upon the accuracy of the curve. Blanks were treated in exactly the same manner as samples because they were unstable over time.

Note: Glassware was always 3x washed with glass distilled water and periodically acid washed to ensure low blank readings.

Nitrite Analysis

Precision: At the 10 ug/l NO_2^- - N level, precision was $\pm$ 5%.

Range: 1.0 - 200 ug/l NO_2^- - N

Equipment:
- 25 ml volumetric flask
- 28 ml glass bottles with rubber lined screw caps
- stopwatch
- spectrophotometer (Coleman Junior II A, Maywood, Illinois, U.S.A.)
- rounded cuvettes, 19 mm x 105 mm (for above, Coleman)
- Nitra Ver powder pillows (Hach Chemical Co., Ames, Iowa, U.S.A.)

Method: A 25 ml filtered water sample at room temperature (see Preparation of Samples for Analysis, p. 32) was dispensed into a 28 ml bottle by volumetric flask. A powder pillow of Nitra Ver was added to the sample and the bottle capped. The bottle was shaken for exactly one minute and then was allowed to sit for a further 9 minutes. Approximately 20 ml of sample were poured from the bottle into a cuvette which was then put into the spectrophotometer to allow air bubbles to come off. After a 5 minute period in the colorimeter tube, the absorbance was read at 493 mu.

A standard curve was constructed from samples of known concentrations of NO_2^- . Each day that samples were analysed, a NO_2^- standard was included to check upon the accuracy of the curve. Blanks were treated in exactly the same manner as samples because they were unstable over time.

Note: Glassware was always 3x washed with glass distilled water and periodically acid washed to ensure low blank readings.

Soluble and Particulate Nitrogen Analysis

Precision: At the 6.0 ug/l NH_3 - N level, ($(NH_4)_3SO_4$), precision was $\pm .6/n^{1/2}$ ug - N/l where n = number of determinations.

Range: .25 - 2000 ug/l NH_3 - N

Equipment:
- Micro - Kjeldahl heating rack
- Micro - Kjeldahl tubes - pyrex - 30 ml
- Tube buzzer
- Boiling water bath
- Cold water bath
- Spectrophotometer (Unicam S.P. 500, Pye Co., England)
- 1 cm cuvettes
- Test tubes 18 x 150 mm
- Pipettes - 25, 10, 5, 1 ml
- Burette

Reagents:

Digestion Mixture

- dissolve 0.1 g selenium dioxide, SeO_2, in 500 ml H_2O
- add 500 ml sulphuric acid and cool to room temperature
- make to a volume of 1000 ml with distilled H_2O and store in tightly stoppered glass bottle

Phenolphthalein Indicator

- dissolve 0.2 g of phenolphthalein in 50 ml of 80% ethanol

Sodium Hydroxide Solution (80 g/ 500 ml)

- rinse 80 g of sodium hydroxide rapidly in 20 ml of H_2O to lose only a few % of alkalie

Sodium Hydroxide Solution (80g/ 500 ml) cont'd

- discard rinsing
- dissolve in deionized H_2O to make a volume of 500 ml
- store in tightly stoppered polyethylene bottle

Ninhydrin - Hydrindantin Reagent

(A) Preparation of Hydrindantin

- To 20 g ninhydrin in 500 ml of H_2O at 90° C, add 20 g ascorbic acid in 100 ml of H_2O at 40° C
- Crystallization begins at once
- Cool solution
- Filter and wash crystals with distilled H_2O
- Dry over P_2O_5 or H_2SO_4 in the dark

(B) Preparation of Acetate Buffer

- Dissolve 136 g of reagent grade sodium acetate trihydrate ($Na\ C_2\ H_3O_2 \cdot 3\ H_2O$) in 100 ml distilled H_2O
- Add 25 ml glacial acetic acid
- Dilute to 250 ml with distilled H_2O
- Store in glass or polyethylene bottle

(C) Preparation of Ninhydrin - Hydrindantin Reagent

- Dissolve 0.50 g ninhydrin and 0.075 g hydrindantin in 20 ml methylcellosolve (ethylene glycol monomethyl ether)

(C) Preparation of Ninhydrin - Hydrindantin Reagent cont'd

- Add 5.0 ml acetate buffer and stir briefly
- Unless the reagent is to be used within 30 minutes, bubble nitrogen gas through the solution while the flask is kept at room temperature and in the dark. The solution is stable for only about 12 hour.

Ethanol - Water Mixture

- Mix together 250 ml of 95% ethanol and 250 ml of distilled water
- Store in a well stoppered glass bottle. The mixture is stable indefinitely

Boiling Chips

A few fragments of Hengar granules or Fisher's "Boil-easers" are satisfactory provided they are pretreated by heating them in boiling or near - boiling concentrated sulphuric acid for 2 - 3 hr. After the acid treatment, the granules should be boiled with 2 or 3 changes of water, rinsed thoroughly, and oven dried. The later treatment is essential if their antibumping properties are to be restored.

Method:

1. Pipette 25 ml of sample into Kjeldahl tube. (For standard, use 25 ml standard solution at 150 ug - N/l as $(NH_4)_3 SO_4$. For blank, use 25 ml high purity distilled water.)
2. Add 2 ml digestion mixture and 1 boiling chip.

Method: (Cont'd)

3. Heat on Kjeldahl rack until all the water is removed. Allow the mixture (≈ 1 ml) to digest for another 60 min at about 200° C.

4. Cool the tube slightly and dissolve the residue in about 20 ml of distilled water. Warm the solution if necessary to dissolve the residue.

5. Cool the solution in an ice bath and then add one drop of phenolphthaleine indicator.

6. Titrate the solution with strong hydroxide solution (80 g/500 ml) until the solution just becomes pink.

7. Draw off exactly 3.0 ml of the solution in a 5 ml pipette and transfer to a clean 18 x 150 mm test tube. Add 2.0 ml of ninhydrin - hydrindantin and mix the solution immediately on a tube buzzer.

8. Heat the tubes in boiling water bath for 20'. Remove the tubes from the bath.

9. Cool the solution rapidly to <u>room</u> temperature in a water bath. Pipette 1.0 ml of the solution into a clean dry test tube into which has already been added 5.0 ml of ethanol - water mixture.

10. Measure the extinction of the solution in a 1 cm cuvette @ 5700Å.

Calibration:

Five Kjeldahl tubes are required. Three of them are filled with 25 ml of 150 ug - N/l standard solution and

two of them are filled with 25 ml of distilled water. They are all treated by the method given previously for samples. The F factor is then calculated:

$$F = \frac{50}{E_s - E_b} = \underline{ca}.\ 10$$

where E_s = mean of the extinctions of the 3 standards

E_b = mean of the extinctions of the 2 blanks

The concentration of nitrogen in the sample is given by:

$$\text{Concentration} = F \times (E_s - E_b)\ \text{ug - N/l}$$

It is highly recommended that the precautions given by Strickland and Parsons (31) be strictly observed. Both of their methods for 'Soluble Organic' and 'Particulate Organic' nitrogen analysis should be reviewed before attempts are made to do the determinations.

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