COMPARISON OF NITROGEN FIXATION RATE ESTIMATION BY NITROGEN-15 UPTAKE AND ACETYLENE REDUCTION METHODS IN FRESHWATER BLUE-GREEN ALGAE

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BY

BARBARA GRAHAM

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ABSTRACT

The relationship of acetylene reduction to nitrogen-15 uptake was investigated for blue-green algal populations in three lakes in the Experimental Lakes Area of northwestern Ontario and two laboratory chemostats. Nitrogen fixation rates as estimated with both techniques were compared and acetylene to nitrogen ratios defined. Lake ratios ranged from 6.3 to 9.7 moles of acetylene reduced per mole of nitrogen fixed depending upon the method of calculation. The chemostat acetylene to nitrogen ratios varied between the chemostats and with the method of determination, but in all cases exceeded the theoretical value of three. Explanations were proposed for the discrepancies between theoretical and empirical ratios, including hypotheses of excretion of assimilated nitrogen-15 labelled material and interference from nitrogenase-mediated hydrogen production. The relationship of the substrate and analogue was further characterized by investigation of the response of the two techniques to increased epilimnetic depth, seasonal variation, dark incubation, extended incubation and varied substrate (analogue) concentration.

To My Family

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INTRODUCTION

Historical

Following the development and use of the acetylene reduction technique (Dillworth, 1966; Schollhorn and Burris, 1966; Hardy and Knight, 1967) as an alternate to direct estimation procedures utilizing labelled (nitrogen-15) or unlabelled molecular nitrogen, a rapid increase in the number of studies estimating bacterial, algal and plant nitrogen fixation was noted (for example, Hardy et al., 1971, 1973; Stewart et al., 1968; Klucas, 1969; Rice and Paul, 1971; Blasco and Jordan, 1976). Though considerable information accumulated which characterized the response of acetylene reduction to various environmental and physiological parameters (Stewart et al., 1968; Hardy et al., 1968, 1973), relatively little effort was expended to definitely establish the relationship between the nitrogen fixation process and the acetylene reduction process assumed to accurately quantify it. To derive an estimate of the rate of nitrogen fixation from acetylene reduction rate data, it was necessary to incorporate a rate conversion factor based upon the stoichiometry of the two chemical reaction equations involved. The basic reaction equations follow:

$$N_2 + 3H_2 \rightleftharpoons 2\ NH_3$$

$$3C_2H_2 + 3H_2 \rightleftharpoons 3\ C_2H_4$$

Theoretically, three moles of hydrogen (H_2) should be sufficient to reduce one mole of nitrogen (N_2) to two moles of ammonia (NH_3), or three moles of acetylene (C_2H_2) to three moles of ethylene (C_2H_4). Therefore, most acetylene reduction derived nitrogen fixation rates reported were calculated assuming the theoretical 3:1 conversion factor, usually without empirical verification of the applicability of this ratio to the system

examined.

Preliminary acetylene reduction—nitrogen-15 uptake rate comparison studies, summarized for various nitrogen fixing populations, demonstrated an acetylene to nitrogen ratio ranging from 1.7 to 8.6 (Hardy et al., 1973). Rather than abandon the analogue-based technique because of this variability, detailed determination of the relationship for given systems appeared warranted. Moreover, sensitivity, speed, ease, economy, and ready adaptability to field assay conditions (Hardy et al., 1968; Stewart et al., 1967), prompted retention of the method. Routine use of the more cumbersome nitrogen-15 uptake method (Ness et al., 1962; Stewart, 1967; Keeney and Tedesco, 1973; Flett, 1977) required a significantly larger investment in equipment (a mass spectrometer or emission spectrometer being essential) and analysis time, both critical aspects in experimental design. Use of the radioactive nitrogen-13 isotope was not found to be a feasible alternative in field studies due to its extremely short half-life (ten minutes) and the involved method of ^{13}N generation via proton bombardment of carbon-13, but it was used in laboratory autoradiographic studies (Wolk et al., 1974).

Preliminary investigation of the relationship of acetylene reduction to nitrogen-15 fixation has stimulated questions as to the universality of the theoretical value independent of the organism and the environment in which nitrogen fixation was being monitored (Bergersen, 1970; Peterson and Burris, 1976). Ratios in excess of theoretical were most frequently observed, indicating either an overestimation of nitrogen fixation by the acetylene reduction method, an underestimation by the nitrogen-15 uptake method or a combination of these two effects. Establishment of the discrepancy led to the proposal of various explana-

tions for its existence.

In accord with the theory of nitrogen-15 uptake rate underestimation, were reports of significant excretion of assimilated nitrogen by blue-green algal cultures (Fogg, 1966; Walsby, 1974; Walsby and Fogg, 1975; Stewart, 1963). In studies where an extended incubation period was involved, labelled nitrogen loss via excretion thus required investigation as a possible source of the ratio discrepancy.

The concept of acetylene reduction overestimation in in situ nitrogen fixation was supported by evidence indicating that only forty to sixty per cent of the electrons supplied to the nitrogenase were utilized in the reduction of nitrogen to ammonia (Schubert and Evans, 1976). The remainder of the electrons channelled into an acetylene-inhibited proton reduction process, also mediated by the nitrogenase, were available for acetylene reduction but not nitrogen fixation. Acetylene reduction estimates would then overestimate in situ nitrogen fixation by an amount equal to normal nitrogenase hydrogen production. Hydrogen production was observed both in intact nodules, bacteria and algal cells, as well as in prepared enzyme extracts. Therefore, investigations concerning the relationships of the three molecules reduced by nitrogenase, nitrogen, acetylene and hydrogen (Bothe et al., 1977; Schubert and Evans, 1976; Jones and Bishop, 1976; Smith et al., 1976; Schubert et al., 1977; Evans et al., to be published) will assist in the explanation not only of ratio magnitude, but also ratio diversity from system to system.

Research Completed

In the Experimental Lakes Area in northwestern Ontario (Cleugh and Hauser, 1971), the acetylene reduction method was employed to estimate the contribution of freshwater blue-green algal nitrogen fixation

to the nitrogen budget of artificially eutrophied lakes (Flett, 1977). Nitrogen budget calculations were made assuming the theoretical relationship of acetylene reduction to nitrogen fixation, though preliminary evidence for its inapplicability was provided (Flett, 1977). To correctly quantify nitrogen fixation contributions, calibration of the acetylene reduction method with the nitrogen-15 uptake method, a direct estimate of nitrogen fixation, was undertaken. Acetylene reduction and nitrogen-15 uptake rates estimated in the epilimnetic region of three lakes in this region were compared to determine whether a consistent ratio relating the two methods could be established. Were a reasonably reproducible acetylene to nitrogen ratio definable for each lake, a single ratio representative of these lakes and similar environments could then be calculated. Adoption of an empirically-verified ratio would eliminate the necessity of repeated employment of the more involved nitrogen-15 uptake technique in the field.

Investigation of the acetylene to nitrogen relationship in two chemostat-maintained blue-green algal populations was a secondary program completed in conjunction with the lake rate comparison experiments. The chemostats were designed simply as convenient reservoirs of active nitrogen fixing cells, not as rigid lake analogues. The high rates of nitrogen fixation attainable in the chemostat permitted rate comparisons at nitrogen fixation levels unachieved by lake blue-green algal populations. An acetylene to nitrogen ratio was calculated for the individual chemostats, as well as for the combined chemostat data set. Relative differences between chemostat ratios and chemostat and lake ratios were considered.

Methodological and biological explanations for ratio discrepan-

cies both from theoretical predictions and from system to system were proposed based upon empirical evidence collected in this study and previous studies. Excretion or filtration-associated loss of ^{15}N-labelled assimilation products (Walsby and Fogg, 1975; Fogg, 1966; Stewart, 1963), and the possibility of active nitrogen-hydrogen substrate competition (Schubert and Evans, 1976; Bothe et al., 1977; Smith et al., 1976) were the most productive hypotheses.

Additional characterization of the relationship between acetylene reduction and nitrogen fixation, independent of the direct rate comparison, was also attempted. Epilimnetic depth profile, time course, substrate-velocity, and dark fixation experiments were completed to demonstrate to what degree the responses of acetylene reduction and nitrogen-15 uptake are similar.

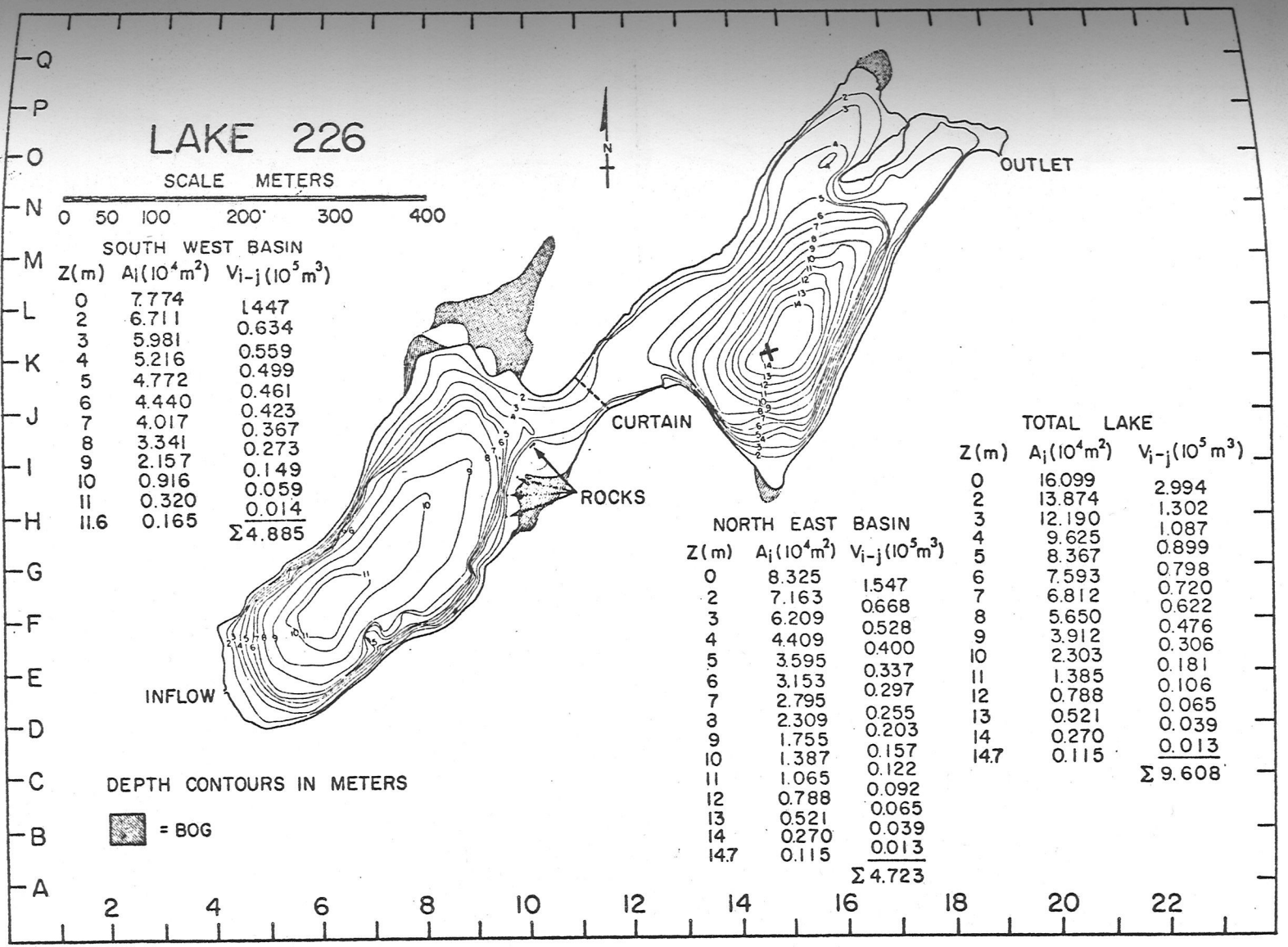
METHODS

Description of Lakes Sampled

Identification of the characteristics of the lake systems involved in acetylene to nitrogen ratio development was necessary if extrapolation of calculated ratios among similar systems was to be contemplated. At various times throughout the sampling season three lakes in the Experimental Lakes Area—Lake 226 NE, Lake 227 and Lake 304 (Figures 1 to 3)—supported blue-green algal populations capable of maintaining nitrogen fixation rates sufficient for rate comparison studies. All three lakes received fertilizer additions whose nitrogen, phosphorus and carbon content was regulated, depending upon the aspect of the eutrophication process to be investigated. During 1976 and 1977, when this study was being completed, the fertilizer ratios for Lakes 226 NE, 227 and 304, respectively, were 10:5:1 (C:N:P); 5:1 (N:P) and 10:1 (N:P) (Schindler, 1977). Blue-green algal bloom development, as a result of these additions, was most successful in Lake 227 in 1976 and 1977. Previously, Lake 226 NE, receiving the same proportion of nitrogen and phosphorus as in 1976 and 1977, supported a much larger Anabaena sp. population (Flett, 1977, Schindler, 1974). Both Lake 226 NE and Lake 304 exhibited evidence of the blue-green algal development in late 1976 and 1977, but acetylene reduction and nitrogen-15 uptake assays revealed relatively low levels of nitrogen fixation. As a result, phytoplankton net haul concentration was adopted to enable acetylene to nitrogen ratio determinations at more well-defined rates.

Aspects of the physical chemistry of these lakes are summarized in a series of figures (Figures 4 to 11) prepared from data collected during intervals representative of peak sampling activity (J. Prokopowich, E.L.A. Chemical Data). The relatively low inorganic nitrogen

Figure 1. Bathymetric map of Lake 226 NE prepared by Experimental Lakes Area personnel.



SOUTH WEST BASIN

Z(m)	$A_i(10^4 m^2)$	$V_{i-j}(10^5 m^3)$
0	7.774	1447
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	Σ4.885

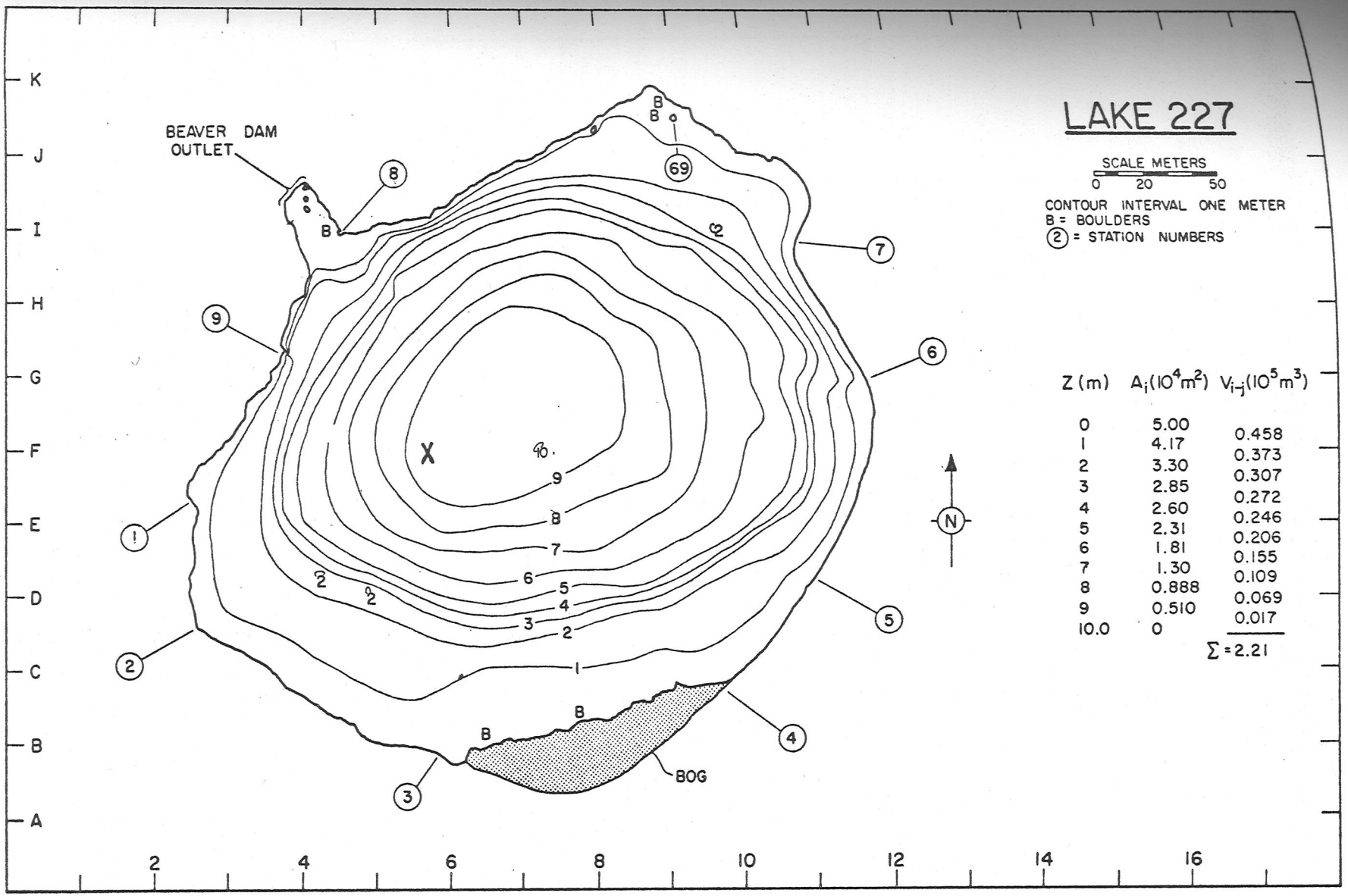
NORTH EAST BASIN

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	Σ4.723

TOTAL LAKE

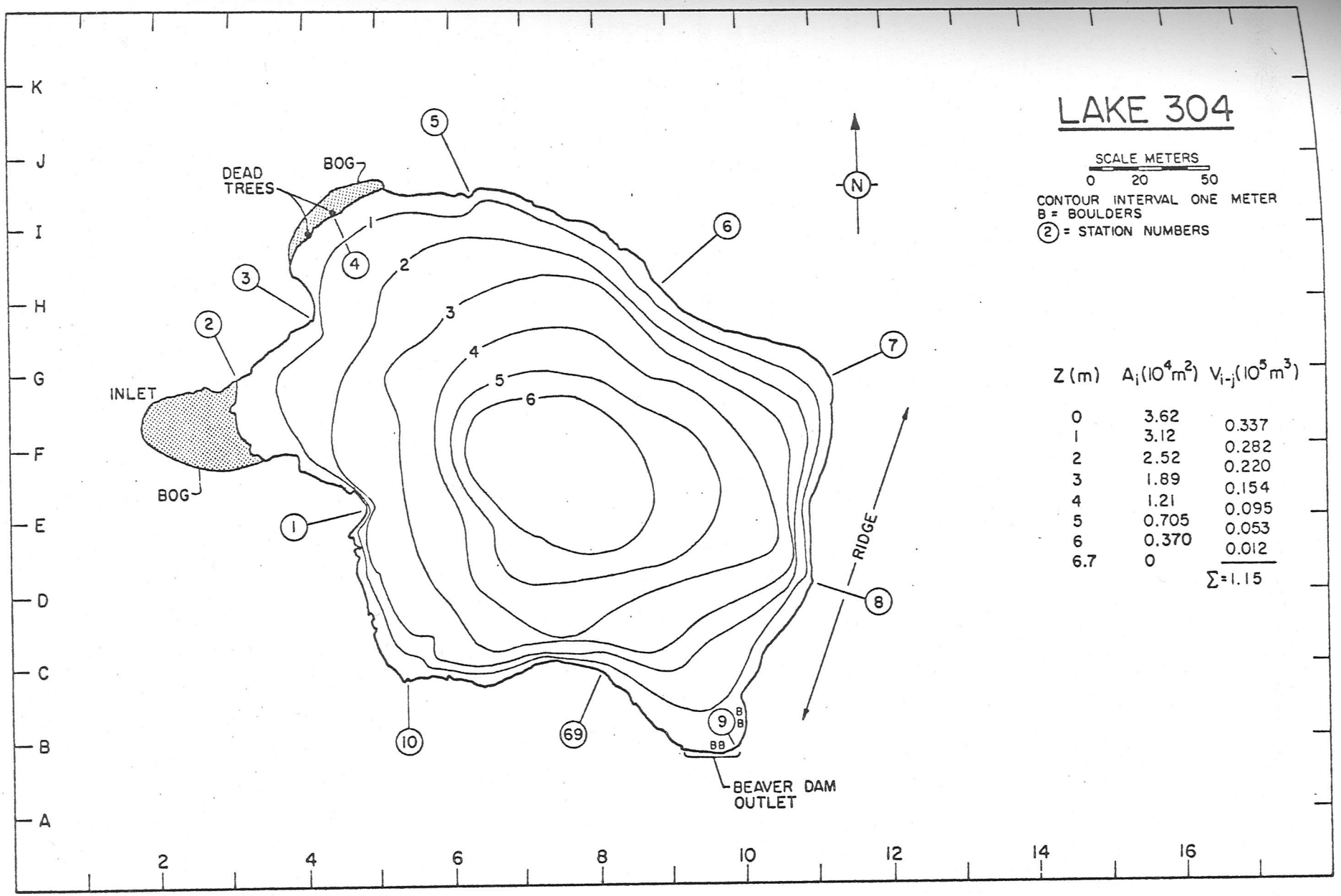
Z(m)	$A_i(10^4 m^2)$	$V_{i-j}(10^5 m^3)$
0	16.099	2.994
2	13.874	1.302
3	12.190	1.087
4	9.625	0.899
5	8.367	0.798
6	7.593	0.720
7	6.812	0.622
8	5.650	0.476
9	3.912	0.306
10	2.303	0.181
11	1.385	0.106
12	0.788	0.065
13	0.521	0.039
14	0.270	0.013
14.7	0.115	Σ 9.608

Figure 2. Bathymetric map of Lake 227 prepared by Experimental Lakes Area personnel.



Z (m)	$A_i(10^4 m^2)$	$V_{i-j}(10^5 m^3)$
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
		$\Sigma = 2.21$

Figure 3. Bathymetric map of Lake 304 prepared by Experimental Lakes Area personnel.



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	
		$\Sigma = 1.15$

Figure 4. Temperature (+) and oxygen (*) concentration depth profiles collected in Lake 227, August 31, 1976, (Experimental Lakes Area Chemistry Laboratory data).

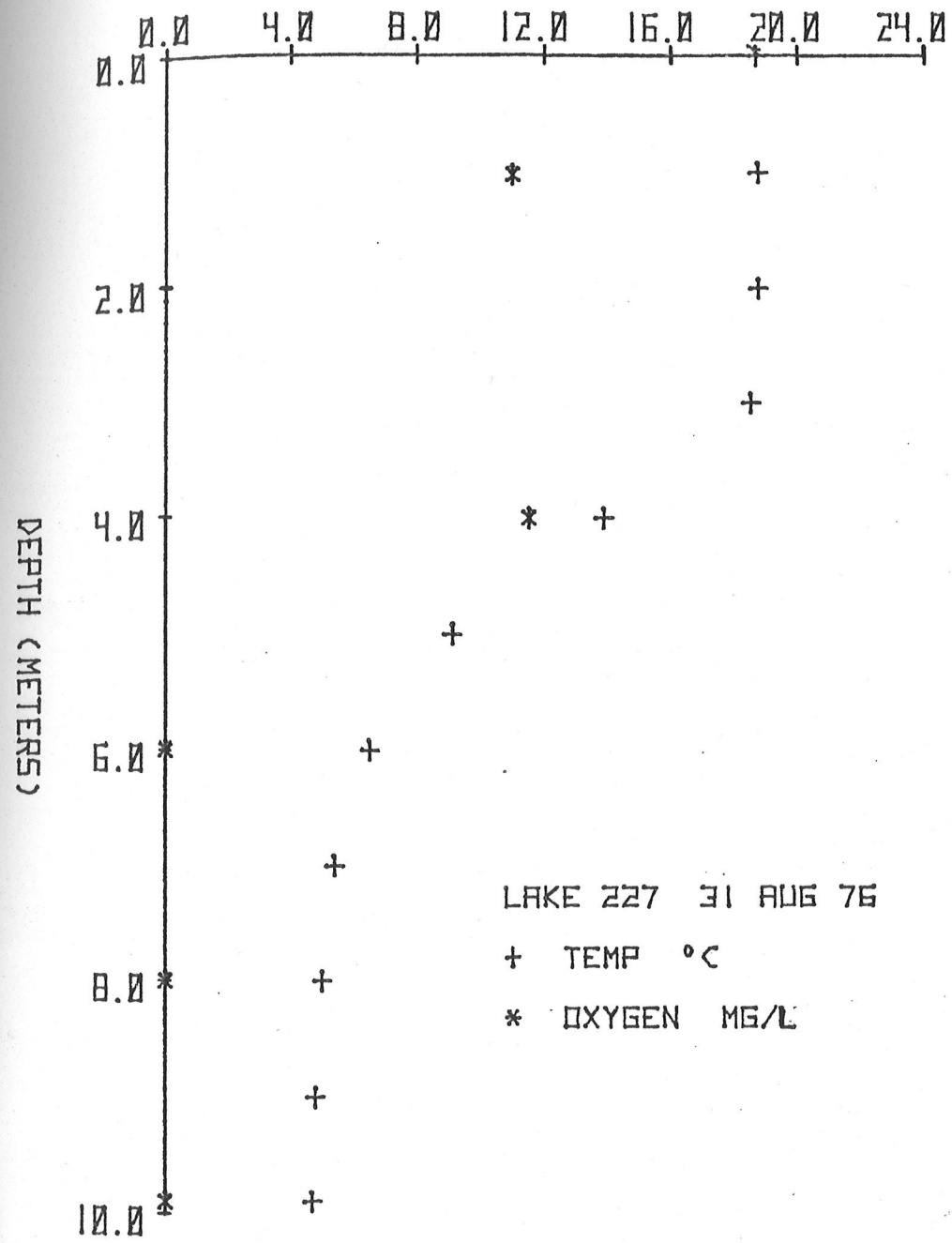

0.0
4.0
8.0
12.0
16.0
20.0
24.0
0.0
2.0
4.0
6.0
8.0
10.0
DEPTH (METERS)
LAKE 227 31 AUG 76
+ TEMP °C
* OXYGEN MG/L

Figure 5. Temperature (+) and oxygen (*) concentration depth profiles collected in Lake 226 NE, July 27, 1977 (Experimental Lakes Area Chemistry Laboratory data).

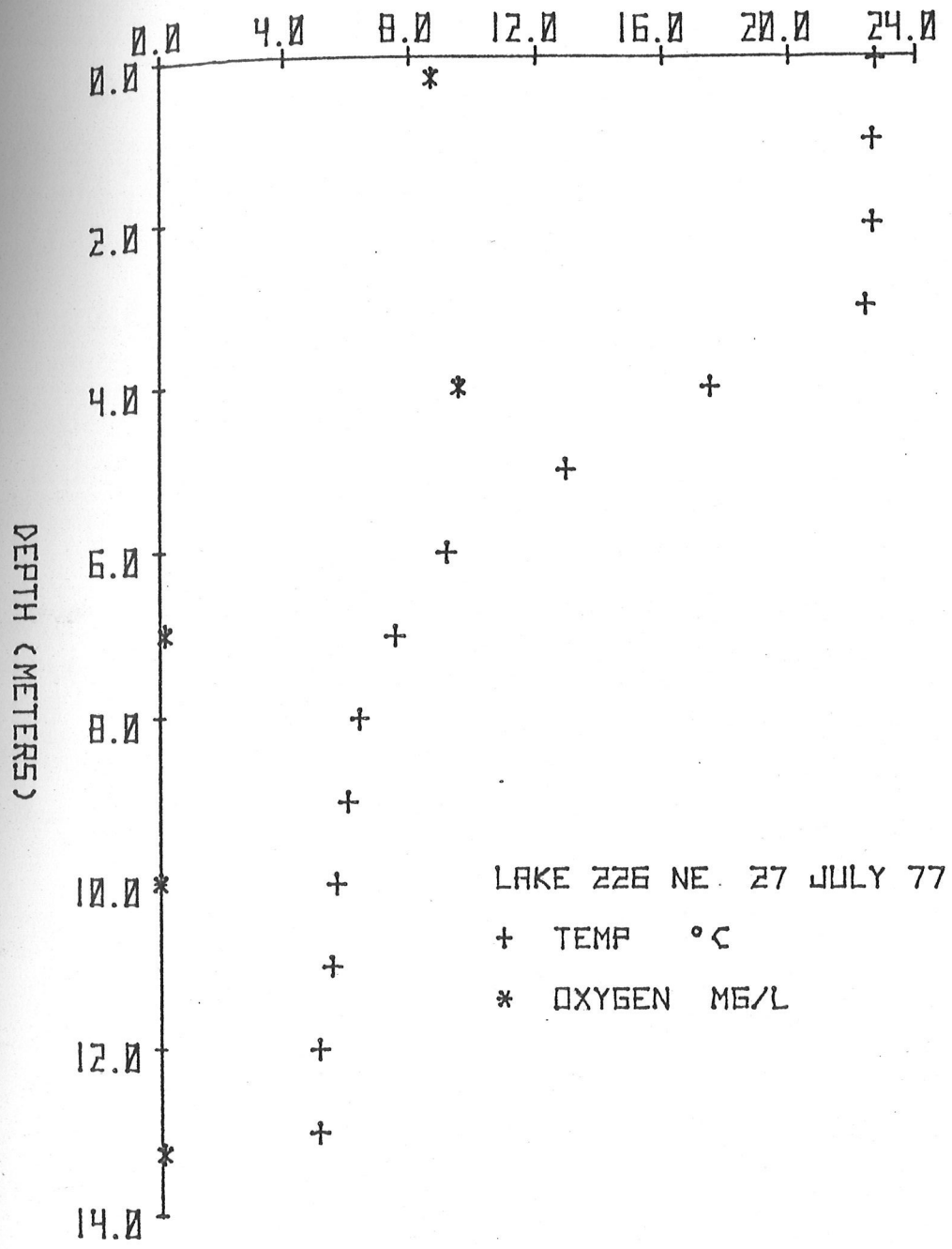

0.0
4.0
8.0
12.0
16.0
20.0
24.0
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
DEPTH (METERS)
LAKE 226 NE 27 JULY 77
+ TEMP °C
* OXYGEN MG/L

Figure 6. Temperature (+) and oxygen (*) concentration depth profiles collected in Lake 227, August 23, 1977 (Experimental Lakes Area Chemistry Laboratory data).

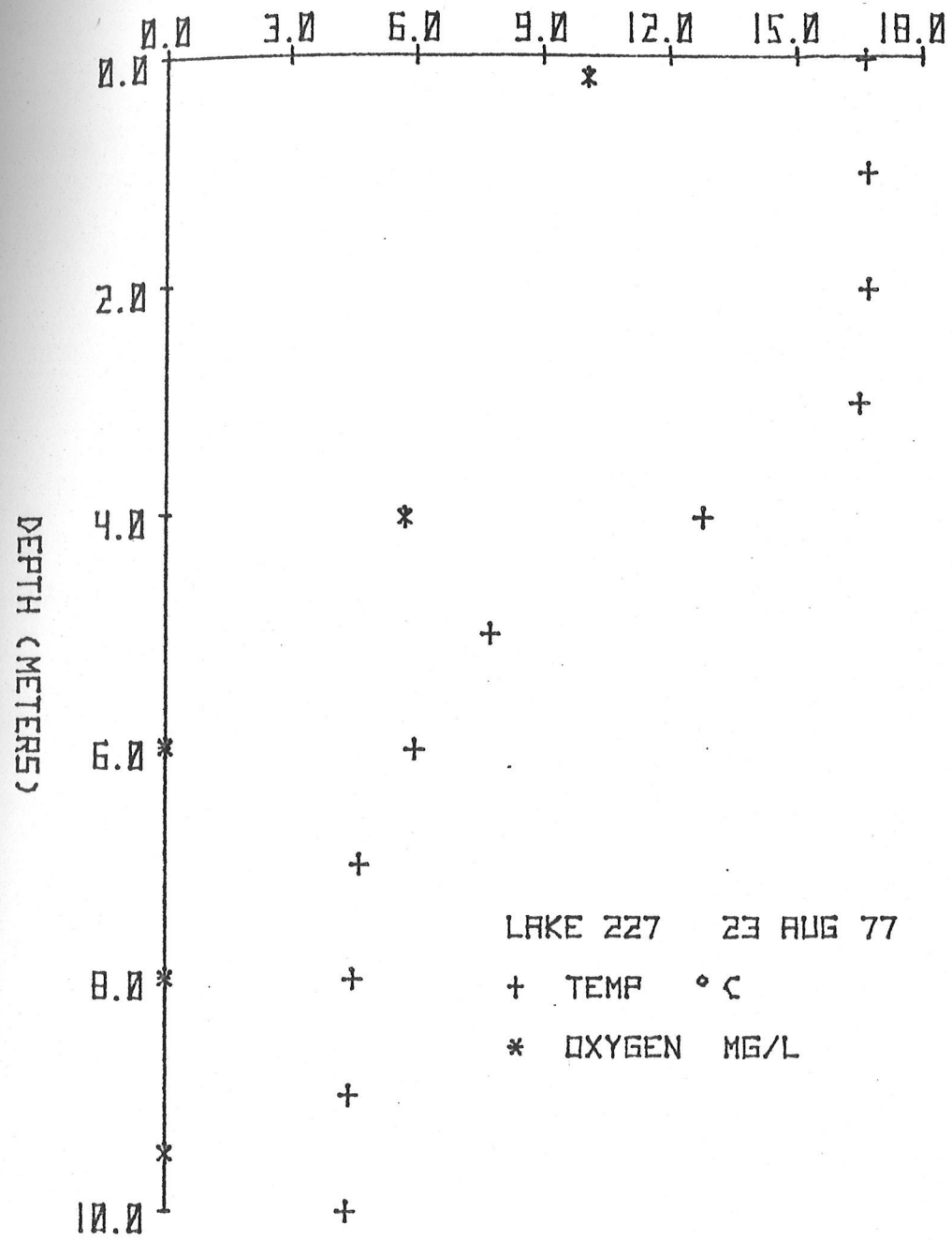

0.0
3.0
6.0
9.0
12.0
15.0
18.0
0.0
2.0
4.0
6.0
8.0
10.0
DEPTH (METERS)
LAKE 227 23 AUG 77
+ TEMP °C
* OXYGEN MG/L

Figure 7. Temperature (+) and oxygen (*) concentration depth profiles collected in Lake 304, July 5, 1977 (Experimental Lakes Area Chemistry Laboratory data).

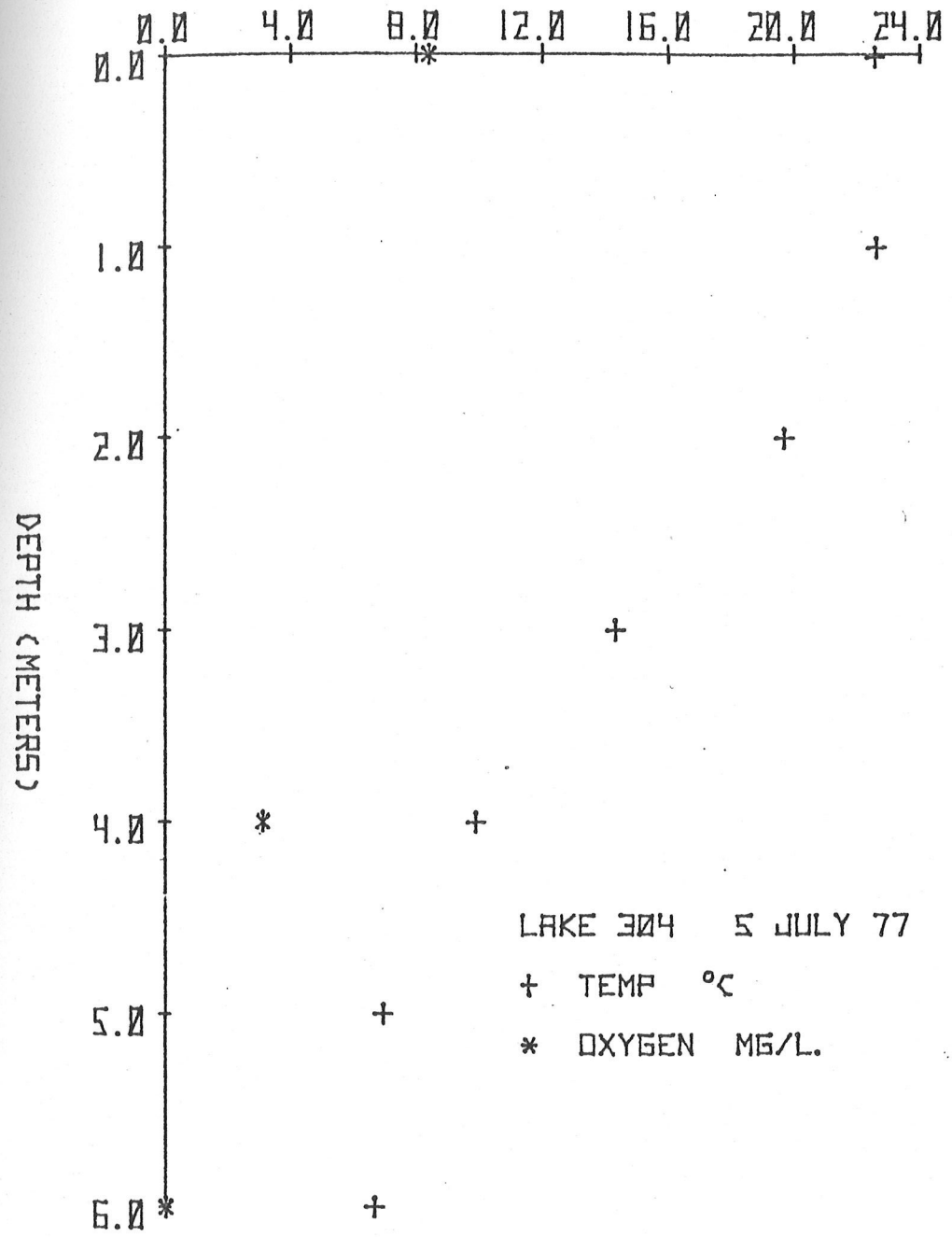

0.0
4.0
8.0
12.0
16.0
20.0
24.0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
DEPTH (METERS)
LAKE 304 5 JULY 77
+ TEMP °C
* OXYGEN MG/L.

Figure 8. Profile of nitrate (+) and ammonia (*) concentration with increasing depth in Lake 227; data collected August 31, 1976 (Experimental Lakes Area Chemistry Laboratory data).

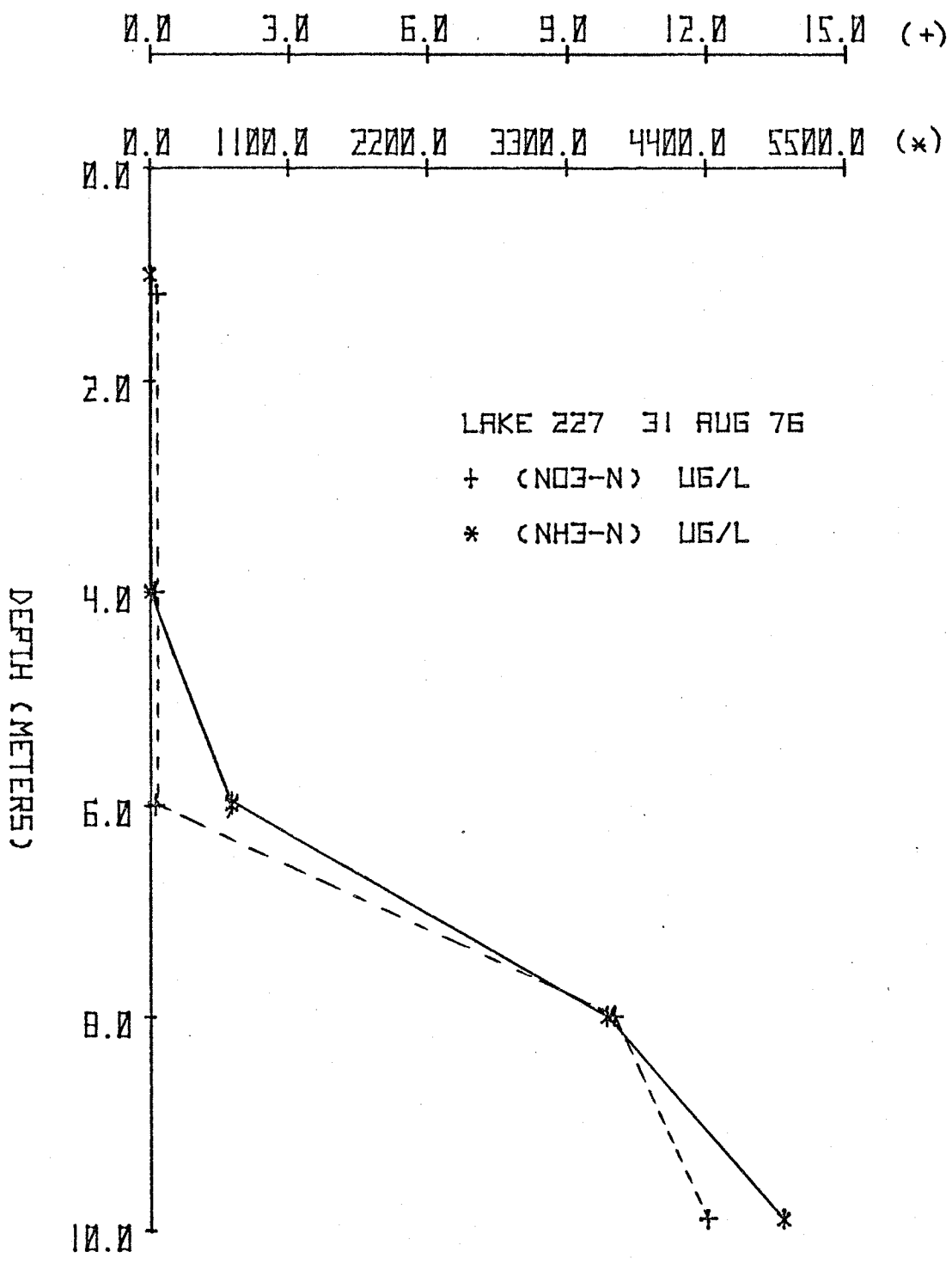

0.0
3.0
6.0
9.0
12.0
15.0
(+)
0.0
1100.0
2200.0
3300.0
4400.0
5500.0
(*)
0.0
2.0
4.0
6.0
8.0
10.0
DEPTH (METERS)
LAKE 227 31 AUG 76
+ (NO3-N) UG/L
* (NH3-N) UG/L

Figure 9. Profile of nitrate (+) and ammonia (*) concentration with increasing depth in Lake 226 NE; data collected July 27, 1977 (Experimental Lakes Area Chemistry Laboratory data).

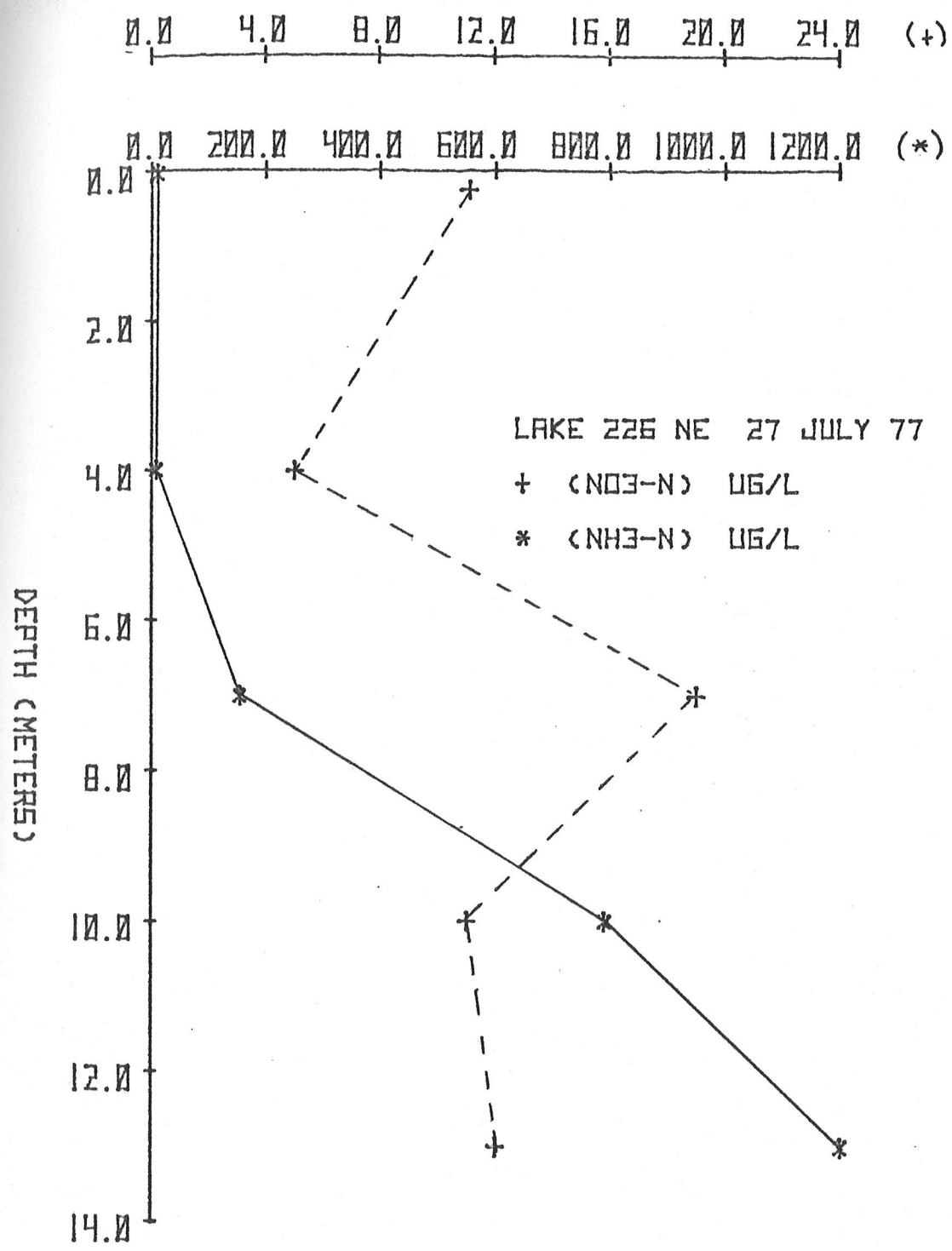
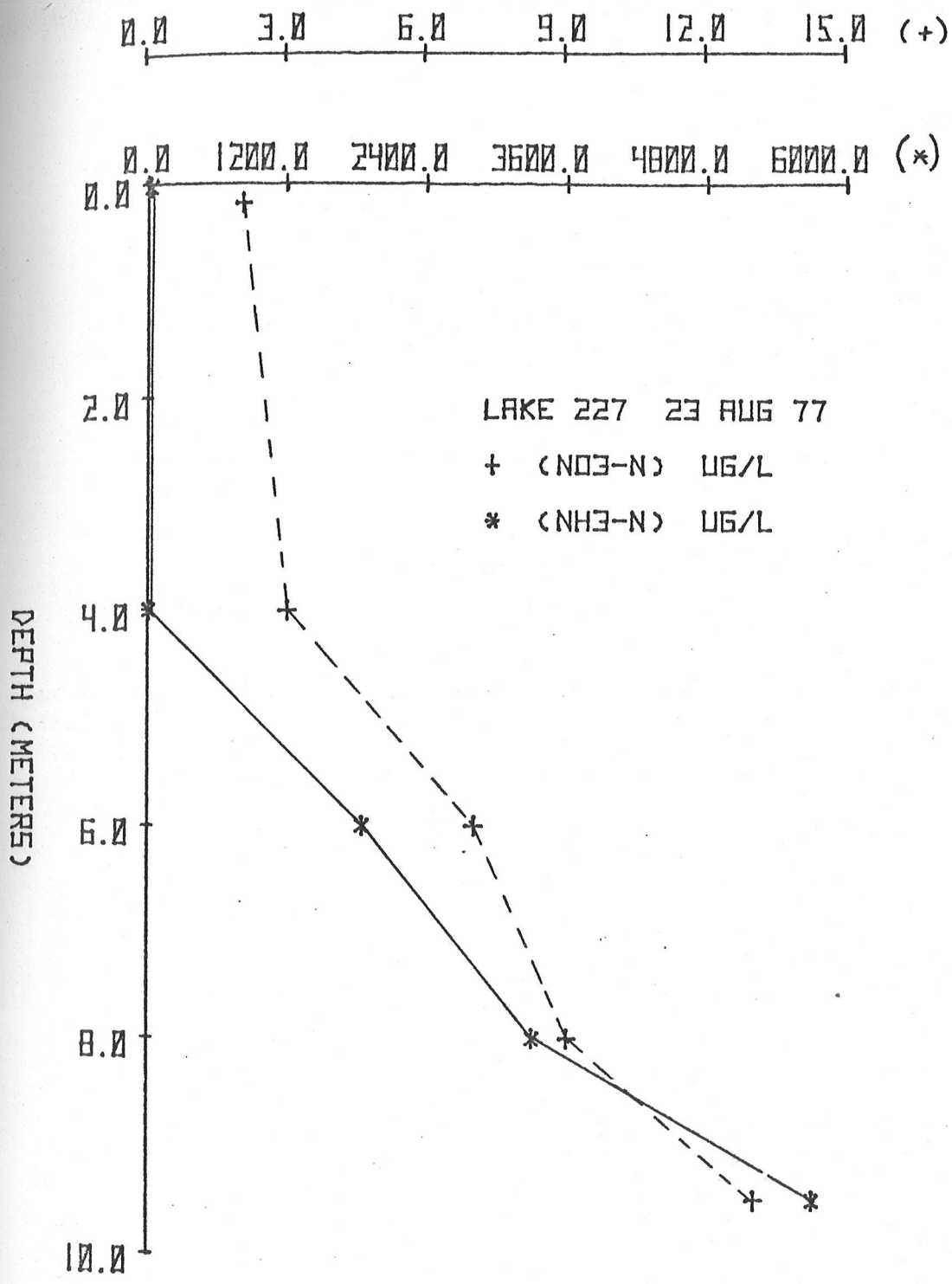
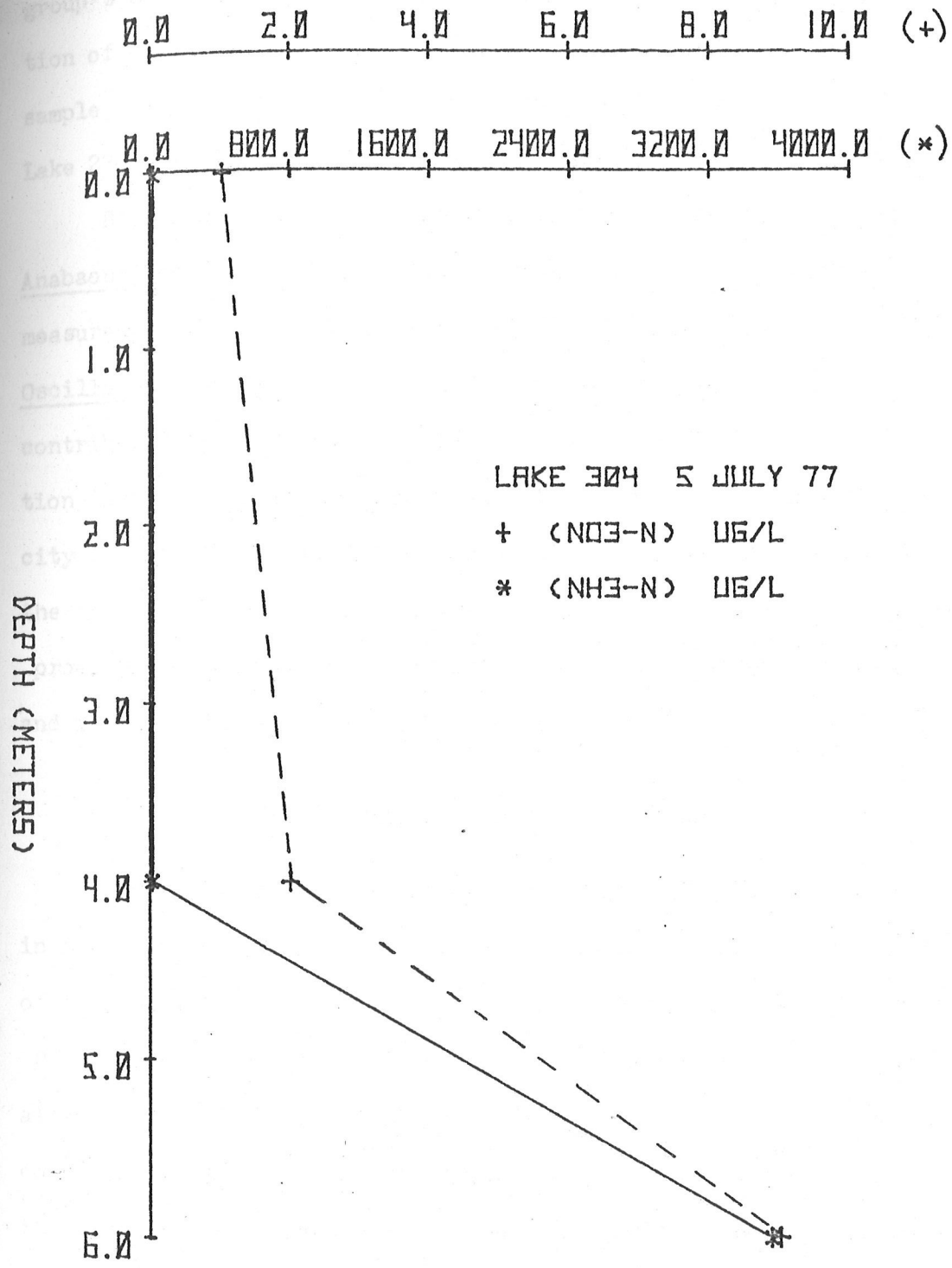

0.0 4.0 8.0 12.0 16.0 20.0 24.0 (+)
0.0 200.0 400.0 600.0 800.0 1000.0 1200.0 (*)
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
DEPTH (METERS)
LAKE 226 NE 27 JULY 77
+ (NO3-N) UG/L
* (NH3-N) UG/L

Figure 10. Profile of nitrate (+) and ammonia (*) concentration with increasing depth in Lake 227; data collected August 23, 1977 (Experimental Lakes Area Chemistry Laboratory data).



ncentra-
collected
istry

Figure 11. Profile of nitrate (+) and ammonia (*) concentration with increasing depth in Lake 304; data collected July 5, 1977 (Experimental Lakes Area Chemistry Laboratory data).


0.0
2.0
4.0
6.0
8.0
10.0
(+)
0.0
800.0
1600.0
2400.0
3200.0
4000.0
(*)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
DEPTH (METERS)
LAKE 304 5 JULY 77
+ (NO3-N) UG/L
* (NH3-N) UG/L

concentrations observed favored blue-green algal development; this group's ability to compete being improved by its capacity for assimilation of atmospheric nitrogen (Fogg, 1971; Fogg et al., 1975). Surface sample chlorophyll concentration measured on the same profile dates were Lake 226NE - 10.3 µg/L, Lake 227 - 28.3 µg/L, and Lake 304 - 8.4 µg/L.

Blue-green algae predominately of two genera, Aphanizomenon and Anabaena, were thought to be responsible for nitrogen fixation activity measured in these three lakes (D. Findlay, E.L.A. Phytoplankton Data). Oscillatoria redekeii, classified as a non-nitrogen fixing blue-green, contributed significantly to the algal biomass, but not to nitrogen fixation levels. Oscillatoria may exhibit a slight nitrogen fixing capacity if high levels of organic material enhance reducing conditions in the surrounding water (Mague, 1977). Blue-green algal activity was most prominent in May through July in Lake 304, in mid-July in Lake 226 NE and in late August and September in Lake 227.

Sample Collection - Lake Sampling

For the most part, sampling for nitrogen fixation determinations in Lake 226 NE, Lake 227, and Lake 304 was co-ordinated with knowledge of impending peaks in primary production (Figure 12 a,b) (J. Shearer and D. De Clercq, personal communication) and the appearance of blue-green algae as one of the dominant phytoplankton groups (D. Findlay, personal communication). Because primary production peaks reflected contributions by many algal groups, only one of which was the blue-green algae, primary production peaks were not always coincident with nitrogen fixation peaks.

In the early stages of the sampling season sparse blue-green algal populations and low nitrogen fixation activity levels prevented adequate

Figure 12. Seasonal variation in primary production (J. Shearer and D. DeClercq, Primary Production Data) and nitrogen fixation.

(a) Lake 304 - Primary Production (x—x); Nitrogen Fixation (o—o). Lake 226 NE - Primary Production (Δ--Δ); Nitrogen Fixation (+--+).

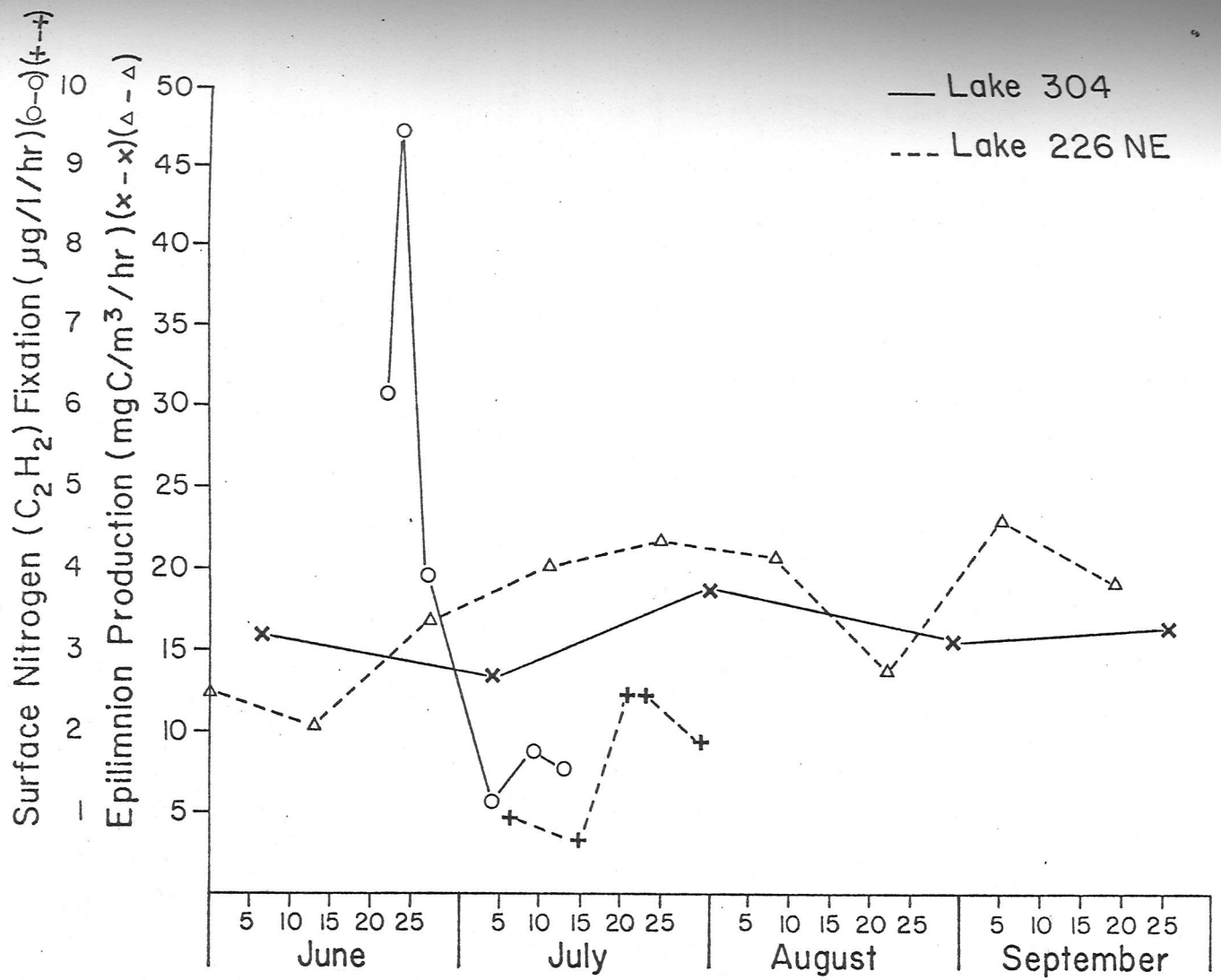
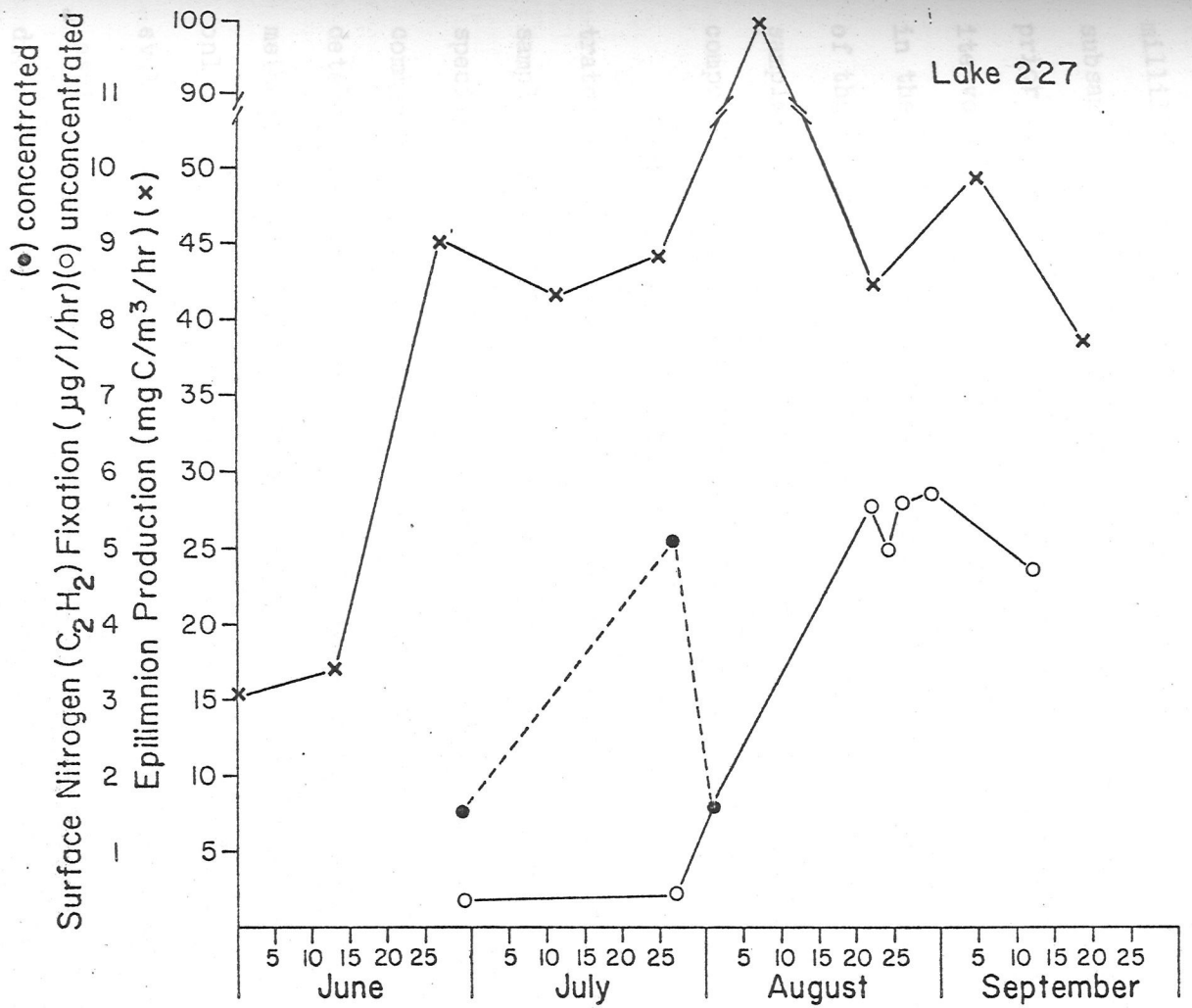

Surface Nitrogen (C_2H_2) Fixation (µg/l/hr)(o–o)(+–+)
Epilimnion Production (mg C/m³/hr)(x–x)(Δ–Δ)
Lake 304
Lake 226 NE
10
9
8
7
6
5
4
3
2
1
50
45
40
35
30
25
20
15
10
5
5 10 15 20 25
June
5 10 15 20 25
July
5 10 15 20 25
August
5 10 15 20 25
September

Figure 12. Seasonal variation in primary production (J. Shearer and D. DeClercq, Primary Production Data) and nitrogen fixation.

(b) Lake 227 - Primary Production (x—x); Nitrogen Fixation - Concentrated (•--•); Unconcentrated (o—o).


Lake 227
(•) concentrated
Surface Nitrogen (C_2H_2) Fixation (µg/l/hr)(○) unconcentrated
Epilimnion Production (mg C/m³/hr) (×)
100
90
50
45
40
35
30
25
20
15
10
5
11
10
9
8
7
6
5
4
3
2
1
5 10 15 20 25
June
5 10 15 20 25
July
5 10 15 20 25
August
5 10 15 20 25
September

methods comparison unless moderate sample concentration was employed. A ten micron mesh phytoplankton net, hauled through the zero to two and one half meter region of the lakes studied, yielded concentrated algal samples. Four to eight hauls, drained to one hundred to two hundred milliliters (mls), were combined in a one liter container from which subsamples could be withdrawn. The composite sample was well mixed, prior to subsample removal, and subsamples rarely depleted the composite volume by more than fifty per cent. The latter precaution assisted in the prevention of sample heterogeneity due to unbalanced reduction of the algal population with repeated subsampling. Thirty milliliter samples for acetylene and nitrogen assay were prepared from the same composite sample to ensure homogeneity among samples.

Examination of a number of parameters characteristic of concentrated and unconcentrated samples allowed estimation of the degree of sample concentration. Analyses completed included phytoplankton counts, specifically blue-green algal cell concentrations (D. Findlay, personal communication), chlorophyll concentration estimations, nitrogen weight determinations and nitrogen fixation rate comparisons (within a given method). Calculation of an approximate concentration factor to be used only as a qualitative indicator of the sample, not for nitrogen budget evaluation, was then possible (Table 1). Lake nitrogen budgets were not estimated because each lake was only sampled during a specific interval during the summer, when nitrogen fixation activity was sufficient for rate comparison studies. The average concentration factor determined for net haul samples collected on one day was less than ten.

As noted above, concentration of late summer blue-green algal populations in Lake 227 was unnecessary, as it had been during the September, 1976 sampling interval. Therefore, direct pump sampling from

TABLE 1

Estimation of Average Factors Describing the Extent of Lake Sample Concentration on Various Dates

		Concentrated/Unconcentrated Indicator					
Date	Lake	Chloro-a	Phyto-Plankton Counts	C_2H_2 Reduction Rate	$^{15}N_2$ Rate	Nitrogen Weight	Average for Given Date[a]
29/6/77	227	-	-	4.3	11.2		7.8
6/7/77	226	-	-			6.7	6.7
9/7/77	304	6.1	-				6.1
13/7/77	304	3.8	-			5.8	4.8
15/7/77	226	2.1	-	10.7	12.1	2.2	6.8
21/7/77	226	-	-	9.2	9.3	1.5	6.7
23/7/77	226	3.1	4.7	7.4	14.7	1.6	6.3
27/7/77	227	3.4	3.7			1.2	2.8
29/7/77	226			6.5	2.1	1.4	3.3

a The value listed is the average concentration factor calculated from the independent estimates for a given date (one horizontal row).

the depths desired was adequate to obtain a large sample ready for immediate subsampling. Tygon[1] tubing, sectioned into connectable meter lengths, was passed through a Cole-Parmer[2] peristaltic pump to facilitate sample collection. The final tubing section was weighted to assist in lowering the sampling hose and maintaining the sampling port at the desired depth. A styrofoam float ensured that the desired length of tubing remained suspended from the surface, compensating for natural fluctuations in lake level during the course of sampling, all depths being measured with reference to the lake surface. Subsequent subsampling procedures were identical to those for concentrated samples. However, the volume of unconcentrated sample prepared for nitrogen-15 uptake incubations was increased from thirty to fifty milliliters to ensure sufficient nitrogen for nitrogen-15 analysis, as will be discussed later.

Chemostat Preparation and Sampling

Chemostat studies were initiated in the attempt to define the acetylene to nitrogen ratio in a system with a blue-green algal biomass and nitrogen fixation activity level unattainable in the lakes examined. It was also thought that the excretion of assimilated nitrogen label, if significant, would be more apparent in such a well-defined system.

Two nitrogen-free, phosphorus-limited chemostats were constructed to provide sources of highly active, nitrogen-fixing blue-green algae (Figure 13). A nine hundred milliliter chemostat was constructed in a 20°C controlled temperature environment, with a bank of fluorescent lights as an energy source for algal photosynthesis. Filter sterilized air, bub-

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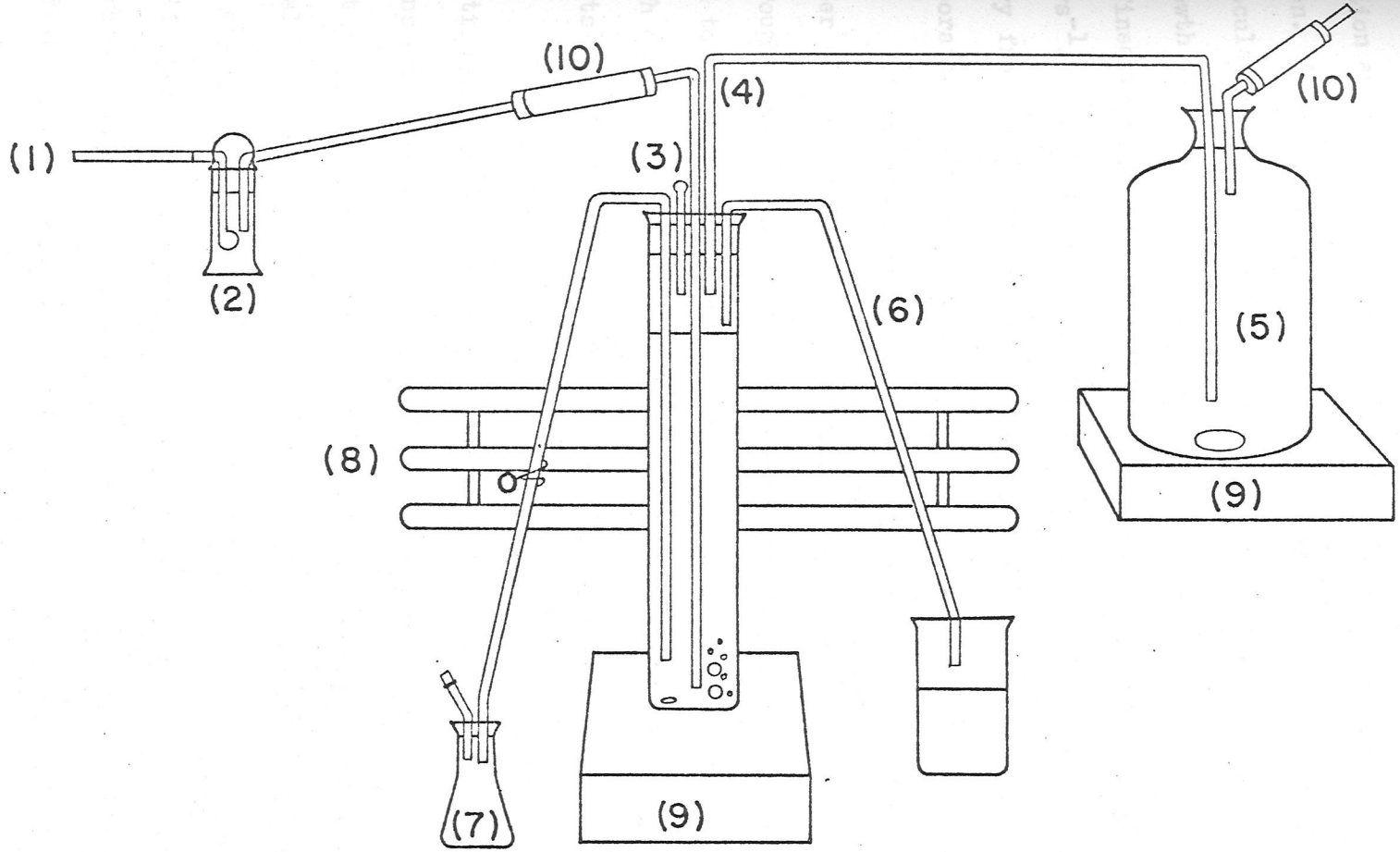
1. Tygon - T.M. of Northern Plastics and Synthetics Division, Akron, Ohio, U.S.A.

2. Cole-Parmer pump - Cole Parmer, Chicago, Illinois, U.S.A.

Figure 13. Schematic diagram of chemostat used for culturing Anabaena variabilis #21 population - (1) Air Inlet, (2) Sparger Flask to moisten air, (3) Injection Port, (4) Medium Inlet, (5) Medium Reservoir, (6) Overflow outlet, (7) Sampling Flask, (8) Fluorescent Light Bank, (9) Magnetic Stirrers, (10) Cotton Air Filter.


(10)
(4)
(10)
(1)
(3)
(2)
(6)
(5)
(8)
(9)
(7)
(9)

bled first through a moisturizing flask prior to passage through the culture, was the only input of nitrogen apart from nitrogenous decomposition and excretion products produced after prolonged chemostat operation. Anabaena variabilis #21 supplied by F. P. Healey was successfully inoculated into both chemostats. The WC medium which supported algal growth was modified for use in these chemostats, medium composition being defined in Table 2. Chemostat dilution rates ranged from 0.31 to 0.33 $days^{-1}$. Chemostat 1 was maintained in the Freshwater Institute Laboratory from March through early May, and Chemostat 2 in the E.L.A. field laboratory from June through September.

Algal growth in Chemostat 1 appeared uniform on visual examination after the initial adjustment period, though some wall growth was apparent throughout the chemostat's operation. The health of the culture was judged to be good on the basis of the bright green cell coloration and the high capacity for nitrogen fixation (Healey, 1973). Trypticase soy broth tests for bacterial contamination proved negative.

The facilities for operation of Chemostat 2 at the E.L.A. field station were less extensive, consequently influencing the growth conditions experienced by the Anabaena variabilis population. Slow development of the population and an unhealthy yellow-brown appearance of the algal cells were characteristic of this chemostat. Close proximity of the culture tube to the Gro-lux[1] light banks, in the confined space of a small incubator, was thought to contribute to this unhealthy response. Therefore, one of the two sets of bulbs was removed. Uniformity of algal distribution in the tube and the level of nitrogen fixation activity were observed to improve, though algal coloration was relatively constant. An

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1 Gro-Lux - Sylvani.

TABLE 2

Modified WC Medium Composition Used for Growth of Chemostat *Anabaena variabilis* Populations

Chemical	Concentrations(mM)
KH_2PO_4	0.15
$MgSO_4 \cdot 7H_2O$	2.00×10^{-3}
$CaCl_2 \cdot 2H_2O$	0.25
$NaHCO_3$	0.15
$Na_2SiO_3 \cdot 9H_2O$	0.10
Trace Elements	a
Vitamins	b
Tris Buffer (pH 7.5)	4.00
NaCl	1.00
KCl	0.05

a 40 mls of trace element solution (provided by F. P. Healey) per 8 liters of medium.

b 8 mls of vitamins solution (provided by F. P. Healey) per 8 liters of medium.

unhealthy yellow color has been attributed to pH elevation due to prolonged aeration of the culture (Nees et al., 1962), however, the Tris buffer system should have minimized pH changes. A decrease in photosynthetic pigments, including chlorophyll and photosynthetic biliproteins, observed to be a general response to nutrient deficiency in blue-green algae (Healey, 1973) may also have been the cause of the unhealthy coloration. Inefficient autoclaving facilities and contamination during medium reservoir changes and sample withdrawal prevented maintenance of the desired axenic condition of the culture.

Chemostat 2 chemistry was defined for an August 25, 1977 subsample (J. Prokopowich, personal communication), the data being summarized in Table 3. An August 18, 1977 sample showed similar chemistry. Lake 227 surface sample chemistry is provided as a comparison.

Chemostat subsampling was limited by the necessity of maintaining the resident Anabaena variabilis population close to a steady state condition. Withdrawal of approximately ten per cent of the total culture volume reduced the disruption of culture growth state, thus allowing daily sampling, if required. Marked fluctuations in culture optical density, a measure of cell concentration, were used to indicate steady state disruption. One hundred to one hundred and twenty milliliter volumes were usually collected from the internal rather than the overflow population following internal scrubbing of the culture tube walls with a magnetic stirring bar-external magnet combination. Medium influx, at the normal flow rate restored the initial resting culture volume after approximately seven hours. Preliminary measurements of algal nitrogen fixation capabilities had indicated that a ten milliliter subsample would provide more than adequate rates and nitrogen pressures (See Nitrogen Analyses discus-

TABLE 3

Analysis of Selected Chemical Species For
Chemostat 2 (August 25, 1977) and Lake 227 (August 23, 1977) Samples

Species	Chemostat 2 [b] Concentration (µg/L)	Lake 227 [c] Concentration (µg/L)
NH_3-N	18,400	6
TDN	65,000	460
TDP	6	10
CHLORO-a	16	28
SUSP N	2,000 [a]	499

a Vacuum guage estimate.

b August 25, 1977 sampling.

c August 23, 1977 epilimnion sampling.

sion) to allow meaningful methods comparisons. Ten milliliter algal samples were diluted to thirty milliliters with chemostat medium to facilitate use of easily measurable gas volumes and employment of available equipment. Dilutions were prepared directly in the fifty milliliter glass syringes used for sample incubation. To promote sample to sample homogeneity, the original sample was kept well mixed throughout the subsampling procedure. Homogeneity was determined by analysis of replicate subsamples for their respective nitrogen contents.

Ten milliliters of culture was pipetted into the stoppered syringe barrel following addition of twenty milliliters of chemostat medium. After insertion of the plunger and expulsion of captured air, the sample was shaken well, prior to acetylene or nitrogen-15 injection. Substrate injections were proceeded with only after completion of the preparation of all samples. Sample dilution was not found to affect rate estimation. Acetylene reduction rates measured for an undiluted sample and for samples diluted 1.5 and 3 times were 21.3, 13.8 and 7.2 μg ethylene produced/L/hr, respectively.

Sample Preparation and Analysis

Sample manipulation for acetylene reduction and nitrogen-15 uptake differed with respect to methods of substrate addition and sample analysis. Methods of substrate addition differed due to the nature of the substrates and the manner in which they were provided by the supplier. Analytical differences were intrinsic to the type of method. The acetylene reduction method measured a released gaseous product, while the nitrogen method estimated the incorporation of a labelled precursor into the particulate fraction. Therefore, each of the techniques will be independently des-

cribed.

Acetylene Reduction Method

A brief summary of the acetylene reduction methodology adopted for this study is outlined below, details having been provided previously by Flett (1976). The importance of consideration of the significant solubilities of both acetylene (C_2H_2) and ethylene (C_2H_4) in sample preparation and analysis is stressed.

Fifty milliliter glass syringes, filled to thirty milliliter sample volumes, were prepared, as described earlier, for injection of acetylene. A small MC refill acetylene cylinder[1], adapted for acetylene withdrawal (Flett, 1976), permitted addition of five milliliters of gas to each syringe. Acetylene was drawn in over the water sample, which was held vertically to prevent additions in excess of five milliliters, due to solubilization of the gas in the sample. Due to its characteristically high solubility in water (1.11 mls acetylene per ml water at 17°C and one atmosphere pressure) almost ninety-five per cent of the added acetylene rapidly dissolved on vigorous shaking for ten seconds. Acetylene was provided in excess to ensure successful competition with nitrogen for reductant and ATP stores (Hardy et al., 1973).

To determine the level of ethylene production achieved during the incubation, gas stripping procedures were necessary. Air stripping into fifteen milliliters total volume was found to be effective. Agitation for thirty seconds of the sample and the air phase introduced after incubation allowed equilibration of ethylene between the aqueous and the vapor phase. Sample temperatures were recorded during gas stripping

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1 Acetylene - Welder's Supply, Winnipeg, Manitoba.

procedures, since the gas solubilities and thus the gas equilibration between phases, was a function of stripping temperatures. Removal of the vapor phase was then accomplished by gas injection into a vented, thirteen milliliter serum bottle, filled with distilled water. Hydrogen flame ionization gas chromatography of 0.1 ml subsamples of this gas mixture provided measurements of ethylene concentration, which were calibrated with a specific 0.893 micromolar ethylene standard. The column packing used for ethylene determination was phenyl isocyanate/Porasil C[1] heated to 45°C.

Correction for ethylene, indigenous to the water sample, or present as a contaminant in injected acetylene, was achieved by running a sample which was air stripped immediately following acetylene injection. Sample ethylene concentrations greater than or equal to twice the background concentration, usually between 0.05 and 0.23 μM, were accepted as being significant. Gas composition analysis was completed within an hour of air stripping procedures.

Estimation of Acetylene Reduction Rates

After correction for ethylene present in the unincubated blank, sample ethylene chart units were converted to a micromolar concentration. The conversion factor applied was calculated from the chart units recorded for the 0.893 μM ethylene standard. A linear relationship between recorder chart units and a specific range of ethylene gas standards was determined. As a result of aqueous-vapor phase partitioning of ethylene and the solubility of ethylene in water (0.122 mls C_2H_4/ml H_2O at 20°C and one atmosphere pressure) the calculated concentration required cor-

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[1] G. C. Packing - Waters Associates, Framingham, Massachusetts, U.S.A.

rection for the fraction of ethylene produced which remained in the water sample following air stripping procedures. Estimation of the fraction transferred from aqueous to vapor phase was described by Flett (1976). Division by this fraction allowed calculation of the actual ethylene production within the entire sample, rather than simply the ethylene production measurable in the vapor phase.

The use of vapor phase and sample volumes in these calculations resulted in a figure presumed to represent the absolute amount of ethylene produced. Division by the time of incubation yielded the rate of ethylene production in μmoles C_2H_4 produced/L/hr, or equivalently, the μmoles C_2H_2 reduced/L/hr. The equivalence was determined by the stoichiometry of the chemical reaction equation for acetylene reduction described in the introduction. Assuming the theoretical acetylene to nitrogen ratio of 3, nitrogen fixation rates were estimated as μg nitrogen fixed/L/hr, after multiplication by the molecular weight of nitrogen, 28 μg/umole.

Nitrogen-15 Uptake Method

Following sample collection, nitrogen-15 uptake sample preparation and analysis differed from acetylene reduction methodology. Unlike acetylene additions, nitrogen-15 gas injections were not direct and were not under pressure. This was due to the nature of the nitrogen-15 gas reservoir[1], a 250 ml or 500 ml glass ampoule, sealed with a large rubber Suba Seal[2]. This type of reservoir closure facilitated subsampling by direct withdrawal of gas into 2.5 or 5 ml Glaspak[3] syringes. Depending

1 Nitrogen-15 gas - Onia-Azote et Produits Chimiques S. A., Paris, France.

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

3 Glaspak - Becton, Dickinson and Company, Rutherford, New Jersey U.S.A.

on the activity of the system studied, four levels of enriched nitrogen-15 gas were utilized for experiments in 1976 and 1977, including 49.9, 94.6, 97.1 and 98 atom per cent. Lack of pressurized injection of the labelled nitrogen-15, from the Glaspak syringe into the sample, caused displacement of a volume of sample equivalent to the injected gas volume.

Aqueous solubilities of acetylene and nitrogen differ significantly. At 17°C and one atmosphere pressure, nitrogen gas is 1.2% as soluble as acetylene. For this reason, only a small fraction of the nitrogen gas bubble introduced, immediately entered the solution. Attainment of gas exchange equilibrium required two to three minutes of vigorous shaking compared to the ten second agitation period required for acetylene samples.

Following parallel incubation with acetylene samples, the amount of the nitrogen isotope fixed in particulate matter was estimated. Filtration through 2.5 cm precombusted Reeve Angel[1] 984H glass fibre filter papers was used to collect the sample. Depending on the cell density of the sample, filtrations under low vacuum could require from thirty to sixty minutes for lake samples. Despite the algal density, chemostat sample filtration was rapid, requiring only two to three minutes for filtration of the thirty milliliter sample; presumably filter clogging by detrital or bacterial matter was much reduced. Filters were rinsed with five milliliters of distilled water, unless filter clogging was prohibitive, and then removed for slow drying at room temperature in a sealed dessicator containing a layer of silica gel dessicant. Removal of unincorporated label was unnecessary, since it was released with atmospheric exposure during filtration. Filtrates were collected and stored for isotope excretion studies, which will be discussed later.

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

Dried filters, which had been stored in the sealed dessicator for from one day to more than a week, were then analyzed for isotopic composition. The procedure and apparatus required were developed and described by Flett (1977). Briefly, what was involved was the oxygen-supported combustion of particulate material in a previously evacuated system, followed by reduction of oxidized nitrogenous compounds to molecular nitrogen.

The sample preparation system was composed of two sections. The combustion unit included a Lindberg Hevi-Duty[1] oven, maintained at 850°C, and a stainless steel combustion tube, with an inner sample chamber, capable of being connected to the evacuation source. A specially designed evacuated glass rack (Flett, 1977), maintained at 1 millitorr, through connection to a Welch Duo-Seal 1405 [2] or Sargent Welch [2] oil diffusion pump, provided the reducing conditions necessary for copper filing reduction of oxidized nitrogen compounds to nitrogen gas. Removable U-shaped liquid nitrogen traps intercepted gaseous contaminants, such as oxygen, water vapor and carbon monoxide, which interfered with isotopic measurements. Detailed sample combustion and oxidation procedures were outlined by Flett (1977).

Following preparatory steps, the nitrogen pressure, from which sample nitrogen weight was derived, was estimated with a Pirani[3] vacuum probe attached to the glass rack and to a Kinney[3] vacuum guage. Nitrogen pressures were recorded from the torr (1 torr = 1 mm mercury) or millitorr scales of the Kinney vacuum guage, prior to release of the gas

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

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

3 Pirani/Kinney Vacuum Guage-Kinney Vacuum Co.,Boston,Mass., U.S.A.

through a copper tubing link to the Statron[1] NOI-5 ^{15}N analyzer discharge tube. Ethylene diamine tetraacetic acid (EDTA) standards of known nitrogen weight, applied in aqueous solution to Reeve Angel filter papers, were used for calculating nitrogen pressure-weight conversions. A representative standard curve is shown in Figure 14. In practice, EDTA standardization was frequent due to instabilities in vacuum guage characteristics, which resulted in a standard curve intercept range of 0.04 to 0.09 torr and a linear slope range of 0.016 to 0.027.

Emission spectroscopy is the basis of Statron analysis of nitrogen isotope compositions of gaseous samples. Excitation of the entering nitrogen gas was initiated by 27.1 megacycle radiation, relaxations to lower energy states being monitored at specific wavelengths, for the molecules of interest. Nitrogen molecules with $^{15}N^{15}N$, $^{14}N^{15}N$ and $^{14}N^{14}N$ combinations were investigaged at 297.7 nanometers (nm), 298.3 nm and 298.9 nm respectively. A ratio of $^{14}N^{14}N$ to $^{14}N^{15}N$ peak heights from recorder output was calculated for use in determination of ^{15}N atom per cent enrichment. The formula utilized is atom % = $\frac{100}{2R+1}$, where R is defined to equal

$$\frac{(\text{Peak Height } ^{14}N^{14}N)\ (\text{attenuation } ^{14}N^{15}N)}{(\text{Peak Height } ^{15}N^{14}N)\ (\text{attenuation } ^{14}N^{14}N)}$$

A typical trace is provided in Figure 15.

To calibrate ^{15}N atom per cent output derived from analyzer traces, known ^{15}N atom per cent ammonium chloride standards were used to construct an actual versus observed atom per cent standard curve (Figure 16). The working range of enriched standards was designed to fall between

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1 P.G.H. Statron, Ehrenfried - Jopp - Str. 59, G.D.R.

Figure 14. Representative standard curve relating the measured nitrogen pressure (Torr) to the weight (μg) of nitrogen present in prepared ethylene diamine tetraacetic acid standards. Pressure-weight standard curves were prepared frequently.

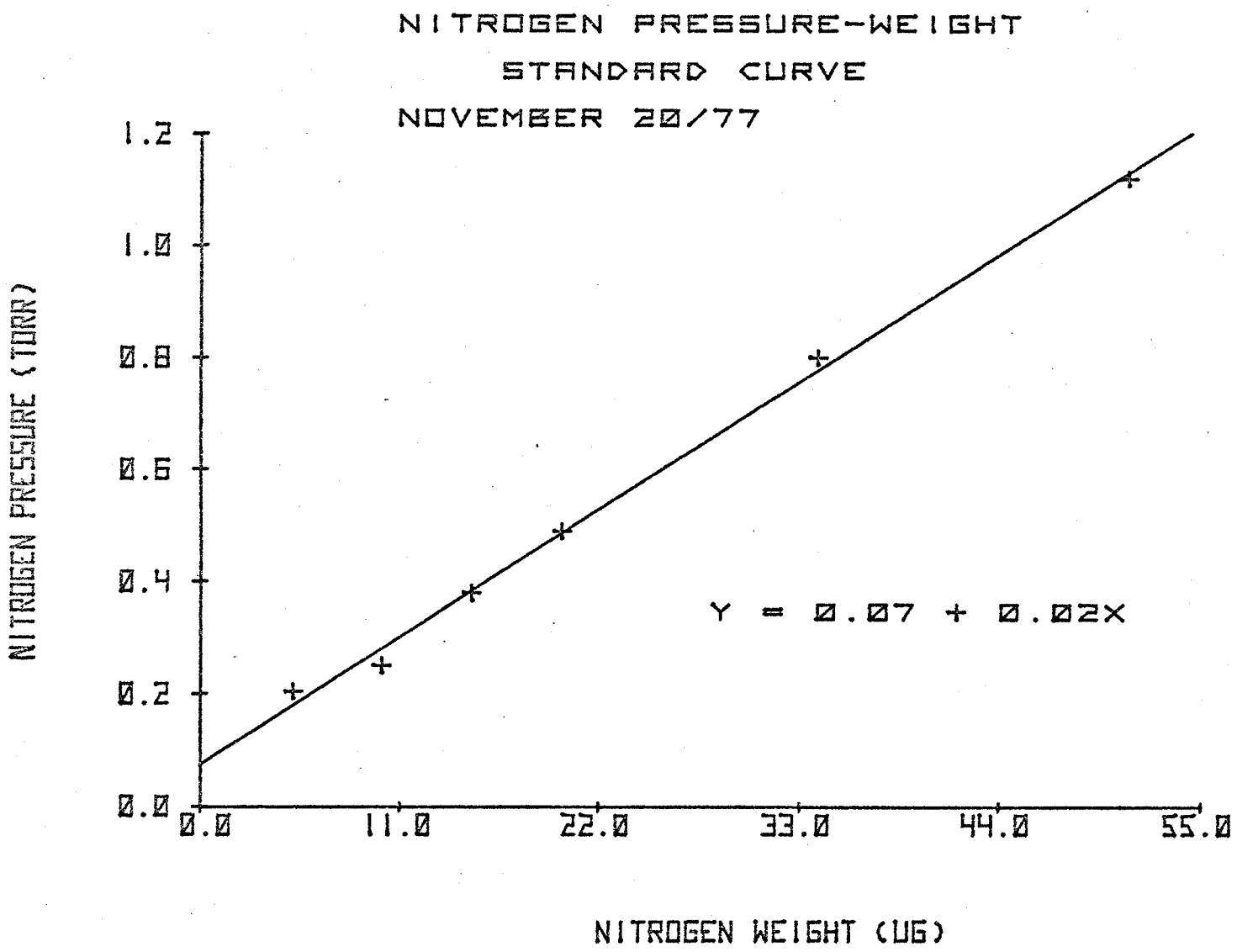

NITROGEN PRESSURE-WEIGHT
STANDARD CURVE
NOVEMBER 20/77
NITROGEN PRESSURE (TORR)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
11.0
22.0
33.0
44.0
55.0
Y = 0.07 + 0.02X
NITROGEN WEIGHT (UG)

Figure 15. Trace of a typical ^{15}N analyzer output obtained on analysis of a natural abundance sample. A shift in attenuation from 10,000x to 300x after the $^{15}N^{14}N$ peak is necessary due to the relative abundances of this molecule and the $^{14}N^{14}N$ molecule. Baseline construction is as described by Flett, 1977.

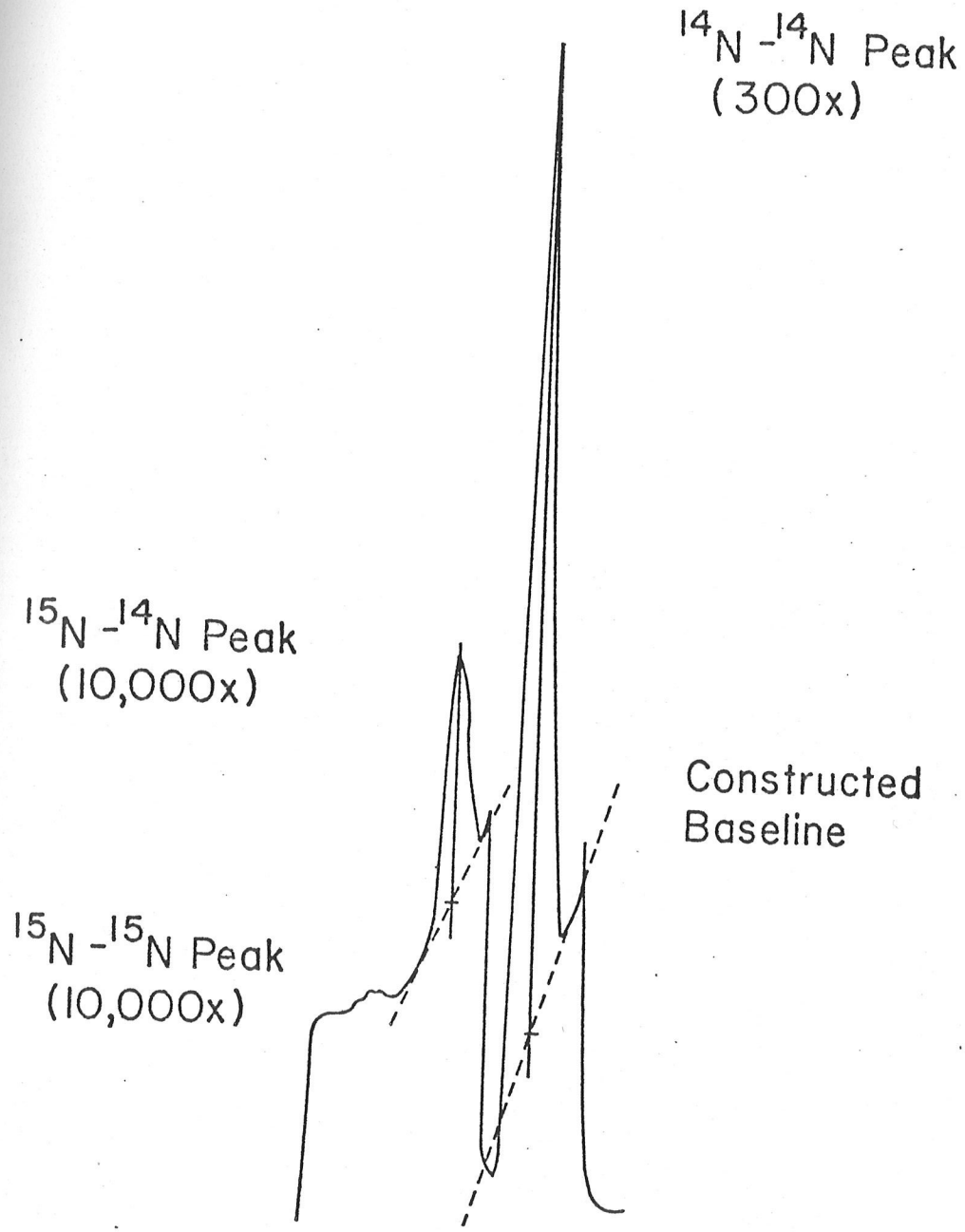
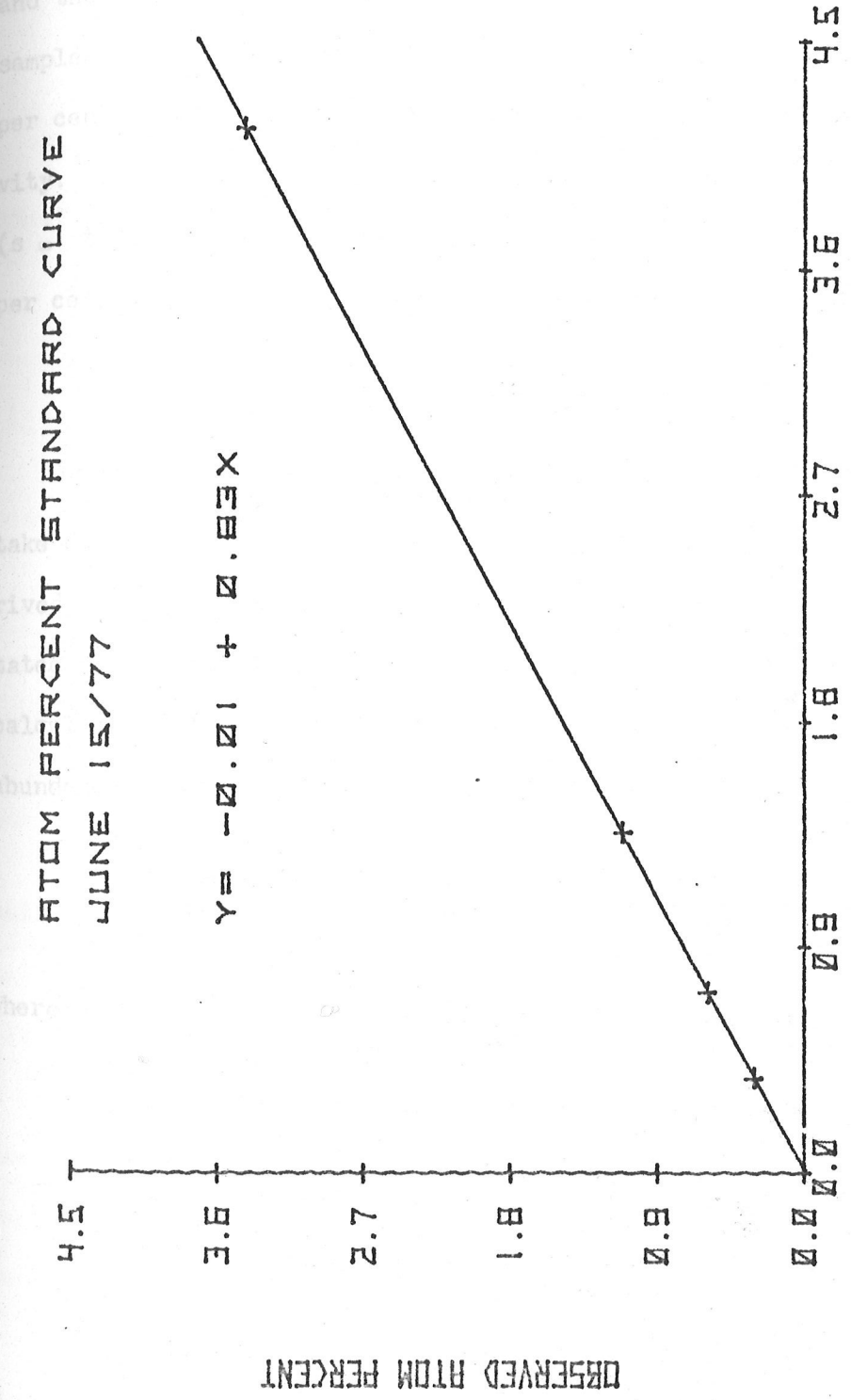

^{14}N - ^{14}N Peak
(300x)
^{15}N - ^{14}N Peak
(10,000x)
Constructed
Baseline
^{15}N - ^{15}N Peak
(10,000x)

Figure 16. Representative standard curve relating actual and observed ^{15}N atom per cent enrichment for four ammonium chloride standards.



actual and
or four ammon-

natural abundance, 0.367 atom per cent and 4.15 atom per cent, which encompassed any enrichment that would likely be attained with lake or chemostat incubations. An unincubated sample, injected with nitrogen-15 and then immediately filtered, served as a blank for each set of ^{15}N samples incubated. Sample atom per cent increases exceeding 0.034 atom per cent were accepted as indicative of positive nitrogen fixation activity. This significance level represents two standard deviations ($s = \pm 0.017$ atom per cent) from the mean natural abundance, 0.393 atom per cent, calculated for a series of unenriched samples.

Nitrogen-15 Uptake Calculations

Estimation of a nitrogen fixation rate from this nitrogen-15 uptake data required no theoretical conversion factor, the rate being derived directly from sample enrichment. The rate calculation necessitated prior determination of the sample specific activity, which involved calculation of the amount of artificial enrichment, plus the natural abundance level characteristic of any unenriched environment.

$$\text{Specific Activity} = \frac{(N)(V_1) + (E)(V_2)}{(V_1 + V_2)}$$

where: N = the fraction of ^{15}N prior to enrichment, i.e., theoretical natural abundance 0.367 atom per cent.

E = the fraction of ^{15}N characteristic of the enriched gas injected.

V_1 = volume (mls) of the unenriched molecular nitrogen dissolved in the water sample. This value was derived from nitrogen gas solubilities, sample temperature and aqueous volume, following ^{15}N injection.

V_2 = volume (mls) of injected nitrogen label.

V_1+V_2 = total volume of molecular nitrogen present in the aqueous-vapor system, (mls).

Utilization of the calculated specific activity along with the empirically determined sample ^{15}N enrichment, nitrogen weight and length of incubation, permitted estimation of the rate of nitrogen fixation in the following manner:

$$N_2 \text{ Fixation Rate } (\mu g/L/hr) = \frac{(A-B)/100\ (W)\ (L)}{(S)\ (H)\ \ (V)}$$

where: A = ^{15}N atom per cent enrichment of the filter prepared for a given sample.

B = background atom per cent determined from the unincubated blank.

W = weight of nitrogen estimated for the sample (µg).

L = mls per liter.

S = sample specific activity (a fraction, therefore no dimension).

H = length of the incubation interval (hrs.).

V = volume of the sample filtered (mls).

Sample Incubation - In Situ and Laboratory

Incubation, which was identical for both acetylene and nitrogen methods, involved suspending the syringes in the water column at desired depths, or placing syringes in a 20°C incubator in the laboratory. Attaching syringe clips to paired clamps, spaced at meter intervals along a polypropylene rope, allowed incubation of acetylene and nitrogen samples at the same depth in the lake and in similar orientations to the lake surface. The buoy from which the sample line was dropped was transparent glass to prevent shading of the suspended samples. Incubation periods of two to three hours were chosen on the basis of time course studies, which will be discussed later. The length of the incubation

was defined by the initiation and termination of light exposure.

Dark Rate Estimation

In addition to incubations carried out in the presence of a solar or fluorescent light source, some nitrogen fixation rates were measured for samples incubated in the dark. Acetylene and nitrogen methods were used to estimate dark fixation of nitrogen, and to observe whether or not the same ratio of acetylene reduction to nitrogen-15 uptake was found in dark and light incubations. Normally, dark samples were not pre-incubated in the dark, prior to acetylene or nitrogen-15 addition, sample preparation procedures being the same as for light samples, except for the lack of the final light exposure. Dark samples were left to incubate in the light-shaded sample box. To determine whether dark fixation was attributable to residual energy reserves of the algae present, some samples were pre-incubated in the dark for four hours prior to acetylene or nitrogen-15 addition. Fixation rates were then measured and compared to rates exhibited by illuminated samples run concurrently.

Experiments With Filtration Procedures

Filtration and drying procedures were found to be important to nitrogen-15 uptake rate determinations. In particular, the nitrogen weight derived from nitrogen gas pressure readings was examined in relation to the volume of sample filtered and the methods of filtration and drying. Samples from Chemostat 1 were filtered utilizing different degrees of vacuum and then air-dried or dried at varying distances from a heat blower. These filters were then combusted and passed through the vacuum rack preparatory system to obtain a nitrogen pressure reading. Increasing aliquots

of culture were also filtered to observe whether the pressures recorded increased in a predictable manner with respect to sample volume. These samples were prepared in duplicate so that a comparison might be made between two different estimations of nitrogen weight; carbon-nitrogen analyzer determinations of nitrogen (B. Hauser, personal communication) as opposed to nitrogen estimates derived from vacuum guage pressures.

Preparation of a filtration error curve (Arthur and Rigler, 1967) for nitrogen-15 uptake in Chemostat 2 samples was also attempted. A one hundred and twenty milliliter serum bottle, filled with sample from the second chemostat, was injected with 4.3 ml of 49.9 atom per cent nitrogen-15 gas. Following a two hour incubation, aliquots of increasing volume, from five to thirty milliliters, in five milliliter increments, were filtered through precombusted Reeve Angel filters. Filters were air-dried in a sealed dessicator, then analyzed for nitrogen weight and atom per cent enrichment for purposes of nitrogen fixation rate determination.

Filtrate Analysis

As was noted in the description of the ^{15}N method, filtrates of ^{15}N samples were recovered following filtration procedures. The filtrates were stored, at 4°C, in plastic or glass scintillation vials, until they could be processed. Storage for up to one month was not uncommon. Processing involved preparation of filtrate samples for ^{15}N analysis. Initially roto-evaporator concentration of filtrate samples at 50 to 55°C, was used as a preliminary to application of the concentrate to a precombusted filter. Roto-evaporation reduced the fifty milliliter filtrate volume to approximately two to three milliliters, which could be collected in a five milliliter Plastipak[1] syringe. A Reeve Angel filter was then

1 Plastipak-Becton, Dickinson and Co.Canada Ltd.,Mississauga, Canada.

repeatedly saturated and dried over a heat blower until all the concentrate had been applied. The temperature within the drying chamber was only 40 to 45°C due to the distance of the drying plate from the heat source.

This procedure was modified as filtrates with higher nitrogen contents were measured. Two alternate methods were adopted. One method involved application of one milliliter of unconcentrated filtrate directly to the filter using the same saturation and drying procedures. As a comparison, the rest of the filtrate was concentrated and then a 0.2 to 0.4 ml aliquot was used to saturate the filter, only once, before a final drying. The reason for the procedural modifications was that concentrates of chemostat and lake sample filtrates, when completely applied, yielded nitrogen pressures which exceeded the capacity of the vacuum guage and contaminated the combustion tube. The desirable filtrate nitrogen pressure fell between 0.3 and 1.0 torr, for which atom per cent characteristics were defined and consistent.

Filters prepared from selected filtrate samples were then analyzed in the same manner as particulate fractions were analyzed. The background ^{15}N enrichment for filtrate samples was established by analysis of the filtrate from the unincubated ^{15}N blank.

Time Course Determinations

Sample preparation for time course studies was identical to that required for normal rate comparison experiments. However, the length and the location of sample incubation, both laboratory and field, varied.

Acetylene Reduction Time Course

The *in situ* determination of the relationship between the amount of

acetylene reduced with increasing time was facilitated by the nature of the air stripping and product collection procedures involved. Samples could be readily stripped in the field and the gases injected into serum bottles, after various lengths of incubation. Incubations from fifteen minutes to four or five hours, at thirty minute intervals, were undertaken to illustrate the relationship over time intervals likely to be applied in day to day sampling.

Time courses were carried out with Chemostat 1 and Chemostat 2, Lake 304 concentrated samples, and Lake 227 unconcentrated samples. All samples for a given system and time course were prepared from the same composite sample. Chemostat samples were incubated in an illuminated 20°C incubator, while lake samples were incubated in situ, syringes being suspended from a horizontal rod 0.25 meters below the lake surface. Incubation was initiated on exposure of all samples to the light source and terminated when samples were stripped, immediately after removal from the light.

Nitrogen-15 Uptake Time Course

Due to procedural problems with sample filtration in the field, samples for nitrogen-15 uptake time courses were incubated in the laboratory-controlled temperature incubator. In this way, access to a filtration unit was rapid, so there would be no lag between removal of the sample from the light (the criterion for end of incubation) and filtration.

Both chemostat and lake time courses were performed and compared. Lake samples were prepared from two liter unconcentrated samples which were drawn from 0.5 meters, and returned to the laboratory in sealed, light-shielded glass containers. Similar to daily samples, nitrogen-15

injections were made, and samples were then incubated for selected time intervals. As with the acetylene reduction time courses, the range of time intervals (fifteen minutes to six hours) encompassed those likely to be used during normal sampling procedures.

Michaelis-Menten Relationships

Relationships of reduction rate and substrate concentration were examined for both substrate and analogue. The intent was to determine whether any differences existed in the uptake response of the blue-green algal population on exposure to either nitrogen or acetylene. Variability in the substrate-velocity response could possibly contribute to explanations of observed rate discrepancies. The concentration-rate experiments were defined independently for each of the substrates.

The Relationship of Acetylene Concentration and Nitrogen Fixation Rate

During the course of a normal assay, acetylene was present in excess so as to compete successfully with the natural substrate, nitrogen. Choice of this concentration was determined through selection of a substrate level corresponding to a plateau reduction rate.

Procedurally, the definition of this plateau region for acetylene reduction rates involved preparation of a series of substrate concentrations to be utilized under normal assay conditions. Substrate dilutions were prepared to obtain a final sample concentration range of 60 to 7500 micromolar. A preliminary experiment was run with a series of lower acetylene concentrations, which assisted in the final selection of a representative concentration range. Volumetric flasks, filled to the prescribed volume with air, were utilized for acetylene dilutions. Flask neckspace

was filled with water. Acetylene volumes to be injected were measured and stored in 1, 2.5, 5 and 10 ml Glaspak syringes. Desired gas volumes were injected into the dilution flasks, through rubber Suba Seal flask stoppers, following removal of an equivalent volume of air from the flask. For purposes of attaining gaseous equilibrium between the small aqueous and large vapor phase flasks were shaken for two to three minutes. This preliminary mixing was necessary for accurate calculation of gas partitioning between phases.

Samples were prepared from a two liter sample drawn from Lake 227, one to two hours prior to substrate injection. This sample was kept closed and light-shielded until subsampling was initiated. Normal subsampling procedures were pursued, however, the sample size prior to injection was thirty-five milliliters, rather than thirty milliliters. The additional five milliliters of sample was displaced on syringe injection of the five milliliter gas bubble prepared from the acetylene dilution flasks. The presence of air in the injected gas caused a smaller percentage of the gas bubble to dissolve on vigorous shaking than when pure acetylene was injected. Incubations, lasting two hours, were not carried out in situ because of the difficulty in sample preparation and gas dilution manipulations in the field. The controlled temperature incubator, fitted with Gro-Lux bulbs, allowed laboratory incubations at in situ temperatures. Gas stripping procedures, following incubation, were identical to those in the standard assay method. Acetylene reduction rates were measured for the acetylene concentrations listed in Table 4. The highest substrate concentration was achieved without dilution procedures. It represented the aqueous concentration achieved in the regular assay by direct injection of five milliliters of acetylene into a thirty milliliter sample.

TABLE 4

Acetylene and Nitrogen Concentrations Employed For Definition of Michaelis-Menten Relationships

C_2H_2 Concentration (μM)	N2 Concentration (μM)
63	47
123	93
244	116
596	225
1198	354
1473	465
2977	495
4402	513
7447	

Acetylene Concentration Calculation

As indicated above, acetylene dilutions were prepared in an air-filled volumetric flask, residual volume in the flask neck being filled with water. Determination of gas-liquid partitioning of the acetylene between the two phases in this flask, and in sample syringes was necessary.

Initial Gas Dilution Determination

The concentration of injected gas was estimated using the following equation:

$$C_g(V_g) + C_l(V_l) = V_T$$

where: C_g = $\frac{ml}{ml}$ concentration of acetylene in the vapor phase at given temperature and one atmosphere pressure.

V_g = volume of the vapor phase (mls).

C_l = $\frac{ml}{ml}$ concentration of acetylene in the aqueous phase at a given temperature and one atmosphere pressure.

V_l = volume of the aqueous phase (mls) - flask neck volume.

V_T = total volume of acetylene initially injected into the dilution flask.

To simplify the above equation $\alpha^{C_2H_2}$, acetylene's solubility coefficient was required.

α = $\frac{V_g}{V_l}$ or equivalently $\frac{C_l}{C_g}$ at a given temperature and one atmosphere pressure.

Symbol definition was as above.

Utilizing a specific α, the first equation was simplified to:

$$\frac{C_l}{\alpha^{C_2H_2}}(V_g) + C_l(V_l) = V_T$$

Since all other parameters were available, C_l could be defined; C_g, of

the diluted gas mixture, then equalled $\frac{C_1}{\alpha}$.

Final Aqueous Acetylene Concentration

After preparation of the initial acetylene/air dilution, five milliliters of this gaseous mixture were injected into a thirty milliliter aqueous sample, (V_1). V_g was assumed to be the volume of injected gas, because the very soluble acetylene contributed only a small proportion of the injected air-acetylene mixture. V_T was calculated from the injected gas volume multiplied by C_g, the estimated concentration of the diluted mixture. Incorporation of these values into the first equation allowed determination of a ml/ml aqueous acetylene concentration for each sample. Micromolar concentrations were obtained following division by the molar volume of the gas at standard temperature and pressure.

All concentrations listed in Table 4, except the highest acetylene concentration, were determined in this manner. Determination of the latter concentration required only the final calculation described, because five milliliters of acetylene were injected directly into this sample, without dilution. V_g and V_1 were 0.25 mls and 29.75 mls, respectively. Because of pure acetylene's high aqueous solubility, all but 0.25 mls of the acetylene injected dissolved, explaining the low V_g volume.

Following incubation and gas chromatographic analysis, acetylene reduction rates were calculated in the same manner as was described in the Rate Estimation Section.

The Relationship of Nitrogen Concentration and Nitrogen Fixation Rate

Determination of the relationship of nitrogen fixation rate and nitrogen concentration was accomplished with Lake 227 samples. Though ni-

trogen dilutions were prepared with nitrogen-15 gas, the use of the labelled gas only, rather than a $^{14}N_2$ - $^{15}N_2$ mixture, was merely a measurement and calculation convenience. Because of the large percentage of nitrogen in air, nitrogen dilutions were prepared in helium-filled volumetric flasks. Prescribed volumes of ^{15}N-nitrogen, prepared in Glaspak syringes, were injected into the independent flasks in a similar manner to that used for acetylene-air dilutions. Calculations were made of the partitioning between the water-filled neckspace volume and the flask gas volume. Some dilutions were prepared directly in the syringe, injection of a given nitrogen-15 gas volume being followed immediately by injection of a given helium volume. To achieve higher nitrogen concentrations a larger volume of pure nitrogen-15 gas was injected, rather than a helium-nitrogen mixture. A combination of these three methods, yielded a final sample nitrogen concentration series which was listed in Table 4. Samples were incubated for two and a half hours under Gro-Lux lighting in the laboratory incubator. Analysis of isotope uptake, and thus nitrogen fixation rates, was identical to that required for normal nitrogen-15 uptake rate estimations.

Nitrogen Concentration Calculation

Calculations for the initial nitrogen-15 gas dilution with helium and the final sample nitrogen concentration were identical to those employed in the acetylene experiment. The equation

$$\frac{C_1 (V_g)}{\alpha N_2} + C_1(V_1) = V_T$$

was used, but with nitrogen gas volumes and concentrations substituted for acetylene terms, in both the preliminary dilution calculation and the final nitrogen calculation. The aqueous solubility of nitrogen, αN_2, rather than

acetylene's solubility coefficient, was required.

When gas dilutions were prepared within the sample itself, a predetermined volume of nitrogen-15 gas and helium were injected directly into the syringe, the aqueous sample volume, V_l, being measured following displacement of some sample with gas injection. For this method, modification of the above equation was required with the following interpretation of terms:

$$C_g\ (V_g) + C_l\ (V_l) = V_T$$

where: $C_g = \frac{C_l}{\alpha N_2}$ = final vapor phase nitrogen concentration in the sample $\frac{ml}{ml}$.

V_g = the total volume of helium and nitrogen injected (mls).

C_l = final aqueous nitrogen concentration $\frac{ml}{ml}$.

V_l = sample volume corrected for water displaced by gas injection (mls).

V_T = volume of nitrogen-15 gas injected (mls). ($V_T = V_g$ when only nitrogen-15 gas injected.)

After estimation of nitrogen weight and ^{15}N enrichment, nitrogen-15 uptake calculations were accomplished using normal rate formulae. Individual specific activities were estimated incorporating the different volumes of nitrogen-15 gas injected, and the effect of ^{14}N nitrogen dilution.

RESULTS AND DISCUSSION

Introduction

Acetylene reduction rate and nitrogen-15 uptake data are presented and discussed for the lakes studied in the Experimental Lakes Area and for Chemostats 1 and 2. Acetylene to nitrogen ratios, calculated by average, weighted average, linear regression and weighted linear regression methods, are indicated both for individual systems and related systems, and the merits of each method of calculation are examined. Explanations for intrasystem variability are suggested. Ratios estimated in specific systems are compared to determine if a relationship exists between the ratios calculated and the environments examined. Conclusions are reached concerning the applicability of the theoretical versus the empirically-derived ratios and discussion of attempts to explain demonstrated differences are included. Also reported are the results of additional experiments further defining the relationship of acetylene reduction and nitrogen-15 uptake. Epilimnetic depth profiles, sampling season ratio variability, dark nitrogen fixation estimation, time course assays and substrate-velocity responses are discussed.

Lake Nitrogen Fixation Rate Comparisons

Nitrogen fixation was estimated by acetylene reduction in Lake 226NE and Lake 227 in the Experimental Lakes Area to establish its contribution to the lake nitrogen budgets (Flett, 1977). Though preliminary nitrogen-15 calibration of the acetylene reduction technique was commenced, acetylene reduction estimates of nitrogen fixation were not corrected for acetylene to nitrogen ratios found. Calibration was completed in this study, acetylene to nitrogen ratios being determined both for unconcen-

trated and concentrated lake samples.

Acetylene reduction and nitrogen-15 uptake rate data accumulated for Lake 226 NE, Lake 227 and Lake 304 in 1976 and 1977 is summarized in Tables 5 to 8. In these tables the acetylene-derived nitrogen fixation rate and the acetylene reduction ratewere distinguished. The former, calculated assuming the stoichiometric acetylene to nitrogen ratio, was only included in the rate percentage estimation. Definition of the dimensionless acetylene to nitrogen ratio required prior conversion of µg/L/hr rates, to µmoles/L/hr by inclusion of the molecular weights of ethylene and nitrogen. Also necessary was the assumption based on the theoretical acetylene-ethylene equation (see Introduction), that an equivalent amount of acetylene was reduced for a given amount of ethylene produced. Rates reported for the individual depths are the averages of rates calculated for duplicate samples. Division of acetylene reduction rates by nitrogen-15 uptake rates yielded a direct estimate of acetylene to nitrogen ratios.

None of the lakes examined exhibited reduction rates exceeding 10 µg ethylene produced/L/hr or 2 µg nitrogen fixed/L/hr. Maximum rates for concentrated lake samples, 2.5 µg ethylene/L/hr (Lake 226 NE), 9.4 µg ethylene/L/hr (Lake 304) and 5.1 µg ethylene/L/hr (Lake 227) were of the same order as acetylene reduction rates measured for unconcentrated Lake 227 samples assayed during the course of the late summer Anabaena bloom, (6.4 µg ethylene/L/hr). From this comparison it would appear that net haul concentration procedures described earlier were not unreasonable. A few of the samples exhibiting low nitrogen fixation activity (for example, July 29, 1977, Lake 226 NE - Table 7) showed some inconsistency in ratio magnitude, but no obvious correlation between

TABLE 5

$^{15}N_2$ Uptake Rate and Acetylene Reduction Rate Estimates Accumulated For Lake 226 NE and Lake 227 During the Summer of 1976. Rate percentages and C_2H_2:N_2 Ratios Are Calculated Directly.

Date	Sample Description		$^{15}N_2$ Rate ($\mu gN_2/L/hr$)	C_2H_2 Rate[a] ($\mu gC_2H_4/L/hr$)	$\frac{^{15}N_2 \text{ Rate}}{N_2(C_2H_2)\text{Rate}^b}$ %	$\frac{C_2H_2}{N_2}$
7/7/76	226NE	0 m	0.080	0.449	53.5	5.6
		1 m	0.045	0.564	23.9	12.6
		2 m	0.072	0.432	50.0	6.0
30/8/76	227	0 m	0.174	1.239	42.1	7.1
		1 m	0.169	1.281	39.6	7.6
		2 m	0.121	1.035	35.1	8.6
7/9/76	227	0 m	0.059	0.528	33.5	8.9
		1 m	0.049	0.524	28.1	10.7
		2 m	0.071	0.500	42.6	7.0
10/9/76	227	0 m	0.166	0.821	60.7	4.9
		1 m	0.089	0.542	49.3	6.1
		2 m	0.080	0.509	47.2	6.4
11/9/76	227	0 m	0.112	0.608	55.3	5.4
		1 m	0.100	0.551	54.4	5.5
		2 m	0.076	0.447	51.0	5.9
14/9/76	227	0 m	0.092	0.372	74.2	4.0
		1 m	0.074	0.356	62.4	4.8
		2 m	0.079	0.287	82.6	3.6
16/9/76	227	0 m	0.054	0.402	40.3	7.4
		1 m	0.052	0.354	44.1	6.8
		2 m	0.072	0.300	72.0	4.2

NOTE 1: a C_2H_2 Rate - Acetylene reduction rate not converted to nitrogen fixation rate.

b $N_2(C_2H_2)$ Rate - Acetylene reduction rate converted to nitrogen fixation rate by assuming the theoretical C_2H_2:N_2 ratio of 3.

TABLE 6

$^{15}N_2$ Uptake Rate and Acetylene Reduction Rate Estimates Accumulated For Lake 304 During the Summer of 1977. Rate Percentages and C_2H_2:N_2 Ratios Are Calculated Directly.

Date	Sample Description		$^{15}N_2$ Rate ($\mu gN_2/L/hr$)	C_2H_2 Rate[a] ($\mu gC_2H_4/L/hr$)	$\frac{^{15}N_2 \text{ Rate}}{N_2(C_2H_2)\text{Rate}^b}$ %	$\frac{C_2H_2}{N_2}$
22/6/77	304	0 m	0.843	6.150	41.1	7.3
		1 m	0.508	4.617	33.0	9.1
		2 m	0.407	3.423	35.7	8.4
	304[c]	0 m	0.012	0.105	34.3	8.9
24/6/77	304	0 m	1.528	9.416	48.7	6.2
		1 m	0.893	7.490	35.8	8.4
		2 m	0.579	4.406	39.4	7.6
27/6/77	304	0 m	0.500	3.911	38.4	7.8
		1 m	0.409	3.003	40.9	7.3
		2 m	0.210	1.913	32.9	9.1
4/7/77	304	0 m	0.218	1.149	56.9	5.3
		1 m	0.196	0.672	87.5	3.4
		2 m	0.135	0.545	74.3	4.0
9/7/77	304	0 m	0.460	1.754	78.7	3.8
		1 m	0.302	1.338	67.7	4.4
		2 m	0.155	0.935	49.7	6.0
13/7/77	304	0 m	0.374	1.560	71.9	4.2
		1 m	0.332	1.649	60.4	5.0
		2 m	0.214	0.780	82.3	3.6

a See Note 1, Table 5.

b See Note 1, Table 5.

c Unconcentrated Sample.

TABLE 7

$^{15}N_2$ Uptake Rate and Acetylene Reduction Rate Estimates Accumulated For Lake 226 NE During the Summer of 1977. Rate Percentages and C_2H_2:N_2 Ratios Are Calculated Directly.

Date	Sample Description		$^{15}N_2$ Rate ($\mu gN_2/L/hr$)	C_2H_2 Rate[a] ($\mu gC_2H_4/L/hr$)	$\frac{^{15}N_2 \text{ Rate}}{N_2(C_2H_2)\text{Rate}}$[b] %	$\frac{C_2H_2}{N_2}$
6/7/77	226NE	0 m	0.117	0.963	36.4	8.2
15/7/77	226NE	0 m	0.133	0.641	62.2	4.8
		1 m	0.136	0.719	56.7	5.3
		2 m	0.084	0.474	53.2	5.6
	226NE[c]	0 m	0.011	0.060	55.0	5.5
21/7/77	226NE	0 m	0.324	2.447	39.7	7.6
		1 m	0.345	2.447	42.3	7.1
		2 m	0.246	1.436	51.4	5.8
	226NE[c]	0 m	0.035	0.267	39.3	7.6
23/7/77	226NE	0 m	0.398	2.444	48.9	6.1
		1 m	0.203	2.451	24.8	12.1
		2 m	0.217	1.790	36.4	8.2
	226NE[c]	0 m	0.027	0.330	24.5	12.2
29/7/77	226NE	0 m	0.176	1.880	28.1	10.7
		1 m	0.098	1.179	24.9	12.0
		2 m	0.037	0.663	16.7	17.9
	226NE[c]	0 m	0.085	0.288	88.5	3.4

a See Note 1, Table 5.

b See Note 1, Table 5.

c Unconcentrated Samples.

TABLE 8

$^{15}N_2$ Uptake Rate and Acetylene Reduction Rate Estimates Accumulated For Lake 227 During the Summer of 1977. Rate Percentages and C_2H_2:N_2 Ratios Are Calculated Directly.

Date	Sample Description		$^{15}N_2$ Rate ($\mu gN_2/L/hr$)	C_2H_2 Rate[a] ($\mu gC_2H_4/L/hr$)	$\frac{^{15}N_2 \text{ Rate}}{N_2(C_2H_2)\text{Rate}}$[b] %	$\frac{C_2H_2}{N_2}$
29/6/77	227	0 m	0.257	1.544	49.9	6.0
		1 m	0.209	1.338	46.9	6.4
		2 m	0.135	0.782	51.8	5.8
	227[c]	0 m	0.023	0.363	19.0	15.8
27/7/77	227	0 m	0.551	5.141	32.2	9.3
		1 m	0.497	4.727	31.5	9.5
		2 m	0.250	3.215	23.3	12.9
1/8/77	227[c]	0 m	0.187	1.625	34.5	8.7
		1 m	0.056	1.044	16.1	18.6
		2 m	0.087	0.855	30.5	9.8
		3 m	0.031	0.447	20.8	14.4
22/8/77	227[c]	0 m	0.826	5.550	44.6	6.7
		1 m	0.579	5.004	34.7	8.6
		2 m	0.268	2.673	30.1	10.0
24/8/77	227[c]	0 m	0.800	4.995	48.0	6.8
		0 m	0.756	5.085	44.6	6.7
		0 m	0.943	4.626	61.2	4.9
26/8/77	227[c]	0 m	0.829	5.598	44.4	6.8
		0 m	0.802	6.181	38.9	7.7
		0 m	0.845	6.294	40.3	7.4
		0 m	0.687	6.418	32.1	9.3
29/8/77	227[c]	0 m	0.860	5.738	45.0	6.7
		1 m	0.739	5.279	42.0	7.1
		2 m	0.346	3.428	30.3	9.9
		3 m	0.120	1.391	25.9	11.6
12/9/77	227[c]	0 m	0.522	4.731	33.1	9.1

a See Note 1, Table 5.

b See Note 1, Table 5.

c Unconcentrated Samples.

rate level and ratio magnitude was apparent within the lake data subset.

All individual sample acetylene to nitrogen ratios listed for Lake 226 NE, Lake 227 and Lake 304 differ significantly from the theoretical value. Determination of representative ratios for each lake and the combined lake data set is discussed following presentation of chemostat data.

Chemostat Rate Comparisons

Though establishment of an acetylene to nitrogen ratio for freshwater blue-green algal populations was the primary purpose of this research, restricting sampling to lakes in the Experimental Lakes Area would have prevented estimation of ratios for systems capable of maintaining very high nitrogen fixation rates. A spectrum of rate levels was considered necessary to determine whether ratio magnitude is a function of nitrogen fixation activity level. If the acetylene to nitrogen ratio is a function of nitrogen fixation rate, application of a single ratio to lakes exhibiting various levels of nitrogen fixation is unjustified.

Measurement of nitrogen fixation rates similar to those anticipated in a eutrophic lake, with a well-developed blue-green algal bloom, was possible in chemostat Anabaena variabilis populations. It must be emphasized, however, that the chemostat was used as a densely populated reservoir of actively fixing cells, rather than a lake analogue from which extrapolations to a eutrophic lake were attempted.

Chemostat 1 and Chemostat 2 differed both in the magnitude of their maximum nitrogen fixation rates and in their predictions of acetylene to nitrogen ratios (Table 9 and Table 10). Table headings are as defined in Tables 5 to 8, however, rates listed are the result of individual, rather

TABLE 9

$^{15}N_2$ Uptake Rate and Acetylene Reduction Rate Estimates Accumulated For Chemostat 1 During the Winter of 1977. Rate Percentages and C_2H_2:N_2 Ratios Are Calculated Directly.

Date	Chemostat Number	$^{15}N_2$ Rate ($\mu gN_2/L/hr$)	C_2H_2 Rate[a] ($\mu gC_2H_4/L/hr$)	$\frac{^{15}N_2 \text{ Rate}}{N_2(C_2H_2)\text{Rate}^b}$ %	$\frac{C_2H_2}{N_2}$
18/3/77	1	9.132	40.455	67.7	4.4
		14.091	33.966	124.5	2.4
23/3/77	1	26.922	87.048	92.8	3.2
		20.145	98.100	61.6	4.9
		17.385	87.048	59.9	5.0
		18.297	98.100	55.9	5.4
19/4/77	1	6.210	43.920	42.4	7.1
		5.301	36.036	44.1	6.8
	1(Dark)	0.705	15.696	13.5	22.3
		7.998	43.920	54.6	5.5
25/4/77	1	9.567	58.644	48.9	6.1
		10.749	57.492	56.1	5.3
	1(Dark)	3.105	27.117	34.3	8.7
		9.807	58.644	50.2	6.0
		12.219	57.492	63.8	4.7
4/5/77	1	8.277	37.146	66.8	4.5

a See Note 1, Table 5.

b See Note 1, Table 5.

TABLE 10

$^{15}N_2$ Uptake Rate and Acetylene Reduction Rate Estimates Accumulated For Chemostat 2 During the Summer of 1977. Rate Percentages and C_2H_2:N_2 Ratios Are Calculated Directly.

Date	Chemostat Number	$^{15}N_2$ Rate ($\mu gN_2/L/hr$)	C_2H_2 Rate[a] ($\mu gC_2H_4/L/hr$)	$^{15}N_2$ Rate $N_2(C_2H_2)$ Rate[b] %	$\frac{C_2H_2}{N_2}$
13/6/77	2	0.945	13.752	20.6	14.6
		1.227	16.236	22.7	13.2
19/6/77	2	0.390	5.076	23.1	13.0
		0.585	4.752	37.0	8.1
		0.522	4.878	32.0	9.4
		0.561	3.321	50.6	5.9
		0.612	4.167	44.1	6.8
30/6/77	2	0.393	5.742	20.6	14.6
		0.789	5.139	46.0	6.5
7/7/77	2	1.179	11.286	31.4	9.6
		1.578	12.636	37.5	8.0
		1.608	11.187	43.1	7.0
		1.977	10.251	57.8	5.2
11/7/77	2	0.222	3.591	18.7	16.1
		0.279	2.952	28.4	10.6
		0.135	3.474	11.8	25.5
		0.249	2.547	29.2	10.3
18/7/77	2	1.104	22.653	14.6	20.5
		2.496	21.321	35.1	8.5
		2.412	22.167	32.6	9.2
		1.545	20.133	23.0	13.0
26/7/77	2	1.044	15.255	20.5	14.6
		1.527	14.580	31.4	9.5
		1.167	13.617	25.7	11.7
		0.948	12.798	22.2	13.5
		1.095	14.040	23.4	12.8

a See Note 1, Table 5.

b See Note 1, Table 5.

than duplicate analyses. Maximum acetylene reduction rates for Chemostats 1 and 2, 98.1 µg ethylene/L/hr and 22.6 µg ethylene/L/hr, exceeded maximum lake rates by greater than a factor of ten in Chemostat 1 and a factor of 2 in Chemostat 2. The differences in rate level between Chemostats 1 and 2 were not anticipated on the basis of the similarity in biological and chemical design of the chemostats. Both chemostats were supplied with the same algal species, limiting nutrient and medium composition and supply. Differences in maximum rates may have been the result of variations in individual cellular capacities for nitrogen fixation, or differences in population nitrogen fixation level based on unequal cell concentration. Support for concentration-induced inequalities was obtained on comparison of particulate nitrogen weights, one indicator of cell density. Ten milliliter samples collected and analyzed for particulate nitrogen April 27, 1977 (Chemostat 1) and September 1, 1977 (Chemostat 2) demonstrated nitrogen weights of 37.0 µg N and 19.3 µg N, respectively, a difference of approximately a factor of two in favor of Chemostat 1. Activity levels differed by a factor of approximately four or five, thus activity level inequality appears to be subject to more than simple population density influences.

An additional explanation proposed for activity differences between the chemostats was based upon the availability of inorganic nitrogen, specifically ammonia, as a nitrogen source more readily assimilated than molecular nitrogen or as an inhibitor of the nitrogen fixation process. Preferential assimilation of ammonia has been observed (Hardy et al., 1973), but its influence on the present study is only supported by inference. The axenic state of the cultures, tested by trypticase soy broth inoculation, the extent of ammonia release mediated by bacterial activity, assuming bacterial contamination, and the degree to which the

released ammonia would affect nitrogen fixation activity are critical to this proposal. Only Chemostat 1 was maintained in an axenic state. Therefore, bacterial decomposition of organic nitrogen with release of ammonia was possible in Chemostat 2. An ammonia concentration of 18.4 µg/L/hr is representative of the significant levels of inorganic nitrogen measurable in Chemostat 2. Suspicion of higher ammonia release per cell in low density Chemostat 2 may be supported by the observation that passage of a small molecule (such as ammonia) from a cell to the environment will be enhanced in less dense natural populations compared to more concentrated populations, though this has not been demonstrated in blue-green algae (Fogg, 1966). Comparison to axenic Chemostat 1 ammonia levels was impossible, though, as an analysis of Chemostat 1 chemistry was unavailable. Ammonia, if present in Chemostat 1 medium, would presumably have been released by algal cells. However, without an estimate of Chemostat 1 ammonia concentration responsibility for observed levels in Chemostat 2 cannot be assigned either to the algae or bacteria.

Experiments completed with Lake 227 samples in 1976 indicated that very high ammonia concentrations decreased the rate of acetylene reduction. Over a concentration range of 590 µM to 4730 µM ammonia, acetylene reduction exhibited a gradual decline to approximately sixty per cent of the rate of a sample receiving no ammonia addition (Table 11). The background ammonia concentration in the control sample was 0.29 µM. As an approximation, the relationship derived with this data set was applied to the Chemostat 2 ammonia concentration, 18,400 µg/L or 1082 µM. The percentage rate decrease, assuming the presence of 1082 µM ammonia and an exponential decline in rate with increasing ammonia concentration, was twenty-four per cent, insufficient to account for the large rate difference between Chemostats 1 and 2. This explanation being inadequate, it

TABLE 11

The Effect of Increased Ammonia Concentration on Acetylene Reduction Rate, as Estimated August 17, 1976 for Lake 227 Samples.

[Ammonia] (μM)	Acetylene Reduction Rate ($\mu gN_2/L/hr$)	Per Cent of Control Rate
0.29	1.40	100
590	1.17	84
1180	0.99	71
1770	0.98	70
2370	0.77	55
2950	0.72	51
3550	0.74	53
4140	0.57	40
4730	0.61	44

would appear that there must be some other reason for rate differences, whether it be related to the influence of different external factors, or to the specific physiology of the algae in Chemostats 1 and 2.

Acetylene to Nitrogen Ratio Estimation

Low nitrogen-15 uptake rate to acetylene-derived nitrogen fixation rate percentages estimated for lake and chemostat samples (Tables 5 to 10) indicated that use of the theoretical factor for acetylene reduction rate conversion was inadequate to achieve balanced estimates of nitrogen fixation activity. Calculation of an empirical acetylene to nitrogen ratio appeared necessary if acetylene reduction rate data were to be used to predict rates of nitrogen fixation. The nitrogen-15 uptake rate and acetylene reduction rate data described above were used to calculate this ratio for each lake and chemostat system. More than one method of calculation was included to demonstrate that the ratio obtained was dependent upon the manner of estimation. The choice of calculation procedure is significant if comparison to previously estimated acetylene to nitrogen ratios is anticipated.

Calculation of a simple mean ratio to describe the acetylene to nitrogen relationship for each system was the most direct procedure, ratios ranging from 6.3 to 11.9 for the individual systems (Table 12). Mean determination in this manner assumed that each value incorporated was characterized by an equal uncertainty of estimation. This assumption was not valid for all sample rates. This assumption may be avoided by inclusion of a weighting factor in the mean calculation. The weighting factor chosen was the reciprocal of the sample variance, s^2, (Bevington, 1969). Contributions to the sample variance included an estimate of the standard de-

TABLE 12

C_2H_2:N_2 Ratios Estimated by Mean, Weighted Mean, Linear Regression and Weighted Linear Regression Analyses. Ratios Are Calculated Independently for Each System Prior to Determination of Overall Ratios For The Lake, Chemostat and Complete Data Subsets.

System	Sample Size	Mean Ratio	Weighted Mean Ratio[a]	Regression Ratio	Correlation Coefficient	Weighted Regression Ratio[b]	Correlation Coefficient
L.226-227 (1976)	21	6.6	7.2	8.8	0.83	9.2	0.78
L. 304	19	6.3	6.9	7.7	0.96	8.3	0.92
L. 226	17	8.2	9.2	8.3	0.90	9.3	0.88
L. 227	26	9.1	9.7	7.2	0.94	8.0	0.95
Chem 1	16	6.4	9.4	4.3	0.87	4.1	0.97
Chem 2	26	11.9	13.2	12.4	0.81	12.4	0.85
All Lakes	83	7.6	8.5	7.5	0.96	8.4	0.92
All Chem	42	9.8	12.4	4.4	0.95	10.5	0.82
All Data	125	8.4	10.3	4.8	0.95	10.9	0.86

a Weighting $= 1/\sigma^2 = 1/\left[(.017)^2 + (\%\ \text{Error} \times \text{Ratio})^2\right]$

b Weighting $= 1/\sigma^2 = 1/\left[(.017)^2 + (\%\ \text{Error} \times {}^{15}N\ \text{Rate})^2\right]$

viation associated with the average of a series of natural abundance samples and a term reflecting the error associated with estimation of a given sample ratio.

$$\text{Weighting Factor} = 1/s^2 = 1/\left[(\text{Standard Deviation})^2 + (\%\ \text{Error} \times \text{Ratio})^2\right]$$

The first term corrected for the larger uncertainty associated with samples whose ^{15}N atom per cent value approached the natural abundance level, which averaged 0.393 ± 0.017 atom per cent. The second term incorporated an estimate of per cent error in the measurement procedure, which was obtained from a series of sample replicates for which nitrogen-15 uptake and acetylene reduction rate analyses were available. Average rates and their associated standard deviations were calculated for nine replicate series (Table 13). The per cent error is equivalent to the ratio of the standard deviation to the average rate. Nitrogen-15 uptake rates exhibited the largest per cent errors, so these values were used in weighting factor determination. Because specific values were available for the lakes, Chemostat 1 and Chemostat 2, data from each system was weighted by its characteristic average per cent error. The following equation yielded the weighted mean ratio:

$$\text{Mean} = \frac{\sum \text{Ratio}/s^2}{\sum 1/s^2}$$

Weighting elevated all the mean acetylene to nitrogen ratios, especially that of Chemostat 1 whose ratio increased by 3.0 slope units.

Linear regression analysis of acetylene reduction and nitrogen-15 uptake data was also used to quantify the acetylene to nitrogen relationship. Regression analysis requires assignment of rate data as either dependent or independent variables. When, as in this case, both parameters

TABLE 13

Determination of Mean Nitrogen-15 Uptake and Acetylene Reduction Rates For a Number of Sets of Sample Replicates. The Standard Deviation of Each Mean and the Per Cent Error ($S/\bar{x}$)(100) Are Included.

Date	System	Mean ^{15}N Rate (µg/L/hr)	Standard Deviation (µg/L/hr)	% Error	Mean C_2H_2 Rate (μgC_2H_4/L/hr)	Standard Deviation (μgC_2H_4/L/hr)	% Error
24/8/77	227	0.833	0.098	11.8	4.902	0.243	5.0
26/8/77	227	0.791	0.071	9.0	6.123	0.363	5.9
23/3/77	Chem 1	21.483	4.906	22.8	92.574	6.380	6.9
19/4/77	Chem 1	6.503	1.372	21.1	41.292	4.552	11.0
25/4/77	Chem 1	10.586	1.203	11.4	58.068	0.665	1.1
19/6/77	Chem 2	0.534	0.087	16.0	4.439	0.711	16.0
7/7/77	Chem 2	1.586	0.326	20.6	11.339	0.982	8.7
11/7/77	Chem 2	0.221	0.062	28.1	3.141	0.484	15.4
26/7/77	Chem 2	1.356	0.381	28.1	14.058	0.934	6.6

exhibit an observable error in estimation (Table 13), the parameter with the largest relative error is usually designated the dependent variable. The average per cent error associated with nitrogen-15 uptake measurements, 18.8%, exceeded that associated with acetylene reduction, 8.5%. Therefore, nitrogen-15 uptake rate was examined as a function of the independent acetylene reduction rate variable. A linear equation $y = mx + b$ was then calculated, such that the discrepancy between measured nitrogen-15 uptake rate values and those predicted by this equation was minimized. The reciprocal of the slope "m", which characterized the relationship of nitrogen-15 uptake (y) to acetylene reduction (x), yielded an estimate of the acetylene to nitrogen ratio. Acetylene to nitrogen ratios calculated by linear regression analysis of the individual data subsets are included in Table 12. The mean ratios determined previously were less than regression estimates, with the exception of ratios calculated for Lake 227 and Chemostat 1.

Regression-derived ratios calculated for each of the lake systems were similar, but chemostat ratios exhibited differences from lake values and between chemostats. Individually, Chemostat 1 rate data yielded the lowest ratio, 4.3, while Chemostat 2 data yielded the highest ratio, 12.4. The distribution of data around linear regression lines and within ninety-five per cent confidence intervals, calculated assuming applicability of the small sample-based Student t distribution, is also shown (Figures 17 to 22).

A similar data weighting procedure was adopted for linear regression analyses as for mean determination, to generate a final series of ratios. Again, use of a weighting factor allowed inclusion of the uncertainty associated with individual estimations. Determination of the sample variance (s^2) preceded introduction of the weighting factor ($1/s^2$)

Figure 17. A plot of the linear regression analysis of acetylene reduction rate and nitrogen-15 uptake rate data collected in the summer of 1976 for Lake 226 NE and Lake 227. The regression equation, correlation coefficient (R) and sample size (N) are provided. Dotted lines indicate the 95% confidence interval calculated for the given line.

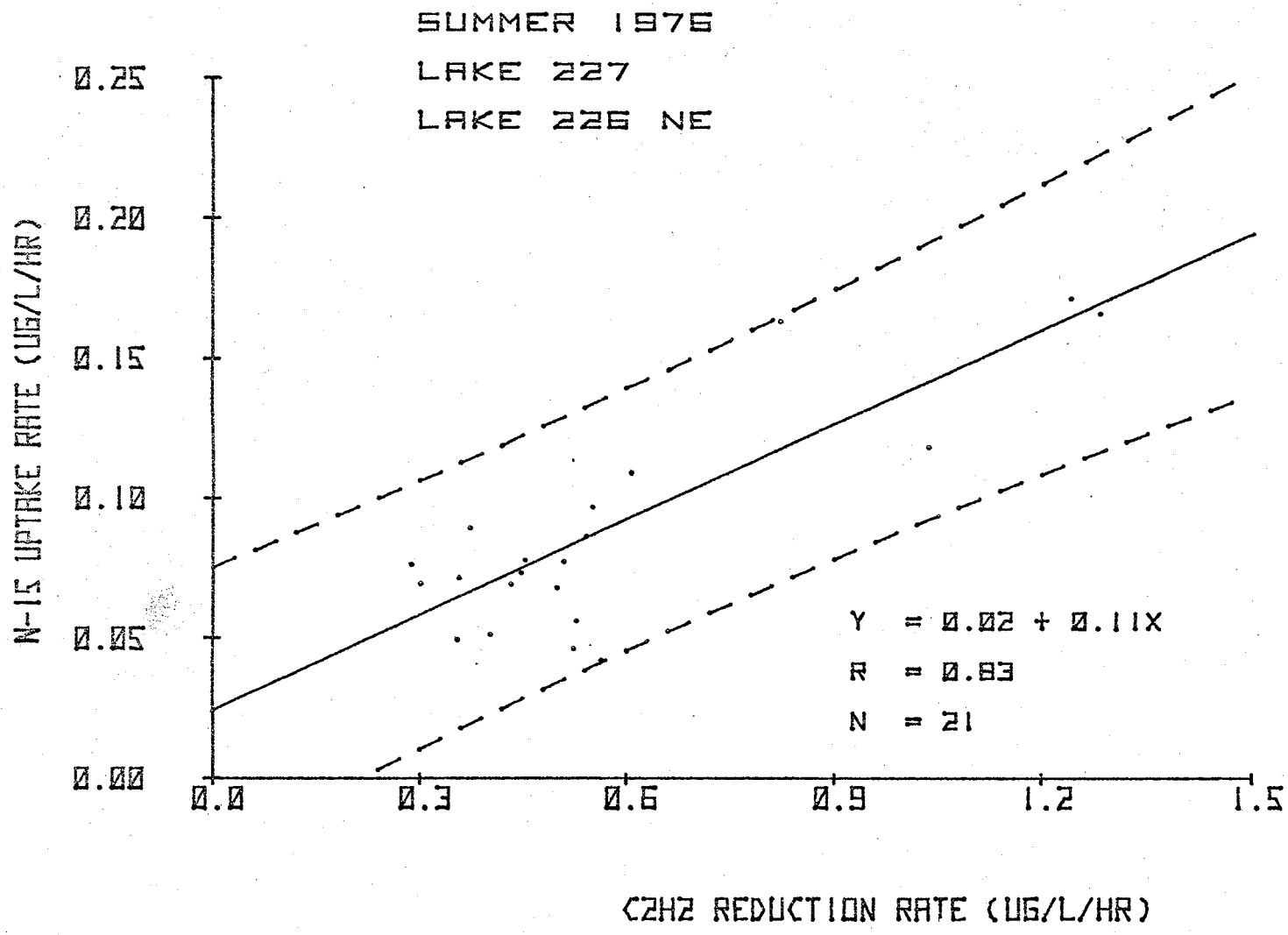

SUMMER 1976
LAKE 227
LAKE 226 NE
0.25
0.20
0.15
0.10
0.05
0.00
N-15 UPTAKE RATE (UG/L/HR)
0.0
0.3
0.6
0.9
1.2
1.5
C2H2 REDUCTION RATE (UG/L/HR)
Y = 0.02 + 0.11X
R = 0.83
N = 21

Figure 18. A plot of the linear regression analysis of acetylene reduction rate and nitrogen-15 uptake rate data collected in the summer of 1977 for Lake 226 NE. The regression equation, correlation coefficient (R) and sample size (N) are provided. Dotted lines indicate the 95% confidence interval calculated for the given line.

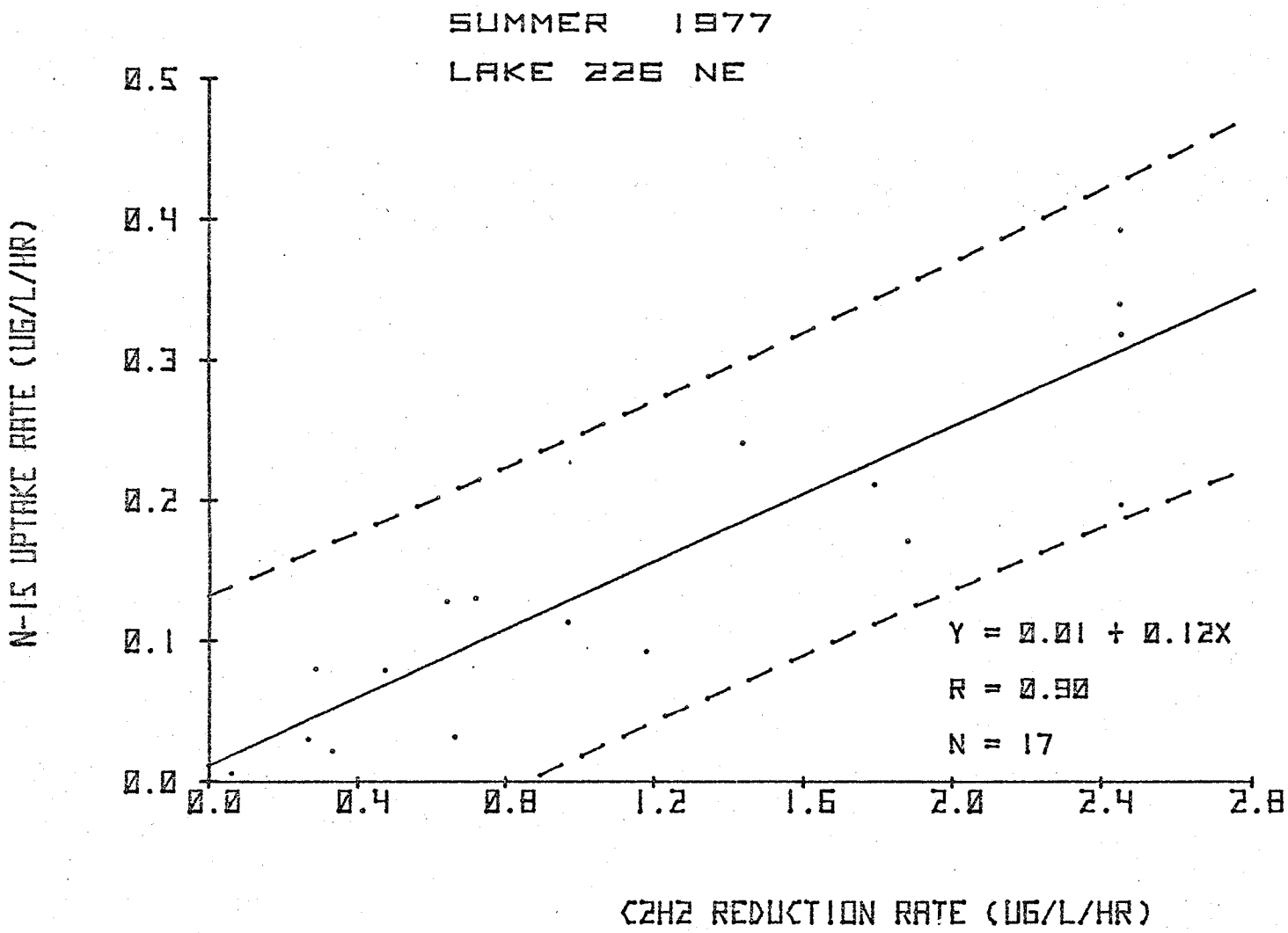

SUMMER 1977
LAKE 226 NE
N-15 UPTAKE RATE (UG/L/HR)
0.5
0.4
0.3
0.2
0.1
0.0
0.0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
Y = 0.01 + 0.12X
R = 0.90
N = 17
C2H2 REDUCTION RATE (UG/L/HR)

Figure 19. A plot of the linear regression analysis of acetylene reduction rate and nitrogen-15 uptake rate data collected in the summer of 1977 for Lake 227. The regression equation, correlation coefficient (R) and sample size (N) are provided. Dotted lines indicate the 95% confidence interval calculated for the given line.

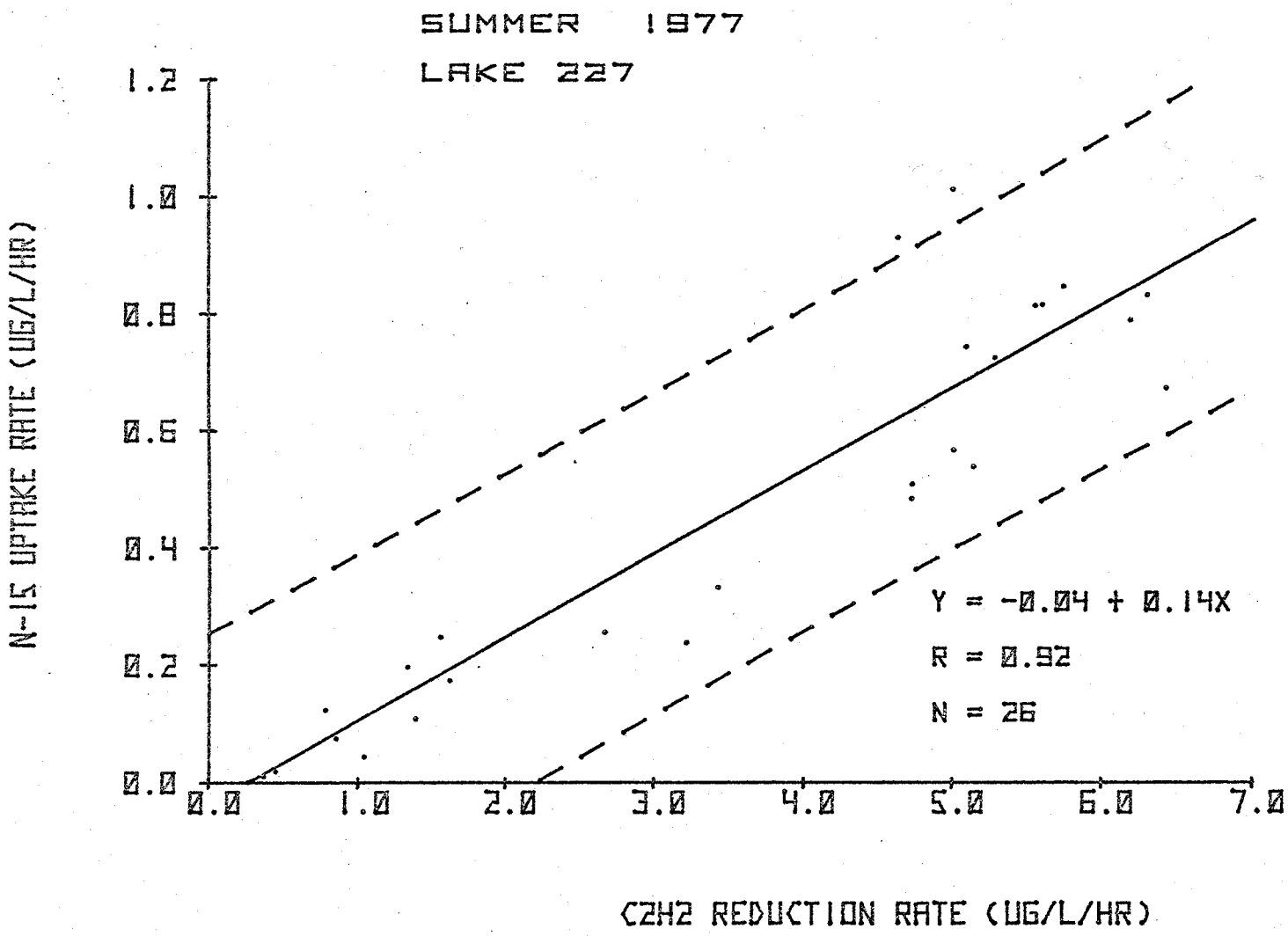

SUMMER 1977
LAKE 227
N-15 UPTAKE RATE (UG/L/HR)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Y = -0.04 + 0.14X
R = 0.92
N = 26
C2H2 REDUCTION RATE (UG/L/HR)

Figure 20. A plot of the linear regression analysis of acetylene reduction rate and nitrogen-15 uptake rate data collected in the summer of 1977 for Lake 304. The regression equation, correlation coefficient (R) and sample size (N) are provided. Dotted lines indicate the 95% confidence interval calculated for the given line.

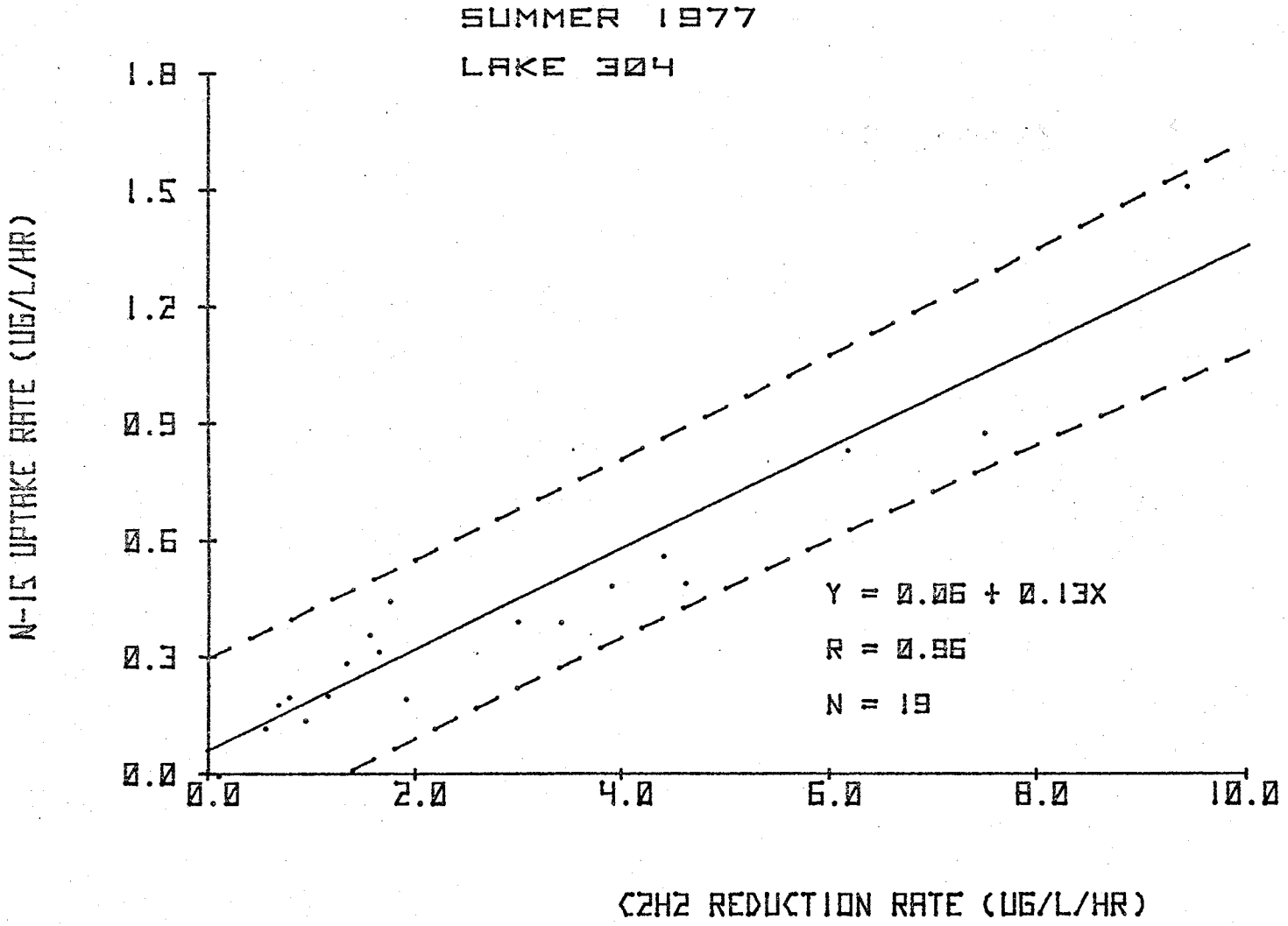

SUMMER 1977
LAKE 304
N-15 UPTAKE RATE (UG/L/HR)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
0.0
2.0
4.0
6.0
8.0
10.0
Y = 0.06 + 0.13X
R = 0.96
N = 19
C2H2 REDUCTION RATE (UG/L/HR)

Figure 21. A plot of the linear regression analysis of acetylene reduction rate and nitrogen-15 uptake rate data collected in the winter of 1977 for Chemostat 1. The regression equation, correlation coefficient (R) and sample size (N) are provided. Dotted lines indicate the 95% confidence interval calculated for the given line.

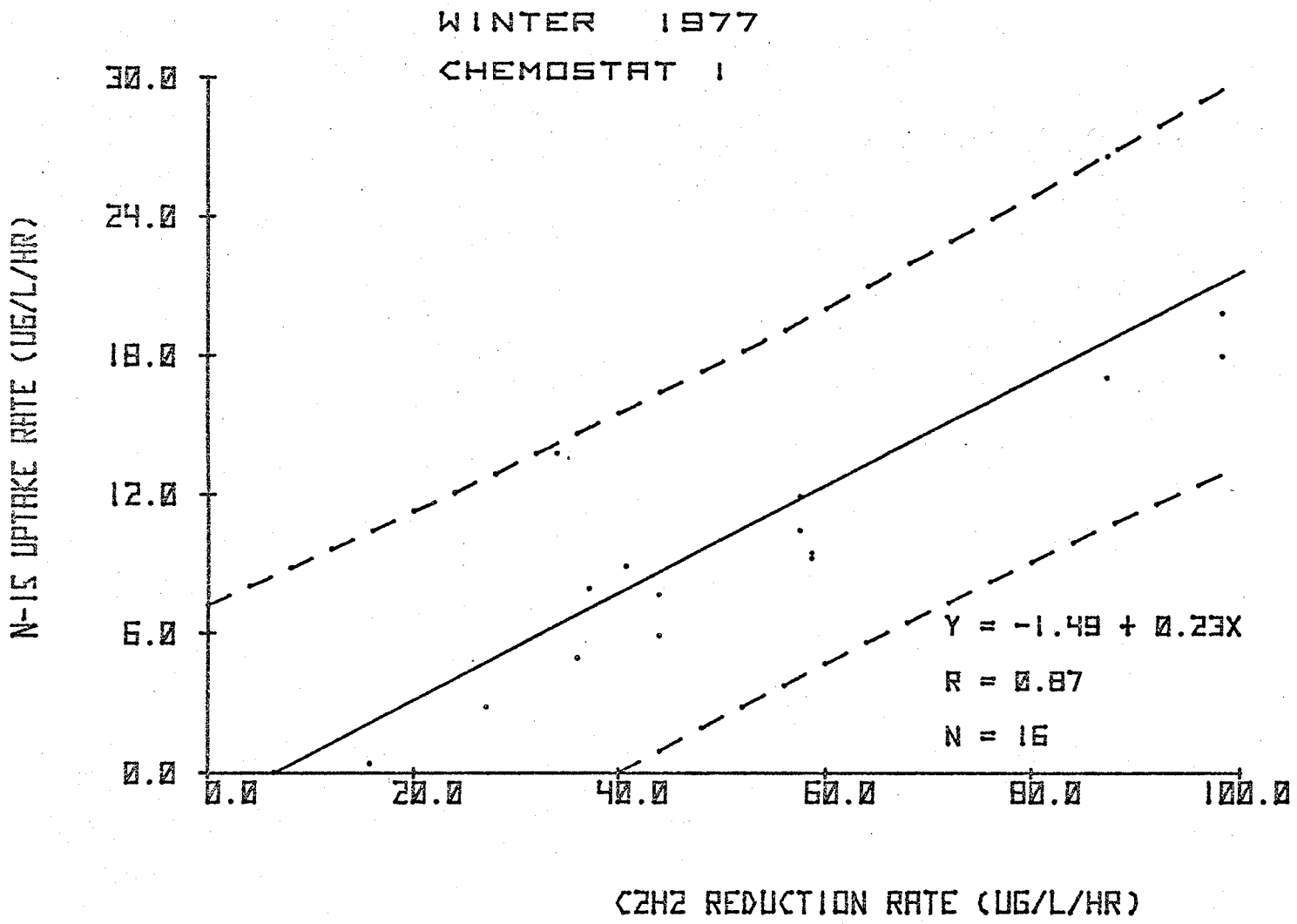
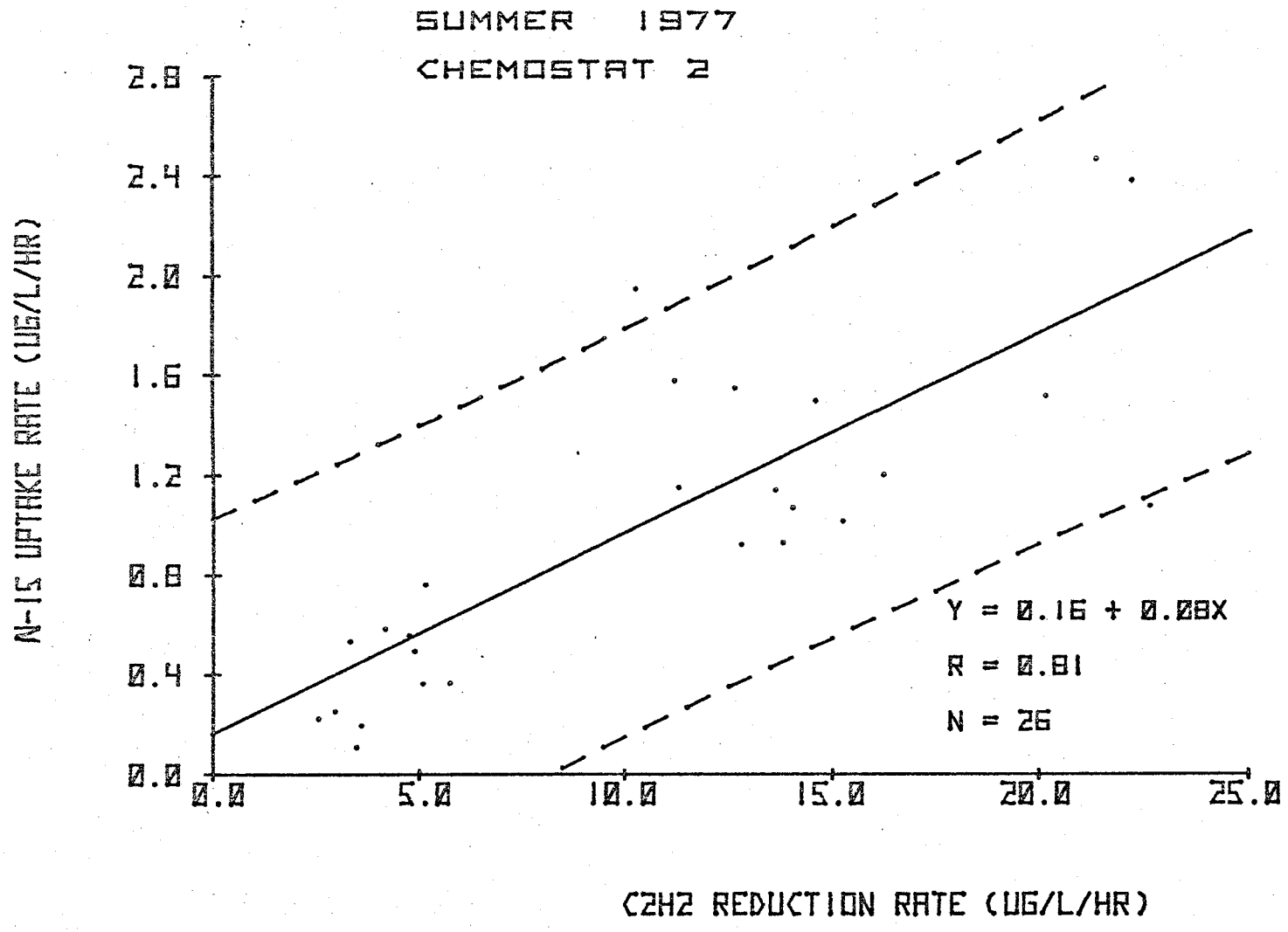

WINTER 1977
CHEMOSTAT 1
30.0
24.0
18.0
12.0
6.0
0.0
N-15 UPTAKE RATE (UG/L/HR)
0.0
20.0
40.0
60.0
80.0
100.0
C2H2 REDUCTION RATE (UG/L/HR)
Y = -1.49 + 0.23X
R = 0.87
N = 16

Figure 22. A plot of the linear regression analysis of acetylene reduction rate and nitrogen-15 uptake rate data collected in the summer of 1977 for Chemostat 2. The regression equation, correlation coefficient (R) and sample size (N) are provided. Dotted lines indicate the 95% confidence interval calculated for the given line.


SUMMER 1977
CHEMOSTAT 2
N-15 UPTAKE RATE (UG/L/HR)
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
0.0
5.0
10.0
15.0
20.0
25.0
Y = 0.16 + 0.08X
R = 0.81
N = 26
C2H2 REDUCTION RATE (UG/L/HR)

into the regression formulae.

$$\text{Weighting Factor} = 1/s^2 = 1/[(\text{Std. Deviation})^2 + (\%\text{Error} \times {}^{15}N_2 \text{ Rate})^2]$$

The only modification in the above equation was the replacement of the ratio values included in the mean calculations, with individual nitrogen-15 uptake rates. Thus the weighting was linked with the rate level observed in the individual systems. Low rates did not have the largest per cent error of estimation as might be anticipated. Both standard deviation (0.017 atom per cent) and per cent error values (Table 13) were identical to those incorporated into weighted mean calculations. The factor derived was then used to weight measured rates prior to the summing involved in regression equation slope calculations.

The response of the regression-derived lake acetylene to nitrogen ratios to weighting was similar to that observed when weighting was incorporated into mean calculations (Table 12). Ratios again increased by up to one slope unit. Both Chemostat 1 and Chemostat 2 reacted differently, exhibiting no response to the weighting procedure, thus maintaining their position as ratio extremes. Because of the magnitude of chemostat nitrogen fixation rates, the second term in the weighting factor was most significant to chemostat rate correction. The correction then was proportional to the rate, therefore the slope calculated on regression analysis of corrected rates was not significantly affected.

Rate differences as a function of biomass increases alone, appeared to provide an inadequate explanation of acetylene to nitrogen ratio discrepancies between the chemostats and the lakes. Use of chlorophyll and particulate carbon concentrations for selected samples to standardize rate estimates to a common unit (Table 14) indicated that rate elevations

TABLE 14

Standardization of Chemostat and Lake Rate Estimates to Rate Per Unit Carbon or Rate Per Unit Chlorophyll Values to Facilitate Comparison of Rate Magnitude in These Two Systems.

Date	Lake	Factor	Concentration (µg/L)	$^{15}N_2$ Rate (µg/unitC/hr)[a]	C_2H_2 Rate (µg/unitC/hr)[a]	Chemostat Rate / Lake Rate ($^{15}N_2$)	Chemostat Rate / Lake Rate (C_2H_2)
5/7/77	304	Carbon	790.0	2.76×10^{-4}	1.45×10^{-3}	2.3	2.2
27/4/77	Chem 1	Carbon	17,800.0	5.71×10^{-4}	3.26×10^{-3}		
3/5/77	Chem 1	Carbon	11,400.0	7.26×10^{-4}	3.26×10^{-3}		
	Average			6.49×10^{-4} [b]	3.26×10^{-3} [b]		
5/7/77	304	Chloro-a	8.4	2.60×10^{-2}	1.37×10^{-1}	6.8	6.9
27/7/77	226	Chloro-a	10.3	3.86×10^{-2}	2.37×10^{-1}	4.6	4.0
23/8/77	227	Chloro-a	28.3	2.92×10^{-2}	1.96×10^{-1}	6.1	4.8
1/9/77	Chem 2	Chloro-a	16.1 [c]	2.89×10^{-1}	-		
26/7/77	Chem 2	-	-	6.48×10^{-2}	9.48×10^{-1}		
	Average			1.77×10^{-1}	9.48×10^{-1} [d]		

a C represents carbon for the first three rates and chlorophyll for the last four rates.
b Average rates calculated for the two Chemostat 1 samples - to be used in the calculation of the chemostat to lake ratio.
c Chemostat 2 Chloro-a concentration measured August 25, 1977.
d Average rates calculated for the two Chemostat 2 samples - to be used in the calculation of the chemostat to lake ratio.

from lake to chemostat were a function of more than an increase in algal density. Following rate correction with particulate carbon concentrations, Chemostat 1 nitrogen fixation activity was still approximately twice that of a Lake 304 sample. This chemostat to lake ratio is a minimum because the Lake 304 fixation rate was calculated for concentrated samples, while the particulate carbon value was obtained on analysis of an unconcentrated lake sample. The result is an enhanced lake rate which reduces the chemostat to lake ratio. Though particulate carbon measurements were unavailable for Chemostat 2, relative chlorophyll concentrations were used to compare cell concentrations. Because of the infrequency of chlorophyll measurements in Chemostat 2, the dates for rate estimation and chlorophyll measurements do not coincide. Therefore, Chemostat 2 to lake ratios calculated here are approximations. Chemostat 2 rates exceeded those of Lake 226 NE, Lake 227 and Lake 304 by a factor ranging from four to seven times, when this correction was applied (Table 14). Therefore, though lower acetylene to nitrogen ratios were estimated for the dense, rapidly fixing Chemostat 1 population than for less concentrated lake samples, the difference in ratio was not solely a function of enhanced rates resulting from an increase in cell density.

Though differences in the absolute magnitude of individual ratios exist, all ratios calculated by the above four procedures exceeded the stoichiometric acetylene to nitrogen ratio. Only regression estimates of the Chemostat 1 acetylene to nitrogen relationship were less than twice the theoretical value indicating that a significant underestimation of nitrogen fixation by nitrogen-15 uptake, or overestimation by acetylene reduction was occurring. Explanations proposed for acetylene to nitrogen ratios observed are discussed later.

Overall Ratio Determinations

The similarity of acetylene to nitrogen ratios obtained for Lake 226 NE, Lake 227 and Lake 304 in 1976 and 1977 promoted determination of an overall ratio characterizing this type of lake system. Estimates derived by all four calculation procedures are included in Table 12, the unweighted regression being illustrated in Figure 23. Moreover, acetylene reduction and nitrogen-15 uptake data collected independently but analyzed similarly, for Lake 226 NE and Lake 227 (Flett, 1977) yielded ratios of the same magnitude as those obtained in this study (Table 15). Both sets of values are larger than average ratios predicted for Lake Mendota samples, 4.8 (Peterson and Burris, 1976) and Bay of Quinté enclosures, 5.7 (Lean et al., 1976). Only two values determined by Flett were observed to lie outside the ninety-five per cent confidence interval calculated for the lake ratio (Figure 24). It would appear that both the type of lake examined, as well as the rate estimation procedures employed influence the magnitude of ratios determined.

Estimation of an overall acetylene to nitrogen ratio summarizing Chemostat 1 and Chemostat 2 data did not have the justification of similar individual ratios, which supported definition of an overall lake ratio. However, the similarity in algal species and original environmental conditions prompted its calculation. Values obtained by the different calculation procedures did not exhibit the same uniformity characteristic of overall lake ratios, ratios ranging from 4.4 to 12.4 (Table 12). The influence of the low ratio calculated for Chemostat 1 was noticeable when unweighted (Figure 25) and weighted regression estimates were compared. The weighting factor correction affected the high rate Chemostat 1 values to a larger extent, attenuating their dominant influence in the overall ratio determination. This weighted estimate was much closer to the ratios

Figure 23. A plot of the linear regression analysis of acetylene reduction rate and nitrogen-15 uptake rate data accumulated for all the lakes studied in 1976 and 1977. The regression equation, correlation coefficient (R) and sample size (N) are provided. Dotted lines indicate the 95% confidence interval calculated for the given line.

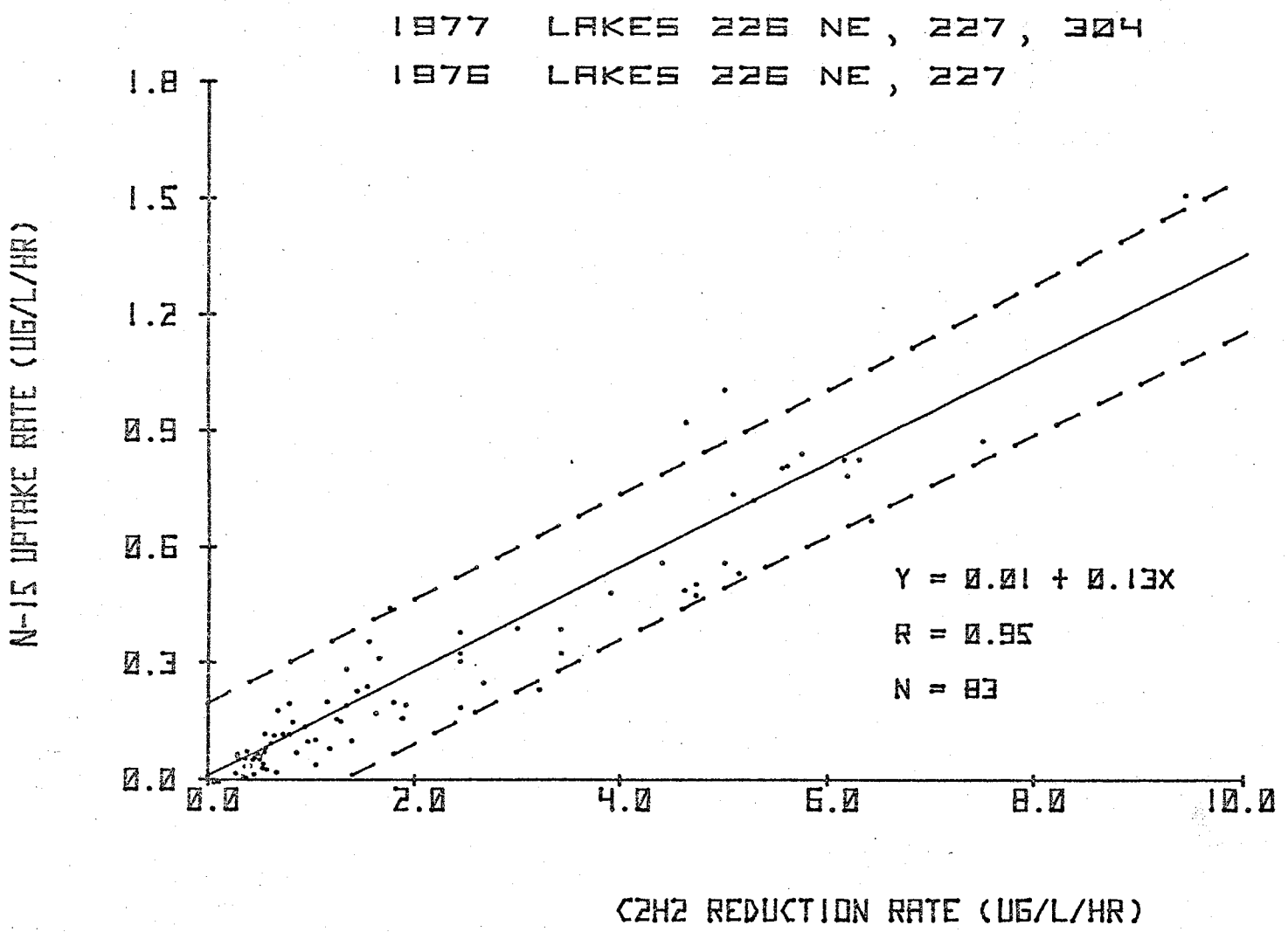

1977 LAKES 226 NE, 227, 304
1976 LAKES 226 NE, 227
N-15 UPTAKE RATE (UG/L/HR)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
0.0
2.0
4.0
6.0
8.0
10.0
Y = 0.01 + 0.13X
R = 0.95
N = 83
C2H2 REDUCTION RATE (UG/L/HR)

TABLE 15

Acetylene Reduction and Nitrogen-15 Uptake Rate Data Collected For Lake 226 NE and Lake 227 in 1974 and 1975 (Flett, 1977). Associated C_2H_2:N_2 Ratios Are Calculated.

Date	Lake	C_2H_2 Rate ($\mu gC_2H_4/L/hr$)	$^{15}N_2$ Rate ($\mu gN_2/L/hr$)	$\frac{C_2H_2}{N_2}$
4/9/74	226NE	6.06	0.51	11.9
4/9/75	226NE	0.78	0.12	6.4
12/9/75	226NE	1.72	0.19	8.9
		1.73	0.17	10.5
		1.30	0.14	9.1
		0.97	0.04	6.9
18/9/75	226NE	0.74	0.11	6.9
24/9/75	226NE	0.35	0.05	7.4
21/7/75	227	2.97	1.40	2.1
18/9/75	227	0.72	0.09	8.2
25/9/75	227	0.29	0.05	6.2

Figure 24. Superimposition of Lake 227 and Lake 226 NE acetylene reduction rate and nitrogen-15 uptake rate data collected by Flett (1977), on the all lake data plot (Figure 23). Flett's data is designated by a square plotting symbol.

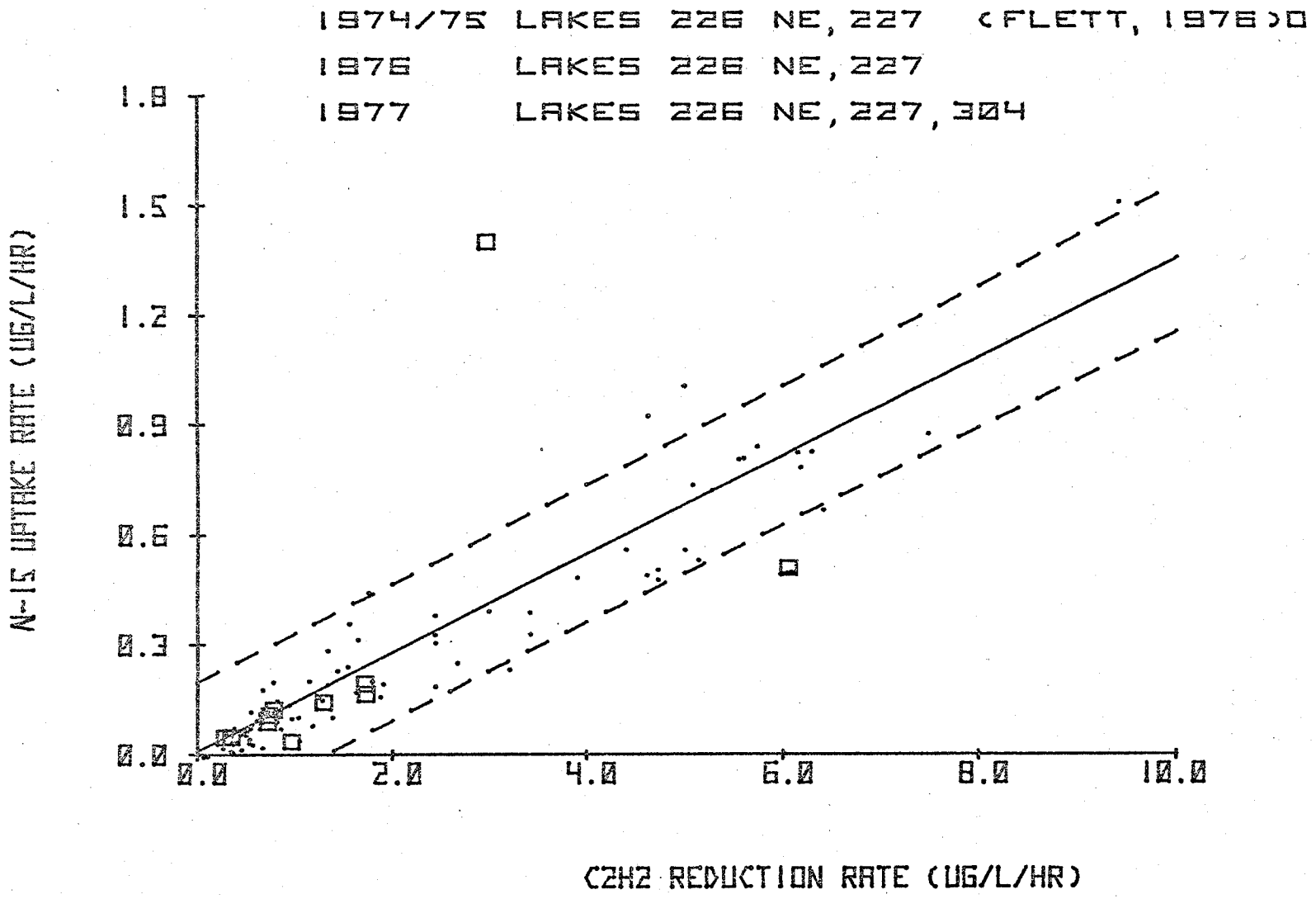
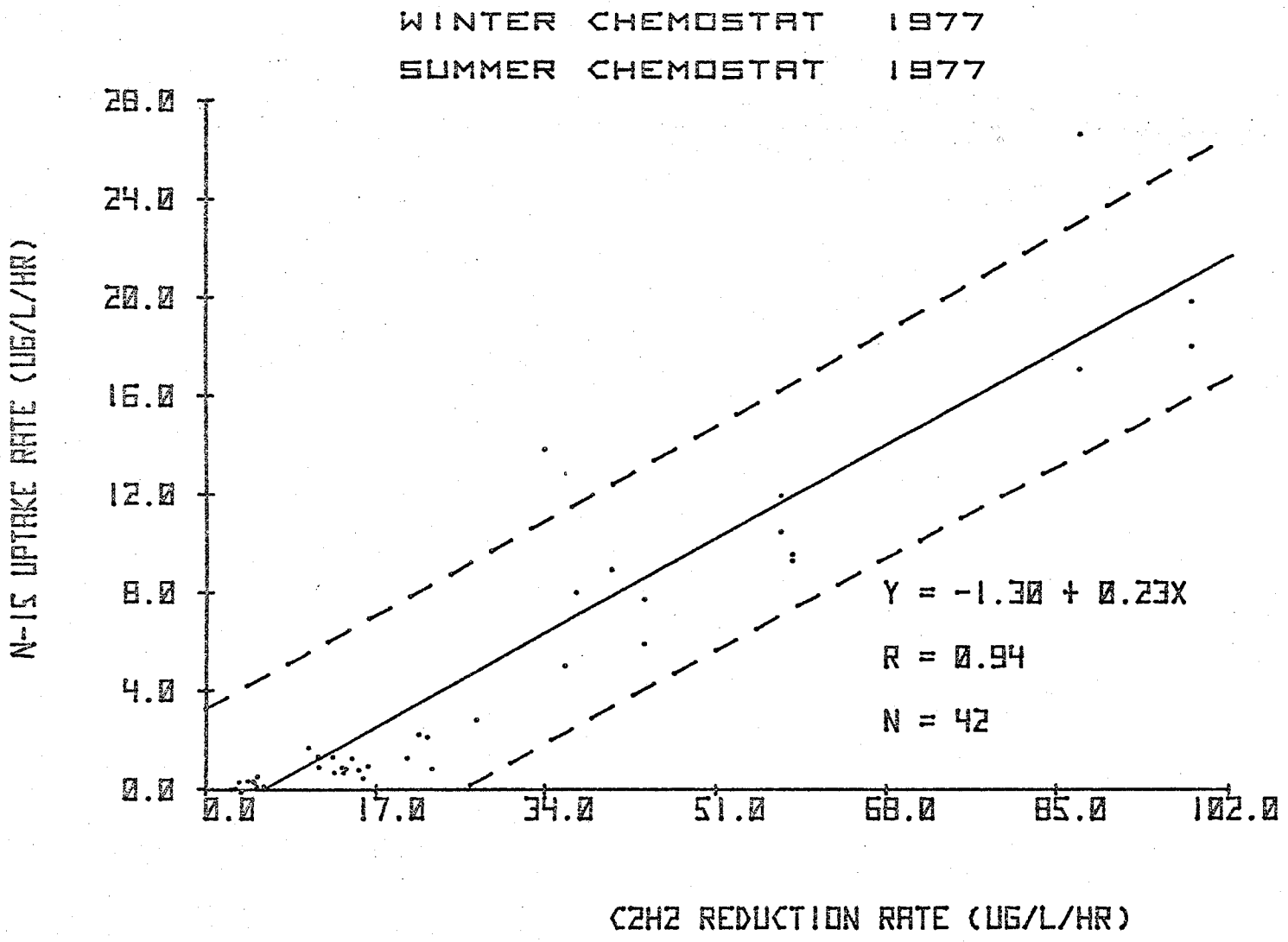

1974/75 LAKES 226 NE, 227 (FLETT, 1976)
1976 LAKES 226 NE, 227
1977 LAKES 226 NE, 227, 304
N-15 UPTAKE RATE (UG/L/HR)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
0.0
2.0
4.0
6.0
8.0
10.0
C2H2 REDUCTION RATE (UG/L/HR)

Figure 25. A plot of the linear regression analysis of acetylene reduction rate and nitrogen-15 uptake rate data accumulated for both Chemostat 1 and Chemostat 2 in 1977. The regression equation, correlation coefficient (R) and sample size (N) are provided. Dotted lines indicate the 95% confidence interval calculated for the given line.


WINTER CHEMOSTAT 1977
SUMMER CHEMOSTAT 1977
28.0
24.0
20.0
16.0
12.0
8.0
4.0
0.0
N-15 UPTAKE RATE (UG/L/HR)
0.0
17.0
34.0
51.0
68.0
85.0
102.0
C2H2 REDUCTION RATE (UG/L/HR)
Y = -1.30 + 0.23X
R = 0.94
N = 42

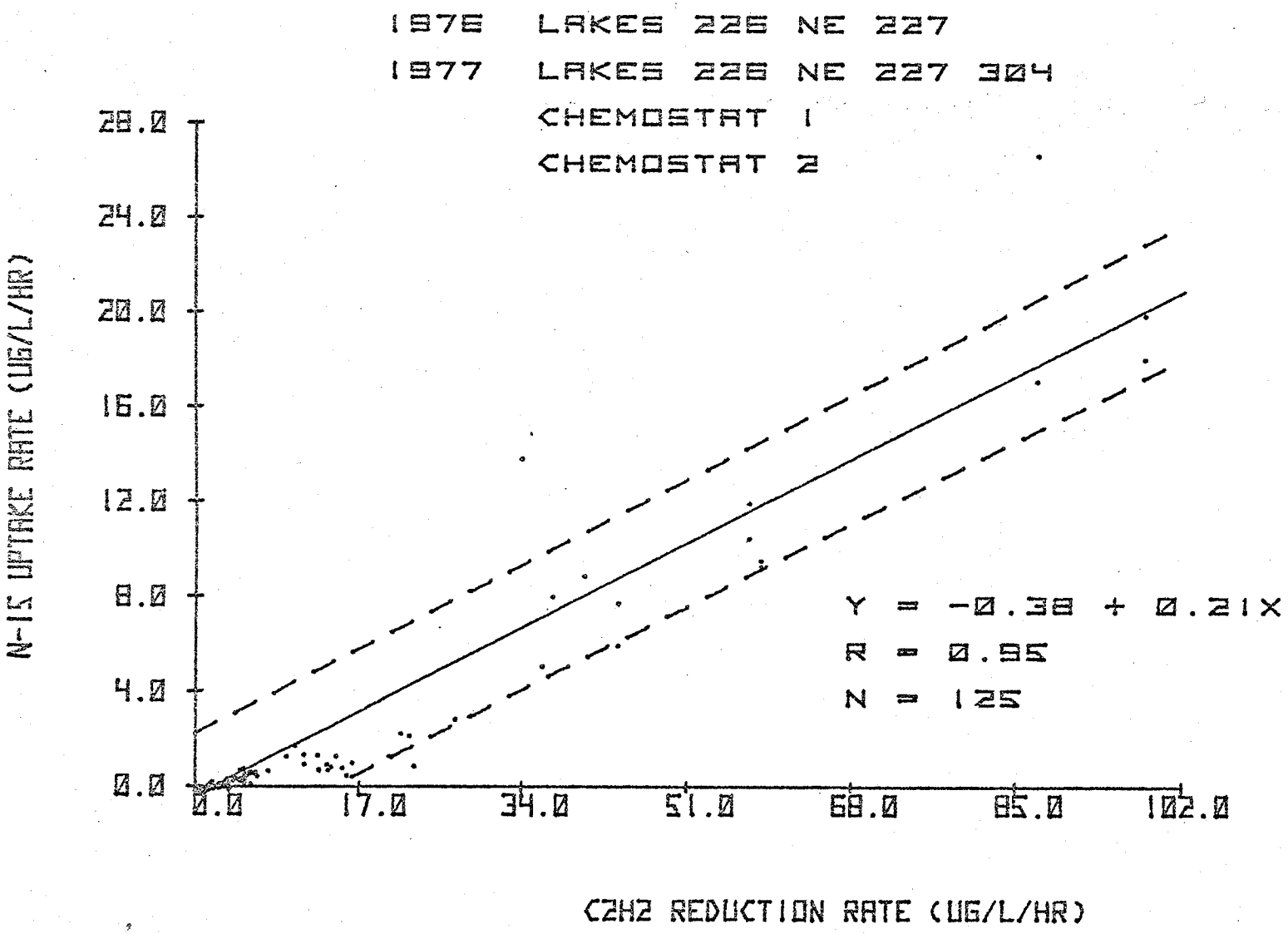
predicted when a mean was calculated. Ratios determined previously for chemostat algal populations were much lower, including values of 2.8 (Anabaena cylindrica), 3.2 (Anabaena flos-aquae) and 3.6 (Nostoc muscorum) (Stewart et al., 1968).

Following determination of the acetylene reduction to nitrogen-15 uptake relationships in the chemostat and lake systems, an overall ratio was defined for the complete data set. Calculation of this ratio was an attempt to ascertain the consistency of the acetylene to nitrogen relationship over a rate range of two orders of magnitude. Because this rate range was unavailable in any one of the systems studied, combination of lake and chemostat rates was the necessary alternative. Unfortunately this prevented clear differentiation of effects due to rate level from those introduced as a consequence of environmental differences.

Ratios calculated by weighted and unweighted mean and regression procedures (Table 12), exhibited a similar pattern to overall chemostat ratios. The low acetylene to nitrogen ratio estimated by unweighted linear regression analysis (Figure 26) again emphasized the unequal influence of the Chemostat 1 ratios, which was counteracted by introduction of the calculated weighting factor. All other estimates exhibited a much larger discrepancy between the theoretical and empirical ratios.

Though an overall ratio may be calculated, its applicability as a conversion factor to be used, independent of the environment sampled, is questionable. The diversity of ratios estimated for individual chemostat and lake systems indicate that use of a single ratio for acetylene reduction rate conversion would lead to significant inaccuracy in nitrogen fixation approximations. Lake nitrogen fixation will be overestimated if the "all data" acetylene to nitrogen ratio, estimated by non-weighted regression

Figure 26. A plot of the linear regression analysis of all acetylene reduction rate and nitrogen-15 uptake rate data collected for chemostat and lake systems in 1976 and 1977. The regression equation, correlation coefficient (R) and sample size (N) are provided. Dotted lines indicate the 95% confidence interval calculated for the given line.


1976 LAKES 226 NE 227
1977 LAKES 226 NE 227 304
CHEMOSTAT 1
CHEMOSTAT 2
28.0
24.0
20.0
16.0
12.0
8.0
4.0
0.0
N-15 UPTAKE RATE (UG/L/HR)
0.0
17.0
34.0
51.0
68.0
85.0
102.0
C2H2 REDUCTION RATE (UG/L/HR)
Y = -0.38 + 0.21X
R = 0.95
N = 125

analysis (Table 12), is used for rate conversion. Use of the same ratio will also greatly overestimate Chemostat 2 nitrogen fixation, but it will provide a reasonable estimate for Chemostat 1 nitrogen fixation.

The variability in the acetylene to nitrogen relationship from system to system would appear to imply the necessity for acetylene reduction precalibration, prior to its use as an estimator of nitrogen fixation activity in independent systems. Even differences in the "health" and contamination of the same chemostat-maintained algal species led to a variable response to acetylene reduction and nitrogen-15 uptake, preventing determination of a consistent ratio for Chemostat 1 and Chemostat 2 populations. Use of the "all lake" ratio for conversion of acetylene reduction rates measured in Lake 226 NE, Lake 227 and Lake 304 introduces less error because of the similarity of individual ratios. However, extrapolation of this ratio to other lake systems is unadvisable without confirmation of its appropriateness for the lake environment to be studied and algae present in that lake. Peterson and Burris (1976) also concluded that conversion factors determined depend upon the organism and environment investigated, and, therefore, should be applied with caution.

Linear Regression Equation Intercepts

When linear regression analysis was used for acetylene to nitrogen ratio definition, the relationship obtained was characterized both by a slope and intercept. If the slope is to be utilized independently as an estimate of the acetylene to nitrogen ratio, the significance of the intercept must be defined. The similarity of the intercepts, calculated for non-weighted and weighted regressions, and their associated errors (Table 16), suggests that the intercepts are not significantly different

TABLE 16

Ordinate Intercepts and Their Associated Error Obtained on Linear Regression Analysis of Lake and Chemostat C_2H_2 Reduction and Nitrogen-15 Uptake Rate Data.

System	Regression Intercept ± Error (µg/L/hr)	Weighted Regression Intercept ± Error (µg/L/hr)
L.226-227(1976)	.02 ± .01	.03 ± .01
L. 304	.06 ± .04	.06 ± .02
L. 226	.01 ± .02	.01 ± .02
L. 227	- .04 ± .04	- .03 ± .02
Chem 1	-1.49 ± 2.13	-3.08 ± .36 *
Chem 2	.16 ± .15	- .03 ± .06
All Lakes	.01 ± .01	- .02 ± .01
All Chem	-1.30 ± .49 *	- .10 ± .07
All Data	- .38 ± .14 *	.03 ± .01

* Intercept significantly larger than associated error.

than zero. Only in two non-weighted regressions and one weighted regression did the intercepts differ significantly from their calculated error. The occurrence of large negative intercepts was associated with the lower ratios calculated for Chemostat 1 (non-weighted and weighted regressions), all chemostat data (non-weighted regression) and all data (non-weighted regression). In these cases, rate conversion utilizing regression-derived ratios alone, rather than the complete regression equation will overestimate nitrogen fixation by an amount dependent upon the intercept magnitude. The intercept correction is more significant at low acetylene reduction rates.

Characterization of the Acetylene to Nitrogen Relationship

To better define the acetylene reduction to nitrogen fixation relationship within lake systems, acetylene reduction and nitrogen-15 uptake rate comparisons were examined with regard to increasing epilimnetic depth and sampling season variations.

To establish a rate versus depth response both concentrated and unconcentrated lake samples, collected in Lake 226 NE, Lake 304 and Lake 227, were incubated at one meter intervals from zero to three meters. If significant ratio variability was observed it would suggest inconsistency in the depth response of one or both of the techniques.

Representative rate versus depth plots (Figures 27 to 30) illustrate that both methods demonstrated a rate decline with depth, in the zero to three meter region. Often the difference in rate between zero and one meter samples was not a substantial one, one meter rates occasionally exceeding those estimated for surface samples. This inversion has been attributed previously to photoinhibition (Mague, 1977; Golterman, 1975). The

Figure 27. The distribution of acetylene reduction rate (+) and nitrogen-15 uptake rate (*) with epilimnetic depth in Lake 227 (September 11, 1976). An acetylene:nitrogen ratio of 3 is used to convert acetylene reduction rates to nitrogen fixation rates.

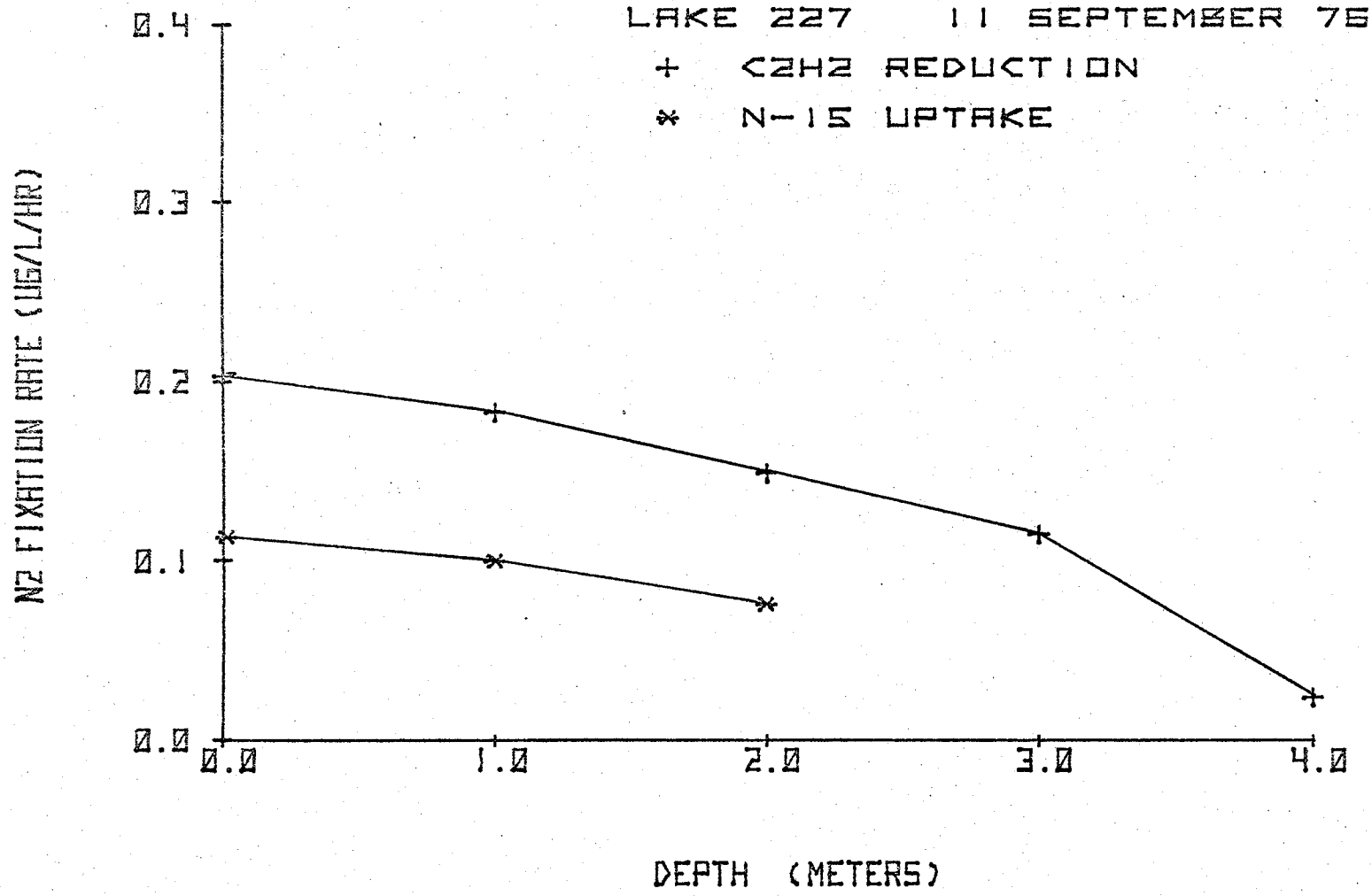

LAKE 227 11 SEPTEMBER 75
+ C2H2 REDUCTION
* N-15 UPTAKE
N2 FIXATION RATE (UG/L/HR)
0.4
0.3
0.2
0.1
0.0
0.0
1.0
2.0
3.0
4.0
DEPTH (METERS)

Figure 28. The distribution of acetylene reduction rate (+) and nitrogen-15 uptake rate (*) with epilimnetic depth in Lake 226 NE (July 15, 1977). An acetylene: nitrogen ratio of 3 is used to convert acetylene reduction rates to nitrogen fixation rates.

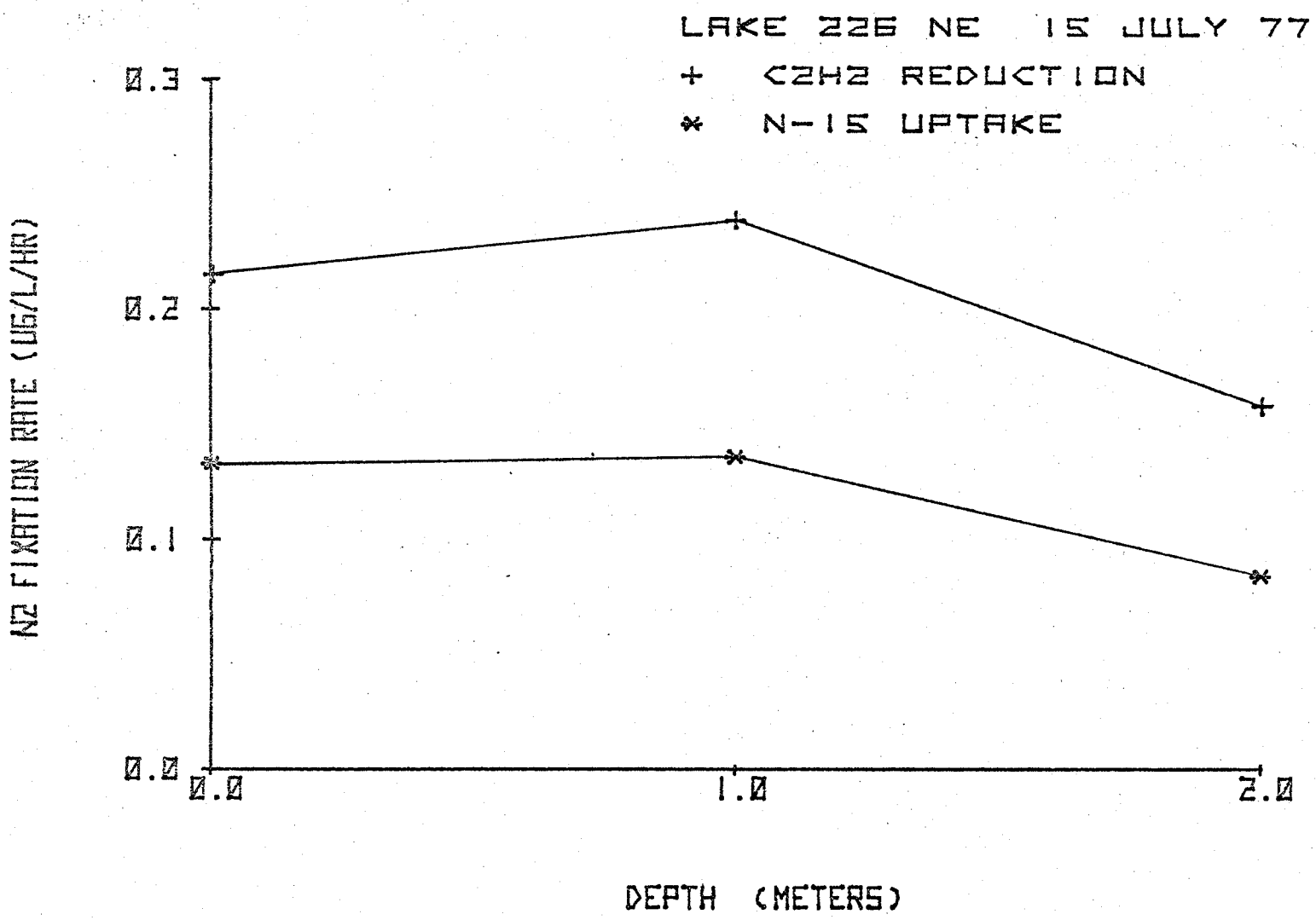

LAKE 226 NE 15 JULY 77
+ C2H2 REDUCTION
* N-15 UPTAKE
N2 FIXATION RATE (UG/L/HR)
0.3
0.2
0.1
0.0
0.0
1.0
2.0
DEPTH (METERS)

Figure 29. The distribution of acetylene reduction rate (+) and nitrogen-15 uptake rate (*) with epilimnetic depth in Lake 227 (August 29, 1977). An acetylene: nitrogen ratio of 3 is used to convert acetylene reduction rates to nitrogen fixation rates.

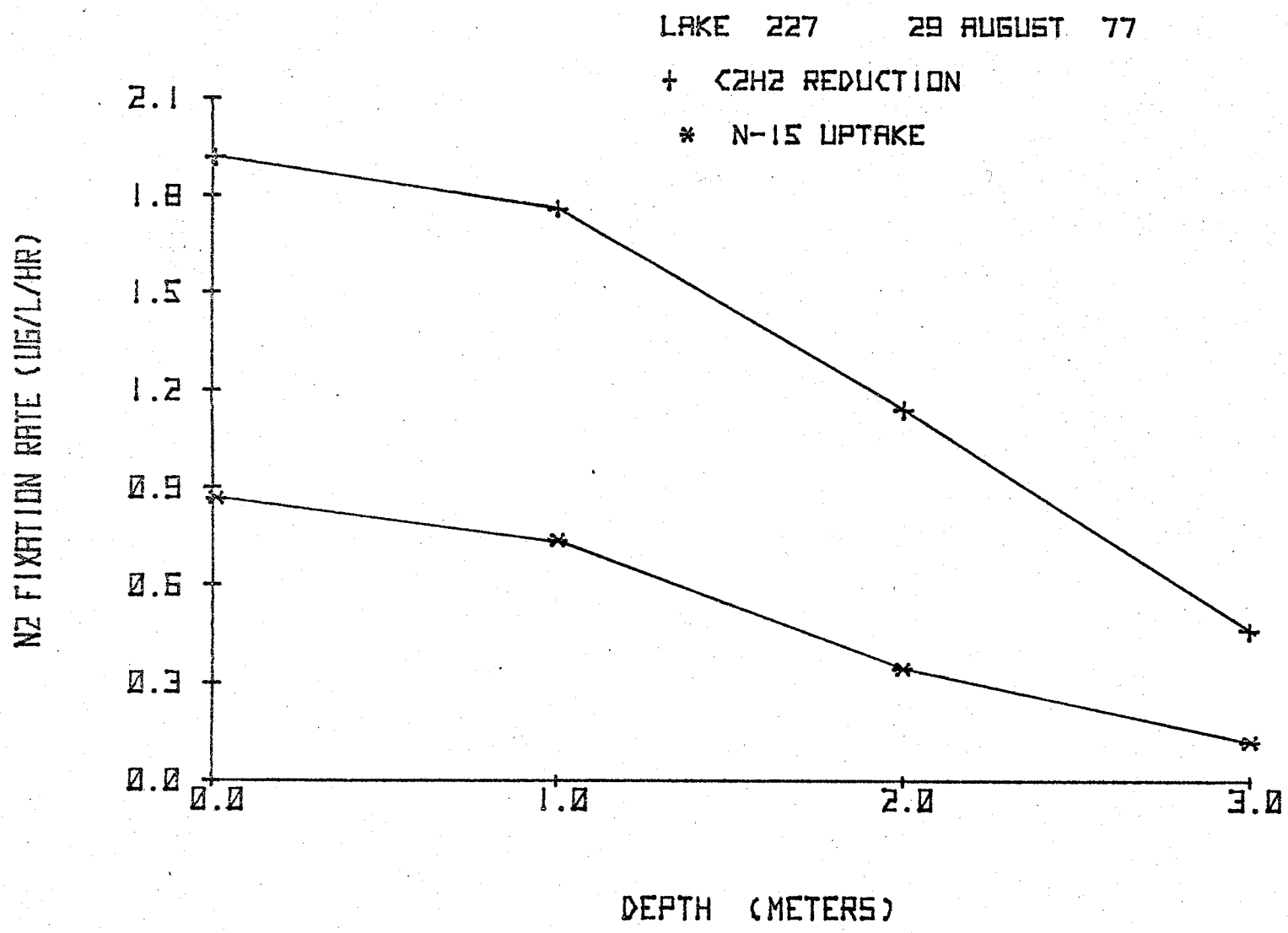
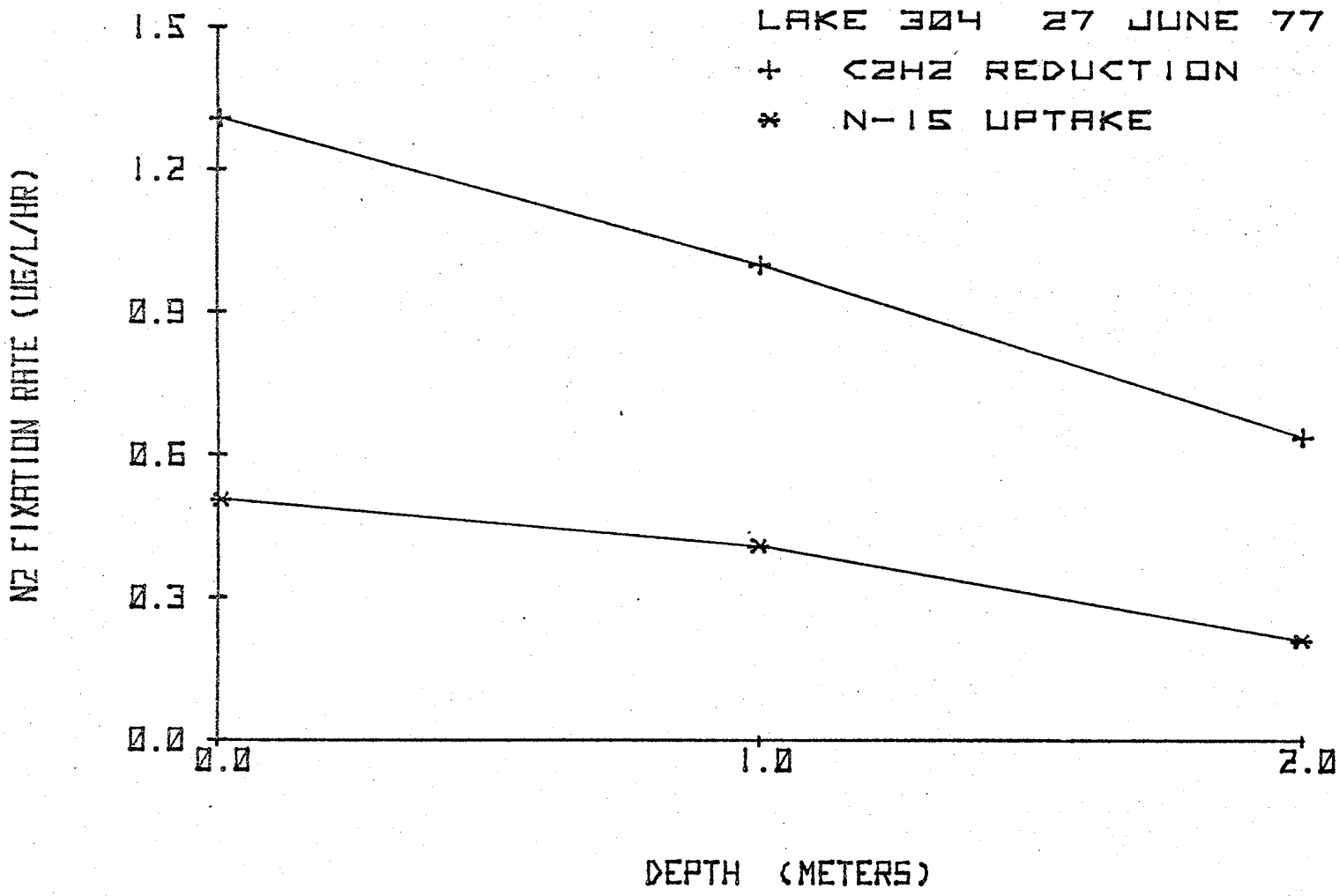

LAKE 227 29 AUGUST 77
+ C2H2 REDUCTION
* N-15 UPTAKE
N2 FIXATION RATE (UG/L/HR)
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0.0
0.0
1.0
2.0
3.0
DEPTH (METERS)

Figure 30. The distribution of acetylene reduction rate (+) and nitrogen-15 uptake rate (*) with epilimnetic depth in Lake 304 (June 27, 1977). An acetylene: nitrogen ratio of 3 is used to convert acetylene reduction rates to nitrogen fixation rates.


LAKE 304 27 JUNE 77
+ C2H2 REDUCTION
* N-15 UPTAKE
N2 FIXATION RATE (UG/L/HR)
1.5
1.2
0.9
0.6
0.3
0.0
0.0
1.0
2.0
DEPTH (METERS)

plots presented include acetylene reduction-derived nitrogen fixation rates rather than acetylene reduction rates, therefore plotted differences between nitrogen-15 uptake and acetylene reduction rates are one-third of the actual values.

Though in situ incubations of concentrated samples occurred at discrete epilimnetic depth intervals, the samples were not collected from individual depths. Subsampling from the one liter samples prepared by net haul concentration eliminated differences among individual samples incubated at specific depths. Therefore, changes observed in acetylene reduction and nitrogen-15 uptake versus depth plots which utilize concentrated samples are better attributed to physical effects such as light attenuation and temperature change, rather than biological factors presumably homogenized through composite sample preparation.

Unconcentrated Lake 227 samples also demonstrated concurrent nitrogen-15 uptake and acetylene reduction rate decline with increasing epilimnetic depth. Because of the similarity in rate versus depth response of both techniques, ratios calculated from these rate pairs exhibited no consistent relationship with epilimnetic depth.

Sampling Season Variation in Acetylene to Nitrogen Ratios

Seasonal variation in the acetylene to nitrogen ratio was difficult to define due to the nature of the bloom/non-bloom characteristics of the individual systems. None of the three lakes examined supported a blue-green algal population capable of measurable nitrogen fixation throughout the summer. A similar observation was made in 1974 and 1975; peaks in nitrogen fixation were only reported in mid-July and the latter half of August and September (Flett, 1977). Therefore, it was impossible to establish an acetylene to nitrogen ratio applicable in each lake over the entire sampling season.

Exceptionally high acetylene to nitrogen ratios (values exceeding 9), many of which were reported in the late July-early August period, appear to be a function of low nitrogen-15 uptake rates rather than high acetylene reduction rates. During this interval, nitrogen-15 substrate was withdrawn from an almost depleted nitrogen-15 gas reservoir. Consequently, nitrogen-15 substrate injections assumed to represent a given nitrogen-15 enrichment may have been deficient in the heavier isotope. If isotope depletion was significant, sample specific activity would be affected. Division by an erroneously high specific activity would cause a low nitrogen-15 uptake estimate of nitrogen fixation and an enhanced rate discrepancy. However, even when these anomolous values were excluded, empirical acetylene to nitrogen ratios still exceeded the theoretical value.

Intrasystem Variability in Acetylene to Nitrogen Ratios

Some of the variability among sample ratios observed within the given systems may be attributable to sample-to-sample nitrogen weight fluctuation. Replicability of sample nitrogen weight was influenced not only by analytical difficulties, but also by problems associated with preparative filtration. Nitrogen analysis of replicate sample filters utilizing only vacuum guage pressure measurements, were completed in order to estimate the variability of this particular procedure. Eight filters were analyzed, five of which were heater-dried, and three air-dried. Table 17 lists the individual weights, the average weight, 8.5 μg N and the standard deviation, ± 1.7 μg N, comprising twenty per cent of the average weight. Because the average weight for the heat-dried filters (7.8 μg N) was less than the average for air-dried filters (9.7 μg N) nitrogen-15 uptake sam-

TABLE 17

Determination of the Replicability of Nitrogen Weight Estimates. Samples Were Collected from Lake 227, May 28, 1977.

Drying Method	Nitrogen Weight (µg)
Heat Gun	10.5
	8.0
	7.0
	7.5
	6.0
Air Dry	8.5
	10.5
	10.0
Average	8.5
Standard Deviation	± 1.7

ples collected in 1977 were air-dried in a closed dessicator.

Lack of a consistent vacuum guage zero and a reproducible nitrogen pressure:weight standard curve contributed to this uncertainty in particulate nitrogen estimates. To establish the accuracy of guage nitrogen estimates, replicate filtered samples were analyzed for nitrogen weight both by vacuum guage pressure and carbon-nitrogen analyzer. On average, guage weights were 22.3% lower than carbon-nitrogen analyzer estimates (Tables 18a and 18b). Only two samples exhibited guage weights which exceeded carbon-nitrogen analyzer estimates. If the carbon-nitrogen analyzer values accurately described the nitrogen weight characteristics of the given samples, an increase in nitrogen-15 uptake rates, proportional to the weight correction, would be expected, as illustrated in Table 18c. These increased nitrogen-15 uptake rates would cause a corresponding decrease in acetylene to nitrogen ratios. However, this correction is insufficient to explain empirical versus theoretical ratio discrepancies.

The effect of the filtration procedure on the determination of nitrogen weight was examined with respect to the volume of sample filtered. A series of chemostat samples of increasing volume were filtered and analyzed for nitrogen weight, April 27, 1977 (Table 18a). A second series, with the same sample volumes diluted to a common total volume (fifteen milliliters) was analyzed May 3, 1977 (Table 18b). The latter sample set was prepared to eliminate differences in sample contact with the filtration apparatus, encountered when undiluted samples were filtered. Though an increase in nitrogen weight with volume filtered was apparent in both experiments, the incremental increases were not uniform. Sample particulate carbon content analyzed simultaneously exhibited a similar relationship to increased volume filtered (Table 18a,b). Though the absolute amount

TABLE 18(a)

Comparison of Nitrogen Weight Estimation by Carbon/Nitrogen (C/N) Analyzer and Vacuum Guage Methods. No Dilution of Two to Ten ml Samples Was Employed. C/N Analyzer Estimates of Carbon Weight Are Included. The Effect of Increasing the Sample Volume Filtered Is Also Demonstrated.

April 4, 1977

Volume (mls)	C/N Nitrogen Estimate (µg)	(µg/ml)	Guage Nitrogen Estimate (µg)	(µg/ml)	C/N Carbon Estimate (µg)	Nitrogen / Carbon %
2	17.0	8.5	13.5	6.8	48.0	35
4	21.0	5.3	18.3	4.6	80.0	26
6	24.0	4.0	29.0	4.8	104.0	23
8	29.0	3.6	33.0	4.1	148.0	20
10	45.0	4.5	37.0	3.7	178.0	25

TABLE 18(b)

Comparison of Nitrogen Weight Estimation by Carbon-Nitrogen (C/N) Analyzer and Vacuum Guage Methods. Two to Ten ml Samples Were All Diluted to a Constant Total Volume of Fifteen mls. C/N Analyzer Estimates of Carbon Weight Are Included. The Effect of Increasing the Sample Volume Filtered Is Also Demonstrated.

May 3, 1977

Volume (mls)	C/N Nitrogen Estimate (µg)	(µg/ml)	Guage Nitrogen Estimate (µg)	(µg/ml)	C/N Carbon Estimate (µg)	Nitrogen / Carbon %
2	14.4	7.2	12.0	6.0	47.0	31
4	17.2	4.3	12.0	3.0	66.0	26
6	17.8	3.0	14.5	2.4	71.0	25
8	25.9	3.2	17.0	2.1	98.0	26
10	26.5	2.7	19.5	2.0	114.0	23

TABLE 18(c)

The Effect of C/N Analyzer-Based Nitrogen Weight Corrections on Nitrogen-15 Uptake Rate and C_2H_2:N_2 Ratio Estimates for Lake 227 and Chemostat 1.

Original Weight (µg)	Corrected Weight N[a] (µg)	Original $^{15}N_2$ Rate (µg/L/hr)	Corrected $^{15}N_2$ Rate (µg/L/hr)	Original Ratio R_O	Corrected Ratio R_C	Difference (R_O-R_C)
Lake 227 - August 22, 1977						
408.4	499.5	0.785	0.960	7.7	6.3	1.4
408.4	499.5	0.866	1.059	5.8	4.8	1.0
385.3	471.2	0.608	0.744	8.5	7.0	1.5
385.3	471.2	0.549	0.672	8.8	7.2	1.6
366.3	448.0	0.260	0.318	10.8	8.9	1.9
366.3	448.0	0.276	0.337	9.2	7.5	1.7
Chemostat 1 - March 23, 1977						
1614.5	1974.6	26.92	32.93	3.2	2.6	0.6
1614.5	1974.6	20.15	24.64	4.9	4.0	0.9
1614.5	1974.6	17.39	22.03	5.0	4.0	1.0
1615.5	1974.6	18.30	23.19	5.4	4.2	1.2

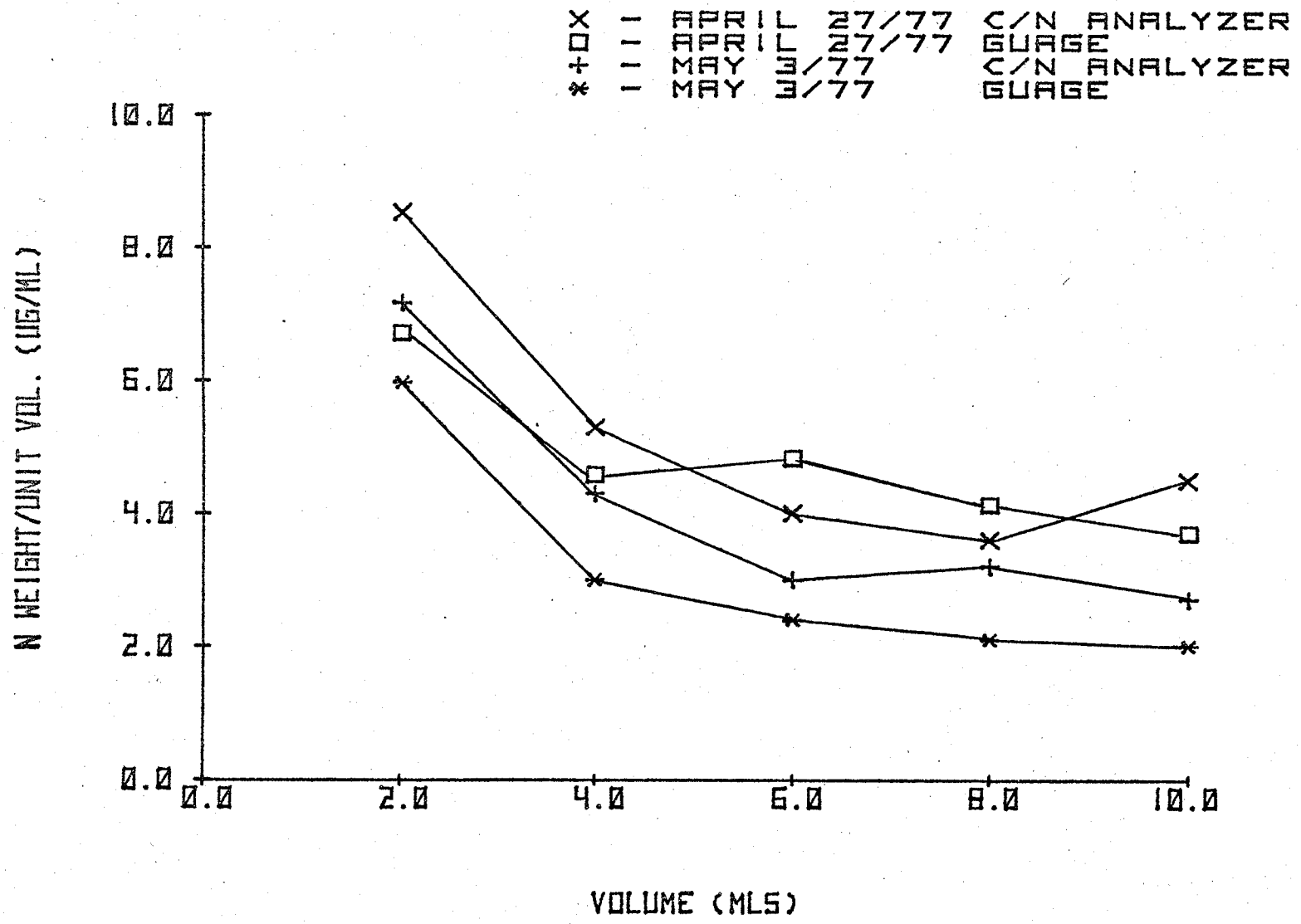
a Correction based on C/N analyzer Nitrogen Weights, which then influence Rate and Ratio Estimates.

of carbon and nitrogen varied with volume filtered, the percentage of nitrogen to carbon remained reasonably constant; the average nitrogen to carbon percentage was twenty-four per cent. The consistent nitrogen to carbon percentage implies a connection between the losses of both cellular components.

Graphs of nitrogen weight per unit volume versus the volume of sample filtered (Figure 31) exhibited similar trends to data collected in filtration experiments involving carbon-14 uptake estimation of primary productivity (Arthur and Rigler, 1967). The carbon-14 experiments implied that rates calculated for filtered samples must be corrected for losses of labelled molecules due to cell rupture and release of cellular contents during the course of sample preparations. The extent of the loss depended upon the harshness of the filtration process, for example, the vacuum applied, and the susceptibility of given species to filtration pressures (Arthur and Rigler, 1967). Carbon-14 estimates were corrected for this loss by extrapolating to zero volume a curve relating the quantity per unit volume to the volume of sample filtered.

This type of analysis was adopted for nitrogen-weight—sample volume data provided in Table 18a and Table 18b. However, to obtain a linear equation from which the zero volume intercept was more readily determined than by subjective extrapolation of the exponential curves, linear regression analyses of the logarithms of the nitrogen weight data versus the volume filtered were completed. The antilog of the regression equation y-intercept yielded an estimate of the nitrogen weight per milliliter anticipated in a sample of zero thickness (Arthur and Rigler, 1967), presumably eliminating filtration effects. This analysis resulted in corrected weight per milliliter nitrogen estimates which included 8.1 µg/ml

Figure 31. Filtration error curves for nitrogen weight as measured by C/N analyzer and vacuum guage. X - undiluted samples (April 27, 1977) analyzed by C/N analyzer. □ - undiluted samples (April 27, 1977) analyzed by vacuum guage. + - samples diluted to 15 mls (May 3, 1977) analyzed by C/N analyzer. * - samples diluted to 15 mls (May 3, 1977) analyzed by vacuum guage.


X – APRIL 27/77 C/N ANALYZER
□ – APRIL 27/77 GUAGE
+ – MAY 3/77 C/N ANALYZER
* – MAY 3/77 GUAGE
N WEIGHT/UNIT VOL. (UG/ML)
10.0
8.0
6.0
4.0
2.0
0.0
0.0
2.0
4.0
6.0
8.0
10.0
VOLUME (MLS)

(non-diluted carbon-nitrogen analyzer samples), 6.9 µg/ml (non-diluted guage samples), 7.5 µg/ml (diluted carbon-nitrogen analyzer samples) and 6.1 µg/ml (diluted guage samples). Comparing these values to measured nitrogen weight/ml values associated with ten milliliter sample volumes, allowed calculation of correction factors including 1.8, 1.9, 2.7 and 3.1, which appeared to be necessary for nitrogen weight calibration. This correction was not applied to the data collected in this study because as shown by the following discussion, analyses of sample filtrates and experiments by McMahon (1973) do not support the hypothesis of filtration-induced cell content loss.

Investigation of the relationship of nitrogen-15 uptake rate to sample volume filtered demonstrated the responses of both nitrogen-15 enrichment and nitrogen weight to filtration. Filtration error curves (Figure 32) were constructed for nitrogen fixation rate per unit volume and nitrogen weight per unit volume for a series of six Chemostat 2 samples of increasing volume (Table 19). As the volume filtered increased from five to thirty milliliters, both nitrogen fixation rate per ml and nitrogen weight per ml exhibited a gradual non-linear decrease. Rate data was treated similarly to nitrogen weight data above, curve extrapolation to zero volume presumably yielding values independent of filtration effects. The equations obtained following logarithmic transformation of rate per ml and weight per ml data were $y = -0.035x + 0.103$ and $y = -0.022x + 0.477$ for the rate per milliliter and weight per milliliter parameters respectively. The zero volume intercepts were calculated from the antilogs of the y-intercepts, 0.103 and 0.477. Values determined were 1.27 µg/L/hr per ml and 3.00 µg per ml. Comparing these values to the rate per ml and weight per ml values estimated for the

Figure 32. Filtration error curve for nitrogen fixation rate (x) and nitrogen weight (□). Samples collected from Chemostat 2 September 1, 1977.

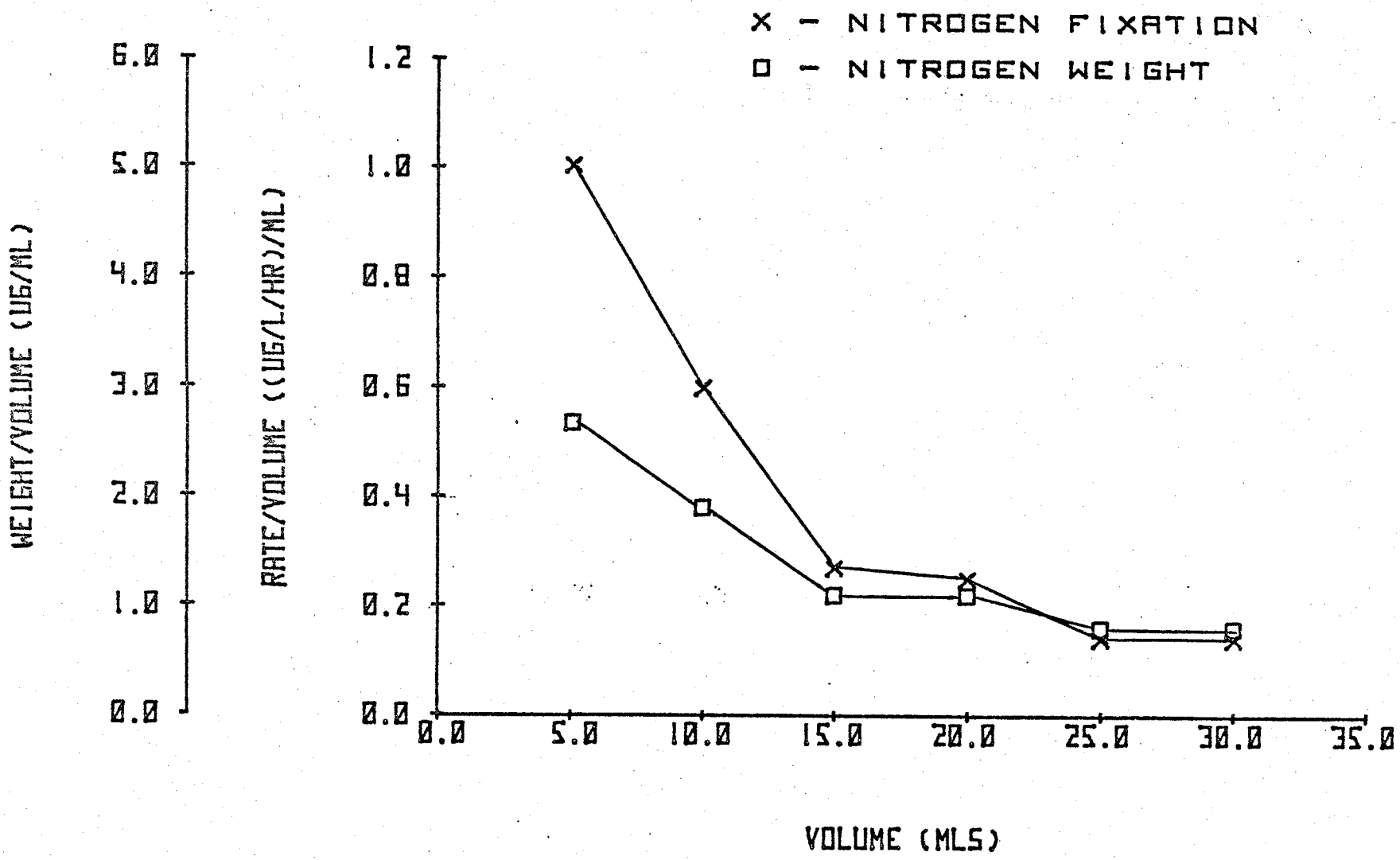

X - NITROGEN FIXATION
□ - NITROGEN WEIGHT
WEIGHT/VOLUME (UG/ML)
RATE/VOLUME ((UG/L/HR)/ML)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
VOLUME (MLS)

TABLE 19

The Effect of Filtration of Increasing Sample Volumes on Nitrogen Weight and Nitrogen Fixation Rate Estimates.

Volume (mls)	Nitrogen Weight (µg)	Weight/ Volume (µg/ml)	Nitrogen Fixation Rate (µg/L/hr)	Rate/Volume (µg/L/hr)/ml
5	13.4	2.7	5.1	1.00
10	19.3	1.9	6.0	0.60
15	16.1	1.1	4.1	0.27
20	22.1	1.1	5.0	0.25
25	20.4	0.8	3.5	0.14
30	23.7	0.8	4.3	0.14

thirty milliliter sample, a factor was calculated which, when applied to samples of equal volume, should correct rate and weight values for observed filtration effects. The factor estimated for rate correction, 9.1, exceeded the factor estimated for weight correction, 3.8, the difference being attributed to filtration-induced loss of nitrogen-15 labelled material, as well as unlabelled material. However, comparison of Chemostat 2 acetylene reduction rates and nitrogen-15 uptake rates, following introduction of the rate correction, did not yield the theoretical acetylene to nitrogen ratio. Correction for the filtration effect resulted in a decrease in the mean Chemostat 2 acetylene to nitrogen ratio from 11.9 to 1.3. The extensive filtration-induced cell breakage and label loss indicated by such a drastic difference in the ratio should be verifiable by a large accumulation of nitrogen-15-labelled material in sample filtrates. No nitrogen-15 enrichments significantly different than the measured natural abundance atom per cent 0.393 ± 0.034 (2σ) were determined in sample filtrates (Table 20). This suggested that label loss on filtration was not occurring to a measurable extent, at least not in a form detectable with the methods employed. Further discussion of filtrate analysis and its implications accompanies discussion of the excretion hypothesis for rate discrepancies.

The hypothesis of cell breakage and label loss on vacuum filtration was also criticized by McMahon (1973), whose filtration experiments indicated that physical adsorption of carbon-14 label on membrane filters was responsible for filtration error curve characteristics. Washing of filters with a large volume of non-labelled water led to elimination of the filtration effect. In addition, the activity per ml decrease for samples exceeding twenty-five milliliters appeared to begin levelling,

TABLE 20

Analyses of Selected Filtrate Samples for ^{15}N Atom Per Cent Enrichment Above Background and Nitrogen Weight. Filtrates Receiving Preliminary Roto-Evaporator Concentration Are Noted (conc.).

System	Date	Atom% Difference	N Weight (mg/L)	Sample Type [a]
Chem 2	13/6/77	0.006	52.4	1 ml unconc.
Chem 2	13/6/77	-	92.9	1 ml unconc.
Chem 2	19/6/77	-	27.7	1 ml unconc.
Chem 2	19/6/77	-	122.9	0.3 ml conc.
L.304	22/6/77	-	68.0	1 ml unconc.
L.304	22/6/77	0.002	16.6	1 ml unconc.
L.304	22/6/77	-	9.8	1.4 ml conc.
L.226NE	6/7/77	0.006	28.7	1 ml unconc.
L.304	9/7/77	0.003	11.3	1 ml unconc.
L.226NE	15/7/77	0.007	16.1	1 ml unconc.
Chem 2	18/7/77	0.003	23.9	1 ml unconc.
L.227	27/7/77	0.022	13.5	1 ml unconc.
L.227	27/7/77	0.016	0.2	1 ml unconc.
L.227	24/8/77	-	7.6	1 ml unconc.

a Differentiation between filtrates which were concentrated and those which were not.

approaching the values measured for washed samples. If the latter observation was also characteristic of nitrogen-15 uptake samples, then the significance of filtration effects would be minimized in routine rate comparison sampling. Nitrogen fixation rate comparison samples were thirty or fifty milliliters depending upon cell densities. Rate per unit volume values characteristic of these sample sizes would appear to lie in the region of curve levelling (Figure 32), where filtration effects resulting from adsorption of label to filters would be expected to be reduced.

To confirm that filter adsorption of nitrogen label does occur in a similar manner to carbon-14 retention, further filtration error experiments are required. Estimation of nitrogen-15 retention on filtration of raw and prefiltered samples and following adequate washing is necessary to characterize filtration effects. The filtration error relationship should also be examined for lake nitrogen-15 samples to determine whether a similar response to that of Chemostat 2 is noted.

Acetylene to Nitrogen Ratio Explanation

Of greater importance than ratio variability among lake samples or chemostat samples is the discrepancy between the empirical acetylene to nitrogen ratios and the ratio predicted on the basis of chemical reaction stoichiometry. Empirical ratios listed in Table 12 exceed the theoretical value by 1.4 to 4.4 times. More than one hypothesis has been forwarded to account for similar acetylene to nitrogen relationships described previously. Proposed explanations include suggestions of differences in (1) technique sensitivities, (2) oxygen sensitivity (Bergersen, 1970), (3) response to zooplankton ammonia excretion (Lean et al., 1976),

(4) partial release of accumulated product (Fogg, 1966; Walsby, 1974; Walsby and Fogg, 1975) and (5) the effects of associated hydrogen reduction (Schubert and Evans, 1976; Bothe et al., 1977; Evans et al., to be published). A brief discussion of these possibilities with respect to their applicability to the present study follows.

The first explanation suggests that ratio differences may be attributable to differing capacities of the acetylene reduction and nitrogen-15 uptake techniques to estimate nitrogen fixation in a system characterized by low blue-green algal biomass or activity. Because of the nature of the analytical procedure, the acetylene reduction technique exhibits a lower limit for detection of nitrogen fixation activity (Hardy et al., 1968; Klucas, 1969). Nitrogen-15 uptake rates measured at activities close to that detection limit may tend to underestimate nitrogen fixation rates, elevating the calculated acetylene to nitrogen ratios. However, in the present study ratio enhancement persisted at rate levels where sensitivity limitations no longer applied (Table 10). Therefore, explanation of rate discrepancies, simply on the basis of differing sensitivity limits for the two methods, is inadequate.

Manipulation of sample oxygen partial pressures and substrate concentrations was also found to cause ratio variability (Bergersen, 1970). Oxygen response curves were observed to differ depending upon the substrate level present. At a high acetylene concentration (0.1 atm) no oxygen inhibition of acetylene reduction was apparent. At a similar nitrogen concentration nitrogen-15 uptake was inhibited, above 0.5 atm oxygen. Inhibition of acetylene reduction at oxygen pressures above 0.5 atm was apparent at low acetylene concentrations (0.005 atm) as well, though the shape of the oxygen response curve differed from that observed for

nitrogen-15 uptake oxygen response. Therefore, depending upon the acetylene concentration and the oxygen partial pressure, rate comparisons could yield a non-constant ratio. In the presence of 0.2 atm oxygen an acetylene to nitrogen ratio of 6.2:1 was calculated for samples experiencing low acetylene levels, and a ratio of 3:1 was estimated for similar samples exposed to 0.7 atm oxygen (Bergersen, 1970).

In the rate comparisons completed in this study, *in situ* gas composition was not manipulated, with the exception of the addition of the gaseous substrate. Some gas stripping out of solution into the injected gas bubble was a possibility, but the bubble itself comprised only 0.8% and 5% of the sample volume in acetylene and nitrogen studies so that large gas concentration changes would not be anticipated. Oxygen present at *in situ* concentrations would not inhibit algal acetylene or nitrogen-15 uptake, assuming the normal oxygen scavenging processes maintained anoxic conditions at the nitrogenase site (Burris and Peterson, 1976). Because acetylene was provided in quantities sufficient to saturate the nitrogenase, the variable response of acetylene reduction to oxygen partial pressure at low acetylene concentrations observed by Bergersen (1970) cannot be adopted here to explain the observed ratio enhancement.

The third explanation for the ratio difference is dependent upon the method of sample preparation, specifically whether extensive concentration of algal samples was employed. Lean *et al.*, (1976) criticized excessive concentration, because simultaneous concentration of zooplankton enhanced grazer-related ammonia release, attenuating the necessity for nitrogen fixation, while not necessarily affecting a corresponding decrease in acetylene reduction. This selective effect, if significant, would lead to increased acetylene to nitrogen ratios. Concentration pro-

cedures were adopted in some lake rate determinations, but sample concentration did not exceed a factor of ten (Table 1). In addition, the phytoplankton net haul method of concentration used for this study should have prevented excessive zooplankton concentration, since many zooplankton should have been able to excape by passively riding the pressure wave developing in front of the fine mesh net. Therefore, the grazer-related explanation would not appear applicable in this situation.

The two most promising explanations for the observed differences between empirical and theoretical ratios include excretion of assimilated label and involvement of hydrogen as a competitor with nitrogen for necessary reductant and energy resources.

Excretion of nitrogenous material was examined by a number of investigators, often in association with studies on phytoplankton-fixed carbon release. Fogg, (1966), Walsby (1974), Walsby and Fogg (1975), Jones and Stewart (1969) and others contributed evidence supporting the release of nitrogenous material from actively growing, as well as senescent blue-green algal cultures. Ammonia and amide release were reported for short term experiments with Westiellopsis prolifica, a nitrogen-fixing alga from rice field soil (Pattnaik, 1964), but the most frequently observed extracellular products were amino acids and small peptides predominantly comprised of serine, threonine and basic amino acids (Fogg, 1966; Jones and Stewart, 1969). Walsby (1974) also noted the release of brownish pigments and substances fluorescing white and blue in ultraviolet light.

In this study, where particulate fraction nitrogen-15 atom per cent enrichment was used to reflect the quantity of nitrogen fixation per unit time loss of nitrogen-15-labelled product from the particulate fraction

may have resulted in the enhanced acetylene to nitrogen ratios estimated. Nitrogen-15 enrichment would then indicate net nitrogen fixation rather than the total nitrogen fixed during the incubation interval. Acetylene reduction, due to the nature of the assay would presumably represent total nitrogen fixed. The significance of the excretion of organic nitrogen to the isotope method depends upon the turnover time of the nitrogen in the cell, assuming the cell structure is not disrupted. If labelled nitrogen assimilated during a given two to three hours interval is excreted as ammonia or organic nitrogen within this incubation period, then the effect of excretion on ratio calculations will be significant.

Two considerations, one empirical and one based upon a turnover time estimate, prevented acceptance of this hypothesis as an adequate explanation of the difference between experimental and theoretical ratios. The empirical approach adopted to investigate this hypothesis involved analysis of sample filtrate enrichments. When labelled particulate material yielded nitrogen fixation rates lower than acetylene reduction-derived estimates, it was hypothesized that filtrates, collected simultaneously, should account for at least part of the missing isotope. On the basis of filtrates analyzed (Table 20) this assumption was unsubstantiated. Though sufficient nitrogen was present, concentrations ranging from 7.6 mg/L (a Lake 227 non-net haul filtrate) to 92.9 mg/L (Chemostat 2 filtrate), the natural abundance value, rather than nitrogen-15 enrichment, characterized the isotopic composition. That extracellular nitrogen release was occurring was suggested by the high dissolved nitrogen concentrations, especially those of Chemostat 1 filtrates, whose initial medium was nitrogen-free. However, the lack of enrichment in incubated sample filtrates appeared to indicate that the organic nitrogen released was the

product of prior nitrogen assimilation, rather than assimilation occurring during the incubation interval. Loss of label in the form of volatile nitrogen-15 labelled ammonia appears less likely as organic nitrogen is indicated to be the primary form of excreted nitrogen observed for most blue-green algal species (Fogg, 1952).

Calculation of an approximate turnover time of the incorporated nitrogen, patterned on a similar calculation described by Walsby and Fogg, (1975), supported the above conclusions. To estimate this turnover time, the values required were the nitrogen mass per cell and the rate of nitrogen fixation per cell. Though per cell values were unavailable, per liter estimates were, allowing the following calculation:

$$\text{Turnover time} = \frac{\text{mass nitrogen/cell}}{\text{nitrogen fixation rate/cell}}$$

$$= \frac{(\text{mass nitrogen/liter}) \,/\, (\text{cell/liter})}{(\text{nitrogen fixation rate/liter})/(\text{cells/liter})}$$

Thus an estimate of cells per liter was not required.

Calculation of the turnover time for a representative unconcentrated Lake 227 sample yielded an estimate of approximately seventeen days, which greatly exceeded the maximum incubation interval employed. Walsby's estimate of the turnover time for nitrogen assimilated by Anabaena cylindrica Lemm. cells was on the order of nine days (Walsby and Fogg, 1975). Fogg (1952) reported that the extracellular nitrogenous products of A. cylindrica did not appear to include any appreciable proportion of substance specifically associated with the fixation process because the relative amount and composition of the fraction remained much the same when the alga was cultivated on nitrate or ammonia, as when only elementary nitrogen was supplied. Each of these observations reinforce the

conclusion that the label excretion hypothesis for ratio discrepancy is not tenable.

A number of laboratory excretion experiments reporting enriched filtrates did involve prolonged incubation periods often exceeding one or two days (Jones and Stewart, 1969; Stewart, 1964; Walsby and Fogg, 1975). Filtrate labelling was also observed in an experiment involving phytoplankton ^{15}N-nitrate uptake (Chan, 1977), ^{15}N-labelled dissolved organic nitrogen being collected by passing sample filtrates through cation-exchange resin. Chan indicated that significant phytoplankton excretion of labelled amino acids was occurring, but it was insufficient to completely explain differences in chemical and isotopic estimates of nitrate uptake.

If label loss was a consequence of cell breakage on filtration, rather than active excretion of assimilated nitrogen, turnover time estimates would be irrelevant. Information collected concerning this aspect is contradictory. A filtration error curve (Arthur and Rigler, 1967), Figure 32) apparently supported the proposal that filtration-induced nitrogen-15 uptake rate underestimation contributed significantly to the enhanced ratio. However, as determined earlier when the filtration error curve was discussed, the correction introduced by the filtration error curve approach was excessive, leading to Chemostat 2 acetylene to nitrogen ratios less than the stoichiometric value of three. Examination of filtration error determinations such as that of McMahon (1973) suggested study of filter retention of ^{15}N label by selective filtration and washing experiments, rather than estimation of label loss on vacuum filtration. Also, cell damage on filtration was demonstrated to be minimal, evidence of structural cell fragments being absent on electron microscopic examina-

tion of precipitated materials from filtered lake samples (Chan, 1977).

Hydrogen Production Hypothesis

Though the final hypothesis concerning nitrogen-hydrogen competition was not investigated here, support for its contribution to the acetylene reduction-nitrogen-15 uptake comparison has increased in the literature. A large proportion of the research on this aspect was completed in nodulated symbionts (Schubert and Evans, 1976; Bethlenfalvay and Phillips, 1977; Schubert et al., 1977; Evans et al., to be published) and bacterial systems (Bulen et al., 1965; Brotonegoro, 1974; and Smith et al., 1976). Empirical evidence for hydrogen production in blue-green algae and descriptions of the process and its responses to various environmental manipulations has also accumulated more recently (Jones and Bishop, 1976; Bothe et al., 1977; Burris and Peterson, 1977).

Concurrent nitrogen fixation and hydrogen formation by blue-green algal nitrogenase necessitates the sharing of both energy (adenosine triphosphate) and reductant reserves. Competition for these resources implies that neither process will function at maximal rates, when both substrates are available (Jones and Bishop, 1976). One third of the energy (ATP) and reductant used by nitrogenase in vivo may be lost in hydrogen production (Anderson and Shanmugam, 1977). Measurement of hydrogen evolution by legume nodules in the presence and absence of nitrogen (Schubert and Evans, 1976) demonstrated that although hydrogen evolution was apparent under an atmosphere of air, evolution was approximately 3.3 times greater when a nitrogen-free environment was employed. The relevance of hydrogen production to rate comparisons would be negligible if substrate competition with hydrogen occurred during acetylene reduction, as well.

However, it was determined that acetylene, supplied in concentrations sufficient for nitrogen fixation assays, inhibited nitrogenase-mediated hydrogen formation, allowing reservation of ATP and reductant for reduction of acetylene (Schubert and Evans, 1976; Bothe et al., 1977). Therefore, acetylene reduction rates would not reflect a balance between hydrogen formation and ethylene production, consequently a maximal reduction rate would be anticipated. Assuming that hydrogen production is characteristic of the species for which the rate comparison was undertaken in this study, responsibility for enhanced ratios might reasonably be attributed to this process. Nitrogen-15 uptake rates would be reduced relative to acetylene reduction estimates by an amount dependent upon the extent of hydrogen formation.

If this explanation is accepted, it is then necessary to ascertain the reason for ratio variability between the lake and chemostat systems. Schubert and Evans (1976) demonstrated that the relative efficiency of hydrogen evolution in leguminous and non-leguminous plants, capable of nitrogen fixation, differed depending on the symbiont examined. The efficiency of hydrogen evolution is influenced by the presence within a given algal specie of a hydrogenase capable of uptake and oxidation of hydrogen produced by nitrogenase (Evans et al., to be published; Anderson and Shanmugam, 1977). Blue-green algal hydrogenases were observed to vary both in type and activity not only from organism to organism, but also from strain to strain (Bothe et al., 1977). Lake and chemostat populations differed in their predominant blue-green algal species.

If the nitrogenase-mediated hydrogen formation and the hydrogenase-mediated hydrogen uptake by the algal species in the lake (predominantly Anabaena solitaria v. planctonica and Aphanizomenon flos-aquae) and chemo-

stat (Anabaena variabilis) differ, the ratio characteristic of each system would be affected. An efficient hydrogenase would be expected to effectively recycle hydrogen as well as ATP, preventing restriction of the reduction of nitrogen to ammonia. In such a system nitrogen-15 uptake rates would approach acetylene reduction rates, consequently yielding acetylene to nitrogen ratios closer to theoretical. Assuming that the Chemostat 1 population either exhibited a lower level of hydrogen formation or possessed a more efficient hydrogen retrieval system than the lake populations, ratio differences observed between it and the lake populations might be plausibly explained.

Interpretation of the ratio discrepancy between Chemostats 1 and 2 is more difficult, since the inoculum for each chemostat was an Anabaena variabilis strain. Differences in the response of the nitrogen-hydrogen relationship to environmental conditions in each chemostat may yield a possible explanation. Such a response to an external factor was reported by Jones and Bishop (1976). They indicated that photosynthesizing populations subject to carbon dioxide limitation would possess a large excess of low potential electrons, which would be shunted through the hydrogen production circuit to eliminate the electrons from the cell. If this electron removal depleted energy resources at the expense of nitrogen-15 fixation, a high acetylene to nitrogen ratio would result. Carbon dioxide limitation should not have been a factor in either chemostat, both systems receiving a similar nutrient input, including atmospheric carbon dioxide and sodium bicarbonate.

Extracellular release of organic carbon favored under conditions of photoinhibition (Fogg, 1966), suspected to occur during early stages of Chemostat 2 operation, could have created restrictions on the availability

of carbon compounds required for nitrogen assimilation. In order that amino acids necessary for synthesis of cellular protein may accumulate, ammonia, the product of nitrogen fixation, must be incorporated into these carbon compounds synthesized via photosynthetic reduction of carbon dioxide. Organic carbon limitation, significant only to nitrogen-15 uptake estimation, would enhance acetylene to nitrogen ratios in Chemostat 2. The effect of light inhibition on the photosynthetically-derived electron supply concerns both nitrogen and proton reduction processes, presumably preventing rate discrepancies caused by unequal electron distribution. Observation of the nitrogen:hydrogen relationship under varying environmental stresses is necessary to characterize the extent of its influence on the acetylene to nitrogen ratio.

Ancillary Experiments

Though rate comparison does provide a numerical characterization of the acetylene to nitrogen relationship, the latter may also be described by ancillary experiments. Acetylene reduction and nitrogen-15 uptake are compared with respect to their response to a period of dark incubation, an extended series of light incubations and a range of their specific substrate concentrations. Differences apparent in the response of either technique may contribute to the possible explanations for acetylene reduction and nitrogen-15 uptake rate discrepancies evident in calculated acetylene to nitrogen ratios.

Dark Nitrogen Fixation

Following incubation, lake samples collected in the field required transport back to the laboratory prior to filtration and analysis. Samples

were shielded from the light to eliminate further light-supported acetylene reduction or nitrogen-15 uptake, but were not killed to prevent subsequent dark fixation. To determine the amount of dark nitrogen fixation which may have occurred during the transport period, dark rate estimation experiments, utilizing both methods, were carried out. Nitrogen-15 uptake and acetylene-derived nitrogen fixation rates were measured and compared to corresponding light rates estimated simultaneously over equivalent intervals (Table 21). From these data, dark rate to light rate percentages were calculated for both acetylene and nitrogen-15. An additional dark-light comparison was completed for one light acetylene reduction sample and one dark acetylene reduction sample, the latter having previously undergone a four hour dark preincubation (Table 21).

Dark acetylene reduction in both chemostat and lake samples, was observed to represent an average of forty per cent of simultaneously measured light acetylene reduction. Based on the single estimate calculated April 25, 1977, dark nitrogen-15 uptake represented twenty-nine per cent of the corresponding light uptake. The dark nitrogen-15 uptake rate estimated April 19, 1977 was not included in the percentage calculation because of the uncertainty in its magnitude, the result of an atom per cent enrichment not significantly different from natural abundance (based on a significance level of two standard deviations -- 0.393 ± 0.034 atom per cent). These dark rate/light rate percentages are comparable to those found by Horne and Fogg (1970), surface sample dark fixation measured by acetylene reduction amounting to fifty per cent of that in the light. Dark preincubation markedly reduced the dark acetylene reduction, dark rates in preincubated samples representing only two per cent of the measured light rate. The result of this preincubation experiment supports the con-

TABLE 21

Comparison of Dark Rate and Light Rate Estimates of Lake and Chemostat Nitrogen Fixation For Given Sampling Dates. C_2H_2 Reduction Rate Data is Converted to Nitrogen Fixation Rates Using a C_2H_2:N_2 Ratio of 3.

Date	Average Dark C_2H_2 Rate (µg N_2/L/hr)	Average Light C_2H_2 Rate (µg N_2/L/hr)	Average Dark $^{15}N_2$ Rate (µg N_2/L/hr)	Average Light $^{15}N_2$ Rate (µg N_2/L/hr)	Dark/Light % C_2H_2	Dark/Light % N_2
19/4/77(Chem)	5.23	13.76	0.71[b]	6.50	38	11
25/4/77(Chem)	9.04	19.36	3.11	10.59	47	29
20/6/77(227)	0.02	0.06	-	-	43	-
2/6/77(304)	0.48	2.05	-	-	23	-
27/6/77(304)[a]	0.02	1.30	-	-	2	-

a Lake 304 sample was preincubated in the dark for four hours prior to C_2H_2 injection.

b Atom per cent enrichment does not exceed natural abundance plus two standard deviations.

tention that the extent of dark fixation depends upon the conditions prevailing during the period preceding dark incubation (Fay, 1976).

The dark rate-light rate percentages calculated for each method apparently indicate a difference in the response of each technique following termination of light exposure. However, because of the small magnitude of the correction for dark nitrogen-15 uptake and acetylene reduction relative to the rates of light fixation, the significance of this difference in percentage between the two techniques is reduced. A maximum dark rate correction for each of the techniques was calculated for the two dark incubation intervals of ten and forty-five minutes, which occurred during routine rate comparisons in the chemostat and lake, respectively. This calculation assumed that the dark acetylene reduction and nitrogen-15 uptake rates equalled their respective light rates throughout the ten and forty-five minute intervals. Comparison of the quantity of acetylene or nitrogen-15 fixed in the dark during these intervals (assuming light fixation rates), to the quantity of these substrates fixed in the light, as illustrated below, indicated that the dark fixation contributions were 8.3% for the ten minute interval and 37.5% for the forty-five minute interval.

$$\frac{(\text{Dark Rate} = \text{Light Rate})(\text{Incubation Time})}{(\text{Light Rate})(\text{Incubation Time})} \times 100 = \text{Per Cent Contribution by Dark Fixation}$$

Example: $$\frac{(19.36\ \mu g/L/hr)(10/60\ \text{hours})}{(19.36\ \mu g/L/hr)(2\ \text{hours})} \times 100 = 8.3\%$$

However, it has been demonstrated that dark acetylene reduction exhibits an exponential decrease in rate with time, falling to fifty per cent of the original value within thirty minutes of termination of light exposure

(Fay, 1976). If it may be assumed that dark nitrogen-15 uptake responds similarly, then the maximum percentage contribution of dark acetylene reduction and dark nitrogen-15 uptake should decrease with time in proportion to the decreasing rates, thus reducing the above dark rate-light rate percentages.

If the relative differences in the average dark rate-light rate percentages for nitrogen-15 and acetylene calculated for the two hour incubations is maintained independent of the length of dark incubation, then a more specific estimate of the dark fixation contribution may be approximated. Introduction of the dark to light acetylene reduction rate percentage of 40% and the dark to light nitrogen-15 uptake rate percentage of 29% to the calculations for the ten and forty-five minute intervals allowed estimation of dark fixation contributions for acetylene of 3.3% (ten minutes) and 15% (forty-five minutes) and for nitrogen-15 of 2.4% (ten minutes) and 10.9% (forty-five minutes). Again, inclusion of the effect of an exponential rate decline (Fay, 1976; Lannergren, 1974) would reduce the dark fixation contribution. Even without this correction, though, the maximum difference in the acetylene reduction and nitrogen-15 uptake dark fixation contributions was less than five per cent. Therefore, the rate discrepancies indicated by acetylene to nitrogen ratios are apparently little affected by the differences in dark fixation response observed between acetylene reduction and nitrogen-15 uptake from the termination of light exposure to the commencement of sample processing.

Time Course Experiments

Acetylene reduction and nitrogen-15 uptake rates were examined during incubations of increasing length, to determine whether both assays

responded similarly during incubation intervals normally employed for rate comparison studies. Azotobacter nitrogenase exhibited linear acetylene reduction and nitrogen-15 uptake behavior for up to forty-five minutes (Hardy et al., 1968). Following an initial lag, nitrogen-15 uptake in Alnus rugosa nodules was also observed to be linear for one hour (Stewart et al., 1967) and acetylene reduction in Myrica cerifera root nodules was shown to be linear for at least four hours (Sloger and Silver, 1967). Sample incubations from fifteen minutes to six hours demonstrated the rate versus time response characteristic of chemostat and lake blue-green algal populations examined in this study.

Acetylene reduction was observed to increase linearly on extended incubation of samples collected in Chemostat 1, Lake 304 (concentrated samples) and Lake 227 (Figures 33 and 35 to 38). Acetylene reduction rates were converted to nitrogen fixation rates, utilizing the theoretical acetylene to nitrogen ratio, prior to inclusion in time course plots. A similar assay completed for Chemostat 2 samples displayed non-linear behavior (Figure 34). Acetylene reduction began to plateau at incubations exceeding 2.5 hours.

Three assays defining the time dependent response of nitrogen-15 uptake were completed in Chemostat 1, Chemostat 2 and Lake 227 (Figures 39, 40 and 41). Significant uptake of nitrogen-15 was not observed immediately in Chemostat 1 and Chemostat 2 samples, a lag of from thirty to sixty minutes being evident. Following this initial lag, linear uptake commenced in both chemostats, but only persisted in Chemostat 1. Nitrogen-15 uptake rates levelled after Chemostat 2 samples had been incubated three to four hours. Nitrogen-15 uptake was linear for Lake 227 samples throughout the incubation interval, no lag or plateau being apparent.

Figure 33. Acetylene reduction time course data for samples collected on April 29, 1977 from Chemostat 1. Acetylene reduction-derived estimates of the quantity of nitrogen fixed ($C_2H_2:N_2 = 3$) are included.

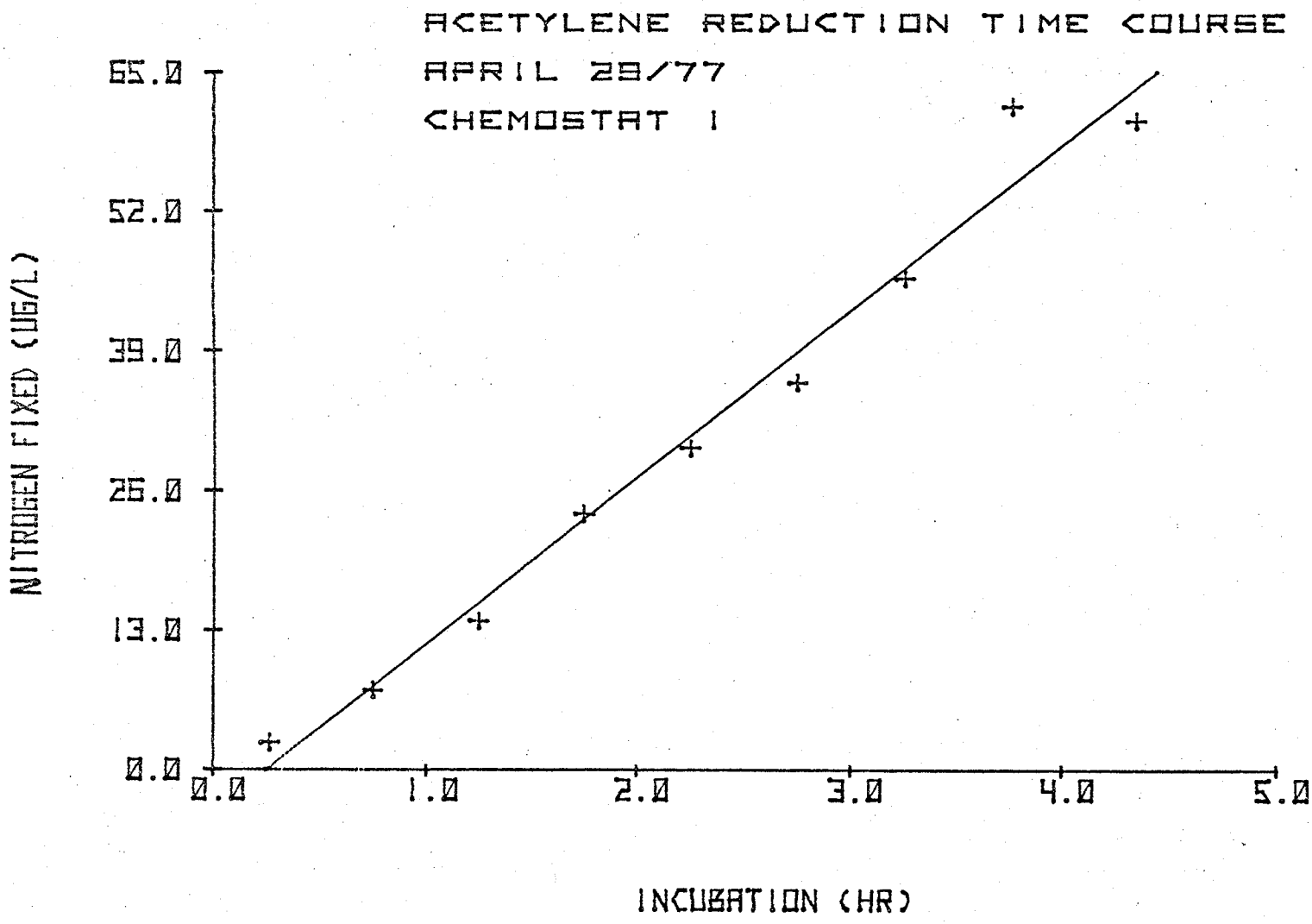

ACETYLENE REDUCTION TIME COURSE
APRIL 29/77
CHEMOSTAT 1
NITROGEN FIXED (UG/L)
65.0
52.0
39.0
26.0
13.0
0.0
0.0
1.0
2.0
3.0
4.0
5.0
INCUBATION (HR)

Figure 34. Acetylene reduction time course data for samples collected on June 15, 1977 from Chemostat 2. Acetylene reduction-derived estimates of the quantity of nitrogen fixed (C_2H_2:N_2 = 3) are included.

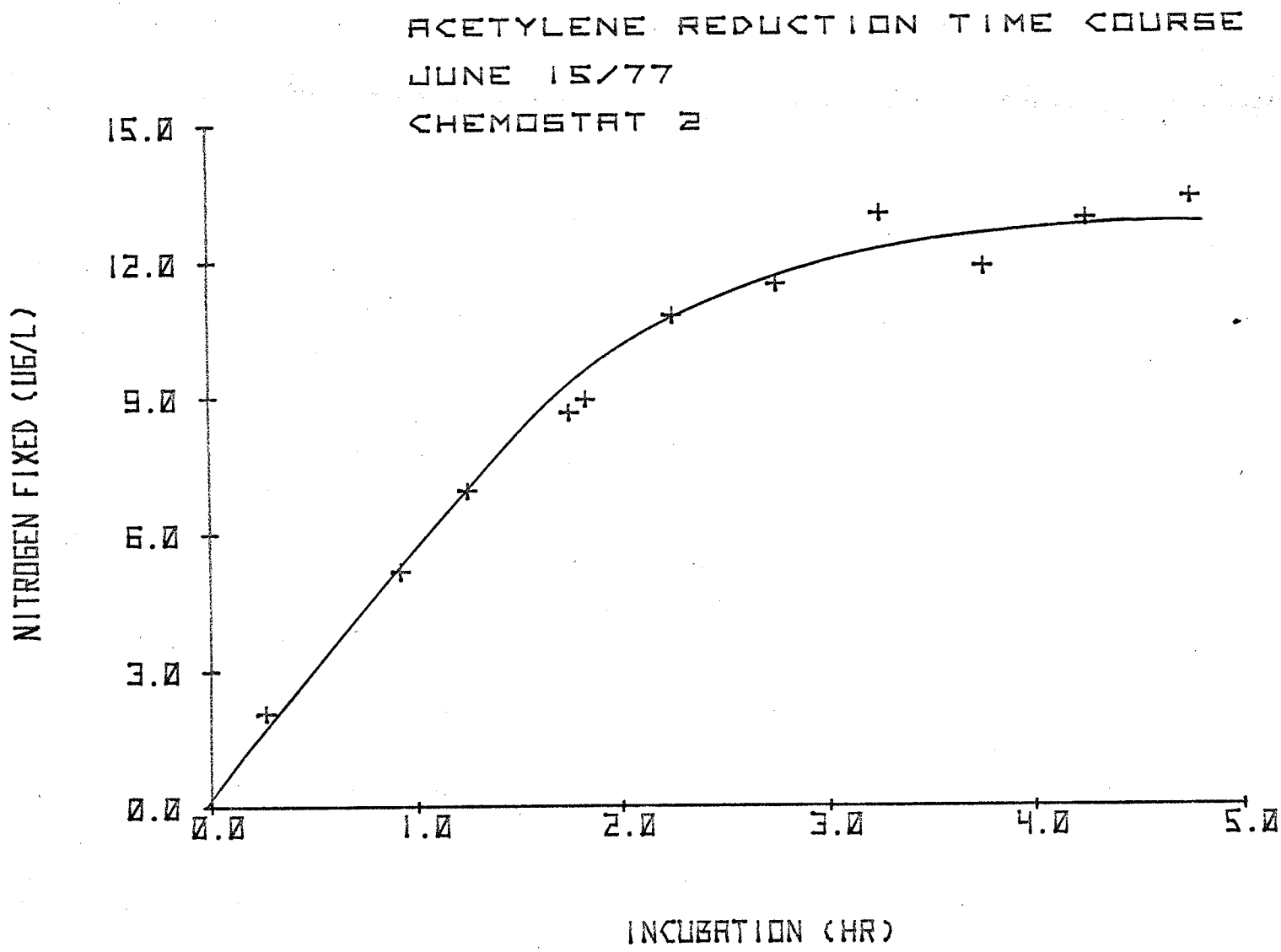

ACETYLENE REDUCTION TIME COURSE
JUNE 15/77
CHEMOSTAT 2
NITROGEN FIXED (UG/L)
15.0
12.0
9.0
6.0
3.0
0.0
0.0
1.0
2.0
3.0
4.0
5.0
INCUBATION (HR)

Figure 35. Acetylene reduction time course data for concentrated samples collected on July 19, 1977 from Lake 304 (*in situ* incubation). Acetylene reduction-derived estimates of the quantity of nitrogen fixed ($C_2H_2:N_2 = 3$) are included.

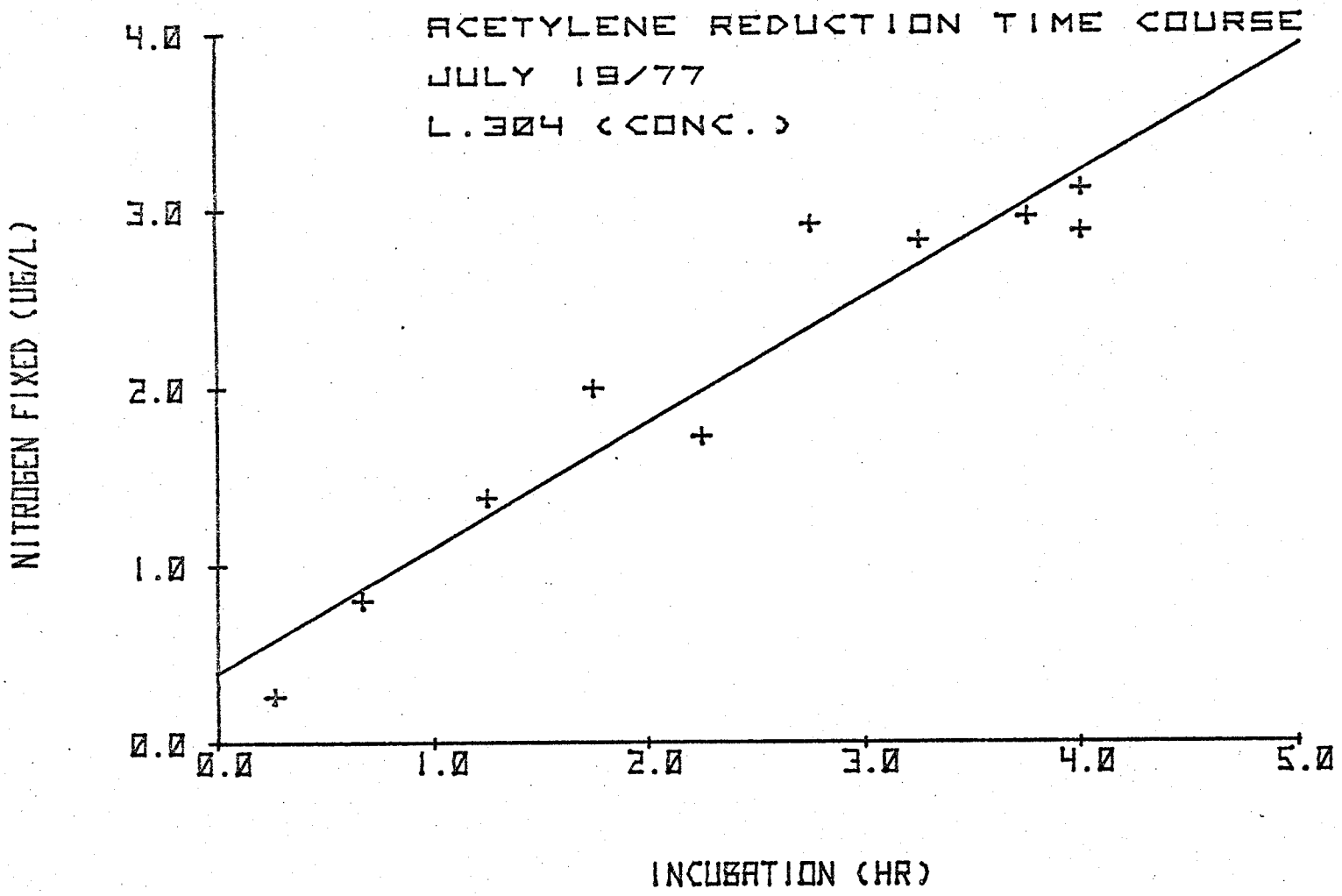

ACETYLENE REDUCTION TIME COURSE
JULY 19/77
L.304 (CONC.)
NITROGEN FIXED (UG/L)
4.0
3.0
2.0
1.0
0.0
0.0
1.0
2.0
3.0
4.0
5.0
INCUBATION (HR)

Figure 36. Acetylene reduction time course data for samples collected on August 10, 1977 from Lake 227 (*in situ* incubation). Acetylene reduction-derived estimates of the quantity of nitrogen fixed ($C_2H_2:N_2 = 3$) are included.

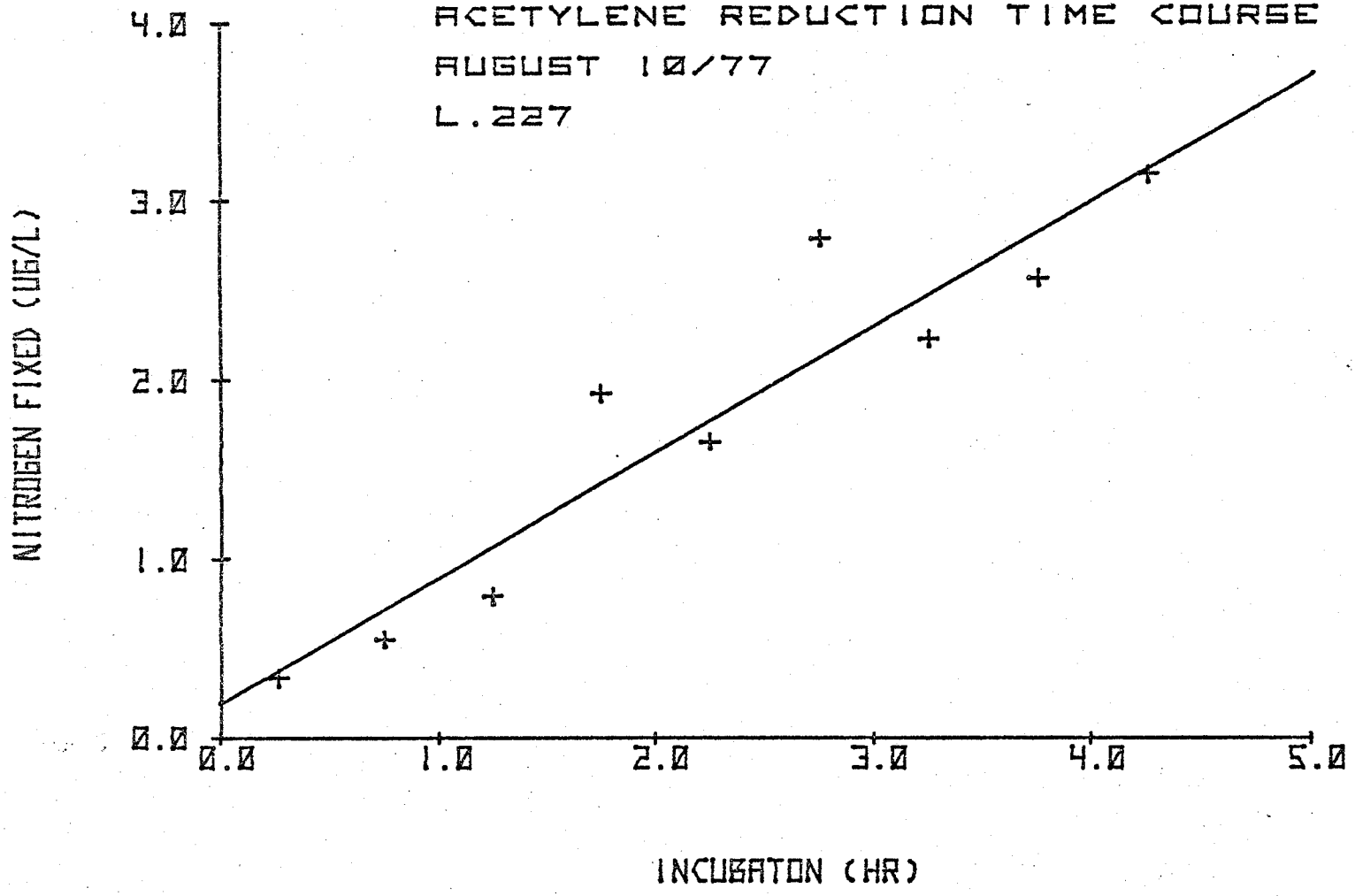

ACETYLENE REDUCTION TIME COURSE
AUGUST 10/77
L.227
NITROGEN FIXED (UG/L)
4.0
3.0
2.0
1.0
0.0
0.0
1.0
2.0
3.0
4.0
5.0
INCUBATON (HR)

Figure 37. Acetylene reduction time course data for samples collected on August 17, 1977 from Lake 227 (*in situ* incubation). Acetylene reduction-derived estimates of the quantity of nitrogen fixed (C_2H_2:N_2 = 3) are included.

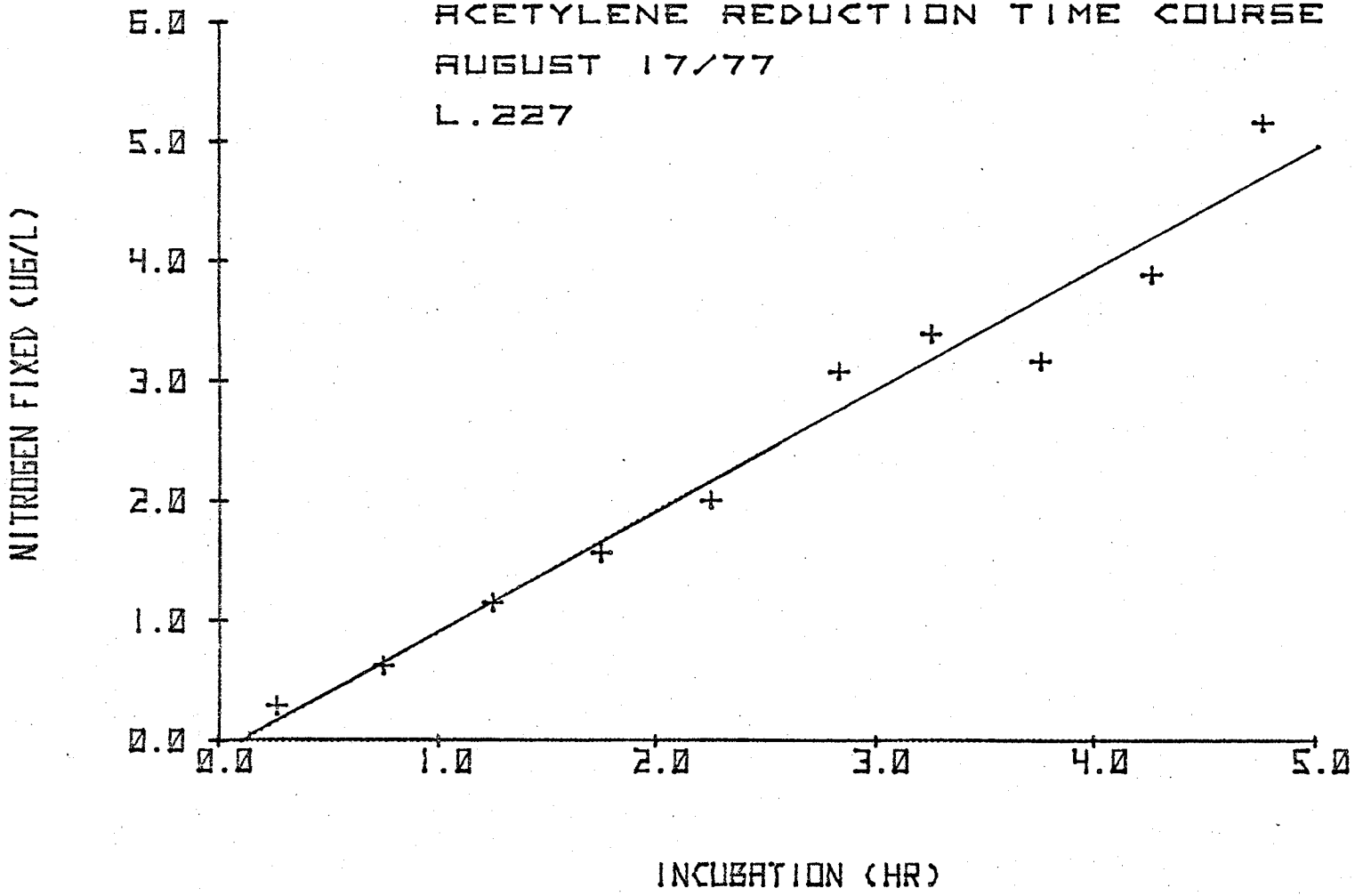

ACETYLENE REDUCTION TIME COURSE
AUGUST 17/77
L.227
NITROGEN FIXED (UG/L)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.0
1.0
2.0
3.0
4.0
5.0
INCUBATION (HR)

Figure 38. Acetylene reduction time course data for samples collected on September 12, 1977 from Lake 227 (laboratory incubation). Acetylene reduction-derived estimates of the quantity of nitrogen fixed ($C_2H_2:N_2 = 3$) are included.

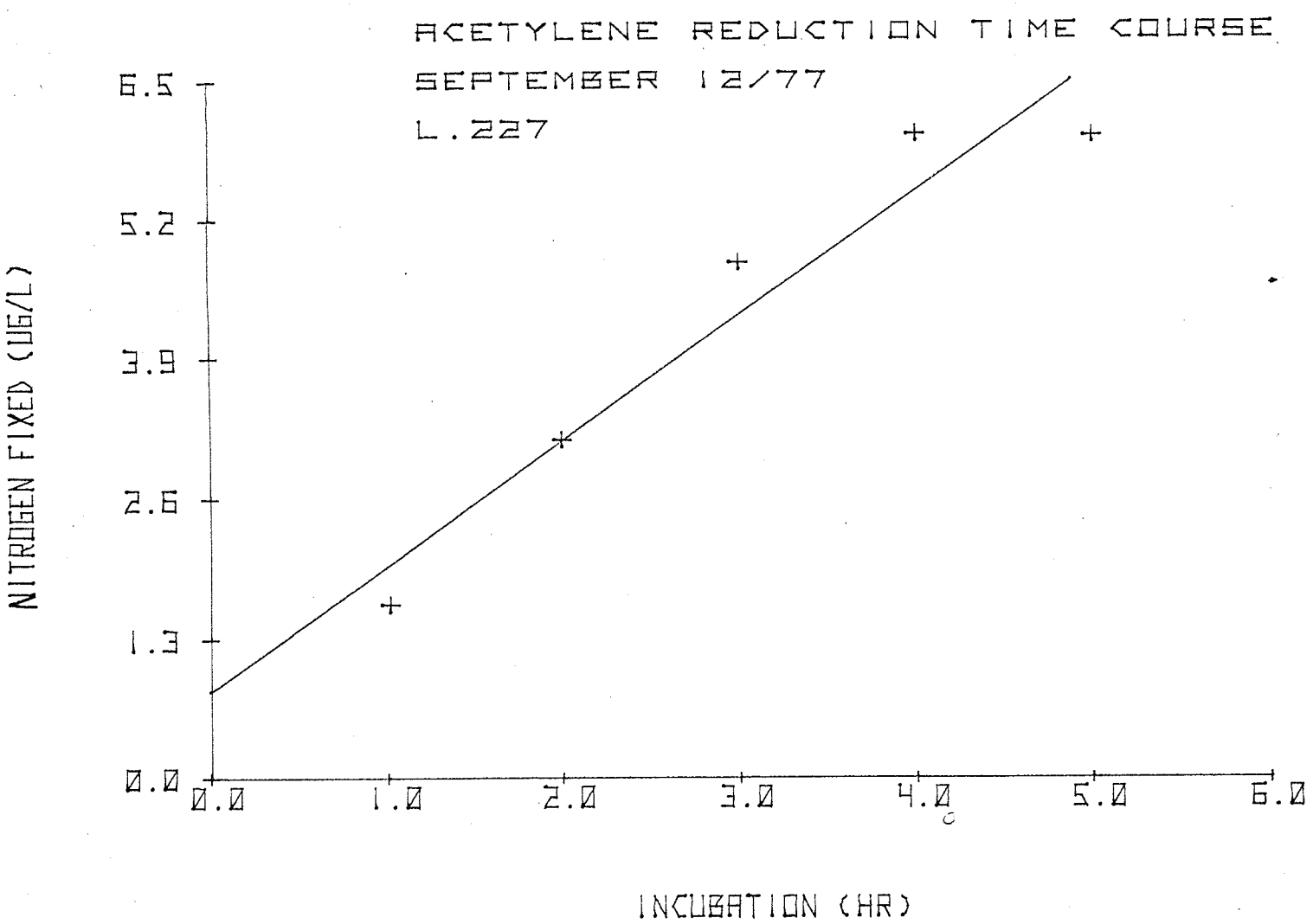

ACETYLENE REDUCTION TIME COURSE
SEPTEMBER 12/77
L.227
NITROGEN FIXED (UG/L)
6.5
5.2
3.9
2.6
1.3
0.0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
INCUBATION (HR)

Figure 39. Nitrogen-15 uptake time course data for samples collected on May 4, 1977 from Chemostat 1.

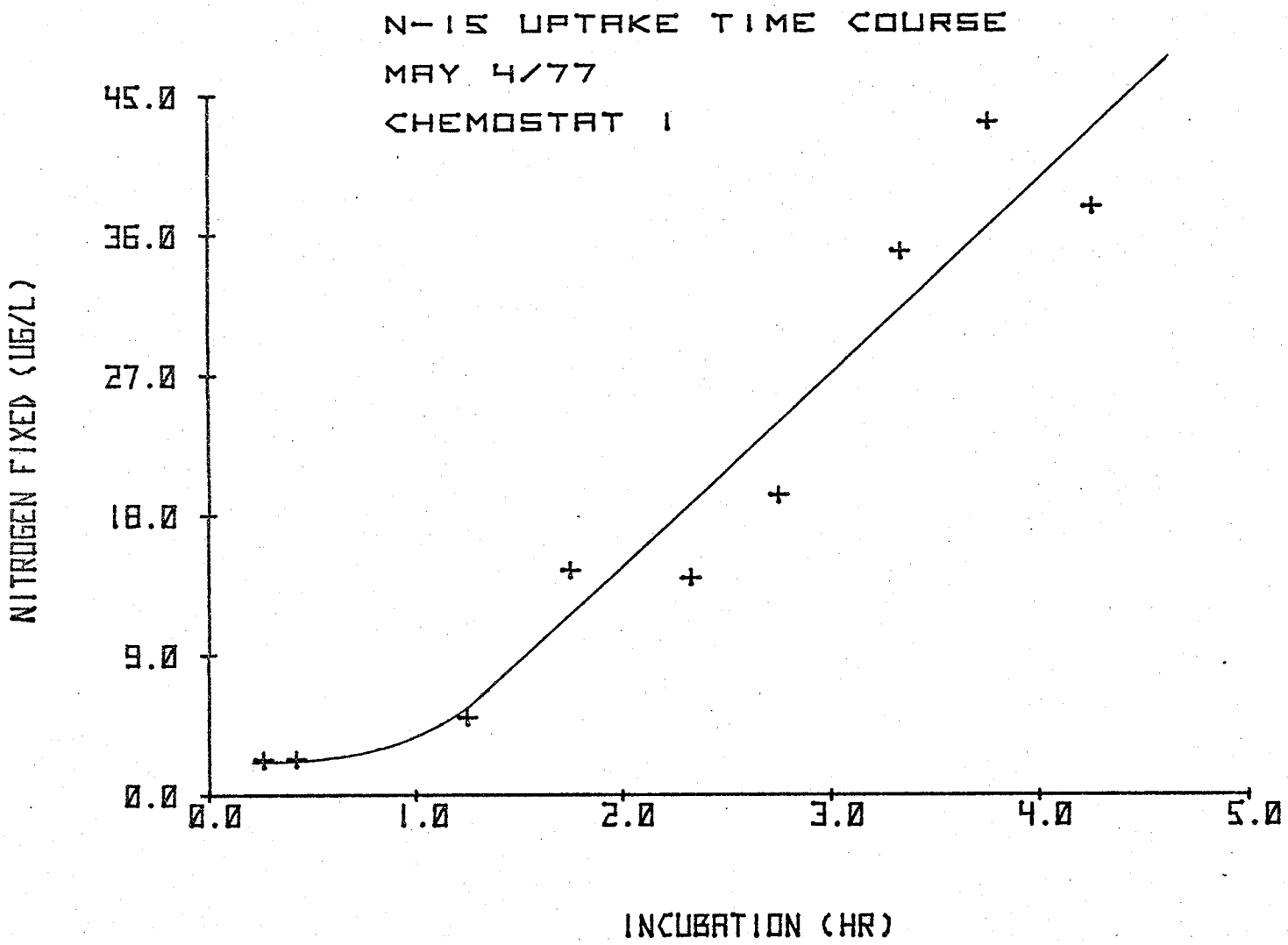

N-15 UPTAKE TIME COURSE
MAY 4/77
CHEMOSTAT 1
NITROGEN FIXED (UG/L)
45.0
36.0
27.0
18.0
9.0
0.0
0.0
1.0
2.0
3.0
4.0
5.0
INCUBATION (HR)

Figure 40. Nitrogen-15 uptake time course data for samples collected on June 16, 1977 from Chemostat 2.

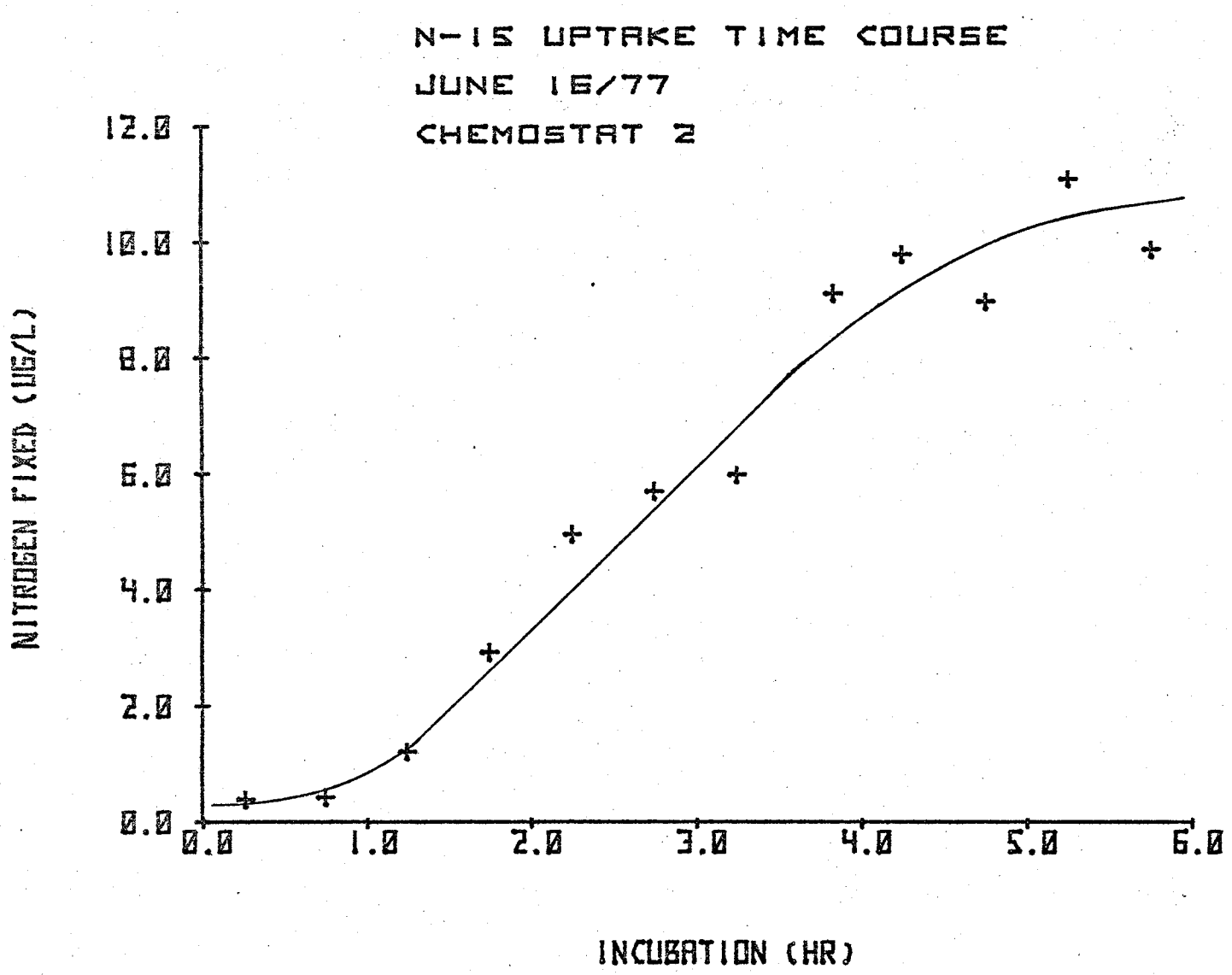
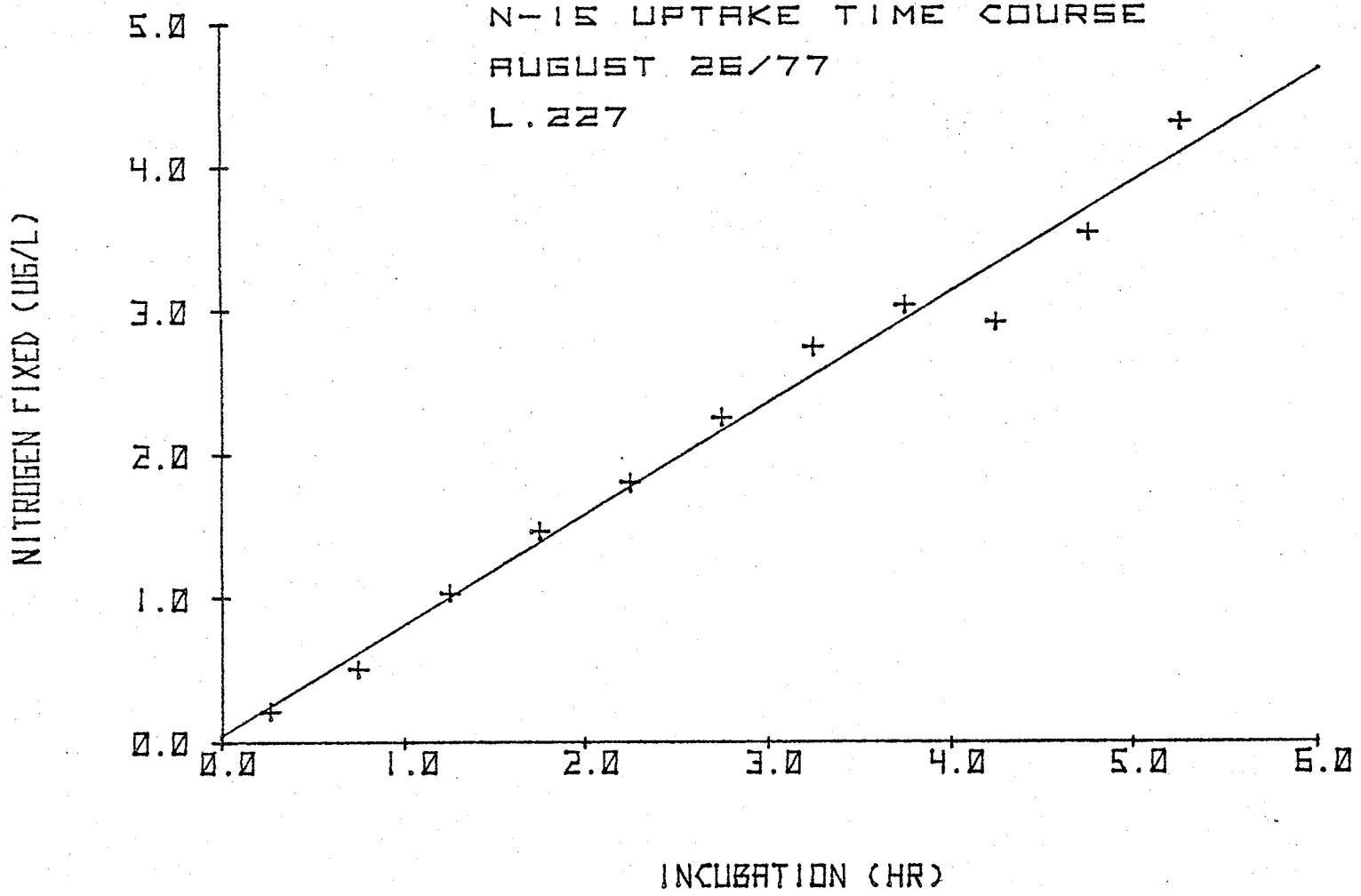

N-15 UPTAKE TIME COURSE
JUNE 16/77
CHEMOSTAT 2
NITROGEN FIXED (UG/L)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
INCUBATION (HR)

Figure 41. Nitrogen-15 uptake time course data for samples collected on August 26, 1977 from Lake 227 (Laboratory incubation).


N-15 UPTAKE TIME COURSE
AUGUST 26/77
L.227
NITROGEN FIXED (UG/L)
5.0
4.0
3.0
2.0
1.0
0.0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
INCUBATION (HR)

Non-linear behavior characteristic of the latter stages of incubation for acetylene reduction and nitrogen-15 uptake in Chemostat 2 samples suggests the development of some physical or chemical limitation. The influence of cell density independent of the algal environment is an inadequate explanation as Chemostat 1, the most concentrated system, exhibited a linear acetylene reduction response over the entire incubation interval. Possible restrictions limiting acetylene reduction and nitrogen-15 uptake include depletion of an essential nutrient, perhaps involved in reductant or energy generation, or accumulation of toxic wastes after extended incubation, such as an excess of photosynthetically-produced oxygen (Mague, 1977). Neither of these potential problems were examined here.

Changing light characteristics during the Lake 304 *in situ* acetylene reduction incubations may have been critical; a dark cloud cover developed during the intermediate stages of the time course assay. Therefore, samples left for longer incubations received reduced solar input. However, the existence of cellular reserves of ATP and reductant should attenuate the effect of this light reduction (See, for example, the Dark Fixation Studies). Constancy of illumination was not a problem during Chemostat 2 acetylene reduction and nitrogen-15 uptake time courses, incubations being carried out in the laboratory incubator. One series of Lake 227 samples collected September 12, 1977, were prepared and incubated in the laboratory to determine whether the incubator lighting would affect the time response. *In situ* (Figures 36 and 37) and incubator (Figure 38) Lake 227 time courses demonstrated a qualitatively similar linear response independent of the location of incubation. Due to the difficulty of sample processing following incubation, incubator rather than

in situ incubations were employed for both chemostat and lake nitrogen-15 uptake time course samples. Therefore, plateau versus linear anomolies in nitrogen-15 uptake time course results for Chemostat 2 samples versus Chemostat 1 and Lake 227 samples were not a function of incubation lighting characteristics.

The existence of a lag in chemostat nitrogen-15 uptake is significant to calculation of nitrogen-15 uptake rates and consequently acetylene to nitrogen ratio estimation. Low initial uptake would reduce the final nitrogen-15 uptake rate estimate, thus increasing the acetylene to nitrogen ratio determined. There is apparently no biological explanation for this delayed assimilation of nitrogen-15; isotopic fractionation effects involving algal preference for the lighter molecule, nitrogen-14, in nitrogen fixation are negligible with respect to the nitrogen-15 enrichments involved (Focht, 1973). However, delayed uptake may be an artifact of the sample preparation procedure. Due to the low water solubility of nitrogen, nitrogen-15 injections required time for attainment of $^{14}N_2$- $^{15}N_2$ isotopic equilibrium, possibly delaying the exposure of the algae to labelled substrate. However, preliminary agitation following substrate injection should minimize this delay. Because no lag in nitrogen-15 uptake was observed in samples collected from Lake 227, which were prepared in the same manner as chemostat samples exhibiting the initial lag, its existence is suspect.

If the variable response in acetylene reduction and nitrogen-15 uptake was limited only to extended incubation anomolies, use of short term incubations, during which both methods exhibit a linear response, would be a sufficient precaution for accurate estimation of nitrogen fixation rates (Steyn and Deliwiche, 1970). Incubations carried out in

rate comparison experiments were chosen to fall within the linear response region. However, if, as in Chemostat 1 and Chemostat 2, a difference occurs in the response of acetylene reduction and nitrogen-15 uptake during initial incubations, the calculated acetylene to nitrogen ratio will be affected. A high acetylene to nitrogen ratio would be anticipated as a result of the lag in nitrogen-15 uptake. The only system for which simultaneous estimation of acetylene reduction and nitrogen-15 uptake with time was completed was Lake 227, which demonstrated no lag. Acetylene to nitrogen ratios calculated for samples incubated from one to four hours were 6.8, 7.7, 7.4 and 9.3, the latter ratio being the result of a low nitrogen-15 uptake rate. Because these ratios differ from theoretical in a system exhibiting no preliminary lag in nitrogen-15 uptake, the ratio discrpancy is apparently a function of parameters other than differing initial time response.

An approximation of the effect of lag and plateau characteristics on acetylene to nitrogen ratio determination may be demonstrated if Chemostat 2 samples collected June 15, 1977 for acetylene reduction and June 16, 1977 for nitrogen-15 uptake are assumed to represent a series of rate pairs. Acetylene to nitrogen ratios were calculated for rates with matched incubation lengths (Table 22). The general trend was a decline in the ratio with time, the ratios being influenced by low initial nitrogen-15 uptake rates (1.4, 0.6, and 1.0 μg/L/hr) and the more rapid levelling of acetylene reduction compared to nitrogen-15 uptake for incubations up to 4.75 hours. If the June 15, 1977 and June 16, 1977 data are representative of the time dependent responses anticipated for Chemostat 2 sample, it suggests that a variable ratio would be obtained depending on the incubation interval adopted. Therefore, definition and comparison of

TABLE 22

Estimation of C_2H_2:N_2 Ratios From Chemostat 2. C_2H_2 Reduction and Nitrogen-15 Uptake Time Course Data.

Incubation Time (Hr)	Acetylene Reduction Rate[a] ($\mu gC_2H_4/L/hr$)	Nitrogen-15 Uptake Rate[b] ($\mu gN_2/L/hr$)	C_2H_2/N_2
0.25	24.2	1.4	17.3
0.75	16.9	0.6	28.2
1.25	16.7	1.0	17.0
1.75	14.9	1.7	8.8
2.25	14.4	2.2	6.5
2.75	12.6	2.1	6.0
3.25	12.0	1.8	6.5
3.75	9.5	2.4	3.9
4.25	9.1	2.3	4.0
4.75	8.5	1.9	4.5
5.25	-	2.1	-
5.75	-	1.7	-

a Chemostat 2 - June 15, 1977 - Acetylene Reduction Time Course

b Chemostat 2 - June 16, 1977 - Nitrogen-15 Uptake Time Course

the time responses for acetylene reduction and nitrogen-15 uptake and, in cases where anomolies exist, standardization of the length of incubation appear to be advisable precautions when rate estimates are to be compared.

K_T and V_{Max} Determinations

Ratio analysis, dark fixation and time course experiments were all completed assuming that the algal nitrogenase enzyme responded similarly to both the natural substrate nitrogen and its analogue, acetylene. Lake 227 samples were exposed to a series of increasing nitrogen and acetylene concentrations in an attempt to define whether both acetylene and nitrogen-15 uptake did function similarly as nitrogen fixation estimators. Blue-green algal nitrogenase is capable of reduction of either substrate, but the affinity of the enzyme for the individual molecules differs (Hardy et al., 1968; 1973; Bergersen, 1970). Experiments patterned on the Michaelis-Menten enzyme-substrate interaction were designed. Direct comparison to the defined enzyme system was unintended, whole cell uptake studies being influenced by processes which do not affect the isolated enzyme preparation (MacIsaac and Dugdale, 1969). However, the information derived from this type of experiment does provide desired indications of the response of the algae to the natural substrate and its analogue.

Previous studies of this nature utilized partial pressure estimates of nitrogen or acetylene, rather than aqueous concentration estimates, when determining the substrate-rate relationships (Hardy et al., 1968, 1973). Rather than define the vapor phase-aqueous phase characteristics of the systems examined, aqueous acetylene and nitrogen contents were calculated utilizing gas partitioning laws and differential solubilities

(see Methods) and reported as micromolar concentrations.

The acetylene concentration range was chosen on the basis of the results of two preliminary experiments, one employing very low concentrations (Table 23), defining only the linear portion of the curve, and the second employing very high concentrations (Table 23), defining the plateau region. Rate estimation for samples in an intermediate concentration range, 62.9 to 7447 μM, allowed better definition of the overall substrate-rate relationships. Michaelis-Menten $\left[v = \frac{V_{Max}\ [S]}{K_T + [S]} \right]$ (Figure 42) and Wolfe $\left[\frac{[S]}{v} = \frac{[S]}{V_{Max}} + \frac{K_T}{V_{Max}} \right]$ (Figure 43) plots were constructed from acetylene reduction rate (v) and acetylene concentration [S] data, to facilitate estimation of the half saturation constant (in uptake studies usually denoted a transport constant, MacIsaac and Dugdale, 1969) K_T, and the maximum velocity, V_{Max}, characteristic of the substrate-rate relationship. Acetylene reduction derived nitrogen fixation rates are included in both plots, the theoretical acetylene to nitrogen ratio being employed for acetylene reduction rate conversion. A computer program utilizing the Chi Squared Goodness of Fit statistical procedure (M. A. Turner, personal communication) was used to discover the Michaelis-Menten curve which best fit the collected data. Estimates of K_T and V_{Max} were generated in the program (Table 24). K_T and V_{Max} estimates were also obtained on linear regression analysis of the data adapted for use in the Wolfe Plot; this K_T value exceeded the Michaelis-Menten curve estimate (Table 24). The average K_T and V_{Max} values were 1504 μM and 1.5 μg/L/hr, respectively.

A concentration distribution spanning 47 to 513 μM established the substrate-rate relationship for nitrogen. Michaelis-Menten and Wolfe

TABLE 23

Substrate and Rate Data Collected in Preliminary Definition of Michaelis-Menten Relationship for Acetylene Reduction in Lake 227 Samples.

September 6/77		August 17/77	
C_2H_2 Concentration (μM)	C_2H_2 Reduction Rate (μg/L/hr)	C_2H_2 Volume[a] (mls)	C_2H_2 Reduction Rate (μg/L/hr)
24	5.90×10^{-3}	1	6.87×10^{-1}
62	2.80×10^{-2}	3	1.05
122	8.70×10^{-2}	5	9.31×10^{-1}
243	1.74×10^{-1}	7	9.79×10^{-1}
601	3.72×10^{-1}	10	9.46×10^{-1}
1202	6.87×10^{-1}	15	9.28×10^{-1}
6449	1.37	20	9.30×10^{-1}

a C_2H_2 concentration was not calculated because the volumes of final aqueous and gas phases were not recorded.

Figure 42. A Michaelis-Menten plot of the velocity versus substrate concentration relationship for acetylene. Samples were collected from Lake 227, September 10, 1977. Acetylene reduction rates are converted to nitrogen fixation rates using the C_2H_2:N_2 ratio of 3.

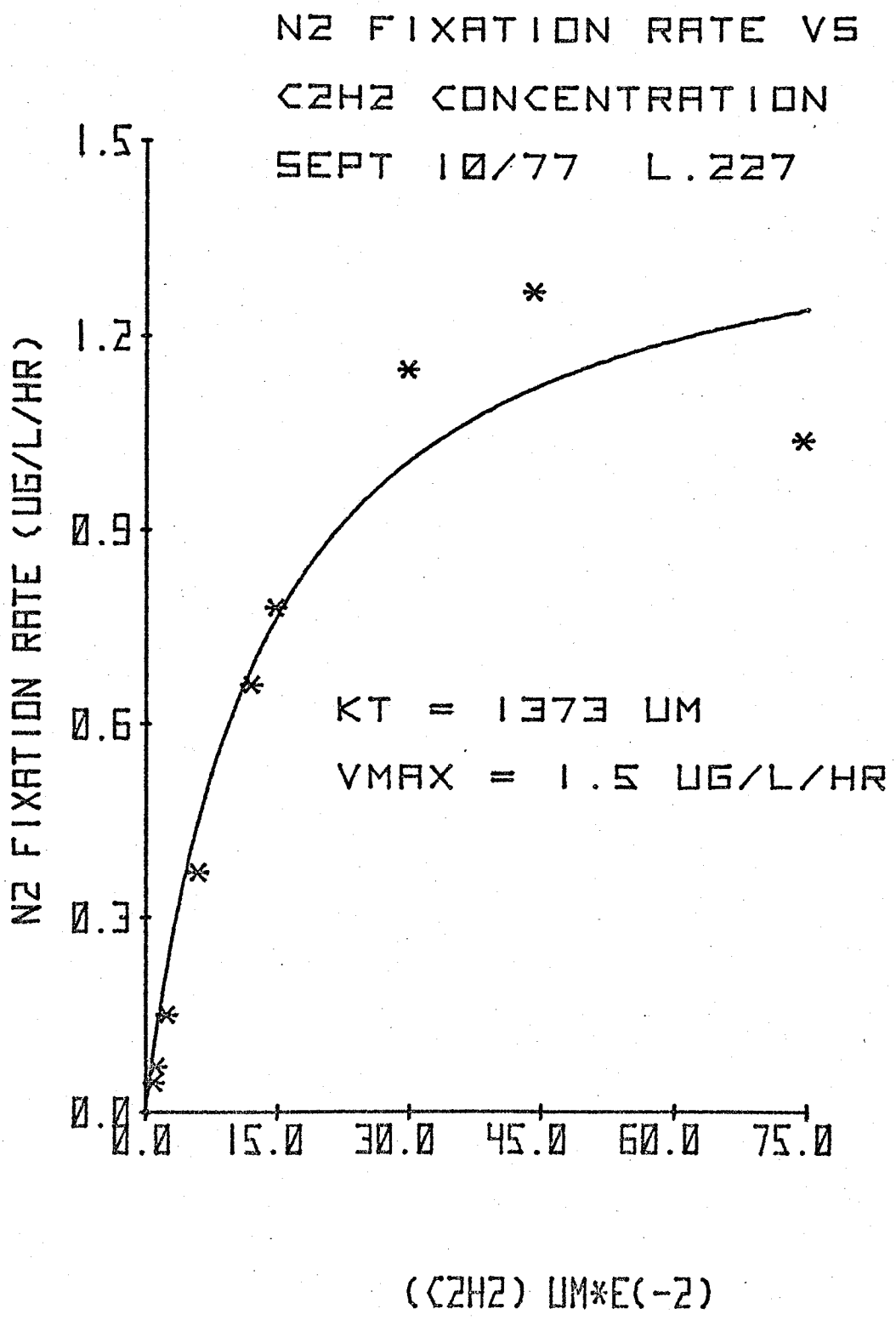

N2 FIXATION RATE VS
C2H2 CONCENTRATION
SEPT 10/77 L.227
N2 FIXATION RATE (UG/L/HR)
1.5
1.2
0.9
0.6
0.3
0.0
0.0
15.0
30.0
45.0
60.0
75.0
KT = 1373 UM
VMAX = 1.5 UG/L/HR
((C2H2) UM*E(-2)

Figure 43. A Wolfe plot of substrate/velocity versus substrate concentration for acetylene. Samples were collected from Lake 227, September 10, 1977. Acetylene reduction rates are converted to nitrogen fixation rates using the C_2H_2:N_2 ratio of 3.

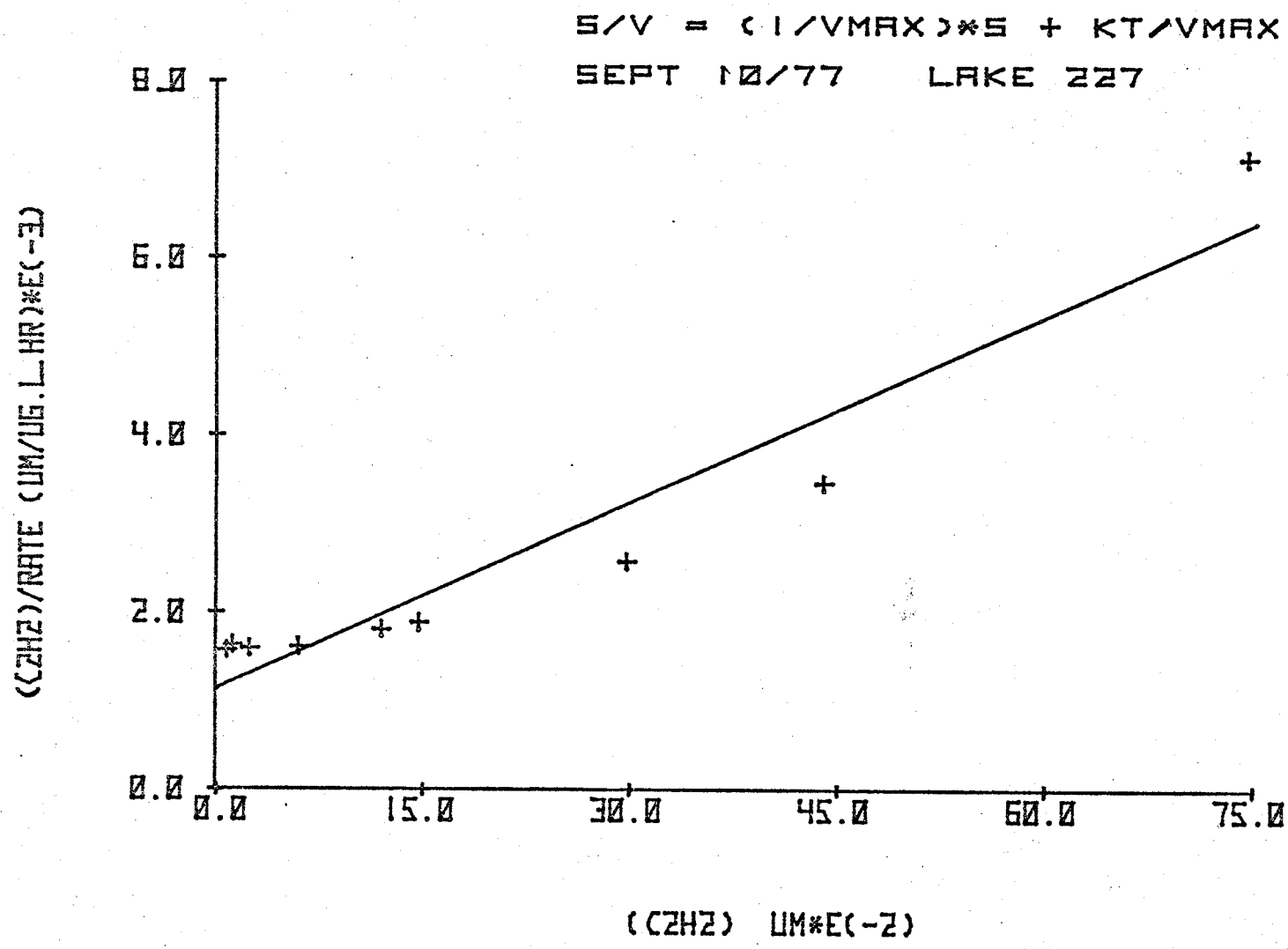

S/V = (1/VMAX)*S + KT/VMAX
SEPT 10/77 LAKE 227
8.0
6.0
4.0
2.0
0.0
(C2H2)/RATE (UM/UG.L_HR)*E(-3)
0.0
15.0
30.0
45.0
60.0
75.0
(C2H2) UM*E(-2)

TABLE 24

Half-Saturation Constant (K_T) and Maximum Velocity (V_{Max}) Values Determined for Lake 227 Acetylene Reduction and Nitrogen Fixation By Michaelis-Menten and Wolfe Plots.

Date	Substrate	Plot Used	K_T (μM)	V_{Max}(μg/L/hr)
10/9/77	C_2H_2	Michaelis-Menten	1373	1.5
10/9/77	C_2H_2	Wolfe	1634	1.6
10/9/77	C_2H_2	Michaelis-Menten[a]	2507	2.1
12/9/77	N_2	Michaelis-Menten	145	0.7
12/9/77	N_2	Wolfe	150	0.7

a Plot of September 10, 1977 data minus the highest concentrations.

plots (Figures 44 and 45) were again useful in the definition of characteristic K_T and V_{Max} parameters for nitrogen uptake (Table 24). Definition of the Michaelis-Menten curve would be improved by introduction of higher substrate concentrations yielding additional plateau nitrogen fixation rates. The average K_T and V_{Max} values were calculated to be 150 μM and 0.7 μg/L/hr, respectively.

Blue-green algal average K_T values for acetylene, 1.5×10^{-3} M, and nitrogen, 1.5×10^{-4} M, calculated above compare favorably with previous K_T estimates. Bacterial and nodule K_T values summarized by Zumft Mortenson (1975) range from 3.6×10^{-4} to 1.2×10^{-2} M for acetylene, and 2.1×10^{-5} to 1.2×10^{-4} M for nitrogen.

Comparisons of the transport constants for acetylene reduction and nitrogen fixation indicate that the K_T for acetylene reduction is almost an order of magnitude greater than the K_T for nitrogen fixation. This implies a stronger affinity for nitrogen than acetylene, which appears reasonable as nitrogen is the natural substrate. However, conclusions based on K_T data, concerning nitrogenase's affinity for specific substrates, must be made with caution because, as mentioned above, algal population substrate uptake was determined, rather than uptake by the isolated enzyme (Mague, 1977). Kinetic studies of reductions catalyzed by nitrogenase must also take into account that the proton, a potentially reducible substrate, is always present and capable of receiving electrons (Zumft and Mortenson, 1975). However, at high acetylene concentrations, acetylene inhibition eliminates hydrogen's interference.

Michaelis-Menten estimates of K_T and V_{Max} values for September 10, 1977 data, excluding the final concentration are also included in Table 24, an increase in both K_T and V_{Max} being observed. Use of direct

Figure 44. A Michaelis-Menten plot of the velocity versus substrate concentration relationship for nitrogen. Samples were collected from Lake 227, September 12, 1977.

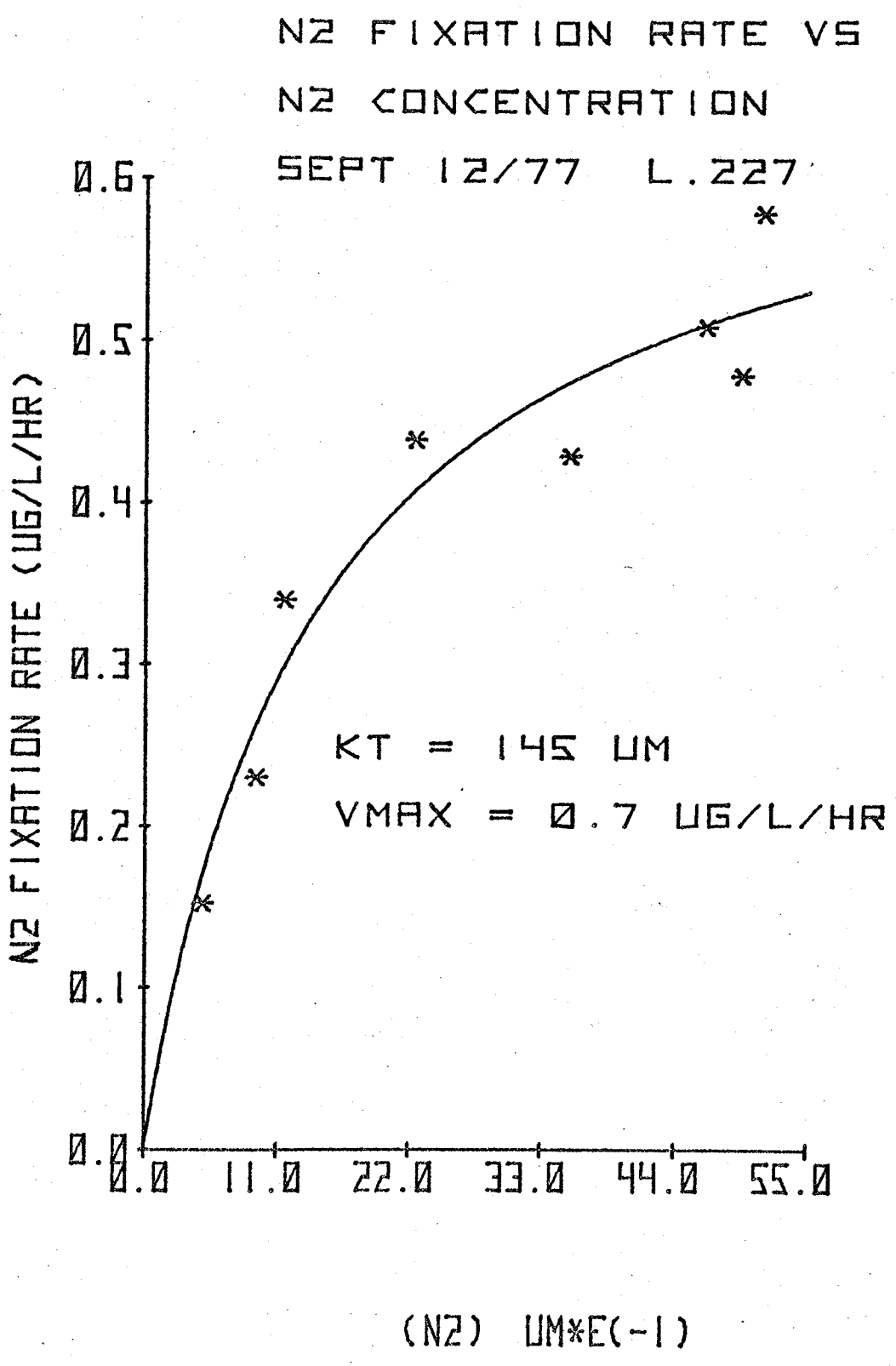
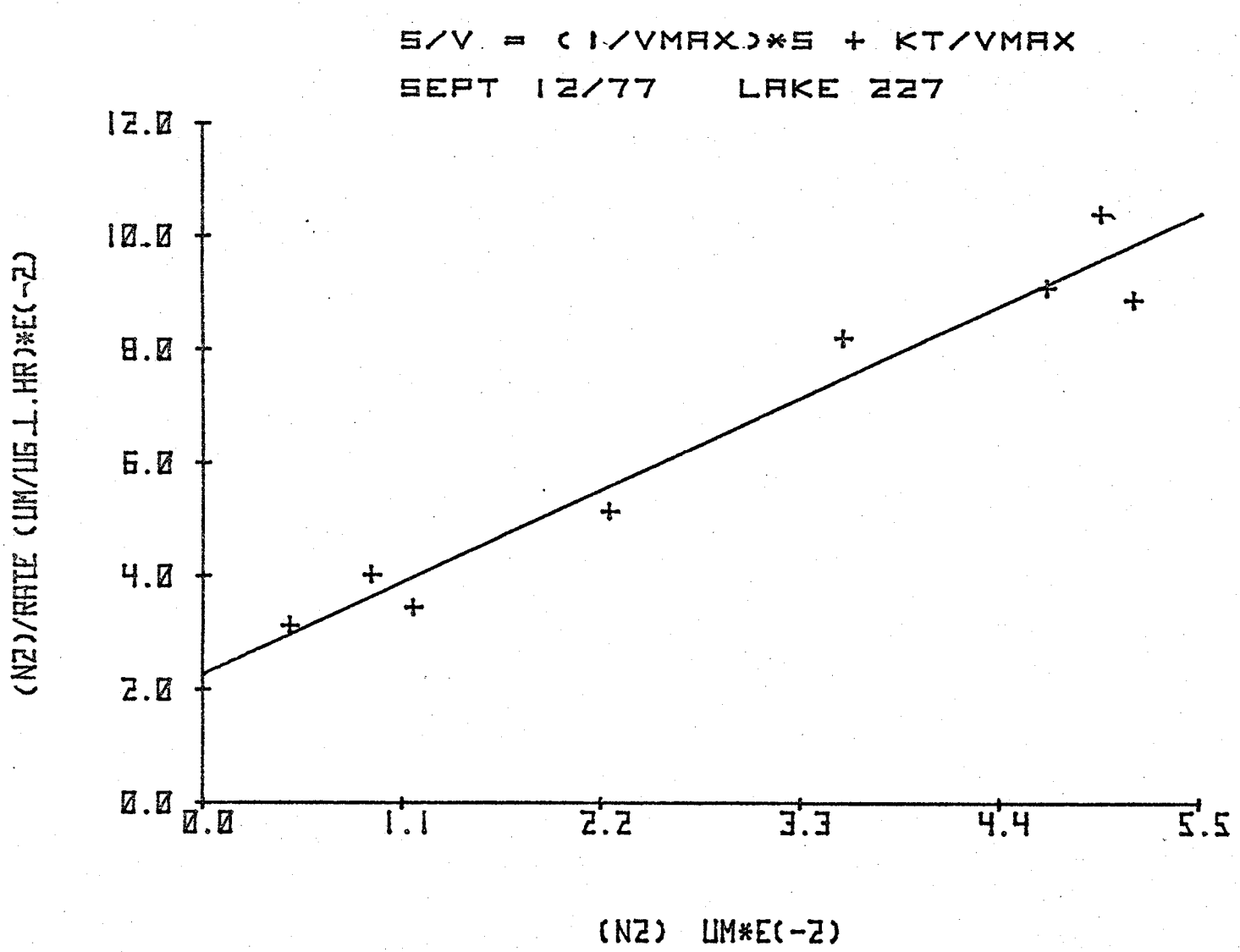

N2 FIXATION RATE VS
N2 CONCENTRATION
SEPT 12/77 L.227
N2 FIXATION RATE (UG/L/HR)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.0
11.0
22.0
33.0
44.0
55.0
KT = 145 UM
VMAX = 0.7 UG/L/HR
(N2) UM*E(-1)

Figure 45. A Wolfe plot of substrate/velocity versus substrate concentration for nitrogen. Samples were collected from Lake 227, September 12, 1977.


S/V = (1/VMAX)*S + KT/VMAX
SEPT 12/77 LAKE 227
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0.0
1.1
2.2
3.3
4.4
5.5
(N2)/RATE (UM/UG.ATOM.HR)*E(-2)
(N2) UM*E(-2)

acetylene gas injection to achieve the highest acetylene concentration, rather than injection of an air-acetylene dilution combination, apparently caused a variation in the response of the system. Perhaps the volume and composition of the gas phase resulting from air-acetylene injections affected the algal nitrogen fixation capacity. The sample receiving the injection of undiluted acetylene demonstrated an acetylene reduction rate below that anticipated on extrapolation of rates measured for lower concentrations. Elimination of this value increased K_T and V_{Max} values to 2507 μM and 2.1 μg/L/hr. The influence of the air-acetylene mixture on sample nitrogen fixation should perhaps be weighed prior to acceptance of K_T and V_{Max} values derived in this manner.

The difference in nitrogen and acetylene K_T values demonstrated here is not a unique observation. A larger K_T for nitrogen was also observed in the early stages of development and promotion of acetylene reduction as a useful *in situ* nitrogen fixation estimator (Hardy *et al.*, 1968, 1973). However, because the acetylene reduction assay utilizes an acetylene concentration sufficient to saturate the nitrogenase, it is probable that substrate affinity differences between acetylene and nitrogen would not be critical during routine field assays. That recent kinetic studies (such as this one) and inhibitor studies (Zumft and Mortenson, 1975) suggest independent sites for acetylene and nitrogen reduction is important, though, since one of the original assumptions of the acetylene reduction assay was the existence of a competition between acetylene and nitrogen for the same nitrogenase binding site. Further investigation of the binding sites of the nitrogenase complex may invalidate the use of acetylene reduction as a nitrogen fixation estimator.

CONCLUSIONS

Use of an analogue-based technique for nitrogen fixation estimation is a convenient and economical method of analysis for a wide range of organisms, demonstrating the capacity to assimilate atmospheric nitrogen. Acetylene reduction has been increasingly applied to estimate nitrogen fixation by soil organisms, aquatic bacteria and algae and leguminous and non-leguminous symbiotic associations. However, it has been determined in this study and others (Peterson and Burris, 1976; Bergersen, 1970; Lean et al., 1976) that assumption of a fixed acetylene to nitrogen conversion factor, which has been common practice, is unjustifiable. Depending on the method of calculation, an acetylene to nitrogen ratio of 6.3 to 9.7 characterizes the relationship of acetylene reduction and nitrogen-15 fixation for blue-green algal populations resident in the freshwater environments examined. Introduction of this ratio rather than the stoichiometric value, will reduce estimates of the contribution of nitrogen fixation to lake nitrogen budgets by a factor of two to three times. Nitrogen fixation contributions estimated in 1975 (Flett, 1977) will decrease from 19% to 8% for lake 226 NE and from 14% to 6% for Lake 227, a significant correction.

Though the similarity among lake acetylene to nitrogen ratios determined here may suggest the possibility of ratio extrapolation among lake systems, it should be remembered that the lakes examined exhibited many common characteristics, enhancing the probability of a common response to both nitrogen fixation estimators. As discussed earlier, independent nitrogen-15 uptake calibration of the acetylene reduction method for a given system is desirable due to the variable nature of the ratio, and the individuality of investigator interpretation of the two techniques. Acetylene to nitrogen ratios calculated for two chemostat Anabaena

variabilis populations demonstrated that ratio variability may even be evident in environments presumed similar. Comparing Chemostat 1 and Chemostat 2 ratios suggests that ratio extrapolation is also subject to restrictions based upon the observed "health" of the algal populations, healthy Chemostat 1 having the lowest ratio (regression estimate) and unhealthy Chemostat 2 the highest. Previously determined ratios for healthy blue-green algal cultures approached the theoretical value (Stewart et al., 1968).

Calibration of the acetylene reduction method has also been stressed by investigators studying simultaneous reduction of hydrogen and nitrogen by cellular nitrogenase. Sharing of available energy and reductant between these two substrates, which is eliminated in the presence of acetylene (Schubert and Evans, 1976), has been demonstrated in symbionts, algae and bacteria. Depending on the presence and activity of a cellular hydrogenase, in situ hydrogen production could decrease nitrogen fixation significantly. Because the acetylene reduction assay is unaffected by hydrogen competition, overestimation of nitrogen fixation appears inevitable. As the efficiency of hydrogen production varies, so will the magnitude of nitrogen fixation and acetylene to nitrogen ratios.

Though excretion of assimilated nitrogen-15 during the course of sample incubations is also suggested to contribute to acetylene to nitrogen ratio discrepancies (Fogg, 1966; Walsby, 1974; Walsby and Fogg, 1975) evidence collected here fails to support this hypothesis. Both filtrate nitrogen-15 analysis and nitrogen turnover time estimates (Walsby and Fogg, 1975) indicate that loss of labelled material is not apparent. Design of an improved method for filtrate analysis may demonstrate some label accumulation in the filtrate undetectable by presently

employed methods.

Analysis of nitrogen-15 assimilation by the particulate fraction necessitated involvement of filtration procedures, introducing difficulties associated with filtration-induced label loss (Arthur and Rigler, 1967) and retention of unincorporated label on the filters (McMahon, 1973; Jordan et al., 1978). Corrections for nitrogen-15 losses due to the rigors of the filtration process, patterned on those suggested by Arthur and Rigler (1967), appear excessive, reducing acetylene to nitrogen ratios to values less than three. Characteristics of filter retention of nitrogen-15 on filtration of small sample volumes are not specifically examined here. Filter retention of label would not account for the enhanced acetylene to nitrogen ratios observed.

Application of the acetylene reduction method as an adequate estimator of nitrogen fixation requires more than simply derivation of an appropriate acetylene to nitrogen conversion factor. The response of acetylene reduction and nitrogen-15 uptake to various parameters was also investigated to determine the suitability of both techniques as nitrogen fixation estimators. The extent of dark fixation, as measured by acetylene reduction was observed to exceed nitrogen-15 uptake estimates, but the significance of this difference to rate comparison studies is reduced, when it is considered in the context of the short dark exposures normally occurring, and the exponential decline in dark fixation with time after light termination (Fay, 1976; Lannergren et al., 1974).

Examination of the time dependent responses of acetylene reduction and nitrogen-15 uptake for lake samples generally indicates a similar linear increase in acetylene reduced and nitrogen-15 fixed with increasing incubation length. Thus elevated acetylene to nitrogen ratios

characteristic of lake samples cannot be attributed to a variable time response between the techniques. The response of the two techniques to extended incubation is not as straightforward when examined in Chemostat 1 and Chemostat 2. Early incubation lags in Chemostat 1 and Chemostat 2 nitrogen-15 uptake and levelling in Chemostat 2 acetylene reduction and nitrogen-15 uptake on extended incubation may lead to some variability in calculated acetylene to nitrogen ratios. Though standardization of a short incubation length alleviates deviations in the late stages of incubation, early incubation anomolies should be investigated as to cause and consistency in additional time course assays.

The above discussions of the responses of acetylene reduction and nitrogen-15 uptake depend on the applicability of acetylene as a nitrogen analogue. Substrate-velocity experiments completed in this study and elsewhere (Zumft and Mortenson, 1975) indicate some difference in the affinity of the nitrogenase, or more accurately, the algal population, for nitrogen and acetylene. Transport constants (K_T) derived differ by approximately a factor of ten and maximum velocities of uptake by a factor of two. These values would appear to support suggestions of different nitrogenase sites for binding of acetylene and nitrogen, implying a non-competitive rather than competitive interaction between the analogue and substrate (Zumft and Mortenson, 1975). However, until studies of the nature of the binding sites of nitrogenase and their response to substrate and analogue definitely demonstrate otherwise, it may be assumed that K_T differences are not critical to the acetylene reduction assay, where acetylene is supplied in excess of saturation requirements of nitrogenase.

Though it appears that the acetylene reduction technique as orig-

inally developed with its assumption of direct competition of acetylene and nitrogen and rigid stoichiometric conversion factor is inadequate, elimination of the technique is not suggested. Use of the acetylene reduction method to provide rapid sensitive nitrogen fixation estimates will still be an advantage, especially in field studies. However, if an accurate estimate of nitrogen fixation is required, use of the isotope uptake method is preferable. If, for expediency, the acetylene reduction method is adopted as a routine nitrogen fixation assay procedure, the importance of precalibration of this method in the given system with the nitrogen-15 uptake method is stressed. When an empirical acetylene to nitrogen ratio is introduced, the method of calculation should be identified (for example, mean or regression slope) as ratios were found to differ depending on the estimation procedure adopted. Without this preliminary method calibration, acetylene reduction rates should be reported as μg ethylene produced/L/hr, rather than μg nitrogen fixed/L/hr following introduction of the untested theoretical ratio.

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