1967

The Ecology of the Nitrogen-fixing Azotobacter and Klebsiella in Selected South Dakota Lakes

Paul A. Gillespie

THE ECOLOGY OF THE NITROGEN-FIXING *AZOTOBACTER* AND *KLEBSIELLA* IN SELECTED SOUTH DAKOTA LAKES

BY

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A thesis submitted
in partial fulfillment of the requirement for the
degree Master of Science, Department of
Bacteriology, South Dakota State
University

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IN SELECTED SOUTH DAKOTA LAKES

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser

Thesis Adviser

Head, Bacteriology Dept.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. Robert M. Pengra and Dr. Paul R. Middaugh for their much needed counsel throughout the course of this investigation. I am particularly indebted to Dr. Middaugh for his assistance during the preparation of the written report.

The financial support of the College of Agriculture and Biological Experiment Station is greatly appreciated.

Grateful acknowledgement is made to Dr. Donald R. Progulske and the Wildlife Department of South Dakota State University for the provision of equipment necessary to carry out the sampling procedures used in this study.

I wish to thank my typists, Miss Jean Wainscott for the typing of the rough draft and Miss Theresa Erschens for the typing of the final copy.

A vote of thanks is also due to my wife, Mary Kay, for her patient understanding and helpfulness.

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INTRODUCTION

The importance of nitrogen in an aquatic ecosystem is quite well known. Nitrogen, being originally derived from the atmosphere, enters into a complex cycle involving both the plant and animal components of the ecosystem. The nitrogenous compounds of natural waters may be broken down into two main categories according to their source, either allocthonous or autochthonous. Allochthonous nitrogen is that derived from outside sources and includes those compounds carried into the lake by precipitation, by surface runoff containing terrestrial nitrogen, or by the inflow of ground water. Autochthonous nitrogenous compounds are those which result from the fixation of elemental nitrogen within the body of water itself. This investigation concerns the possible significance of bacterial nitrogen fixation in the over-all nitrogen balance of a lake.

Very little work has been reported demonstrating the numbers of the various nitrogen-fixing bacteria in fresh waters. For this reason the populations of two genera of bacteria which are capable of nitrogen fixation have been followed in five lakes within a 40-mile radius of Brookings, South Dakota. This study was initiated on a preliminary basis using samples taken from Lake Cochrane (Fig. 1). After a period of four months it was decided to expand the study to include an additional four lakes. It was not the purpose of this study to achieve a representative sampling of each lake, but only to take a composite sample of the same sites in each lake at designated time intervals.

In a survey of the genus Azotobacter in soils, Pengra (1965) found that an inverse relationship existed between the number of organisms and the amount of fixed nitrogen present. This may be due to the inability of Azotobacter to compete with other soil organisms under such conditions. In laboratory studies, however, Rabotnova et al. (1959) found ammonium to be the favored form of nitrogen for Azotobacter. Cells grown on ammonium nitrogen exhibited a much shorter lag phase than did those utilizing molecular nitrogen. It has also been noted that Klebsiella will grow preferentially on a fixed source of nitrogen rather than atmospheric nitrogen. To clarify these relationships it was intended to demonstrate any correlations between numbers of these bacteria and the total (combined) nitrogen content of the environment. Upon compiling these data and considering the effects of such variables as pH, temperature, and weather conditions, it was hoped to determine the effect of these genera on the nitrogen content and consequently the productivity of the lake. Also, because this was a preliminary study, it was hoped to uncover and recommend further avenues of approach to the problem.

LITERATURE REVIEW

In 1862 Jodin, a French scientist, measured the disappearance of gaseous nitrogen from a closed system in which "vegetoux mycodermiques" and "les mucedenees" were growing. From this he concluded that those organisms fixed elemental or gaseous nitrogen. Berthelot (1885) was able to demonstrate that unsterilized soils were capable of fixing nitrogen whereas sterilized soils were not. An anaerobic organism capable of fixing molecular nitrogen, Clostridium pastorianum (now called C. pasteurianum) was isolated by Winogradsky in 1893. Beijerinck (1901) showed that the aerobic Azotobacter chroococcum and Azotobacter agilis would also fix nitrogen. In 1928 Skinner demonstrated nitrogen fixation by Bacterium aerogenes (Klebsiella pneumoniae). This was validated by Hamilton and Wilson (1955) using an isotopic tracer technique to demonstrate nitrogen fixation in eight of sixteen strains tested. Vagn Jensen (1956) demonstrated fixation in two strains of Aerobacter aerogenes (K. pneumoniae) isolated from a Danish water course.

With this foundation, further studies and screening experiments were carried out until at present the list of free-living, nitrogen-fixing microorganisms contains 14 genera of blue-green algae, 18 genera of bacteria (Stewart, 1966), two genera of yeasts (Metcalfe et al., 1954) and two species of the actinomycete Nocardia (Metcalfe and Brown, 1957).

It has been shown using N^{15} tracer techniques that nitrogen fixation by aquatic microorganisms can, under certain conditions, contribute significantly to the nitrogen budget of a lake (Dugdale et al., 1959; Dugdale and Dugdale, 1962). In each case, however, the responsible organisms were assumed to be species of the blue-green algae (Myxophyceae). Goering and Neess (1964) indicated the possibility of obligate nitrogen-fixers, such as certain of the bacteria, as being a factor worthy of consideration in understanding the over-all significance of nitrogen fixation in fresh water.

M. E. Gambarian (1958) made a survey of the bacterial nitrogen fixation in Seven Lake in the Armenian highlands. In view of the results of this survey, Gambarian assumed that the assimilation of molecular nitrogen in the water areas of the lake was insignificant because of the small numbers of nitrogen-fixing bacteria present. He considered this to be one of the main reasons for the nitrogen-poor condition of the water of Seven Lake. He did believe, however, that the numbers of these bacteria in the bottom sediments were great enough (up to 10,000 per gram of moist sediment) to have some value in the enrichment of the lake with nitrogen. By using cultures of his own isolates of aerobic and anaerobic nitrogen-fixing microorganisms, Gambarian calculated that one cell of A. chroococcum, after 5.5 days incubation at 25 C, fixed 6.1×10^{-11} mg of nitrogen and C. pasteurianum, after 6.5 days, fixed 3.8×10^{-11} mg of nitrogen.

In a study of the quantitative distribution of Azotobacter and Clostridium in the region of the Phyllophora fields of the Black

Sea, Pshenin (1959) indicated that there was a definite influence of these organisms on the biological productivity of this area. He found that the surface of the Phyllophora thallus was inhabited by microcolonies or diffuse masses of cells which were morphologically identical to Azotobacter species. This bacterial growth, which was similar to that found on artificial agar media, was assumed to be the result of a metabiotic or symbiotic relationship.

This study has been concerned only with the nitrogen-fixing bacteria in the genus Azotobacter and the genus Klebsiella. The family Azotobacteraceae is composed of strictly aerobic, Gram negative, heterotrophic rods of the genera Azotobacter, Beijerinckia and Derxia. The genus Azotobacter, which is commonly found in both soil and water, is generally considered to contain three species, A. chroococcum, A. agilis, and A. vinelandii. The Seventh edition of Bergey's Manual of Determinative Bacteriology (1957), however, does not recognize the latter as a distinct species. This genus is characterized by highly pleomorphic cells ranging from 4-6 u X 2-3 u in size. In pure culture, under optimal conditions, strains of Azotobacter vinelandii have been found to possess the ability to fix from 10 to 20 mg of nitrogen per gram of sugar consumed (Stewart, 1966). Optimum conditions for fixation in laboratory cultures consist of a slightly alkaline pH and a temperature of from 25 to 28 C.

The taxonomic status of the nitrogen-fixing bacteria which have previously been classified as Aerobacter aerogenes is at the present

time still dubious. The most recent trend in their classification has been to place them in the genus Klebsiella. A scheme for the classification of the Klebsiella and Aerobacter taxa was proposed by Edwards and Ewing (1962). Using this scheme, Mahl et al. (1965) concluded that the nitrogen-fixing strains of this organism should, on the basis of their serotypes, be placed in the genus Klebsiella. Therefore, in the presentation of this study the precedent of Mahl and co-workers will be followed by replacing A. aerogenes with K. pneumoniae.

These organisms are facultatively anaerobic, Gram negative, non-spore forming rods (Breed et al., 1957) which, in this case, possess the ability to fix atmospheric nitrogen. When supplied with a source of ammonium nitrogen they will grow either aerobically or anaerobically. It was noted by Hamilton and Wilson (1955), however, that only small quantities of nitrogen were fixed under aerobic conditions whereas a much higher fixation rate was possible anaerobically. This oxygen inhibition was substantiated by Pengra and Wilson (1956). In the laboratory, actively fixing strains of Klebsiella pneumoniae will fix nitrogen in an amount of from 1 to 5 mg per gram of sugar consumed (Stewart, 1966). Jensen (1958) demonstrated an optimum temperature of from 15 to 18 C for nitrogen fixation, whereas for growth the optimum temperature was somewhat higher. He also noted an optimum pH of from 6 to 8 with no fixation taking place below pH 3.5 to 3.6.

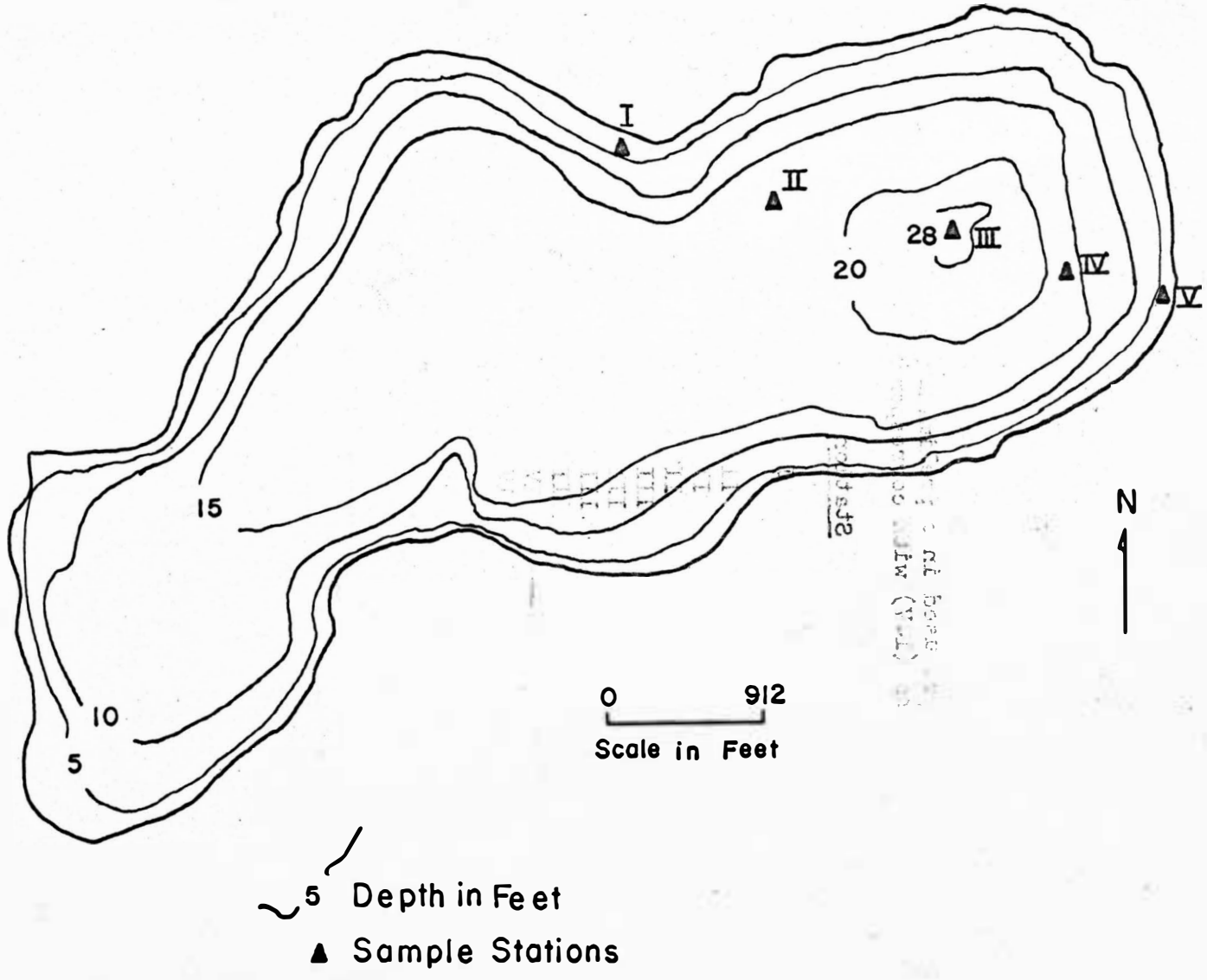
MATERIALS AND METHODS

Sampling Procedures

In a preliminary study, samples were taken at five stations along a previously drawn transect of Lake Cochrane (Fig. 1). At each station samples were taken at three-meter depth intervals including a surface and a bottom sample. This gave a total of 14 sample sites. To minimize sampling error, two samples were taken at each site providing a total of 28 samples which were representative of the profile of the lake.

After a four-month period this study was expanded and altered to include four additional lakes: Fish, Oak, Tetonkaha, and Campbell (Fig. 2). Samples were taken at three stations on each lake as indicated. Since little variation was noted between duplicates in the preliminary survey, this control was abandoned in favor of taking a composite sample of the profile of the lake at each station. One composite water sample was taken which consisted of equal portions of the surface, central, and lower water levels. A bottom sample was also taken at each station. The sample sites were numbered as indicated on the following maps with odd numbers denoting water samples and even numbers denoting bottom samples.

Water samples were taken with a plexiglass, two-liter Van Dorn sampler, model #120 (Fig. 3), and siphoned off into sterile one-quart jars. Bottom samples were taken with an Eckman dredge, model #196 (Fig. 4) and the sediment was poured or scraped into similar


I
II
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▲ Sample Stations

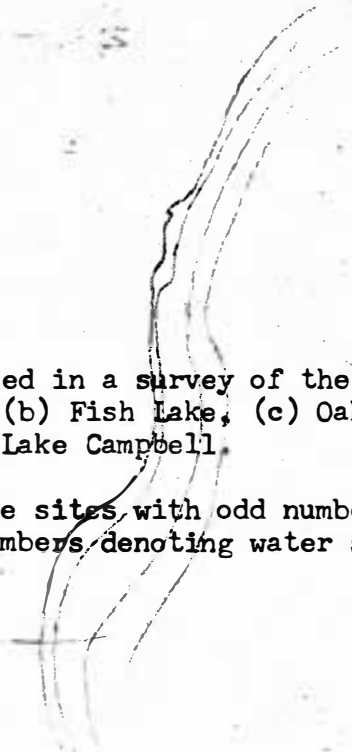


Figure 2. Sample sites used in a survey of the following lakes: (a) Lake Cochrane, (b) Fish Lake, (c) Oak Lake, (d) Lake Tetonkaha, and (e) Lake Campbell.

● designates sample sites with odd numbers denoting bottom samples and even numbers denoting water samples.

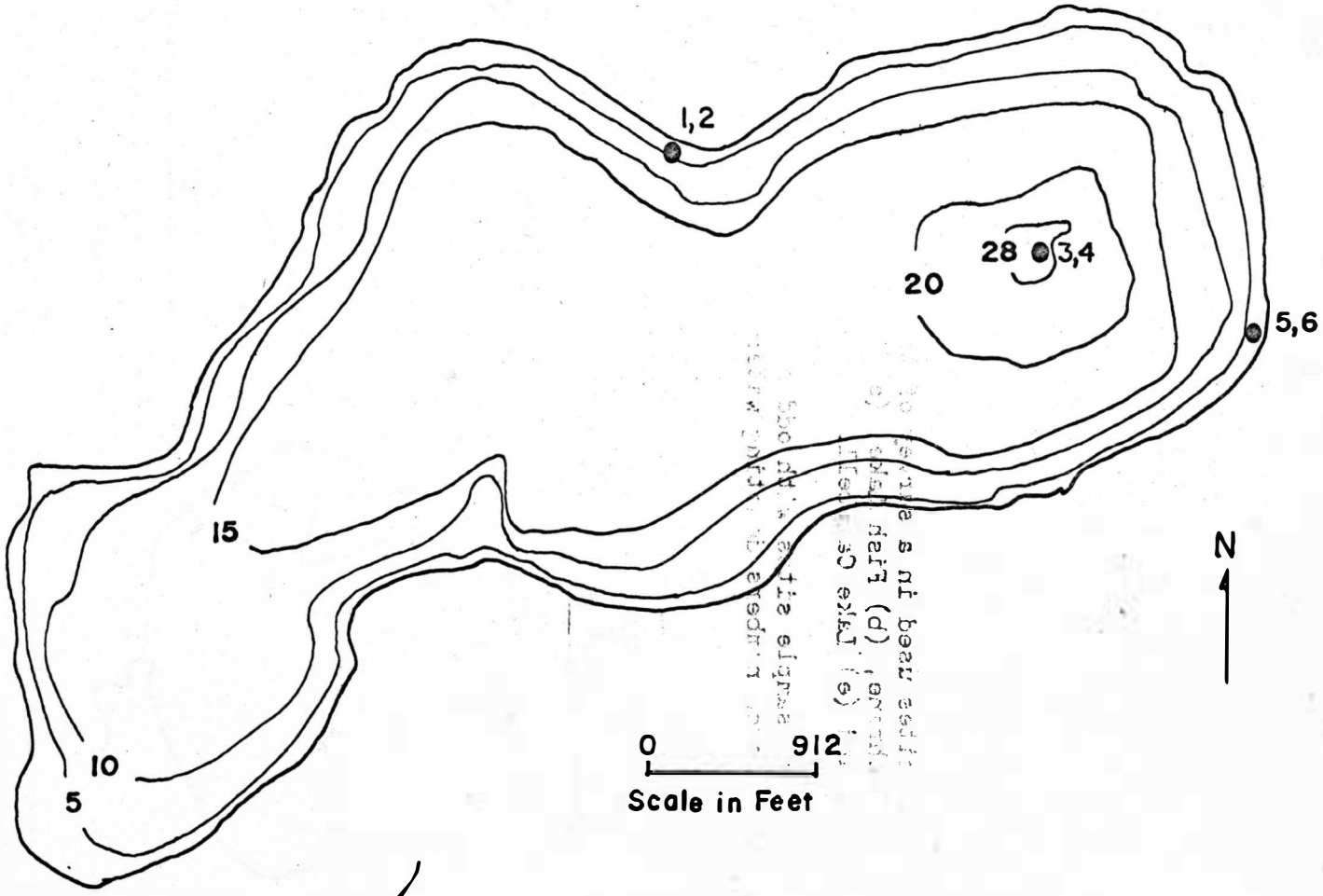


Figure 2a. Lake Cochrane

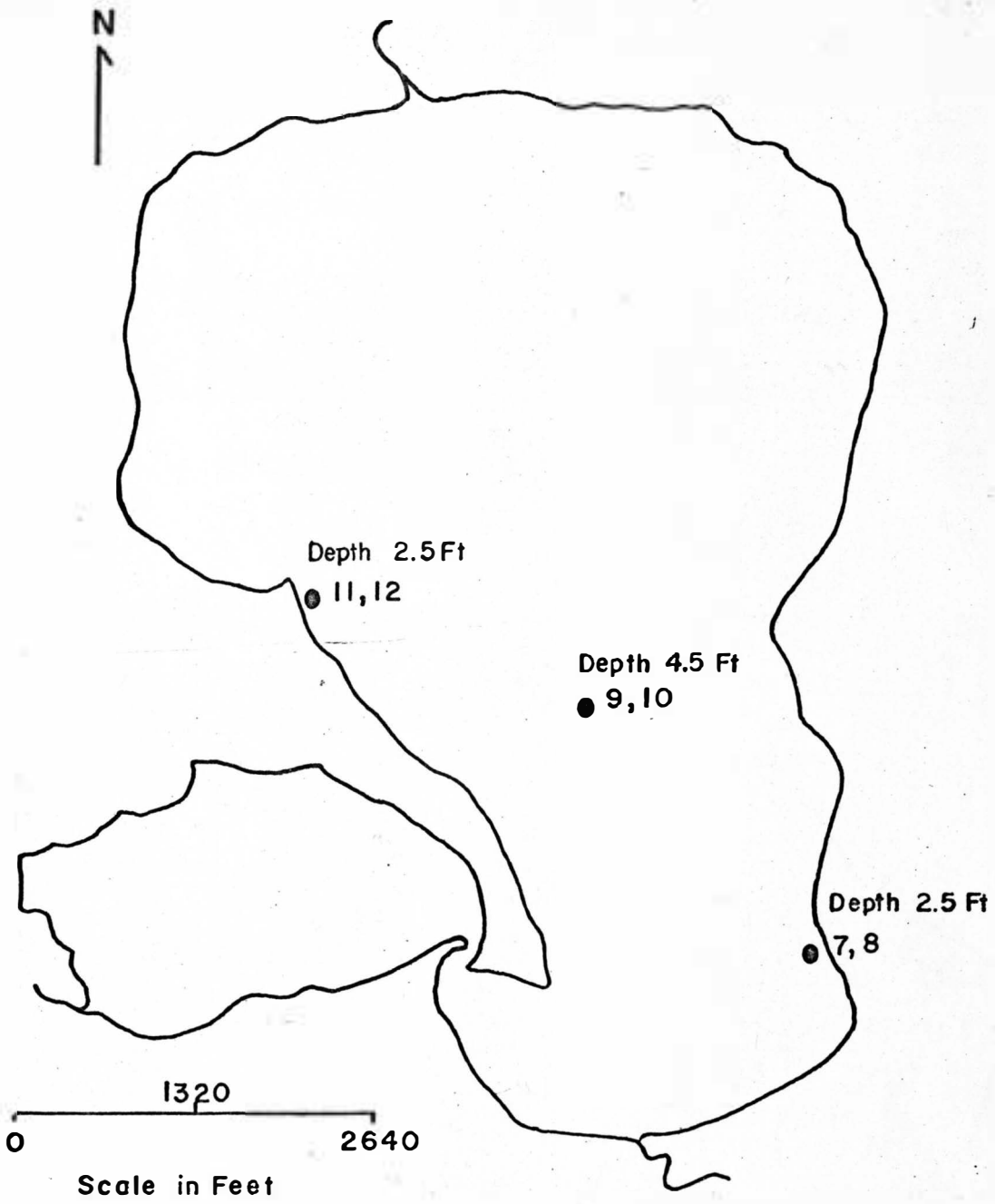


Figure 2 b. Fish Lake

● Sample Sites

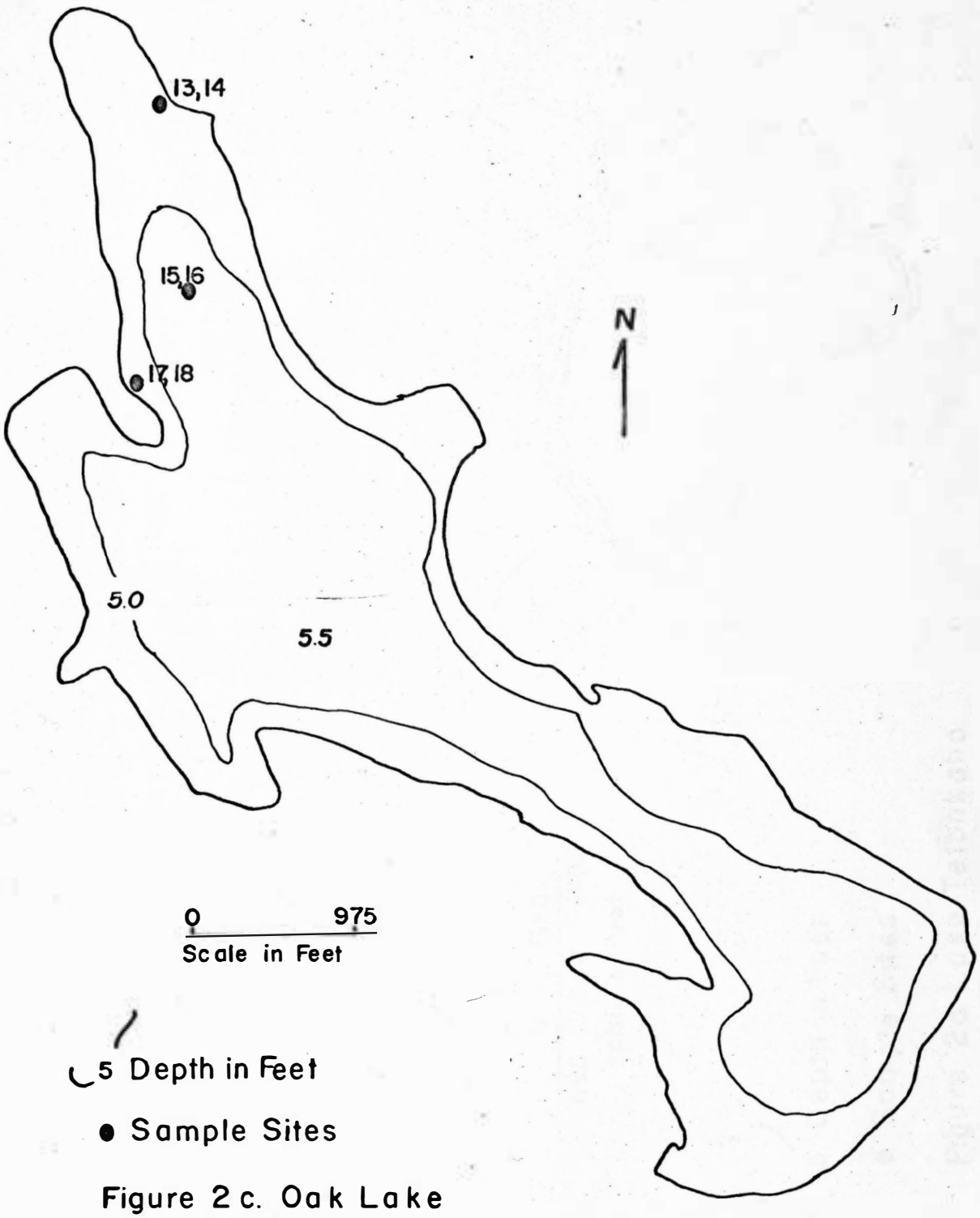


Figure 2 c. Oak Lake

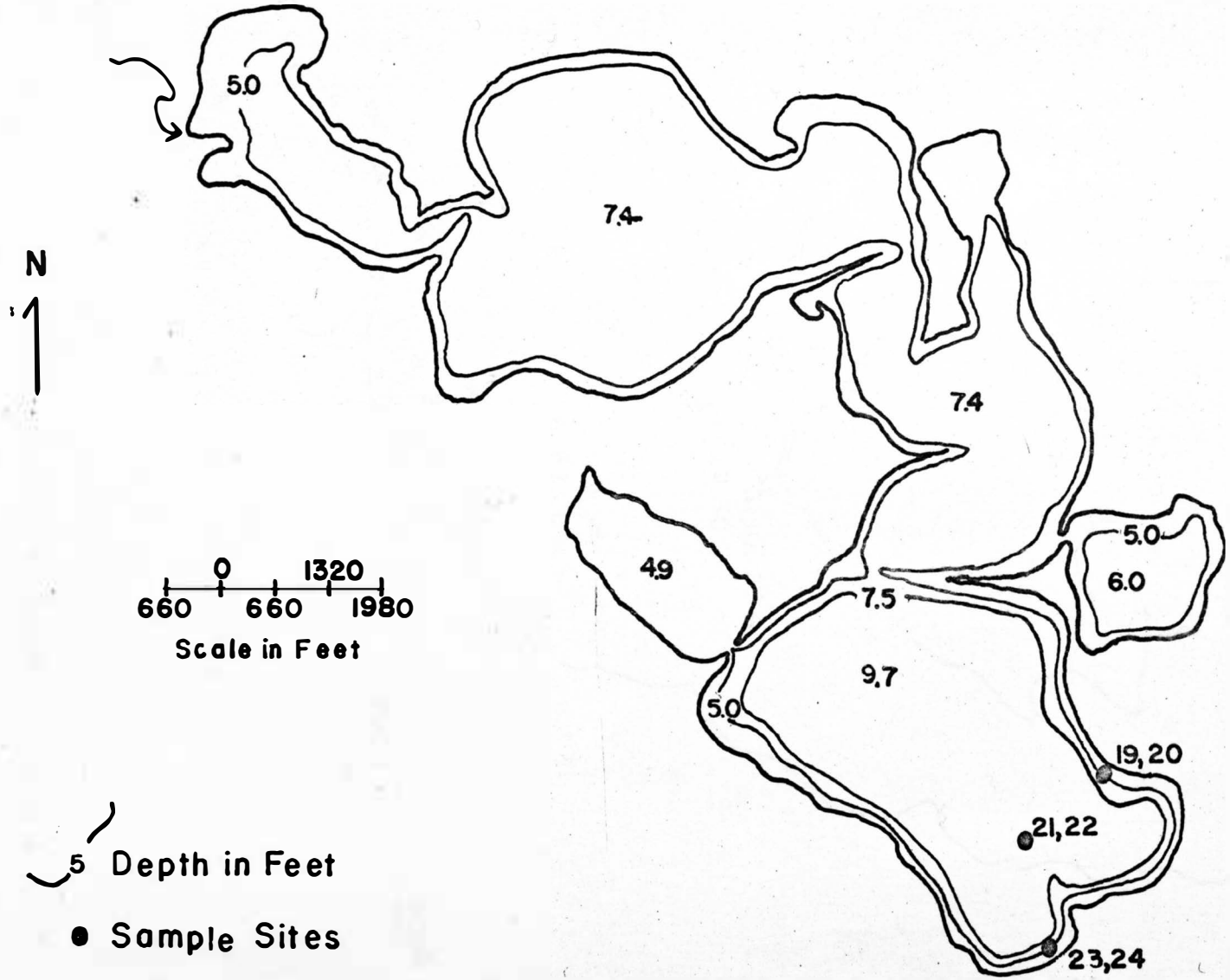


Figure 2d. Lake Tetonkaha

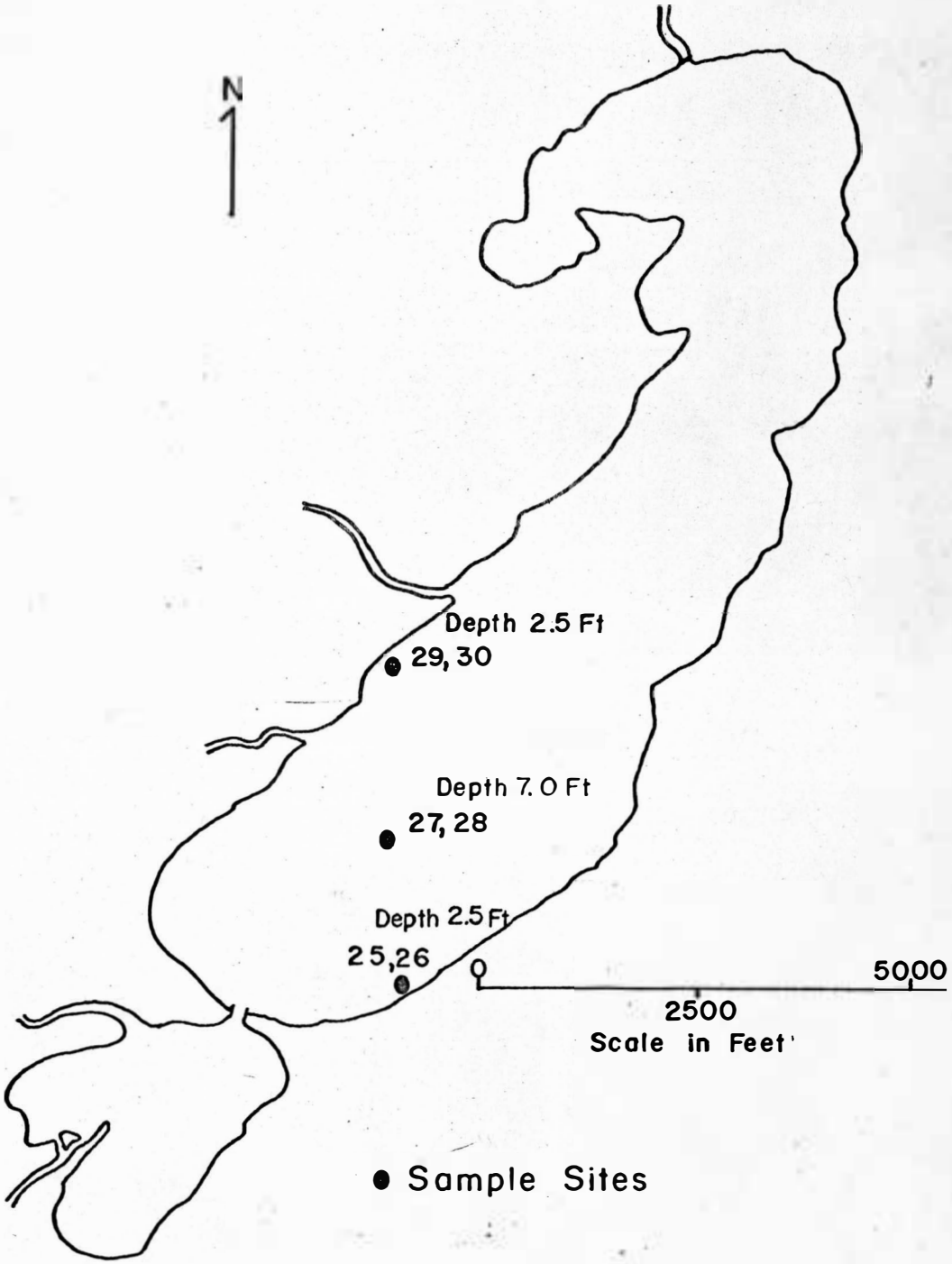


Figure 2e. Lake Campbell

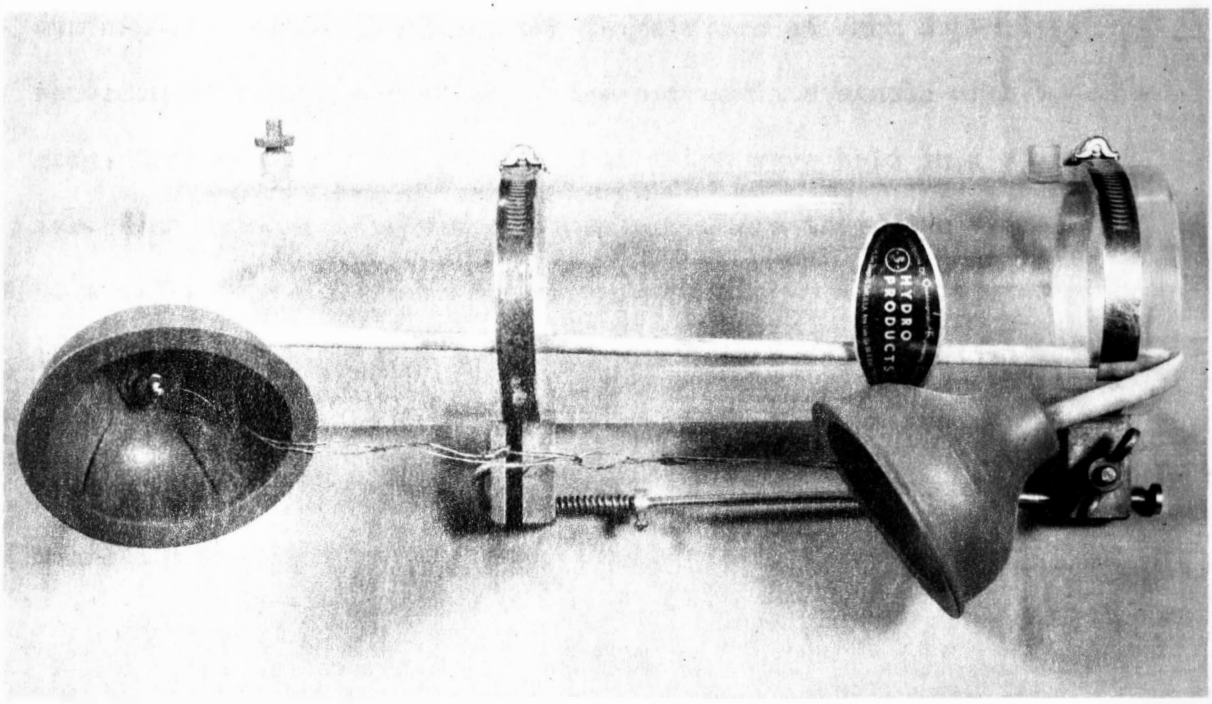


Figure 3. Van Dorn Water Sampler

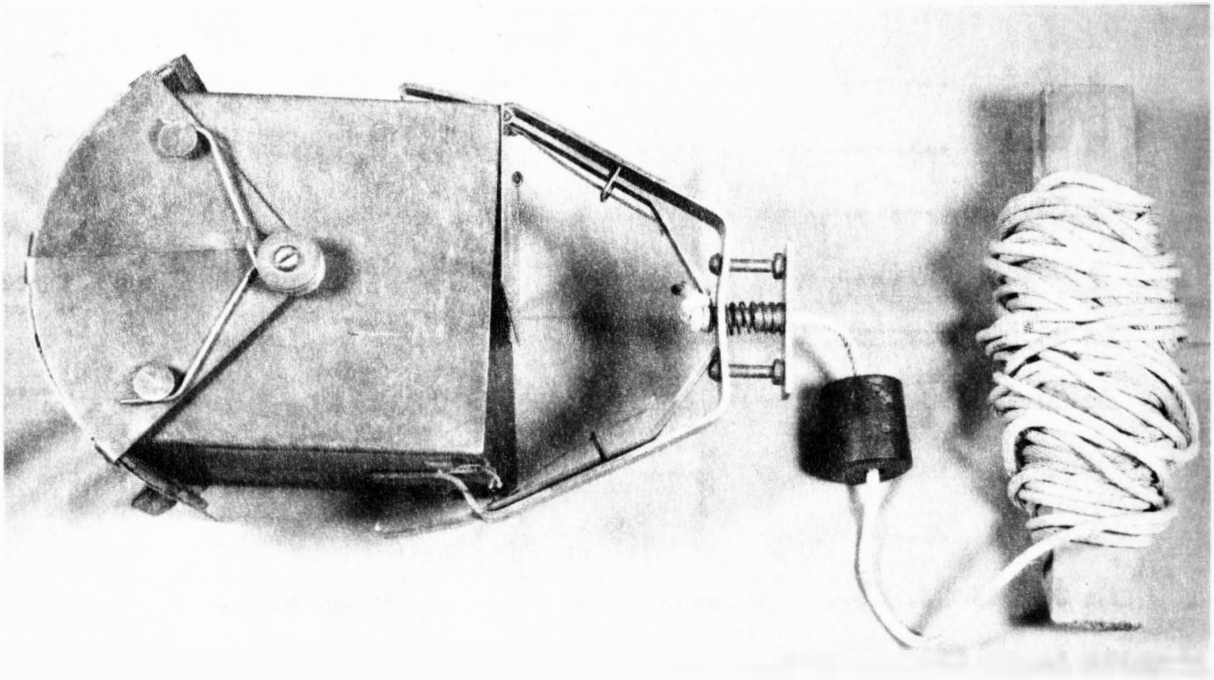


Figure 4. Eckman Dredge

containers. While sampling, the temperature at each depth level was recorded by taking a reading of the appropriate sample with a centigrade thermometer. The samples thus taken were held in a styrofoam ice-chest until reaching the laboratory where they were transferred to a walk-in cooler (4 C) to await processing. After returning to the laboratory, the following analyses were made: pH readings (made immediately to minimize error), plate counts of the genera Azotobacter and Klebsiella, and total nitrogen determination via a reduction modification of the macro-Kjeldahl procedure.

Media

The following medium, which was proposed by Brown et al. (1962) was used in the plating of Azotobacter:

Solution I

Sucrose	5.00 g
$MgSO_4 \cdot 7\ H_2O$	0.20 g
$CaCl_2$	0.15 g
Agar	15.00 g
Deionized water	900.00 ml
Fe-Mo solution (Wilson and Knight, 1952)	1.00 ml

Solution II

K_2HPO_4	0.80 g
Deionized water	100.00 ml

Solutions I and II were autoclaved separately and mixed after cooling to approximately 45 C.

The following medium, used in the plating of Klebsiella, was a modification of the medium used by Yoch and Pengra (1966):

Na_2HPO_4	12.5 g
KH_2PO_4	1.5 g
$MgSO_4 \cdot 7\ H_2O$	0.2 g
NaCl	8.5 g
Fe-Mo solution (Wilson and Knight, 1952)	1.0 ml
Mannitol	20.0 g
Agar	15.0 g
Deionized water	1000.0 ml

Filtration and Plating Techniques

One set of 140 mm sterile petri dishes was poured with Azotobacter medium and one set of 60 mm sterile petri dishes was poured with the above medium for Klebsiella. The following filtration technique was then carried out in duplicate to accommodate the plating of both genera. Five, 10, and 50 ml portions (lesser amounts in highly turbid samples) of each water sample were drawn through separate Millipore filters (pore size 0.45 u). A somewhat lesser amount was used of a 1:10 dilution (w/w) of the bottom samples depending upon the amount of colloidal material present. To prevent contamination of one sample from the previous one, the filtration apparatus was dipped in 95 per cent ethyl alcohol and flamed after each sample. Fifty ml of sterile water were drawn through each of two filters (one for each medium) to check for equipment or air contamination. The filters were

then placed directly upon the agar with one similarly treated filter for each set of plates. All Klebsiella plates were incubated with a control at 32 C in Brewer's jars with an atmosphere of nitrogen gas. The jars were evacuated four times followed by flushing with high-purity nitrogen. After the final evacuation, 0.9 of an atmosphere of nitrogen was placed in each jar. Azotobacter plates were incubated at room temperature. After four to seven days of incubation, counts were made on each set of plates and populations of organisms were expressed as cells per liter of water or per gram of dry sediment.

pH Measurement

The pH readings of all water samples were read directly using a Sargent Model PL portable pH meter. In measuring the pH of the bottom material, 20 g of sediment were mixed with 20 ml of distilled water and allowed to equilibrate with intermittent stirring for 20 minutes. The pH of this mixture was then measured.

Macro-Kjeldahl Procedure

A 300 ml aliquot of a water sample or a weighed portion of sediment (one to three grams) was put into an 800 ml Kjeldahl flask. To this 40 ml for water samples and 50 ml for bottom samples of a salicylic acid-concentrated sulfuric acid mixture (2 g:40 ml) was added and mixed well by swirling. This was allowed to stand overnight or for approximately 12 hours. Five grams of sodium thiosulfate were then added and the flask was heated gently for five minutes. Upon cooling, a Kelpak containing 0.30 g $CuSO_4$ and 10 g K_2SO_4

was added. The flask was then boiled, using glass beads as boiling chips, for approximately two hours or until a bluish-green liquor was obtained. After allowing the flask to cool, the neck was washed down with distilled water and then reheated and boiled for an additional hour to complete digestion. This was allowed to cool and then diluted with 300 ml of distilled water. At this point, three or four pieces of mossy zinc were added along with sufficient NaOH to make the solution strongly basic. This was mixed and approximately 250 ml were distilled into a titration flask containing 50 ml of a four per cent boric acid solution. Eight drops of Tashiro's indicator were then added and the resulting green solution was titrated to a bright purple color with N/14 HCl. The levels of nitrogen were expressed as mg per liter or mg per gram of dry sediment.

Effect of Storage Time

To determine the effects of storage time on the results of this study, an experiment was performed making the various analyses repeatedly at prearranged timed intervals. Four water samples and four bottom samples were collected at regular sample sites at Lakes Campbell and Oakwood. The pH readings were taken in the field. Upon returning to the laboratory the samples were immediately plated and the appropriate portions measured out and acidified in preparation for the Kjeldahl procedure. In each case this was taken as the initial time and successive duplicate analyses were made at timed intervals. The samples were kept under refrigeration (4 C) at all times during the experiment.

RESULTS AND DISCUSSION

Effect of Storage Time

In considering the effects of storage time, it was decided that within the period tested, there was no significant increase or decrease in the numbers of organisms or nitrogen content of the samples to invalidate the assumptions to be made from the results of this study (Table I).

Preliminary Survey

The preliminary study of Lake Cochrane presented a number of questions which warranted further inquiry. It was evident that this study would have to be altered and expanded in order to fulfull the original goals. During the first four months of sampling the numbers of organisms were not at high enough levels to add significantly to the nitrogen content of the lake (Fig. 5).

Because counts were low and varied extremely from month to month, it was difficult to show any definite correlation between the numbers of organisms and the chosen variables. Tables II and III contain data concerning the climatic conditions during the period of sampling. Since the wind effect during the ice-free period prevented any layering of the lake, over-all trends, rather than individual areas of the profile, were examined in order to determine the subsequent plan of study. It was noted during these first four months that the trends in numbers of both Azotobacter and Klebsiella in water samples were inversely related to the amount of nitrogen present. Counts of both the Azotobacter and

Table I. Effect of Time of Sample Storage at 4 C

	Sample Sites**	0 Days	3 Days	7 Days
No. of Azotobacter/l or g *	19	30.8	15.4	0.0
	20	38.0	32.0	33.4
	23	0.0	0.0	0.0
	24	6.0	4.0	2.6
	25	15.4	46.2	15.4
	26	8.3	7.7	7.4
	29	15.4	46.2	30.8
	30	27.1	30.0	29.1
No. of Klebsiella/l or g *	19	61.5	15.4	30.8
	20	0.0	0.0	0.3
	23	215.0	200.0	169.0
	24	0.6	0.0	1.0
	25	538.5	415.4	600.0
	26	0.0	2.9	0.6
	29	7666.7	7266.7	6600.0
	30	1.1	0.0	0.6
Mg of Total N/l or g*	19	0.25	---	0.23
	20	0.19	---	0.06
	23	0.18	---	0.16
	24	0.13	---	0.10
	25	5.06	---	4.33
	26	3.83	---	4.00
	29	2.00	---	2.33
	30	2.17	---	2.00
pH	19	8.30	8.50	8.60
	20	7.35	7.35	7.30
	23	8.30	8.50	8.60
	24	7.40	7.50	7.55
	25	9.00	8.85	8.60
	26	7.65	7.50	7.55
	29	9.00	8.85	8.60
	30	7.60	7.70	7.40

* Analyses of bottom samples were calculated per gram of moist sediment.

** The sample sites correspond with those of Lakes Tetonkaha and Campbell (Figures 2d and 2e).

--- No analysis.

Table II. Water temperatures, thickness of ice, and depth of snow cover on ice on the dates of sampling

Date	Lake	Water Temp. (C) Surface	Middle	Bottom	Depth of Ice Cover (inches)	Depth of Snow on Ice (inches)
1966						
Apr. 14	Cochrane	5.0	4.0	4.0	0	0
May 16	Cochrane	11.0	10.0	10.0	0	0
June 24	Cochrane	21.0	21.0	21.0	0	0
July 23	Cochrane	23.0	23.0	23.0	0	0
Aug. 30	Cochrane	*	*	*	0	0
Oct. 8	Cochrane	18.5	18.5	18.5	0	0
Oct. 8	Fish	13.0	13.0	13.0	0	0
Oct. 8	Oak	12.0	12.0	12.0	0	0
Oct. 8	Tetonkaha	12.0	12.0	12.0	0	0
Oct. 8	Campbell	12.5	12.5	12.5	0	0
Nov. 11	Cochrane	2.0	3.0	5.0	2-4	Trace
Nov. 11	Fish	1.0	2.0	4.0	8-10	Trace
Nov. 11	Oak	2.0	3.0	3.0	4-6	Trace
Nov. 11	Tetonkaha	1.0	2.0	3.0	6	Trace
Nov. 11	Campbell	1.0	1.0	3.0	7	Trace
Dec. 10	Cochrane	1.0	2.0	4.0	8	4-6**
Dec. 10	Fish	1.0	2.0	3.0	12	4-6**
Dec. 10	Oak	1.0	2.0	3.0	10	4-6**
Dec. 10	Tetonkaha	1.0	2.0	3.0	10	4-6**
Dec. 10	Campbell	1.0	2.0	3.0	10	4-6**
1967						
Jan. 14	Cochrane	1.0	3.0	5.0	24	4-6***
Jan. 14	Fish	1.0	2.0	3.0	24-30	4-6***
Jan. 14	Oak	1.0	2.0	3.0	24-30	4-6***
Jan. 14	Tetonkaha	1.0	2.0	3.0	24-30	4-6***
Jan. 14	Campbell	1.0	2.0	3.0	24-30	4-6***
Mar. 4	Cochrane	0.5	2.0	3.5	30-36	6-12**
Mar. 4	Fish	0.5	1.0	2.0	30-36	6-12**
Mar. 4	Oak	0.5	1.0	2.0	30-36	6-12**
Mar. 4	Tetonkaha	0.5	2.5	3.0	30-36	6-12**
Mar. 4	Campbell	0.5	2.5	3.0	30-36	6-12**
Apr. 15	Cochrane	8.0	7.0	7.0	0	0
Apr. 15	Fish	10.5	10.5	10.5	0	0
Apr. 15	Oak	10.0	10.0	10.0	0	0
Apr. 15	Tetonkaha	10.0	10.0	10.0	0	0
Apr. 15	Campbell	11.0	11.0	11.0	0	0

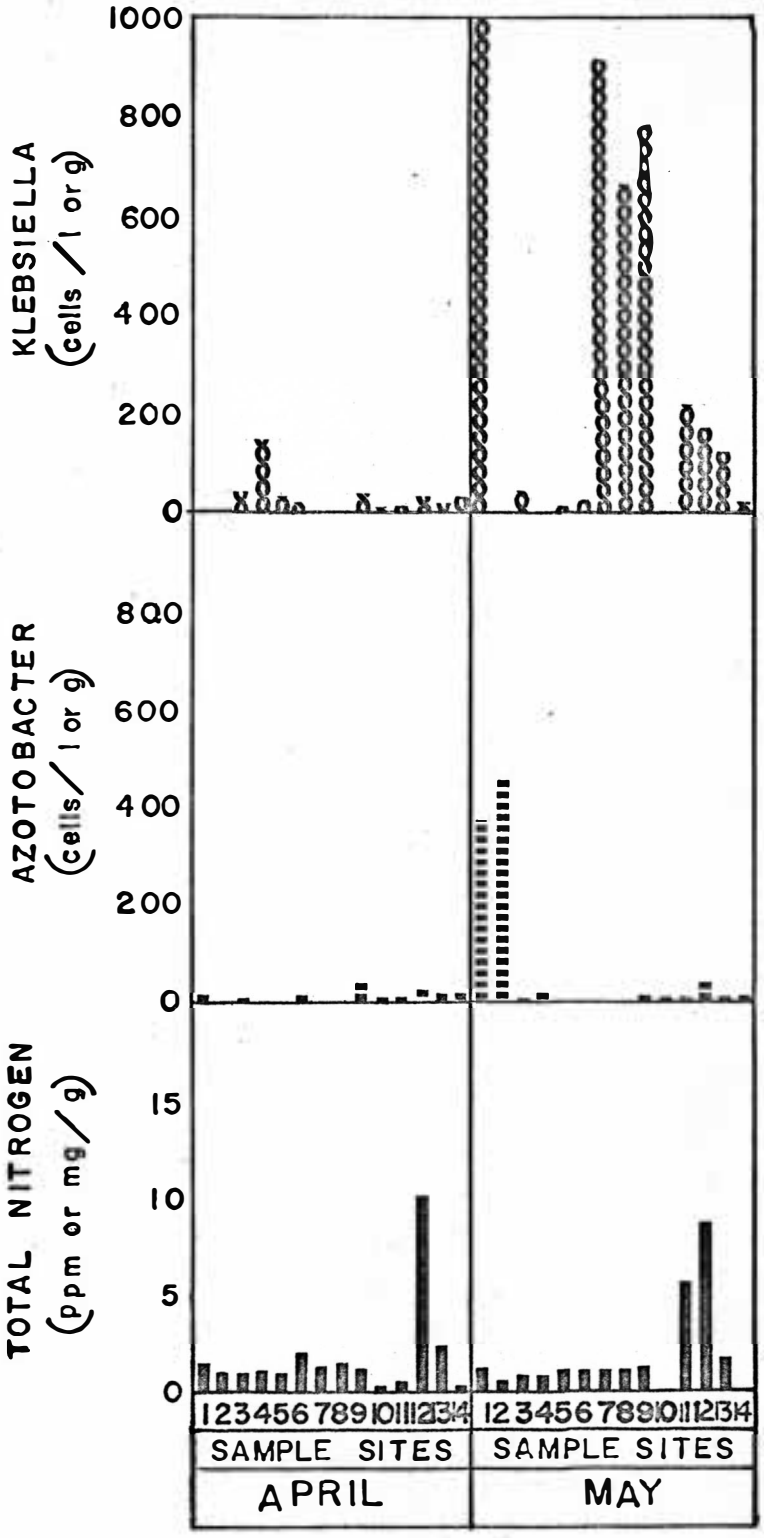
* No data
** Some areas clear of snow
*** Drifts up to 24 inches

Table III. Wind velocities and amounts of precipitation throughout the sampling period**

Date	Precipitation (inches)						Wind Velocity (mph)		
	Total	Greatest Amount	Day	Snow Total	Max. on Ground	Day	Ave.	Max.	Day
Mar. 1966	0.82	*	*	*	*	*	13.5	40	05
Apr.	1.76	0.61	28	7.0	3.0	20	12.3	26	01
May	1.31	0.41	11	trace	trace	11	12.8	31	15
June	5.21	1.52	21	0.0	0.0	--	11.9	44	13
July	1.39	0.45	14	0.0	0.0	--	11.8	46	04
Aug.	3.01	1.04	21	0.0	0.0	--	10.6	43	14
Sept.	1.35	0.48	02	0.0	0.0	--	9.8	25	24
Oct.	0.86	0.44	14	0.0	0.0	--	12.6	38	14
Nov.	0.12	0.12	10	4.0	4.0	10	12.2	31	29
Dec.	0.41	0.40	08	6.0	5.0	12	10.1	31	05
Jan. 1967	0.23	0.14	31	1.5	1.0	20	12.5	45	16
Feb.	0.55	0.12	05	12.5	6.0	21	12.7	35	23
Mar.	0.23	0.18	20	3.0	3.0	20	*	*	*
Apr.	1.69	0.95	02	*	*	*	*	*	*

* No data

** Precipitation values were obtained from the Agricultural Engineering Department, South Dakota State University. Wind velocities were recorded at the Sioux Falls Airport.


KLEBSIELLA (cells / 1 or g)
1000
800
600
400
200
0
AZOTOBACTER (cells / 1 or g)
800
600
400
200
0
TOTAL NITROGEN (ppm or mg / g)
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
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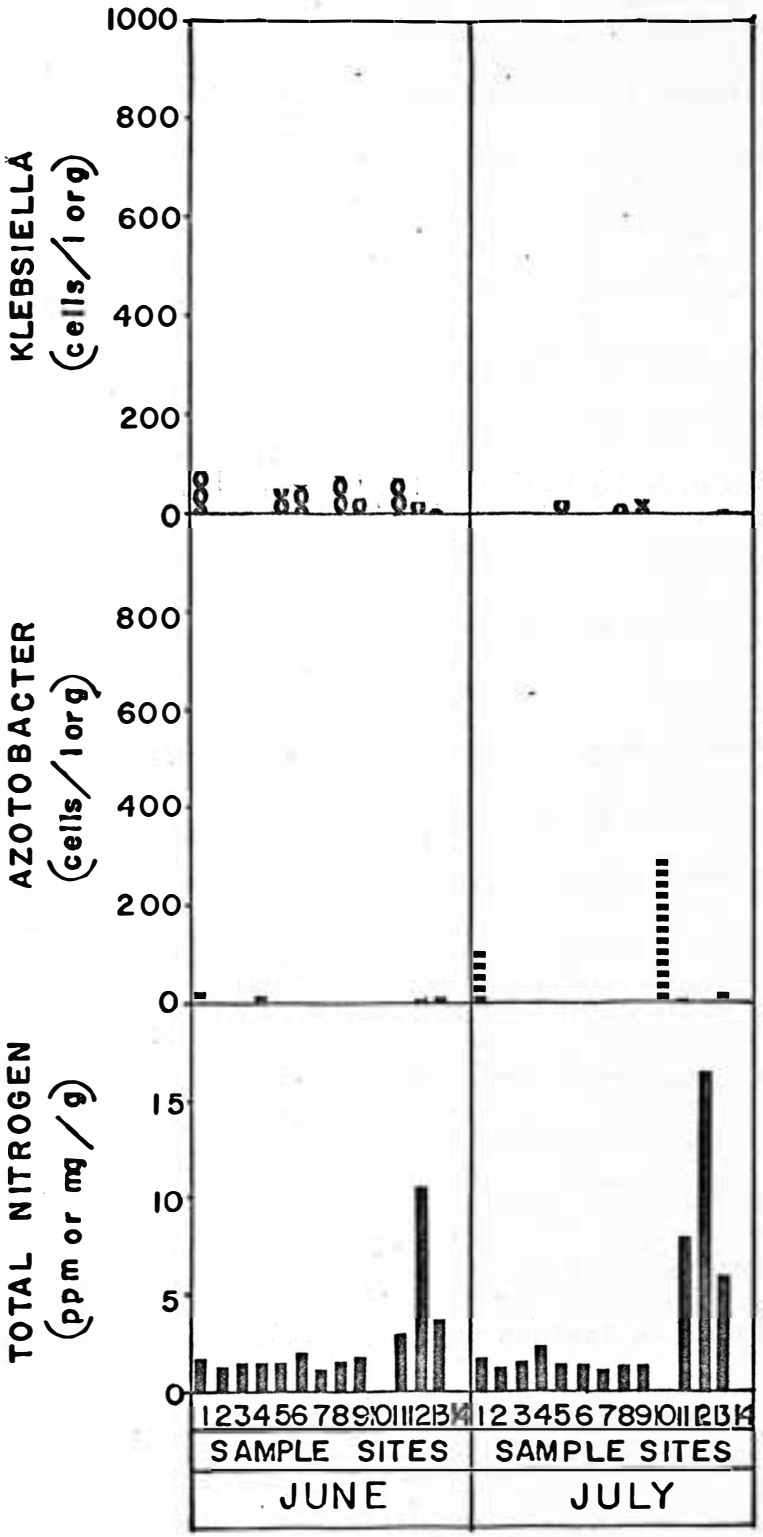
KLEBSIELLA (cells/1 org)
1000
800
600
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AZOTOBACTER (cells/1 org)
800
600
400
200
0
TOTAL NITROGEN (ppm or mg/g)
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 1 2 3 4 5 6 7 8 9 10 11 12 13 14
SAMPLE SITES
SAMPLE SITES
JUNE
JULY

Klebsiella were decidedly higher during the May sixteenth sample period than during the other three months. This was accompanied by a correspondingly low nitrogen content during the same period.

The question of whether or not these microorganisms were reproducing and actively fixing atmospheric nitrogen was also created in looking at the preliminary results. The surge in population of both the Azotobacter and Klebsiella between the April and May sample periods could have been caused solely by the wash-in of soil organisms during the spring thaw. It was also noted that the Azotobacter appeared most frequently and in the highest concentrations in the bottom sediment and shore-line water samples. The organisms may have been washed in by rain water and wave action and deposited in the bottom sediment. Since Azotobacter has the highest oxygen requirement of any organism known, it is doubtful that these bacteria could have been in a state of active fixation in the anaerobic or low-oxygen environment found in the bottom mud. There is a possibility, however, that the organism could have been growing at the water-sediment interface where oxygen would be supplied by the constant movement of the water. As the lakes chosen are characteristically quite shallow, the mixing effect caused by wind during the ice-free period would keep the dissolved oxygen content at fairly high levels throughout the profile of the water layer.

As stated previously, Klebsiella, although capable of aerobic growth, only fixes appreciable amounts of nitrogen under anaerobic conditions. In this preliminary study, Klebsiella was found in the

highest numbers in the water samples. As this is an aerobic environment, it is doubtful that these organisms would be actively fixing nitrogen under such conditions.

Five Lake Survey

By expanding the study to include four additional lakes, it was hoped to either substantiate or disprove the above preliminary indications.

These data further substantiate previous evidences that these organisms are not found in sufficiently high numbers and under the proper environmental conditions to add significant amounts of nitrogen to the lake. The results again show that the Azotobacter are found most often and in the highest numbers in the bottom sediment. This fact is most clearly shown in samples taken from Lake Campbell. This lake, having a dam at the north end, is characterized by a relatively high rate of inflow in comparison to the others which remain essentially closed for the major portion of the year. For this reason there is a higher rate of siltation and sedimentation filling in the central portion of the lake. Therefore, it was assumed that the relatively high numbers of organisms found in the bottom mud from the central portion of the lake were due predominantly to wash-in and are not indigenous to this environment. Further study using more precisely defined sampling techniques will be necessary to explore the possibility of growth and fixation of Azotobacter at the mud-water interface. Other than in this limited area or in other specialized

Figure 6. Monthly variations in numbers of Klebsiella and Azotobacter and total nitrogen contents in samples taken from the following lakes: (a) Lake Cochrane, (b) Fish Lake, (c) Oak Lake, (d) Lake Tetonkaha, and (e) Lake Campbell.

* The designation of the times of sampling is as follows:
S -- August 30, 1966
O -- October 9, 1966
N -- November 12, 1966
D -- December 11, 1966
J -- January 15, 1967
M -- March 5, 1967
A -- April 16, 1967
No data were obtained for the month of February.

** Calculations of analyses of all bottom samples were made on a dry weight basis.

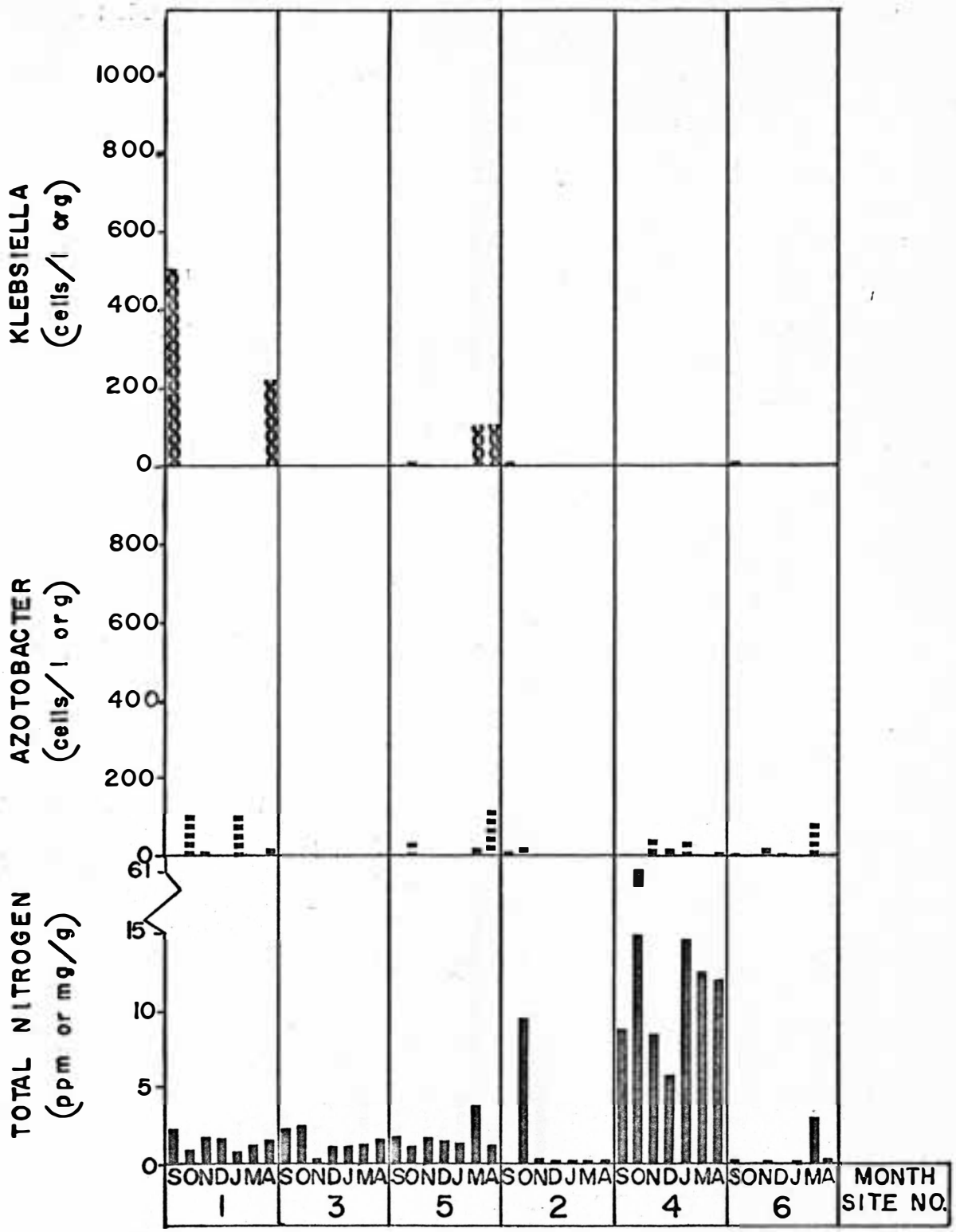

Figure 6a. Lake Cochrane

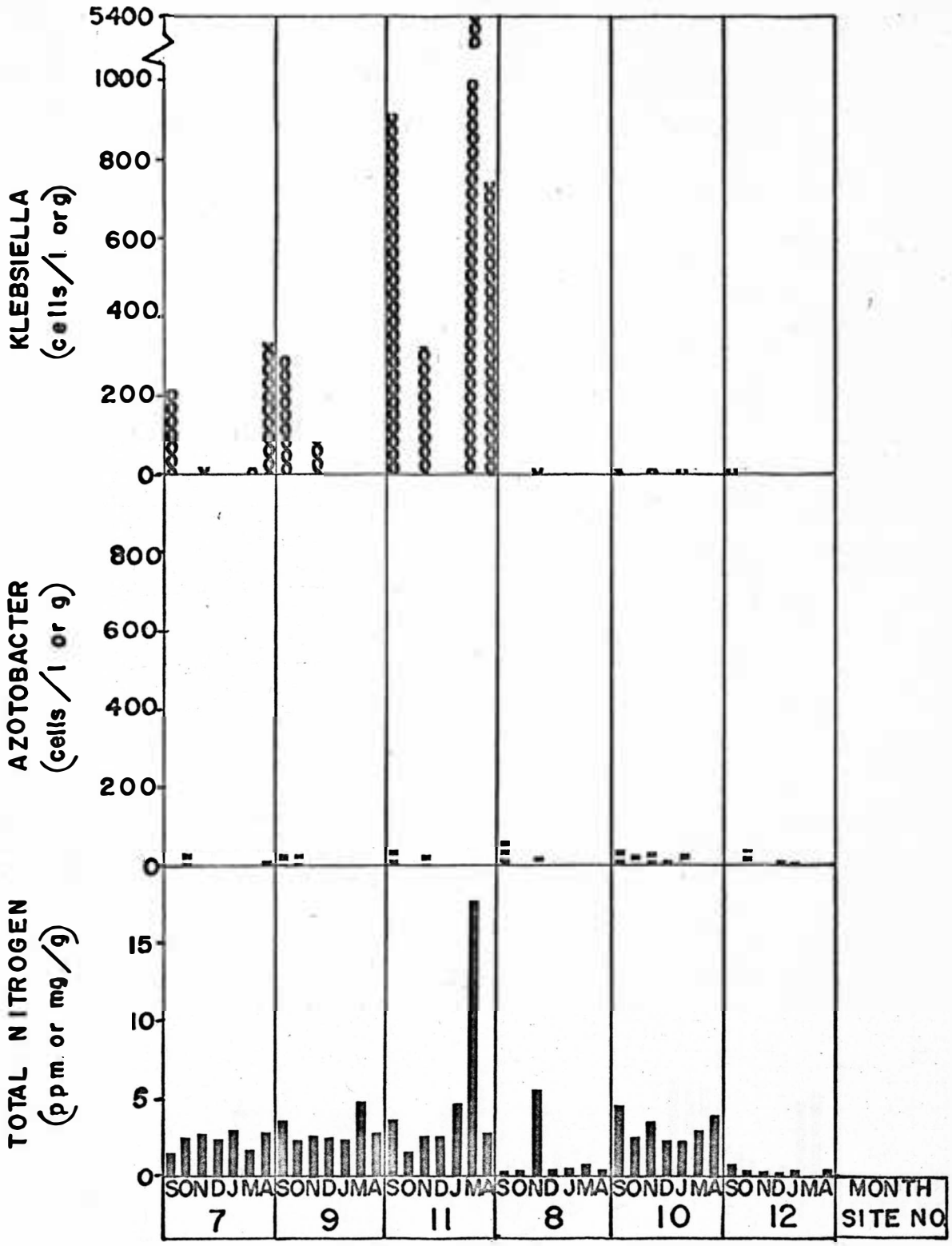

Figure 6b. Fish Lake

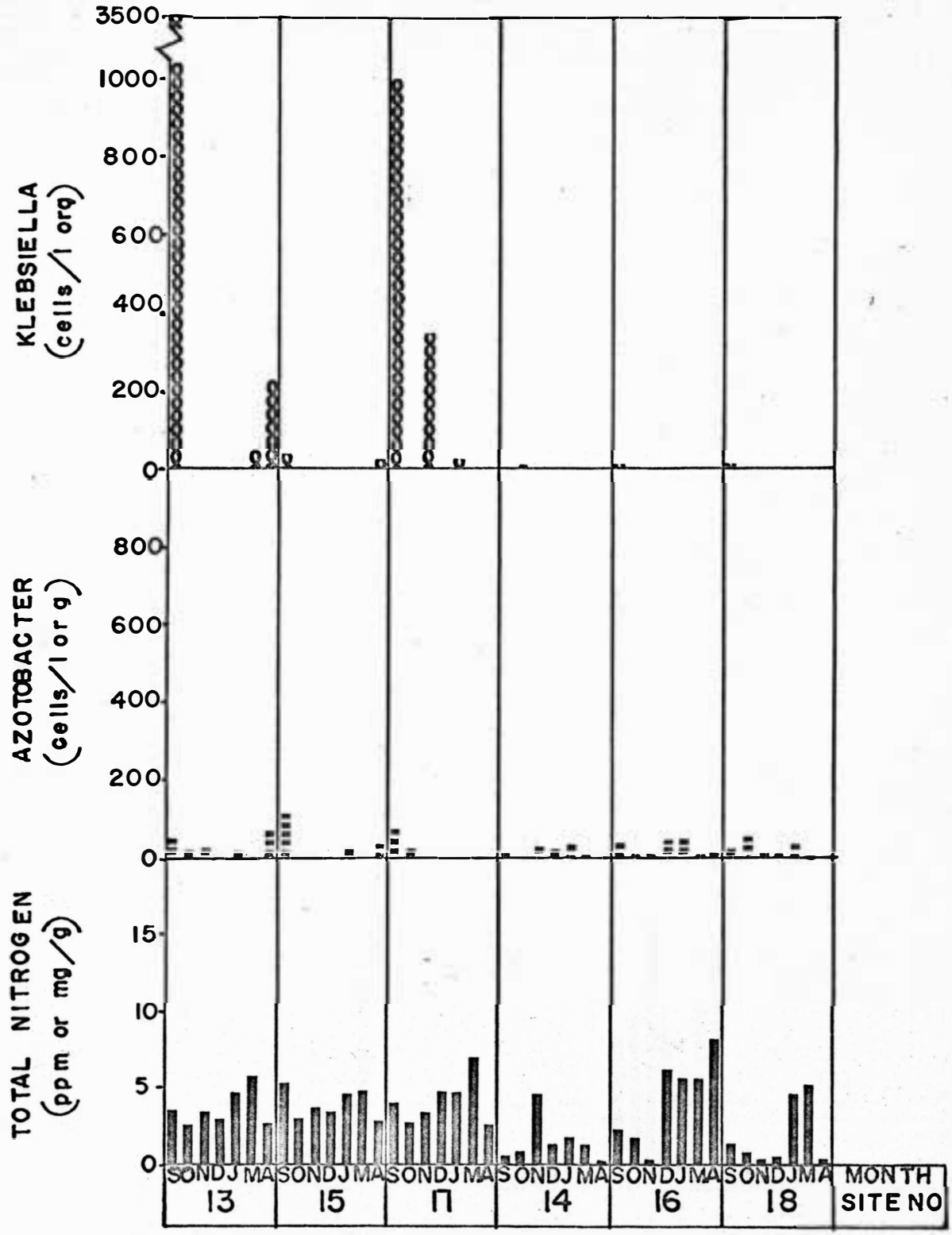

Figure 6c Oak Lake

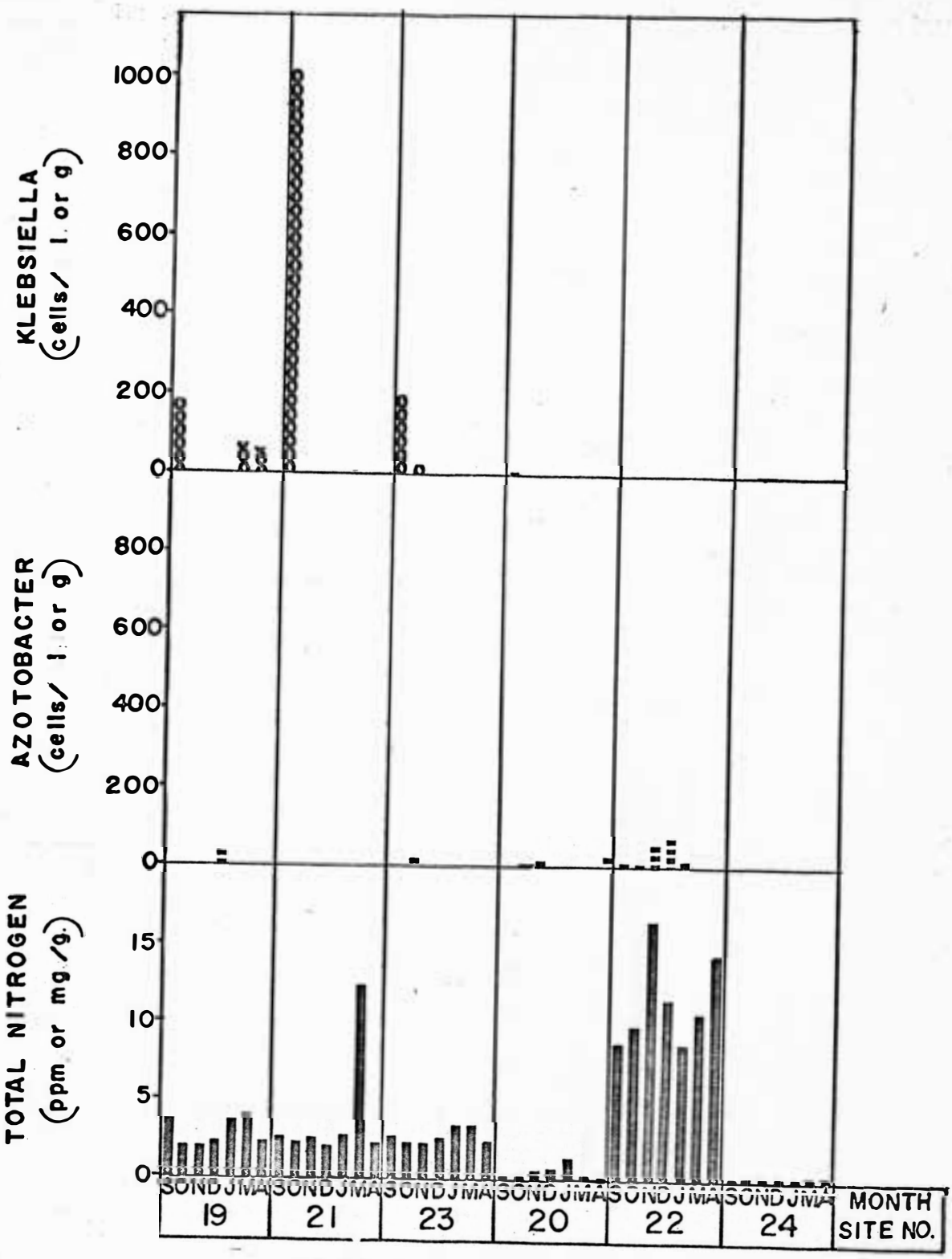

Figure 6d. Lake Tetonkaha

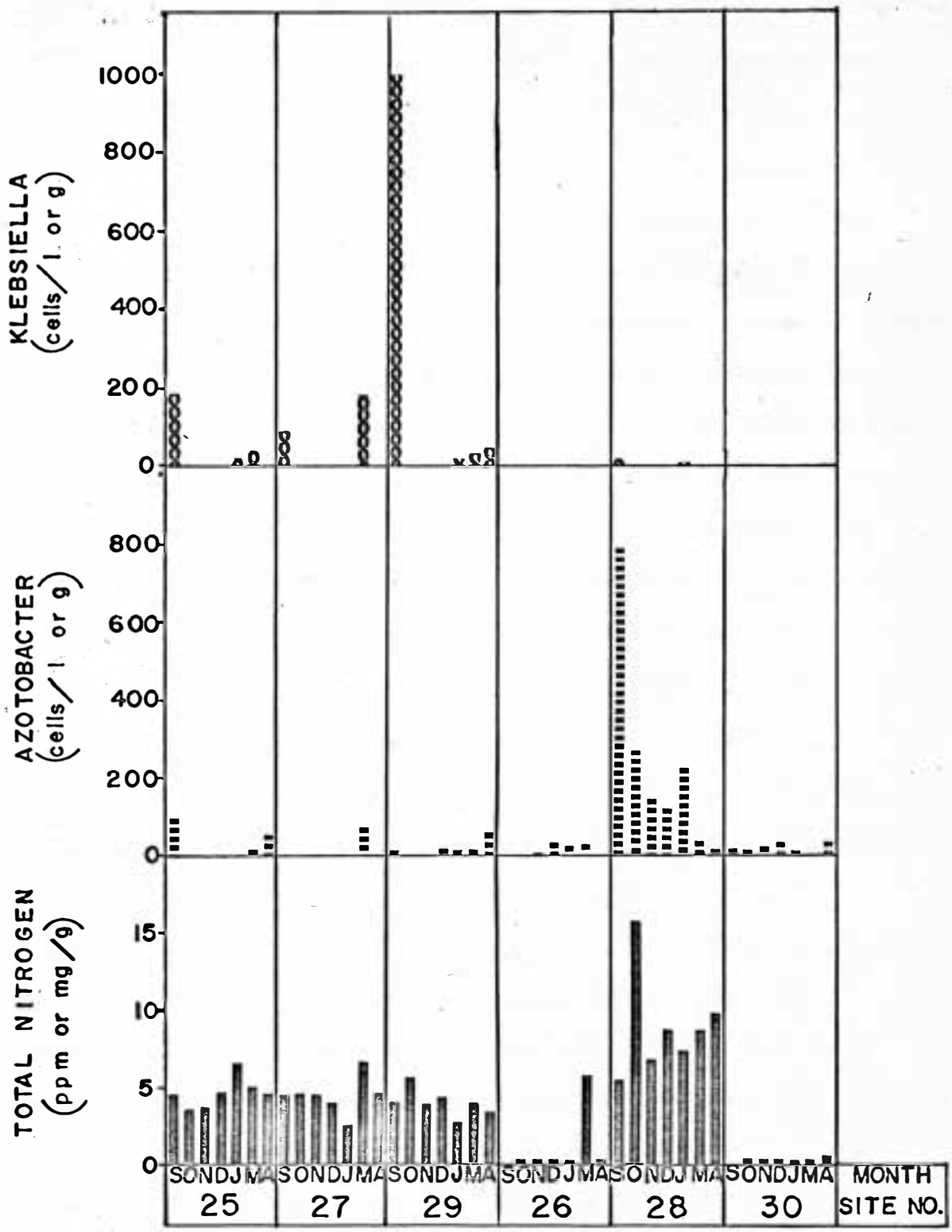

Figure 6e. Lake Campbell

environments as mentioned previously in the work of Pshenin, it is doubtful that the high oxygen requirements of the Azotobacter would permit growth and nitrogen fixation in the bottom sediment.

The numbers of Klebsiella found were quite inconsistent and were, as a whole, quite low. It was evident, however, that these organisms were not surviving to any great extent in the bottom sediments. Counts in every case were almost negligible in samples taken from this area. The highest count was less than 20 colonies per gram of dry sediment. Counts taken from the water samples, although still quite low, were consistently higher than this. Numbers ranging up to 5,400 per liter of water were found. These results further substantiate the assumption that the nitrogen-fixing strains of Klebsiella which were found in an aerobic environment were not in an active state of fixation. The question still remains, however, as to whether or not these organisms were growing heterotrophically on fixed sources of nitrogen or merely existing for a period of time after being washed in from the soil. To give an indication of the answer to this question an attempt was made to correlate numbers of Klebsiella with weather conditions for the corresponding time periods. There was a trend toward higher Klebsiella counts during the September, March, and April sampling periods. This seemed to correlate with a heavy rainfall of 1.04 inches just nine days prior to the August 30 collection date indicating that the wash-in of soil organisms may have been an important factor. The high counts in

March and April may have been the result of the spring thaw which began in early March and continued intermittently until ice-out in early April.

Other than in the preliminary study, no inverse relationship was noted between the numbers of Klebsiella and the amount of nitrogen in the samples. Contrary to this the highest numbers of both Azotobacter and Klebsiella were most often associated with samples having a relatively high nitrogen content. In order to determine the relationship here, laboratory studies will have to be performed under strictly controlled conditions.

As the pH readings of all but a few samples were characteristically quite alkaline, it is difficult to evaluate the effect of this variable on the numbers of organisms. In comparing the resulting readings with the pH optima for the organisms we may be able to expect some inhibition, particularly of Klebsiella, at pH readings approaching 9. On certain occasions, pH readings above 9.0 were noted in both Lake Campbell and Lake Tetonkaha. Throughout the entire study, pH levels in the water layer varied from a high of 9.5 to a low of 7.25. In the bottom sediment, readings of 7.15 to 8.65 were found. No definite correlation was evident between the numbers of organisms and the above mentioned changes in pH.

CONCLUSIONS

1. The numbers of *Klebsiella* and *Azotobacter* were not found to be high enough to be significant in the over-all nitrogen balance of the lakes.

2. *Azotobacter* were found most consistently and in the highest concentrations in the bottom sediments.

3. *Klebsiella* were found most consistently and in the highest concentrations in the water samples.

4. Both genera of bacteria were found predominantly under environmental conditions which were unfavorable for nitrogen fixation.

5. The existing correlations between climatic conditions and bacterial numbers indicates that the wash-in of soil organisms is at least partially responsible for increases in numbers.

6. No definite correlations were noted between the numbers of bacteria and the total nitrogen content of the samples.

7. Further investigation, using more precisely defined sampling techniques, is necessary to explore the possibility of specialized environments which may be favorable for the growth of these organisms in natural waters.

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