ACETYLENE-ETHYLENE ASSAY FOR NITROGENASE ACTIVITY IN KEYSTONE RESERVOIR, OKLAHOMA

By

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CHAPTER I

INTRODUCTION

No known information is available on the importance of nitrogen fixation in reservoirs of the Great Plains region of the United States. Presently, over 1000 reservoirs with a total storage capacity of 360 million acre-feet exist in the United States (Frey 1967, Woodbury 1967). By the year 2000 the states on the Great Plains will require more than 410 million acre-feet of water for agricultural, municipal, and industrial needs (Dominy 1967). Because of the mandatory need for the multiple reuse of water, one must evaluate the consequences of various management strategies formulated to maximize the chemical and biological benefits in terms of water quality. The management of one criterion of water quality, nitrogen, requires an understanding of the dynamics of this element in reservoirs: its inputs, outputs, and transformations. The present study evaluates the importance of nitrogen fixation as a nitrogen input into one reservoir system.

Three methods used to determine the rate of nitrogen fixation lend themselves to <u>in situ</u> estimations: Kjeldahl, nitrogen-15 method, and acetylene reduction techniques. The Kjeldahl method, which depends upon the changes in

total nitrogen, is insufficiently accurate and sensitive. The nitrogen-15 method is expensive and time consuming. The acetylene method, however, provides a quick, inexpensive, and sensitive technique for estimation of fixation rates.

Stewart, Fitzgerald, and Burris (1967, 1968) first used the acetylene reduction technique to perform in situ studies with lake algae. They correlated daily and hourly variation in ethylene production with the nitrogen fixing algae Gloetrichia echinulata and demonstrated the effects of light on reduction. Brezonik and Harper (1969) demonstrated acetylene reduction, probably by nitrogen fixing bacteria, in the trophylytic zone of lakes. Hardy et al. (1968) performed an extensive evaluation of the acetylene reduction technique and concluded that the method was a valid measurement of nitrogen fixation. The variability of correlations of acetylene reduced to nitrogen fixed (Porter 1969) and the ubiquitous occurrence of ethylene formation (Burg 1962) may invalidate the method for precise estimations of nitrogen fixation rates, but the technique does provide a method for a quick assay of fixation potential.

Temperature (Fogg and Than-Tun 1960, Goering and Neess 1964) and light (Fogg and Stewart 1965, Fogg and Than-Tun 1960, Dugdale and Dugdale 1962, Goering and Neess 1964) affect nitrogen fixation; an increase of either factor within physiological limits increases the rate of

nitrogen fixation. Combined inorganic nitrogen, especially ammonia, inhibits nitrogen fixation in laboratory culture (Fogg 1956); but more recent in situ studies have shown simultaneous uptake of combined nitrogen and elemental nitrogen (Dugdale and Dugdale 1965, Goering and Neess 1964, Billaud 1966, 1968).

In the present study, assays for acetylene reduction were made; values of combined nitrogen, temperature, and light determined; and the possible relationships between these environmental variables and acetylene reduction evaluated in Keystone Reservoir. This work is a contribution to an understanding of this reservoir, as prior limnological investigations have not emphasized nitrogen. The work of Eley et al. (1967), Eley (1970), Ransom (1969), Spangler (1969), Kochsiek (1970), Burks (1969), and Falls (1969), however, has provided valuable data upon which this study is based.

CHAPTER II

DESCRIPTION OF AREA AND PROCEDURES

Keystone Reservoir

Keystone Reservoir was formed by impounding the Arkansas River at a point 3.2 km below the confluence of the Arkansas and Cimarron rivers. The reservoir reached power pool level (220.37 m MSL) in April, 1965. The Arkansas and Cimarron rivers form the two main arms of the reservoir extending up the rivers for 45 and 49 km, respectively. At power pool level the reservoir has a surface area of 10,648 ha (Eley 1970).

Sampling Areas

One sampling station was established over the old river channel on the Arkansas (station 2) and Cimarron (station 1) arms of the reservoir, respectively (Figure 1). The mean depth at the Cimarron station was 15 m and at the Arkansas station, 11 m.

Sampling Procedures and Field Measurements

Temperature and Secchi disk transparency were determined, water samples for chemical analyses collected, and

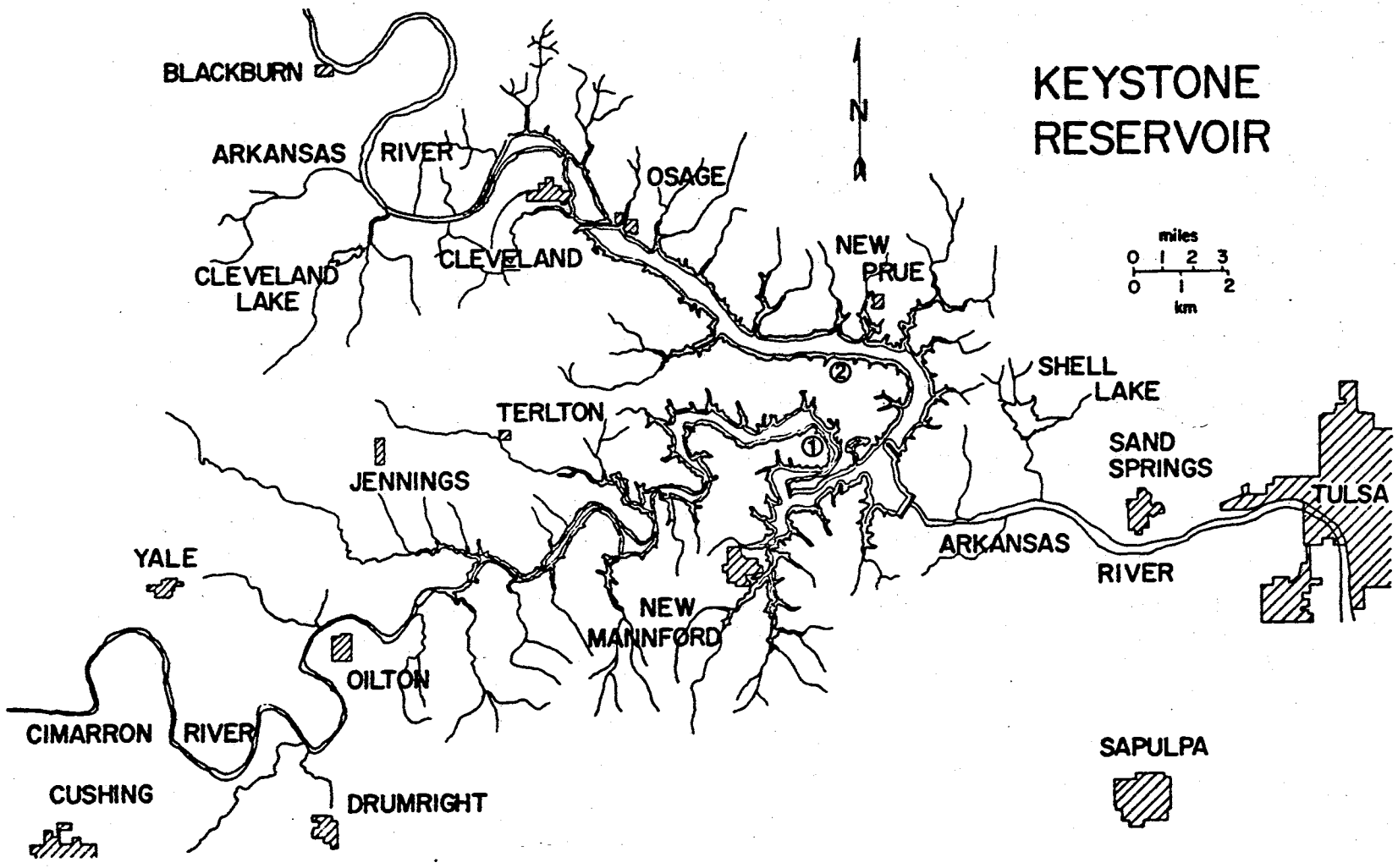

Figure 1. Keystone Reservoir, Oklahoma

acetylene reduction assays performed at two to four week intervals at both stations on Keystone Reservoir from February 12, 1969 to January 28, 1970.

Surface water samples for chemical analyses were collected with a non-toxic plastic Kemmerer bottle. Water for ammonia, nitrate, and nitrite analyses was filtered through 450 nm membrane filters, transferred into polyethylene bottles, and transported to the laboratory on dry ice where the samples were stored at -20°C until time of analyses. Particulate matter for chlorophyll analyses was retained on 5000 nm membrane filters sandwiched over one of pore size 450 nm. The sandwiched filters permitted filtering of a greater volume of turbid lake water. Particulate nitrogen in 300 to 1000 ml of lake water was retained on Reeve Angel 984 H Ultra Glass Fiber filters.

Water samples for the acetylene reduction experiments were collected with a non-toxic Kemmerer bottle, transferred into polyethylene bottles, and brought to shore for processing.

Water temperature was measured with an Applied Research Model FT 3 Hydrographic thermometer. Secchi disk transparency was determined using a 20 cm disk.

Acetylene Reduction Experiments

The particulate material in 100-500 ml aliquots of the water sample was concentrated in the field on 450 nm membrane filters by gentle filtration. The particulate

matter was gently scraped and washed into 5 ml serum bottles and the water volume in the bottles made to 2 ml using water from the same collection. Two experimental and two control bottles were prepared for each sampling station.

The samples were processed as outlined by Stewart et al. (1967) but using 2N hydrochloric acid as a fixative instead of trichloroacetic acid. The samples were incubated in situ suspended from an anchored buoy at a depth of one-half the Secchi disk transparency for 0.5 to 1.0 hours.

Laboratory Analysis

Ammonia Nitrogen

Ammonia nitrogen was determined for duplicate 50 ml subsamples by the distillation method of Riley (1953) as modified by Crowther and Large (1956) but substituting ethanol as a solvent for phenol.

Recovery (ammonia nitrogen in distillate/known amount of nitrogen distilled) was inconsistent between series of analyses (range=0.73 to 0.99, σ^2=0.13) and tended to increase within a series. The average recovery of 0.88 was used to correct all ammonia concentration estimates.

Standard curves were prepared for each day of analyses by using standard solutions of ammonium sulfate at concentrations from 0.08 to 1.00 mg at -N m^{-3}. Standards followed Beer's Law throughout the concentrations used.

Absorbancies of unknowns were converted to concentrations using equations based upon least squares linear regressions of absorbance on concentrations of standards.

The inhomogeneity of variances of subsamples between analysis did not permit pooling of variances, but the average variance among subsamples over all analyses is 128 (mg at-N m^{-3})^2. The variance of subsamples increased with an increase of their mean.

Nitrate Nitrogen

Nitrate nitrogen was determined for duplicate subsamples using the method of Mullin and Riley (1953) with the substitution of the nitrite color developer, N-(1-naphthyl)-ethylenediamine dihydrochloride, of Strickland and Parsons (1968) for the 1-napthylamine reagent.

Standard curves for nitrate analyses were prepared for each day of analyses using potassium nitrate at concentrations of 0.02 to 0.32 mg at-N m^{-3}. Absorbance of standards adhered to Beer's Law throughout the concentration range.

The average variance of subsample values over all analyses is 369 (mg at-N m^{-3})^2. The variance of subsamples increased with their mean.

Nitrite Nitrogen

Nitrite nitrogen was determined using the method of Strickland and Parsons (1968). Standard curves for nitrite

analyses were prepared using sodium nitrite in concentrations of 0.01 to 0.30 mg at-N m^{-3}. Absorbance of standards adhered to Beer's Law throughout the concentration range.

Particulate Nitrogen

Particulate nitrogen concentrations for samples collected between February 12, 1969 and August 14, 1969 were determined for duplicate samples by Kjeldahl digestion of the particulate material collected on glass filters. Particulate nitrogen for samples collected between September 5, 1969 and January 28, 1970 was determined by using a Coleman Nitrogen Analyzer. Subsample glass filters were combined, wrapped in aluminum foil, and placed in the combustion tube of the analyzer. The aluminum foil wrapping was used to prevent fusing of the glass filters to the quartz combustion tube, but the trapped nitrogen may not have been purged prior to combustion. This trapping may account for the variability of the glass filter blanks (σ^2=0.04 (mg at-N)^2) and may have resulted in overestimation of particulate nitrogen concentrations.

Ethylene

The gas phase in the acetylene reduction experimental vials was analyzed for ethylene using a F and M Model 810 gas chromatograph with dual flame hydrogen detectors. Separation of the gas components was achieved using a six foot, 1/8 inch stainless steel column packed with 80/100

mesh Poropak-R. Nitrogen was used as a carrier gas at 25 ml min^{-1}. Hydrogen flow was 50 ml min^{-1}, and air flow was 400 ml min^{-1}. Operating temperatures for the injection port and oven were ambient and for the detectors, 250°C. Each vial was subsampled twice using a 1.0 ml syringe with water displacement prior to gas removal.

Calibration of recorder response with syringe injections of a premixed gas standard containing 500 ppm ethylene in nitrogen was performed each day of analyses. The electrometer range and attenuation setting were adjusted so that calibration peak heights were 50 to 90 per cent of full deflection. Three methods were tried for defining detector response: disk-chart integrator, peak height x width at peak base, and peak height x width at half-peak height. The use of peak height x width at half-peak resulted in the greatest reduction in sum of squares (99.9 per cent) by the linear regression of concentration on response. The response curve exhibits a linear relationship at concentrations less than 250 nl ethylene with a flattening at higher concentrations. Since all experimental ethylene concentration injection estimates are less than 250 nl, the values obtained for concentrations greater than 250 nl were not used in least squares estimation of regression curves.

The ethylene concentration estimates, measured gas phase volume, and particulate nitrogen content in the experimental vials were used to estimate ethylene production rates normalized with respect to particulate nitrogen.

CHAPTER III

RESULTS AND DISCUSSION

Physicochemical Conditions and Phytoplankton Structure

Surface temperatures varied from a low of 2.1°C at station 2 to a high of 31°C at both stations (Table I). Maximum heating rate occurred during March and April with maximum cooling during October through December.

Secchi disk transparency varied from 1.85 m at station 1 to 0.13 m at station 2 (Table I). Variation of transparency is mainly attributed to reservoir inflow high in suspended clays and silts (Figure 2).

Surface nitrate nitrogen concentrations were high in the winter and spring months (400-600 mg m^{-3}) and low in the summer months (4-50 mg m^{-3}) (Table II). The reduction of nitrate nitrogen at station 1 was coincident with an increase in particulate nitrogen (Figure 3), but the spring peak in particulate nitrogen at station 2 preceded the reduction in nitrate nitrogen (Figure 4). The spring high of particulate nitrogen at both stations during spring was characterized by very few algae but much detrital material. The autumal peak of particulate nitrogen preceded the increase in nitrate at station 1 but occurred simultaneously

TABLE I

SURFACE TEMPERATURES AND SECCHI DISK TRANSPARENCIES

Date	CIMARRON STATION(1)		ARKANSAS STATION (2)	
	Temperature(^{o}C)	Transparency(m)	Temperature(^{o}C)	Transparency(m)
February 12, 1969	4.5	1.22	3.5	1.20
March 12, 1969	6.0	1.24	6.5	0.59
April 9, 1969	13.2	0.63	13.0	0.25
April 23, 1969	16.0	0.52	17.2	0.21
May 14, 1969	23.0	0.14	23.0	0.23
May 26, 1969	24.5	0.20	23.8	0.14
June 11, 1969	24.5	0.38	24.5	0.13
June 27, 1969	26.0	0.47	26.0	0.44
July 11, 1969	31.0	0.36	31.0	0.21
July 25, 1969	29.5	0.64	29.5	0.87
August 14, 1969	28.5	0.96	28.0	0.62
September 5, 1969	28.0	0.94	27.5	0.75
September 18, 1969	25.0	0.69	25.2	0.45
September 30, 1969	25.0	0.86	24.0	0.24
October 30, 1969	14.8	0.65	14.0	0.46
November 25, 1969	11.0	1.03	10.5	0.61
January 2, 1970	4.1	*	4.0	*
January 28, 1970	2.4	1.85	2.1	1.64

*Samples lost.

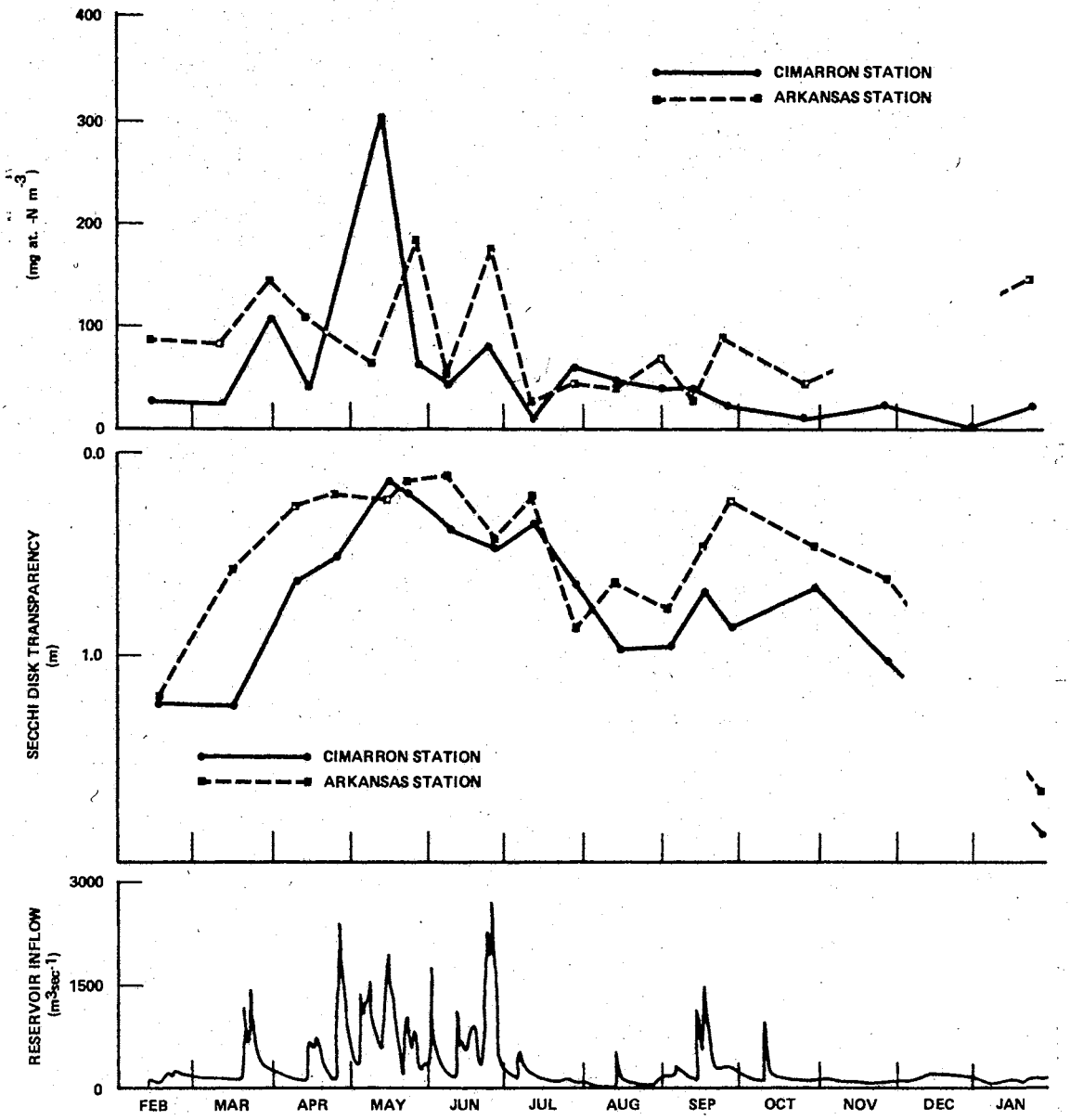

Figure 2. Secchi Disk Transparency, Ammonia Concentrations, and Reservoir Inflow 1969-1970

TABLE II

SURFACE NITROGEN CONCENTRATIONS AS mg m^{-3}

Date	CIMARRON STATION(1)				ARKANSAS STATION(2)			
	NO^3-N	NO^2-N	NH^4-N	PN*	NO^3-N	NO^2-N	NH^4-N	PN*
February 12, 1969	340	54	30	**	476	126	87	**
March 12, 1969	246	95	27	**	604	106	84	**
April 9, 1969	470	**	110	85	458	**	144	120
April 23, 1969	460	**	42	230	151	**	110	250
May 14, 1969	294	42	307	200	426	21	64	250
May 26, 1969	416	64	63	250	374	36	183	130
June 11, 1969	459	10	45	200	403	39	51	200
June 27, 1969	206	17	79	235	504	58	178	350
July 11, 1969	22	2	16	520	427	14	28	265
July 25, 1969	20	7	60	360	42	41	43	120
August 14, 1969	4	5	50	170	32	169	40	120
September 5, 1969	8	12	42	88	32	17	70	6
September 18, 1969	18	26	42	47	57	21	29	173
September 30, 1969	20	12	25	134	78	29	91	11
October 30, 1969	46	14	13	118	67	20	46	41
November 25, 1969	49	16	24	313	77	23	24	300
January 2, 1970	354	3	0	120	321	7	**	345
January 28, 1970	**	3	26	127	637	9	144	281
Average								

*PN=particulate nitrogen.
**Samples lost.

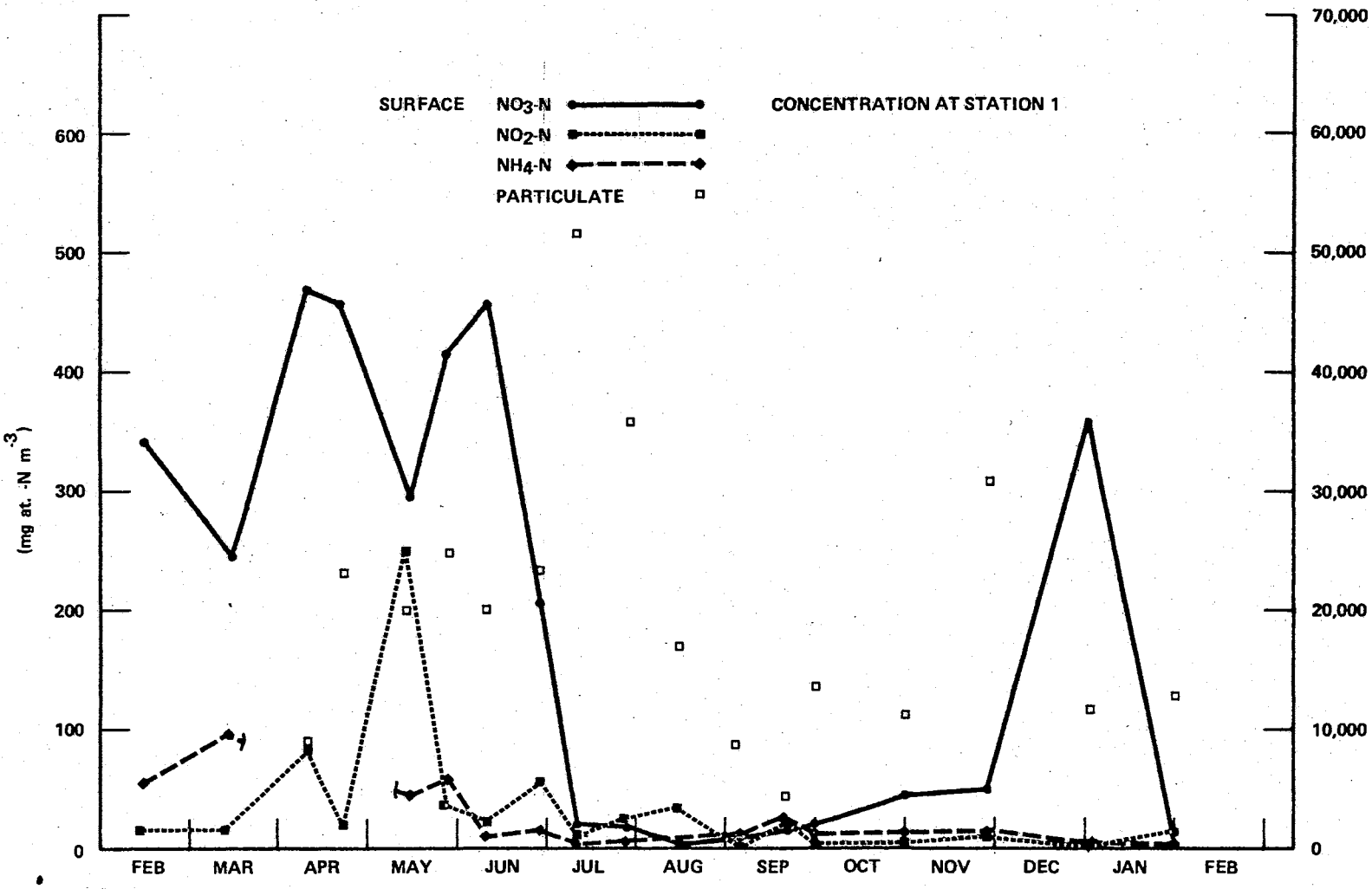

Figure 3. Surface Nitrogen Concentrations at the Cimarron Station 1969-1970

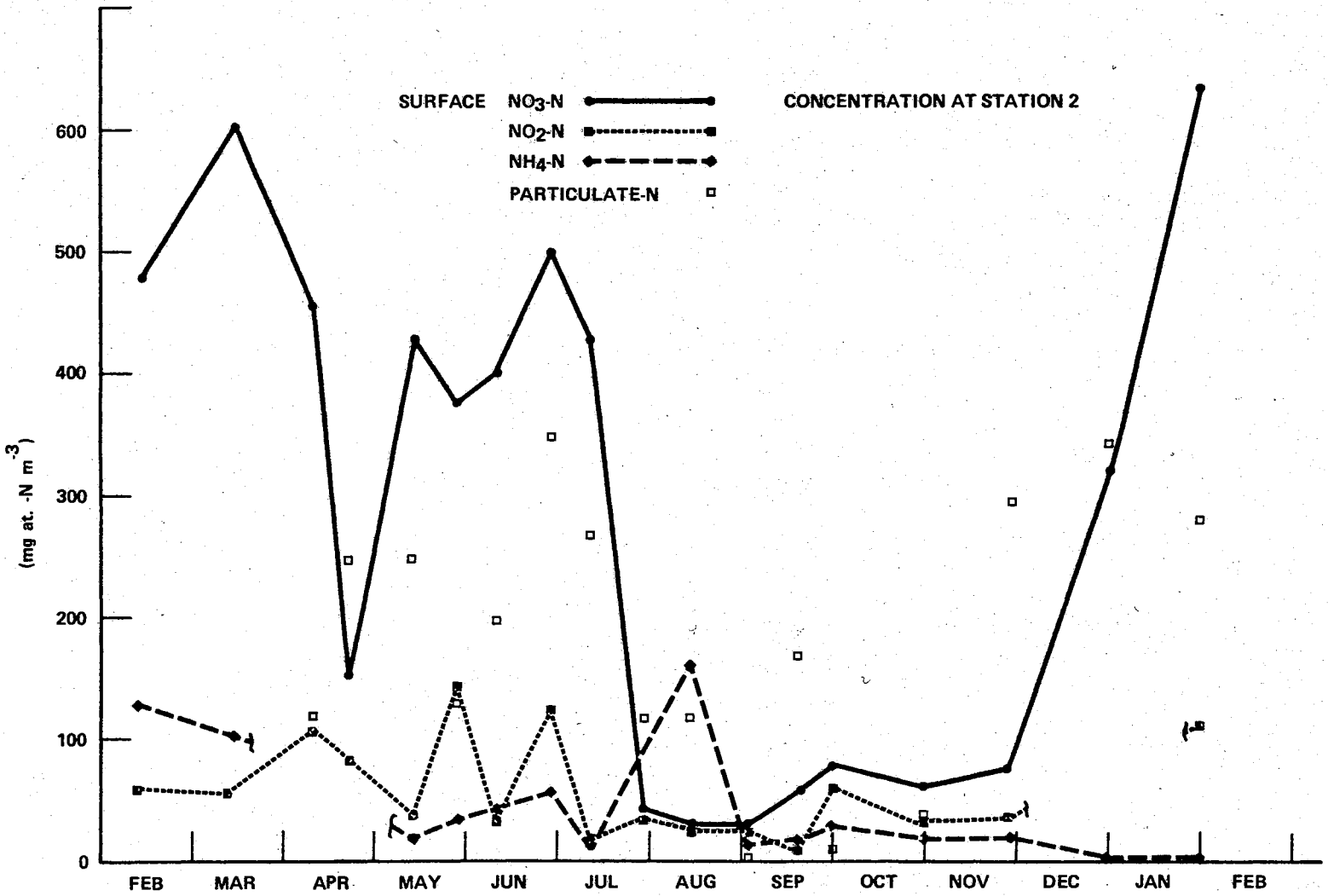

Figure 4. Surface Nitrogen Concentrations at the Arkansas Station 1969-1970

with nitrate increase at station 2. The sum of all measured nitrogen forms decreased during the time nitrate was decreasing, suggesting change of nitrogen into forms which were not measured. The nitrate concentrations at both stations remained low between July and November with rapid increased during December and January. Prochazkova (1966) suggests that reduction in nitrate can not be explained on the basis of phytoplankton uptake since nitrate may not be utilized significantly by phytoplankton in reservoirs, but he noted greater nitrate than ammonia assimilation by species groups dominated by Chlamydomonas during photosynthesis. Recent work (Dugdale and Dugdale 1965, Billaud 1966, 1968) indicates that ammonia is a dominant algal nitrogen source and that there is a very rapid turnover of ammonia. In Keystone Reservoir a rapid uptake of nitrate by phytoplankton may occur, followed by a reduction in the length of the recyling path by shunting nitrification, resulting not only in more rapid nitrogen turnover but also a decrease of the size of the nitrate pool.

Ammonia nitrogen concentrations were substantially lower than nitrate nitrogen concentrations during most of the study period. At both stations ammonia concentrations increased, but greatly fluctuated during the months March through June, remained at low levels from July to November, and increased during December and January (Table II). Mean concentrations of ammonia were greater throughout the year

at station 2 than at station 1. Increases of ammonia at both stations appear to be associated with increased reservoir inflow (Figure 2).

Nitrite concentrations at both stations were erratic with a general decrease during the study period (Table II). The mean concentrations of nitrite nitrogen was greater at station 2 than at station 1.

Microscopical examination of water samples was performed to determine the presence or absence of heterocystous blue-green algae, the organisms found responsible for significant nitrogen fixation in lakes. Rather complete genera and relative abundance lists were made for the months May through September. The unicellular Volvocales (Chlamydomonas and Trachelomonas) and Euglenales were the most abundant algae at both stations except during late July and August. Scenedesmus and Ankistrodesmus greatly dominated the non-motile Chlorophyta. The Chroococcales were the most prevalent Cyanophytes with Chroococcus and/or Gloeocapsa more abundant in early summer and Merismopedia more abundant in late summer and early autumn. Oscillatoria and/or Lyngbya were present during late June at station 2. Diatoms were scarce during early summer but rapid increases during July and August at station 1 and August at station 2 resulted in relatively large diatom populations. A small Centrales, (probably Actinocyclus) was dominant at station 1. Actinocyclus and Melosira were codominant at station 2.

The rate of water exchange and turbidity may dominate the inorganic and particulate nitrogen concentrations in Keystone Reservoir. Prochazkova (1966) found that variations in inflow and surface level influenced nitrate concentrations more than nitrate turnover within the reservoir. High flushing rates reduce the phytoplankton standing crop and selectively favor the nannoplankton (Dickman 1969). Turbidity also limits production in main stem reservoirs on the Missouri River (Benson and Cowell 1967). The rapid rate of water exchange in Keystone Reservoir (Eley 1970) may account for the observed paucity of phytoplankton during periods of high reservoir inflow, the small size of the individual phytoplankters, and the turbidity which may limit or retard the development of phytoplankton populations. The greater abundance of particulate nitrogen at station 1 may be due to lower rates of flushing and greater transparency of water in the Cimarron Arm. Detrital material was more common in the Arkansas Arm.

Acetylene Reduction Experiments

Keystone Reservoir

No significant acetylene reduction was observed in Keystone Reservoir. The absence of nitrogenase activity was probably due to the lack of heterocystous blue-green algae. Also, the high concentrations of inorganic nitrogen

present in the reservoir may prevent induction of the nitrogenase enzyme system if potential nitrogen fixers were present (Stewart 1966). Bacterial nitrogen fixation may occur in anoxic waters (Brezonik and Harper 1969) or sediments, but fixation by photosynthetic sulfur bacteria in the metalimnion is unlikely in Keystone Reservoir due to restricted light penetration. Kuznetsov (1969) suggested that bacterial nitrogen fixation is insignificant in Russian reservoirs.

Concentration Effects

Concentration of the particulate matter in samples for nitrogenase assay was necessary because of the low particulate nitrogen concentrations of the lake water. An experiment was conducted using Anabaena cylindrica cultured in combined-nitrogen free cyanophycean solution (Starr 1960) to determine the effects of concentration by filtration on ethylene production.

The Anabaena culture was divided into three aliquots. One aliquot was diluted 1:25 and a second 1:50 with sterile culture media. The third aliquot was not diluted. The initial Anabaena culture and subsequently the three aliquots were continuously stirred using magnetic stirrers.

Samples of 100 ml from each aliquot was filtered through 0.45μ membrane filters. The collected particulate material was scraped and washed into 5 ml serum bottles

and processed by purging of atmospheric gases and injecting acetylene gas as usual. The samples were incubated under florescent lighting for one hour.

The concentration experiment consisted of three dilution treatments replicated three times in a randomized block design. Each replicate was processed before beginning the next. The gas phase in the vials was analyzed for ethylene. An analysis of variance performed with the ethylene estimates showed no significant temporal differences ($P > 0.1$). No significant differences were found among treatments ($P > 0.1$).

The experiment failed to demonstrate that concentration of the particulate material affects estimates of ethylene production. Stewart et al. (1968) suggests that failure to demonstrate nitrogen fixation in Aphanizomenon flos-aquae using the nitrogen-15 method is due to the fragility of the alga and the harshness of the method. The use of Aphanizomenon in a similar experiment might indicate significant effects of concentration on ethylene production.

Storage Effects

The long storage of sample vials before ethylene analysis made necessary a study of the effects of storage on ethylene concentration. After return to the laboratory, vials used in acetylene reduction experiments were stored at $2^{\circ}C$. Bottled acetylene contains small amounts of

ethylene which results in measurable ethylene peaks on chromatograms during gas phase analysis. The initial concentration of ethylene in the control vials was assumed constant and a plot of ethylene concentration as a function of storage time was made (Figure 5). The plot indicates that the relationship between ethylene concentration and storage time is erratic with no indication of change of ethylene with time. A new bottle of acetylene was used beginning on January 2, 1970. The discussion of Hardy et al. (1968) suggesting that ethylene contamination of acetylene is dependent upon tank pressure, invalidates this approach.

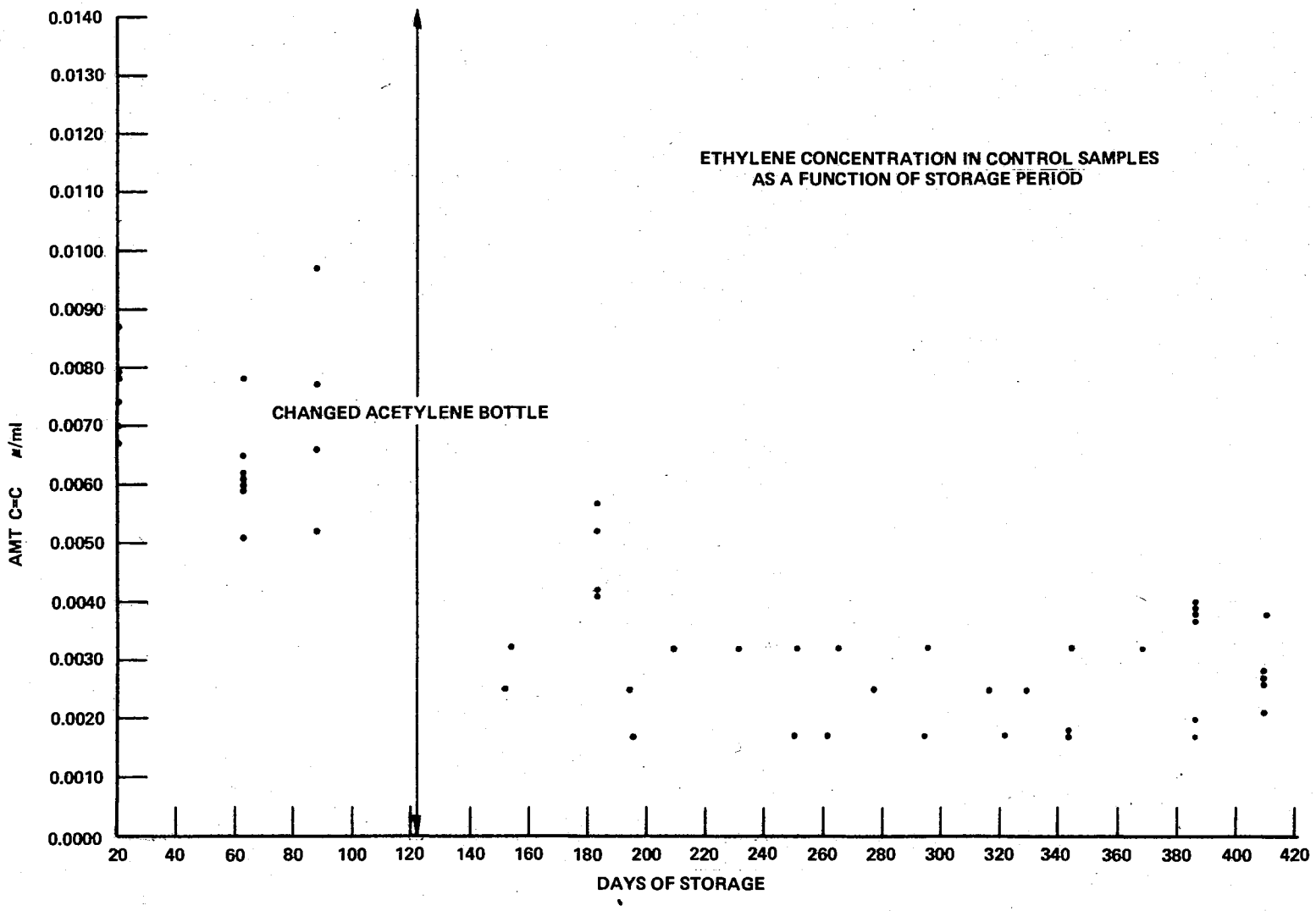

Figure 5. Ethylene in Control Samples as a Function of Storage Period

CHAPTER IV

SUMMARY

1. Two sampling stations were established on Keystone Reservoir, one each on the Arkansas and Cimarron Arms. Each station was sampled at two to four week intervals from February 12, 1969 to January 28, 1970. Assays for acetylene reduction were made; values of combined nitrogen, temperature, and light determined; and the possible relationships between these environmental variables and acetylene reduction evaluated.

2. Surface nitrate nitrogen concentrations were high during the winter and early spring (400-600 mg-N m^{-3}) and were low during summer and early autumn (4-80 mg-N m^{-3}). Ammonia and nitrite nitrogen generally decreased during the study but fluctuated widely. Increases of ammonia appear to be associated with increased reservoir inflow. Maximum particulate-nitrogen concentrations occurred during periods in which there were much detrital material and few algae.

3. Secchi disk transparency at the Cimarron station varied from 0.14 to 1.85 m and at the Arkansas station from 0.14 to 1.64 m. Greatest transparency at both

stations occurred during the winter months. Variation in transparency is mainly attributed to reservoir inflow high in suspended clays and silts.

4. The unicellular Volvocales (Chlamydomonas and Trachelomonas), Euglenales, and the diatoms Melosira and Actinocyclus were the most prevalent algae. Scenedesmus and Ankistrodesmus dominated the non-motile Chlorophyta. Chroococcales were the most prevalent Cyanophytes with Chroococcus more abundant in early summer and Merismopedia more abundant in late summer and early autumn. No heterocystous Cyanophytes were seen.

5. No significant acetylene reduction by surface particulate matter larger than 0.45μ was observed in Keystone Reservoir. The absence of nitrogenase activity was probably due to the lack of heterocystous blue-green algae. Also, the high concentrations of inorganic nitrogen present in the reservoir may prevent induction of the nitrogenase enzyme system if potential nitrogen fixers were present. Fixation by photosynthetic sulfur bacteria in the metalimnion is unlikely in Keystone Reservoir due to restricted light penetration.

6. The rapid rate of water exchange may account for the observed paucity of phytoplankton during periods of high reservoir inflow, the small size of the individual phytoplankton cells, and the turbidity which may limit or retard the development of phytoplankton populations.

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