Evaluation of the Acetylene Inhibition Technique for Estimating Denitrification in Freshwater Epilimnetic Sediments Using Nitrogen-15.

A Thesis Submitted to
the Faculty of Graduate Studies
University of Manitoba

In Partial Fulfillment
of the Requirements for the Degree of
Master of Science

by
Nancy R. Loewen

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ABSTRACT

To determine if the acetylene inhibition method is applicable to denitrification studies in freshwater sediments it was compared to a $^{15}N_2$ production assay. Denitrification rates for pure cultures of denitrifying bacteria isolated from lake sediments, sediment slurries, and intact sediment cores as estimated by both methods were compared. Differences in rates varied from total agreement between the two methods for certain isolated bacteria and sediments, to greater than a 90% underestimation by the acetylene inhibition method. In intact sediment core incubations denitrification rates in the presence of acetylene were consistently lower than in its absence. The indisciminate use of the acetylene inhibition method for estimating denitrification rates in freshwater sediments is discouraged.

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INTRODUCTION

INTRODUCTION

Denitrification is defined as the dissimilatory reduction of nitrate or nitrite to gaseous nitrogen endproducts (N_2, N_2O). In the reaction nitrate and nitrite serve as the terminal electron acceptors in the respiratory generation of adenosine triphosphate (ATP). The reaction is generally considered to be an anaerobic process but has been reported for denitrifying cultures grown in the presence of up to 5 % O_2 (Delwiche and Bryan 1976). The sequence of events in the reaction has not yet been fully clarified. The currently favoured sequence of intermediates involved is thought to be :

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

Denitrification plays an significant role in the global nitrogen cycle. The reaction results in the loss of fixed nitrogen to the atmosphere as gaseous nitrogen. In agricultural practices caution is taken to minimize these nitrogen losses and increase the effiencency of nitrogen fertilizer applications, whereas in waste management the process is beneficial for the removal of nitrogen from high-nitrogen waste materials. In lake systems nitrate concentrations can influence the total lake productivity. These concentrations are the net result of nitrfication, immobilization and denitrification. Recently denitrification in lake sediments has gained recognition as a potential source of neutralization in lakes with low buffering capacity receiving acid precipitation. Acid rain results in increased loading of nitrate and sulfate ions as well as hydrogen ions to the surface waters of

lakes. In lakes low in natural alkalinity , such as those located along the Precambrian Shield, it is thought that the microbial reduction of nitrate and sulfate ions could result in at least transient increases in alkalinity (Kelly et al. 1981; Kelly et al. in press). In the Experimental Lakes Area of Northern Ontario, this concept is being studied in Lake 302 whose double basin is divided by a sea curtain. Since midsummer 1982, the southern basin has received sulfuric acid additions to its surface water and the northern basin nitric acid. The purpose of the experiment is to study the relative acidification differences of nitric and sulfuric acids and the contribution of nitrate and sulfate reducing bacteria to lake buffering.

My first problem in experimental design for this study was to decide on what quantitative method for "in situ" measurement of denitrification would be appropriate for Lake 302 sediments. Methods based on the observation that acetylene inhibited the reduction of nitrous oxide to nitrogen gas (Fedorova et al. 1973; Balderstone et al. 1976; Yoshinari and Knowles 1976) have been used for estimating rates in soils (Klemedtsson et al. 1977; Lensi and Chalamet 1982; Smith et al. 1978), marine sediments (Sorensen 1978a; Haines et al. 1981), and freshwater sediments (Chan and Knowles 1979; Knowles 1979). The validity of the method in freshwater sediments, however, has not been rigorously verified. The purpose of this study is to determine if the method is reliable for denitrification measurements in epilimnetic sediment. Denitrification rates were measured by the acetylene

inhibition method and compared to rates determined using N-15 labelled nitrate.

Comparisons were done on pure cultures isolated from lake sediment and on epilimentic sediment slurries. Concurrently, a core incubation technique was developed for estimating denitrification in freshwater sediments.

A major portion of the research for this thesis was performed in the Experimental Lakes Area (ELA) in northwestern Ontario (Johnson and Vallentyne, 1971) where initial research concerned nutrient cycling and eutrophication (Schindler et al. 1973). Presently the response of aquatic ecosystems to acidification (Schindler et al., 1980; Schindler and Turner, 1982) is being examined.

HISTORICAL

HISTORICAL

Davy (Delwiche 1965) reported on the evolution of dinitrogen in organic decomposition as early as 1841. In an intensive study in the 1880's, Gayton and Dupeit demonstrated that nitrous oxide as well as nitrogen gas was evolved and at the same time pointed out that nitrate was serving as an electron acceptor for the oxidation of organic matter by organisms.

Interest in denitrification has increased since the realization that it could result in the loss of fixed nitrogen from various ecosystems. This is of particular importance in fertilizer applications in agriculture. Concern over the deleterious effect of N_2O to the atmospheric ozone layer has also drawn attention to denitrification and the environmental factors regulating it. At present it is generally accepted N_2O release to the atmosphere, via denitrification, does not pose a threat to the ozone layer. However it is speculated that as the use of nitrogen fertilizers increase in agricultural practices, N_2O generation could be significant enough to cause alarm (Delwiche 1981). Because the reaction can be deliberately used to remove nitrate from systems wherein it would be considered a pollutant, it may be a valuable tool in combating problems of eutrophication, or acidification of lakes due to increased deposition of nitrate and sulfate ions associated with acid rain. The microbial reduction of nitrate and sulfate deposited in the form of acid rain is thought to have a potential neutralizing effect (Kelly et al. 1981).

Denitrification is defined as the dissimilatory reduction of nitrate or nitrite coupled to the respiratory generation of ATP and the evolution of gaseous products (nitrous oxide and dinitrogen) under low oxygen tensions (< 5.0% O_2 saturation) or in the complete absence of oxygen (Delwiche and Bryan 1976; Knowles 1981a and 1982; Carlson and Ingraham 1983). The reaction could be described as a cascade of anaerobic processes, the sequence of intermediates currently thought to be:

nitrate -> nitrite -> nitric oxide -> nitrous oxide -> dinitrogen

NO_3^- NO_2^- NO N_2O N_2

(Payne 1976; Payne and Balderston 1978). Each step along the pathway is catalyzed by a complex enzyme system (Fewson and Nicholas 1961; Payne et al. 1971; Radcliffe and Nicholas 1970).

The first step in the denitrification sequence, the reduction of nitrate to the free intermediate nitrite , is mediated by the enzyme nitrate reductase. This reaction is the most studied of the sequence (Payne 1973; Stouthamer 1976). Electrons are transported by a b-type cytochrome to nitrate which serves as an electron acceptor (Bryan 1981).

Activity of the enzyme is regulated by oxygen and nitrate but the mode of action is not understood. As little as 0.35 % O_2 repressed denitrification in continuous cultures of Azospirillium brasilense while enzyme activity was derepressed when the O_2 concentration was

reduced to 5% in cultures of Pseudomonas denitrificans and 1.3% for Thiobacillus denitrificans (Bryan 1981).

The effect of nitrate on enzyme synthesis is complicated. In Bacillus sterothermophilus synthesis of nitrate reductase occurs only in the presence of nitrate and the amount of enzyme synthesized was found to be directly proportional to the nitrate concentration (Downey and Numer 1967). In E. coli nitrate reductase activity is derepressed by anoxia alone; however, in the presence of nitrate the synthesis is initiated before anoxia is reached Bryan 1981).

Nitrite reductase has not been as well studied as nitrate reductase. Unlike nitrate reductase where the enzyme is similar among organisms capable of dissimilatory nitrate reduction, there are two distinct types of enzymes catalyzing the reduction of nitrite. One is a copper containing nonheme protein and the other is a noncopper containing protein in which c- and d-type cytochromes are involved in the transfer of electrons. The physiological endproduct of nitrite reduction is still an issue for debate. It has not been confirmed whether NO or N_2O is the endproduct. In pure culture studies Barbaree and Payne (1967) and Garber and Hollocher (1981) were able to detect NO as a free intermediate in the denitrification pathway in certain denitrifying bacteria. In lake sediments , Chen and his co-workers (1972) were unable to identify NO as an intermediate in denitrification. It is possible that these latter results arise from the fact that the sediments are composed of a mixed population of bacteria including certain strains which could reduce the NO as rapidly

as it was produced thus preventing its accumulation.

Garber and Hollocher (1981) suggest that the existence of metabolic diversity in the pathway among organisms may account for the conflicting findings on the participation of NO in denitrification. They found that free NO seems not to be an obligatory intermediate for all denitrifiers but does exist as an enzyme bound intermediate thus avoiding detection because it does not escape from the cell.

On the other hand, nitrous oxide has been well established as a free obligatory intermediate (Allen and van Neil 1952; Blackmer and Bremner 1978; Boogerd et al. 1980). Koike and Hattori (1975) have suggested that nitrous oxide escapes from the cell as a means of discharging surplus energy under conditions where nitrate concentrations are high since both nitrate and nitrous oxide compete as electron acceptors but nitrate reduction has higher energy yields. Very little is known about the enzyme involved in the reduction of N_2O to N_2. Matsubara (1975) found evidence for the involvement of type b and c cytochromes in denitrifying species of Alcaligens and Achromobacter. Nitrous oxide reduction in Pseudomonas denitrificans is inhibited by $CuSO_4$ and monoiodocetate suggesting involvement of heavy metals and thiol groups in the N_2O reductase (Matsubara and Mori 1968).

Regulation of the enzyme systems is complex and not fully understood. It is controlled by factors such as oxygen and nitrate concentrations, pH and temperature. The reader is referred to the extensive reviews on the subject that have recently been published

(Payne 1973, 1976, 1981; Delwiche and Bryan 1976; Firestone 1981; Jeter and Ingraham 1981; Knowles 1981a,b, 1982).

Actual rates and the exact conditions for denitrification in various areas of the biosphere are still unknown. In order to quantify the role of denitrification in the nitrogen cycle reliable methodologies are required.

Indirect methods have been adapted for studying denitrification in a wide variety of ecosystems. Lack of direct measurements of natural denitrification for undisturbed systems is partially due to the high ambient concentration of nitrogen gas making it difficult to detect small increments of nitrogen in systems as a measure of denitrification.

Early measurements of denitrification were based on mass balance approaches where nitrogen losses, not accounted for within the system were ascribed to denitrification (Bremner and Shaw 1958 a, b). This method is restricted by the problems of accurately monitoring all nitrogen inputs and outputs which include leaching of soils or sediments, ground water influences and nitrogen uptake by the biota present.

Indirect measurements based on the disappearance of added nitrate, nitrite, or nitrous oxide under anaerobic conditions (Konrad et al. 1970; Keeney et al. 1971; Chen et al. 1972) are considered unreliable. The assumption that nitrate and nitrite are reduced exclusively to nitrous oxide and nitrogen gas could result in an overestimation of

denitrification as significant portions of nitrate have been reported to be reduced to ammonium under certain conditions (Hasan and Hall 1975; Buresh and Patrick 1978; 1981; Koike and Hattori 1978a; Tiedje et al. 1981).

Direct measurements of nitrous oxide and nitrogen gas in closed systems where the atmospheric concentration of dinitrogen has been reduced by flushing with an inert gas such as helium, have provided estimates of the rate of denitrification in soils (Arnold 1954; Hauck and Melsted 1956; McGarity and Hauck 1969; McGarity and Rajaratnan 1973), in freshwater sediments (Seitzinger et al. 1980), and marsh sediments (Kaplan et al. 1977, 1979). The replacement of the atmosphere above a denitrifying system with an inert gas has been critized because by doing so the reducing conditions within the closed system may increase, possibly stimulating denitrification and leading to an overestimation of activity. To eliminate this problem, Seitzinger et al. (1980), replaced the headspace above the sediments to be measured fo denitrification activity with a mixture of 21 % O_2 and 312 ppm CO_2 in a balance of He.

Using a Warburg respirometer, Bartlett and his co-workers (1979) were able to measure denitrification in wetland soils by performing a mass balance measuring N_2, N_2O, CO_2, total Kjeldahl nitrogen, nitrate, and ammonium. They concluded that 90% of the supplemented nitrate could be removed via denitrification. These results demonstrate the potential of denitrification but are difficult to relate to field conditions where numerous enviromental parameters, which cannot be

simulated in the laboratory, influence the process.

Nitrogen/argon ratio measurements in sediment pore waters have been used in an attempt to quantify denitrification (Richards and Benson 1961; Nishio et al. 1981; Barnes et al. 1975). The underlying assumption is that the N_2/Ar ratio should remain constant if the increase in N_2 is due solely to partial pressure differences, therefore any increase in the ratio above the value expected from solubility and atmospheric pressure implies an increase in nitrogen, presumably via denitrification. The method is useful for assaying the presence of activity but quantification of rates is difficult as excess nitrogen in the sediments is rapidly lost to the overlying waters due to physical and biological disturbances of the near surface sediments resulting in an underestimate of denitrification rates by the N_2/Ar ratio (Nishio et al. 1982).

Recent applications of radioisotope tracer techinques in pure culture studies (Sorensen et al. 1980), and soils (Gersberg et al. 1976; Tiedje 1978; Tiedje et al. 1979) have been convincing. The longest lived radioisotope of nitrogen is N-13 and its half life is only 10 minutes. This in addition to the requirement for cyclotron facilities for its production restricts its application for field studies.

Nitrogen-15, a stable isotope of nitrogen with a natural abundance of 0.367 atoms % ^{15}N, provides a means of directly measuring fluxes of labelled nitrogen in an closed system without the concern for

contamination by atmospheric nitrogen. Wijler and Dlewiche (1954) performed early work using ^{15}N to study denitrification and since then ^{15}N has been applied to studies of pure cultures (Il'ina et al. 1978), soils (Rolston et al. 1976; Oren and Blackburn 1979; Cho et al. 1979; Siegel et al. 1982), freshwater systems (Goering and Dugdale 1966; Keeney et al. 1971; Chen et al. 1972; Chan and Campbell 1980), and marine sediments (Koike and Hattori 1978 a, b; Sorensen 1978a; Nishio et al. 1983). The method has been criticized due to the need to add nitrate to the system which may lead to increased activity. Although this may be so in some cases, the addition of labelled nitrate allows the production of other products of dissimilatory nitrate reduction to be identified. The contribution of nitrate reduction to dinitrogen gas and nitrate reduction to ammonium can be determined simultaneously.

Relatively recently Fedorova et al. (1973) during the developement of gas exchange assays for its possible use in detection of extraterrestrial life, noted that the reduction of nitrous oxide by soil bacterium was inhibited in the presence of acetylene. These findings were confirmed in pure cultures of Pseudomonas perfectomarinus , Pseudomonas aeruginosa , and Micrococcus denitrificans (Yoshinari and Knowles 1976), and in sediments (Balderston et al. 1976). Acetylene concentrations as low as 0.01 atm were found to cause stoichiometric accumulation of N_2O in denitrifying cultures. The acetylene inhibition technique provides a means of quantifying denitrification by following the progressive accumulation of nitrous oxide. Nitrous oxide can be accurately measured by gas chromotography and because of its low

atmospheric concentration small increases in nitrous oxide can be detected in the presence of air.

The discovery of the inhibitory effect of acetylene has lead to its widespread use in denitrification studies in soils (Smith et al. 1978; Ryden et al. 1979; Yeomans and Beauchamp 1978; Watanabe and DeGuzman 1980; Ryden 1982), and in sediments (Sorensen 1978 a, b; Chan and Knowles 1979; Van Raalte and Patriquin 1979; Sorensen et al. 1979; Haines et al. 1981; Kaspar 1982). Because the method does not require nitrate additions and the analysis of nitrous oxide is relatively simple and quick, the method is appealing. Caution is warranted against the method as acetylene is known to inhibit nitrification, methane production and oxidation, and nitrogen fixation (Walter et al. 1979 ; Hynes and Knowles 1978 ; Knowles 1979). It appears to be a general poison for microbial communities and as such, its presence could alter the total microbial community structure in the system under study.

There is only a limited amount of evidence to demonstrate that the rate and extent of denitrification measured in the presence and absence of acetylene is equivalent (Ryden et al. 1979). The purpose of this study is to evaluate the acetylene inhibition technique for quantifying denitrification in eplimnetic sediments using a ^{15}N tracer technique.

GENERAL METHODS

GENERAL METHODS

Description of Lakes Studied

Epilimnetic sediments were collected from four lakes located in the Experimental Lakes Area, Northwestern Ontario. The region is situated on the precambrian shield and consequently all of the lakes possess waters of realatively low alkalinity (Johnson and Vallentyne, 1971). Morphometric maps for the four lakes involved in this study are shown in figures 1 and 2. Sediments were collected from 3-5 m depths in each of the lakes.

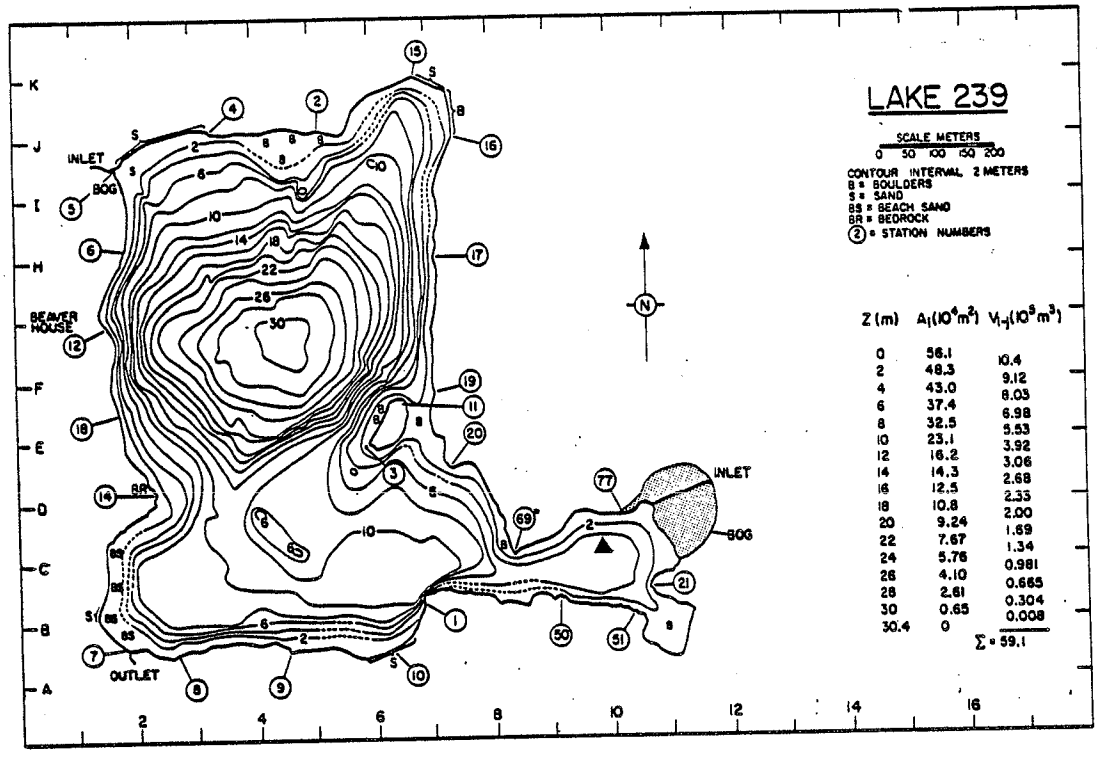
Lake 239 is a stratified lake which has not been experimentally manipulated but its watershed has suffered the destructive effects of a windstorm in 1973 and forest fires which occurred in 1974 and 1980 (Schindler et. al 1981). During the sampling period involved in this study the nitrate concentration in the epilimnion declined rapidly from 4.5 umoles NO_3^--N/L in early May to 0.1 umoles NO_3^--N/L by June. The lake pH was in the range of 6.5-7.0.

Lake 114 is a small nonstratified lake. The lake has received additions of sulfuric acid since 1979 (Schindler, 1980). The nitrate concentration in the lake at the time of sampling was below the detection limits (0.07 umol NO_3^--N/L) and the pH was at pH 5.9.

Lake 302 is a stratified double basin lake. The basins are separated by a plastic sea curtain. From 1973-1975 the lake was used in a fertilization experiment in which the north basin received

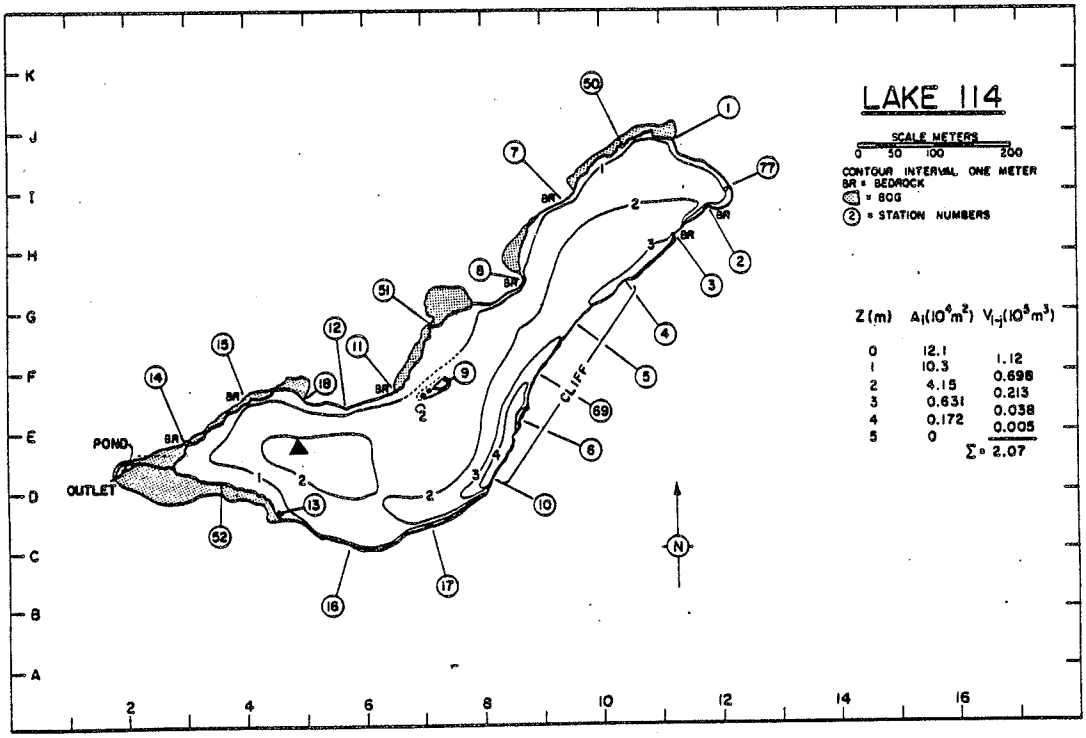
Figure 1a. Morphometric map of Lake 239 (Brunskill and Schindler 1971). The sampling location is indicated by (▲). (3 M)

1b. Morphometric map of Lake 114 (Brunskill and Schindler 1971). The sampling location is indicated by (▲). (2 M)



Z (m)	$A_i (10^4 m^2)$	$V_{i-j} (10^3 m^3)$
0	56.1	10.4
2	48.3	9.12
4	43.0	8.03
6	37.4	6.98
8	32.5	5.53
10	23.1	3.92
12	16.2	3.06
14	14.3	2.68
16	12.5	2.33
18	10.8	2.00
20	9.24	1.69
22	7.67	1.34
24	5.76	0.981
26	4.10	0.665
28	2.61	0.304
30	0.65	0.008
30.4	0	

$\Sigma = 59.1$

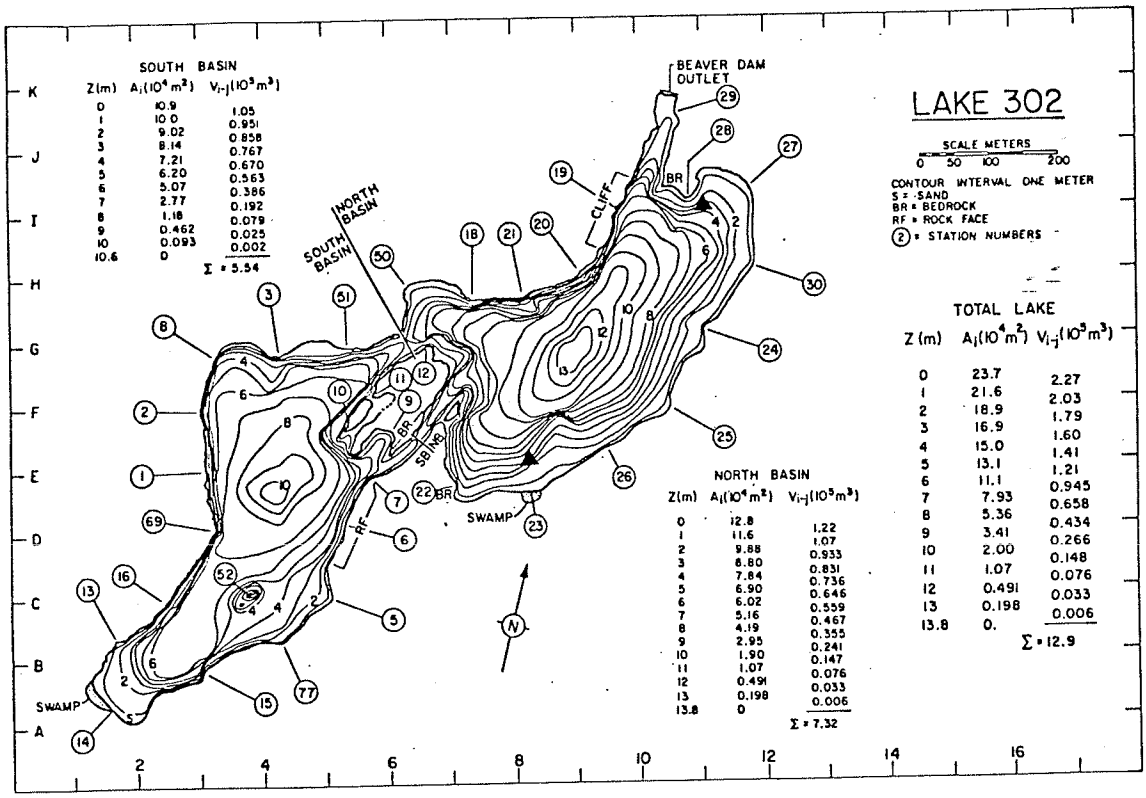


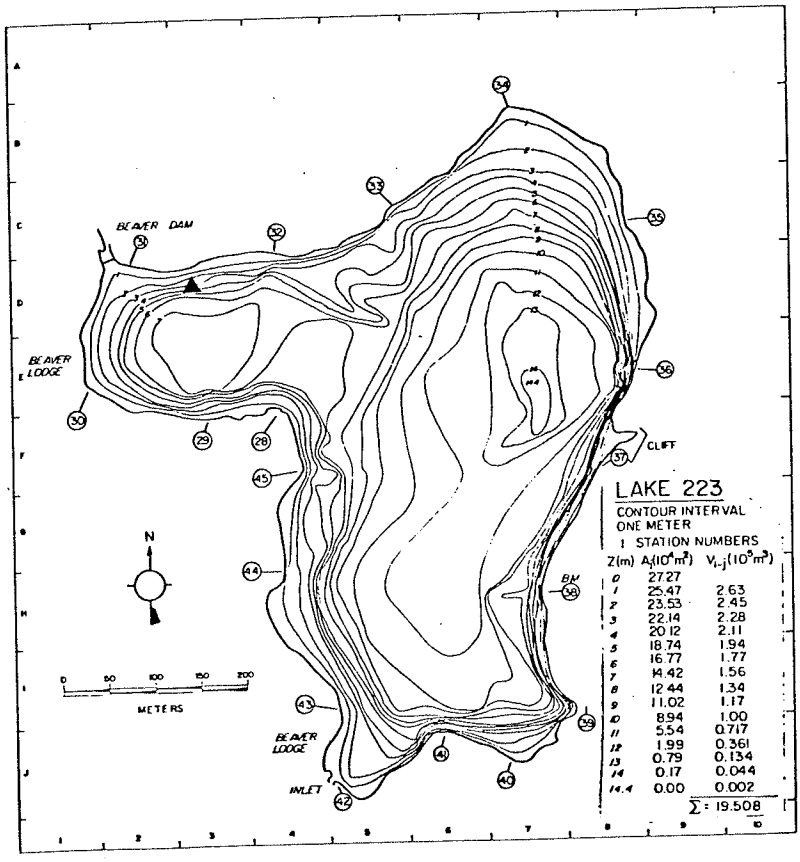
Z (m)	$A_i (10^4 m^2)$	$V_{i-j} (10^3 m^3)$
0	12.1	
1	10.3	1.12
2	4.15	0.698
3	0.631	0.213
4	0.172	0.038
5	0	0.005

$\Sigma = 2.07$

Figure 2a. Morphometric map of Lake 302 (Brunskill and Schindler 1971). The sampling locations are indicated by (▲). (3 and 5 M)

2b. Morphometric map of Lake 223 (Brunskill and Schindler 1971). The sampling location is indicated by (▲). (4 M)


LAKE 302
SCALE METERS
0 50 100 200
CONTOUR INTERVAL ONE METER
S = SAND
BR = BEDROCK
RF = ROCK FACE
(2) = STATION NUMBERS
SOUTH BASIN
Z(m) Ai(10⁴m²) Vi-j(10³m³)
0 10.9 1.05
1 10.0 0.951
2 9.02 0.858
3 8.14 0.767
4 7.21 0.670
5 6.20 0.563
6 5.07 0.386
7 2.77 0.192
8 1.18 0.079
9 0.462 0.025
10 0.093 0.002
10.6 0 0
Σ = 5.54
NORTH BASIN
Z(m) Ai(10⁴m²) Vi-j(10³m³)
0 12.8 1.22
1 11.6 1.07
2 9.88 0.933
3 8.80 0.831
4 7.84 0.736
5 6.90 0.646
6 6.02 0.559
7 5.16 0.467
8 4.19 0.355
9 2.95 0.241
10 1.90 0.147
11 1.07 0.076
12 0.491 0.033
13 0.198 0.006
13.8 0 0
Σ = 7.32
TOTAL LAKE
Z(m) Ai(10⁴m²) Vi-j(10³m³)
0 23.7 2.27
1 21.6 2.03
2 18.9 1.79
3 16.9 1.60
4 15.0 1.41
5 13.1 1.21
6 11.1 0.945
7 7.93 0.658
8 5.36 0.434
9 3.41 0.266
10 2.00 0.148
11 1.07 0.076
12 0.491 0.033
13 0.198 0.006
13.8 0. 0
Σ = 12.9
BEAVER DAM OUTLET
NORTH BASIN
SOUTH BASIN
CLIFF
BR
SAND
RF
SWAMP
N


LAKE 223
CONTOUR INTERVAL ONE METER
STATION NUMBERS
Z(m) Ai(10⁴m²) Vi-j(10⁵m³)
0 27.27
1 25.47 2.63
2 23.53 2.45
3 22.14 2.28
4 20.12 2.11
5 18.74 1.94
6 16.77 1.77
7 14.42 1.56
8 12.44 1.34
9 11.02 1.17
10 8.94 1.00
11 5.54 0.717
12 1.99 0.361
13 0.79 0.134
14 0.17 0.044
14.4 0.00 0.002
Σ = 19.508
BEAVER DAM
BEAVER LODGE
BEAVER LODGE
INLET
CLIFF
BM
N
METERS

additions of carbon, nitrogen and phosphorus to the hypolimnion while the south basin was left unaltered (Schindler, 1975). At present the lake is the subject of an acidification study. The south basin is being acidified with sulfuric acid and the north basin with nitric acid. Additions commenced in midsummer of 1982. Prior to the nitrate additions to the north basin, nitrate concentrations in the epilimnion of this basin during sampling for this study were below detection limits (<0.07 umol NO_3^--N/L). After additions, the nitrate concentration fluctuated between 15-30 umoles NO_3^--N/L. The pH of the basin remained unchanged at pH 6.4 - 6.7.

Lake 223 is a stratified lake which has received sulfuric acid additions since 1976 (Schindler, 1980; Schindler and Turner, 1982). Since that time the pH of the lake has dropped from an initial pH of 6.6 to 5.2 at the time of sampling. Epilimnetic nitrate concentrations during the sampling period were less than 0.07 umoles NO_3^--N/L.

A more detailed discription of the lakes is given by Brunskill and Schindler (1971).

Sampling

A brief discription of the incubation vessels will be given here and a more precise discription will accompany each of the relevant sections. In general the incubation vessels consist of sealed containers with a liguid phase (porewater or culture medium) and a gas

phase (N_2 or Ar/CH_4). At regular time intervals the two phases were equilibrated by shaking the vessel vigorously for one to three minutes. A 5 ml subsample was withdrawn anaerobically from the headspace of the incubation vessel. The subsample was then analyzed. Samples taken from incubation vessels receiving C_2H_2 additions were analyzed for N_2O within four hours and samples from vessels in which C_2H_2 was omitted and $^{15}N_2$ production was followed were analysed within one to two weeks.

Analyses

N_2O Determinations

Nitrous oxide concentrations were determined using a Varian 3700 gas chromatograph equipped with a Ni-63 (8 mCi) electron capture detector operated at 350 C. Separation of the gases in the samples was achieved using a Porapak Q glass column (2.44 m long ; O.D. 0.635 cm) controlled at 60 C with an injection port temperature of 100 C. The carrier gas used was a mixture of 5 % CH_4 in a balance of Ar, with a flow of 40 ml/min.

The instrument was calibrated with N_2O standards ranging in concentration from 0.113 to 1.67 ppm obtained from Scott Environmental. Higher N_2O concentration standards were prepared using a mixture of hospital grade N_2O in a balance of ultra pure N_2.

The response of the detector was determined to be linear for the range of N_2O concentrations being measured. Therefore, the concentration of N_2O in a sample could be determined from the peak height response of the recorder.

One or two millitres of the sample headspace was injected through a heated injection port leading to the Porapak Q column. The concentration of nitrous oxide was calculated using the following set of equations :

$$\frac{A}{\text{standard pk ht}} = \frac{B}{\text{sample pk ht}} \qquad (1)$$

where A= concentration of N_2O in gas standard (ppm)

B= concentration of N_2O in sample (ppm)

pk ht = peak height (chart units)

therefore :

$$B = \frac{A \times \text{sample pk ht}}{\text{standard pk ht}} \qquad (2)$$

Since one mole of gas at STP occupies 22.4 litres, the concentration of nitrous oxide in the gas sample can be expressed as moles N_2O/L at STP (B') given in the equation :

$$B' = Bx10^{-6} \times P/760 \text{ mm} \times 273 \text{ K/T} \div 22.4 \text{ L/moles} \quad (3)$$
$$= \text{moles } N_2O/L$$

where P = pressure (mm)

T = equilibration temperature (K)

This value gives the concentration (moles/L) of nitrous oxide in the subsample after equilibration of the liquid and gas phases in the incubation vessel. To determine the total concentration of N_2O generated by the denitrifying system it is necessary to determine the number of moles of N_2O in the gas phase and the number of moles of N_2O in the liquid phase and add the two together to determine the final mass of N_2O present. This was calculated using the following set of equations :

$$\text{moles } N_2O \text{ liquid phase} = B'(\text{moles/L}) \times \alpha \times Vl \quad (4)$$

where B' was determined in eq. 3

α = solubility coefficient (mL N_2O/mL H_2O)

Vl = volume of liquid phase (L)

$$\text{moles } N_2O \text{ gas phase} = B'(\text{moles/L}) \times Vg \qquad (5)$$

where Vg = volume of gas phase (L)

The total mass of N_2O generated is determined by combining equations 4 and 5 to yield :

$$\text{total moles } N_2O = B'[(\alpha \times Vl) + (Vg)] \qquad (6)$$

Mass Spectrometric Analysis for $^{15}N_2$

After equilibration of the gas and liquid phases, subsamples, obtained from the headspace of the incubation vessels not supplemented with acetylene, were analyzed for $^{15}N_2$ content using an Isotope Micromass 602E mass spectrometer. A two millilitre volume of the subsample was injected through a rubber septum into a sidearm emerging from a three-way stopcock which joined with the vacuum line and mass spectrometer. The sidearm was immersed in a liquid nitrogen bath to trap contaminating gases (eg. O_2, CO_2, N_2O) which could interfere with the analysis. The gas sample was then introduced into a gas inlet reservoir where the pressure of the sample could be reduced to 0.5 to 1.0×10^{-7} Torr to accommodate the requirements of the mass spectrometer. The sample was then passed into the ion source where the nitrogen molecules are ionized by bombardment with electrons thus becoming accelerated. The movement of the gas through the system is controlled by a series of on/off valves (Figure 3). As the molecules become accelerated the detected ion beams are individually amplified and the signal fed to a recorder which registers the amplified discharge currents at the masses scanned (masses 28 to 30). A trace of a typical output is shown in Figure 4.

Masses 28 ($^{14}N^{14}N$), 29 ($^{14}N^{15}N$), and 30 ($^{15}N^{15}N$)were scanned. The peak heights of each are proportional to the relative quantity of each of the three types of molecules. The quantity of $^{15}N_2$ evolved from a sample was calculated from the excess over time zero of ^{15}N as determined from the peak heights.

Figure 3. Mass spectrometer inlet and vacuum system.

S1 - S5 valves controlling movement of sample to the mass spectrometer
R1 - R5 valves controlling movement of reference gas to the mass spectrometer
V1 and V2 valves controlling vacuum pumps

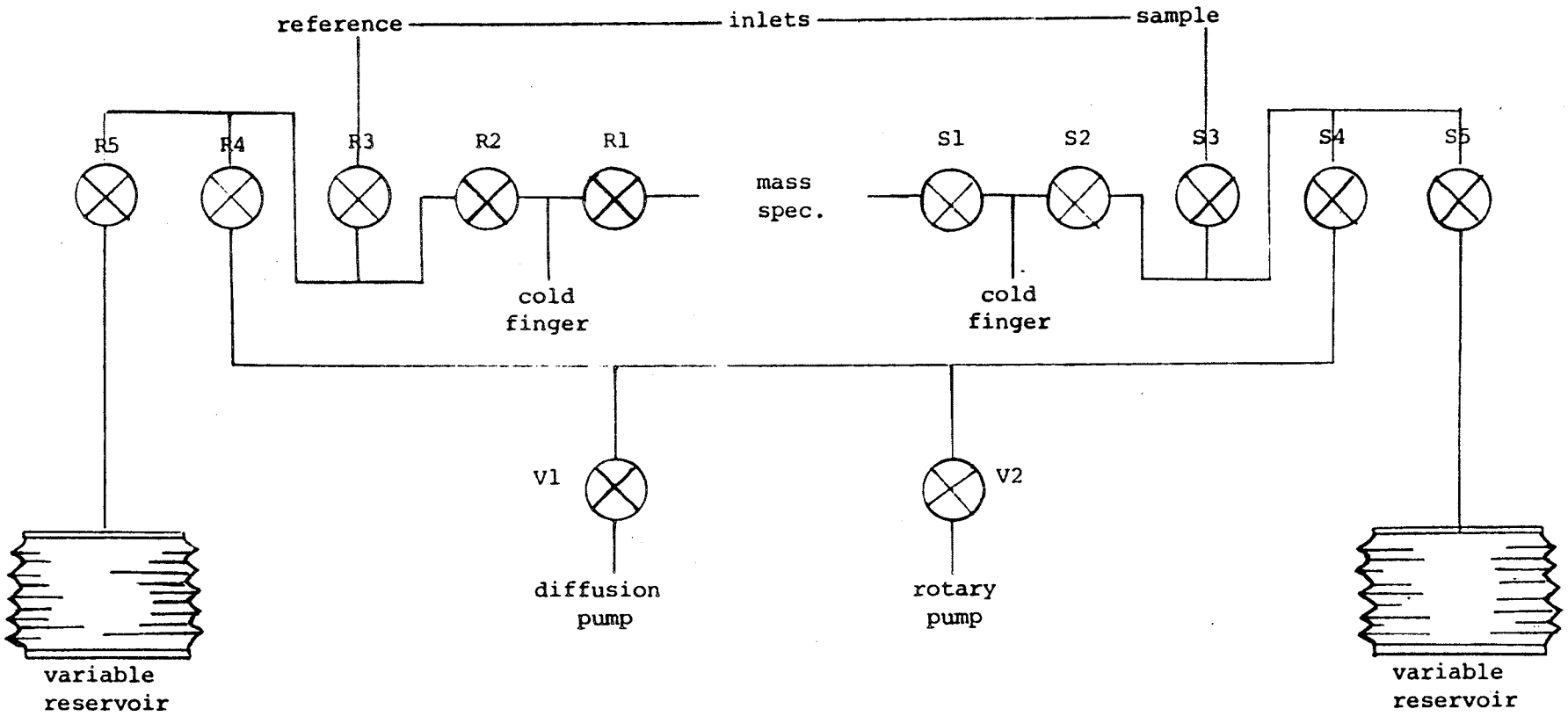
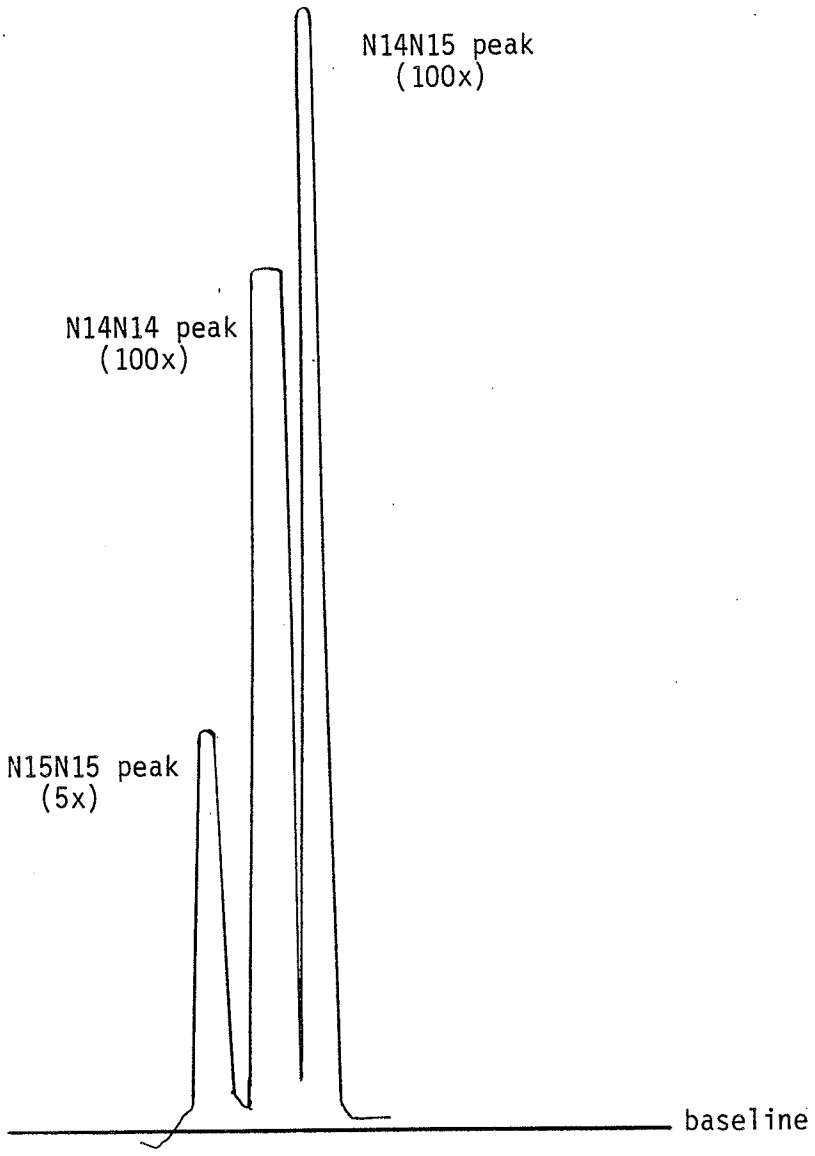

reference
inlets
sample
R5
R4
R3
R2
R1
S1
S2
S3
S4
S5
mass
spec.
cold
finger
cold
finger
V1
V2
diffusion
pump
rotary
pump
variable
reservoir
variable
reservoir

Figure 4. Trace of a typical output obtained on analysis of a time zero gas sample. A shift in attenuation from 5x to 100x after the $N^{15}N^{15}$ peak is necessary due to the relative abundance of $N^{14}N^{14}$ and $N^{14}N^{15}$.


N14N15 peak
(100x)
N14N14 peak
(100x)
N15N15 peak
(5x)
baseline

Calculations

The N-15 content of a sample is calculated by determining the differences in mass 29 and 30 from the time zero sample using the following equations :

time zero gas

$$XN29' = \frac{XN29}{XN28 + XN29 + XN30} \times 100\% \qquad (7)$$

$$XN30' = \frac{XN30}{XN28 + XN29 + XN30} \times 100\% \qquad (8)$$

where XN28 = pk ht mass 28 ($^{14}N^{14}N$) of time zero gas

XN29 = pk ht mass 29 ($^{14}N^{15}N$) of time zero gas

XN30 = pk ht mass 30 ($^{15}N^{15}N$) of time zero gas

XN29'= ratio of $^{14}N^{15}N$ molecules to total number of N molecules for time zero gas

XN30'= ratio of $^{15}N^{15}N$ molecules to total number of N molecules in time zero gas

The change in N-15 in an enriched gas sample can then be calculated using the following equations :

$$SN29' = \frac{SN29}{SN28 + SN29 + SN30} \times 100\% \qquad (9)$$

$$SN30' = \frac{SN30}{SN28 + SN29 + SN30} \times 100\% \qquad (10)$$

where SN28 = pk ht at mass 28 ($^{14}N^{14}N$) of sample

SN29 = pk ht at mass 29 ($^{14}N^{15}N$) of sample

SN30 = pk ht at mass 30 ($^{15}N^{15}N$) of sample

SN29'= ratio of $^{14}N^{15}N$ molecules to total number N molecules in sample

SN30'= ratio of $^{15}N^{15}N$ molecules to total number of N molecules in sample

By subtracting the time zero ratios from the sample ratios, the increase in mass ratios can be determined :

$$\Delta 29mass = SN29' - XN29' \qquad (11)$$

$$\Delta 30mass = SN30' - XN30' \qquad (12)$$

The total change in N-15 molecules can be calculated from :

$$\Delta N15 = (29mass/2 + 30mass) \times 0.367 \text{ atoms } \% N15/(XN29/2) \qquad (13)$$

assuming that the natural abundance of N-15 is 0.367 atoms % ^{15}N and that the number of mass 30 ($^{15}N^{15}N$) molecules in the time zero sample is negligible.

The concentration of labelled N_2 in the gaseous subsample was calculated from the change in N15 using the following equations :

$$N_2/(\text{moles/L}) = N15/100\% \times 273\ K/T \times P/760mm \div 22.4L/mole \quad (14)$$

where T = temperature of equilibration (K)

P = pressure (mm)

This value gives the concentration of N_2 in the subsample of the headspace. To calculate the total number of moles of N_2 evolved by the denitrifying system the following equations were used :

$$\text{moles } N_2 \text{ (gas phase)} = N_2(\text{moles/L}) \times Vg \quad (15)$$

where Vg = volume of gas phase (L)

$$\text{moles } N_2 \text{ (liquid phase)} = N_2(\text{moles/L}) \times \alpha \times Vl \quad (16)$$

where Vl = volume of liquid phase (L)

α = solubility coefficient (ml N_2/ ml H_2O)

By combining equations 15 and 16 the total number of moles of labelled N_2 evolved can be calculated :

$$\text{total moles } (^{15}N_2) = N_2(\text{moles/L}) \times [(\alpha \times V1) + V] \quad (17)$$

This gives a value for N_2 produced from N-15 labelled nitrate. It is necessary to correct for dilution of this nitrate by N-14 nitrate. For example if the nitrate present in a system consisted of 9.6 atoms % ^{15}N then the final value from equation 17 would need to be multiplied by a correction factor (C) to obtain a total value for all the nitrate present where :

$$C = \frac{100 \text{ atoms \%}}{\text{atoms \% N15}} \quad (18)$$

PURE CULTURE STUDIES

PURE CULTURE STUDIES

Introduction

The number of bacterial species capable of denitrification is numerous and includes both heterotrophic and autotrophic organisms (Jeter and Ingraham 1981). These organisms are generally found in nature interacting with other bacterial species to form metabolically diverse microbial communities. Pure culture studies eliminate these interactions and allow the worker to concentrate on a specific process. Although pure culture studies are not comparable to natural ecosystems , they do provide comprehensive knowledge of the mechanisms of different microbial processes and factors which influence their rates of activity.

There is still much that is unknown about denitrification , probably due to a lack of a reliable rapid means of measuring denitrification rates. Since the discovery that acetylene inhibits the reduction of nitrous oxide to dinitrogen gas causing the accumulation of nitrous oxide (Fedorova et al. 1973), acetylene inhibition has been suggested as a relatively easy and fast method for measuring denitrification activity. The exact mechanism of blockage is not known, however Yoshinari and Knowles (1976) have suggested that acetylene acts as noncompetitive inhibitor of nitrous oxide reductase. Acetylene does not appear to influence the activity of nitrate and nitrite reductases (Balderstone et al. 1976). Because of these properties it has been postulated that rates of denitrification could

be estimated by following the accumulation of nitrous oxide over time. The advantage of this method is that it eliminates the analytical problem of measuring relatively small increases in N_2 concentration in a 80% N_2 atmosphere.

The acetylene inhibiton method for estimating rates of denitrification for pure cultures of denitrifiers has not previously been rigorously evaluated to determine if rates measured in the presence and absence of acetylene are equivalent. Balderston et al. (1976) followed the accumulation of N_2O over time for cultures of Pseudomonas aeruginosa and marine sediments in the presence of 0.1 % KNO_3 and KNO_2, and 0.01 to 0.02 atm C_2H_2 but did not compare their results using any other methods. Yoshinari and Knowles (1976) followed the disappearence of NO_3^- and NO_2^- and the appearence of N_2O in the presence of 0.1 atm C_2H_2 but did no comparisons to the accumulation of gaseous endproducts in its absence.

The purpose of this study was to determine the reliability of the acetylene inhibition method by comparison with $^{15}N_2$ production by pure cultures grown in the absence of acetylene but provided with N-15 enriched nitrate as the sole electron acceptor. Five denitrifying bacteria isolated from E.L.A. lake sediments were tested to determine the accuracy of the acetylene inhibition method by comparison with $^{15}N_2$ production.

Methods

Sediment Sampling

Epilimnetic sediments were collected from Lake 114 and the north basin of Lake 302 (302N) at 4 meter depths by means of an Eckman Grab (Burton and Flannagan 1973). The top 1-2 cm of sediment was transfered to a 500 ml Nalgene bottle by sweeping up the sediment through a length of thick walled 6 mm ID Tygon tubing connected to the bottle which was continuously evacuated by a hand pump. The bottles were completely filled to exlude air. The sediments were stored in the dark at 4 C for up to a week before isolation procedures were begun.

Isolation and Purification

Denitrifying bacteria were isolated from epilimnetic sediments collected from Lake 114 and the north basin of Lake 302 using a Winogradsky column enrichment technique (Aaronson 1970; Chan 1977).

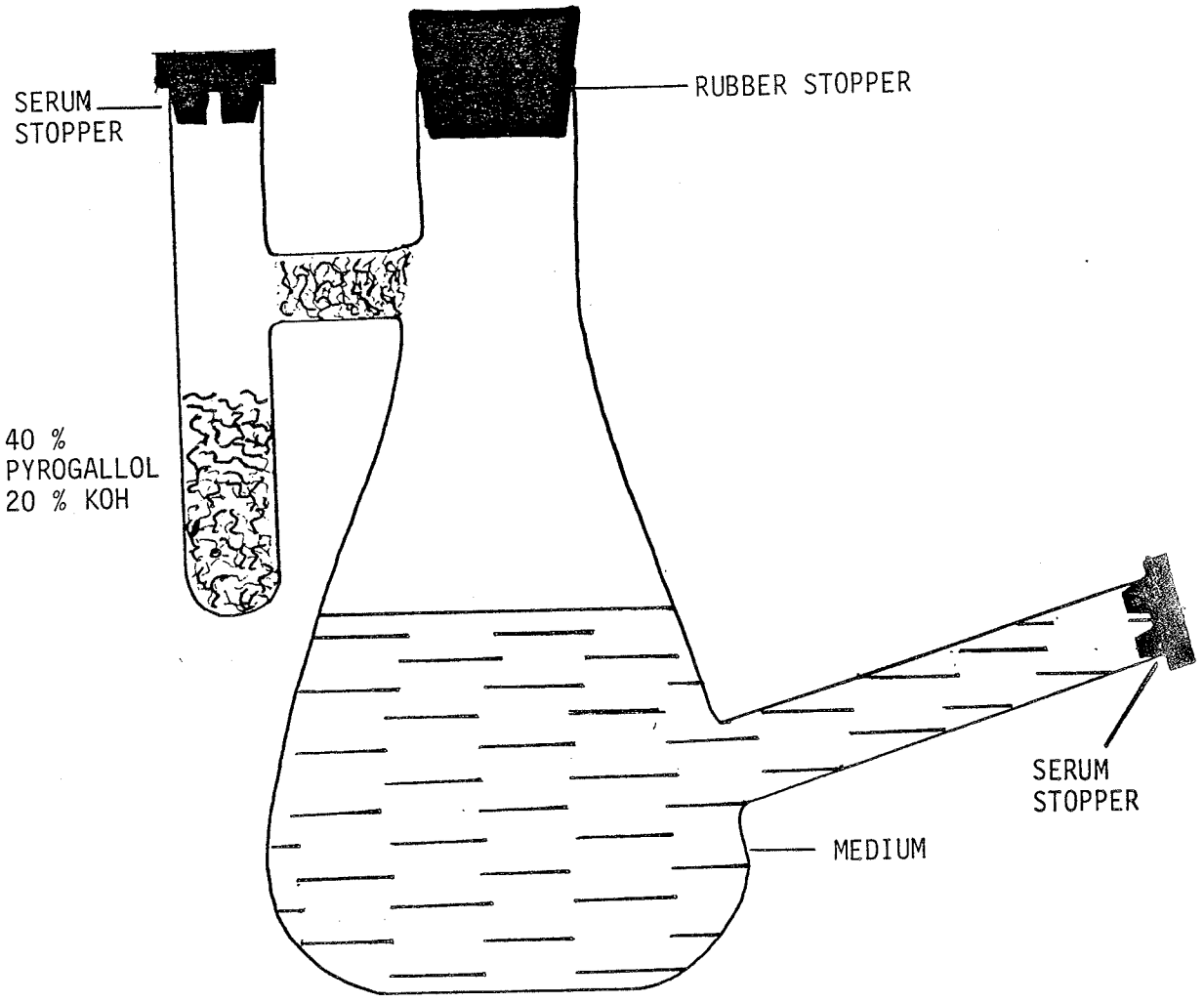
Winogradsky columns were prepared for the two lake sediments using sterile 250 mL glass graduated cylinders. A mixture of sediment, shredded filter paper, fibrous cellulose and sterile enrichment solution containing 0.5% KNO_3 and 0.2% D-L malic acid, the pH adjusted to 7.2 with NaOH was prepared. 25 mL of this mixture formed the bottom layer of each column. Each column was then packed with sediment to 2/3 full and the remaining portion of the column was filled with the

remaining enrichment solution. A no. 9 rubber stopper was loosely placed on top of the column allowing for gas ebullition. The columns were incubated at 21 +/- 1 C in the dark for 10 days.

Enrichment cultures were started by transferring 2 mL of sediment slurry from the bottom third of the column to an anaerobic flask (figure 5) containing 150 mL of sterile enrichment medium (1L : 3.0 g KNO_3, 2.0 g D-L malic acid, 1.0 g peptone, and 0.5 g Yeast extract; pH 7.2). The oxygen concentration of the medium was reduced by scrubbing with N_2 for 15 minutes before inoculation and 5 minutes following inoculation. One mL of 40% pyrogallol and 2 mL of 20% KOH were added to the side arm of the anaerobic flask. At regular time intervals the medium from each of the flasks was subsampled for NO_3^- determinations. To maintain anaerobosis within the flasks the samples were withdrawn through the serum stopper sealing the sidearm, using a sterile 10 mL Plastipak syringe. The subsamples were analyzed for nitrate using Nitraver V nitrate reagents (Hach Chemical Company). The disappearance of nitrate over time was recorded.

Pure cultures were isolated by streaking serial dilutions of enrichment culture onto plates of an enrichment medium solidified with 1.5% Bacto agar. The plates were incubated in Gaspak anaerobic jars at 22 C for 2 weeks. Isolated colonies were randomly selected and successively transfered to Trypticase Soy agar plates amended with 0.1% KNO3. Cultures were tested for their ability to reduce nitrate to gaseous nitrogen by culturing on nutrient broth containing 0.1% KNO_3 as the sole electron acceptor. Stock cultures were maintained in

Figure 5. Anaerobic enrichment flask modified from a 250 mL Erlenmeyer flask. The flask was flushed for ten minutes before inoculation and five minutes after.


SERUM
STOPPER
RUBBER STOPPER
40 %
PYROGALLOL
20 % KOH
SERUM
STOPPER
MEDIUM

Trypticase deep agar tubes amended with 0.1% KNO_3 and 0.5% glycerol. Transfers to fresh medium were carried out monthly. After growth at 22 C, the cultures were kept at 4 C and used to prepare liquid cultures.

Characterization of Cultures

Five isolates were chosen for further study based on their morphological and physiological differences.

Cell Morphology

Isolates grown on Trypticase soy agar amended with 0.1% KNO_3 were examined for colony characteristics and pigment production.

Physiology

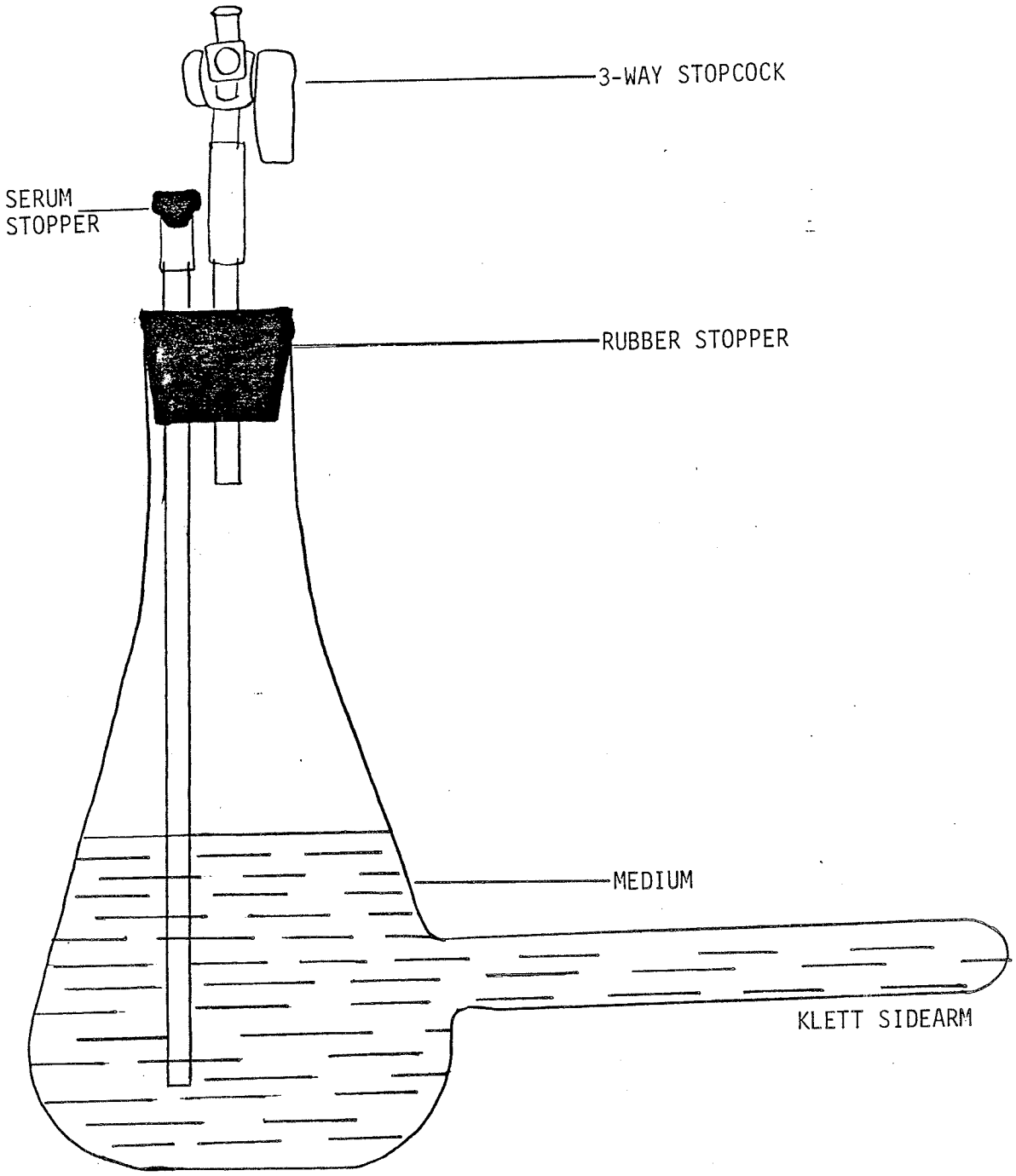
Biochemical tests carried out on the organisms included Gram's stain, citrate utilization, nitrate reduction, methyl red, Voges Proskauer, catalase, oxidase, hydrogen sulfide production (TSI), and glucose, sucrose and lactose utilization (Rohde 1968; Skerman 1967).

Assay of Denitrification

500 mL Erlenmeyer flasks equipped with Klett side arms, containing 250 mL of test medium (1L : 2.0 mg KNO_3, 2.0 g D-L malic acid, 1.0 g peptone, and 0.5 g K_2HPO_4; final pH adjusted to 7.0-7.4 with NaOH) were inoculated from the stock cultures to provide liquid cultures for subsequent evaluation of the C_2H_2 inhibition assay. Each of the flasks was sealed with a rubber stopper. Two pieces of glass tubing were inserted through the stopper, one extending into the medium and the other into the neck of the flask. The former was sealed with a serum stopper and the latter with a Teflon three-way stopcock (Figure 6). Anaerobiosis was established by flushing the medium with sterile O_2-free N_2 for 10 minutes prior to inoculation and 5 minutes after innoculation. Oxygen was removed from the N_2 gas by passing the gas over a heated reduced copper column (Hungate 1969). The flasks were incubated at 22 C on a rotary shaker for 24 hours to provide a population density of approximately 10^6 cells per ml. Growth of the cultures was monitored by measuring absorbancy using a Klett photoelectric colorimeter.

Evaluation of the acetylene inhibition method by comparison to dinitrogen production was carried out in duplicate or triplicate 50 ml sterile glass syringes containing 25 mL of liquid culture. Air contamination was avoided by continuously flushing the flask with N_2 during the transfer. The syringes were sealed with serum stoppers and preincubated at 22 C for two hours to avoid a lag phase which was observed in initial experiments when a preincubation period was not used. This lag was probably caused by the introduction of small

Figure 6. Anaerobic culturing flask modified from a 250 mL Erlenmeyer flask. The klett side arm allows monitoring of growth by turbidity. Liquid cultures were removed by inverting the flask and attaching a sterile 50 ml syringe to the three way stopcock. The system was scrubbed with sterile N2 before and after inoculation.


3-WAY STOPCOCK
SERUM STOPPER
RUBBER STOPPER
MEDIUM
KLETT SIDEARM

amounts of O_2 during transfer of the cultures to the syringes. Care was taken to minimize this effect.

Labelled KNO3 (96 atoms% ^{15}N : ICN, Calif., U.S.A.) was injected into all of the syringes through the serum stoppers, to give a final NO_3^- concentration in the range of 10 - 80 umoles NO_3^- - N / L culture. A 25 mL head space of N_2 gas was introduced into each of the syringes. For measurements of denitrification rates by the acetylene inhibition method, a volume of C_2H_2 was injected into each of the syringes and dissolved into solution by vigorously shaking the syringe for one minute. The injected volume of acetylene gave a 5% concentration (vol/vol) in the gas phase above the culture. Other workers have used C_2H_2 concentrations ranging from 1% to 10% (vol/vol). In preliminary tests on the isolates used in this study, no differences in the rate of N_2O accumulation were encountered for C_2H_2 concentrations in the range of 5 to 10% (vol/vol).

These syringes were incubated at 20 C in the dark for up to ten hours on a rotary shaker. At regular time intervals starting with time zero, the syringes that received acetylene additions were sampled for N_2O production. Sampling consisted of withdrawing a 5 mL gas sample from the headspace above the culture after equilibration of the liquid and gas phases by manually shaking the syringe for one minute. It was previously determined that one minute was sufficient to achieve complete equilibrium between the two phases. The sample was withdrawn using a 5 mL glass syringe fitted with a 25 G needle. To ensure that no contamination by atmospheric gases occurred during subsampling, the

syringe was flushed with O_2free-N_2 prior to sampling. The needle was inserted through the serum stopper sealing the syringe containing the bacterial culture. Five millilitres of headspace gas were forced into the sample syringe by creating a positive pressure in the culture syringe by applying force on the the barrel of the latter. The syringe containing the gas sample was sealed by partially inserting the needle into a silicone stopper.

The nitrous oxide samples were analyzed within four hours of sampling as described in the general methods section. The total number of moles of nitrous oxide in each subsample taken from the headspace above the pure cultures receiving acetylene additions were calculated as described previously in the general methods section. These values are expressed as the number of moles of N_2O per litre culture.

The rate of denitrification was also measured by the rate of $^{15}N_2$ production for cultures receiving $^{15}NO_3^-$ additions for comparison with the acetylene inhibition method. Incubation procedures were the same with the exception that acetylene was omitted. The headspace above the culture was sampled in the same manner as for the C_2H_2 method using a 10 ml glass syringe fitted with a 23 G needle. The syringes containing the N15 gas samples were stored submerged in water for up to two weeks before being analyzed. As long as the barrels of the syringes remained moistened sample leakage was not observed. The quantity of dinitrogen gas evolved from the cultures was calculated as discribed in the general methods section.

Accumulation of N_2O above cultures receiving acetylene additions and N_2 for those not, versus time was presented graphically.

Results and Discussion

A number of strains of bacteria were isolated from Lake 114 and 302N sediments using the Winogradsky enrichment technique. The isolates were characterized according to their cellular, cultural and physiological characteristics. Five were selected to study the reliability of the acetylene inhibition method for measuring denitrification by comparison to the N-15 method. Since the microorganisms were not taxonomically identified they have been designated as isolates A, F, G, 4, and 9. Isolates A, F, and G were isolated from Lake 114 sediments and Isolates 4 and 9 from Lake 302N sediments. All are gram negative, motile, rod shaped bacteria. Their physiological characteristics are listed in Table 1. Isolate G was the only isolate able to produce a diffusible nonflourescent green pigment when grown anaerobically on Trypticase Soy agar.

The five isolates were grown under anaerobic conditions with N-15 labelled nitrate provided as sole electon acceptor. Under these conditions each isolate was tested for its ability to produce nitrous oxide in the presence of 5% acetylene and labelled dinitrogen gas in the absence of acetylene. The concentration of acetylene applied in this study was determined in preliminary work where no difference in nitrous oxide accumilation was observed at acetylene concentrations of one, five and ten percent.

Table 1. Physiological properties of denitrifiers isolated from Lake 114 and Lake 320N sediments.
+ positive results ; - negative results

test	Isolate A	F	G	4	9
oxidase	+	+	+	+	+
catalase	+	-	+	+	+
acid from:					
glucose	-	-	-	-	-
sucrose	-	-	-	+	-
lactose	-	-	-	+	-
citrate	-	+	+	+	+
MR	-	-	-	+	-
VP	-	-	-	-	-
H_2S	-	-	-	+	-
NO_2^- from NO_3^-	-	-	-	-	-
pigment	-	-	+	-	-

Denitrification rates as determined by the N-15 method were taken as the standard for comparison with denitrification rates determined by the acetylene inhibition method .

Rates of denitrification were calculated from the accumulation of N_2O or N_2 over the first four to six hours of incubation.

An initial lag in denitrification activity was observed for isolate A (Figure 7) both in the presence and absence of 5% C_2H_2. Taking this into account, initial rates of denitrification (0-6 hours) for isolate A were 0.90 umoles N_2/L culture/hr as determined by the N-15 assay and 0.82 umoles N_2O/L culture/hr by the C_2H_2 inhibition technique. These rates do not differ significantly ($p=.05$). After six hours, there was a three fold increase in denitrification for the culture without acetylene. At this same time the culture with acetylene showed a decrease in activity. It appears that the high nitrate concentrations (~80 umol) induced enzyme production in the one culture and in the second culture, C_2H_2 may have inhibited enzyme induction. Bacterial counts determined microscopically for each of the cultures showed no apparent differences in numbers of cells.

Denitrification activity for isolate F (Figure 8) did not have an initial lag as Isolate A was seen to have. Rates of denitrification as determined by both methods remained steady for the first eight hours at which time nitrate was no longer available. Comparison of the two methods showed agreement between the rates obtained. Denitrification rates for Isolate F were 5.38 umole N_2O/ L culture/hr as measured by

Figure 7. Production of N2 in the absence of C2H2 and N20 in the presence of 5% C2H2, with an initial NO_3^- concentration of 80 umol NO_3^- -N / L culture, for isolate A (Apr. 27/82).

_____ N2 production 0.90 umol/L culture/hr (0-6 hr)

__.__ N20 production 0.82 umol/L culture/hr (0-6hr)

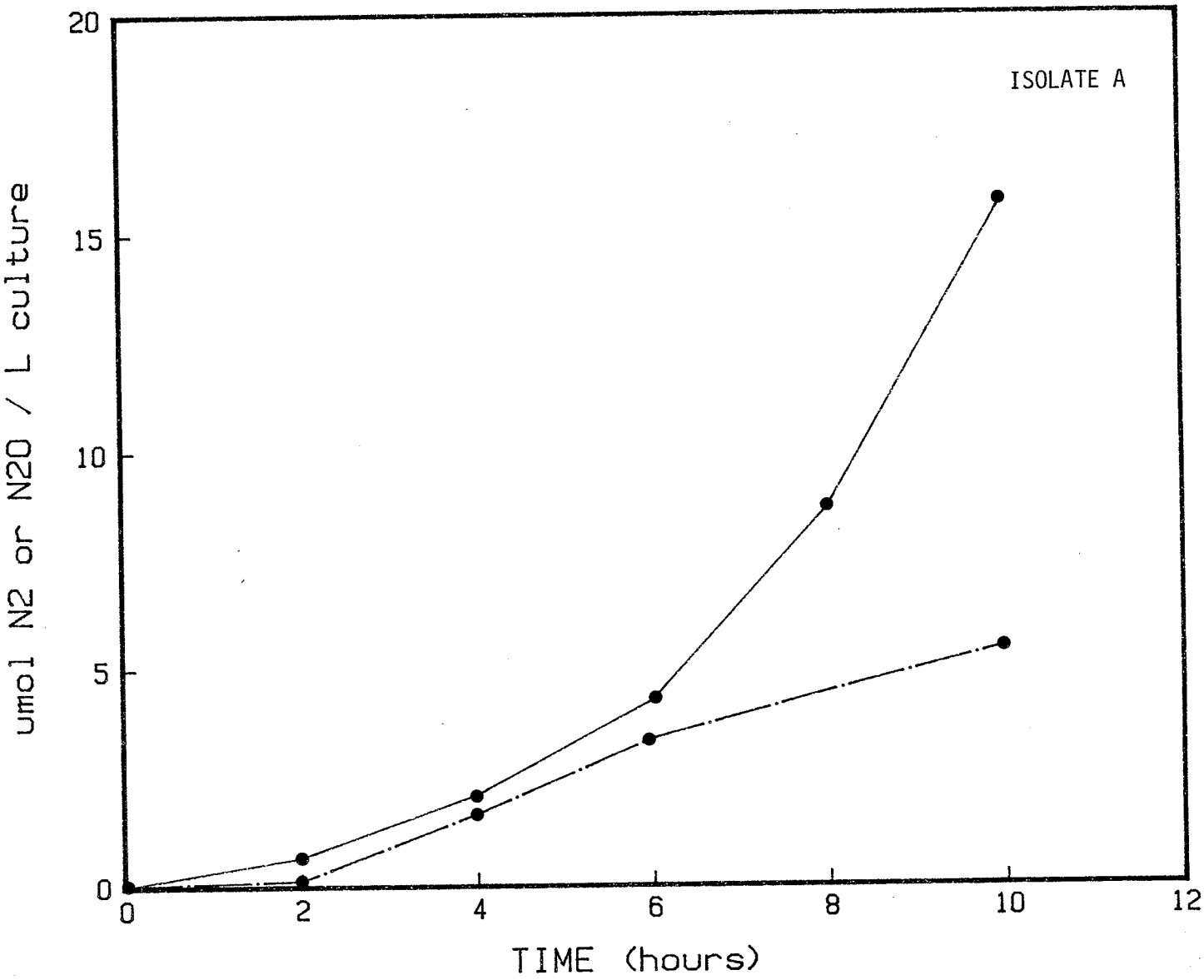
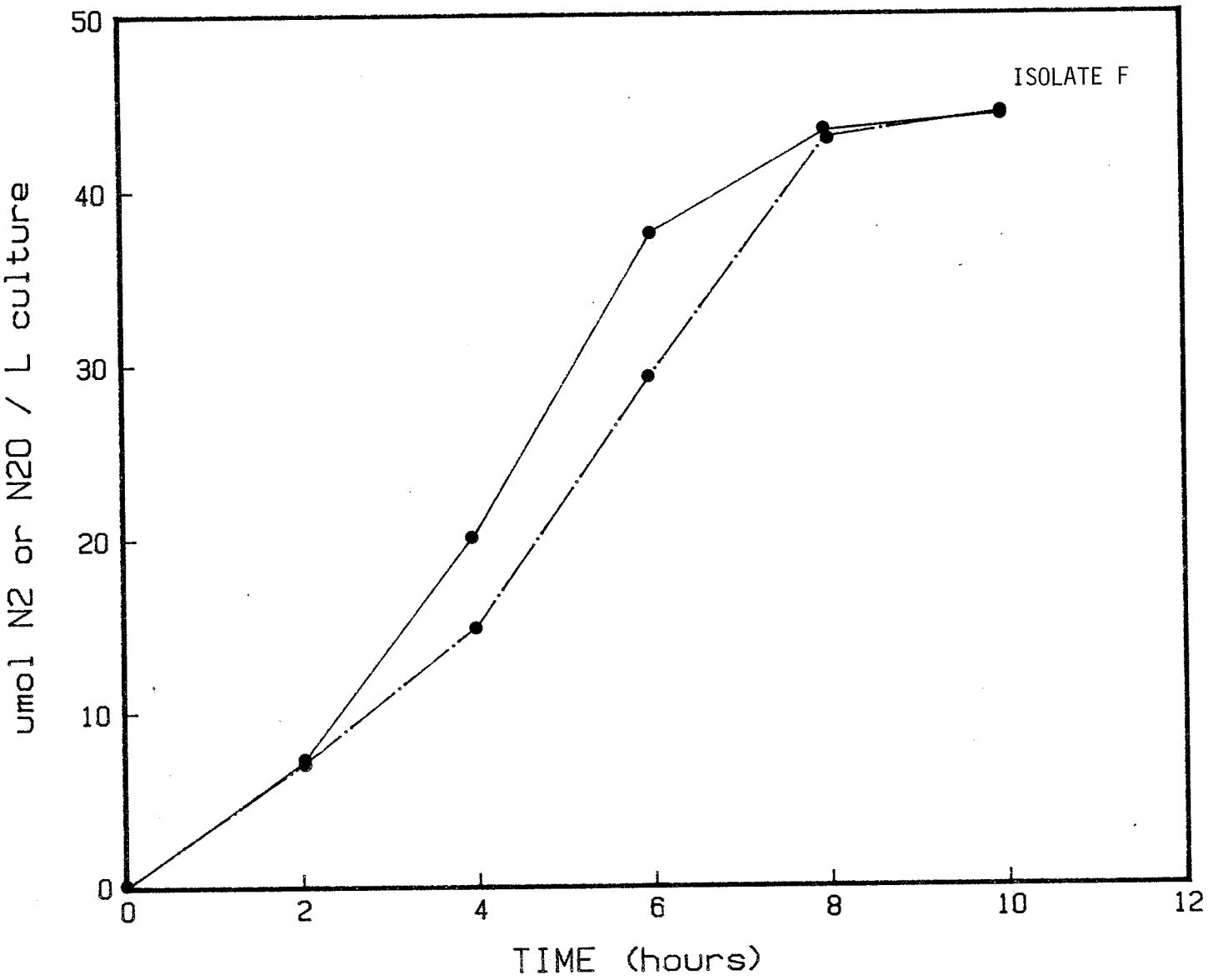

ISOLATE A
umol N2 or N2O / L culture
20
15
10
5
0
0
2
4
6
8
10
12
TIME (hours)

Figure 8. Production of N_2 in the absence of C_2H_2 and N_2O in the presence of 5% C_2H_2, with an initial NO_3^- concentration of 80 umol NO_3^--N/L culture, for isolate F (Feb. 28/82).

_____ N_2 production 5.42 umol/L culture/hr (0-8 hr)

__.__ N_2O production 5.38 umol/L culture/hr (0-8 hr)


ISOLATE F
umol N2 or N2O / L culture
TIME (hours)
0
10
20
30
40
50
0
2
4
6
8
10
12

C_2H_2 inhibition and 5.42 umole N_2/L culture/hr by $^{15}N_2$ production.

Comparisons of denitrification rates for Isolate G (Figure 9) resulted in good agreement between the two methods. Rates were calculated for the 2 to 6 hour interval, disregarding the initial lag in activity. These rates were 7.40 umoles N_2/L culture/hr and 8.60 umoles N_2O/L culture/hr as determined by the N-15 and acetylene inhibition methods. In both incubation systems all of the nitrate had been consumed after 6 hours.

No significant nitrous oxide production was observed for cultures of Isolate 4 incubated in the presence of 5% C_2H_2 (Figure 10). N_2 production in the absence of C_2H_2 was constant for the first three hours of incubation , 0.38 umoles N_2/L culture/ hr, then increased by 60 % in the next hour. Comparisons of bacterial counts for both cultures at the finish of the experiment did not differ from each other. These results support the suggestion that acetylene in some cases may inhibit total denitrification activity. A second possibility is that N_2O reduction is not completely inhibited and some is being further reduced to N_2.

Evaluation of the acetylene inhibition assay indicated denitrification rates approximately ten times lower than rates obtained by the N-15 method for isolate 9 (Figure 11). These rates were 0.47 umoles N_2O/L culture/hr and 4.36 umoles N_2/ L culture/hr respectively. During this experiment $^{15}N_2$ production in the presence of C_2H_2 was assayed. No appreciable accumulation of $^{15}N_2$ was detected therefore

Figure 9. Production of N2 in the absence of C2H2 and N20 in the presence of 5% C2H2, with an initial NO_3^- concentration of 80 umol NO_3^- -N/L culture, for isolate G (Apr. 27/82).

_____ N2 production 7.40 umol/L culture/hr (2-6 hr)

__.__ N20 production 8.60 umol/L culture/hr (2-6 hr)

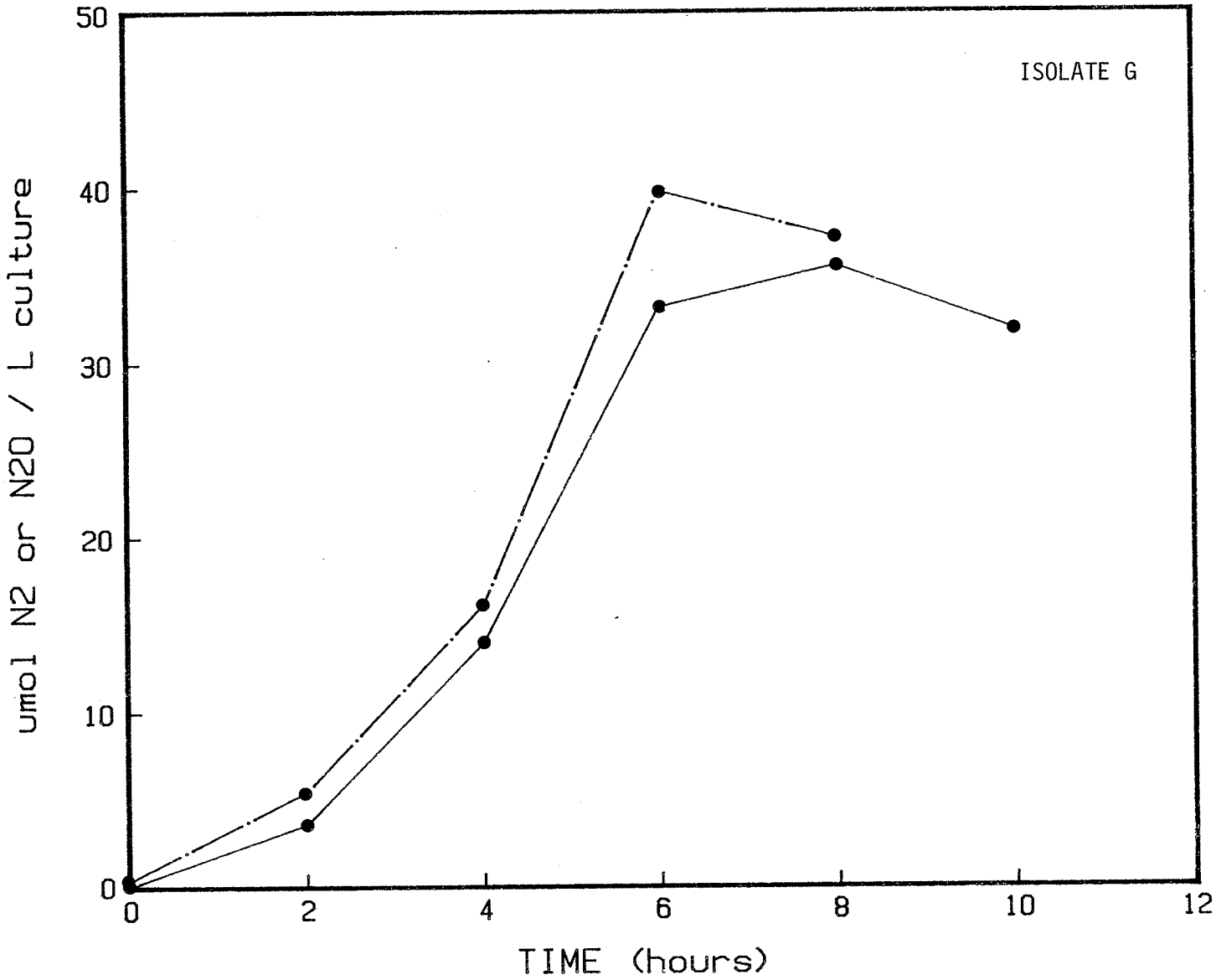

ISOLATE G
umol N2 or N20 / L culture
50
40
30
20
10
0
0
2
4
6
8
10
12
TIME (hours)

Figure 10. Production of N2 in the absence of C2H2 and N20 in the presence of 5% C2H2, with an initial NO_3^- concentration of 10 umol NO_3^- -N/L culture, for isolate 4 (Jun. 12/82).

_____ N2 production 0.38 umol/L culture/hr (0-3 hr)

__.__ N20 production 0

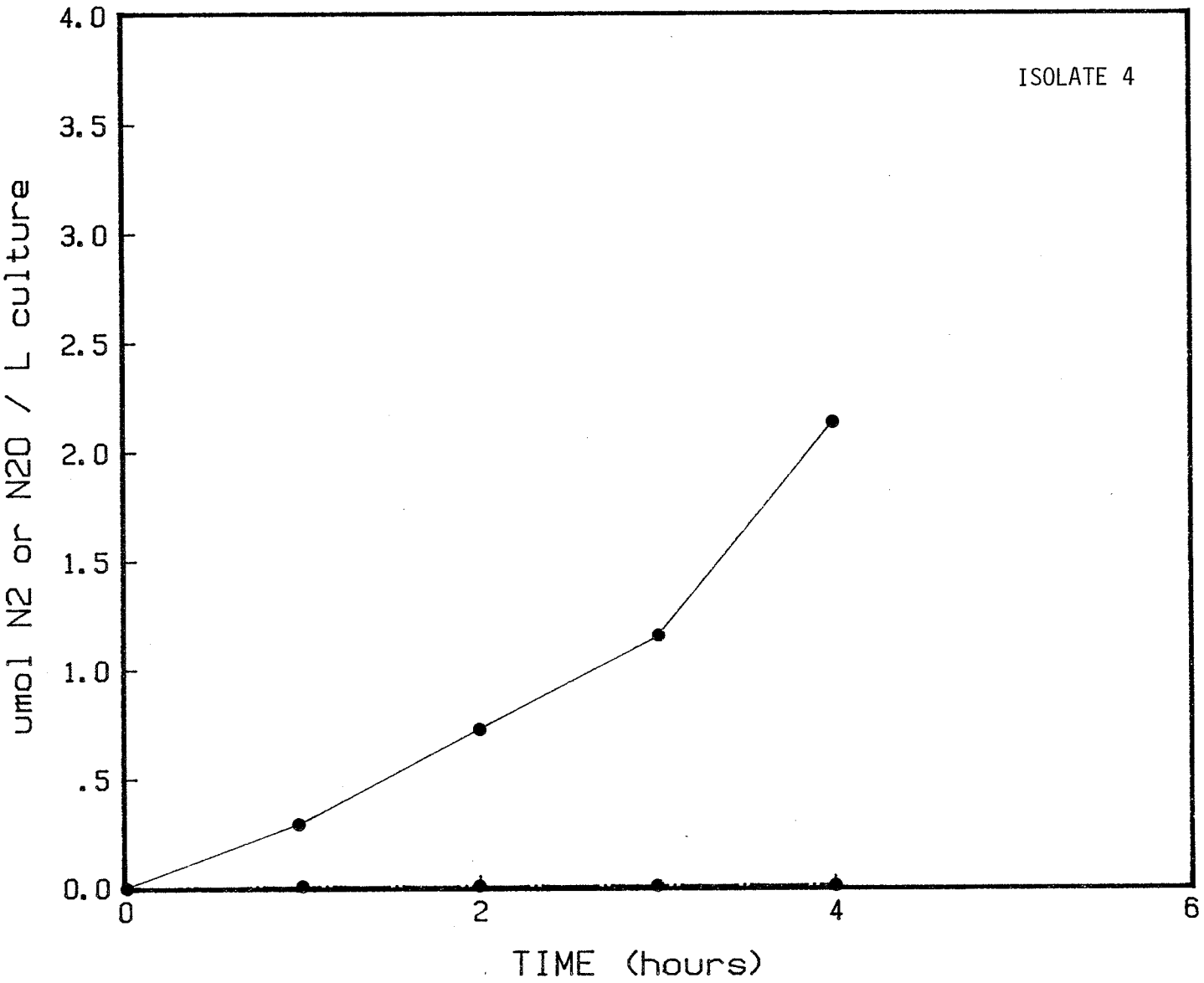

ISOLATE 4
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
0.0
umol N2 or N2O / L culture
0
2
4
6
TIME (hours)

Figure 11. Production of N2 in the absence of C2H2 and N20 in the presence of 5% C2H2, with an initial NO_3^- concentration of 20 umol NO_3^- -N/L culture, for isolate 9 (July 12/82).

_____ N2 production 4.36 umol/L culture/hr (1-3 hr)

__.__ N20 production 0.47 umol/L culture/hr (1-3 hr)

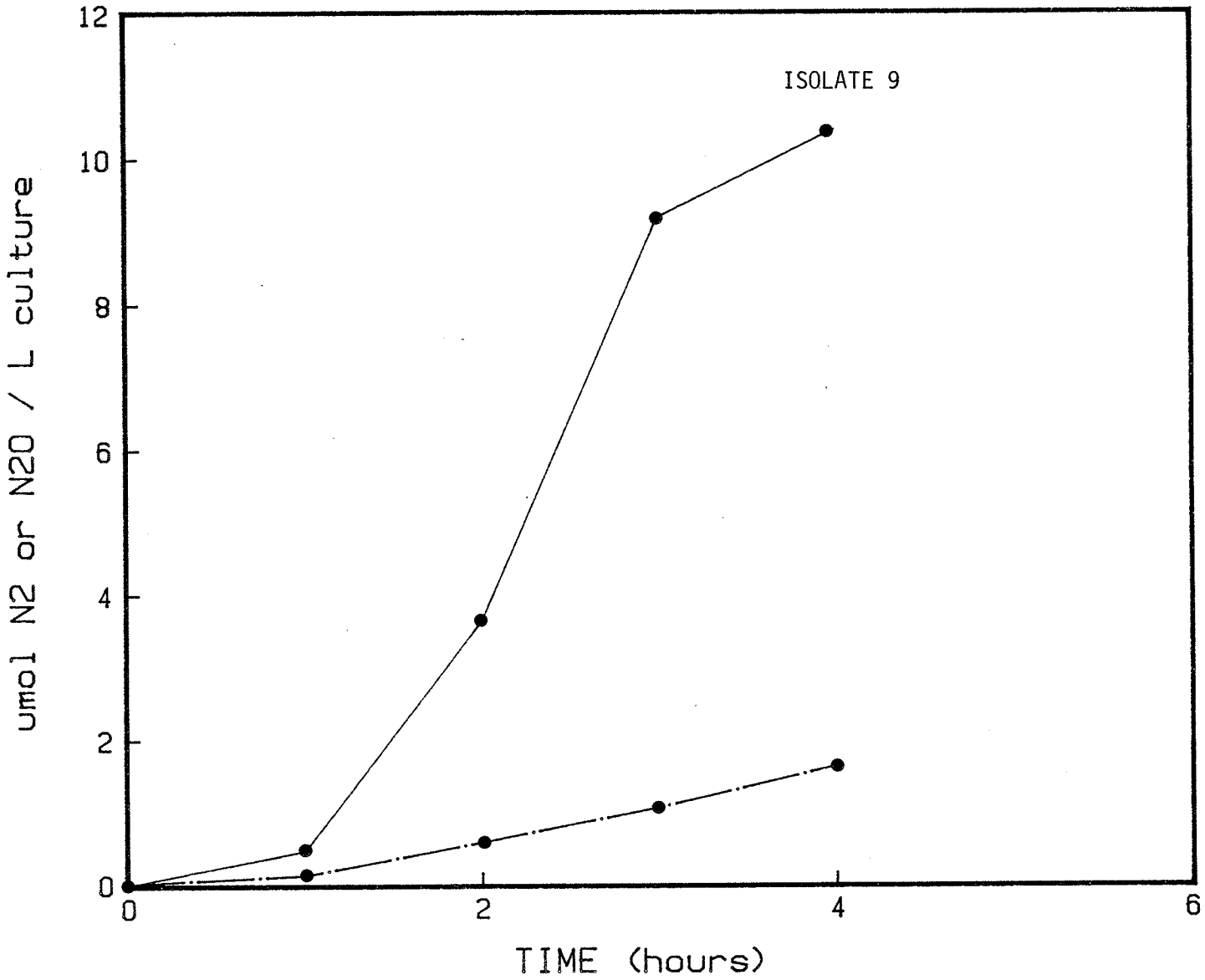

ISOLATE 9
umol N2 or N2O / L culture
TIME (hours)
0
2
4
6
0
2
4
6
8
10
12

Table 2. Summary of comparisons of denitrification rates for denitrifing bacteria isolated from Lake 114 and Lake 302N sediments as measured by the acetylene inhibition method (N_2O production) and the N-15 method (N_2 production).

Isolate	initial NO_3^- umol	moles N_2/ L culture/hr	umoles N_2O/ L culture/hr
A	80	0.90	0.82
F	80	5.42	5.38
G	80	7.40	8.60
4	10	0.38	0
9	20	4.36	0.47

the lower rates in the presence of C_2H_2 appear to be a result of overall inhibition of the denitrification pathway.

A summary of rates obtained for each of the cultures is given in table 2.

Pure culture studies provide an easy means of studying factors influencing particular microbial processes without complications by interactions with other microbial species which would be present in mixed cultures or natural systems. In this study pure cultures were used to determine the accuracy of the acetylene inhibition technique by comparison with N-15 dinitrogen production.

The results of this study suggest that the reliability of acetylene inhibition as a means of estimating denitrification rates is species dependent. Acetylene appears to slow the overall denitrification process for certain strains of bacteria. It also appears that initial nitrate concentration may influence the degree to which the C_2H_2 method underestimates rates. The descrepancy between the two methods increased as the initial nitrate concentration decreased from 80 umol for Isolate A to 10 umol for Isolate 4 (Table 2). This is difficult to confirm from these results because different bacterial strains were involved in each of the assays. It has however, been previously demonstrated that the effectiveness of C_2H_2 to inhibit N_2O reduction decreases in marine sediments at low nitrate concentrations (Kaspar 1982).

These results indicate that due to the species dependency of the acetylene inhibition and the influence by the nitrate concentration the acetylene inhibition method could result in underestimates of rates of denitrification.

SEDIMENT SLURRY STUDIES

SEDIMENT SLURRY STUDIES

Introduction

Direct methods for measuring denitrification rates in natural sediment samples are lacking. This is partially due to the high ambient concentration of nitrogen gas making it difficult to measure denitrification directly from its endproduct dinitrogen gas. As a result indirect methods have been developed. These techniques include measurement of nitrate disappearence (Keeney et al. 1971; Chen et al. 1972), measurement of dinitrogen production in an inert gas above a denitrifying system (Seitzinger et al.1980), production of dinitrogen relative to argon in sedimentary pore waters (Nishio et al. 1981; Barnes et al. 1975), ^{13}N tracer techniques (Tiedje et al. 1979 ; Sorensen et al. 1980), production of $^{15}N_2$ from labelled nitrate (Goering and Dugdale 1966; Chen et al. 1972; Chan and Campbell 1980), and the acetylene inhibition method (Yeomans and Knowles 1976 ; Balderston et al. 1976 ; Chan and Knowles 1979). The latter two methods are widely used for studying denitrification. Neither is without its faults. The N-15 technique requires that nitrate be added to the system; however, despite the disadvantage of perturbing the N balance by the addition of even small amounts of nitrate, this method enables one to follow the different nitrogen pathways that could be simultaneously occurring in nature (Chen et al. 1972 ; Buresh and Patrick 1981 ; Haines et al. 1981 ; Koike and Hattori 1978a, b). The acetylene blockage method has been applied to soils (Ryden 1982 ; Smith

et al. 1978 ; Yeomans and Beauchamp 1978 ; Yoshinari et al. 1977), freshwater sediments (Chan and Knowles 1978) and marine sediments (Sorensen 1978a,b) since it was found that acetylene inhibits the reduction of nitrous oxide to nitrogen gas in the denitrification pathway (Fedorova et al. 1974). The accumulation of nitrous oxide is taken as a measure of denitrification. Although the method does not require nitrate addition, acetylene is known to influence other microbial processes which could, in turn, influence denitrification rates. The most significant reaction affected would be nitrification. It is known that acetylene inhibits nitrifying bacteria (Walter et al. 1979) which under nitrate limiting conditions could be the controlling process for denitrification. Acetylene has also been found to inhibit CH_4 production and oxidation, as well as N_2 fixation (Walter et al. 1979 ; Hynes and Knowles 1978 ; Knowles 1979). Because of the toxicity of acetylene to various microbial groups, its presence in a mixed microbial community will influence the flow of electrons within the system. If the microbial metabolism of the mixed population were to be reduced due to the presence of acetylene, the denitrification population will face less competition for carbon sources therefore measurements of activity could yield rates greater than those that would naturally occur.

Studies in which the rate and extent of denitrification in the presence and absence of acetylene was measured have resulted in conflicting results. Sorensen (1978a) evaluated the C_2H_2 method in marine sediments using $^{15}NO_3^-$ methods and Ryden et al. (1979a) compared

C_2H_2 methods in soils with ^{13}N methods. Both studies concluded that acetylene had no inhibitory effect on the rate of denitrification. In more recent studies VanRaatle and Patriquin (1979) in a study of salt marsh sediments and Yoshinari and Beauchamp (1978) who studied soil systems, only limited inhibition of N_2O reduction was observed following acetylene additions. These results suggest that the applicability of the method is dependent on the system under study.

Evaluation of the acetylene inhibition method in freshwater sediments has not previously been reported. The purpose of this study is to determine the accuracy of the actylene inhibition method for measurement of denitrification rates in several ELA lakes by comparing rates of nitrous oxide production in the presence of acetylene to rates of $^{15}N_2$ production in the absence of acetylene as a standard.

Previously Chan and Campbell (1980) determined that the epilimnetic sediment-water interface was a more significant site of natural denitrification when compared to the hypolimnion of ELA lake 227. In this study an evaluation of the acetylene inhibition method using a $^{15}N_2$ assay will be carried out on epilimnetic sediments obtained from four ELA lakes.

Methods

Epilimnetic sediments were collected from four ELA lakes, Lakes 239, 114, 223, and 302, by means of an Eckman grab. The top 1-2 cm of sediment was transfered to a 500ml Nalgene bottle by sweeping up the sediment through a length of thick walled Tygon tubing (ID 6mm) connected to the bottle which was continuously evacuated by using a hand vacuum pump. The bottles were completely filled to exclude air. Two 500 mL Nalgene bottles were filled during each sampling. Upon return to the laboratory the two bottles were pooled in a one liter beaker and mixed under an atmosphere of tank nitrogen using a magnetic stirrer. As the sediments were being mixed, 25 mL were transfered to a series of sterile 50 mL glass syringes. The tip of each syringe was sealed with a tightly fitting Suba Seal. The syringes containing sediment were preincubated for two hours at *in situ* lake temperatures so that O_2 which may have entered the syringes during the transfer of sediment into the syringes was consumed.

40 umoles $^{15}NO_3^-$/L wet sediment of labelled KNO_3-N (96 atoms % ^{15}N ; ICN Calif., USA) were injected into each of the syringes and mixed into the sediment by shaking each syringe vigorously for one minute. A 25 mL headspace of deoxygenated nitrogen gas was then introduced into each syringe. Acetylene was injected into each syringe containing sediment in which nitrous oxide accumulation was to be followed. The acetylene was mixed into solution by vigorously shaking each syringe for one minute. The injected volume of acetylene gave a 5% concentration(v/v) in the gas phase above the sediment. This

concentration of acetylene was decided on after preliminary studies showed no differences in the rate of nitrous oxide accumulation by denitrifying sediments in the presence of 1, 5, and 10% acetylene. The syringes were incubated at in situ temperatures for up to ten hours. At time zero and at regular time intervals during incubation the syringes were sampled for nitrous oxide production as was described previously in the pure culture section.

Nitrous oxide samples were analysed within 24 hours of experiment initiation. The sediments were then dried at 60 C overnight. Denitrification rates of sediments assayed by the acetylene inhibition method were calculated from the accumulation of nitrous oxide over time and expressed as rates of umoles N_2O/L g dry wt. sediment/hour.

Rates of denitrification as determined by the N-15 method for comparison with the acetylene inhibition were carried out on sediments which received only $K^{15}NO_3$ additions. Time zero samples and samples at regular time intervals thereafter were withdrawn from each syringe. Sampling for $^{15}N_2$ production was the same as for the pure cultures in the previous section. $^{15}N_2$ samples were stored submerged in water for up to two weeks before being analyzed. At the end of the incubation the sediment in each syringe was dried and weighed. The quantity of dinitrogen evolved was calculated as described in the general methods section.

Rates of denitrification by the N-15 method were determined from the accumulation of N_2 over time and expressed as umoles N_2/g dry wt. sediment/hour.

Unamended sediments from each of the sampling sites were dried and analyzed for total nitrogen, carbon, and phosphorus, and carbonate on a CN analyzer in the Freshwater Institute laboratory in Winnipeg.

Results and Discussion

Results for the chemical analysis of surface sediments collected from the five sampling sites are given in Table 3. With the exception of the two sites in lake 302N, the sediments varied between the different sampling sites. Lake 114 sediments are fine and flocculent in structure and have a high, 174 mg C/g dry sediment, organic carbon content while Lake 302N sediment consists of a coarse gravel with a relatively low organic content, 6-8 mg C/g dry sediment. The remaining sites are intermediate to Lakes 114 and 302N.

Sediment slurries collected from the five sites were assayed for their denitrification potentials by following the accumulation of N_2O from slurries treated with 5% C_2H_2 and $^{15}N_2$ from $^{15}NO_3^-$ in the absence of acetylene. Nitrous oxide production was disregarded in the N-15 method because of its transient nature in the reaction (Sorensen 1977). Isotopic fractionation does not proceed at an appreciable rate, the fractionation factor being less than 1.023 (Blackmer and Bremner 1977), therefore it was neglected.

Denitrification potentials were calculated from the total accumulation of N_2O or N_2 during the first 4 to 6 hours of incubation. Beyond this time, rates for some of the sediments decreased dramatically as illustrated in Figures 12 to 18. A summary of denitrification potentials for the different lake sediments is given in Table 4.

Table 3. Sediment analysis for total nitrogen, phosphorus and organic carbon and carbonate on samples collected from the five sampling sites.

Lake	Total Nitrogen mg/g dry sediment	Total Phosphorus mg/g dry sediment	Carbonate Carbon mg/g dry sediment	Organic Carbon mg/g dry sediment
239	3.4	0.6	2	40
114	17.1	1.1	7	174
223	1.9	0.6	1	16
302 (N)	0.6	0.2	1	6
302 (S)	0.7	0.2	1	8

Figure 12. Production of N-15 N2 in the absence of C2H2 and N20 in the presence of 5% C2H2 in Lake 239 sediment slurries (May 26/82).

_____ N2 production 20.56 nmol/gm dry sediment/hr
__.__ N20 production 11.63 nmol/gm dry sediment/hr

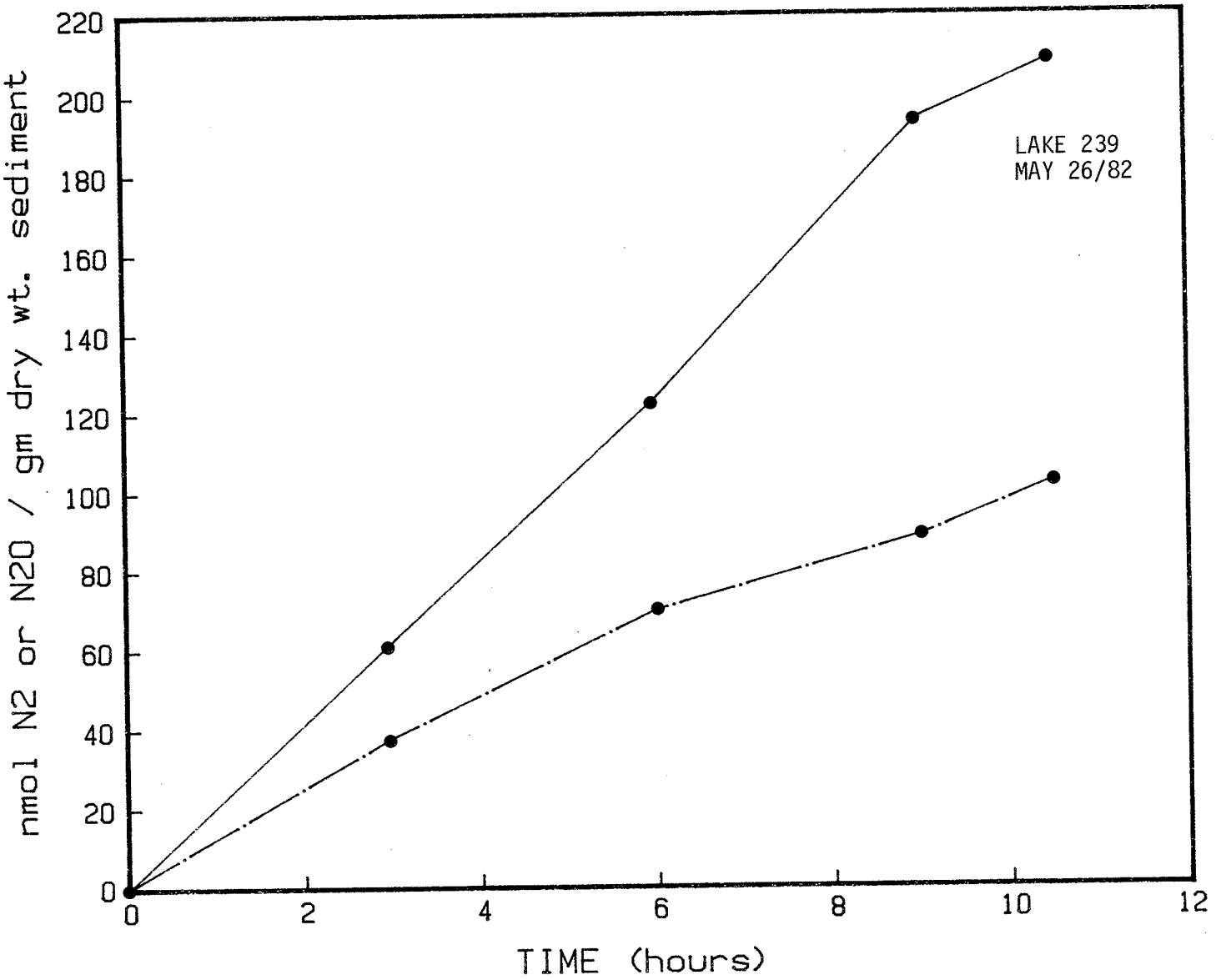

220
200
180
160
140
120
100
80
60
40
20
0
nmol N2 or N2O / gm dry wt. sediment
0
2
4
6
8
10
12
TIME (hours)
LAKE 239
MAY 26/82

Figure 13. Production of N-15 N2 in the absence of C2H2 and N20 in the presence of 5% C2H2 in Lake 239 sediment slurries (June 11/82).

_____ N2 production 36.31 nmol/gm dry sediment/hr
__._ N20 Production 12.72 nmol/gm dry sediment/hr

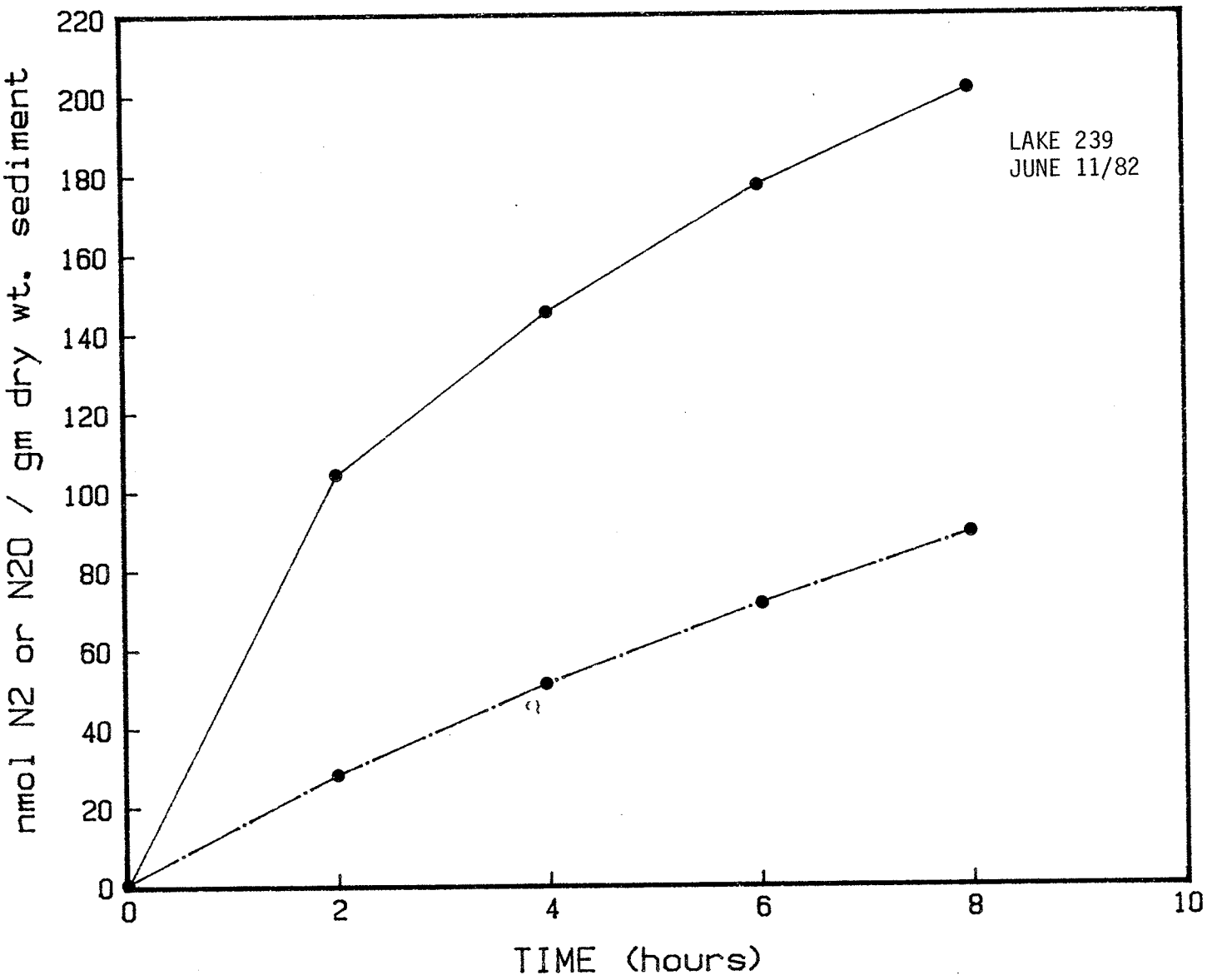

220
200
180
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10
nmol N2 or N2O / gm dry wt. sediment
TIME (hours)
LAKE 239
JUNE 11/82

Figure 14. Production of N-15 N2 in the absence of C2H2 and N20 in the presence of 5% C2H2 in Lake 239 sediment slurries (July 15/82).

_____ N2 production 30.54 nmol/gm dry sediment/hr
__.__ N20 production 16.62 nmol/gm dry sediment/hr

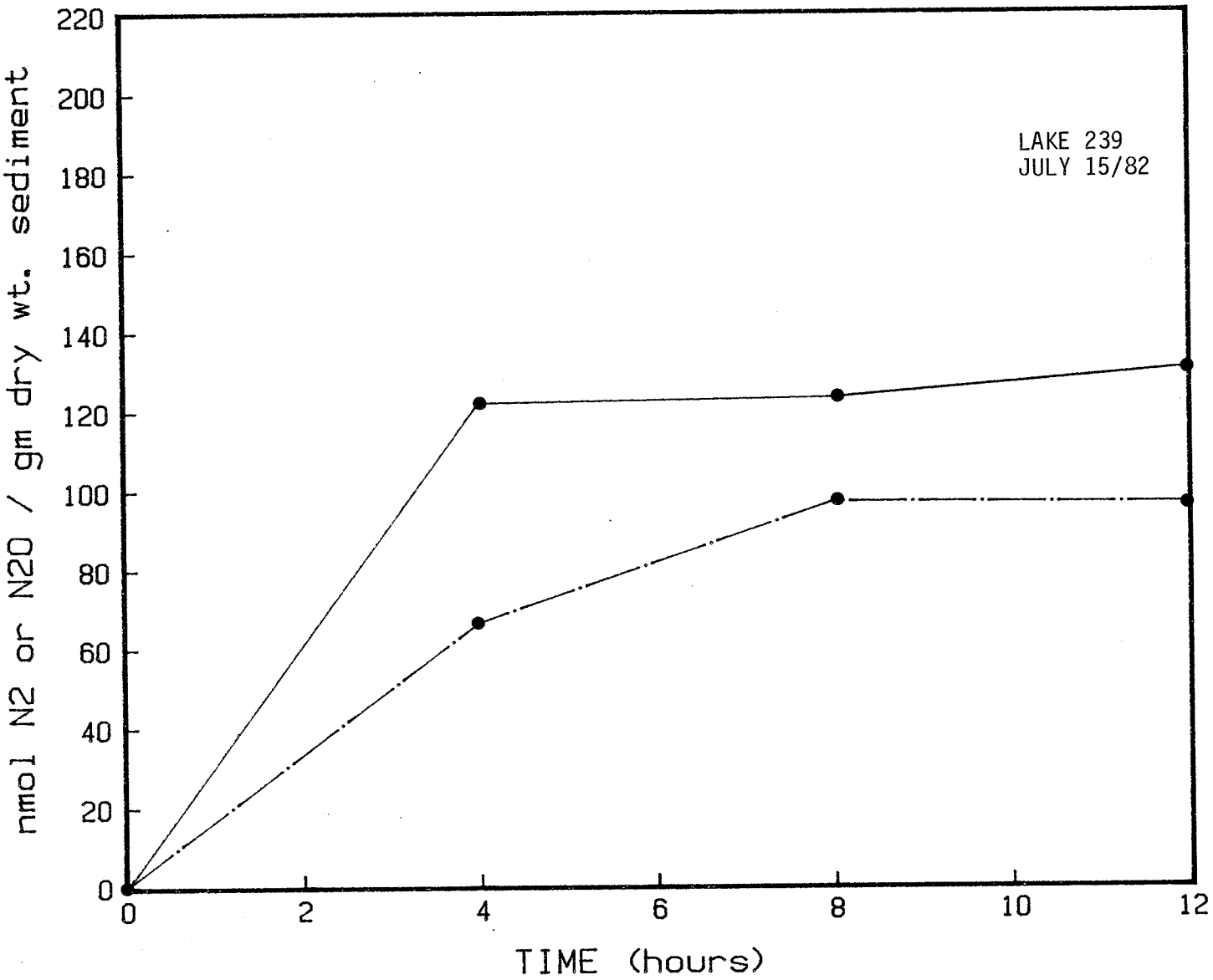
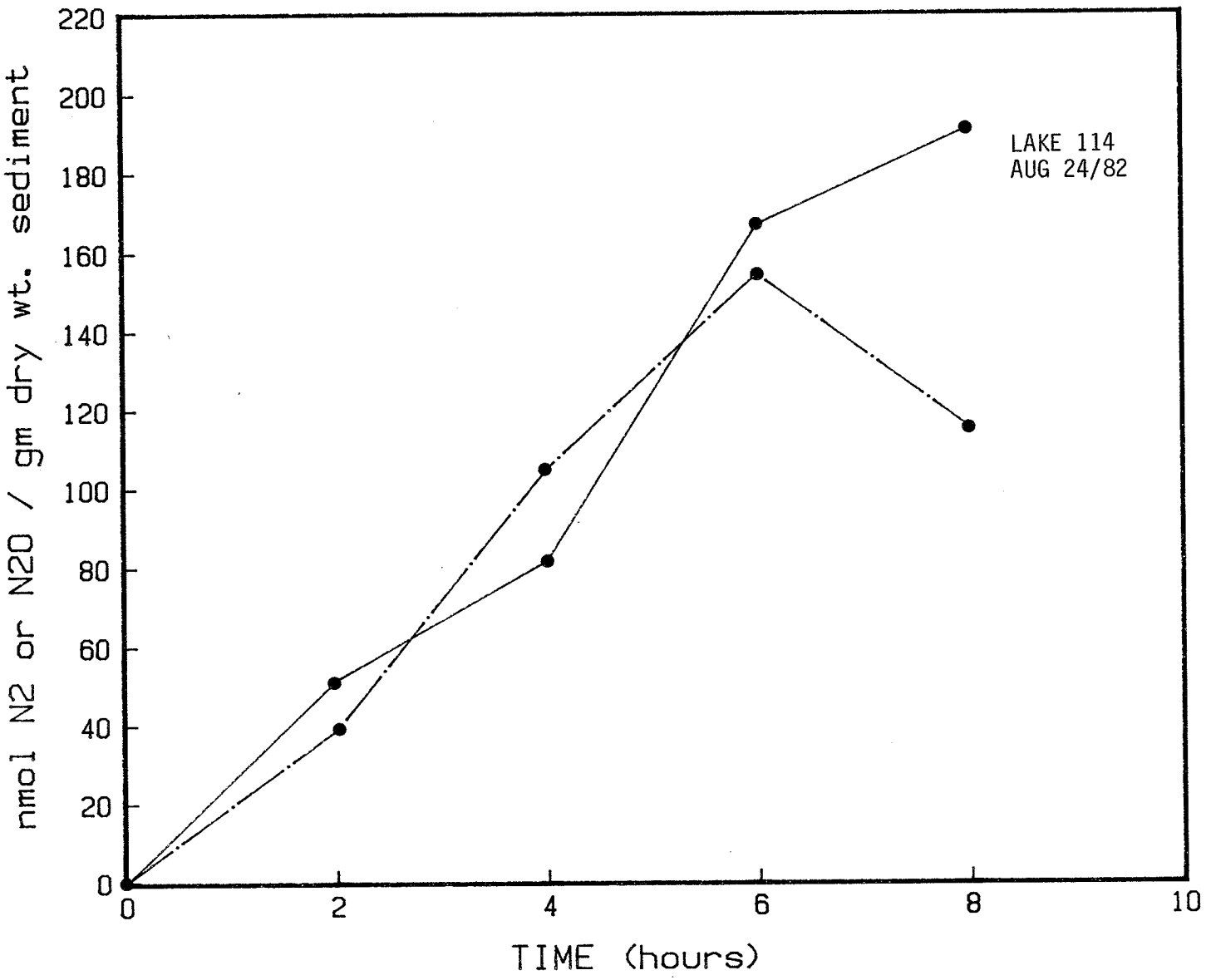

LAKE 239
JULY 15/82
nmol N2 or N20 / gm dry wt. sediment
TIME (hours)
0
20
40
60
80
100
120
140
160
180
200
220
0
2
4
6
8
10
12

Figure 15. Production of N-15N2 in the absence of C2H2 and N20 in the presence of 5% C2H2 in Lake 114 sediment slurries (Aug. 24/82).

_____ N2 production 27.81 nmol/gm dry sediment/hr
__.__ N20 production 25.71 nmol/gm dry sediment/hr


220
200
180
160
140
120
100
80
60
40
20
0
nmol N2 or N2O / gm dry wt. sediment
0
2
4
6
8
10
TIME (hours)
LAKE 114
AUG 24/82

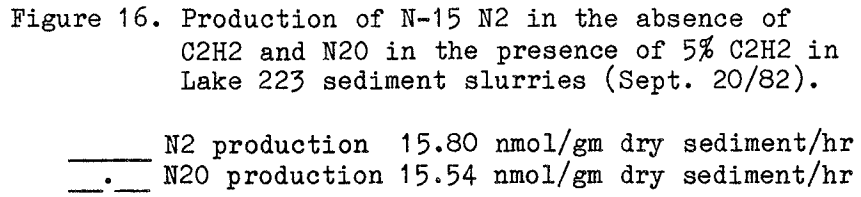


Figure 16. Production of N-15 N2 in the absence of C2H2 and N20 in the presence of 5% C2H2 in Lake 223 sediment slurries (Sept. 20/82).

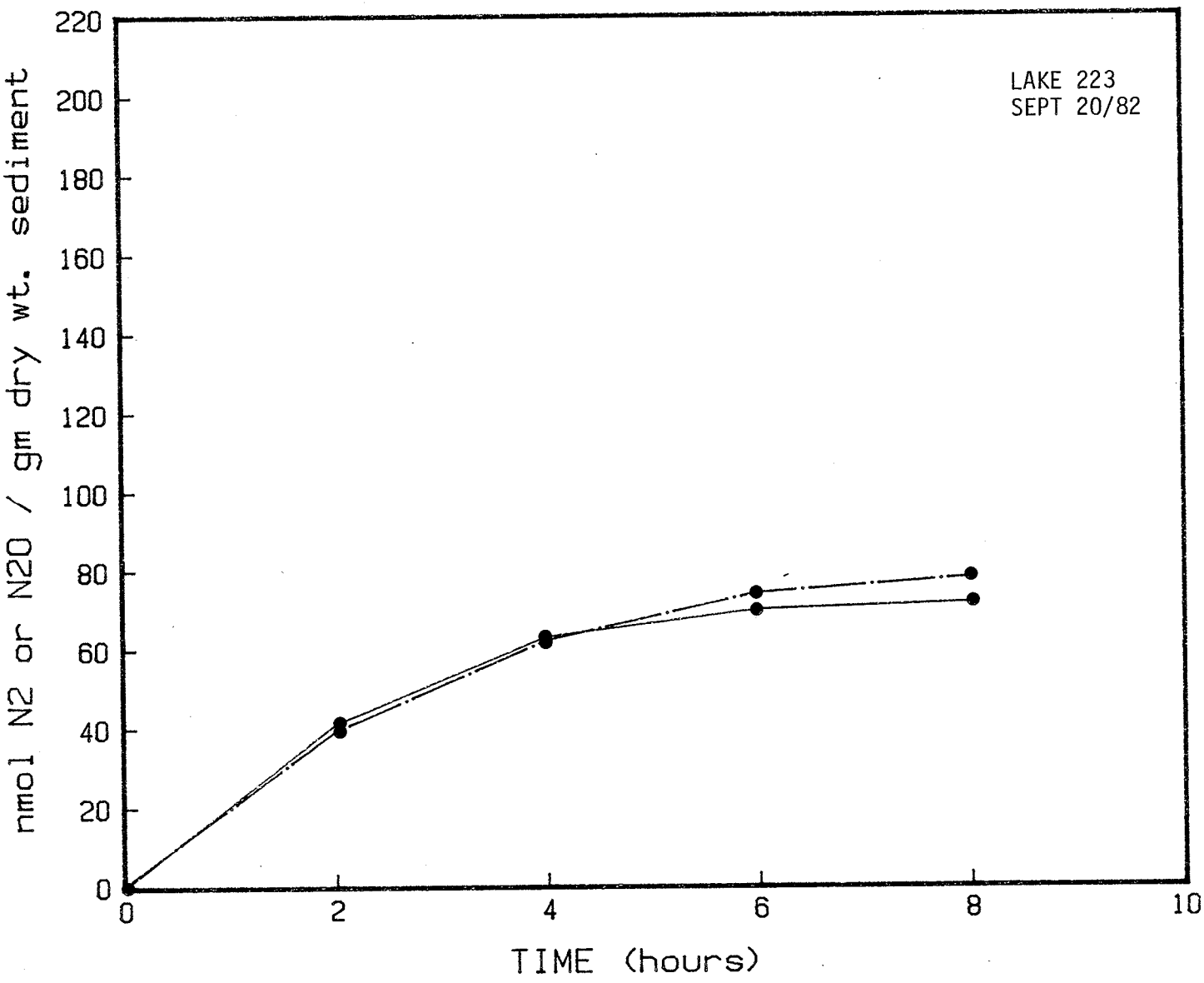

LAKE 223
SEPT 20/82
nmol N2 or N2O / gm dry wt. sediment
TIME (hours)
0
20
40
60
80
100
120
140
160
180
200
220
0
2
4
6
8
10

Figure 17. Production of N-15 N2 in the absence of C2H2 and N20 in the presence of 5% C2H2 in Lake 302N (5 M) sediment slurries (Sept. 21/82).

_____ N2 production 20.91 nmol/gm dry sediment/hr
__.__ N20 production 17.48 nmol/gm dry sediment/hr

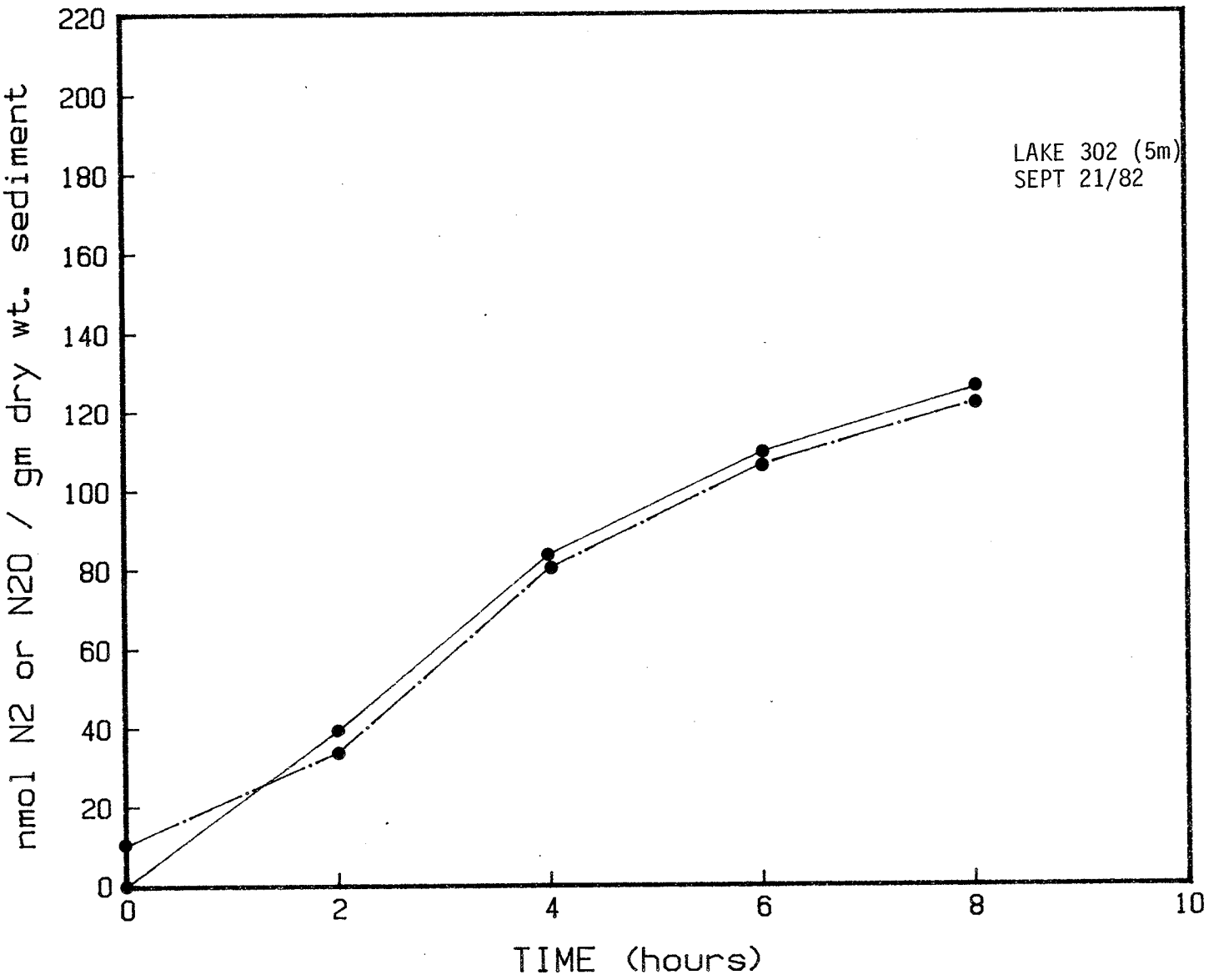

LAKE 302 (5m)
SEPT 21/82
nmol N2 or N20 / gm dry wt. sediment
TIME (hours)
0
20
40
60
80
100
120
140
160
180
200
220
0
2
4
6
8
10

Figure 18. Production of N-15 N2 in the absence of C2H2 and N20 in the presence of 5% C2H2 in Lake 302N (3 M) sediment slurries (Sept. 21/82).

_____ N2 production 8.73 nmol/gm dry sediment/hr
__.__ N20 production 9.21 nmol/gm dry sediment/hr

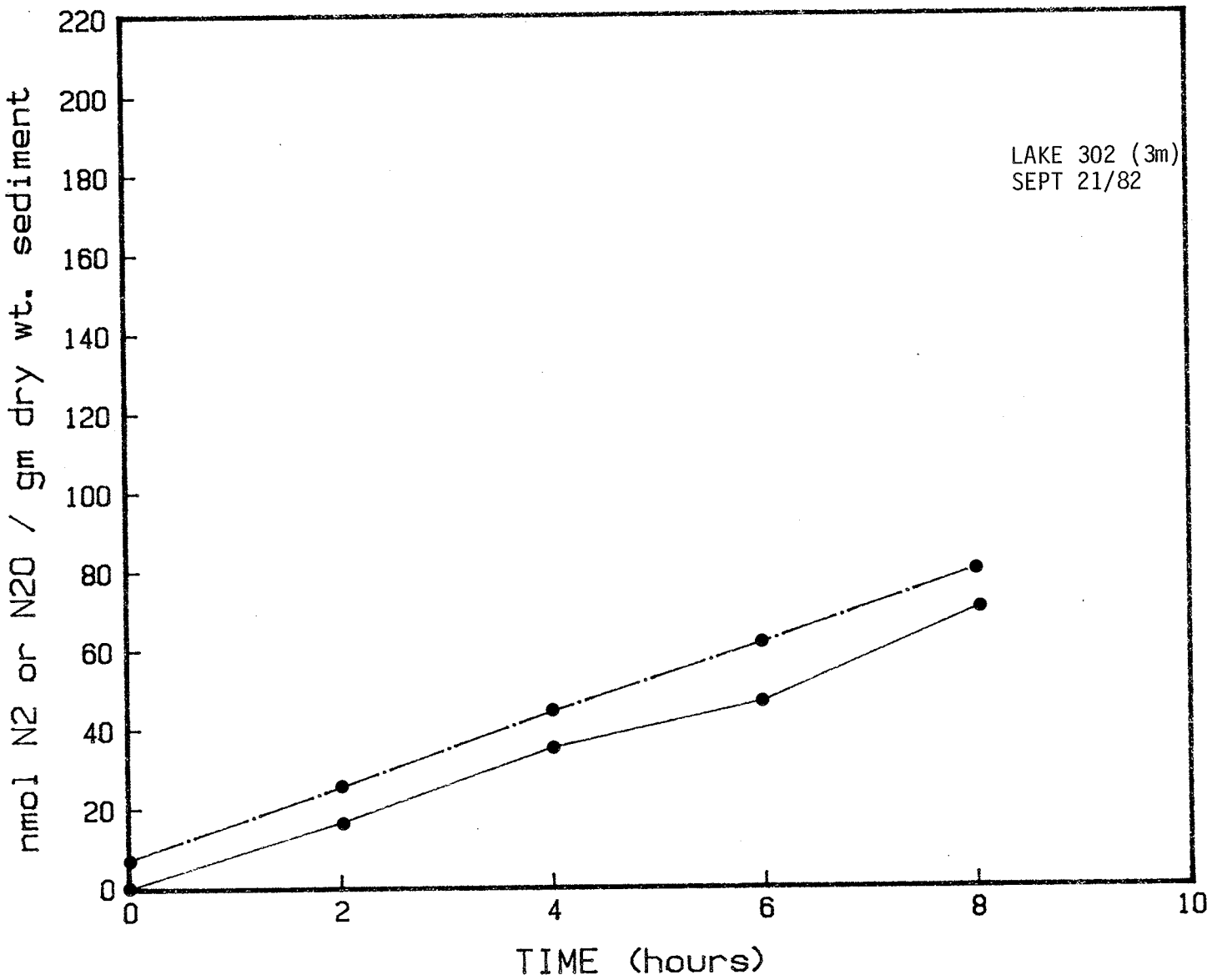

LAKE 302 (3m)
SEPT 21/82
nmol N2 or N2O / gm dry wt. sediment
TIME (hours)
0
20
40
60
80
100
120
140
160
180
200
220
0
2
4
6
8
10

Table 4. Summary of denitrification rates for epilimnetic sediments as measured by the acetylene inhibition technique (N_2O production) and the N-15 method

Lake	Date	initial nitrate nmol/g dry wt	N_2 nmol/g dry wt/hr	N_2O nmol/g dry wt/hr	N_2O/N_2
239	26/05/82	613	20.56	11.63	.57
239	11/06/82	559	36.31	12.72	.35
239	15/07/82	382	30.54	16.62	.54
114	24/08/82	134	27.81	25.71	.92
239	20/09/82	62	15.80	15.54	.94
302(3m)	21/09/82	380	20.91	17.48	.84
320(5m)	21/09/82	324	8.73	9.21	–

In comparing the two methods in Lake 239 sediment slurries (Figures 12 to 14), the acetylene inhibition method consistently resulted in denitrification rates 2 to 3 times lower than those determined by the N-15 method. During another assay (May 26, 1982 ; Table 4), rates determined by the C_2H_2 and N-15 method were 11.63 nmoles N_2O/g dry sediment and 20.56 nmoles N_2/g dry sediment respectively. During the course of the incubation these rates remained constant. In the same sediments assayed on June 11 and July 15 the inital rates were of the same order of magnitude (Table 4) however after four hours of incubation these rates decreased. In the July 15 assay there was almost a complete shutoff of the N_2 production. It is not sure why these systems shut down as there was still sufficient nitrate (>150 nmol /g dry sediment).

This descrepency between the two methods was not observed for Lakes 114, 223 and 302N sediments tested (Figures 15 to 18; Table 4). Rates determined by the acetylene inhibition for sediment slurries from these lakes were within 10% agreement with the rates of N_2 production.

Previous comparisons of the acetylene inhibition method to N-15 and N-13 tracer studies in marine sediment slurries (1.5 umol NO_3^-/g sediment) ,and soils slurries (8 ppm NO_3^-) respectively (Sorensen 1978a; Smith et al. 1978) concluded that the acetylene inhibition technique was reliable for estimating rates of denitrification in these systems. More recently workers have found that the acetylene inhibition method could result in an underestimate of denitrification

capacities. VanRaalte and Patriquin (1979) applied the acetylene inhibition technique to salt marsh sediments and found that total gaseous nitrogen production was as much as 20 times higher in the absence of acetylene than in its presence. Messer and Brezonik (1983) in a comparison of denitrification rate estimate techniques in Lake Okeechobee, Florida found that the acetylene inhibition method generally underestimated the rate of denitrification by .1 to .5 times. Both studies concluded that acetylene was an inconsistent inhibitor of nitrous oxide reduction. VanRaalte and Patriquin (1979) suggest that the lower rates measured by the acetylene inhibition method may be due to a change in the microbial population such that bacteria are capable of reducing N_2O in the presence of C_2H_2.

In this study the validity of the acetylene inhibition method appears to be dependent on the nature of the sediment. It is not certain if the discrepancy between the acetylene inhibtion technique and the N-15 method in Lake 239 sediments is a result of the chemical characteristics of the sediment or, as indicated by the pure culture work in this study, it could be a result of the particular denitrifying species present in the sediments microbial population. In an experimental trial in which slurries were analyzed for the production of N-15 dinitrogen in the presence of 5% acetylene no N-15 dinitrogen was detected. This finding suggests that the lower rates of denitrification determined by the acetylene inhibition method resulted from an inhibition of the overall reaction and is not the consequence of limited inhibition of nitrous oxide reduction to

dinitrogen gas. It has been established that acetylene inhibits nitrifying bacteria in soils (Walter et al. 1979) and methane producers and oxidizers as well as N/2/ fixing bacteria (Knowles 1979 ; Flett et al. 1975). Knowles (1979) found that concentrations as low as 0.1 atm C_2H_2 completely inhibited methane production in freshwater sediments. Therefore it is not unrealistic to think that acetylene could be toxic to certain denitrifying bacteria or to other members of the community on which denitrifiers are dependant. This could account for the Lake 239 results. This suggestion requires further investigation. A point that should be brought forth at this time is that because of the inhibitory influence of acetylene on microbial processes which occur in the same vicinity as denitrification and which could influence denitrification rates, the technique should be limited to short term incubations.

Sediment slurry studies used in this work have provided a means for comparing the acetylene inhibition technique using the N-15 method for comparison. Sediment slurries however do not always provide a reliable method for estimating 'in situ' rates of denitrification. The method causes disruption of the sediment's chemical and physical structure when the sediments are homogenized. From sedimentary pore water studies in Lake 302N (J. W. M. Rudd, unpublished data) it was observed that nitrate concentrations within the epilimnetic sediments were greatest at the sediment water interface and decreased rapidly down into the sediment to less than 0.07 umoles NO3-N / L at 1 cm in some instances. In sediments such as these diffusion of nitrate into

the sediments is likely a limiting factor for denitrification. Therefore, in sediment slurries where the sediments are homogenized denitrification rate estimates could result in higher rates due to thorough mixing of nitrate throughout the sediment. It is important that not only a reliable method for estimating denitrification be found but also a reliable incubation technique.

From this study it was concluded that the acetylene inhibition method is not always appropriate for estimating denitrification rates in freshwater sediments and that the ^{15}N method is a more reliable method.

CORE INCUBATIONS

CORE INCUBATIONS

Introduction

Denitrification in lake sediments is mediated by denitrifying bacteria where rates of activity are controlled by the distribution of bioavailable organic matter, nitrate and nitrite in the sediment (Kaspar 1982). Distribution of nitrate and nitrite in sediment pore waters is a function of their concentrations in the overlying water, their diffusion rates in the sediment, and rates of bacterial nitrification and denitrification (Nishio et al. 1983). Disruption of the sediment structure can enhance denitrification by disturbing chemical and physical parameters. Mixing of sediments increases the availability of nitrate and/or organic matter throughout the sediment (Messer and Brezonik 1983). To obtain realistic values for rates of *in situ* denitrification it is necessary that the integrity of the sediment be maintained.

Various methods have been developed to estimate denitrification in sediments which minimize the disturbance of the sediment structure and associated chemical and physical changes. Chan and Knowles (1979) rested a plexiglas chamber on the sediment surface and circulated water containing acetylene through the chamber and estimated denitrification rates by the accumulation of N_2O. Previous to this work Chan and Campbell (1980) used the same system following $^{15}N_2$ production from labelled nitrate in the absence of acetylene. Kaplan et al. (1979) set a bell jar on the sediments and measured N_2 production. Seitzinger et

al. (1980) measured N_2 production from undisturbed cores in a gas tight chamber. Nishio et al. (1982) followed N_2 production in undisturbed cores using a continuous- flow sediment water system and a N-15 tracer technique. Sorensen (9178b) injected acetylene saturated distilled water directly into sediment cores and followed nitrous oxide production.

The use of acetylene in intact cores has not been previously evaluated. The purpose of this study is to determine the applicability of the acetylene in undisturbed cores using a N-15 technique in the absence of acteylene, as a standard for comparison.

Presently at ELA Lake 302 is being used in a whole lake acidification study. The two basins are separated by a plastic curtain. Since the midsummer of 1982 the south basin has received additions of sulfuric acid to the epilimnion and the north basin nitric acid. The purpose of the whole lake experiment is to study the limnological processes which could result in consumption or removal of these acid species from the system.

In the north basin emphasis is being placed on the removal of nitrate from the system which could contribute to lake alkalinity (Kelly et al. 1982). Theoretically denitrification could result in a permanent removal of nitrate from the system as nitrogen gas which could escape to the atmosphere. To study the contribution of denitrification in the system, reliable in situ methodologies are required. For this reason the use of the acetylene inhibition method

in core incubations is evaluated using a N-15 technique for L302N sediments.

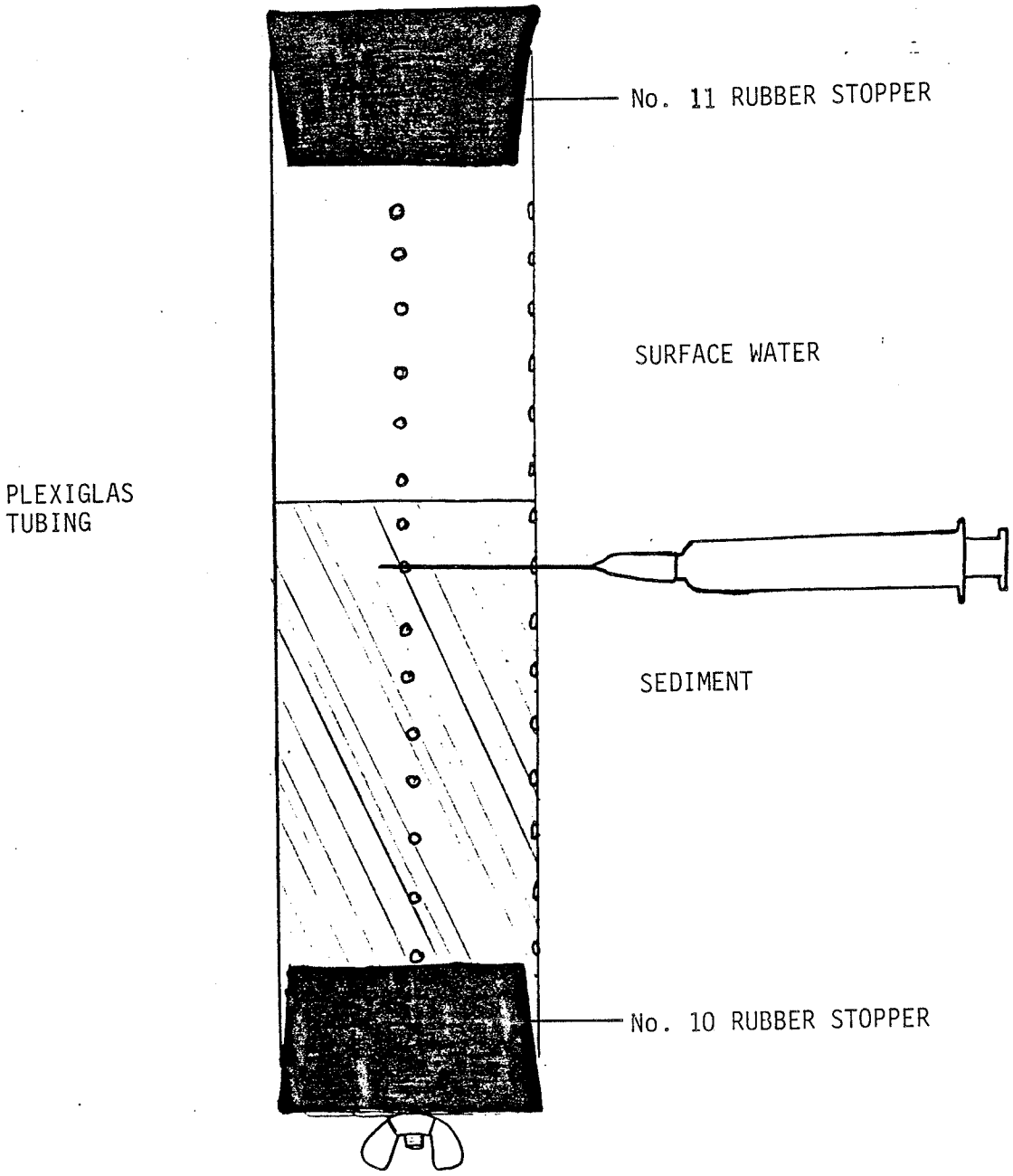
Methods

SCUBA was used to collect cores from the north basin of Lake 302 at a depth of three meters. The core tubing was constructed from Plexiglas tubing (ID 5 cm) cut into 25-30 cm lengths. The cores were sealed with a no. 11 stopper at the top and a no. 10 stopper at the bottom. The no. 10 stopper was modified by drilling a hole through the centre of the stopper and layering two washers, a 4 cm and a 2 cm washer on either side of the stopper held secure with a bolt and wing nut. Tightening or loosening of the wing nut adjusted the diameter of the stopper to facilitate tightening or loosening of the stopper. Intact cores consisted of 6-7 cm of sediment with 5-6 cm of water above.

The cores were returned to the laboratory submerged in lake water to eliminate problems of leakage of pore water through the bottom of the core. Experiments were initiated within two hours after the cores were collected.

The core tubing had two vertical series of 3 mm holes drilled at 1 cm intervals and at 45 degree angles to each other. These holes were filled with silicone sealant and served as ports for injecting acetylene into the cores (Figure 19). For cores in which denitrification rates were to be assayed by the acetylene inhibition method, 5 injections of .2mL of acetylene saturated distilled water were injected horizontally through each pair of ports. The injections were made in five directions at each 1 cm interval by slowly

Figure 19. Injection of core through the silicone ports along the side of the core.


No. 11 RUBBER STOPPER
SURFACE WATER
PLEXIGLAS
TUBING
SEDIMENT
No. 10 RUBBER STOPPER

withdrawing the needle through the sediment as the plunger was depressed (Figure 19). This facilitated diffusion of acetylene throughout the core sediment. At the time that the acetylene was injected into the sediments it was added to the overlying water. The final concentration of acetylene in the core was 10% (v/v).

Cores in which denitrification rates were estimated by the N-15 method received injections of degassed distilled water in the same manner that acetylene was added to the other cores to maintain consistency between the two treatments.

All cores received additions of N-15 labelled KNO_3 (96 atoms% N-15) to their surface waters yielding a final concentration of 20-100 umoles NO_3^--N / L surface water. Denitrification activity tends to be confined to the surface layers of the sediment, the optimal location being the sediment-water interface (Sorensen 1978b). Therefore diffusion of the $K^{15}NO_3$ into the active zone of denitrification should not be unrepresentitive; NO_3^- concentration is probably the limiting factor. Diffusion studies in sediment cores utilizing tritium demonstrated that diffusion of ions into these sediment is representative in 3-4 hours (C. Kelly personal communications).

Surface waters were sampled for nitrate concentration at the start of each experiment. The samples were analyzed by the automated method of Stainton et al. (1974). Before the cores were resealed, a volume of surface water was removed from each core so that a 1-2 cm air pocket remained at the top after the stopper was replaced. This was to

prevent the suface water from going anaerobic during the incubation. Periodically the surface water was analyzed at the end of an incubation for oxygen using a semi-micro technique (Rudd et al. 1976) to ensure that O_2 was not depleted.

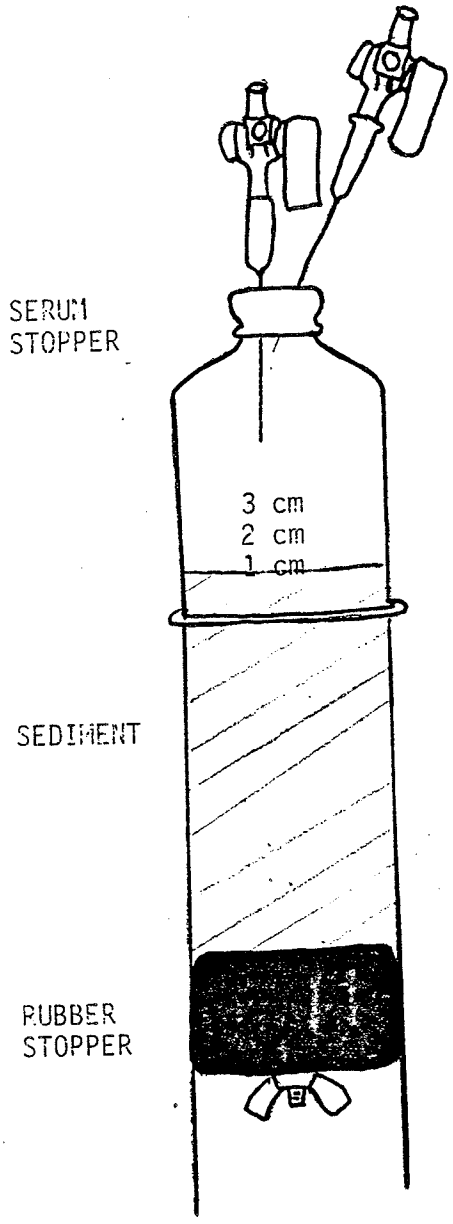
The cores were resealed and incubated at in situ lake temperatures submerged in water. Incubations were carried out for 12 or 24 hours at which time the air pocket and surface water were sampled for analyses. With the stopper in place a 5 mL gas sample was withdrawn from the air pocket using a 5 mL glass syringe fitted with a 23 G needle which was inserted through one of the sideports. The syringe was sealed by partially inserting the needle into a silicone stopper. The top stopper sealing the core was removed and after gently stirring the surface water 25 mL of the surface water was sampled using a 50 mL glass syringe fitted with an 18 G needle. Cores receiving acetylene plus nitrate additions were analyzed for nitrous oxide production and cores recieving additions of nitrate and not acetylene were analyzed for N-15 dinitrogen production. For water samples to be analyzed for nitrous oxide a 25 mL headspace of 5 % CH_4 in a balance of Ar was introduced in to the syringe and the liquid and gas phases equilibrated by shaking the syringe vigorously for 1-2 minutes. A subsample of the gas phase was analyzed and the concentration of nitrous oxide in the surface water was calculated as described in the general methods section. For $^{15}N_2$ analysis on the surface water the same procedure was employed with the exception that a headspace of ultra pure nitrogen gas was used.

After all of the surface water was removed from the core, a gas equilibration vessel was placed on the top of the core tubing (Figure 20a). This vessel was designed so that its inner diameter was equivalent to the inner diameter of the core tubing. Two 18 G, half inch needles fitted with teflon 3-way stopcocks were inserted through the Suba Seal which sealed the top of the vessel. To prevent atmospheric contamination during extrusion of the core sediment into the vessel, the vessel was continuously flushed. This was accomplished by having both 3-way stopcocks opened to the vessel and the atmosphere and flowing the gas through one into the vessel and out the other. For cores which received acetylene additions to be analyzed for nitrous oxide production , the system was flushed with 5 % CH_4 in a balance of Ar and for cores not receiving acetylene additions ultra pure nitrogen was used.

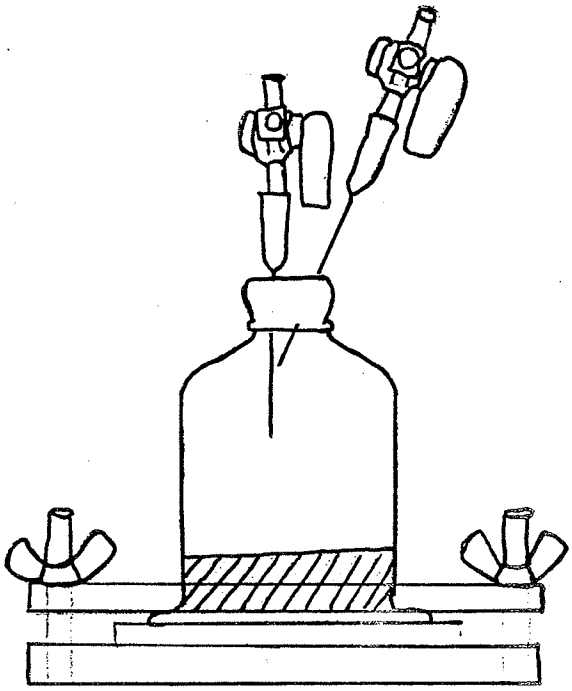
With the equilibration vessel in place and being continuously flushed the sediment core was forced up into the vessel to the desired depth by loosening the wing nut in the bottom stopper and applying an upward pressure on the stopper. The sediment segment was then sliced using two stainless steel knives lying flat against each other. One edge of each of the knives was sharpened to facilitate in slicing of the sediment. The width of the knives was greater than the diameter of the core tubing. The two knives were slipped between the equilibration vessel and the plexiglas core tubing. The equilibration vessel was then lifted off the core tubing with one of the knives serving as the bottom of the vessel and the other sealing off the top of the remaining

Figure 20 a. Extrusion of core into the gas equilibration vessel. The two needles at the top of the vessel were used to flush the core continuously during the process.

b. Equilibration vessel held secure in plexiglas holder



A.



B.

sediment core to prevent diffusion of gases in or out of the sediment.

Flushing was discontinued at this point by closing both 3-way stopcocks to the vessel. The vessel containing the sediment segment was transferred from the stainless steel knife to a ground glass plate smeared with silicone grease. The lip around the bottom of the glass equilibration vessel was constucted of ground glass so that a gas tight seal could be obtained. The whole apparatus was held secure between two plexiglass plates held together with screws positioned at of the corners (Figure 20 b).

To facilitate in extraction of the gases from the sediment 10 mL of degassed distilled water was injected into the equilibration vessel through one of the 3-way stopcocks while the other was opened during the injection to prevent a build up of gas pressure in the vessel. Both valves were then closed and the vessel shaken for 2-3 minutes to strip the gases from the core segment into the headspace above. A 5 mL sample of the headspace was withdrawn using a 10 mL glass syringe fitted with a 3-way stopcock. This was connected to one of the stopcocks of the equilibration vessel. Both of these were opened to the vessel and a gas sample was withdrawn. As the gas was drawn out of the vessel degassed distilled water was injected into the vessel through the other 3-way stopcock to maintain a positive pressure in the equilibration vessel.

One to 3 cm segments were extruded from each core such that the top 3-4 cm of sediment were analyzed. In cores receiving acetylene additions nitrous oxide production was assayed and those not receiving C_2H_2 were assayed for $^{15}N_2$ production. Both analyses were described previously in the general methods section.

Rates of denitrification were expressed per as rates of N_2O or N_2 accumulation per m^2 sediment surface.

Results and Discussion

In the initial development of the core incubation methodologies, cores were collected from four limnocorrals being used for an acidification experiment. In brief. the limnocorrals or tubes as they shall be referred to as, consisted of plastic tubing anchored into the epilimnetic sediment in Lake 302N. The average depth of each tube was 1.4 m with a diameter of 5m. Two of the tubes (tube 2 and4) were acidified with HCl the remaining two were left at lake pH (~pH7). Attempts were made to try and maintain tubes 2 and 4 at a pH below 6 by almost daily additions of HCL. All four tubes received sulfate (100 umol) and nitrate (100 umol) additions to their surface waters. The disappearence of the two ions was followed during the period extending from June 10/82 when the additions were made, to the end of the experiment on July 19/82. Cores were collected from two or all of the tubes at three different times during the course of the experiment. All cores collected from the tubes were assayed using the C_2H_2 inhibition method. No nitrate additions were made to any of these cores.

Results of these incubations are given in Table 5. The first set of cores assayed was collected prior to any nitrate additions to the tubes to determine background measurements. Distribution of N_2O production within this set of cores is shown in Figure 21. Figure 22 illustrates the distribution of activity for cores collected on 28/06/82, 18 days after additions of nitrate to the tubes. In both cases N_2O production was greatest in the upper most cm of sediment.

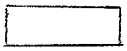
Table 5. N_2O Production in cores collected from tubes in Lake 302N during June 9, 1982 to July 20, 1982.

* single cores analyzed

** duplicate cores analyzed

date	denitrification rate determined by C2H2 method umoles $N_2O/m^2/d$	surface water nitrate (umol)	porewater (0-1cm) nitrate (umol)
9/06/82**			
tube 1	1	<.07	<.07
tube 2	2	<.07	<.07
28/06/82*			
tube 1	229	55	46
tube 2	294	53	15
tube 3	149	51	23
tube 4	246	69	34
20/07/82*			
tube 1	93	23	4
tube 2	173	32	1

Figure 21. Distiburtion of N_2O production in single cores collected from Tubes 1 and 2 (June 9, 1982) and incubated in the presence of 10% C_2H_2.

Tube 1 

Tube 2 

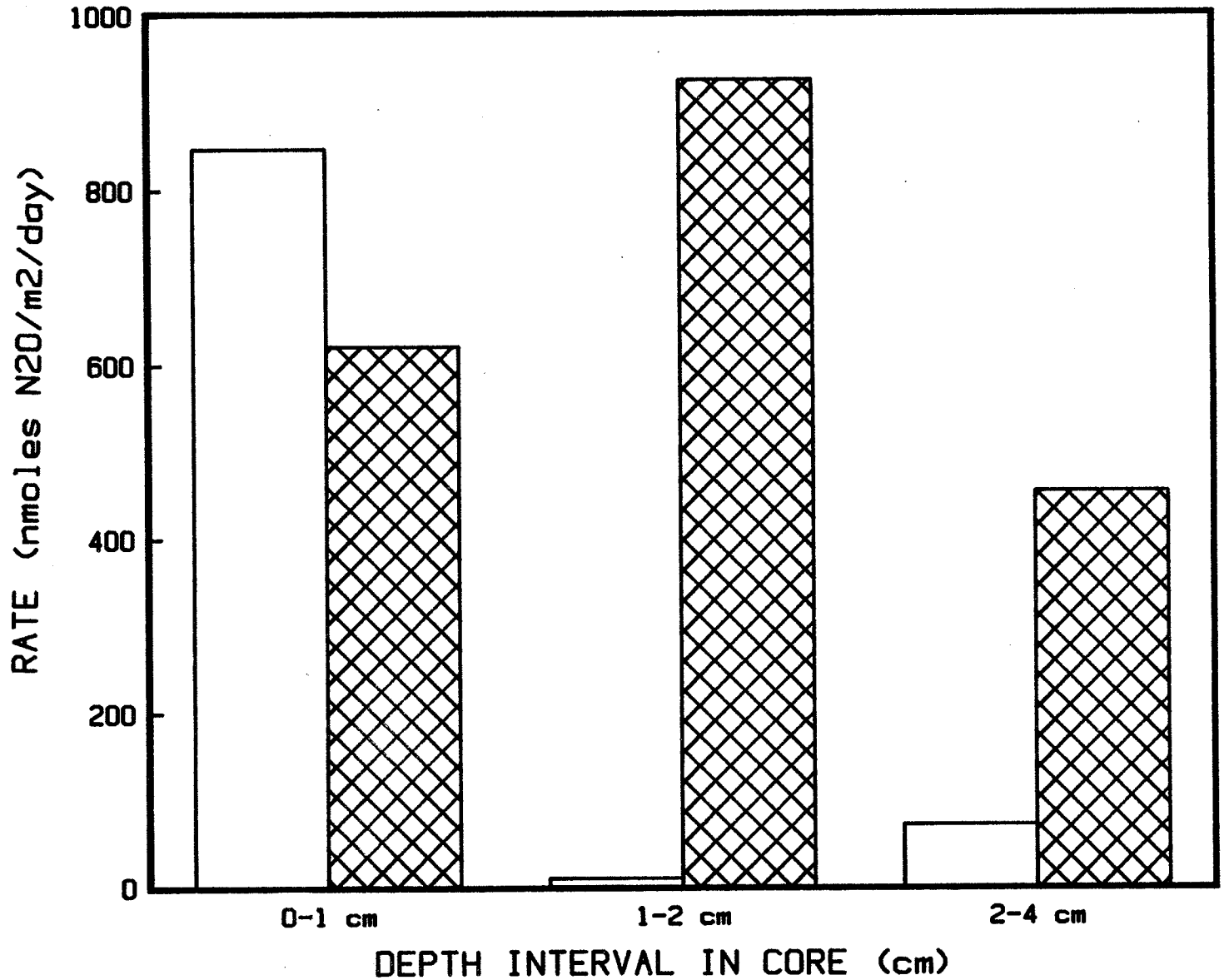
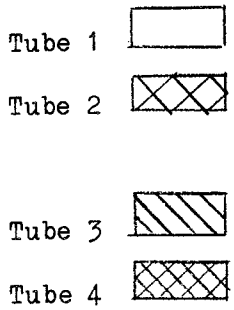
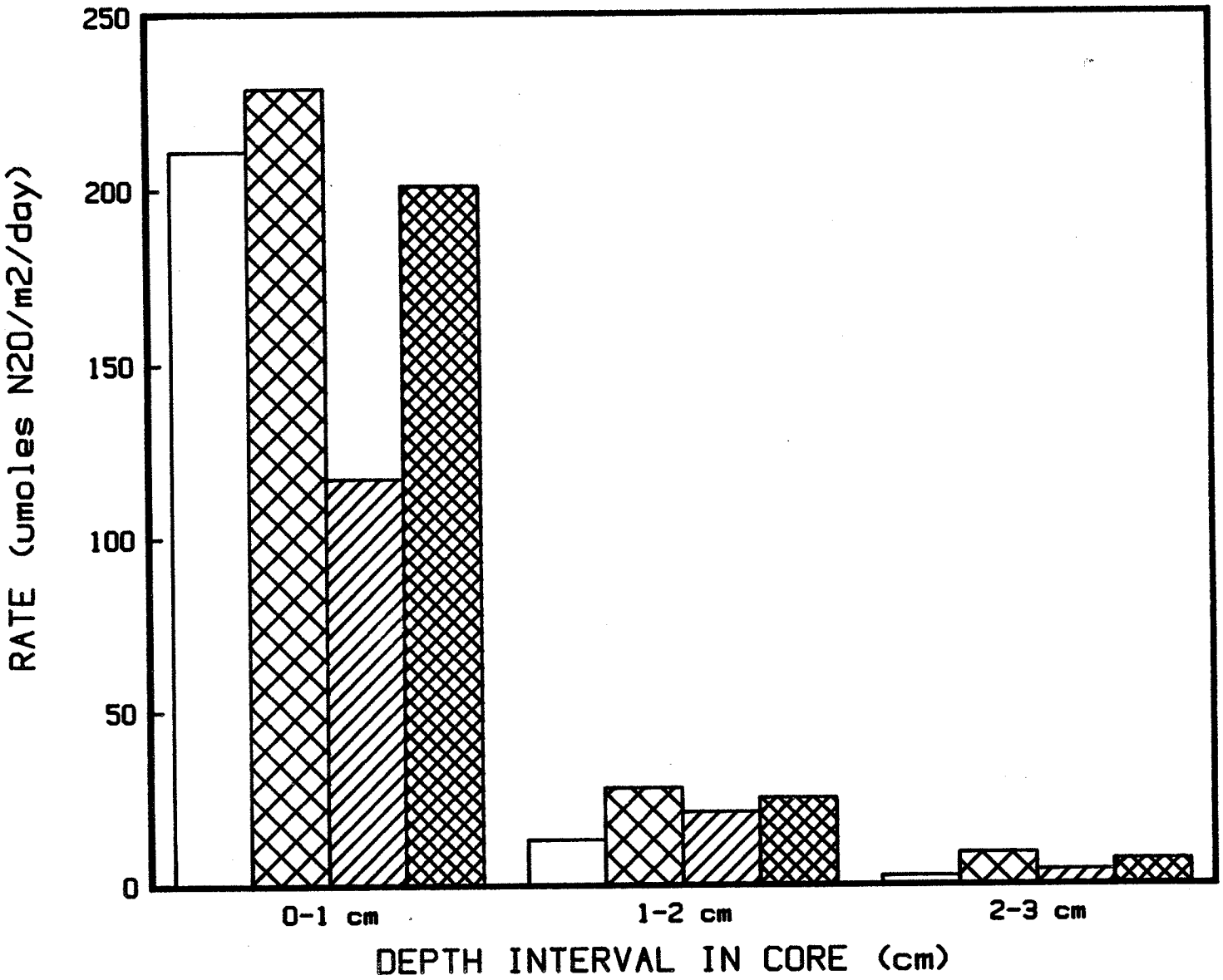

1000
800
600
400
200
0
RATE (nmoles N20/m2/day)
0-1 cm
1-2 cm
2-4 cm
DEPTH INTERVAL IN CORE (cm)

Figure 22. Distribution of N_2O production in single cores collected from Tubes 1, 2, 3, and 4 (June 28, 1982) and incubated in the presence on 10% C_2H_2.




250
200
150
100
50
0
RATE (umoles N20/m2/day)
0-1 cm
1-2 cm
2-3 cm
DEPTH INTERVAL IN CORE (cm)

The purpose of these initial experiments was to develop the coring methods fully before attempts were made to evaluate use of the C_2H_2 inhibition method in cores using the N-15 methods. At the same time it is evident from these results that denitrification rates within the tube sediments increased following the nitrate additions however there were no distinct differences between the acidified and the nonacified tubes.

Evaluation of the actylene inhibition method and its application to core incubations were performed on cores collected from Lake 302 N outside the region of the tubes.

Collection of the cores was coordinated with days that in situ pore water samplers in the vicinity of the sampling site were being analyzed.

In cores collected from Lake 302N on July 16, 1982 which received additions of 100 umoles 15N03-N / L to the surface , peak denitrification activity as determined by both the acetylene inhibition technique and the N-15 method was observed in the top first cm of sediment (Figure 23). The rates expressed for the top cm were calculated as a total of the rate of accumulation of N-15 dinitrogen in the air pocket, surface water and top cm of sediment for cores that did not receive additions of acetylene and the rate of nitrous oxide production in these three compartments for cores that did receive acetylene additions. In cores analyzed by the N-15 method approximately 95 % of the denitrification activity was observed in the

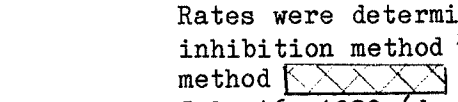
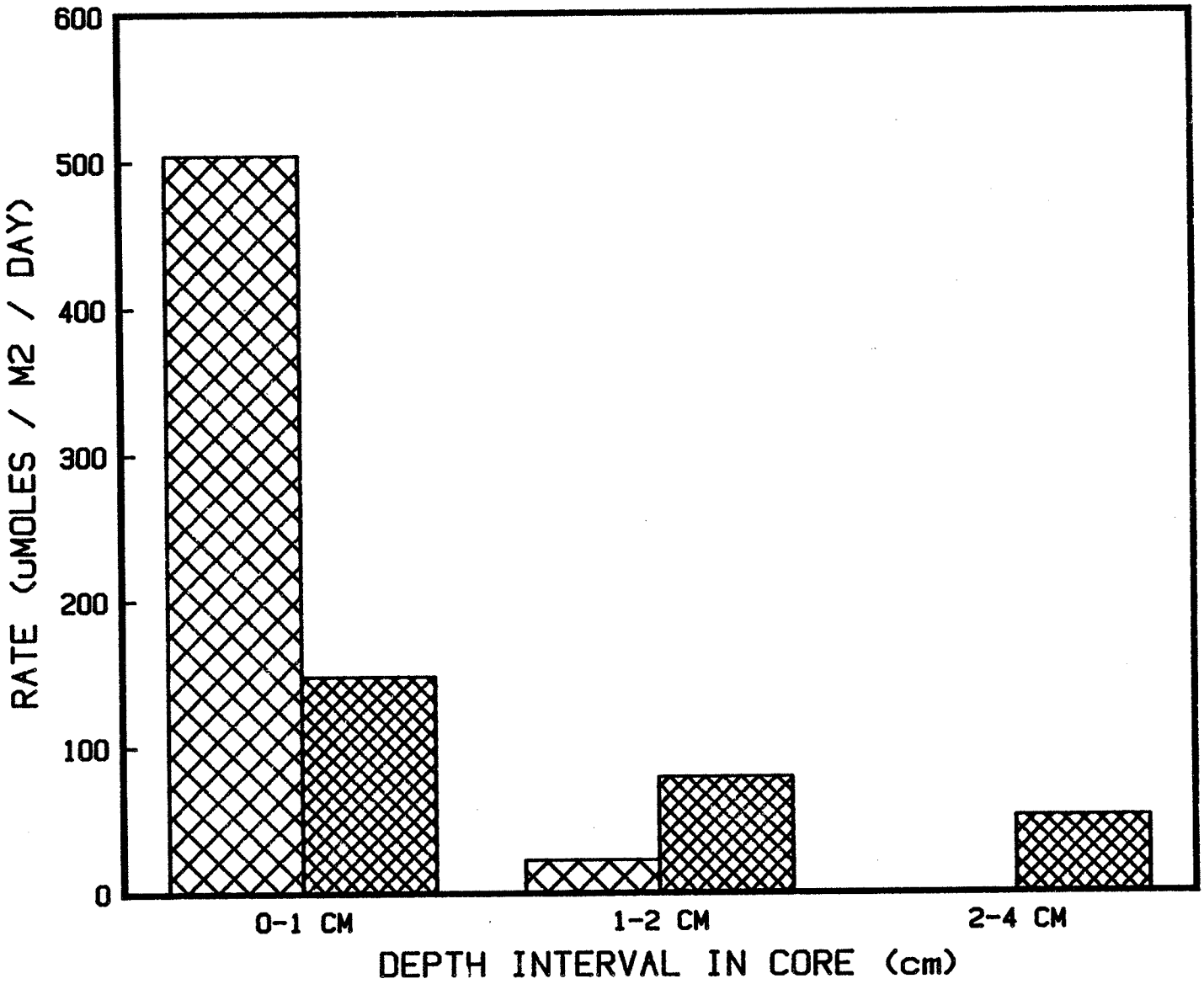


Figure 23. Average distribution of denitrification activity in lake 302 sediments with 100 umoles NO3- -N added to core surface waters. Rates were determined by the acetylene inhibition method and the N-15 method for cores collected July 16, 1982.(duplicate cores for each analysis)


600
500
400
300
200
100
0
RATE (uMOLES / M2 / DAY)
0-1 CM
1-2 CM
2-4 CM
DEPTH INTERVAL IN CORE (cm)

top cm of sediment and no dinitrogen production was observed below 2 cm. In cores analyzed by the acetylene inhibition method total rates of denitrification were lower and only 65 % of the total activity was found in the top cm of sediment.

Denitrification is an anaerobic process therefore these results suggest that O_2 is depleted just below the sediment-water interface. The occurrence of maximum activity in the top cm of sediment agrees with nitrate pore water profiles in the same area from which the cores were obtained. A typical nitrate profile is illustrated in Figure 24. Peak nitrate concentrations were found in the water immediately overlying the sediment and decrease sharply with increased depth into the sediment to below detection limits (1ug NO3-N / L) at one cm. The steep gradient suggests that most of the nitrate reduction occurs in the top cm of sediment showing agreement with the core incubations.

Table 6 lists the incubation data for the distribution of N-15 dinitrogen production throughout a core receiving additions of 100 umoles $^{15}NO_3^-$-N / L surface water and the distribution of nitrous oxide production in a typical core receiving acetylene as well as nitrate additions.

Rates of denitrification for all the Lake 302 cores collected in late summer of 1982 are summarized in Table 7. The N-15 method resulted in rates of denitrification 2 -20 times higher than the acetylene inhibition method. Rates measured in these cores showed a high degree of variability between replicate cores for the N-15

Figure 24. Nitrate concentration in lake 302 sediments at 3 M measured with an in situ pore water sampler (Hesslein 1976). Profile is for July 16, 1982.

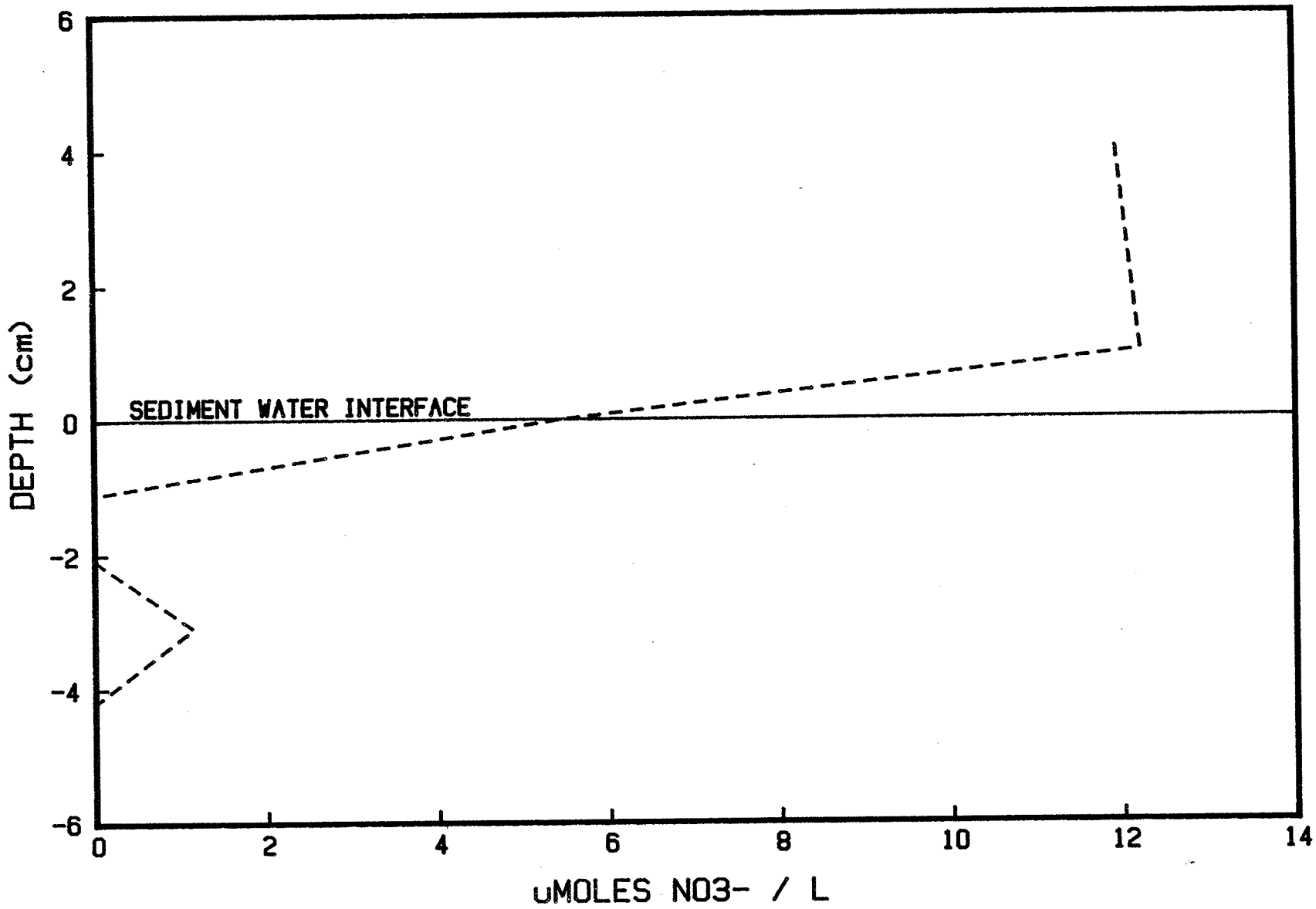

6
4
2
0
-2
-4
-6
DEPTH (cm)
SEDIMENT WATER INTERFACE
0
2
4
6
8
10
12
14
uMOLES NO3- / L

Table 6. Distribution of denitrification activity throughout single cores as determined by the acetylene inhibition and N-15 methods. The two cores compared recieved additions of 100 umoles NO_3^- -N/L to the surface water.

core segment	nmol N_2/core segment/day	% total	nmol N_2O/core segment/day	% total
air pocket	6.9	0.7	6.4	1.2
H20	769.6	74.0	201.6	37.6
0-1 cm	220.3	21.2	83.2	15.5
1-2 cm	43.5	4.2	155.1	29.0
2-4 cm	0	0	102.0	19.0

Table 7. Summary of denitrification rates for Lake 302N core incubations using the C_2H_2 inhibition (N_2O production) and the N-15 method (N_2 production). (duplicate or triplicate cores analyzed)

Date	NO3- in surface water (uM)	% N-15 enrichment	N2 production umol/m2/day	N20 production umol/m2/day
Jul 16/82 (2 cores)	100	96	551 +/- 322	237 +/- 23
Aug 19/82 (3 cores)	20	15	319 +/- 25	64 +/- 6
Sept 15/82 (3 cores)	43	44	350 +/- 100	53 +/- 10

analyses. The variability is of the same order of magnitude as found by Kaspar (1982) in sediment cores and previously reported in soils (Rolston et al. 1979). This high degree of variability could be attributed to sediment heterogenety as differences in the amount of sedimented algal material and distribution of small invertebrates between cores was observed.

In summary the results show lower denitrification rates obtained by the acetylene inhibition method. Injections of acetylene were done through the side of the core in five directions to facilitate an even distribution throughout the sediment. Because 5 % acetylene was effective at inhibiting nitrous oxide reduction in Lake 302N sediment slurries (previous section) it was assumed that 10 % acetylene would be effective in cores, however the acetylene inhibition rates were consistently lower than rates determined by the N-15 method. Referring back to Figure 21 denitrification activity within cores receiving acetylene occurred deeper in the sediment than in cores not receiving acetylene. This suggests that NO3 was not reduced as quickly in this core such that it diffused deeper into the sediment for activity to occur. The decreased activity in these cores agrees with the findings of VanRaalte and Patriquin (1979) who found that the presence of acetylene depressed total nitrogenous gas production in salt marsh sediments.

The core incubation technique provides a realistic approach for estimating in situ denitrification rates but the use of the acetylene inhibition method in this type of incubation was found to be

unreliable.

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

Various techniques have been developed for estimating denitrification rates in sediments. Most measurements rely on indirect methods because of the difficulties in measuring dinitrogen production directly. Messer and Brezonik (1983) used a mass balance approach in which losses of nitrogen not accounted for in a freshwater lake were attributed to denitrification . Nishio et al. (1981) attempted to measure denitrification activity in marine sediments from increases in the N2/Ar ratio in the sediment pore water. The use of N-15 tracer techniques for estimating denitrification rates in sediments has been widespread (Chen et al. 1972; Koike and Hattori 1978a,b; Oren and Blackburn 1979). Since the finding that acetylene inhibits the reduction of nitrous oxide to dinitrogen gas in soils (Fedorova et al. 1973) and verification of the inhibition in pure cultures of denitrifying bacteria (Yoshinari and Knowles 1976; Balderston et al.1976) the progressive accumulation of nitrous oxide in denitifying systems incubated in the presence of acetylene has been used as a measure of denitrification. The actylene inhibition method has been favoured in many studies (Sorensen 1978a, b; Chan and Knowles 1979) because it does not require addition of nitrate to the system under study and it is easy to assay for N_2O. There is a limited amount of evidence to demonstrate that the rate and extent of denitrification in the presence and absence of acetylene are equivalent.

In early studies evaluating the accuracy of the acetylene inhibition method in soil slurries using N-13 (Smith et al. 1978) and N-15 (Ryden et al. 1979) the acetylene yielded denitrification results equivalent to the tracer measurements. Sorensen (1978) evaluated the acetylene inhibition method in marine sediment slurries using N-15 nitrate as a standard for comparison and also concluded that the methods yielded similar rates. More recently VanRaalte and Patriquin (1979) have reported that the acetylene inhibition technique can result in underestimates of denitrification in salt marsh sediments. Messer and Brezonik (1983) observed that rates of denitrification as measured in intact cores by the acetylene inhibition method were as much as 50% lower than rates determined by a mass balance approach in a freshwater lake sediment.

In this study the acetylene inhibition method was applied to pure cultures, sediment slurries, and intact cores. Inconsistent results as to its accuracy for estimating rates of denitrification by evaluation with N-15 nitrate were observed. It is apparent from the pure culture study in this work that the acetylene inhibition technique is not reliable for certain denitrifying species. For isolates F and G the acetylene and the N-15 method showed good agreement but for isolates 4 and 9 the acetylene inhibition method resulted in rates > 90% slower than rates determined from $^{15}N_2$ production. For Isolate A initial rates of denitrification were similar, however, a three fold increase in activity after 6 hours was observed only for the culture grown in the absence of acetylene. The effectiveness of the method in sediment

slurries varied from lake to lake, agreement between the two methods was found for sediments collected from Lakes 114, 223, and 302. In Lake 239 sediments the acetylene method consistently underestimated rates by as much as 46 %. The results found here agree with those of Chan and Knowles (1979) who found that the degree of inhibition is dependent on the nature of the sediment. It cannot be concluded from this study if the discrepency between the two methods is related to the chemical nature of the sediment or to the species of denitrifiers present in lake 239 sediment. Yeomans and Beauchamp (1978) previously postulated that the effectiveness of the acetylene inhibition may be related to the number of denitrifiers and the presence of species capable of reducing nitrous oxide to dinitrogen in the presence of normally inhibitory concentrations of acetylene.

Tam and Knowles (1979) have suggested that the presence of sulfide in anaerobic sediments may account for the ineffectiveness of the acetylene inhibition method in certain sediments after determining that the presence of sulfide can relieve acetylene inhibition. Acid volatile sulfur is a magnitude higher in concentration in Lake 223 sediments than Lake 239 sediments(P. Ramlal personal communications). Free H_2S is almost nonexistent in E.L.A. lakes (C. Kelly personal communications). Therefore this cannot account for the Lake 239 results.

In situ denitrification is a function of carbon and nitrate availability (Firestone and Tiedje 1979) and sulfide distribution in sediments (Sorensen 1980) as well as the exclusion of oxygen.

Acetylene is known to be toxic to various microbial processes which occur concurrently with denitrification including nitrification, methane production, and nitrogen fixation (Hynes and Knowles 1978; Walter et al. 1979; Knowles 1979). Because of its toxic nature acetylene could slow down the whole sediment microbial community limiting the flow of energy, bioavailable carbon, and nitrate to denitrification resulting in an underestimate of activity.

To obtain realistic rates of denitrification it is necessary that the integrity of the sediment be maintained as disruption of the chemical and physical; incubations are generally carried out with homogenized sediments in determination of denitrification rates. More recently intact cores have been used for estimating the denitrification (Sorensen 1978b; Messer and Brezonik 1983). In this study intact cores collected from Lake 302 N were used to study denitrification. The core system was similar to that used by Sorensen (1978b) with the exception that a gas equilibration vessel was designed so that gases were analyzed immediately following incubation whereas Sorensen froze cores for later analyses. Sorensen using the acetylene technique in marine sediment cores with a maximum nitrate concentration of 160 uM in the upper 2 cm of sediment found rates of denitrification of 0.99mmoles N / m2 /day. These rates were not compared to any other techniques. To my knowledge the use of acetylene inhibition method in intact cores has not been rigorously evaluated. In this study rates of denitrification in cores as analyzed by the acetylene inhibition method were compared to rates measured using N-15 nitrate and following the production on

N-15 dinitrogen gas. Rates were underestimated by the acetylene inhibition method when compared to rates of N-15 dintrogen production in cores collected in the same region of Lake 302N. The discrepency between the methods in core incubations ranged from rates 2 -20 times higher being obtained by the N-15 method.

Reasons for the discrepency are not clear. Kaspar (1982) who applied acetylene to core sections observed that acetylene was not distributed thoughout the sediment in core incubations. In his study acetylene was added as a single injection to the sediment surface. In our study acetylene was added as a series of five injections in different directions at one cm intervals to facilitate diffusion and it was assumed that acetylene was evenly dispersed throughout the core. Based on rates of $^{3}H_2O$ diffusion into cores it appears that during the period of incubation used in this study, that C_2H_2 would have been completely distributed throughout the core (R. H. Hesslein and J. W. M. Rudd personal communications). Lower activity was consistently found in the cores receiving acetylene additions suggesting that acetylene may have a toxic effect or is not reaching the sites of active denitrification. The reaction between acetylene and sediment has not been studied but it is possible that the sediment particles may adsorb the acetylene preventing it from interacting with the denitrifying population.

Pure culture and sediment slurry studies are useful for studying individual microbial processes but are difficult to relate to environmental conditions. It is important that the chemical and

physical parameters of the sediment be disturbed as little as possible when estimating *in situ* rates of denitrification. Undisturbed cores permit incubation of the sediments with minimal disturbance to the sediment.

The results of this study indicate that the acetylene inhibition method, because of its inconsistent results is not a reliable technique for estimating denitrification in ELA lake sediments. The method could result in an underestimate of the denitrification potential of these sediments therefore use of the N-15 method is recommended.

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