DENITRIFICATION AND PHYTOPLANKTON ASSIMILATION OF NITRATE IN LAKE 227 DURING SUMMER STRATIFICATION

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

In Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

by
Yiu-Kwok Chan
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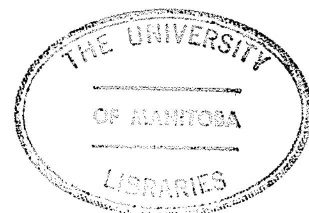

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A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

A rapid gas-extraction technique was developed and applied to assay *in situ* denitrification with ^{15}N-labeled NO_3^- in a small fertilized Canadian Shield lake during summer stratification periods in 1973, 1974, and 1975. The littoral sediment-water interface was assessed to be the most significant site of natural denitrification when compared with the oxygen-limiting (DO less than 0.2 mg liter^{-1}) upper hypolimnion and the anoxic hypolimnion. Dissolved oxygen and NO_3^- concentration were the main factors controlling denitrification in the water column and in sediments. Dinitrogen was consistently the predominant product of denitrification although NO_2^- and N_2O were occasionally formed in detectable quantities.

Littoral sediment denitrification rates estimated by *in situ* experiments at a high enrichment level averaged about 15 mg m^{-2} day^{-1}. Almost all of the NO_3^- that mixed into the surface sediment was denitrified. Alone, littoral denitrification was capable of removing 14% of the added NO_3^- annually although the observed disappearance of NO_3^- in the epilimnion largely resulted from phytoplankton assimilation during summer blooms. Results indicate that, below the euphotic zone, sediments provide an efficient sink for NO_3^- without simultaneous initiation of eutrophication.

As in limnetic denitrification, both the psychro-

trophic and mesophilic species of *Pseudomonas* isolated from lake sediments released N_2 as end product. A species difference was disclosed in that at the low temperatures equivalent to that prevailing in the hypolimnion of a stratified lake, little N_2O was accumulated by the psychrotroph while the mesophile accumulated N_2O at these temperatures.

Ambient NO_3^- concentration in Lake 227 surface waters was largely controlled by phytoplankton in the summer periods. Prior to 1975, the weekly NO_3^- addition was consumed through algal assimilation in about three days. Excretion of assimilated NO_3^- represented a significant portion of the nutrient incorporated by the cells. Hence, uptake rates of NO_3^- were underestimated when measured by ^{15}N retained in the particulate fraction alone. Only about 10% of the total NO_3^- assimilated could be accounted for as dissolved organic nitrogen by isotope assays. These excreted organic forms were predominantly serine and glycine in the dissolved free amino acid fraction. Bacteria as well as algae might be expected to contribute to and modify the extracellular nitrogen pool.

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ABBREVIATIONS

ATP....................Adenosine 5'-triphosphate

BOD....................Biochemical oxygen demand

DCMU...................3-(3,4-Dichlorophenyl)-1,1-dimethylurea

DFAA...................Dissolved free amino acids

DNA....................Deoxyribonucleic acid

DO.....................Dissolved oxygen

DON....................Dissolved organic nitrogen

E'_o....................Oxidation-reduction potential at pH 7.0

$\Delta F'$....................Free-energy-change at pH 7.0

K'_{eq}....................Apparent equilibrium constant

K_m....................Michaelis constant

K_t....................Half-saturation transport constant

NAD^+...................Nicotinamide adenine dinucleotide, oxidized form

NADH...................Nicotinamide adenine dinucleotide, reduced form

$NADP^+$..................Nicotinamide adenine dinucleotide phosphate, oxidised form

NADPH..................Nicotinamide adenine dinucleotide phosphate, reduced form

RNA....................Ribonucleic acid

TDN....................Total dissolved nitrogen

TPN....................Total particulate nitrogen

Wherever the symbol 'NO_3^-' is used it denotes nitrate without isotope enrichment.

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INTRODUCTION

INTRODUCTION

A major portion of this research thesis is based on investigations carried out at the Experimental Lakes Area (ELA) in northwestern Ontario (Johnson and Vallentyne 1971). Since 1966, an experimental limnology project launched there has been directed by the Freshwater Institute, Environment Canada. Initially, its primary objective was to study interactions between nutrients and lake eutrophication as well as means of eutrophication control. The background environmental data on ELA has been documented in a special issue of the Journal of Fisheries Research Board of Canada[1]. Progress in the various aspects of the research project has been reported in a series of thirteen papers published in 1973[2] and summarized by Schindler and Fee (1974).

Lake 227 (Fig. 1), one of the first experimental lakes of the area selected for long-term studies, has had its major nutrient budgets accurately manipulated. Prior to artificial nutrient loading, Lake 227 was oligotrophic by all standards. From 1969 to 1974 inclusive, the lake

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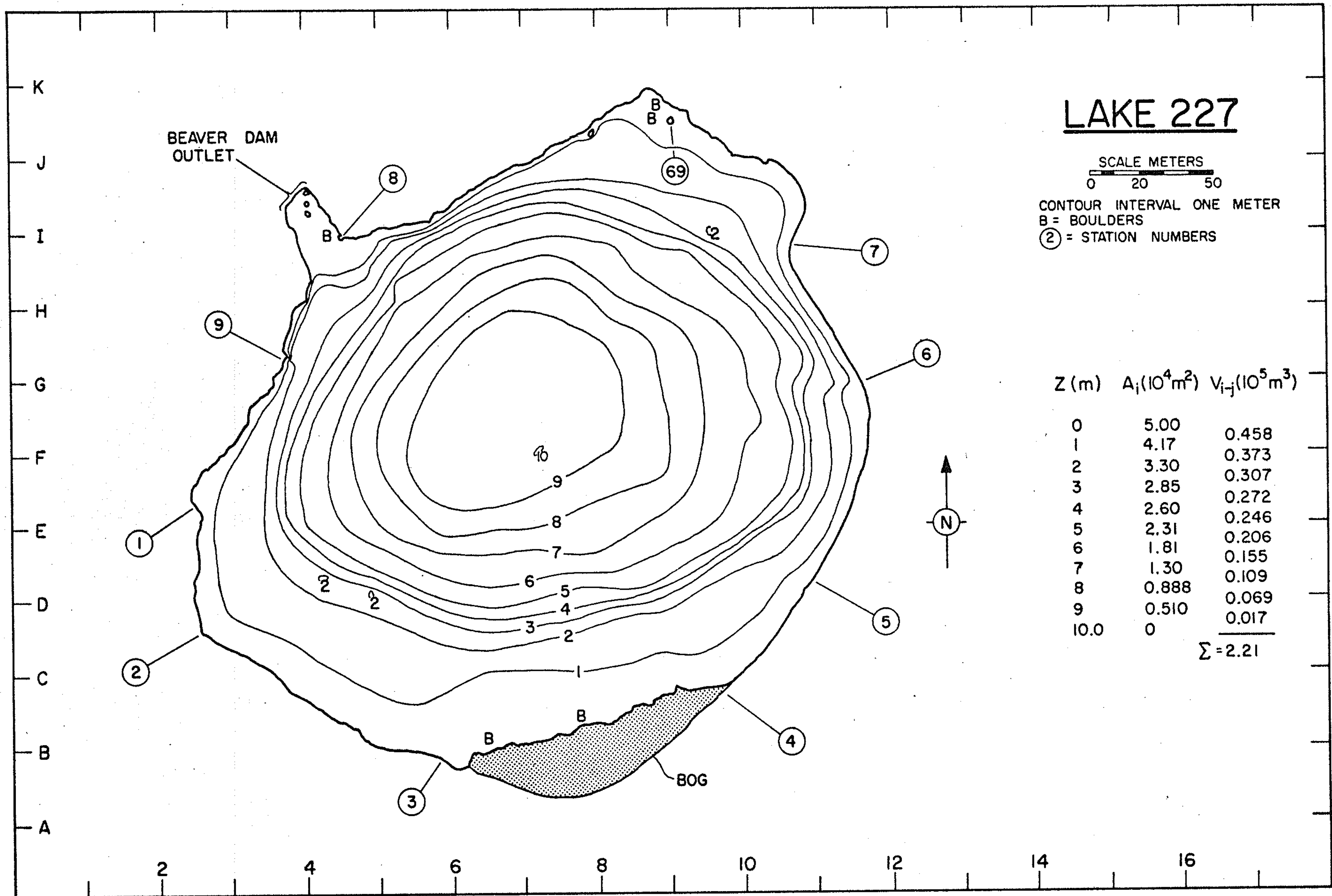
[1]Vol. 28, No. 2, February 1971.

[2]J. Fish. Res. Board Can. 30 (10), p.1409-1552 (1973).

Fig. 1. Bathymetric chart of Lake 227 (Brunskill and Schindler 1971).



LAKE 227
SCALE METERS
0 20 50
CONTOUR INTERVAL ONE METER
B = BOULDERS
(2) = STATION NUMBERS
BEAVER DAM OUTLET
BOG
N
Z (m) | A_i(10^4 m^2) | V_i-j(10^5 m^3)
0 | 5.00 | 0.458
1 | 4.17 | 0.373
2 | 3.30 | 0.307
3 | 2.85 | 0.272
4 | 2.60 | 0.246
5 | 2.31 | 0.206
6 | 1.81 | 0.155
7 | 1.30 | 0.109
8 | 0.888 | 0.069
9 | 0.510 | 0.017
10.0 | 0
Σ = 2.21

was enriched weekly with nitrogen as nitrate and phosphorus as phosphate (6.29 g N m^{-2} yr^{-1} and 0.48 g P m^{-2} yr^{-1}) during the ice-free periods (Schindler et al. 1971, 1973). The immediate effect can be described as experimental eutrophication and to date, Lake 227 remains highly eutrophic. Phytoplankton standing crop was near the theoretical maximum (Schindler et al. 1973).

Although the fertilizers added to the lake might provide adequate nitrogen, a supplementary input via biological fixation of atmospheric nitrogen had to be considered in drawing up a whole-lake nitrogen budget. Moreover, this nitrogen budget could also be complicated by denitrification, a process that releases dinitrogen from nitrate. This study was undertaken to assess the potential of denitrification as one component of nitrate-nitrogen removal. Emphasis was given to direct evaluation of in situ denitrification for the determination of sites of significant activity. The relatively stabilized conditions that pertain during summer stratification provide a defined period convenient for monitoring in situ denitrification rates.

Ambient nitrate and phosphate concentrations in Lake 227 remained low despite the artificial supply of these compounds, presumably due to phytoplankton consumption. A kinetic approach was adopted to characterize the algal population in Lake 227 with respect to nitrate

assimilation by means of a ^{15}N tracer technique. These kinetic data and evidence for a causal relationship between nitrate assimilation and excretion of organic nitrogen by the indigenous phytoplankton population are presented. An estimation of the comparative rates of denitrification and nitrate assimilation under natural conditions can be made from these studies.

HISTORICAL

HISTORICAL

Denitrification

Pioneers in the investigation of nitrate metabolism include Beijerinck (Verhoeven 1956), Gayon and Dupetit (1886) and Schloesing (1868) who studied the anaerobic conversions of nitrate and nitrite mediated by microorganisms. However, the evolution of dinitrogen in organic decomposition was reported as early as 1841 by Davy (Delwiche 1956). By the latter half of the 19th century, denitrification in soil had been observed and recognised as a reaction of nitrate in the presence of microorganisms. The evolution of nitrous oxide as well as dinitrogen was observed by Dehérain and Maquenne in the 1880's. This was later confirmed in the classical work of Gayon and Dupetit (1886) who further demonstrated that nitrate functioned as an 'hydrogen' acceptor for the microbial oxidation of organic substrates. In this reaction nitrite was formed as the first intermediate. By the beginning of the 20th century, denitrification was characterized as a respiratory process (Jensen 1904).

The capacity to carry out biological denitrification is restricted, as far as is known at the present time, to bacteria. In the most part, denitrifying bacteria are heterotrophic facultative anaerobes. <u>Pseudomonas</u> and

Bacillus species are regarded as preponderant among the nitrate respirers that give off dinitrogen while utilizing sugars, organic acids, and polyols as electron donors (Allen and vanNiel 1952; Federova and Sergeeva 1957; Rhodes *et al*. 1963; Sacks and Barker 1952; Verhoeven 1956). Certain strains of *Pseudomonas*, however, release nitrous oxide as the terminal product of denitrification (Payne 1976). Denitrification has also been found to be coupled with autotrophic processes such as sulfur oxidation in *Thiobacillus denitrificans* and *Thiobacillus thioparus* by Beijerinck in 1904, and with the oxidation of hydrogen in *Paracoccus denitrificans* (formerly *Micrococcus denitrificans*, Davis *et al*. 1969) by Kluyver and Verhoeven in 1954 (Delwiche 1956). A *Hyphomicrobium* species has been demonstrated to reduce nitrate or nitrite stoichiometrically to dinitrogen with the concomitant oxidation of methanol to carbon dioxide (Sperl and Hoare 1971). Kosaric and Zajic (1974) suspected that anaerobic oxidation of hydrocarbons coupled with denitrification might be carried out by certain bacteria that could be responsible for the biodegradation of complex hydrocarbons in nature. Because of the wide range of oxidisable substrates for denitrifiers, their distribution in nature is ubiquitous. They have been isolated from soil, freshwater and sea water, salt marsh, sediment, activated sludge, sewage, and even from pharyngeal and urinary sources (Hollis *et al*. 1972;

Payne 1973b).

Our knowledge of the role played by denitrifiers in their natural habitats has developed largely from studies of their physiological behavior. Although by 1882 denitrification had been reported by Dehérain and Maquenne as being an essentially anaerobic process (Delwiche 1956), the effect of oxygen on the reaction was first studied by Gayon and Dupetit (1868). Early reports on the oxygen effect were conflicting due to inaccurate measurement of dissolved oxygen concentration and to inadequacies in experimental design (Painter 1970). Oxygen has an inhibitory effect on denitrification in in general but anoxia is not a prerequisite for the reaction. The tolerance to oxygen varies among denitrifiers (Mechsner and Wuhrmann 1963). Denitrification in soil often occurs in the vicinity of nitrification activity (Greenwood 1962) which probably provides an anaerobic microenvironment enriched with nitrate for the denitrifiers. In whole-cell systems, synthesis and functioning of the denitrification enzymes are inhibited by oxygen but derepression of synthesis can be achieved by lowering the oxygen tension even in the absence of an oxide of nitrogen (Payne *et al*. 1971).

Biological denitrification by definition is restricted to the production of gaseous dinitrogen or nitrogenous oxides from nitrate, nitrite or any other appropriate oxidized intermediate (Nicholas 1963).

Payne (1973b) presented the currently accepted sequential reduction of nitrate as: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. Each step is catalysed by a complex enzyme system (Fewson and Nicholas 1961; Payne *et al*. 1971; Radcliffe and Nicholas 1970). Electron transport to nitrate and the intermediates is similar to that in aerobic repiration where cytochromes are involved (Nicholas 1963). Oxidative phosphorylation is coupled to the reduction of nitrate (John and Whatley 1970; Yamanaka *et al*. 1962) but phosphorylation linked to the reduction of nitrite and nitrous oxide has only been suggested (Koike and Hattori 1975). The biochemistry of denitrification has been recently reviewed by Delwiche and Bryan (1976) and Payne (1973b, 1976).

Denitrification has long been viewed as a nuisance in terms of soil-nitrogen economy (Delwiche 1970). In addition, recent concern has been expressed over the release of nitrous oxide to the atmosphere by denitrification (Johnston 1972; Junge and Hahn 1971) and hence a possible acceleration of erosion of the protective ozone layer in the stratosphere. This calls for a further more accurate evaluation of biospheric contribution to atmospheric levels of nitrous oxide (Delwiche and Bryan 1976). On the other hand, the seepage of undenitrified anionic nitrate through the soil column to ground water and its eventual entry into adjacent waterways and the sea constitute a source

of water pollution. The sources of this nitrate-nitrogen may not necessarily be solely from synthetic fertilizers. Soil organics, animal and human excreta, food processing residues and industrial wastes all may contribute to the nitrogen budget of water supplies (Alexander 1974). These provide an enormous quantity of nitrogenous material for microbial mineralization and oxidation to nitrate. In view of the hazards posed by the presence of excess nitrate in the human environment (Walters 1973), exploitation of bacterial denitrification to remove nitrate from waste waters including agricultural drainage waters has been well documented (Focht and Chang 1975; Francis and Callihan 1975). On the global scale, denitrification is viewed as a significant sink for fixed nitrogen, keeping the nitrogen cycle in balance. Delwiche (1970) estimated that about 30 million metric tons of nitrate was denitrified annually in the anaerobic marine environment. Evidently, with the ever increasing amount of industrially fixed nitrogen, doubts have been expressed towards the efficiency of natural denitrification in returning gaseous nitrogen to the atmosphere. However, the actual rates and exact conditions of denitrification in various areas of the biosphere are still not known.

Early measurements of denitrification in soils were often done by determining the loss of total Kjeldahl nitrogen from soils after incubation with nitrate or

nitrite(e.g. Bremner and Shaw 1958) before superior techniques by mass spectrometer and infra-red identification of the gaseous products were being developed (Arnold 1954; Wijler and Delwiche 1954). Because of the generally low nitrogen content of natural waters as opposed to soils, total nitrogen determination by the Kjeldahl method is not sensitive enough to be reliable for denitrification studies in these aquatic environments.

Lacustrine denitrification was first inferred from nitrogen balance studies on Lake Windermere (Mortimer 1939, in Hutchinson 1957). The following phenomena have been observed as manifestations of denitrification in natural waters: (a) development of a vertical dichotomic nitrate clinograde during summer stratification of lakes (Hutchinson 1957); (b) increase in dissolved dinitrogen-argon ratio (e.g. Richards and Benson 1961) or supersaturation of dinitrogen (e.g. Brezonik and Lee 1968); and (c) correlation between oxygen depletion and nitrate reduction (e.g. Brezonik and Lee 1968). When modern mass spectrometric and gas chromatographic techniques became widely available, they were applied to denitrification measurements in aquatic and marine systems (Goering and R.C. Dugdale 1966; Goering and V.A. Dugdale 1966) and in salt marsh sediments (Payne 1973a). However, the more sensitive mass spectrometric method is still less frequently employed to quantify denitrification rates in sediments

than in soils. Indeed, an N-15 method suitable for field studies and adaptable for *in situ* measurements of denitrification is a primary requirement. The recent finding that acetylene inhibits nitrous oxide reduction in denitrifying bacteria (Balderston *et al*. 1976; Yoshinari and Knowles 1976) leads to the notion of applying this inhibitory effect as a means of quantifying natural denitrification and for measuring the release of nitrous oxide from the biosphere. With improved techniques, more accurate estimates of potential denitrification in nature and studies of control factors of the process will be advanced.

Keeney (1972, 1973) reviewed the contribution of denitrification to the nitrogen cycle in aquatic ecosystems. Investigations have indicated that this biological process is a significant nitrogen sink in lakes (Brezonik and Lee 1968; Chen *et al*. 1972; Goering and V.A. Dugdale 1966; Keeney *et al*. 1971). To date, only few proper *in situ* ^{15}N denitrification assays have been made. For example, some investigators regarded bottle assays carried out by laboratory incubation as *in situ* denitrification measurements (Goering and V.A. Dugdale 1966). With an indirect ^{15}N tracer technique, Brezonik and Lee (1968) found rates of denitrification in the hypolimnion of Lake Mendota ranged from 8 to 26 μg N $liter^{-1} day^{-1}$. Koike *et al*. (1972) found that, although

denitrifying bacteria were uniformly distributed in the water column in a brackish lake irrespective of seasonal variation, their population density seldom exceeded 40 cells ml^{-1} and did not correlate with denitrification activities as measured by an N-15 tracer technique. Further, little denitrification was evident in waters with more than 1.8 mg dissolved oxygen $liter^{-1}$. Koike *et al*. (1972) concluded that denitrification rates were controlled by nitrate concentration, a point ignored by most workers. Apparently, no *in situ* sediment denitrification measurements by ^{15}N tracer have been documented. By nitrogen mass balance, Anderson (1974) estimated a net denitrification rate of 0-47 g N m^{-2} yr^{-1}(equivalent to 0-54% of total nitrogen input) in six connected non-stratified productive lakes and suggested that denitrification in the surface sediments was responsible. The relation between whole lake denitrification and factors such as lake morphometry and trophic state has not been investigated. When nitrate-containing ground water enters a lake,sediment denitrification plays an important role as a nitrogen sink (Keeney *et al*. 1971). Nitrate added to the sediments may also be immobilized. Apart from denitrification in soils (Broadbent and Clark 1965) and waste waters (Dawson and Murphy 1972), little information is available concerning the low temperature effect on the quantitative aspects of denitrification in aquatic

ecosystems. Chemodenitrification, the non-biological reactions of nitrite to give off gaseous nitrogenous oxide or dinitrogen, was observed in soils (Broadbent and Clark 1956; Delwiche 1956). However, its role in sediment denitrification has not been clarified.

Phytoplankton Assimilation of Nitrate

The first comprehensive studies of phytoplankton nutrition and production began in the 1920's following Naumann's publications on eutrophication of several lakes in Sweden (Hutchinson 1973). By 1941 Hutchinson had devised a bottle assay for measuring plankton production and had demonstrated the concept of cooperative increase in phytoplankton by the application of nitrate and phosphorus to a pond under investigation. Later, he concluded that combined nitrogen, phosphorus and silica were likely limiting production of phytoplankton (Hutchinson 1944). Thomas (1953, in Hutchinson 1967) found that while both nitrogen and phosphorus were limiting nutrients in the Alpine lakes he studied, phosphorus was significantly limiting in winter but nitrate might be more limiting during summer. It has been noted that oligotrophic lakes are not limited by nitrogen; in eutrophic lakes both nitrogen and phosphorus are important limiting factors (Thomas 1969). Recently, evidence is emerging that a natural long term corrective process for nitrogen deficiency in eutrophic lakes can be

achieved through blue-green algal fixation of atmospheric nitrogen (Schindler 1977).

It was previously thought that certain green algae and diatoms demonstrate a preference for nitrate over ammonia as a nitrogen source (Hutchinson 1957); this is now attributed to variations in molybdenum availability (Hutchinson 1967). Molybdenum, an essential constituent of the nitrate reductase enzyme complex, is required for the initial reduction of nitrate to nitrite. Ammonia is readily utilized by algae although many also utilize nitrate. It is the product of nitrate reduction via nitrite as an intermediate (Kessler 1959, in Brown *et al*. 1974). Assimilation of ammonia in eukaryotic algae is distinct from that in the prokaryotic types (Brown *et al*. 1974). Glutamate dehydrogenases are commonly found in eukaryotic algae either grown with nitrate or ammonia as nitrogen sources. In blue-green algae, all the possible enzymes of ammonia assimilation (viz. glutamate dehydrogenase, alanine dehydrogenase and glutamine synthetase/glutamate synthetase system) have been implicated.

According to Healey (1973) both nitrate uptake and reduction that constitute nitrate assimilation *in vivo* are probably separate reactions which may or may not link to each other. Although nitrate uptake and reduction by algae can occur in darkness, light stimulation of these processes has been reported for some time. The mechanisms involved in light stimulation are not fully known. It

has been suggested that in the light, photosynthesis supplies carbon skeletons for the assimilation of ammonia (Grant 1967). In Anabaena, reduction and assimilation of nitrate is stimulated by light through the photoreduction of ferredoxin (Candau 1976). The role of light in promoting nitrate assimilation could also be due to provision of ATP by photophosphorylation. Falkowski (1975) reported that the uptake of nitrate by marine phytoplankton appeared to be mediated by an anion-dependent ATPase. Again, the source of ATP may partly come from photophosphorylation. Observed diurnal periodicity in nitrate reductase activity or in rates of nitrate uptake is partly due to the direct effect of light.

Nitrate and ammonia uptake in phytoplankton cultures has been shown to follow the Michaelis-Menten initial velocity kinetics for enzyme systems (Dugdale 1967; Eppley et al. 1969a). This hyperbolic relation between uptake velocity and nutrient concentration also holds for in situ populations of marine phytoplankton (MacIsaac and Dugdale 1969). Since then, when Michaelis-Menten kinetics are obeyed for nitrate uptake in marine phytoplankton, kinetic parameters are utilized to characterize the species that are of ecological and economic importance. The uptake kinetics are also regulated by light and temperature (Eppley and Thomas 1969; MacIsaac and Dugdale 1972) reflecting that different algal species are well-adapted to their

environments and their needs in competition with one another for limiting nutrients (Eppley *et al*. 1969b). Half-saturation transport constants for nitrate and ammonia uptake by freshwater algal species have not been measured extensively nor used for predicting the relative ability of different species to consume low levels of nutrients. Goering (1972) cautioned that while the kinetic approach potentially helps to predict nutrient limitation in the natural environments, nutrient interaction such as inhibition of nitrate uptake by ammonia and simultaneous limitation of growth by other nutrients or trace elements must be considered in its application.

Most algae appear to excrete small quantities of amino acids, polypeptides, and proteins among other organic compounds (Fogg 1971). Walsby (1974) suggested that the release of certain peptides in *Anabaena* cultures might be linked with the dinitrogen fixation process, but the relationship between assimilation of inorganic nitrogen and the excretion of organic nitrogen has scarcely been examined. To date, only one report has directly related the release of dissolved free amino acids to nitrate assimilation by phytoplankton (Schell 1974). The factors controlling organic nitrogen release and its role remain obscure.

METHODS AND MATERIALS

METHODS AND MATERIALS

PART I. DENITRIFICATION

A. Development of ^{15}N Tracer Technique

Nitrogen-15 methodology for the quantitative study of denitrification mainly involves the determination of the major gaseous product of denitrification, viz. N_2. A procedure suitable for the study of both aquatic denitrification and denitrification by pure cultures has to satisfy the following requirements: (a) prevention of sample contamination by atmospheric O_2 and N_2 during sampling, when applying the labeled NO_3^-, and in all subsequent operations; and (b) reduced tediousness in sample preparation procedures for mass spectrometry. A rapid gas-extraction technique is particularly suited to aquatic profile studies where, usually, a large number of samples must be prepared for analysis in a short period of time. Such a technique was developed and adopted as standard procedure in this part of the study. It had been tested by application to denitrification assays in $K^{15}NO_3$-amended lake water samples and in pure culture experiments.

Extraction of Dissolved Gases

Before gaseous analysis, dissolved gases were extracted from biologically fixed water samples stored in standard 300 ml BOD bottles or from culture flasks. Each sample bottle had been fully filled (see 'Sampling' in

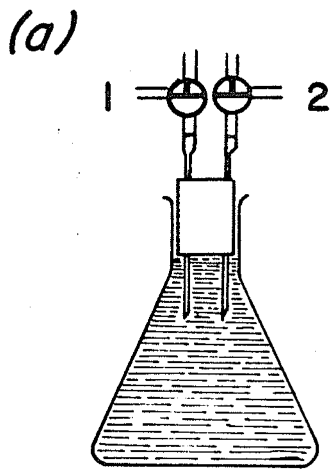
the 'Water Column' section, p.29) and tightly stoppered with a modified #3 rubber stopper. The height of the stopper had been reduced by boring a well halfway down its top to facilitate the insertion and retrieval of hypodermic needles through its core. In principle, the technique developed to extract dissolved gases involved the introduction of a gas phase into the bottle and allowing the dissolved gases to partition into the gas phase for equilibration. The procedure was as follows:

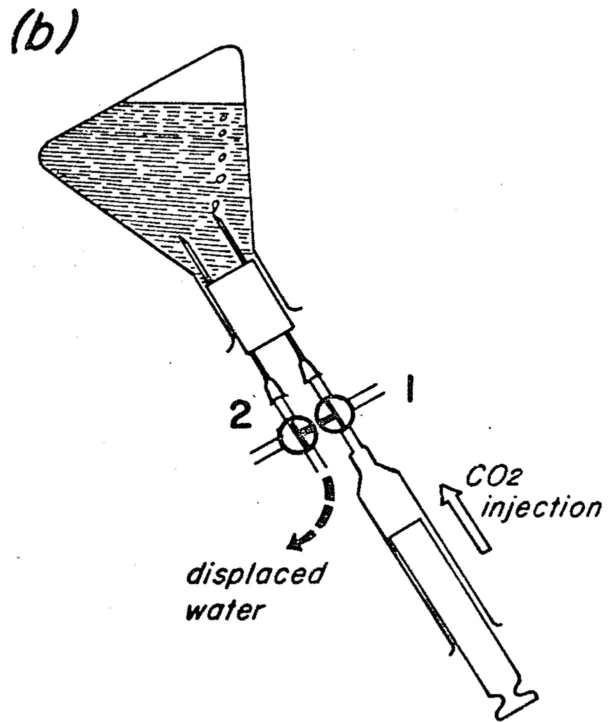
Two 3.8 cm 18-gauge steel hypodermic needles fitted with plastic three-way stopcocks (Ace Glass Inc., Vineland, NJ), No. 1 and No. 2, were inserted through the rubber stopper of the BOD bottle or sample flask with their outlets to the atmosphere closed (Fig. 2a). A 50 cc glass syringe filled with helium[1] at one atmosphere was connected to stopcock No. 1. With stopcock No. 1 open to the syringe, about 50 ml of water sample was slowly displaced from the container by helium via stockcock No. 2 open to the atmosphere (Fig. 2b). Stopcocks No. 1 and No. 2 were then closed and the needles withdrawn from the stopper. Stopcock assemblies were flushed with helium immediately before use to avoid air contamination during injection of helium. To equilibrate the sample gas and

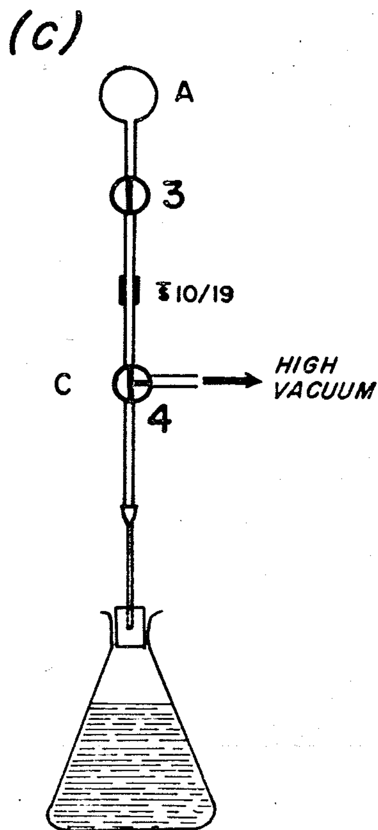
---

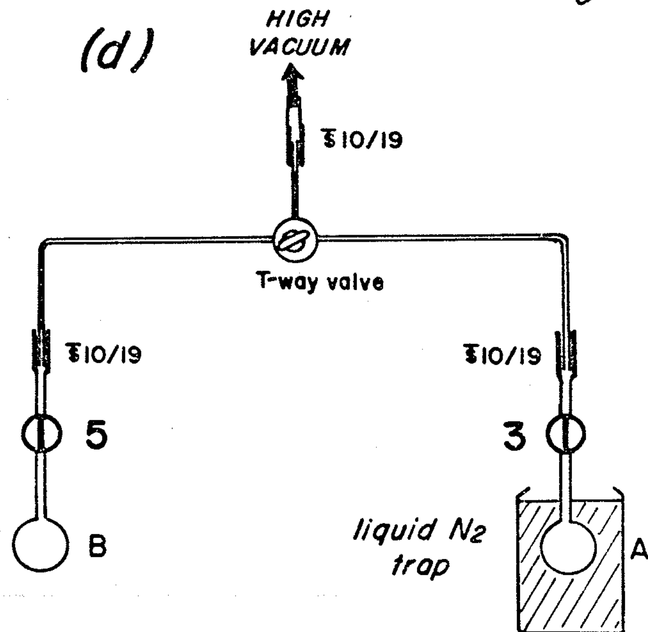
[1]Carbon dioxide was used initially but helium, a light, inert gas was a better choice for both mass spectrometric and gas chromatographic analyses of the extracted gases.

Fig. 2. Sequential steps (a), (b), (c), and (d) in the gas-extraction procedure for analysis of dissolved dinitrogen (N_2) by mass spectrometry.


(a)
1
2


(b)
2
1
CO2 injection
displaced water


(c)
A
3
$10/19
C
4
HIGH VACUUM


(d)
HIGH VACUUM
$10/19
T-way valve
$10/19
$10/19
5
3
B
liquid N2 trap
A

liquid phases, the sample container was vigorously hand-shaken for 2 min. The gas phase after equilibration was directly sampled for mass spectrometric analysis.

Mass Spectrometry for Denitrification Assay

Sample preparation

Before N-isotope ratio analysis was carried out by mass spectrometry, the extracted dissolved gas samples were purified as follows:

An all-glass T connection (Fig. 2c) was used to transfer a fixed portion of the equilibrated sample atmosphere to a 10 ml glass sample bulb. With the syringe needle inserted halfway through the container stopper and with stopcocks No. 3 and No. 4 open, the T connection C and sample bulb A were evacuatedfor 1 min by a high-vacuum system ($<10^{-5}$ Torr) consisting of a mercury diffusion pump in series with a Vac Torr D25 mechanical pump. Stopcock No. 4 was then closed to vacuum and the T connection needle pushed through the stopper into the gas phase of the container. Stopper No. 3 was then closed and the filled sample bulb A was transferred to one side of a T-way assembly (Fig. 2d) connected to the high-vacuum manifold. Carbon dioxide, water vapor, gaseous oxides of nitrogen, and other impurities present in the sample were immobilized by freezing when bulb A was immersed for 3 min in liquid N_2. A similar sample bulb, B, was attached to the opposite arm of the T-way valve with its stopcock No. 5 open. Bulb B

and the T-way assembly were evacuated for 2 min by the high-vacuum system. Then, with the T-way valve closed to vacuum and stopcock No. 3 opened, about one-half of the gas sample was transferred from A to B. Stopcock No. 5 was then closed and bulb B removed for direct attachment to the mass spectrometer. All glass joints and valves were lubricated before use with 'Apiezon N' grease to provide for high-vacuum and gas-tight conditions.

Mass spectrometry procedures

All gas samples were analysed using a Varian MAT-GD 150 spectrometer: N_2 at masses (i.e. M/e ratios) 28, 29, and 30, O_2 at mass 32, and Ar at mass 40 which served as an internal standard. Peak heights as expressions of ion voltages were measured and air leakage corrections were accounted for by masses 32 and 40 according to the procedure of Bremner (1965).

To determine the validity of peak height measurements, serial doubling dilutions of a standard ^{15}N-labeled dinitrogen gas with compressed N_2 were carried out and similarly measured by mass spectrometry.

The formation of NO (measured at mass 30) from N_2 and O_2 introduced into the mass spectrometer was also investigated. This was performed to set a limit on the allowable amount of O_2 present in the sample that would not significantly interfere with peak measurement at mass 30 due to $^{15}N^{15}N$. Gas samples of approximately one

atmosphere were prepared by mixing N_2 and O_2 in various proportions taken from pressurized lecture bottles. Mass peaks from 28 to 32 were then scanned.

Test measurements of ^{15}N natural abundance

The initial test of the gas-extraction procedure was made using nine Erlenmeyer flasks each filled with 300 ml of air-saturated distilled water at room temperature. One manganous sulfate and one alkaline iodide-azide pillow were added to each of the eight flasks to absorb the dissolved oxygen (DO) present (Anon. 1969), the nin th flask remained untreated. The abundance of ^{15}N in the gas phase of each flask was measured after extraction and analysis as previously described. A laboratory air sample was also analysed and served as a control.

Test assays of aquatic denitrification

Replicate water samples contained in BOD bottles collected from a depth of 8 m in Lake 227 in October 1972 were used for test assays of denitrification. To each bottle, 95.0 atom % $K^{15}NO_3$ was added by syringe to provide a final concentration of 500 µg NO_3^--N liter^{-1}. A nitrification inhibitor, 2-chloro-6-(trichloromethyl) pyridine (N-Serve), was also added at a concentration of 1.0 mg liter^{-1}. Control sample bottles similarly amended contained, in addition, 20 mg $HgCl_2$ liter^{-1}. All samples were incubated at 22°C in the dark for 5 days. After incubation, the gas extraction technique described above for denitrification

assay was performed on the samples.

Test denitrification assays in growing cultures

A denitrifying culture, designated B, isolated from Lake 227 sediments (see 'Pure Culture Studies', p.41) was cultured anaerobically in a medium containing in 1 liter, 2.0 mg KNO_3-N, 2.0 g D-L malic acid, 1.0 g peptone, and 0.5 g K_2HPO_4; final pH 7.0-7.2. The sterilized medium (250 ml) was aseptically dispensed in 300 ml Erlenmeyer flasks. Before inoculation the medium in each flask was scrubbed with sterile N_2 for 7 min. After inoculation, a further 3 min of scrubbing to provide anaerobiosis was applied before the flasks were tightly closed with rubber stoppers. Thus treated, each flask contained about 50 ml N_2 gas phase over 250 ml liquid culture. The cultures were incubated at 27°C overnight on a rotary shaker (120 rpm) to provide a 4% by volume inoculum for each subsequent ^{15}N-tracer experiment.

In the ^{15}N-tracer experiments the same medium and culture procedure were used except that the unlabeled NO_3^- was replaced by either 95.0 or 47.5 atom % $K^{15}NO_3$ at the same concentration. At time zero in each experiment the initial population density was about 10^6 cells ml^{-1}. Incubation was as described for inoculum preparation but was extended to 48 h after which N_2 in the gas phase was transferred to sample bulbs as described earlier. Triplicate culture flasks were used per experiment

and their mean results calculated.

Method of calculation

The correction factor required to compute the total moles of dissolved N_2 in V_ℓ ml water before liquid-to-gas transfer in the closed system was derived as follows:

If G ml = volume of total dissolved N_2 in the water from which dissolved gas was to be extracted,

V_g ml = volume of gas phase above the water,

and x ml = amount of N_2 transferred to the gas phase after equilibration,

by Henry's Law, partial pressure of the gas above the water was given by x/V_g.

Assuming α ml = saturating amount of the gas dissolved per ml water at the temperature and pressure prevailing at the time of sampling,

the amount of the gas remained in solution after equilibration was therefore $(\alpha V_\ell)(x/V_g)$ ml.

Hence, $G = x + (\alpha V_\ell) \cdot (x/V_g)$

and

$$\frac{x}{G} = \frac{V_g}{V_g + \alpha V_\ell} \qquad (1)$$

The last equation gave the fraction of the dissolved gas extracted from the water sample.

The fraction, d, of the total moles of dissolved N_2 evolved by denitrification from ^{15}N-labeled NO_3^- in lake water samples was calculated according to Hauck et al. (1958) modified to allow for gas-liquid equilibration

after gas transfer from solution to the gas phase. Using eq. 1 to correct for d, then

$$d_{corr} = \frac{d(V_g + \alpha V_\ell)}{V_g} \qquad (2)$$

which was the corrected d and was used subsequently to calculate the weight of N_2 released through denitrification as explained below. Since 1 gram-mole of a gas at STP measures 22.4 liters, assuming saturation of the water with N_2, the total weight of N_2 in the gas and liquid phases of the closed sample container at equilibration is given by

$$\left(\frac{28}{22.4}\right) \cdot \left(\frac{273}{T + 273}\right) \cdot (\alpha V_\ell) \cdot 10^3 \ \mu g\ N$$

where T is the temperature of equilibration in degrees Celsius. Hence, the weight of N_2 evolved by denitrification would be

$$d_{corr}\left(\frac{28}{22.4}\right) \cdot \left(\frac{273}{T + 273}\right) \cdot (\alpha V_\ell) \cdot 10^3 \ \mu g\ N \text{ per 250 ml water.}$$

To express this in terms of µg N liter^{-1} water sample,

$$W_{N_2} = d_{corr} \left(\frac{28}{22.4}\right) \cdot \left(\frac{273}{T + 273}\right) \cdot (\alpha V_\ell) \cdot 10^3 \cdot 4$$

$$= 5{,}000 \cdot d_{corr}\left(\frac{273}{T + 273}\right) \cdot (\alpha V_\ell) \qquad (3)$$

The method for computing the weight of N_2 evolved by the denitrifying bacterial isolate was similar to that for lake waters except that the weight of N_2 initially present in the gas phase was included as part of the total N_2 in the flask in addition to the dissolved

N_2, i.e.

$$W'_{N_2} = 5{,}000 \cdot d_{corr}\left(\frac{273}{T + 273}\right) \cdot (V_g + \alpha V_\ell) \quad (4)$$

A Fortran program was written for the calculation of aquatic denitrification products from $^{15}NO_3^-$ (cf. Appendix II) and run on the IBM 370 computer at the University of Manitoba Computer Center.

Chemical Analyses

Nitrate and nitrite

Unless otherwise stated, NO_3^- was first reduced with hydrazine and determined colorimetrically as NO_2^- by diazotization and chemical coupling according to the method of Sawicki and Scaringelli (1971) with modifications necessary for microanalysis. The principal modification involved the use of five times the designated volume of the diazotizing-coupling reagent. Sample NO_3^- and NO_2^- concentrations were determined by spectrophotometer at 540 nm without dilution immediately after the color reaction was completed. Before chemical analysis, water samples were prefiltered with Whatman GF/C glass fiber filters which had been ignited at 500°C for 20 h.

Ammonia

The method of Stainton *et al*. (1974) was followed to determine NH_3 concentration in the water samples.

Dissolved oxygen (DO)

Dissolved oxygen was determined using the

modified Winkler method (Anon. 1969). The determination of *in situ* DO concentration of lake waters was done on samples with their DO fixed by addition of manganous sulfate and alkaline iodide-azide upon collection.

B. Lacustrine Studies

a. Water Column

Sampling

For denitrification studies of the water column, water from a specific depth at the center station of Lake 227 was drawn into standard BOD bottles by a portable Masterflex[R] sampling pump (Cole Palmer, Chicago, IL). To avoid atmospheric contamination, each bottle was filled from bottom upwards and the water allowed to overflow for at least twice the bottle volume. The bottle was then tightly stoppered with a modified #3 rubber stopper (see 'Extraction of Dissolved Gases', p.19), avoiding trapped air bubbles. For the depths sampled, water column temperatures were measured at the same station with a FT3 marine hydrographic thermometer(Applied Research, Austin, TX).

Gas chromatography for nitrous oxide determination

Dissolved N_2O was determined by a Beckman GC72-5 gas chromatograph equipped with a helium-ionization detector (HID) and a stainless steel column (366 x 0.32 cm) packed with Porapak R (Water Associates, Inc., Framingham, MA). The operating conditions were as follows: HID

temperature, 150°C; column temperature, 65°C; inlet temperature, 55°C; carrier gas (high purity helium) flow rate, 20 cc min^{-1}; electrode voltage, 215 v; polarizing voltage, 1000 v. A standard curve was obtained by measuring peak heights for the 0-0.82 nmole range of an N_2O calibration gas at an attenuation of 400X. After extraction of dissolved gases by gas-liquid equilibration described previously, the gas phase in the sample container was directly sampled with a gas-tight Hamilton syringe. The sample size for injection into the gas chromatograph was usually not more than 0.2 ml.

Denitrification rate measurements

A set of triplicate water samples contained in BOD bottles from each depth was used for the denitrification assay. To each sample bottle, 200 µg $K^{15}NO_3$-N (95.0 atom %) $liter^{-1}$ was added by syringe immediately after collection. These treated samples were then returned to the depths from which they had been taken or to the field station for immediate incubation in the dark at temperatures within 2°C of *in situ* water temperatures. After an appropriate period of incubation two samples from each depth of the water column were retrieved, biologically fixed by injecting 20 mg $HgCl_2$ $liter^{-1}$ (final concentration), and stored until the time for dissolved gas extraction. One of these samples was reserved for gas chromatographic and the other for mass spectrometric analyses to determine N_2O and N_2 production

respectively. The third sample bottle of each set was used for NO_3^- and NO_2^- determinations at the field station. DO, NO_3^- and NO_2^- concentrations at each sampling depth were also determined. An additional set of bottles containing triplicate water samples from a depth of 6 m was similarly treated and, in addition, each received 20 mg $HgCl_2$ liter^{-1}. These control bottles were incubated simultaneously with the others.

The denitrification assay experiments were usually started early in the afternoon in mid-week when little background NO_3^- was usually found in the surface waters. (Whole-lake fertization was carried out on Tuesdays.)

Distribution of denitrifiers

Enumeration of the denitrifying population in the water column was done using the most-probable-number (MPN) method of Alexander (1965). The enrichment culture tubes were incubated at 22°C for 14 days.

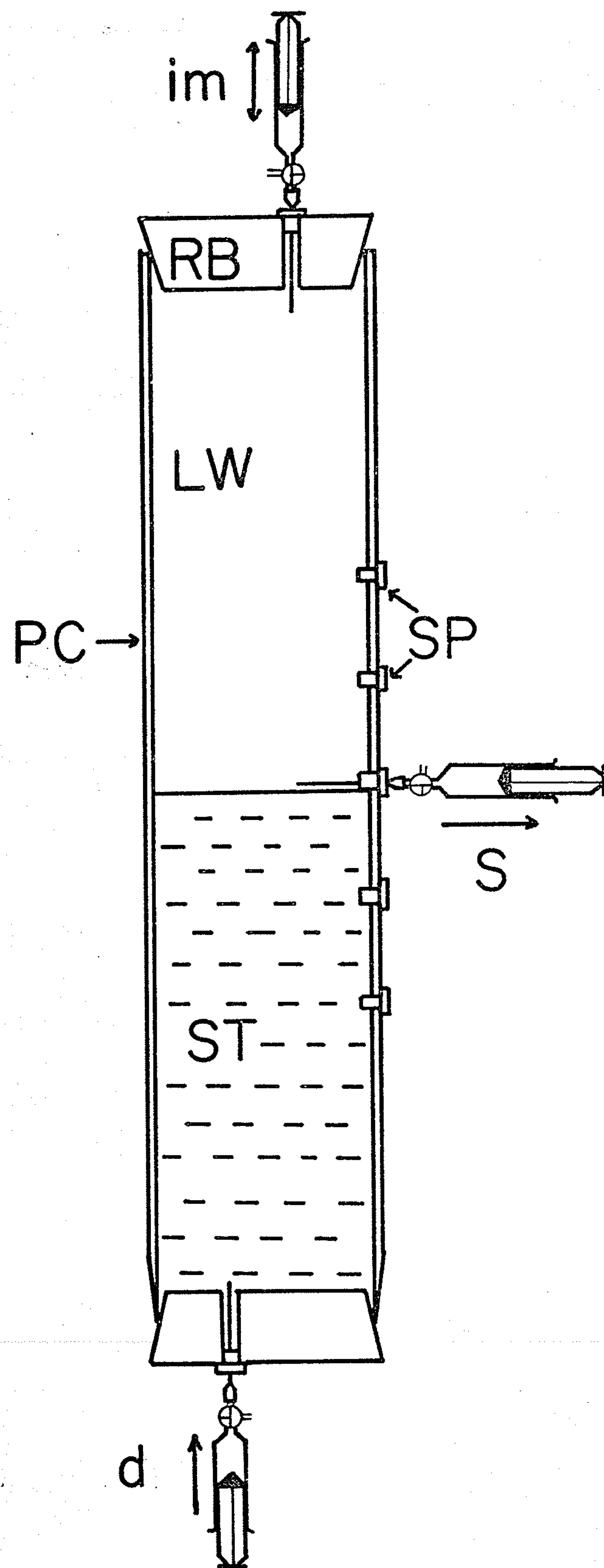
b. Littoral Sediment-Water Interface

Core experiments

Assays of sediment-water denitrification were performed in August 1973 using two plexiglass cores (51 x 7.6 cm) of two-liter capacity to take representative sediment-water samples containing about 1 liter of each component. These samples were taken at a water depth of 1.5 m by pressing the plexiglass cores vertically down into the sediment and then closing the open ends with

rubber bungs by hand. Upon return to the field station, the cores were equilibrated for 24 h at 12°C in the dark. Through a serum stopper in the top bung, Fig. 3, the water phase of each core was injected with 95.0 atom % $K^{15}NO_3$ to give a homogeneous enrichment of 100 µg N liter^{-1} water. One of the two cores received in addition, 20 mg $HgCl_2$ dissolved in a small volume of water injected close to the sediment surface. Each day for up to 4 days of incubation, after mixing the water phase, a total of 130 ml of water samples were withdrawn from each core by a 50 cc syringe through a side port by displacement with an equal volume of helium-flushed distilled water injected slowly via the bottom bung. Dissolved oxygen was determined on a 30 ml water sample with a DO kit (Hach Chemical Co., Ames, IA) while a 20 ml sample was used for NO_3^- determination. Two 40 ml water samples were taken for dissolved gas analyses with the 50 cc syringe prefilled with helium in the following manner: Just before sampling from the side port, 40 ml helium was expelled from the syringe and then the needle was inserted through the sampling port. Hence, after sampling, the syringe contained 40 ml water and 10 ml helium. To extract the dissolved gases, the syringe was immediately closed to the atmosphere and agitated manually for 2 min to facilitate gas exchange. The gas phase was injected into a pre-evacuated 10 ml glass vial closed by a serum stopper. One gas sample

Fig. 3. Closed system for laboratory study of denitrification at the sediment-water interface. Syringes (50 cc, fitted to three-way stopcocks): d, displacement with helium-flushed distilled water; im, injection and mixing; s, sampling. LW, lake water; PC, plexiglass core (51 x 8 cm, 0.6 cm wall); RB, rubber bung; SP, sampling ports fitted with serum stoppers; ST, sediment.

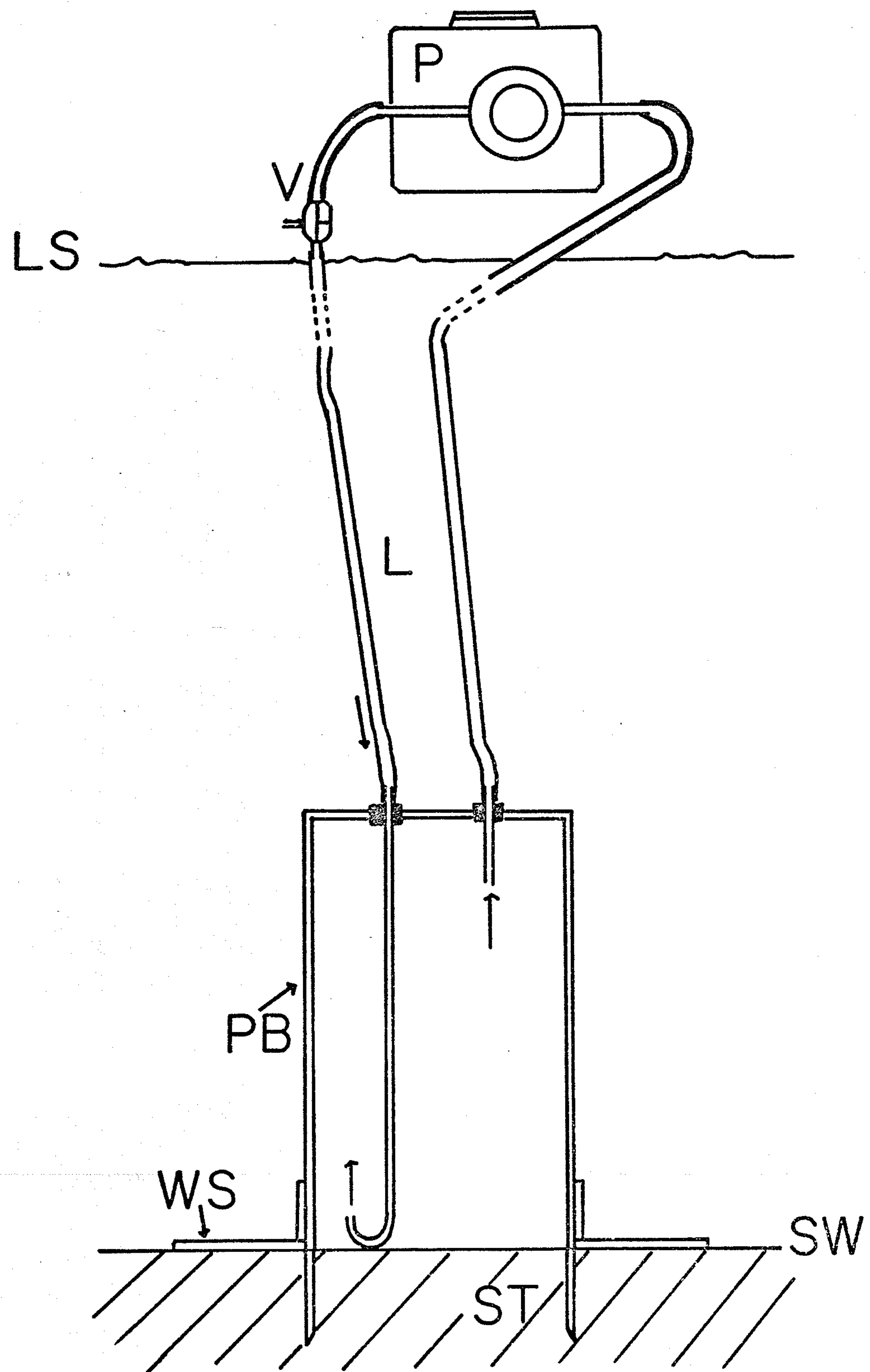

im
RB
LW
PC
SP
S
ST
d

thus prepared was analysed for N_2O by gas chromatography and the other for $^{15}N_2$ by mass spectrometry as previously described.

IN SITU denitrification rate measurements

Assays of *in situ* denitrification at the littoral sediment-water interface were carried out by the following experimental design. A plexiglass chamber with the bottom end open (height, 39 cm; base, 19.8 x 19.8 cm) was lowered from a boat so as to rest on its winged supports at the sediment surface at a depth of less than 2 m (Fig. 4) at a station off the northwestern shore of Lake 227. The chamber enclosed approximately 15 liters of water and 0.04 m^2 of sediment surface. A flexible tube-loop permitted the circulation of water in the chamber overlying the sediment by means of a portable sampling pump operated in the boat moored above the chamber. After 24 h of equilibration, four BOD bottles were used to take water samples from the chamber for background chemical and gaseous analyses before $K^{15}NO_3$ (95.0 atom %) was added to give a final concentration of 500 μg N $liter^{-1}$ in the water enclosed. After one or two days of incubation, the enclosed water was circulated by pump to achieve homogeneity. Four replicate water samples were then taken in BOD bottles from the chamber. A separate set of four BOD bottles were also used to assay for denitrification in water samples collected from the water overlying the sediments in the exterior

Fig. 4. Semi-closed system for *in situ* study of denitrification at littoral sediment-water interface. L, loop of tubings for water circulation and sampling; LS, lake surface; P, portable sampling pump; PB, plexiglass chamber; ST, sediment; SW, sediment-water interface; WS, winged support of chamber; V, T-way valve.


P
V
LS
L
PB
WS
SW
ST

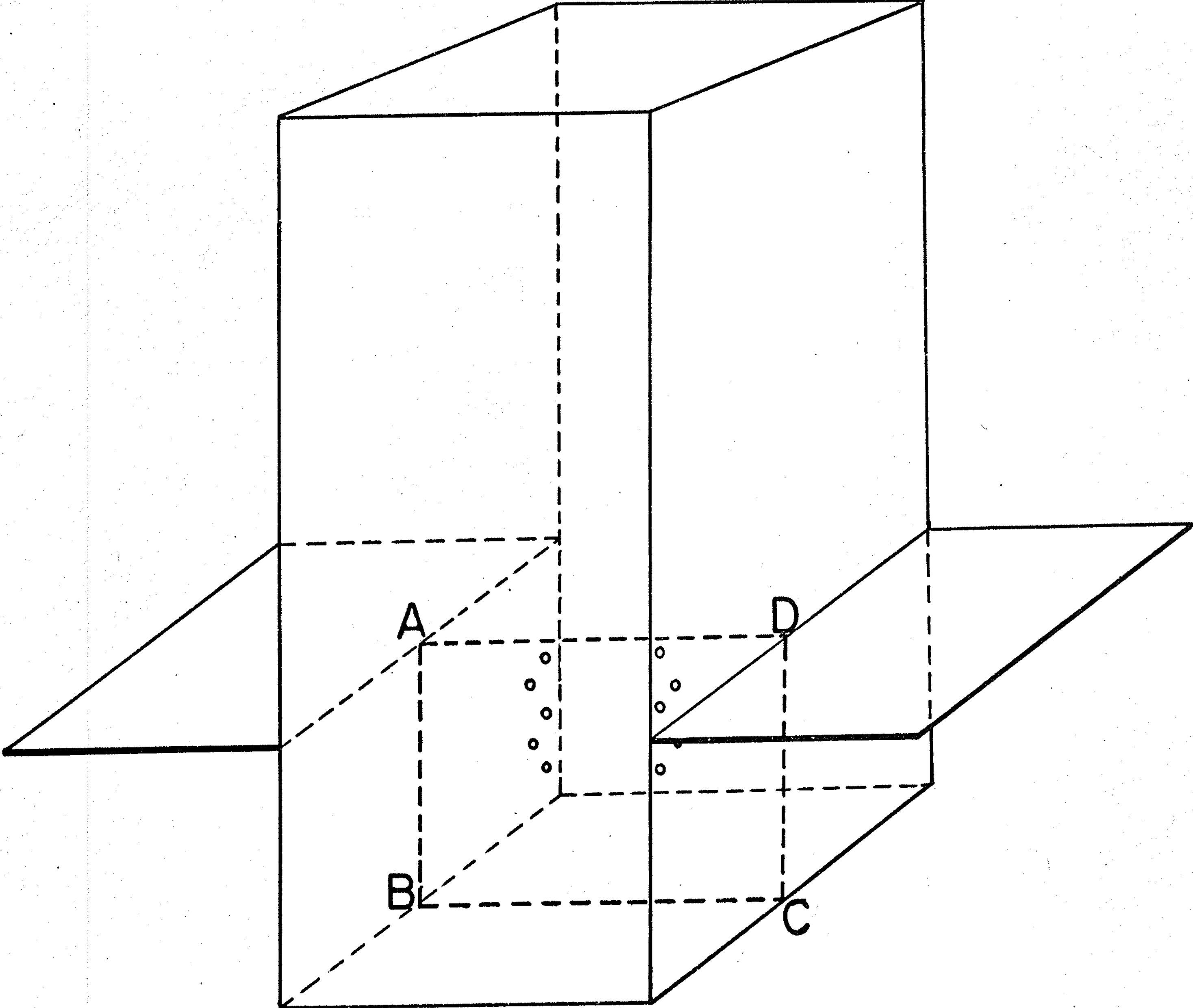
neighborhood of the chamber. These samples were similarly incubated for 48 h. In each set of four water samples, one was used for DO determination, one for NO_3^- and NO_2^- determination, and two for gaseous analyses as described for the water column denitrification studies.

Altogether, three plexiglass chambers were constructed and employed simultaneously in the denitrification assays including control experiments.

Mixing at sediment surface

Simultaneous measurement of sediment interstitial water nutrient concentration and denitrification was carried out to estimate the extent of surface sediment mixing by modifying one of the plexiglass chambers. A plexiglass plate was fixed vertically inside the chamber across its two opposite walls so as to form a partition from the winged-support level to the bottom of the chamber (Fig. 5). Two rows of holes 1.5 cm apart had been drilled in the partition from its top downwards for the insertion of 8 ml plastic vials. The vials containing distilled deionized water covered with 0.45 μm Millipore^R filters were then horizontally embedded in the surface sediments when the plexiglass chamber was resting on its winged supports at the sediment surface. Equilibration with interstitial water during the incubation period by dialysis then permitted chemical analyses of the vial contents for the determination of nutrient concentration profiles

Fig. 5. Diagram of modified chamber for measuring mixing at the sediment surface simultaneously with *in situ* denitrification assay. ABCD, plexiglass plate with holes was installed inside the lower end of the chamber, AD being level with the winged supports.


A
D
B
C

in the interstitial water. Since the water samples in the vials were small (8 ml), NO_3^- and NH_3 concentrations at each sediment depth were determined by automated methods according to Stainton *et al.* (1974).

C. Pure Culture Studies

All operations involved in dilutions and platings described in this section were performed aseptically at not more than 22°C. All media were prepared with distilled water and autoclaved at 121°C for 15 min.

Isolation and Purification of Denitrifiers from Lake Sediments

Denitrifying bacteria from lake sediments were isolated by Winogradsky column enrichment (Aaronson 1970). The sediment sample was collected from Lake 227 with an Eckman dredge and had been stored at 4°C in the dark in an air-tight polyethylene bag for about two weeks. The column was prepared as follows:

A glass column (4.5 x 20 cm) closed at the bottom with a #9 rubber stopper and sealed with vaseline was first layered with a 20 ml thick slurry of sediment and shredded filter paper in an enrichment solution containing 0.5% KNO_3 and 0.2% D-L malic acid adjusted to a pH of 7.2 with NaOH. Two-thirds of the column was then packed with additional slurry taking care to avoid trapped air bubbles.

The rest of the column was then filled to the top with the enrichment solution and a #9 rubber stopper was used to cover the top so as to allow for gas ebullition during incubation. Incubation was carried out in the dark at $20 \pm 1^{o}C$ for one week.

Enrichment cultures for denitrifiers were started by inoculating 150 ml of sterile liquid medium scrubbed with N_2 in an anaerobic flask (Fig. 6) with 2 ml of the incubated sediment taken from the bottom third of the column. The medium contained in 1 liter, 3.0 g KNO_3, 2.0 g D-L malic acid, 1.0 g peptone, and 0.5 g yeast extract; final pH 7.0-7.2. At regular intervals during stationary incubation ($20 \pm 1^{o}C$, 15 days) samples were removed by syringe, appropriately diluted, and analysed for NO_3^- and/or NO_2^- depletion as a measure of culture growth.

To purify the denitrifying species, aliquots of the anaerobic enrichment cultures were appropriately diluted with sterile medium and spread-plated on the same medium solidified with 1.5% Bacto-agar. Incubation was carried out aerobically and anaerobically in a GasPak^R jar for 10 days. No attempt was made to measure the bacterial density of enrichment cultures. Isolated colony types that developed anaerobically on the agar medium were selected at random and successively transferred at regular intervals to separate agar plates containing trypticase soy supplemented with 0.1% KNO_3. Stock cultures were

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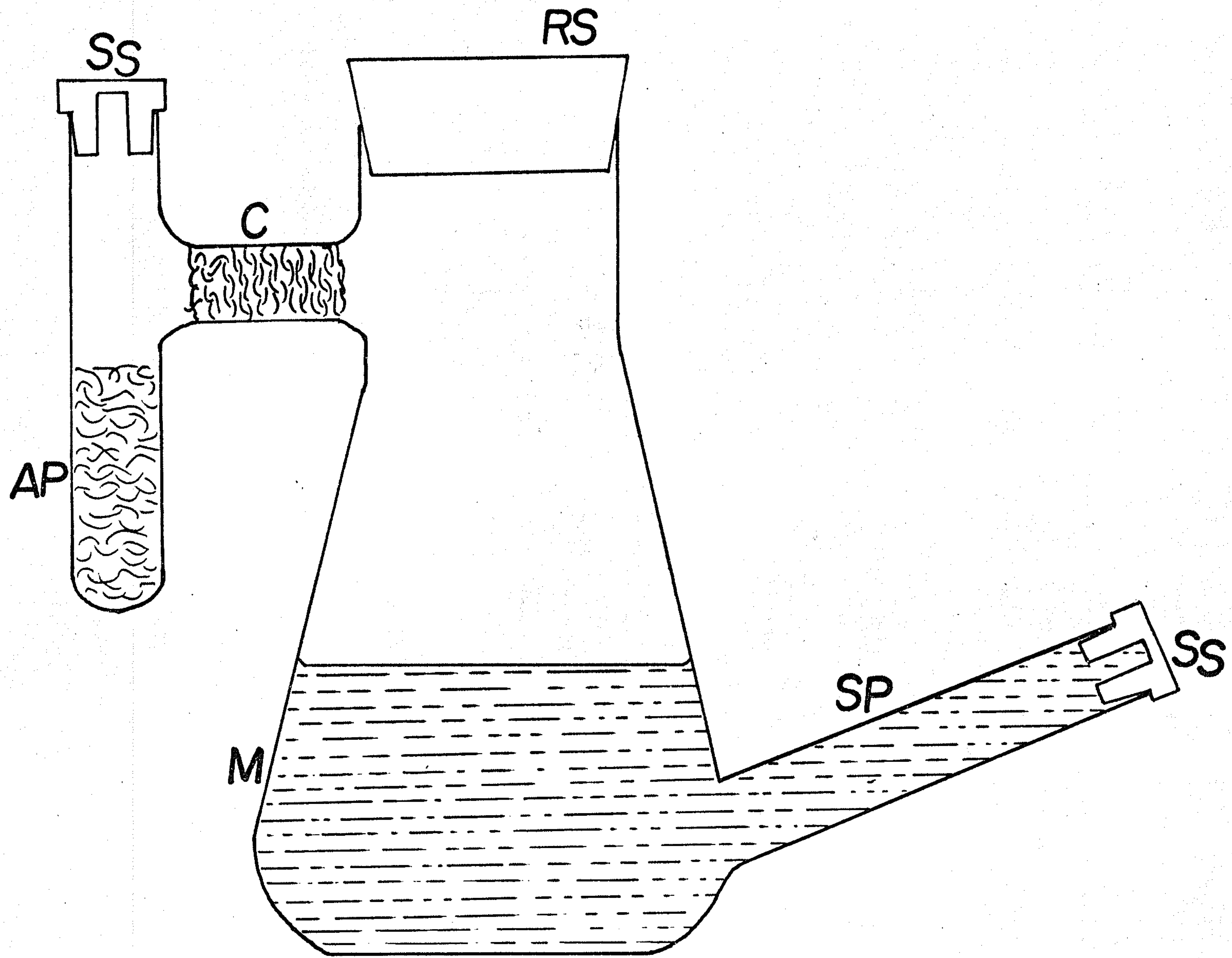
Fig. 6. Anaerobic flask modified from a 250 ml Erlenmeyer flask. AP, alkaline pyrogallol-soaked cotton wool; C, cotton plug; M, medium; RS, rubber stopper; SP, sampling port; SS, serum stopper. Aseptic procedures were followed for the set-up of the experiment. Sampling of the medium was performed by means of a sterile hypodermic syringe via the sampling port through the serum stopper. The system was scrubbed with sterile N_2 before and after inoculation.


SS
RS
C
AP
M
SP
SS

prepared from the anaerobic cultures in screw-capped tubes of trypticase soy deep agar amended with 0.1% KNO_3 and 0.5% glycerol. These were routinely maintained by retransfer every two weeks and occasionally checked for purity by streak-plating onto the same medium.

Characterization of Denitrifying Isolates

Cell morphology

Isolates grown for 24 h at 20°C in screw-capped tubes of trypticase soy broth amended with 0.1% KNO_3 were examined by phase-contrast microscopy. Cell size was estimated with a slide-micrometer. Gram and flagella stains were prepared according to Skerman (1967).

Culture characteristics

The isolates were cultured on the following media for the examination of colony characteristics, pigment production, and poly-β-hydroxybutyrate accumulation respectively:

A. Trypticase soy agar amended with 0.1% KNO_3,

B. King's medium B (King *et al*. 1954),

C. β-hydroxybutyrate medium (Sta nier *et al*. 1966).

Medium A was also used to screen the temperature range of aerobic colony formation at 5, 10, 20, and 30°C for each isolate.

Physiological characteristics

The following biochemical tests were carried

out on the isolates according to the procedures described by Rohde (1968) and Skerman (1967): catalase, oxidase, O/F (Hugh-Liefson), glucose, lactose, β-galactosidase, nitrite, indol, methyl red, Voges-Proskauer, citrate (Koser), hydrogen sulfide (TSI), urease, gelatinase.

Effect of Temperature on Growth and Denitrification Products in a Defined Medium

For temperature studies, flask cultures of two denitrifying species selected from the isolates were prepared. The method described previously in the section on 'Test Denitrification Assays in Growing Cultures' (p.25) was followed with the exception that the cultures were preadapted to the experimental temperatures (5 to 30°C) in a defined medium[1]. At appropriate intervals of incubation, replicate samples of each culture were withdrawn and analysed for cell numbers by spread-plate-count on the same but agar-solidified medium at 20°C, for NO_3^-, NO_2^-, N_2O, and $^{15}N_2$ as described previously.

Chemicals

The chemicals used in this part of the study

---

[1]Prepared in 1 liter distilled water containing 600 μg ^{15}N (95.0 atom%) as KNO_3, 1.0 g NH_4Cl, 1.0 g D-L malic acid, 6.0 g K_2HPO_4, 4.0 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.15 g $Na_2MoO_4.2H_2O$, 0.00076 g $MnSO_4.H_2O$, and 5 ml iron-citrate solution (1.03 g $FeSO_4.6H_2O$ and 1.05 g citric acid in 1 liter distilled water); final pH 7.0-7.2.

were purchased from commercial sources. Specifications and suppliers of some special chemicals are summarized in Table I.

All the reagents and culture media were prepared in double distilled or deionized water.

PART II. PHYTOPLANKTON UPTAKE OF NITRATE

Sampling

Composite water samples for NO_3^- uptake studies were collected between 0800 and 1000 h using an opaque 3 liter van Dorn bottle from a depth of 1.25-1.75 m at the center station on Lake 227. These were stored in the dark at 20°C for 2-4 h before experiments were started in order to deplete the photosynthetically produced energy pool of the algal population.

Kinetic Studies

The 'light-starved' (dark-incubated) bulk samples collected for NO_3^- uptake studies were dispensed into 125 ml Pyrex glass reagent bottles some of which were modified to provide light-proof conditions. Each replicate was then treated with DCMU or PO_4^{3-} according to the particular experimental design after receiving an aliquot of $K^{15}NO_3$ (95.0 atom %). Controls were also set up which contained 20 mg $HgCl_2$ liter^{-1}. Incubation was carried out in an illuminated water-bath incubator (Schindler and Fee 1973)

Table I. Special chemicals used in denitrification studies.

Chemical	Specification	Supplier
Gaseous compounds		
Helium carrier gas	High purity	Welders Supplies, Winnipeg, Manitoba
Nitrous oxide	1000 ppm in helium	Applied Science Laboratories Inc., State College, PA
^{15}N-labeled dinitrogen ($^{15}N_2$)	49.9 atom %	Office National Industrial de l'Azote, Paris, France
^{15}N-labeled compounds		
Potassium nitrate ($K^{15}NO_3$)	95.0 atom %	International Nuclear & Chemical Corp., Irvine, CA
Sodium nitrite ($Na^{15}NO_2$)	99.2 atom %	ditto
Inhibitor		
2-chloro,6-(trichloromethyl) pyridine	Nitrification Inhibitor Formula 2533(TM)	Hach Chemical Co., Ames, IA

at 20 ± 1°C and 0.15 ly·min^{-1} light. After incubation, particulate N was retained on 2.5 cm Reeve Angel glass-fiber filters (grade 984H, preignited at 500°C for 20 h) by vacuum (12.5 cm Hg) filtration and then determined as N_2 by dry combustion (Flett 1976). The ^{15}N abundance of each particulate N sample was determined by emission spectrometry on a Statron NOI-5 ^{15}N Analyser following the procedure developed by Flett (1976). Enrichment in ^{15}N of the particulate fraction over that of background was taken to measure NO_3^- incorporation. Colorimetric determinations of NO_3^-, NO_2^-, and NH_3 were also done on the incubated water samples.

Uptake and Primary Production

To study the effect of DCMU on the kinetics of NO_3^- uptake, primary production was measured simultaneously by the ^{14}C technique (Fee 1973) but acidification and bubbling (Schindler *et al.* 1972) instead of filtration was employed to determine C uptake.

Preferential Uptake of Inorganic Nitrogen

Phytoplankton uptake rates of inorganic N in different forms were compared using the light incubator technique described above (Schindler and Fee 1973). Replicate water samples were separately provided with $^{15}NH_4Cl$ (95.0 atom %), $^{15}NH_4{}^{15}NO_3$ (95.4 atom %), $K^{15}NO_3$

(95.0 atom %), and $Na^{15}NO_2$ (99.2 atom %) each at a final concentration of 50 µg N $liter^{-1}$. After simulataneous incubation for 3 and 5 h, ^{15}N uptake was measured by emission spectrometry.

Examination of Glass-Fiber Filtrates

The extent of possible cell damage during vacuum-filtration of the water samples was investigated by electron microscopic examination of the glass-fiber filtrates. After incubation with $K^{15}NO_3$ in the NO_3^- uptake experiments, the water filtrate that passed through the Reeve Angel filter was subjected to ultracentrifugation at 10^5 x g for 1.5 h. The pelleted material was resuspended in a minimal amount of distilled water, deposited onto carbon-coated copper grids as thin films and negatively stained for 10 sec with 1% potassium phosphotungstate at pH 7.4. After drying in air, the preparations were examined by means of an AEI EM6B electron microscope. The resuspended material of the filtrate was also absorbed on a preignited glass-fiber filter and dried before determination of total N and ^{15}N abundance by dry combustion and emission spectrometry respectively (Flett 1976).

Examination for Extracellular Products of Nitrate Assimilation

Experimental

Experiments were designed to investigate the

problem of possible phytoplankton excretion of NO_3^- assimilation products. The purpose was three-fold: (a) to obtain unequivocal evidence for the existence of such excretion using the ^{15}N tracer method; (b) to identify the chemical forms of these extracellular products; and (c) to estimate the excretion rate under experimental conditions.

The test experiment consisted of a 4 liter epilimnetic water sample in an open cylindrical Pyrex jar. It was constantly mixed by means of a teflon-coated stirring magnet driven by a motor-stirrer at a moderate rate and kept illuminated by two Sylvania Gro-Lux lamps installed 15 cm directly above. The fluorescent light source was enriched in the 425 and 650 nm regions which together made up 35% of the total photo output (C.T. Chow, personal communication). At time zero, the sample was enriched with 100 μg liter^{-1} $K^{15}NO_3$-N (95.0 atom %). During the 4 h incubation period at ambient temperature, the water temperature never rose more than 3°C (i.e.$<25^{o}$C). Control experiments were also set up in parallel which consisted of $K^{14}NO_3$ enriched, DCMU or $HgCl_2$-treated water samples similarly illuminated and a $K^{15}NO_3$ enriched sample incubated in darkness. At the termination of incubation, subsamples were drawn for $^{15}NO_3^-$ uptake measurements in the usual manner before the entire contents of each jar were continuously fed to a Heraeus-Christ Junior 15000

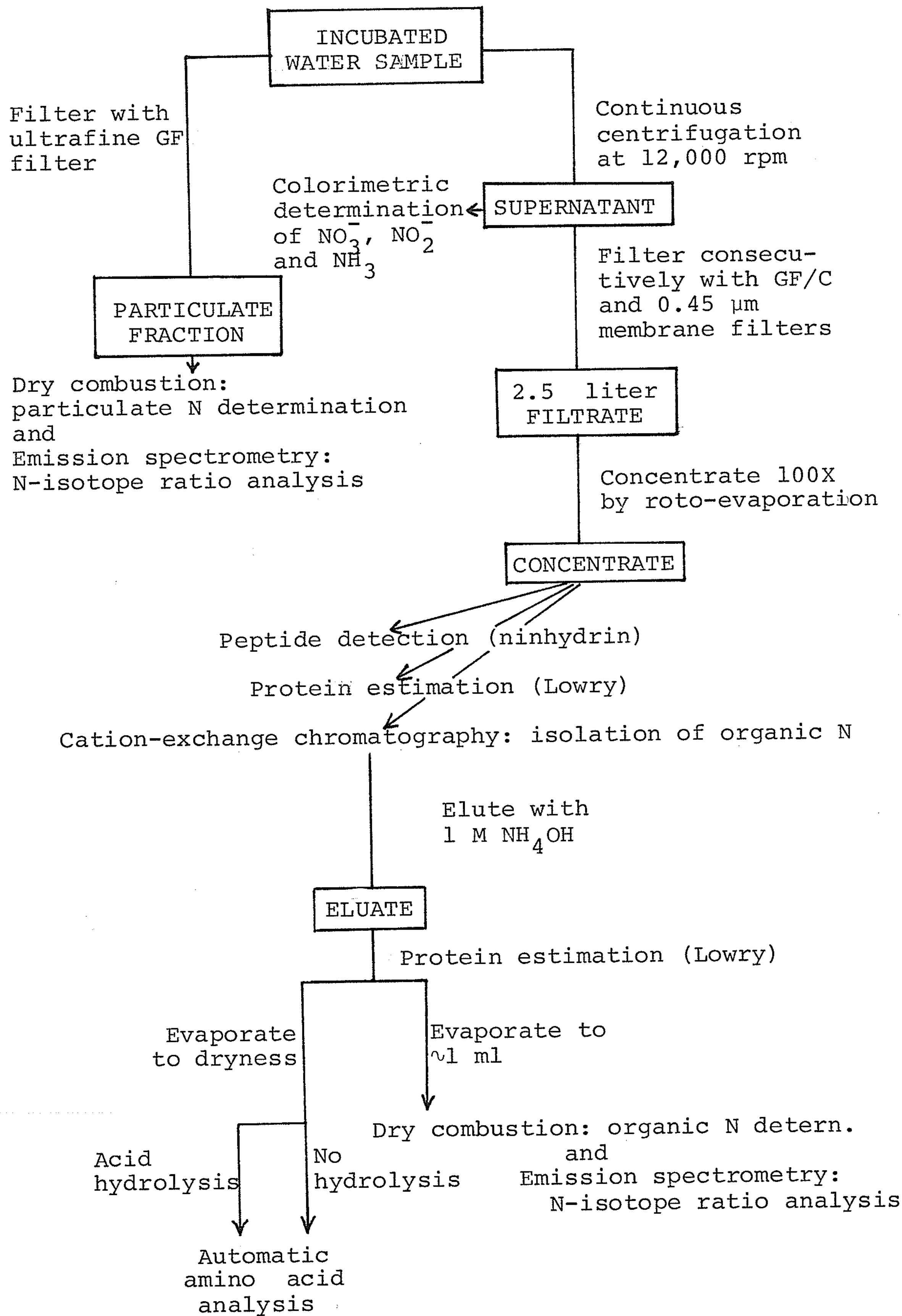
centrifuge to remove the large particulate matter at 12,000 rpm. All samples were centrifuged within 0.5 h of the end of the incubation time and those awaiting centrifugation were kept under diffused light conditions. After centrifugation 2.5 liter of each sample of supernatant was consecutively filtered on preignited 5.5 cm Whatman GF/C filters and 4.7 cm Millipore^{R} HA filters of pore size 0.45 μm. The filtration pressure was maintained at 12.5 cm Hg and the walls of the funnel were washed with distilled water after each filtration.

Samples to be analysed for amino acids and peptides, the suspected dissolved organic forms of excreted N, were first prepared from the filtrates by concentration on a Rotavapor (Brinkman Instruments, Westbury, NY) at 55°C to 1/100 of their original volumes. Aliquots of the concentrates were used for peptide detection, protein estimation, and cation-exchange chromatography. These procedures are described below and outlined in a flow-diagram (Fig. 7). All the apparatus and glass-ware had been acid-washed and thoroughly rinsed with distilled water prior to use.

Detection of Peptides and Protein Determination

The peptide content including dissolved free amino acids (DFAA) in an appropriate aliquot of the concentrated filtrate was estimated by the ninhydrin method

Fig. 7. Summary of procedures used in the examination for extracellular products of nitrate assimilation.


INCUBATED WATER SAMPLE
Filter with ultrafine GF filter
Continuous centrifugation at 12,000 rpm
Colorimetric determination of NO_3^-, NO_2^- and NH_3
SUPERNATANT
Filter consecutively with GF/C and 0.45 μm membrane filters
PARTICULATE FRACTION
Dry combustion: particulate N determination and Emission spectrometry: N-isotope ratio analysis
2.5 liter FILTRATE
Concentrate 100X by roto-evaporation
CONCENTRATE
Peptide detection (ninhydrin)
Protein estimation (Lowry)
Cation-exchange chromatography: isolation of organic N
Elute with 1 M NH_4OH
ELUATE
Protein estimation (Lowry)
Evaporate to dryness
Evaporate to ~1 ml
Dry combustion: organic N detern. and Emission spectrometry: N-isotope ratio analysis
Acid hydrolysis
No hydrolysis
Automatic amino acid analysis

(Hirs 1967) using leucine as standard. Protein determination of the concentrate was by the method of Lowry *et al*. (1951) using bovine serum albumin as standard.

Cation-Exchange Chromatography

Dissolved organic N in the concentrated filtrates was isolated by cation-exchange resin chromatography. Columns (1 x 8 cm) were modified from 10 cc glass hypodermic syringes fitted with three-way stopcocks and packed with Bio-Rad cation-exchange resin (AG^R 50W-X12, 100-200 mesh, H form). Each column was washed with 20 ml of 0.1 N HCl prior to use. Before the sample (concentrated filtrate) was applied to a column, its pH was adjusted to about 2.0 with 0.1 N HCl. After the sample was adsorbed onto the column, it was washed with 40 ml of 0.1 N HCl and the washing applied to a second column. Each of the two columns was then washed with 40 ml deionized water. Cations thus bound were then eluted with 1 M NH_4OH; 25 ml eluate was collected from each column and pooled. Each pooled sample was assayed for protein (Lowry *et al*. 1951) and then divided into appropriate fractions for subsequent amino acid analyses and ^{15}N determination. All the fractions were concentrated by rotary evaporation (Brinkman Evapo-Mix) at 36^oC to yield 1 ml samples before analyses.

The efficacy of this procedure was tested by the separate application of known quantities of L-tyrosine, bovine serum albumin, calf-thymus DNA and yeast RNA to the

cation-exchange columns as described above. In each case, the test amino acid, protein and nucleic acids were bound to the resin. Furthermore, recovery of these standards was close to 100% when the first 25 ml eluate from each column was collected and assayed by absorption measurement at 260 or 280 nm.

Amino Acid Analyses

The fractions from column eluates allocated for amino acid analyses were first evaporated to dryness at 110°C. This step removed the ammonia contained in the eluates and allowed samples to be stored till analysis. A Beckman 121 analyser was employed to determine the amino acid composition of acid-hydrolysed and unhydrolysed duplicate samples following the procedure of Dexter and Dronzek (1975). Calculation of the amino acid concentrations was done knowing the original water filtrate volume represented by the sample analysed.

^{15}N Tracer Determination

The N isotope ratios of the dissolved organic N in the 1 ml fractions of concentrated column eluates were determined as previously described (see 'Examination of Glass-Fiber Filtrates', p. 50) by loading the sample onto a preignited glass-fiber filter. Traces of ammonia on the desiccated filters left from the column elution procedure were removed when the filters were heated and

evacuated in the sample preparation unit before ^{15}N analysis. The dissolved organic N and its ^{15}N content were computed knowing the original water filtrate volume represented by the sample analysed.

Chemicals

The chemicals used in this part of the study are included in Table I. In addition, $^{15}NH_4Cl$ (95.0 atom %) was purchased from International Nuclear and Chemical Corp., Irvine, CA. Ammonia nitrate ($^{15}NH_4{}^{15}NO_3$, 95.4 atom %) was prepared by Merck Sharp & Dohme, Montreal, Quebec; DCMU by BDH Chemicals, London, England. As standards for cation-exchange chromatography, calf-thymus DNA, yeast RNA, L-tyrosine, and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, MO.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

PART I. DENITRIFICATION

A. N-isotope Ratio Analysis by Mass Spectrometry

RESULTS

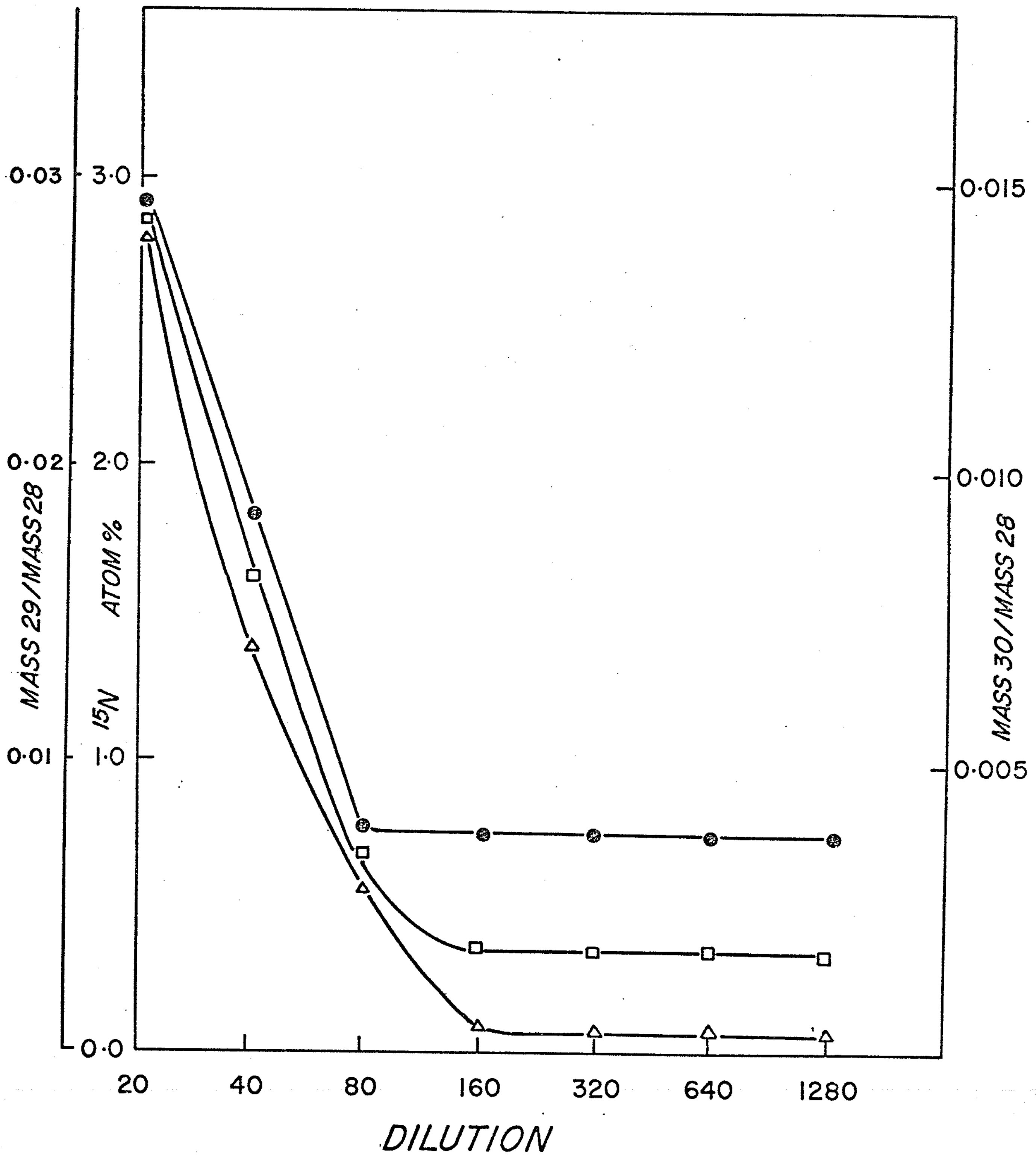
N-isotope Ratios Derived from Peak Height Measurements

Dilution curves of a standard ^{15}N gas sample (Fig. 8) demonstrated that the asymptotes of the ratios, mass 29/mass 28 and mass 30/mass 28, and the calculated ^{15}N atom % gave values of 0.0074, 0.0002, and 0.369 respectively for normal N_2. The lower limits of the linear portion of these curves, 0.008, 0.0003, and 0.0037 respectively were arbirt arily taken as safety limits below which ^{15}N enrichment was not directly proportional to dilution owing to the normal abundance of ^{15}N.

Nitric Oxide Formation

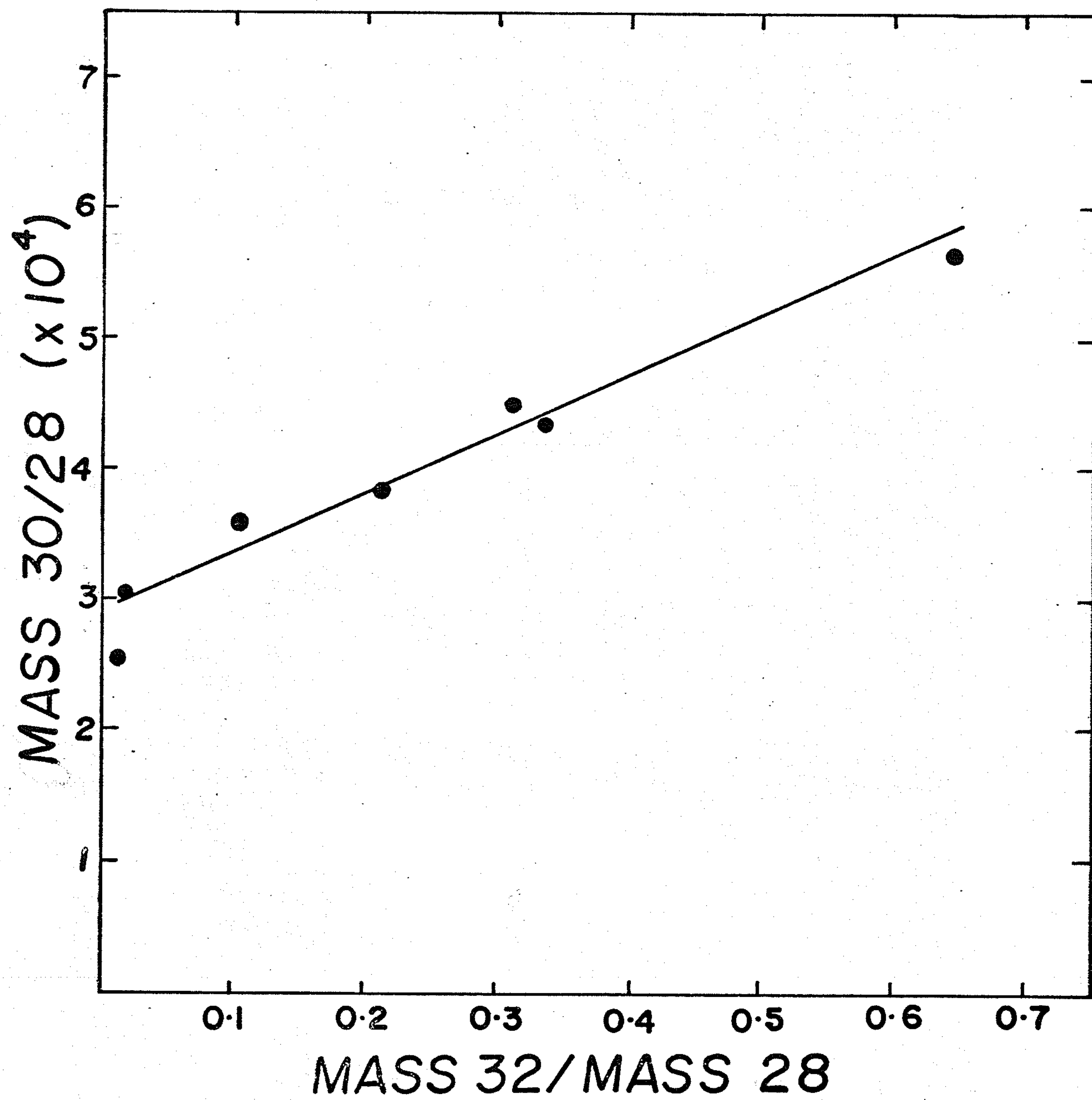
It was found that NO formation in the mass spectrometer on analysis of N_2 and O_2 mixtures contributed to the ion peak at mass 30 in direct proportion to the amount of O_2 present when the latter constituted more than about 3% of the samples (Fig. 9). Hence, samples with mass 32/ mass 28 ratios much greater than 3% were rejected. Minor deviations in mass 32 measurements between replicates indicated little if any atmospheric contamination.

Fig. 8. Dilution curves of a standard $^{15}N_2$ gas sample with normal N_2. Closed circles, mass29/mass 28 ratios; open triangles, mass 30/mass 28 ratios; open squares, the corresponding ^{15}N atom % values calculated.



0·03
3·0
0·02
2·0
0·01
1·0
0·0
MASS 29/MASS 28
15N ATOM %
0·015
0·010
0·005
MASS 30/MASS 28
20
40
80
160
320
640
1280
DILUTION

Fig. 9. Nitric oxide formation in mass spectrometer when N_2 and O_2 mixtures were analysed.


MASS 30/28 (x 10^4)
7
6
5
4
3
2
1
0·1
0·2
0·3
0·4
0·5
0·6
0·7
MASS 32/MASS 28

^{15}N Natural Abundance

Based on the average of eight replicate analyses the atom % ^{15}N in gas samples extracted from air-saturated distilled water treated with the deoxygenating agents was 0.370 ± 0.004 SD (Table II). This value was in close agreement with those determined for controls without chemical treatment and in air. Ion voltage readings determined at mass 28 ranged from 14 to 17 V while the O_2/N_2 ratio measured at masses 32 and 28 was about 3% or about one-seventh of the value found in air.

Test Denitrification Assays

The weight of N_2 evolved by denitrification in the test water samples was estimated to be 8.0 μg liter^{-1} assuming that the lake water was saturated with respect to N_2 and using the documented solubility values of N_2 (Link 1965). Thus about 20% of the NO_3^- taken up was denitrified. In addition to N_2, 12 μg NO_2^--N liter^{-1}, equivalent to 31% of the NO_3^- uptake, had accumulated (Table III).

In the denitrifying bacterial cultures about 90% of the NO_3^- taken up was denitrified (Table IV). Little NO_2^- accumulated at the end of incubation. Similar results were obtained when 47.5 or 95.0 atom % was used in the initial enrichment with $K^{15}NO_3$.

Results were calculated from triplicate incubation

Table II. Natural abundance of ^{15}N in dissolved N_2 extracted from air-saturated distilled water treated with manganous sulfate and alkaline iodide-azide.

Sample Number	Mass 28 ion voltage	$\frac{\text{mass } 32}{\text{mass } 28} \times 100$	^{15}N atom %[a]
1	16	3.3	0.372
2	14	3.2	0.378
3	15	3.0	0.367
4	14	2.7	0.364
5	16	2.9	0.372
6	17	3.2	0.372
7	15	3.2	0.366
8	17	2.8	0.372
9[b]	16	24.0	0.372
10[c]	—	22.0	0.370

[a]Calculated by peak heights from ion voltage measurements at masses 28, 29, and 30 according to Bremner (1965).

[b]From air-saturated distilled water not treated with manganous sulfate and alkaline iodide-azide.

[c]Air, as a control.

Table III. Denitrification in lake water samples amended with 95.0 atom % $K^{15}NO_3$.

Treatment	Incubation days	NO_3^--N µg/ℓ	NO_2^--N µg/ℓ	N_2 µg/ℓ	DO[a] mg/ℓ
Lake water + N-Serve	0	570	0	0	2.0
	5	531	12	8	0.0
Lake water + N-Serve + $HgCl_2$ (20 mg/ℓ)	0	565	0	0	2.0
	5	563	0	0	1.8

[a]Dissolved oxygen (DO) measured according to Anon.(1969).

Table IV. Denitrification of $K^{15}NO_3$ by a *Pseudomonas* species.

Sample Number	NO_3^--^{15}N Enrichment	Incubation days	NO_3^--N μg/0.25ℓ	NO_2^--N μg/0.25ℓ	N_2 μg/0.25ℓ
1	95.0 atom %	0	500	0	0
		2	60	2	416
2	47.5 atom %	0	500	0	0
		2	65	4	408
3	Control[a]	0	500	0	0
		2	502	0	0

[a]Identical with sample 1 except for the addition of 20 mg/ℓ $HgCl_2$.

sets and analyses per experiment and their average taken.

DISCUSSION

Standardization of the ^{15}N Tracer Technique

Consistency of results for the amounts of N_2 analysed from eight replicate samples (average ion voltage was 16 V) suggests that sufficient quantities of N_2 can be consistently recovered from 250 ml water samples and are quite adequate for mass spectrometric analysis. The natural abundance of ^{15}N in aerated distilled water and in air did not vary appreciably from the documented normal abundance value of 0.366 atom % (Junk and Svec 1958). Manganous sulfate and alkaline iodide-azide applied to the water samples reduced O_2 interference to a very low value during mass spectrometric analysis. Measurements of mass 32/mass 28 ratios (Table II) indicated an eight-fold reduction in O_2 content of gas samples by the deoxygenating treatment. The slightly higher mass 32/mass 28 ratio percent observed in dissolved air than that noted for the air sample control (24% as compared to 22%) can be accounted for by the higher solubility of O_2 in water than N_2.

In Fig. 8, the mass 30/mass 28 ratio plot shows a non-linear relation of $^{15}N^{15}N$ abundance with dilution before the level of normal abundance was reached. A

direct linear relation as shown by the mass 29/mass 28 ratio plot would normally be expected. This non-linearity may be accounted for partly by the limited number of $^{15}N^{15}N$ molecules present and partly by the slight reduction in pressure of the gas samples during the process of dilution. Under these circumstances the relative abundance of the various ions produced by electron bombardment of N_2 could be influenced (Bremner 1965). However, as Table II does not show the dependence of ^{15}N determination on the quantity of N_2 analysed, the effect of inlet gas pressure on ion peak measurement is probably insignificant provided the sample gas pressures vary only slightly.

Since the mass spectrometer has been determined to reliably detect about 0.004 atom % excess ^{15}N at the natural abundance level by mass peak measurement, it can be deduced that with the assay technique developed the sensitivity of detection is 0.3 μg N_2-N liter^{-1} with respect to N_2 production[1]. In the adopted method of

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[1] The amount of dissolved N_2 extracted from 250 ml water samples is approximately 3.75 ml or 4.2 mg if 0.015 ml N_2 dissolves in 1 ml water, i.e. assuming saturation and 90% efficiency of extraction. Only about half of the extracted gas sample is analysed by mass spectrometry. For this amount to contain 0.004 atom % ^{15}N excess over natural or background abundance, (2.1 x 0.00004) or about 0.08 μg $^{15}N_2$ has to be derived through denitrification per 250 ml water sample. Hence, about 0.3 μg $^{15}N_2$ liter^{-1} water sample produced by denitrification (when 95.0 atom % $^{15}NO_3$ is initially present) can be detected with the ^{15}N assay technique.

calculation, the total amount of dissolved N_2 in the water sample is estimated using data available in literature. Accuracy will be improved in estimating denitrification rates when the exact amount of dissolved N_2 in the samples is measured.

Test Assay

Active denitrification was not found in the test water samples examined. This was due mainly to O_2 repression since the DO in these samples was 2.0 mg liter^{-1}. In their study of denitrification by indigenous microflora in a brackish lake, Koike *et al.* (1972) observed that little denitrification was evident in waters in which DO exceeded 1.3 ml liter (ca. 1.8 mg liter^{-1}). Earlier, Skerman and MacRae (1957) had reported that NO_3^- was not reduced by *Pseudomonas denitrificans* when cultured in media containing DO more than 0.2 to 0.4 mg liter^{-1}. Although 1 mg liter^{-1} N-Serve, which is known to inhibit NH_3 oxidation (Campbell and Aleem 1965), was added to the water samples, it was unlikely to suppress denitrification at such a low concentration. Recently, Henninger and Bollag (1976) found that N-Serve did not inhibit denitrification in soil or in a *Pseudomonas* culture at concentrations below 30 ppm.

Although the denitrification rate was high in pure culture experiments where conditions for this activity were optimal and where about 80% of applied NO_3^- was de-

nitrified, about 4% of the NO_3^- was not accounted for either as NO_2^- or N_2. An imbalance somewhat smaller than 4% between NO_3^- applied and NO_2^- plus N_2 recovered was also noted in the lake water samples. In this case the imbalance was not likely influenced directly or indirectly by nitrification since N-Serve had been added to the water sample to inhibit nitrification before incubation. Fixation of released N_2 and/or assimilatory NO_3^- reduction were probably insignificant because of the relatively large amount of NH_3 present.

Two possibilities may account for the NO_3^- imbalance: (a) underestimation of N_2 evolution by the extraction and analysis techniques, and (b) the formation of gaseous intermediates such as NO and N_2O.

The ^{15}N atom % in the $K^{15}NO_3$ used was not checked experimentally. The enrichment specified by the supplier (I.C.N. Corp.) was assumed to be correct and was used for all calculations. Analytical data obtained in the calculations of evolved N_2 support the mechanism that this final product of denitrification originated largely from the NO_3^- applied although the possibility of metabolic pooling of cell N and incorporated NO_3^--N is not ruled out. It was assumed that when N_2 volatilized from NO_3^- and pre-existing dissolved N_2 were mixed, no common intermediate was formed and therefore no isotope pooling occurred (Hauck _et al_. 1958). Isotope discrimination was negligible since high

levels (95.0 atom %) of ^{15}N enrichment were used as tracer. If the progressive dilution of the tracer $^{15}NO_3^-$ during incubation by NO_3^- release from organic matter exists, the average ^{15}N atom fraction in the substrate can be calculated (Hauck and Bouldin 1961). However, dilution of $^{15}NO_3^-$ substrate is normally slow and only significant at high O_2 tension.

The error in NO_3^- and NO_2^- determinations was insignificant, therefore the imbalance with respect to NO_3^--N did not likely arise from errors of chemical measurement. It was recognised that gaseous denitrification products other than N_2 were not measured in these initial test assays but N_2O was determined in subsequent experiments by gas chromatography in parallel with mass spectrometric analysis.

B. Lacustrine Studies

RESULTS

a. Water Column

Determination of Incubation Time

The incubation time required for initial denitrification rate measurement was determined in a time-course experiment with anoxic water samples collected at a depth

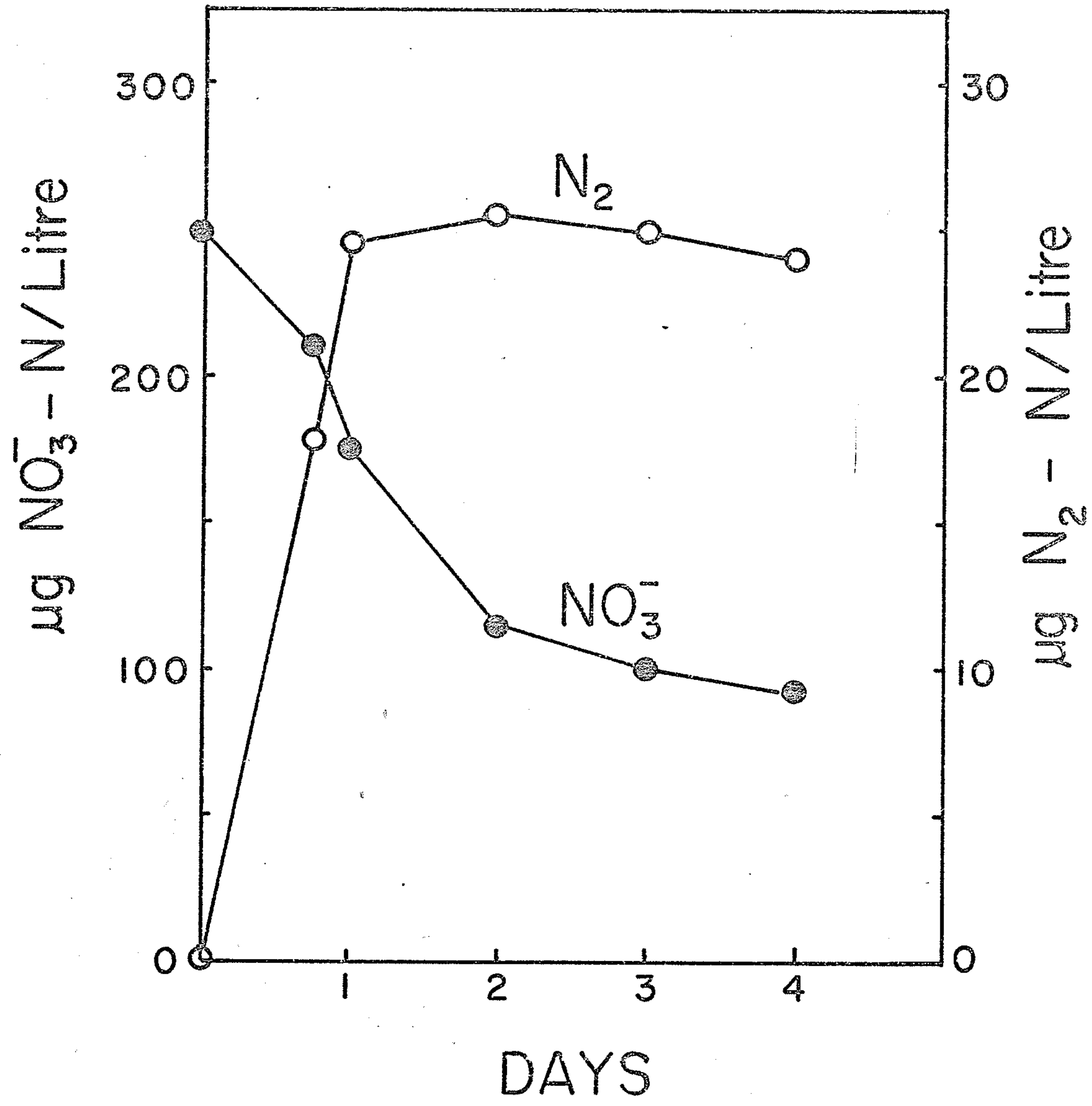
of 5 m. The progress curve of N_2 production (Fig. 10) is linear up to 24 h of incubation while the linear disappearance of NO_3^- extends to 48 h of incubation. A 24 h incubation period was then chosen for the standard aquatic denitrification assay.

Effect of Nitrate Concentration

The dependence of denitrification as well as NO_3^- uptake (measured as its disappearance) rates on NO_3^- enrichment was demonstrated in anoxic hypolimnetic waters (Fig. 11). Rates of denitrification and NO_3^- uptake reached their maxima at 400 and 800 μg N liter^{-1} enrichment levels respectively. Since a subsaturating NO_3^- enrichment (200 μg N liter^{-1}) was used in the standard denitrification assay to simulate natural conditions, the presence of various amounts of background NO_3^- in the lake, depending upon the time and site of sampling, could affect the estimation of aquatic denitrification rates to different extents. Comparison of results obtained in these cases therefore could not be made.

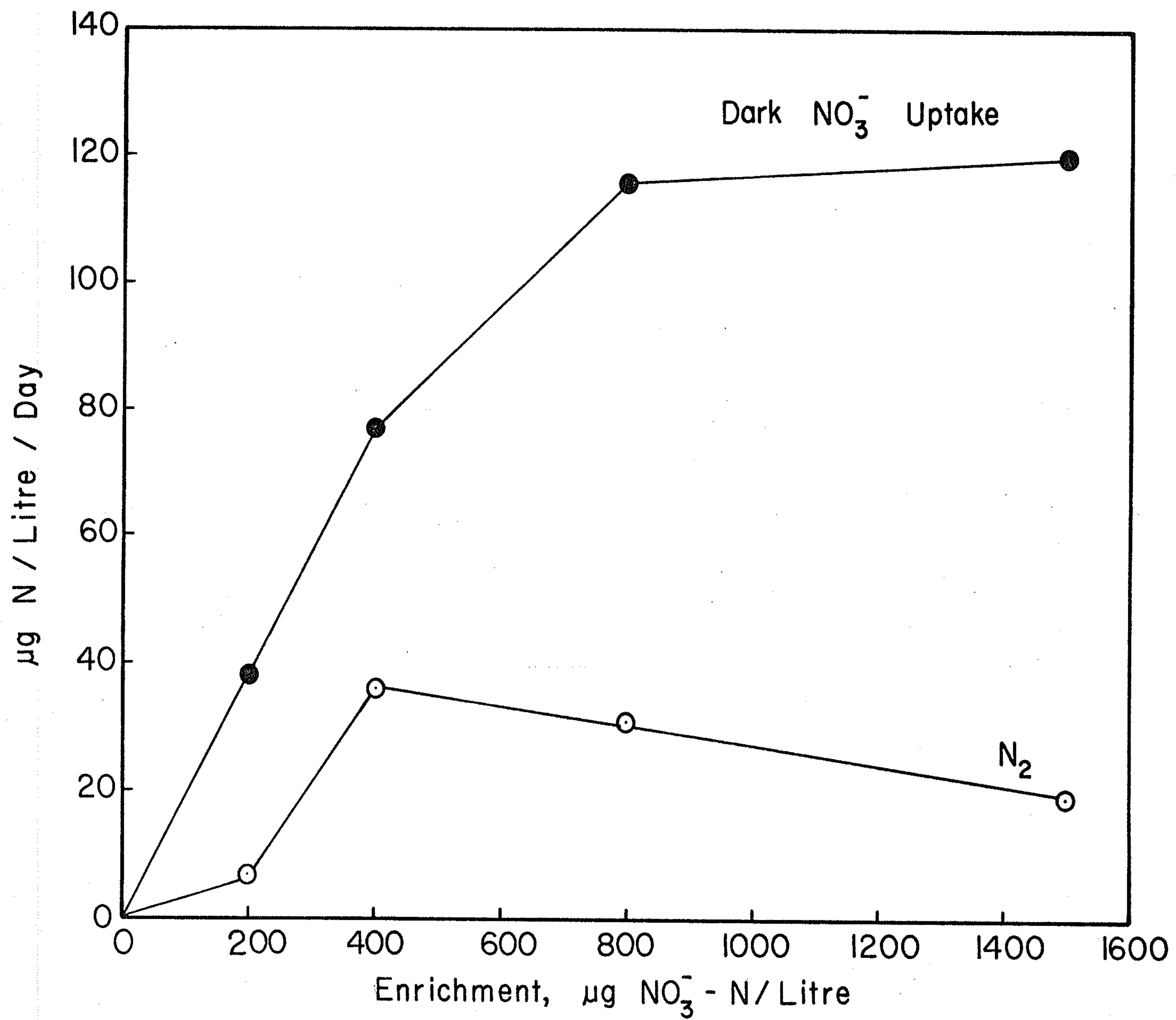
The ambient NO_3^- concentration in the lake waters was monitored after the addition of fertilizer NO_3^-. When dissolved NO_3^- was surface-applied weekly (Schindler et al. 1971) prior to summer 1975, the NO_3^- profile was consistently observed to return from being clinograde after each addition to the dichotomic pattern in 24 h (Fig. 12). Two days after the addition, epilimnetic NO_3^- concentration was reduced,

Fig. 10. Time-course denitrification in 5 m anoxic water samples (June 25, 1973).



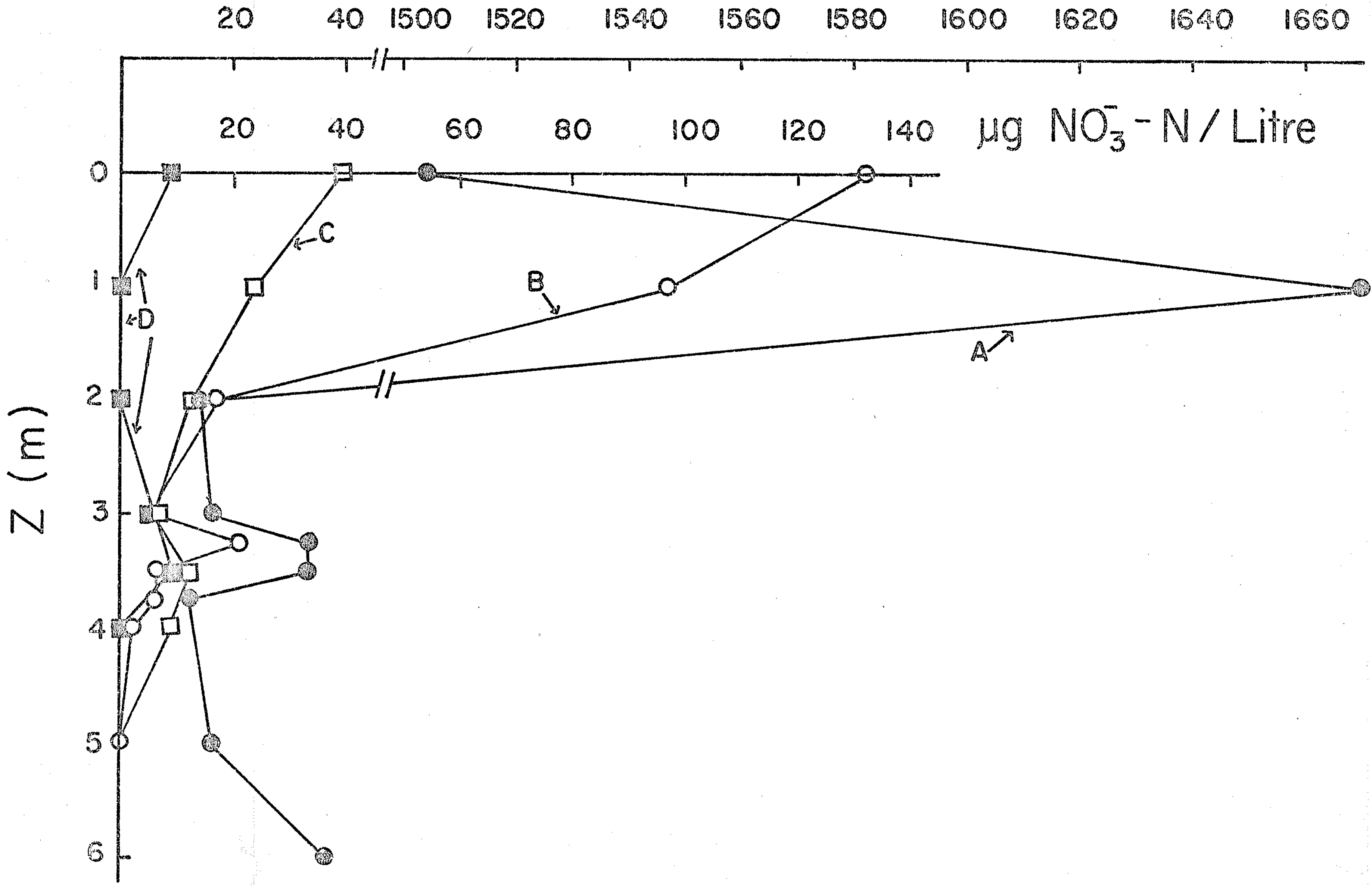
300
200
100
0
30
20
10
0
$\mu g\ NO_3^- - N$ / Litre
$\mu g\ N_2 - N$ / Litre
N_2
NO_3^-
1
2
3
4
DAYS

Fig. 11. Effect of NO_3^- enrichment on the rates of denitrification (open circles) and NO_3^- uptake (closed circles) in 5m anoxic water samples (July 11, 1975).



Dark NO_3^- Uptake
N_2
μg N / Litre / Day
Enrichment, μg NO_3^- - N / Litre
0
20
40
60
80
100
120
140
0
200
400
600
800
1000
1200
1400
1600

Fig. 12. Changes in NO_3^--N profile after weekly fertilization of Lake 227. A and B, 40 min and 5 h after fertilization on July 23, 1974; C and D, respectively 24 h and 7 days after fertilization. The NO_3 concentrations at 0 and 1 m, curve A, refer to the upper x-axis.



20
40
1500
1520
1540
1560
1580
1600
1620
1640
1660
20
40
60
80
100
120
140
μg NO_3^- - N / Litre
Z (m)
0
1
2
3
4
5
6
A
B
C
D

presumably by algal uptake, to detectable levels (ca. 10 μg N liter^{-1}) and NO_3^- was not found below 5 m. A NO_3^- peak less than 40 μg N liter^{-1} usually existed at 3.5 m. The measurements of denitrification rate in the water column were made more than 24 h after fertilization by which time little background NO_3^- remained at the start of the experiments. When NO_3^- loading was reduced by two-thirds to about 2.1 g N m^{-2} yr^{-1} starting in summer 1975, NO_3^- was barely detectable in the lake waters in almost all occasions since it was quickly assimilated by particulate matter. Nitrite did not accumulate in significant quantities in Lake 227.

Effect of Nutrient Enrichment

When glucose, NH_4Cl, and Na_2HPO_4 were individually applied at different concentrations to anoxic waters in addition to 200 μg NO_3^--N liter^{-1}, no significant effect on denitrification was observed (Table V). Addition of C and P did not affect NO_3^- uptake but enrichment with NH_4^+ slightly enhanced NO_3^- uptake. The latter observation may suggest N limitation in Lake 227 plankton.

Effect of Light

Similar denitrification rates were obtained for anoxic hypolimnetic waters (5 m) incubated *in situ* or at the *in situ* temperature in the dark for 24 h. For example, the rate of N_2 production was 12 μg N liter^{-1} day^{-1} when

Table V. Effect of C, N, and P enrichment on denitrification and NO_3^- uptake rates in anoxic hypolimnetic waters (5 m) at 6°C in the dark.

	Enrichment	Rate of N_2 Production μg N $liter^{-1}$ day^{-1}	Dark NO_3^- Uptake μg N $liter^{-1}$ day^{-1}
Glucose	0 μg C $liter^{-1}$	10	42
(July 14, 1975)	60	9	42
	100	11	44
	150	8	47
	250	9	49
NH_4Cl	0 μg N $liter^{-1}$	4	38
(June 30, 1975)	100	4	52
	200	4	52
	600	6	56
	1,000	6	53
Na_2HPO_4	0 μg P $liter^{-1}$	12	44
(July 22, 1975)	0.5	13	40
	1.0	15	41
	5.0	17	44
	20.0	15	43

incubated *in situ* compared to that of 14 μg N liter day^{-1} when incubated in the dark (July 16, 1974). Uptake of NO_3^- was 20 and 24 μg N liter^{-1} day^{-1} respectively in the same experiment.

Distribution of Denitrifiers

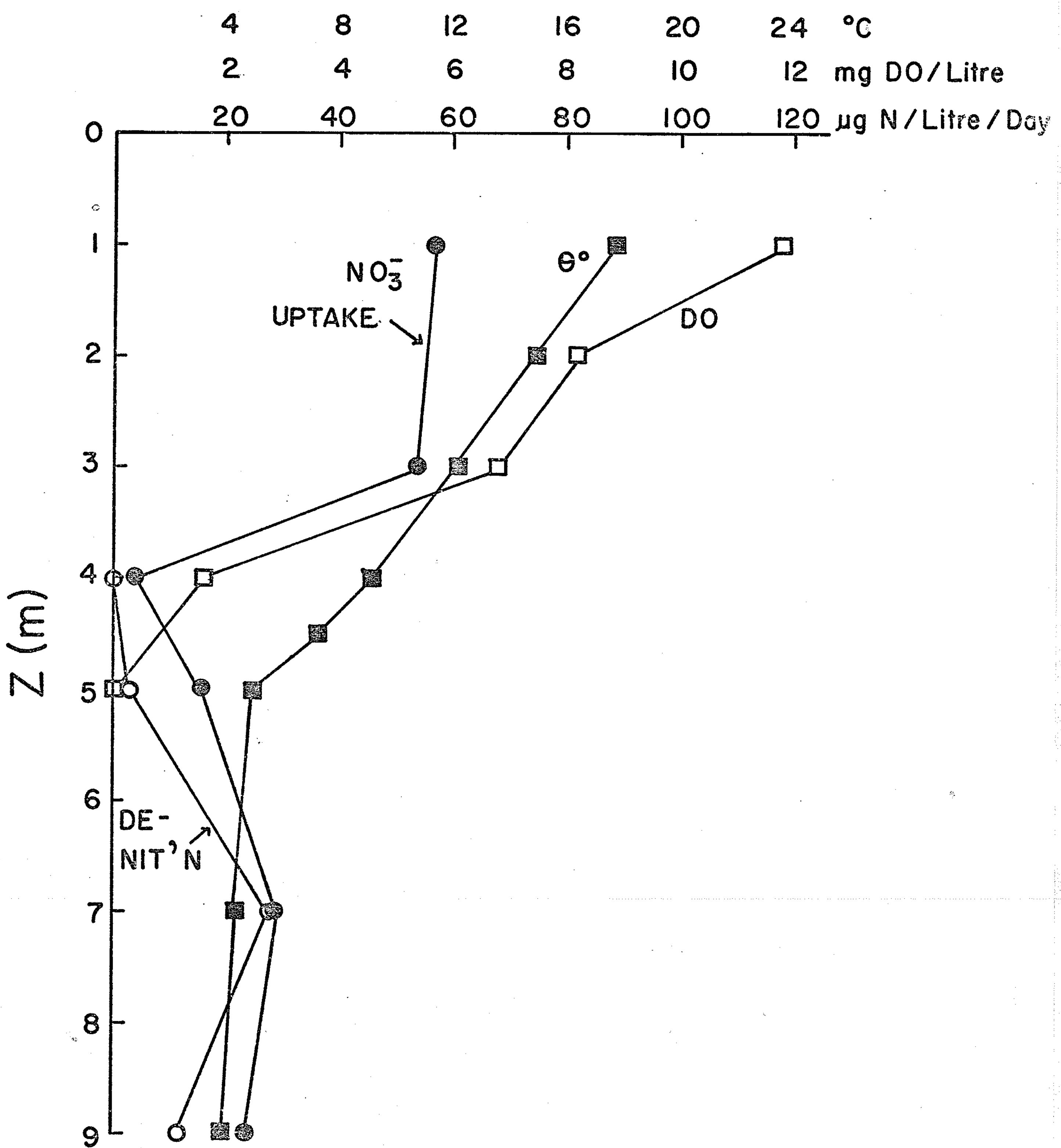
MPN estimates for the density profile of denitrifiers in the water column on two occasions in August 1973 ranged from 20 to 500 cells liter^{-1}(Figs. 13c and 13d). Although there were more denitrifiers in the epilimnion than in the hypolimnion, their number did not correlate with the DO, NO_3^- or denitrification profiles. In the hypolimnion, however, the number of denitrifiers was about 50 cells liter^{-1} or less and demonstrated denitification activities accordingly although the number might not be absolute and possibly underestimated (discussed below).

Estimation of Denitrification Rates

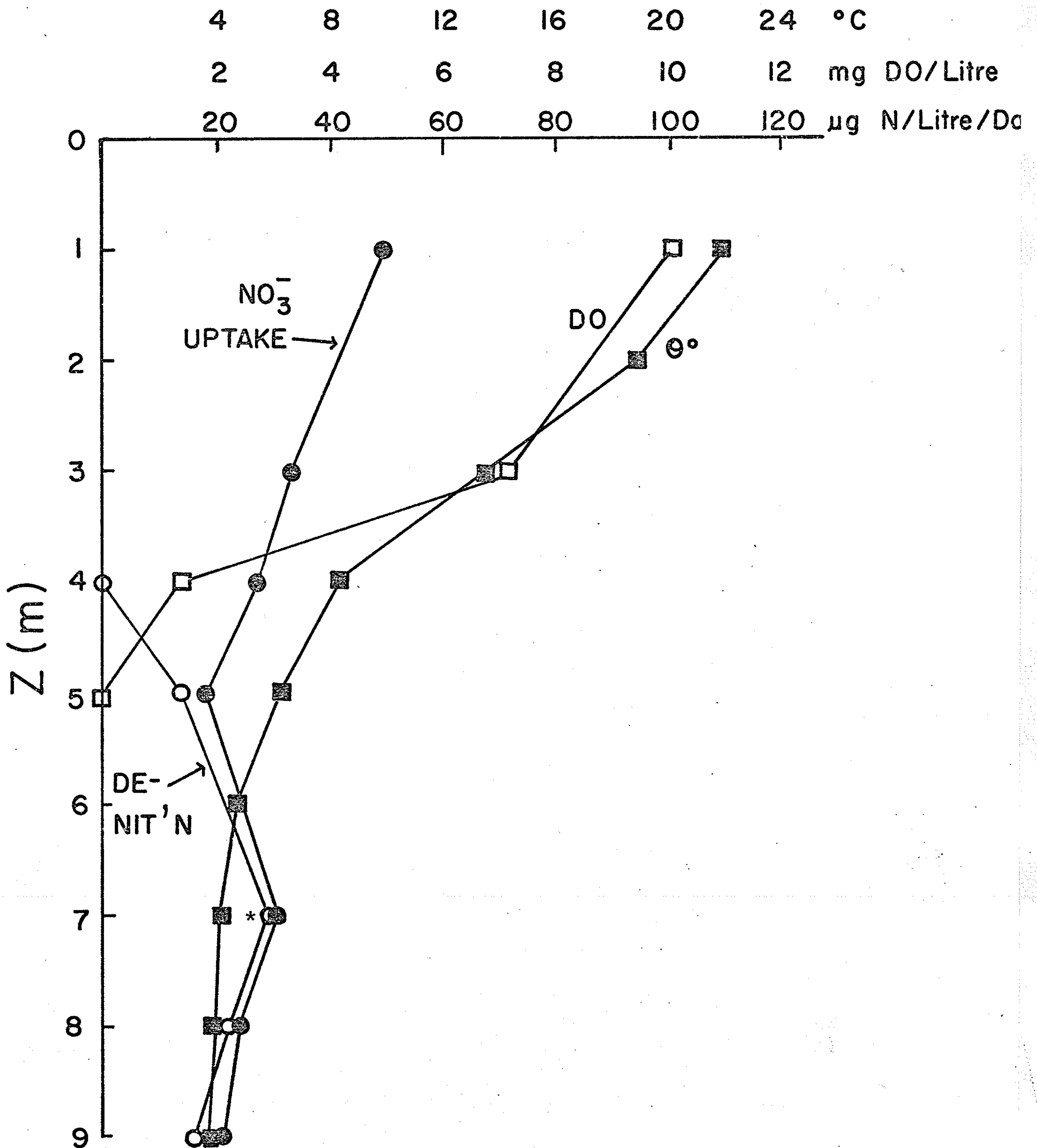
Rate profiles of denitrification and NO_3^- uptake (measured as NO_3^- disappearance after incubation) from June to August in 1973 are shown in Fig. 13. In all cases, denitrification did not occur in the water column where DO was above 0.2 mg liter^{-1}. Below the thermocline where anoxic conditions prevailed, denitrification rates were proportional to the NO_3^- uptake rates. Denitrification rates as high as 30 μg N liter^{-1} day^{-1} were measured. The upper

Fig. 13. a,b,c,d Denitrification profiles and related parameters in the water column during summer 1973. An asterisk denotes detectable N_2O production.

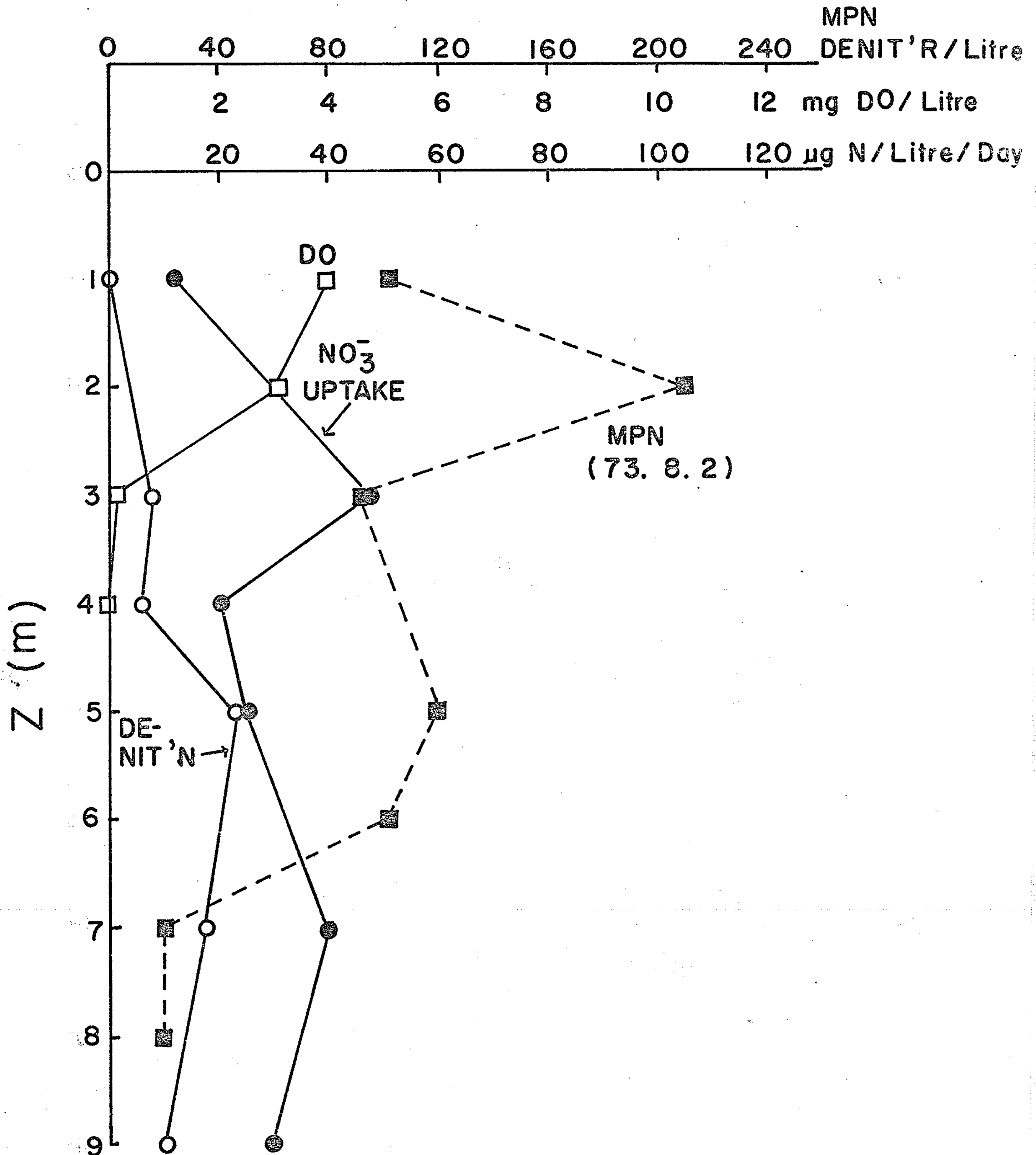
a. JUNE 20, 1973.

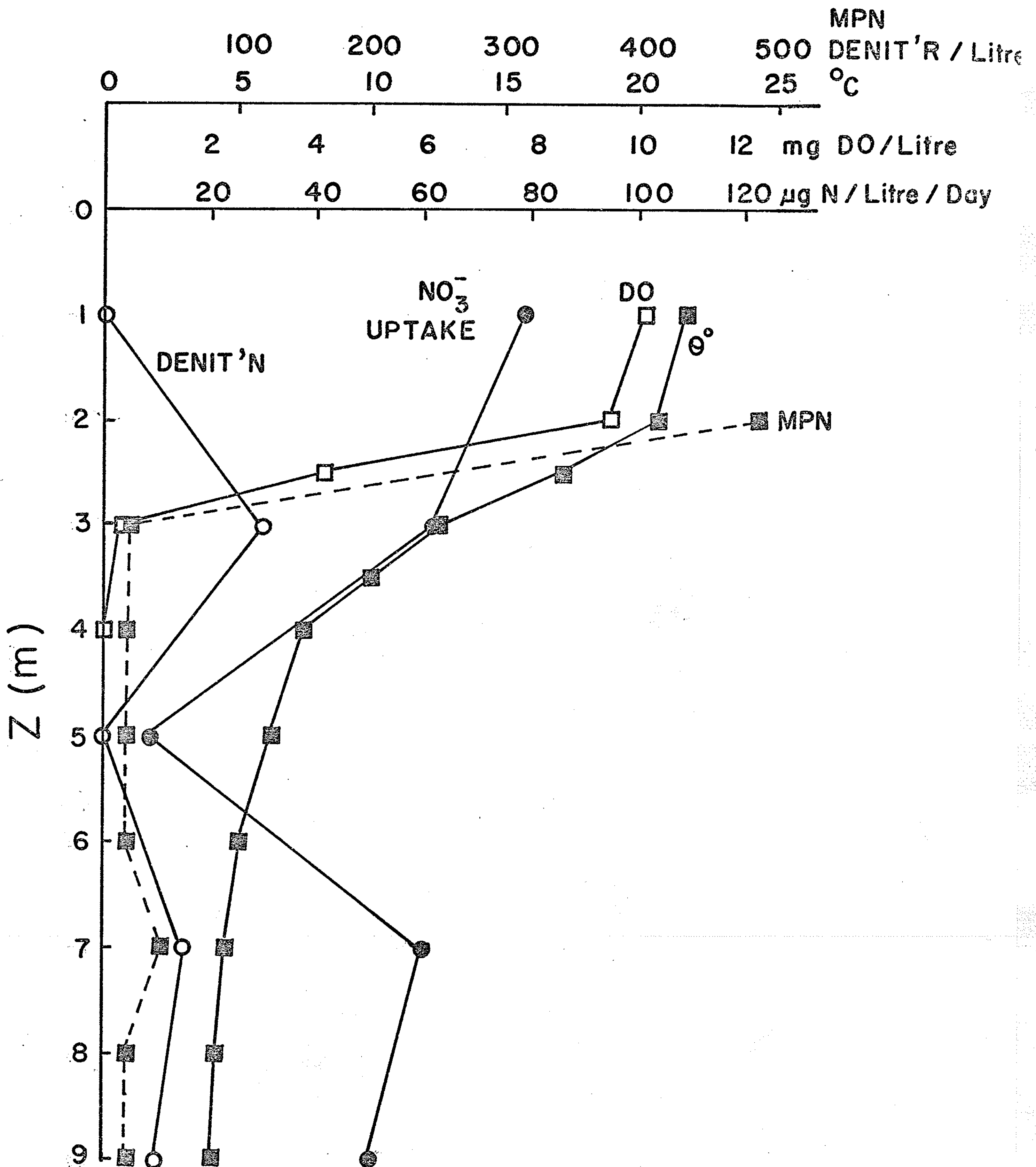


b. JULY 16, 1973.



c. AUG 1, 1973





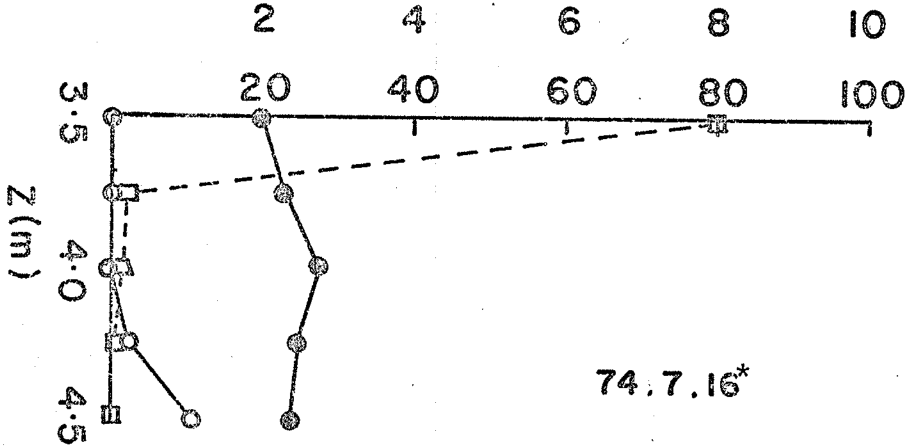
d. AUG 22, 1973
MPN
100 200 300 400 500 DENIT'R / Litre
0 5 10 15 20 25 °C
2 4 6 8 10 12 mg DO/Litre
20 40 60 80 100 120 µg N / Litre / Day
0 1 2 3 4 5 6 7 8 9
Z (m)
NO_3^- UPTAKE
DO
θ°
DENIT'N
MPN

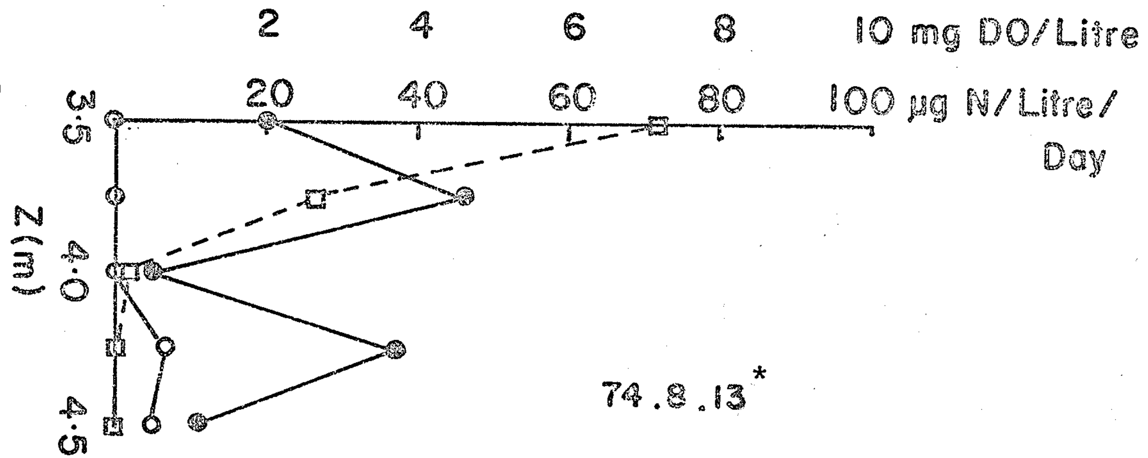
extension of the denitrification region to the three-meter level towards the end of July 1973 coincided with the upward extension of the anoxic zone found on August 1 due to the O_2 demand imposed by the collapsed phytoplankton population (Fig. 13c). Nitrous oxide formation was detected in the hypolimnion but only traces of indigenous N_2O were occasionally detected. The rates of denitrification determined represented the sum of both N_2O and N_2 production with the latter as the consistently predominant product.

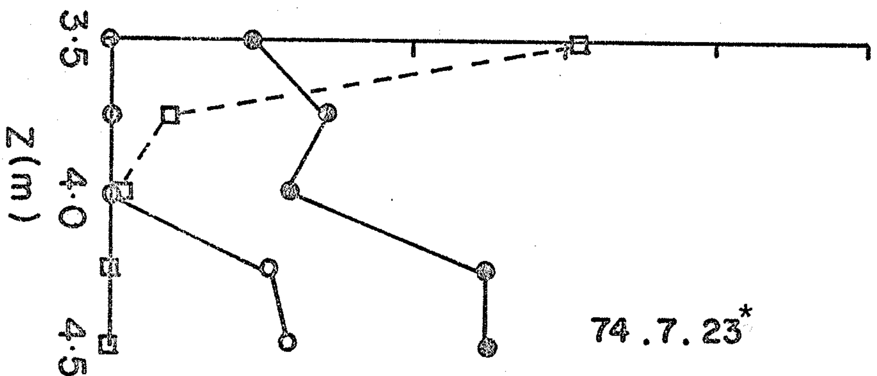
The NO_3^- uptake rate was about 60 μg N liter^{-1}day^{-1} in the surface waters in June and July, 1973 (Fig. 13a and 13b) presumably due to phytoplankton development. This rate decreased drastically in late July and the beginning of August as the phytoplankton collapsed. In late August, when a secondary algal bloom developed, it increased and exceeded its former rate to about 80 μg N liter^{-1} day^{-1}.

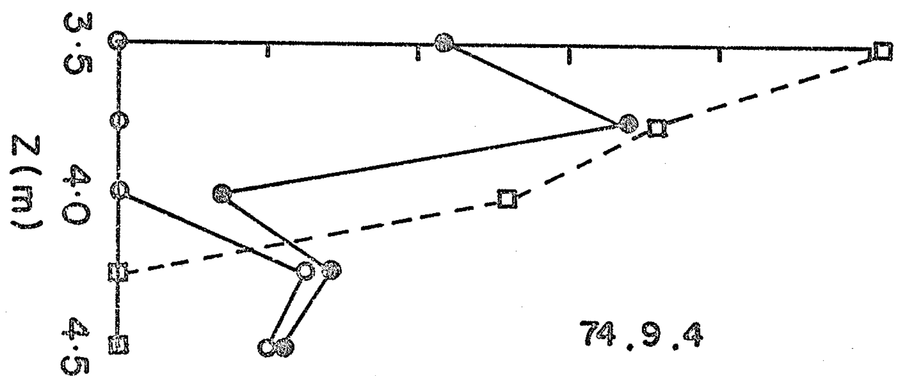
A one-meter water layer between 3.5 and 4.5 m in the thermocline region was examined for denitrification activities during summer 1974. In this layer of water little DO was found and detectable quantities of NO_3^- were present probably as unassimilated fertilizer or product of NH_3 oxidation. Between 4.0 and 4.5 m where DO was less than 0.2 mg liter^{-1} denitrification occurred at rates ranging from 3 to 23 μg N liter^{-1} day^{-1} (Fig. 14). Nitrate uptake varied from 5 to 65 μg N liter^{-1} day^{-1}. In summer 1975, the O_2-limiting layer (where DO was between 0 and 0.2 mg liter^{-1}) was confined to a depth interval of 0.5 m

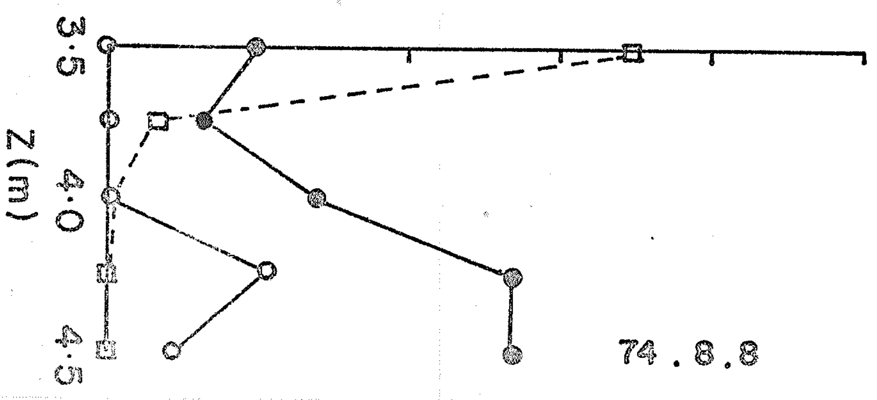
Fig. 14. Denitrification profiles and related parameters in the O_2-limiting region during summer 1974. Open circles, denitrification; closed circles, nitrate uptake; open squares, dissolved oxygen. An asterisk following the date indicates the experiment was set up just before fertilization.


2
4
6
8
10
20
40
60
80
100
3·5
4·0
4·5
Z(m)
74.7.16*


10 mg DO/Litre
100 μg N/Litre/Day
2
4
6
8
20
40
60
80
3·5
4·0
4·5
Z(m)
74.8.13*


3·5
4·0
4·5
Z(m)
74.7.23*


3·5
4·0
4·5
Z(m)
74.9.4


3·5
4·0
4·5
Z(m)
74.8.8

in the thermocline and followed the oscillation of the thermocline in the 3-5 m depth range (Fig. 15). High rates of denitrification and NO_3^- uptake were observed in June but averaged about 10 and 50 μg N liter^{-1} day^{-1} respectively for the rest of the summer period. Fluctuation in the uptake of NO_3^- probably resulted from the oscillation of the algal population in that region. Nitrous oxide formation was occasionally detected in 1975 but not in 1974 in the O_2 minimum layer.

b. Littoral Sediment-Water Interface

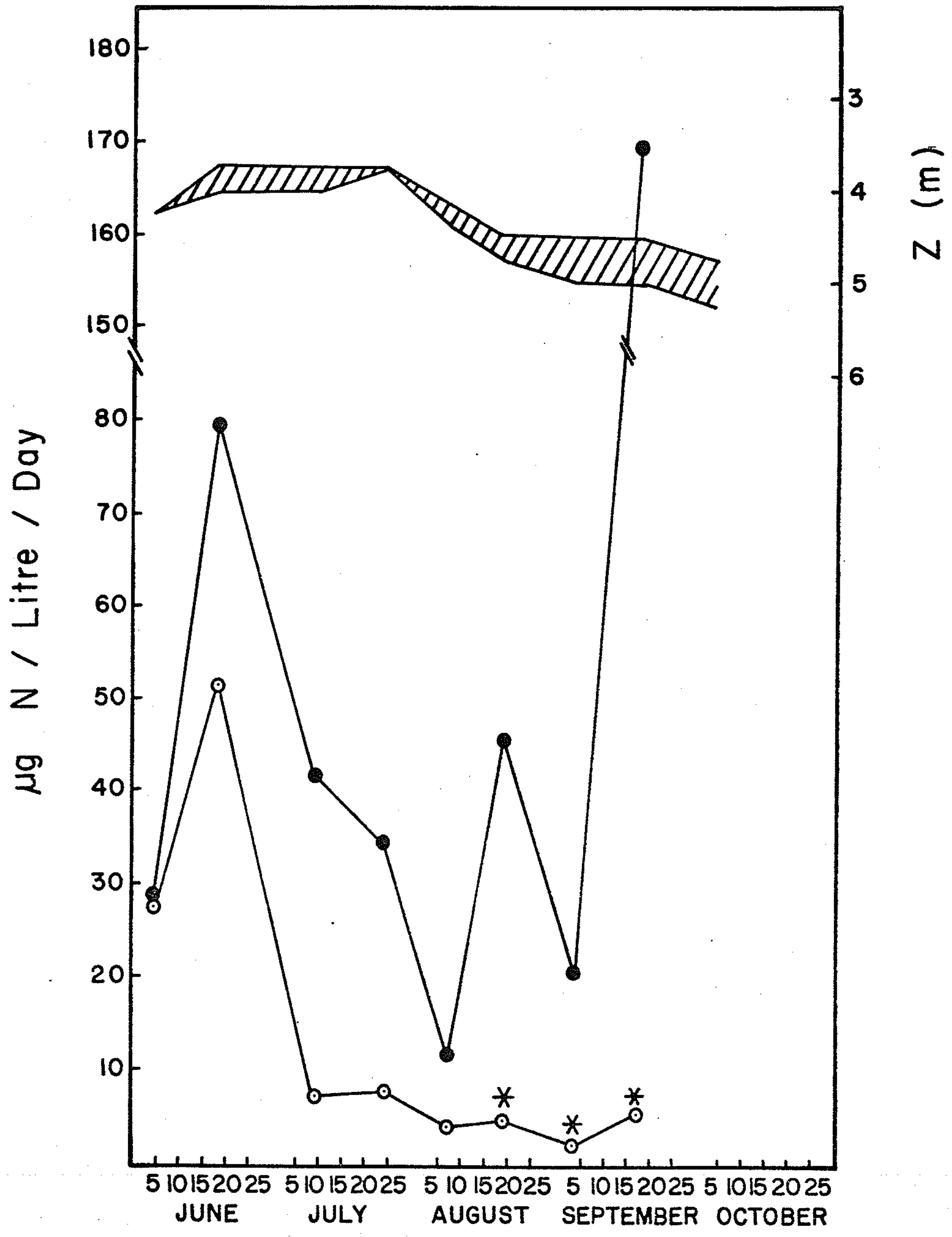
Core Experiments

Sediment-water denitrification assays on two plexiglass cores demonstrated N_2 production in both $HgCl_2$-treated and untreated samples. Similar results were obtained for the two cores. Nitrite and N_2O were not formed during the course of incubation. For reasons discussed below, although the $HgCl_2$-treated sample failed to serve as a proper control, denitrification was assumed to be a biological process.

IN SITU Denitrification Rates

Studies of *in situ* denitrification in semiclosed sediment-water systems with the plexiglass chambers were initiated in summer 1974. A typical set of results is presented in Table VI. After the first and second days

Fig. 15. Denitrification and NO_3^- uptake rates (open and closed circles respectively) in the water column where DO<0.2 mg $liter^{-1}$ and NO_3^--N >10 μg $liter^{-1}$ (hatched area) during summer 1975. Asterisks denote detectable N_2O production.



180
170
160
150
80
70
60
50
40
30
20
10
μg N / Litre / Day
3
4
5
6
Z (m)
5 10 15 20 25 5 10 15 20 25 5 10 15 20 25 5 10 15 20 25 5 10 15 20 25
JUNE
JULY
AUGUST
SEPTEMBER
OCTOBER
1975

Table VI. Denitrification at littoral sediment-water interface studied with an *in situ* semi-closed system (September 2, 1974).

Day 0	BOD Sample 1.5 m water	Chamber Experiment	Chamber Control ($HgCl_2$)
Dissolved O_2	11.3	8.05	6.75 mg liter^{-1}
NO_3^--N	535	529	529 μg liter^{-1}
NO_2^--N	0	0	0 μg liter^{-1}
N_2O-N	0	0	0 μg liter^{-1}
N_2-N	0	0	0 μg liter^{-1}
Day 1			
Dissolved O_2		5.95	3.88 mg liter^{-1}
NO_3^--N		320	329 μg liter^{-1}
NO_2^--N		0	0 μg liter^{-1}
N_2O-N		0	0 μg liter^{-1}
N_2-N		19.5	21.4 mg m^{-2}
Day 2			
Dissolved O_2	9.2	5.70	2.90 mg liter^{-1}
NO_3^--N	42	165	120 μg liter^{-1}
NO_2^--N	0	4	6 μg liter^{-1}
N_2O-N	0	8	10 μg liter^{-1}
N_2-N	0	28.1	27.8 mg m^{-2}

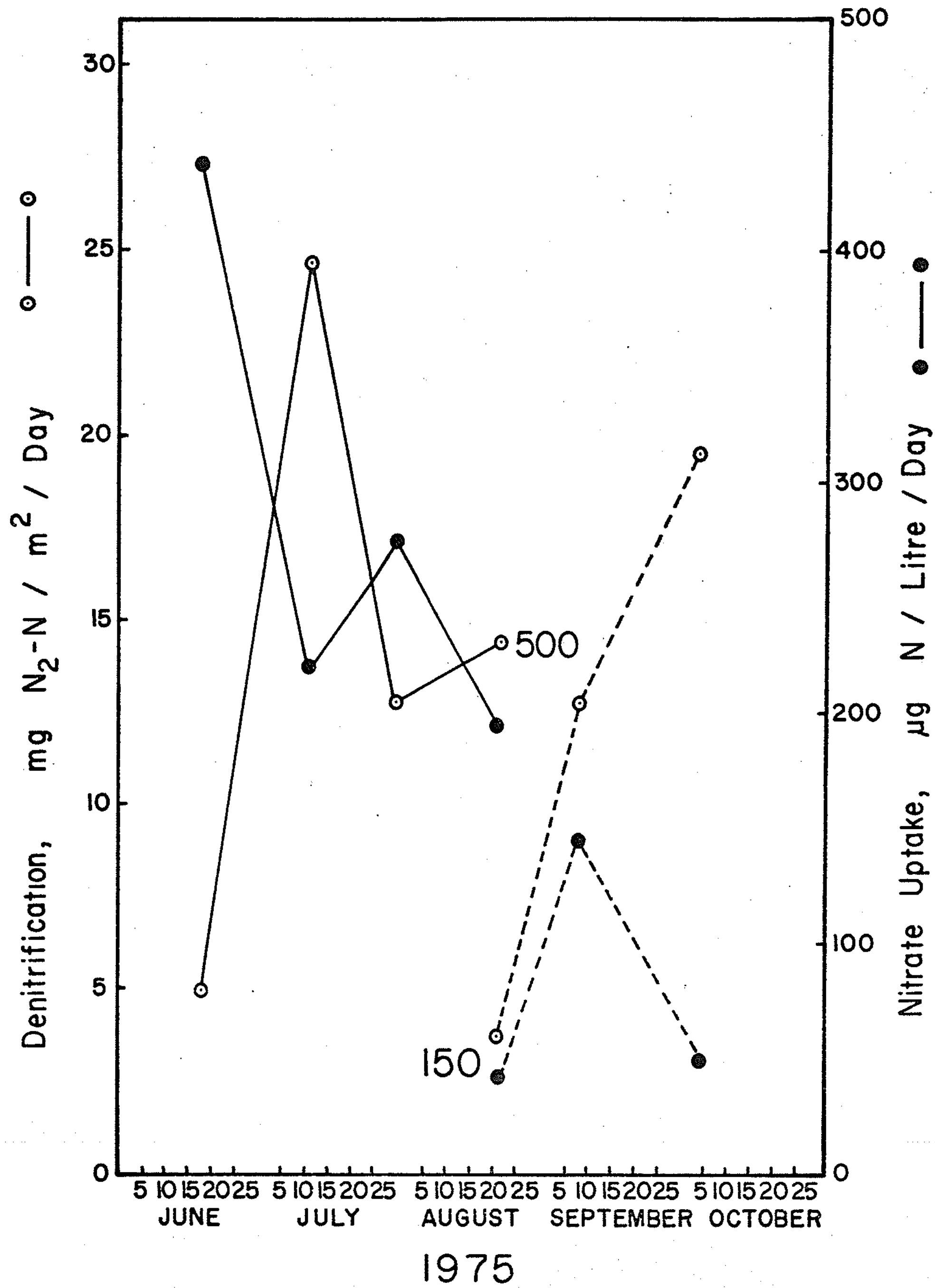
of incubation, 19.5 and 28.1 mg N_2-N m^{-2} were produced respectively. A comparable result was seen in the chamber treated with $HgCl_2$ which again indicated that $HgCl_2$ was an ineffective biological inhibitor for sediment systems. The use of formaldehyde (4% final concentration) was later found to be effective. There was no N_2 production in the bottled epilimnetic waters overlying the sediments, therefore the denitrification observed was effected by bacterial activity in the anoxic sediment. In these *in situ* experiments, 20% of the NO_3^- consumed in 2 days was accounted for by denitrification activity, the remainder was presumably taken up by the phytoplankton. The high NO_3^- uptake rate (ca. 200-300 μg N liter^{-1} day^{-1}) compared to that in the surface waters in the denitrification profile studies (maximum 60 μg N liter^{-1} day^{-1}) was likely due to the high NO_3^- enrichment used in the chamber experiment as well as to additional uptake by benthic organisms.

In 1975, denitrification rates were in the same order of magnitude as those measured in the previous year (Fig. 16). The 'opposite trends' in the magnitude of denitrification and NO_3^- uptake rates in the plexiglass chamber indicated that denitrification was dependent on the amount of residual NO_3^- available to the sediment after assimilation by phytoplankton.

Effect of Nitrate Concentration

The dependence of denitrification rate on NO_3^-

Fig. 16. Denitrification rates in the littoral sediment-water interface during summer 1975. On August 21, both 150 and 500 μg NO_3^--^{15}N liter^{-1} enrichments were used. The high enrichment was used before, and the low after August 21.


Denitrification, mg N2-N / m2 / Day
Nitrate Uptake, µg N / Litre / Day
30
25
20
15
10
5
0
500
400
300
200
100
0
500
150
5 10 15 20 25
JUNE
JULY
AUGUST
SEPTEMBER
OCTOBER
1975

concentration was also demonstrated at the sediment-water interface. When measured with 150 and 500 μg NO_3^--N liter^{-1} enrichments, these rates were 4 and 15 mg N m^{-2} day^{-1} respectively when converted on an areal basis. Traces of NO_2^- and N_2O were formed only with the high enrichment (see also Table VI) and the intermediates of denitrification did not accumulate with the low enrichment.

Effect of Light

Littoral denitrification at the sediment-water interface might be repressed in day-light under *in situ* conditions since photosynthetic generation of O_2 might maintain a high DO concentration in the overlying waters to destabilize the O_2-limiting conditions essential for denitrification. Rates of denitrification measured with one clear and one light-proof dark chamber were compared. Although DO decreased from 7.2 mg liter^{-1} to 5.5 and 3.1 mg liter^{-1} in the clear and dark chambers respectively in 24 h, denitrification rates were similar (ca. 15 mg N m^{-2} day^{-1}). Therefore, even in the absence of photosynthetic generation of O_2 the oxidative processes that took place during the incubation period did not lower DO sufficiently (thus not reducing O_2 supply to the sediments) to promote denitrification significantly in the experimental system.

Rate of Mixing at the Sediment Surface

From the profile of interstitial NO_3^- concentration in the littoral sediments (Table VII), the enrichment NO_3^- in the sediment-water enclosure penetrated only into the upper 2 cm of sediment. When the denitrification rate was reliably measured, the mixing rate of NO_3^- into the anoxic sediments could be estimated by the sum of denitrification, interstitial NO_3^- and sediment NO_3^- uptake. Nitrate uptake in the sediments was probably insignificant since where inorganic N as NH_3 was abundant, NO_3^- was not an important N source. It was found that of the total 7,500 μg N NO_3^--N added to the chamber, 4,995 μg N was lost in one day. About 11% of this loss was accounted for by denitrification and only 0.3% by interstitial NO_3^-. The rate of NO_3^- mixing into the littoral sediments under the experimental conditions was then approximately 13.7 mg N m^{-2} day^{-1} which was essentially the same as the denitrification rate. It implied that almost all the NO_3^- that mixed into the sediments was denitrified. The mechanism of mixing was a combination of diffusion, wind action and bioperturbation.

DISCUSSION

Significant Site of Lacustrine Denitrification

The littoral sediment-water interface was assessed

Table VII. Interstitial NO_3^- and NH_3 gradients in littoral sediments of Lake 227.

Depth, cm	NO_3^--N, μg liter^{-1}	NH_3-N, μg liter^{-1}
1.5	22	145
3.0	6	254
4.5	5	313

to be the most significant site of natural denitrification compared with the O_2-limiting (<0.2 mg liter^{-1} DO) upper hypolimnion and the anoxic hypolimnion for the following reasons. It had been observed that in the water column where the algal primary producers quickly took up most of the added fertilizer NO_3^- in the surface waters, and where the density-discontinuous layer (thermocline) presented a physical barrier to diffusion during summer stratification, only very little unassimilated NO_3^- was available for *in situ* denitrification in the O_2-limiting hypolimnion. In view of the low *in situ* NO_3^- concentrations and hence the low denitrification rates at the oxic-anoxic boundary (see below), this site does not appear to contribute significantly by denitrification to whole lake N budget except, perhaps, for early summer when ambient NO_3^- concentration remains high. Similarly, since the hypolimnion hardly received any fertilizer NO_3^- it is not considered to be a site of natural denitrification in Lake 227, which is not influenced by ground water. On the other hand, the surface-applied NO_3^- that reached the littoral sediments added to conditions conducive to denitrification at the sediment surface. In Lake 227, the surface sediments are of fine silt in texture, rich in organic C and N, reduced in O_2 tension, and probably sufficiently low in redox potential (Brunskill *et al*. 1971) to support bacterial denitrification.

Factors Controlling Denitrification Rates

Of the parameters examined (DO, NO_3^- concentration, light, and denitrifying population) in parallel with denitrification, DO and NO_3^- concentration were the main factors determining denitrification in the water column and in sediments. In the *in situ* sediment-water system although DO was high in the overlying waters (even in the absence of photosynthetic generation of O_2) and might favor entry by diffusion or mixing into the surficial sediments, anaerobic microenvironments were presumably maintained on the surfaces of particulates assisted by oxidative processes.

Denitrification was severely limited by the low *in situ* NO_3^- concentration in the water column. From the data shown in Fig. 11, this process was probably limited at NO_3^- concentrations below 400 μg N liter^{-1}. As pointed out by Koike *et al*. (1972), it becomes more difficult, if not impossible, to measure the absolute *in situ* denitrification rate since the addition of tracer NO_3^- inevitably raises the level of *in situ* NO_3^-. On the other hand, low enrichment could result in N_2 production beyond the sensitivity of analysis. Consequently, a practical compromise for simulating *in situ* concentrations and determining N_2 production within detection limit has to be made to allow valid estimation of relative denitrification rates. The

error of over-estimation involved with the standard 200 μg NO_3^--N liter^{-1} enrichment (about 10 times *in situ* concentration) assay technique for denitrification profile studies was realized. Hence, the estimated denitrification rates at the O_2-limiting layer only demonstrated a potentially significant site of natural denitrification during summer stratification. Prior to 1975, each weekly addition of NO_3^- to Lake 227 surface produced a high NO_3^- concentration state in the epilimnion which lasted for about 5 h (Fig. 12). During this transient period substrate limitation for sediment denitrification was believed to be non-existent and over-estimation of natural denitrification was not anticipated. In the summer of 1975, *in situ* NO_3^- concentrations remained low after the reduction in NO_3^- loading. Enrichment with the standard 500 μg NO_3^--N liter^{-1} in sediment denitrification assays therefore likely over-estimated natural rates (Fig. 16).

The mere presence of denitrifiers (facultative anaerobes) in the water column did not reflect their *in situ* denitrification activity which was largely dictated by DO (Figs. 13a and 13b). Their numbers indicated a denitrification potential upon depletion of DO and correlated positively with denitrification in the anoxic hypolimnion only. Koike *et al*. (1972) found in a brackish lake that denitrifying bacteria were uniformly distributed in the water column irrespective of seasonal variation but their number did not show a positive correlation with

denitrification. It has been realized that natural microbial population is mainly concerned with heterotrophic production and mineralization and only secondarily with the ecological conditions and stress factors to which it is exposed (Jannasch *et al*. 1971). Hence, the number and composition of microorganisms may only reflect their environmental conditions to a certain extent depending on their metabolic alternatives. Enumeration of the denitrifiers in Lake 227 by MPN enrichment technique and gas production indicated uniform distribution in the hypolimnion but low numbers of denitrifiers were found. It was suspected these numbers represented an underestimation. First, incubation at 22°C employed for the MPN method might be too high for the obligate psychrophiles to develope. Secondly, production of N_2O might have proved a more sensitive and reliable criterion for the presence of denitrifiers in the MPN enrichment cultures than general gas production in inverted vials (Patriquin and Knowles 1974). The denitrifying population was not enumerated in the sediments. Their numbers may vary from 10^5 to 10^7 g^{-1} wet weight depending on the sediment chemistry (Patriquin and Knowles 1974).

Although the effect of temperature on the rate of denitrification was not investigated on the field samples, based on pure culture studies (Section C) temperature is believed to exert an effect on the quantitative distribution

of the denitrification products as well as on denitrification rate.

Nitrous Oxide Production

Formation of trace amounts of N_2O was only occasionally observed in hypolimnetic or sediment denitrification. In the sediment-water system, N_2O was detected only in association with the high NO_3^- enrichment (500 μg N $liter^{-1}$) and it was not formed with the low enrichment (150 μg N $liter^{-1}$). When milligram amounts of NO_3^- was added to Lake 227 littoral sediments in the laboratory for long term incubation at 13°C, N_2O was found by mass spectrometry to be accumulating in large quantities although N_2 was still the final product (C.M. Cho, personal communications). Considering the small quantity of N_2O produced in the lake and that its solubility being about 40 times (by volume) that of N_2 in water, this oxide of nitrogen seemed to be reduced easily and escaped detection by gas chromatography. In pure cultures, N_2O production by aquatic denitrifiers was temperature dependent as well as species dependent (see Section C).

Biocontrol

In spite of the failure of $HgCl_2$ to serve as a proper biocontrol for sediment denitrification, this activity is believed to be of biological origin rather than chemodenitrification. First, only NO_2^-, not NO_3^-, is

chemically denitrified, usually at a pH below 5.0, to form gaseous nitrogen compunds (Nömmik and Thorin 1972). Secondly, chemodenitrification should occur irrespective of the presence of O_2. Thirdly, bacterial species in sediments, e.g. Pseudomonas spp., have been demonstrated to be capable of methylating Hg^{2+} at low concentrations and volatilizing Hg^{o} (Jensen and Jernelöv 1969; Spangler et al. 1973). Finally, 4% formaldehyde was shown to stop denitrification in the sediments. It appeared that the concentration of Hg^{2+} might have been quickly lowered by binding to colloidal particles or S^{2-} at the sediment surface (Gavis and Ferguson 1972), thus reducing its bacteriostatic efficiency while formaldehyde did not bind to sediments. However, the Hg^{2+} remaining in the control sediment-water system was not determined after incubation to check for its uptake.

Denitrification as a Potential N Sink

Denitrification in the vicinity of the upper hypolimnion during summer stratification might not be readily apparent as a significant sink for NO_3^--N under natural conditions because of possible overestimation due to addition of tracer NO_3^- dicussed above. The in situ NO_3^- concentration in the surface water was controlled by both phytoplankton uptake (discussed in Part II) and by denitrification at the littoral sediment-water interface.

The maximum NO_3^- removal rate can be computed for ice-free periods prior to 1975 assuming that littoral sediment denitrification rate was essentially constant for water depths <2 m throughout the summer fertilization period. If A (m^2) is the total sediment surface area below 2 m or less of water, L (μg N) is the NO_3^- loading per week, and V_{dn} (μg N m^{-2} h^{-1}) is the rate of sediment denitrification, then the time required for the weekly loading of NO_3^- to be depleted by sediment denitrification alone is given by

$$t_{dn} \text{(in hours)} = L/(A.V_{dn}) \qquad (5)$$

Similarly, if $\overline{V}_{NO_3^-}$ (μg N $liter^{-1}$ h^{-1}) is the maximum NO_3^- uptake rate estimated by chamber experiments, the time required for this process alone to deplete one week's loading of NO_3^- is given by

$$t_p \text{(in hours)} = (3/2).L/(V.\overline{V}_{NO_3^-}) \qquad (6)$$

assuming a 16 h day light period applies and V (liters) is the volume of water in the surface 2 m of the lake. Hence, the time required for both processes together to deplete the weekly NO_3^- loading is given by

$$t_{total} \text{(in hours)} = (t_{dn}.t_p)/(t_{dn} + t_p) \qquad (7)$$

For Lake 227, whose bathymetric data are known (Brunskill and Schindler 1971), t_{total} can be calculated according to the above equations[1]. Values for t_{dn} and t_p were

---

[1]For Lake 227, the symbols in equations 5 and 6 are assigned the following values: A = 2.0×10^3 m^2; L = 1.5×10^{10} μg N; V = 8.31×10^7 liters; V_{dn} = 625 μg N m^{-2} h^{-1}; $\overline{V}_{NO_3^-}$ = 10.0 μg $liter^{-1}$ h^{-1}.

found to be 1.2 x 10^4 and 27.0 h; therefore, t_{total} would be 26.9 h. The value of t_{total} is consistent with the observation that little *in situ* NO_3^- was usually found in the surface waters one day after fertilization when discontinuous addition of NO_3^- was practised (Fig. 12). Although sediment denitrification seems to be insignificant in NO_3^- removal compared with uptake of the nutrient, of the total addition of 3 x 10^5 g NO_3^--N to Lake 227 in 20 weeks perannum, about 14% is estimated to be removed through littoral denitrification. Littoral sediment denitrification may serve as a reserve sink for the added NO_3^--N at times such as a phytoplankton collapse. Other channels of NO_3^- removal besides phytoplankton uptake include uptake by benthic algae, periphyton, macrophytes and,to a lesser extent, sediments in the littoral region. Dinitrogen fixation that may cause denitrification rates to be underestimated at the sediment surface has not been clarified (Flett 1976). It is suspected that N_2 fixation may be considered to be unimportant as NH_3 could be available from mineralization of organic nitrogen. The significance of denitrification during winter anoxic conditions is worth examining. The present findings indicate that, below the euphotic zone, sediments provide an efficient sink for NO_3^--N without simultaneous initiation of eutrophication.

C. Pure Culture Studies

Classification of Isolated Denitrifiers

RESULTS

Three isolates, designated A, B, and C, were selected at random from anaerobic agar-plate cultures for study. When tested for denitrification capability with $^{15}NO_3^-$, all the selected isolates evolved $^{15}N_2$. They were similar in cell morphology, all being non-sporing Gram-negative rods about 2.5 μm long and polarly flagellated. Anaerobically-formed pin-point colonies were observed on trypticase soy-KNO_3 agar at 22°C in 5 days but no pigment was produced. Only isolate C aerobically formed a diffusible non-fluorescent bluish green pigment which was lost after several transfers. Isolate B was distinguished from isolate A by its formation of butyraceous colonies. The physiological properties recorded for these isolates are presented in Table VIII. These together with the morphological properties are of preliminary diagnostic value at the genus level (Skerman 1967).

DISCUSSION

As in lake waters, the presence of denitrifiers in lake sediments indicates potential denitrification activity subject to the environmental factors. It is not known, however, what portion of the benthic microflora

Table VIII. Physiological properties of three denitrifiers isolated from Lake 227 sediments. +: positive result; - : , negative result; o: oxidative metabolism.

Characteristics	Isolates A	B	C
Poly-β-hydroxybutyrate accumulation	-	-	-
Oxidase	+	+	+
Catalase	+	+	+
O/F (Hugh-Liefson)	o	o	o
Hydrolysis of gelatin	-	+	-
Hydrolysis of urea	-	-	-
Acid from glucose	-	+	-
Acid from lactose	-	-	-
β-galactosidase	-	-	-
IMViC*	-	-	-
H_2S production	-	-	-
Nitrite from nitrate	+	+	+
Colony formation at 5°	-	-	+
10°	+	+	+
20°	+	+	+
30°	+	+	+

*Indol production, methyl red test, Voges-Proskauer test, and citrate utilization.

is capable of denitrification. Even when the number and compositon of the denitrifiers are determined, denitrifying activity is not necessarily implicated. For these reasons bacterial density in the sediments was not measured.

Although the screening tests performed merely served to provide a partial characterization of the isolates employed in subsequent temperature and enzyme studies, their preliminary characterization (Gram-negative,non-sporing rods with polar flagellation) points to a tentative identification with the *Pseudomonas* genus (Buchanan and Gibbons 1974). In soil, water purification, and marine environments *Pseudomonas* species have been regarded as the most important denitrifiers (Woldendorp 1966; Fabig and Ottow, *in press*; Baumann *et al*. 1972). Recently, Heitzer and Ottow (1976) isolated 16 denitrifying cultures from Red Sea sediments. Fifteen of these were described as new marine *Pseudomonas* species but not identified with any of the *Pseudomonas* species recognised to date. Denitrifiers belonging to the *Pseudomonas* genus from aquatic environments have not been extensively studied or described. Determination of the taxonomic status of the selected isolates is not intended and is outside the scope of the present investigation.

Temperature Relationship of Two Denitrifiers in Liquid Culture

RESULTS

Growth of the Denitrifiers

Fig. 17 shows the growth characteristics of the two *Pseudomonas* isolates, B and C, under denitrifying conditions in defined medium at temperatures ranging from 5° to 30°C. Starting with 10^{3} colony-forming-units (cfu) ml^{-1}, isolate C reached a population maximum in 6 days at 5°C. It had an optimum growth temperature with respect to growth rate at 25°C and seemed to have a maximum growth temperature between 25° and 30°C as little growth was apparent at the latter temperature. On the other hand, starting with 10^{4} cfu ml^{-1}, isolate B showed little growth at 5°C but growth rate increased progressively with increasing temperature up to 30°C. Higher growth rates were displayed by isolate B at the higher temperatures (20° and 25°C) while C grew faster at the lower temperatures (10° and 15°C) when the two isolates were compared.

When "Arrhenius plots" of the growth rates are made as shown in Fig. 18, the linear functions disclose that C behaves as a typical psychrotroph (Ingraham 1962) which has been defined as a microorganism capable of growing at 5°C or less, disregarding its optimum temperature (Eddy 1960). Isolate C displays a lower slope and thus a lower temperature characteristic, μ, than B although in this plot, the relative specific growth rate of B was

Fig. 17. Effect of temperature on the an̲aerobic growth of Pseudomonas C and B with NO_3^-.

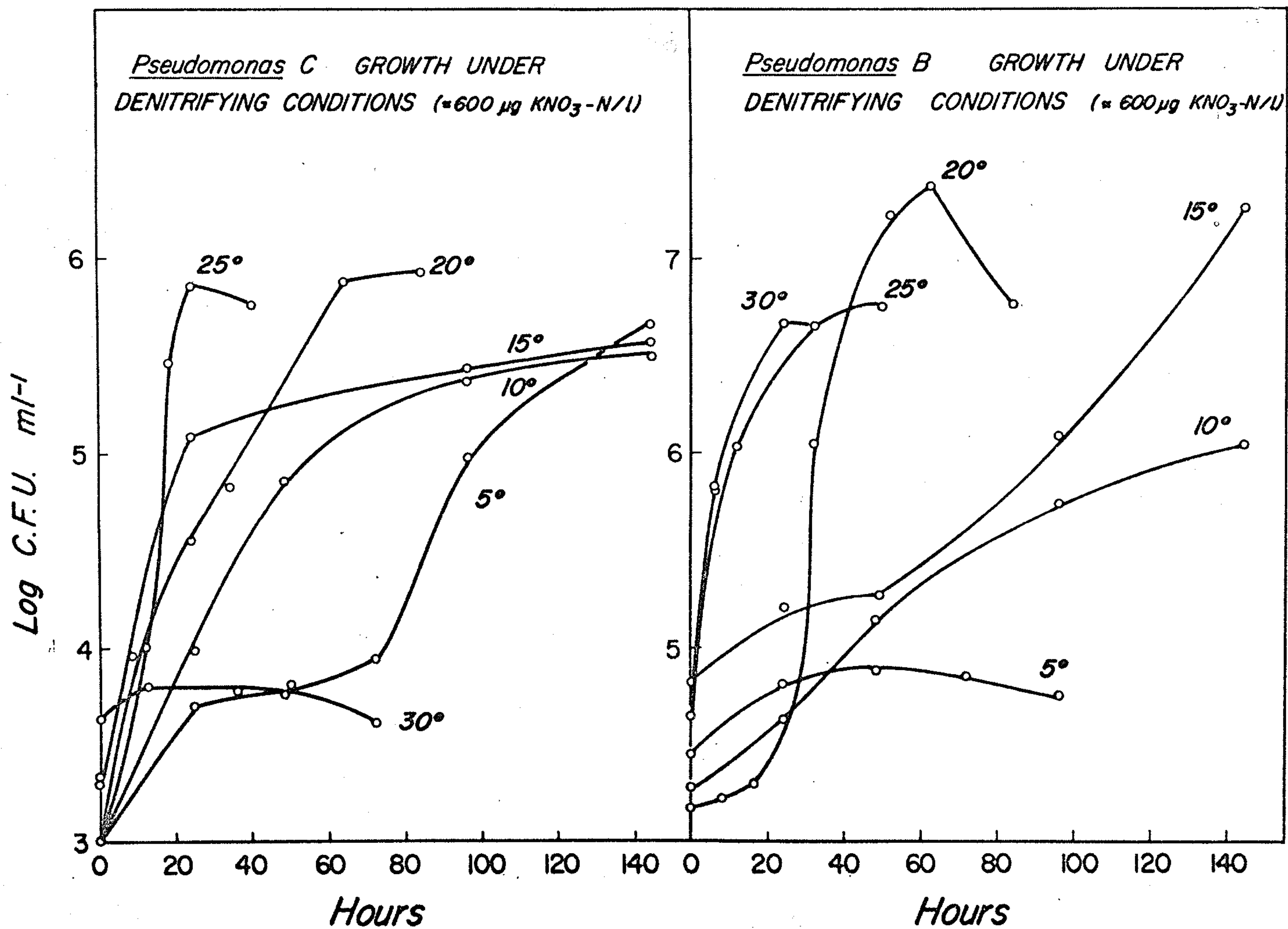
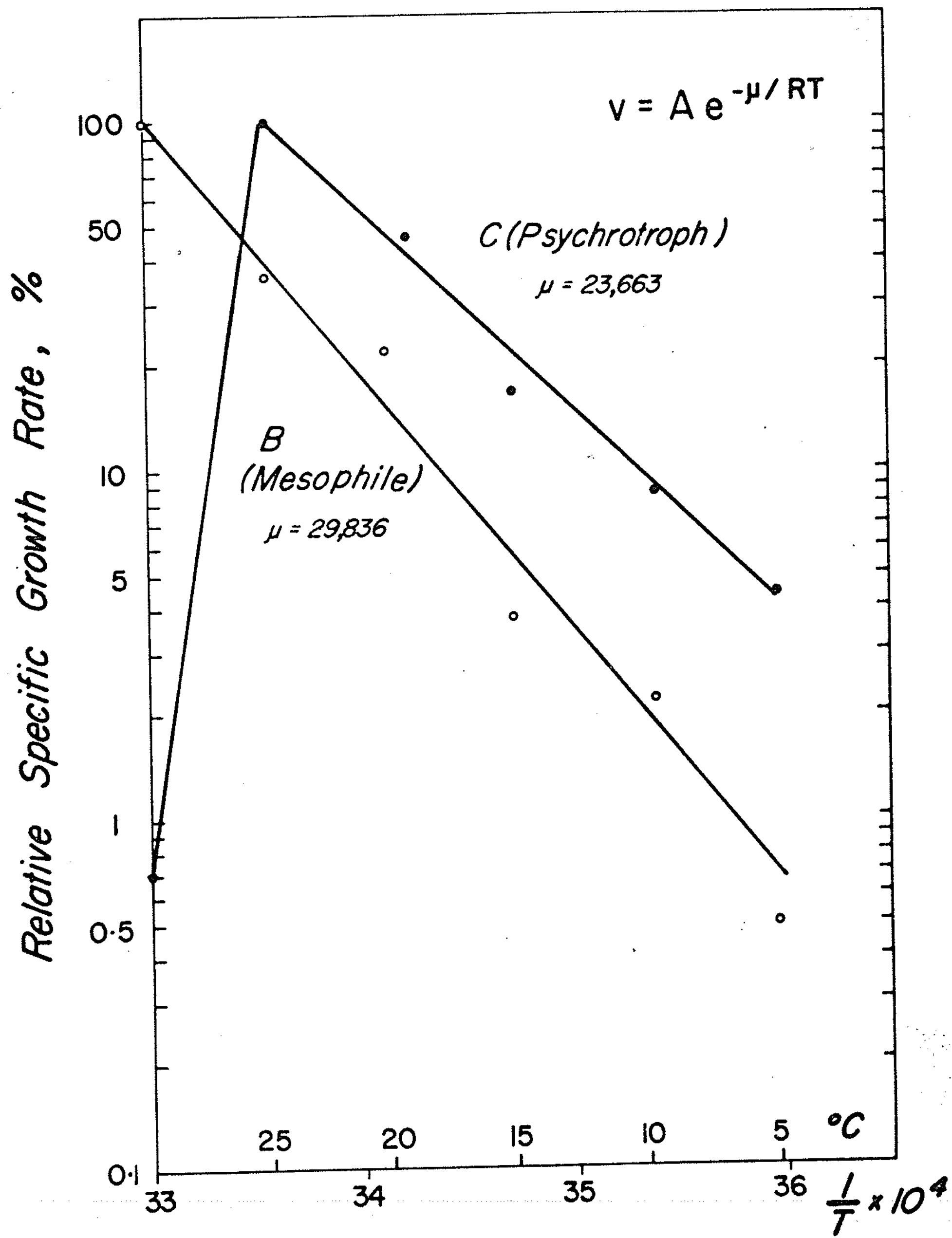

Pseudomonas C GROWTH UNDER
DENITRIFYING CONDITIONS (≈600 μg KNO3-N/l)
Pseudomonas B GROWTH UNDER
DENITRIFYING CONDITIONS (≈ 600μg KNO3-N/l)
Log C.F.U. ml-1
Hours
Hours
25°
20°
15°
10°
5°
30°
20°
15°
30°
25°
10°
5°

Fig. 18. 'Arrhenius plots' of the anaerobic growth rates of the psychrotrophic *Pseudomonas* C and the mesophilic *Pseudomonas* B. This plot of ln v vs. 1/T follows from another form of the Arrhenius equation (shown in figure), viz. $\ln v = -(\mu/RT).A$ (Ingraham 1962).



V = A e^-μ/RT
100
50
10
5
1
0·5
0·1
Relative Specific Growth Rate, %
C (Psychrotroph)
μ = 23,663
B
(Mesophile)
μ = 29,836
25
20
15
10
5
°C
33
34
35
36
1/T x 10^4

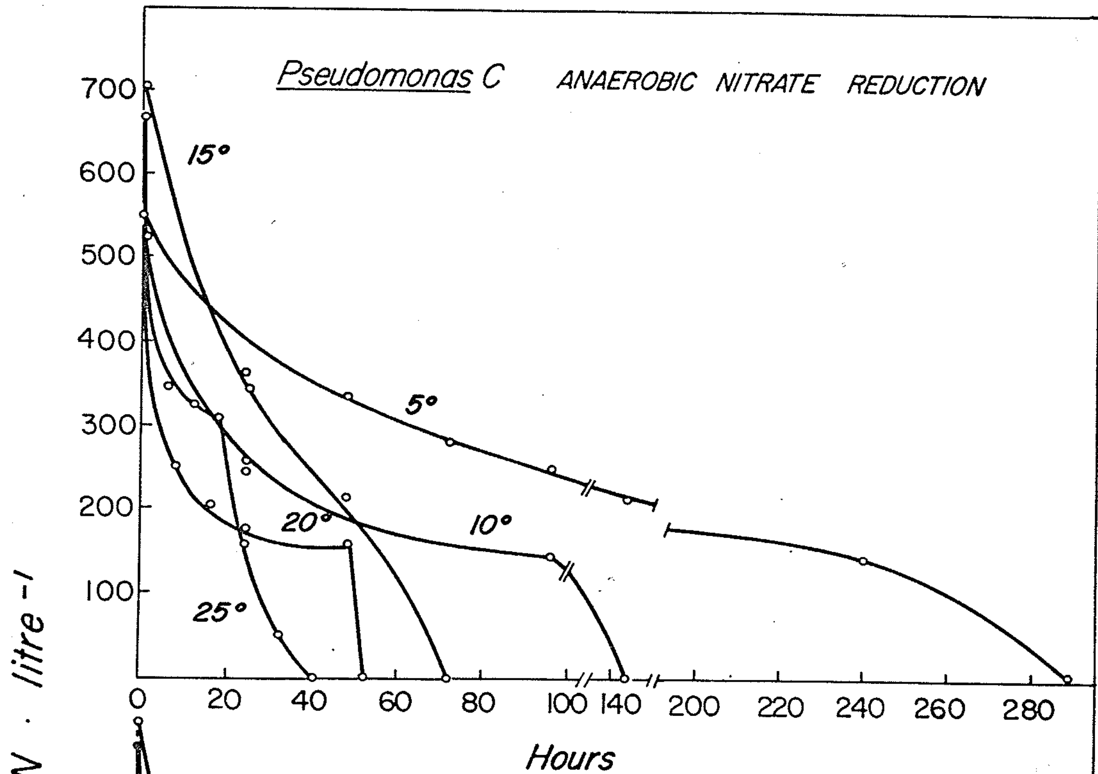
arbitrarily taken to be 100% at 30° and that its growth at 5° is considered as part of the linear function.

Effect on Denitrification Products

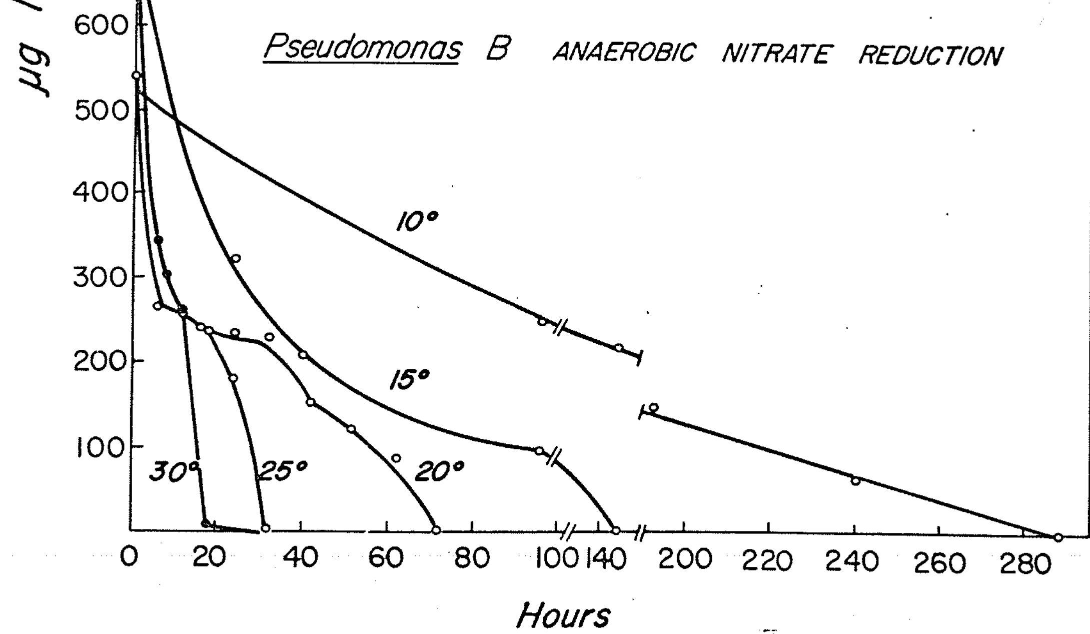
At all growth temperatures, both denitrifying isolates showed similar patterns of intermediate and product formation in the sequence: NO_2^-, N_2O and N_2. Uptake and reduction of NO_3^- were exponential (Fig. 19). In the case of C, NO_2^- accumulated only after more than half of the NO_3^- had been reduced (Fig. 20). At 25°C when growth rates were high for both denitrifiers, the transient accumulation of NO_2^- barely exceeded one-third of the initial NO_3^- concentration. At 20°C and below, C accumulated about 400 μg NO_2^--N liter^{-1}. More NO_2^- was also accumulated by B as the incubation temperature decreased to 15°C although about the same amount was formed at 20° and 25°C. Apparently, the unexpected low accumulation of NO_2^- at 10°C was an anomaly.

At 10° to 25°C, culture C did not form N_2O and only at 5°C that detectable amounts of N_2O was accumulated in 10 days. In culture B, the formation of N_2O was highest at 10° and 15°C although N_2 was the end product (Fig. 21). Recovery of NO_3^--N as N_2 was usually late in the incubation period (Fig. 22). No attempt was made to provide a nitrogen balance in these experiments.

Fig. 19. Effect of temperature on NO_3^- depletion during denitrification by <u>Pseudomonas</u> C and B.

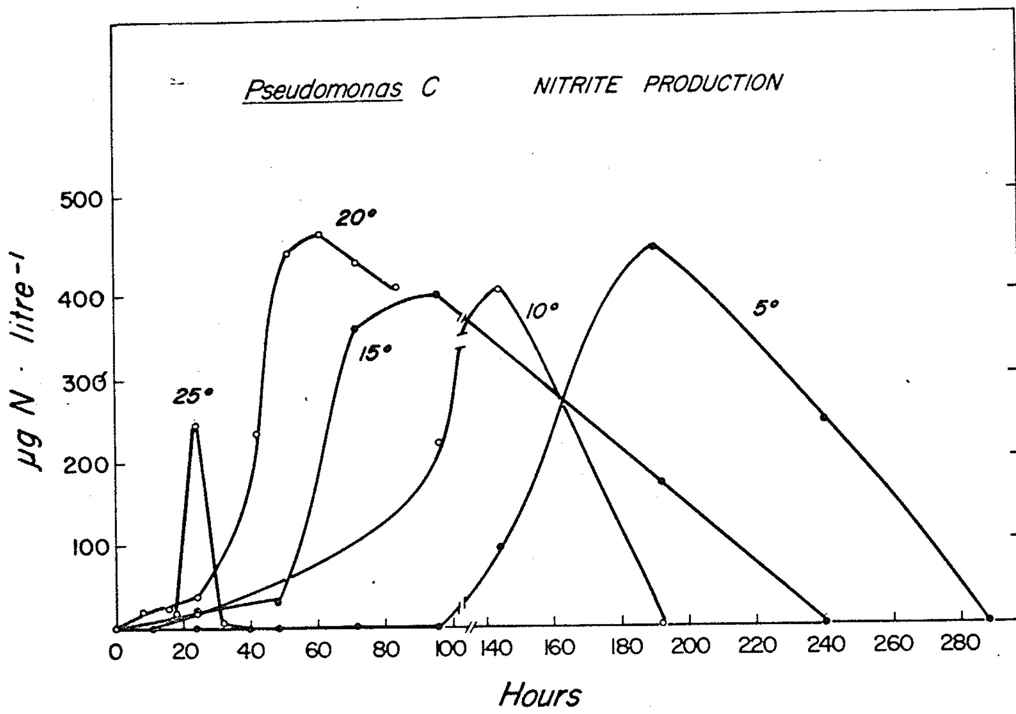


Pseudomonas C ANAEROBIC NITRATE REDUCTION
700
600
500
400
300
200
100
15°
5°
10°
20°
25°
0
20
40
60
80
100
140
200
220
240
260
280
Hours
μg N · litre -1



Pseudomonas B ANAEROBIC NITRATE REDUCTION
600
500
400
300
200
100
10°
15°
20°
25°
30°
0
20
40
60
80
100
140
200
220
240
260
280
Hours

Fig. 20. Effects of temperature on NO_2^- accumulation during denitrification by _Pseudomonas_ C and B.


Pseudomonas C
NITRITE PRODUCTION
500
400
300
200
100
20°
15°
10°
5°
25°
μg N · litre⁻¹
0
20
40
60
80
100
140
160
180
200
220
240
260
280
Hours

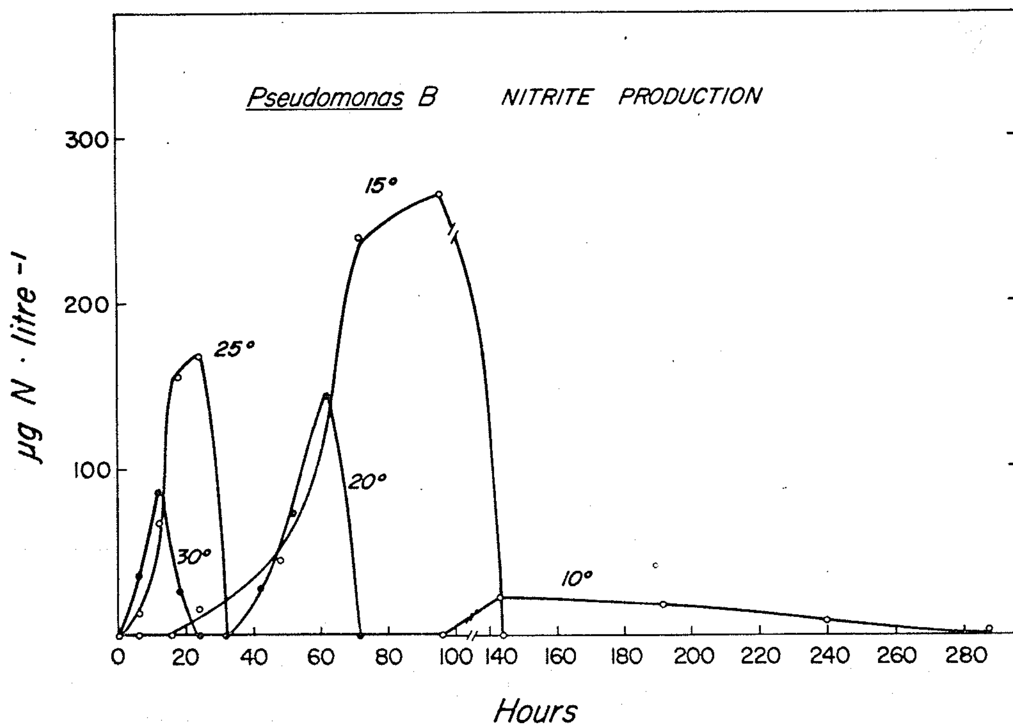
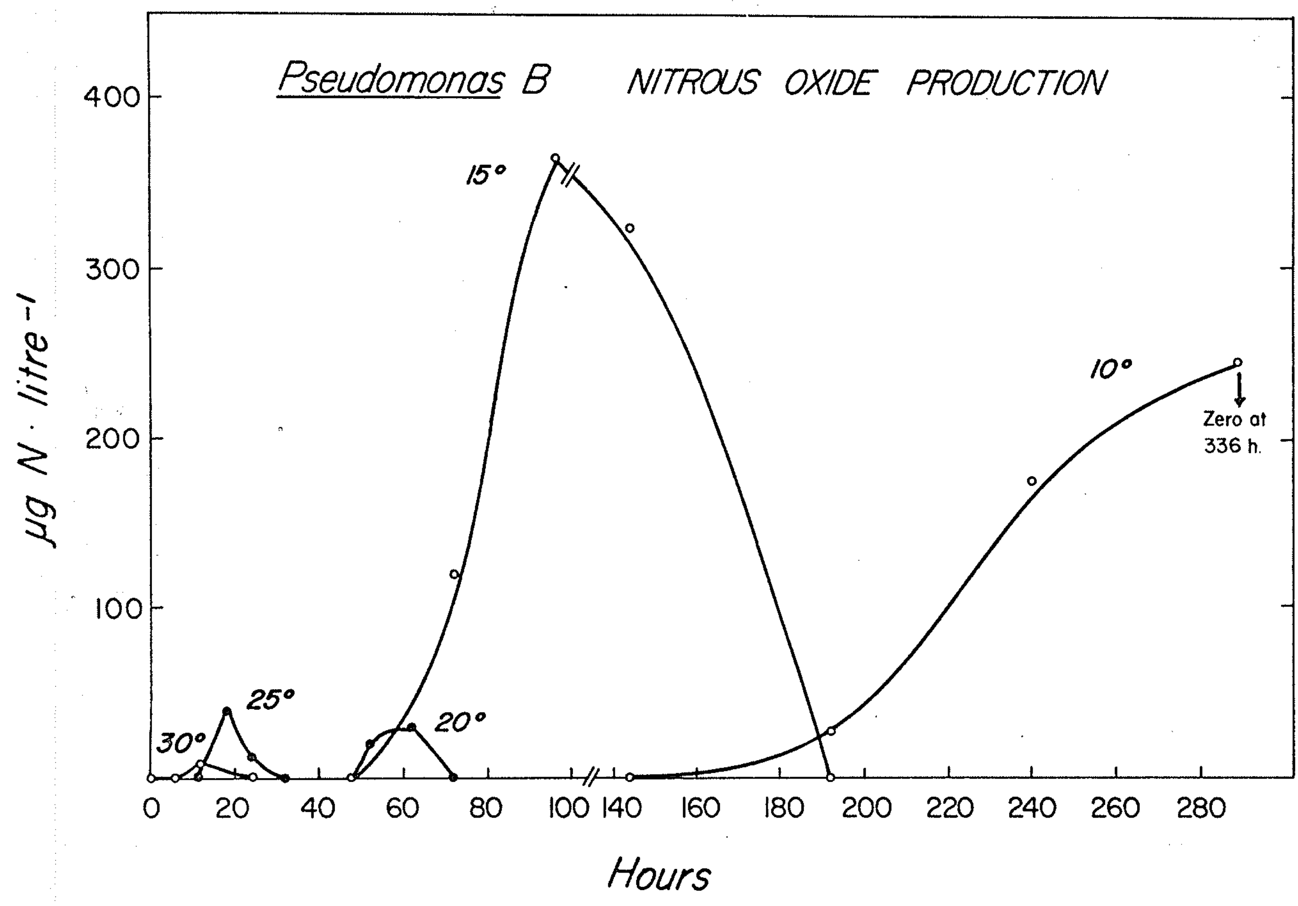
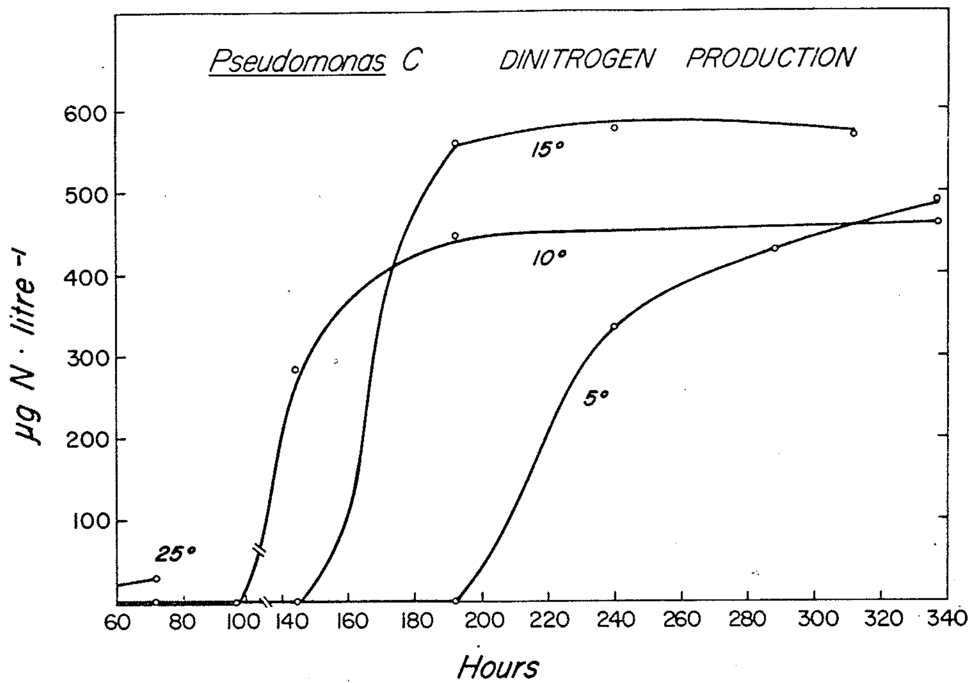

Pseudomonas B
NITRITE PRODUCTION
300
200
100
15°
25°
20°
30°
10°
μg N · litre⁻¹
0
20
40
60
80
100
140
160
180
200
220
240
260
280
Hours

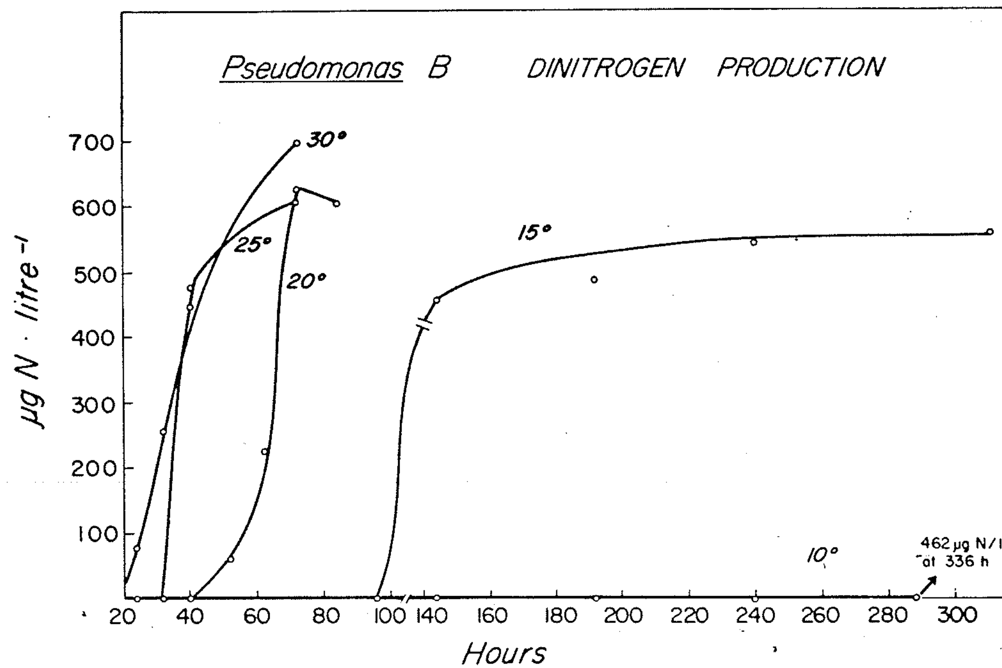
Fig. 21. Effect of temperature on the appearance of N_2O during denitrification by *Pseudomonas* B.



Pseudomonas B
NITROUS OXIDE PRODUCTION
μg N · litre -1
400
300
200
100
15°
10°
25°
30°
20°
Zero at
336 h.
0
20
40
60
80
100
140
160
180
200
220
240
260
280
Hours

Fig. 22. Effect of temperature on N_2 production during denitrification by *Pseudomonas* C and B.


Pseudomonas C DINITROGEN PRODUCTION
600
500
400
300
200
100
μg N · litre⁻¹
15°
10°
5°
25°
60 80 100 140 160 180 200 220 240 260 280 300 320 340
Hours


Pseudomonas B DINITROGEN PRODUCTION
700
600
500
400
300
200
100
μg N · litre⁻¹
30°
25°
20°
15°
10°
462 μg N/l at 336 h
20 40 60 80 100 140 160 180 200 220 240 260 280 300
Hours

DISCUSSION

Ecological Significance of Temperature Response of Denitrifying Isolates

In most Canadian Shield lakes where thermal stratification occurs, dominant low temperatures in the range of 4-10°C persist in the hypolimnion. The denitrifying *Pseudomonas* isolates are representitive of psychrotrophic and mesophilic denitrifiers operating during closed incubation studies of aquatic denitrification at these temperatures. Low temperatures generally retard growth and denitrification. Species differences have also been revealed in these temperature studies regarding the growth rate and the quantitative distribution of denitrification products and thus the contribution to denitrification by these isolates. For example, denitrification rates decreased more slowly with decreasing temperature in the psychrotroph and more rapidly in the mesophile. Although both psychrotrophic and mesophilic pseudomonads released N_2 as end product through N_2O reduction, little N_2O was accumulated by the psychrotroph. On the other hand, the mesophile accumulated N_2O at the lower temperatures, a condition tending to retain N_2O in solution. At the low temperature in the O_2 minimum layer during summer stratification of the lake, both *Pseudomonas* species contribute to denitrification but the mesophile is expected to be responsible for N_2O formation in this region.

PART II. PHYTOPLANKTON UPTAKE OF NITRATE

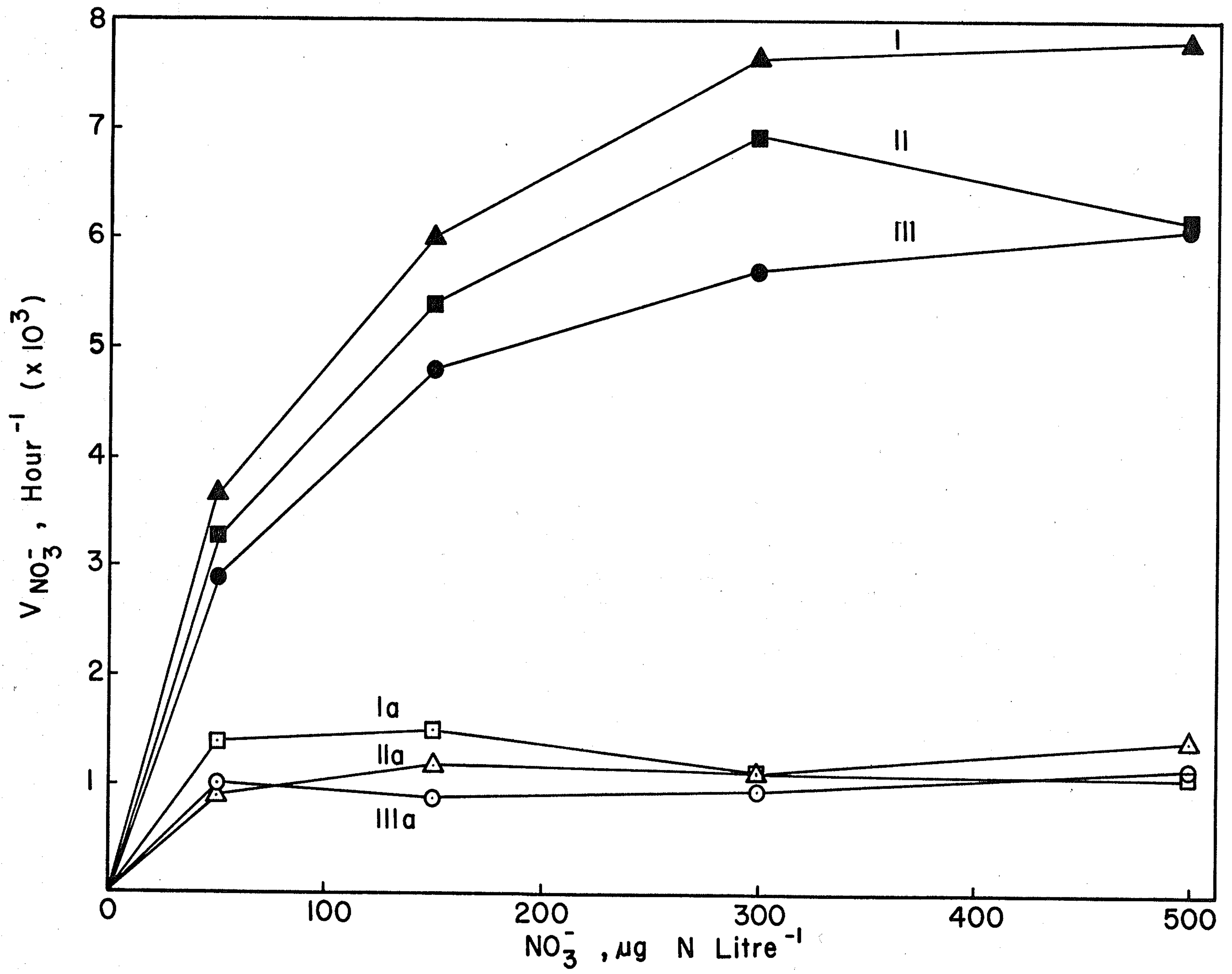
RESULTS

Uptake Kinetics

At 50 µg NO_3^--N liter^{-1} enrich ment, uptake was linear with time during 8 h of incubation on August 23, 1975. No lag period was observed. Nitrate uptake rates in the light, with 10^{-6} M and 10^{-5} M DCMU and in the dark were in the ratio of 8:2:1:1. $V_{NO_3^-}$, the fractional uptake rate of NO_3^-, was 0.005 h^{-1} under light saturation. Dark uptake of NO_3^- was insignificant as compared to that in the light.

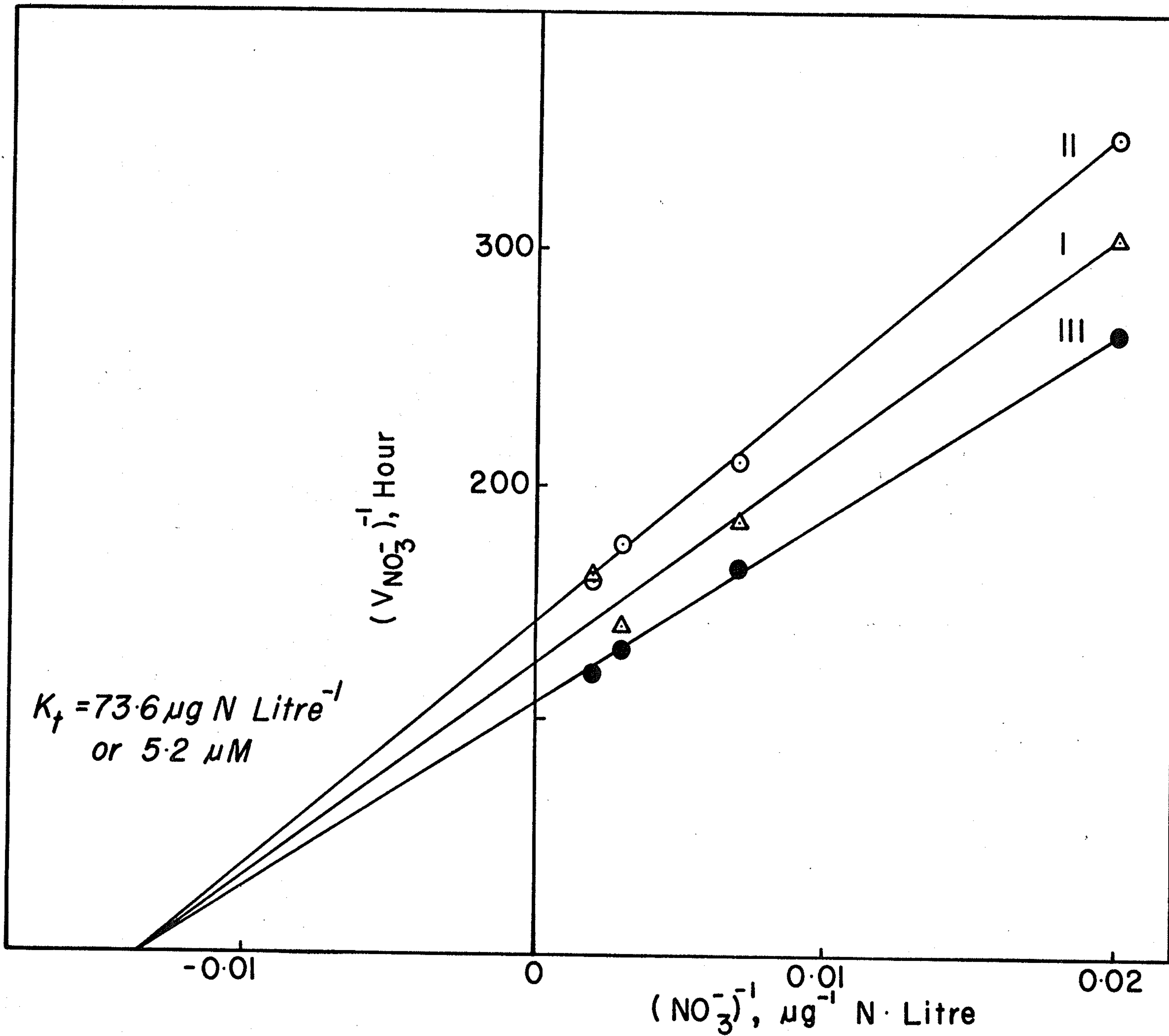
Using enrichment levels up to 500 µg NO_3^--N liter^{-1} on September 5, 1975, the light data displayed the Michaelis-Menten hyperbolic relationship between the rate of uptake and substrate concentration (Fig. 23) and hence could be analysed by the Lineweaver-Burk linear transformation to yield the half-saturation transport constant, K_t, and the maximum fractional uptake rate, V_{max} (Fig. 24). These kinetic constants were determined to be about 73.6 µg N liter^{-1} (or 5.2 x 10^{-6} M) and 0.008 h^{-1}, respectively. No Michaelis-Menten kinetic function was readily discernible from data obtained with the dark-incubated samples because of the low uptake rates. Therefore, these results were not amenable to linear analysis. The effect of 10 and 50 µg

Fig. 23. Effect of NO_3^- concentration on $V_{NO_3^-}$ in Lake 227 epilimnetic waters (September 5, 1975). Incubation was carried out for 4 h under the following conditions: I, light saturation without PO_4^{3-} addition; II and III, with 10 and 50 μg PO_4^{3-}-P $liter^{-1}$ respectively; Ia, IIa, and IIIa were similarly treated but incubated in the dark.



8
7
6
5
4
3
2
1
0
100
200
300
400
500
I
II
III
Ia
IIa
IIIa
$V_{NO_3^-}$, Hour^{-1} ($\times 10^3$)
NO_3^- , μg N Litre^{-1}

Fig. 24. Lineweaver-Burk linear transformation of NO_3^- uptake kinetics data from Fig. 23. Incubation was carried out for 4 h under the following conditions: I, light saturation without PO_4^{3-} addition; II and III, with 10 and 50 μg PO_4^{3-}-P liter^{-1} respectively; Ia, IIa, and IIIa were similarly treated but incubated in the dark.



II
I
III
300
200
$(V_{NO_3^-})^{-1}$, Hour
K_t = 73·6 µg N Litre^{-1}
or 5·2 µM
-0·01
0
0·01
0·02
$(NO_3^-)^{-1}$, µg^{-1} N · Litre

P liter^{-1} on NO_3^- uptake (Fig.23) was considered to be insignificant and was more likely due to experimental variation. Unpublished data from studies of several ELA lakes have shown a phosphorus uptake at as high as 150 μg P liter^{-1} enrichment level for 24 h without any apparent toxic effect due to overloading (F.P. Healey, personal communication). Hence, the P enrichment employed in this experiment could not be expected to produce any adverse effect on Lake 227 algae.

Enrichment with 50 μg N liter^{-1} in different inorganic forms demonstrated decreasing affinity for them by algae in the order, NH_4Cl, NH_4NO_3, KNO_3, and $NaNO_2$. The respective uptake rates for these forms were in the ratio 5:3:2:1.

Uptake and Primary Production

To further characterize the effect of DCMU on the kinetics of NO_3^- uptake, primary production was determined concomitantly Nitrate at various concentrations up to 1 mg N liter^{-1} had no significant effect on phytoplankton production (Table IX). Inhibition of primary production by DCMU was incomplete. DCMU at 10^{-6} M inhibited photosynthesis by 96% but still permitted CO_2 fixation at a rate which was consistently two times higher than that of dark-incubated samples. Since an identical inhibition effect was noted in NO_3^- uptake experiments where 10^{-6} M

Table IX. Maximum primary production (Pn_{max}) with NO_3^- enrichment up to 1 mg N liter^{-1} in Lake 227 epilimnetic waters (September 7, 1975). Water samples were incubated for 3 h. See also Fig. 25.

Treatment	Pn_{max}, µmole C h^{-1} with added NO_3^- at						Mean Pn_{max}, µmole C h^{-1}
	0	50	150	300	500	1,000 µg N/l	
I Light-saturated	2.946	ND*	ND	2.982	2.892	2.726	2.886
II With 10^{-6} M DCMU	ND	0.107	0.118	0.111	0.114	0.110	0.112
III Dark-incubated	0.062	0.072	0.067	0.070	0.064	0.065	0.067

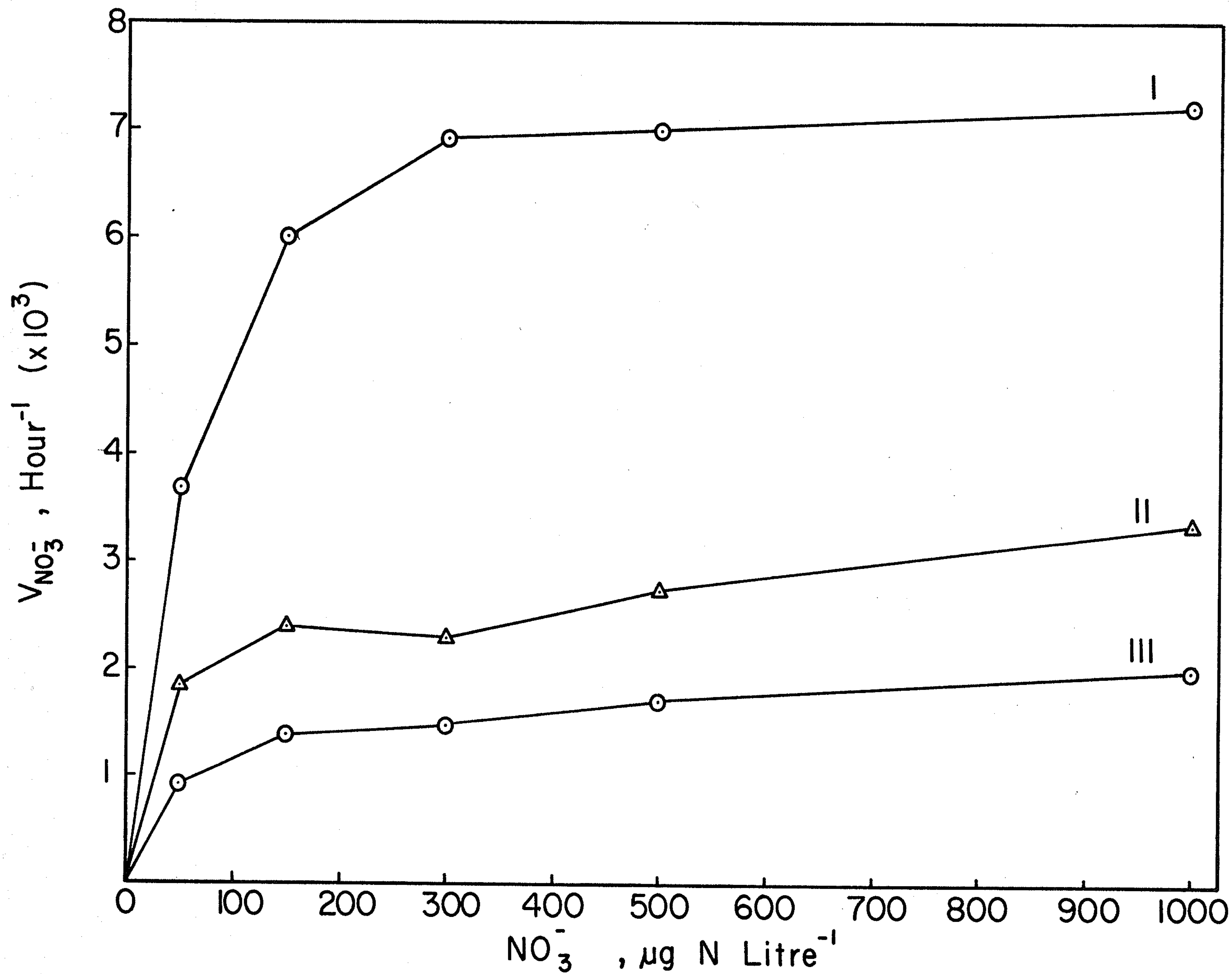
*Not determined.

DCMU was included (Fig. 25), it suggests that NO_3^- uptake is directly dependent on or related to non-cyclic photophosphorylation.

Extracellular Products of Nitrate Assimilation

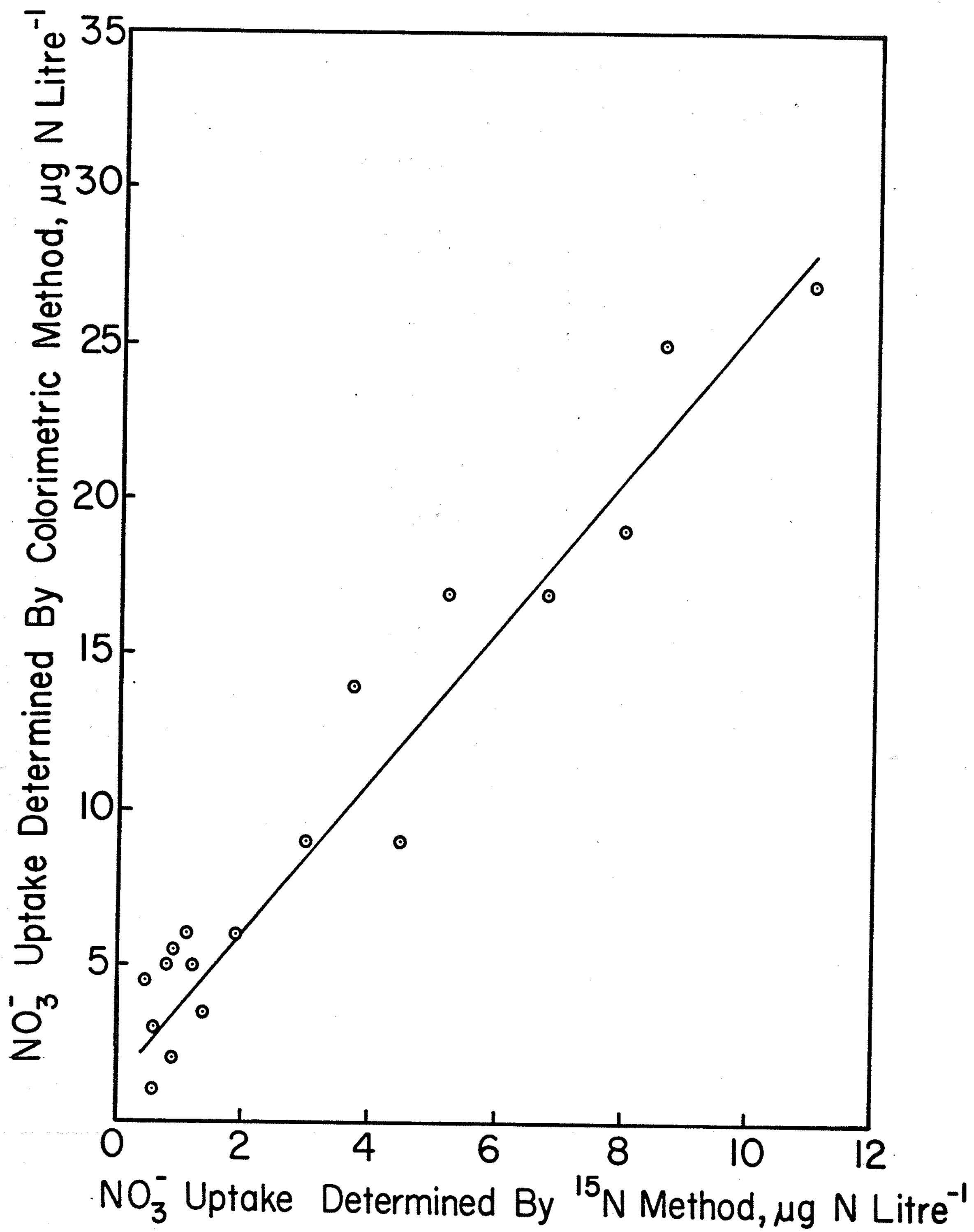
When comparing $^{15}NO_3^-$-incorporation results to those obtained by following the disappearance of NO_3^- chemically in the same experiments, a strong correlation ($r = 0.99$) was found between the two sets of data but uptake values obtained by the ^{15}N method were consistently only about one-third of those obtained by chemical analyses (Fig. 26). Although the correlation coefficient 'r' may vary when samples with widely different population composition and detritus nitrogen are studied, some preliminary results suggested that the discrepancy can also be due to the excretion of soluble organic nitrogen. First, neither NO_2^- nor NH_3 accumulation was detected during NO_3^- uptake. Cell lysis could not be significant during the short-term (3 or 4 h) incubation of fresh water samples. Secondly, electron microscopic examination of ultracentrifuge-precipitated materials from filtered water samples incubated with $^{15}NO_3^-$ did not reveal any structural cell fragments. Hence, cell damage during filtration appeared to be minimal. Little particulate nitrogen was recovered by ultracentrigation and ^{15}N was not incorporated into the sedimented materials. Thirdly, control samples containing 20 mg liter^{-1}

Fig. 25. Effect of DCMU on the kinetics of NO_3^- uptake in Lake 227 epilimnetic waters (September 7, 1975). Incubation was carried out for 3 h under the following conditions: I, light saturation; II, with 10^{-6} M DCMU; III, in the dark. Maximum primary production was also estimated in the experiment using a C-14 method. See Table IX.



8
7
6
5
4
3
2
1
0
100
200
300
400
500
600
700
800
900
1000
I
II
III
$V_{NO_3^-}$, Hour^{-1} (x 10^3)
NO_3^- , μg N Litre^{-1}

Fig. 26. Correlation between ^{15}N tracer and colorimetric determination of NO_3^- uptake in Lake 227 epilimnetic waters.



NO_3^- Uptake Determined By Colorimetric Method, µg N Litre^{-1}
35
30
25
20
15
10
5
0
2
4
6
8
10
12
NO_3^- Uptake Determined By ^{15}N Method, µg N Litre^{-1}

$HgCl_2$ did not take up NO_3^- as determined by chemical or ^{15}N analysis. Therefore, the chemical method of NO_3^- analysis was not at fault. It was already known from the NO_3^- uptake results of light and dark experiments (Figs. 23 and 25) that this process was light-dependent and was largely due to phytoplankton assimilation.

It seemed that the difference in the NO_3^- uptake results obtained by the two methods might be further examined simply by measuring (a) total dissolved nitrogen and (b) the initial uptake velocity for brief incubation periods as in the radio-bioassay of orthophosphate concentration in limnetic water (Rigler 1966). However, it was realized that the first method required a significant loss of dissolved nitrogen (both organic and inorganic) in order to detect a measurable change in total dissolved nitrogen. From experience, the second method was not considered feasible when applied to the $^{15}NO_3^-$ uptake measurements as the amount of ^{15}N incorporated in less than 1.5 h at low enrichments (e.g. 50 μg N liter^{-1}) was usually inadequate for analysis.

The problem at hand was then to obtain unequivocal evidence for the excretion of assimilated NO_3^-. From the preceeding evidence, low molecular weight extracellular nitrogenous products were likely to be considered as reasonable candidates to account for the observed NO_3^- uptake discrepancies. It had been reported that marine phytoplankton populations released at least 10% of the ^{15}N as dissolved organic nitrogen

after assimilation of $^{15}NO_3^-$ (Schell 1974).

The increase of ^{15}N label in the dissolved organic nitrogen (DON) isolated from filtrates of incubated water samples by cation-exchange chromatography and the concurrent uptake of $^{15}NO_3^-$ are presented in Table X. Increase in total particulate nitrogen (TPN) after incubation was estimated to be less than 10%. Isotope analysis of particulate N and DON demonstrated the cycling of NO_3^--N into DON. If the net DON production originated from the particulate fraction alone, about 20-45% of this DON in the light-incubated samples was primarily due to phytoplankton excretion. In the dark-incubated and DCMU-treated samples, respectively 34 and 13% of the DON released was due to phytoplankton and bacteria. It was noted that DCMU inhibited only 50% of NO_3^- uptake in the latter experiment. DCMU appeared to inhibit DON production as well as NO_3^- uptake. The rate of fractional DON release was 1/10 as fast as that of fractional NO_3^- uptake while subsequent heterotrophic utilization of DON was unknown. As previously stated, nitrogen balances measured (a) by NO_3^- disappearance (chemical analysis) and (b) by $^{15}NO_3^-$ uptake showed a discrepancy of about 40% in terms of the total nitrogen applied. Of this, only about one quarter (10% of total) could be accounted for as DON by the isotope assay.

Amino acid analyses of the isolated DON were performed with and without acid hydrolysis. Analytical

Table X

a. Net dissolved organic nitrogen (DON) production in epilimnetic water samples determined by N-15 technique. Incubation time, 4 h; total DON, ca. 80 μg liter^{-1}.

Experiment	Atom % ^{15}N Excess in DON	Net DON Prod. μg liter^{-1}	Rate h^{-1} (x10^{3})
June 22, 1976			
Light	0.211	0.16	0.56
Dark	0.084	0.06	0.21
July 20, 1976			
Light	0.705	0.66	1.85
10^{-6} M DCMU	0.133	0.12	0.34

b. Net $^{15}NO_3^-$ uptake in epilimnetic water samples. Incubation time, 4 h; TPN, ca. 560 μg liter^{-1}.

Experiment	Atom % ^{15}N Excess in Particulates	Net NO_3^- Uptake μg N liter^{-1}	Rate h^{-1} (x10^{3})
June 22, 1976			
Light	1.069	6.23	2.81
Dark	0.248	1.44	0.65
July 20, 1976			
Light	1.560	9.46	4.10
10^{-6} M DCMU	1.085	5.37	2.85

results obtained with acid treatment represent total amino acid content consisting of peptides (combined amino acids) and dissolved free amino acids (DFAA) while those from untreated samples represent DFAA (Table XI). Scrutiny of Table XI discloses a convenient partition of the data into two groups, viz. A and B versus C and D (controls). Each group has commom DFAA and peptide concentrations which are significantly different from those of the other. A comparison of the two groups leads to two prominant features: (a) Light-incubated samples enriched with either $^{15}NO_3^-$ or $^{14}NO_3^-$ are about 150 nM higher in total DFAA concentration than control samples treated with DCMU or $HgCl_2$. This shows that DCMU, although only partially inhibiting NO_3^- uptake, prevented the release of DFAA from the particulate fraction. Treatment with $HgCl_2$ did not promote cell lysis as there was no significant increase in total DFAA or combined amino acids over background levels. (b) Most individual DFAA concentrations are low (usually <50 nM) and similar in all samples except for serine and glycine. The peptides contain more glutamate, glycine and alanine than the other amino acid residues. However, serine concentration is clearly higher in A and B than in the controls while glutamate and alanine content in the peptides of A and B are higher. The phytoplankton in Lake 227 at the time of the experiment was predominantly made up of green algae (Spondylosium and Scenedesmus) and small Chrysophycean species (ELA data).

Table XI. Amino acid composition of DON isolated from filtrates of light-incubated water samples* (July 20, 1976). A, 100 µg $^{15}NO_3^-$-N liter^{-1} enrichment; B, 100 µg $^{14}NO_3^-$-N liter^{-1} enrichment; C, 100 µg $^{15}NO_3^-$-N liter^{-1} and 10^{-6} M DCMU added; D, 100 µg $^{15}NO_3^-$-N and 20 mg $HgCl_2$ liter^{-1} added. Results are expressed in nmoles liter^{-1} water sample.

	A			B			C			D		
	Unhyd.	Hyd.	Diff.	Unhyd.	Hyd.	Diff.	Unhyd.	Hyd.	Diff.	Unhyd.	Hyd.	Diff.
Try	3	--	--	3	--	--	2	--	--	6	--	--
Lys	54	67	13	46	74	28	31	52	21	20	36	16
His	2	3	1	2	5	3	0	2	2	0	2	2
Arg	0	4	4	1	5	4	0	2	2	0	0	0
Asp	33	63	30	29	58	29	20	51	31	11	35	24
Thr	18	37	19	19	30	11	11	32	21	12	26	14
Ser	92	98	6	81	91	10	51	54	3	52	54	2
Glu	27	110	83	34	135	101	21	73	52	15	54	39
Pro	15	30	15	19	30	11	15	20	5	12	25	13
Gly	60	138	78	52	151	99	41	114	73	40	105	65

Table XI (Cont'd).

	A			B			C			D		
	Unhyd.	Hyd.	Diff.	Unhyd.	Hyd.	Diff.	Unhyd.	Hyd.	Diff.	Unhyd.	Hyd.	Diff.
Ala	34	100	66	39	124	85	24	70	46	25	52	27
Val	11	33	22	8	24	16	6	24	18	5	15	10
Ile	11	23	12	9	22	13	7	17	10	4	11	7
Leu	16	33	17	15	36	21	11	24	13	11	18	7
Tyr	6	7	1	3	6	3	0	4	4	0	2	2
Phe	3	24	21	3	22	19	0	6	6	0	5	5
TOTAL	385	770	388	363	813	453	240	545	307	213	440	233

*Cysteine and cystine were not determined. Tryptophan was destroyed by acid hydrolysis. Traces of methionine are not included.

DISCUSSION

Nitrate Uptake and Nitrate Status of Lake 227

The uptake of NO_3^- in epilimnetic waters of Lake 227 during summer stratification was largely phytoplanktonic. It was greatly reduced when the water was treated with DCMU, an inhibitor of photosystem II and noncyclic photophosphorylation, which paralleled the effect of dark incubation. Photosynthetic assimilatory reduction of NO_3^- is generally operative through the supply of reducing power from photosynthates (carbohydrates) and ATP from photophosphorylation. However, it has been recently reported that in blue-green algae the photosynthetic reduction of NO_3^- may be independent of NADPH and ATP through a ferredoxin-NO_3^--reducing system (Candau _et al_. 1976). The small NO_3^- uptake in the dark probably represented the endogenous uptake by phytoplankton as well as uptake by bacteria.

The K_t value of 73.6 μg N liter^{-1} (5.2×10^{-6} M) for NO_3^- uptake by phytoplankton in Lake 227 is high compared to values (generally ca. 1.0×10^{-6} M or less) for natural populations and cultures of marine algal species (MacIsaac and Dugdale 1969; Eppley _et al_. 1969b). It falls in the upper part of the range of K_t's (about 0.1 to 10 $\times 10^{-6}$ M) determined for marine algae (Eppley _et al_. 1969b) and probably reflects the uptake characteristics of the dominant algal species belonging to the genera _Scenedesmus_,

Oscillatoria and *Aphanizomenon* that have adapted to the eutrophic conditions in the epilimnion of Lake 227. This relatively high K_t therefore reflects the NO_3^- limitation, though not nitrogen limitation, in Lake 227 during the summer months when the ambient NO_3^- concentration remained low (<10 μg N liter^{-1}) in spite of the addition of fertilizer NO_3^-. The fertilizers added to the lake had contained adequate nitrogen prior to 1975. In 1975 when the nitrogen-to-phosphorus ratio (by weight) of fertilizer added to Lake 227 was reduced from 14 to 5, there could have been a severe limitation of primary production. However, this nitrogen-deficient fertilization favored the development of blue-green algae (*Aphanizomenon gracile*) that fixed atmospheric N_2, thus alleviating the nitrogen deficiency of the lake (Schinder 1977). Ambient NO_3^- concentration in the surface waters was still largely controlled by phytoplankton during summer stratification. It was estimated by isotope tracer technique that at the maximum uptake rate, the turnover of NO_3^--N could be as long as 5 days without considering subsequent exchange with the particulate fraction (see below).

Excretion of Assimilated Nitrate

Low molecular weight N compounds in the form of DFAA and combined amino acids were released as part of the extracellular DON produced by phytoplankton assimilation of NO_3^- and CO_2 in Lake 227. These are probably metabolic

intermediates and thus belong to type I of extracellular product according to Fogg's classification (Fogg 1966). Since their appearance depends on metabolic activity, the presence of consumers, and other factors, the rate of their release may change even during relatively short experiments. Uptake of NO_3^- and its subsequent excretion as DON were inhibited by DCMU, suggesting that these processes were light-mediated. Hence, phytoplankton played a more important role than bacterioplankton in the contribution to the extracellular DON pool while bacteria might significantly modify DON composition and quantity by heterotrophic uptake. This is of course not the first report on phytoplankton release of DFAA. The phenomenon has been observed in marine phytoplankton populations enriched with NO_3^- (Schell 1974). Evidence from ^{15}N tracer experiments in support of such an excretory process in Lake 227 phytoplankton is unequivocal, however. The possibility that this observation might have been an artifact resulting from cellular fragility and decomposition was ruled out by the use of biostatic controls. It remains unknown whether amino acids were solubilized from colloidal forms when the pH of the concentrated filtrate was lowered to 2.0 for cation-exchange chromatography. Assuming that if such a reaction in fact occurred and that the same amount of colloids released the same amount of amino acids on acidification in both experiments and controls, the observed results would be still meaningful.

The extracellular DON partially identified as DFAA by automated amino acid analyses contained a relatively high abundance of serine and glycine. Serine and glycine have also been found to be the major DFAA in Upper Klamath Lake (Oregon) and Lake Mendota (Wisconsin) surface waters (Burnison and Morita 1974; Gardner and Lee 1975). These amino acids are characterized by their low heat of combustion and therefore are thought to be unsuitable for energy storage (Andrew and Williams 1971). The small amount of dissolved free glutamic acid as opposed to the relatively large amount found in the peptides perhaps reflected the high affinity of the indigenous heterotrophic microflora for glutamate (Burnison and Morita 1974). Though less obvious, a similar relationship was observed in the case of glycine.

The exact nature of the extracellular peptides was not known and forms other than DFAA and peptides might be excreted since, although the latter forms were probably major products they did not fully account for the difference (about 40%) between $^{15}NO_3^-$ uptake and NO_3^- disappearance. Anionic macromolecules, if excreted, could have escaped detection. 'Loss' of amino acid through combination with colloids was also possible. Finally, if it is considered that the turnover and exchange rates of certain amino acids may be fast they would not be detectable in natural aquatic environments. In this case failure

to demonstrate DFAA production does not prove its absence. The present findings indicate that measurement of NO_3^- uptake by phytoplankton as ^{15}N incorporation alone would likely represent an underestimation. Assuming an underestimation of NO_3^- uptake of 40% by the ^{15}N method, the turnover time of NO_3^- in Lake 227 epilimnion will be 3 instead of 5 days.

The presently available techniques are still unsatisfactory for the elucidation of the mechanism and function of the extracellular products of plankton assimilation. Applying microautoradiography and scanning electron microscopy to study phosphorus cycling in plankton, Paerl and Lean (1976) recently suspected that the fine filaments extruded from blue-green algae and a colloidal pool of phosphorus were identical entities. The colloidal P was produced during filtration which stripped off the filaments without concurrent cell damage. The colloidal P might also be a fraction not retained by 0.45 μm filters (Lean 1976). If this is also true for NO_3^- metabolism, it can explain the movement of N between the soluble and particulate fractions in lake waters. Paerl and Lean (1976) further postulated that deposition of P around the algal capsule served to maintain P availability by preventing P loss through sedimentation. The excretion of amino acids may be the result of their production temporarily exceeding consumption because of uncontrolled synthesis in the absence of mechanisms for enzyme repression (Hood *et al*. 1969).

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

Nitrate-nitrogen following its surface application to eutrophic aquatic systems was initially subjected to reductive metabolism. Bacterial denitrification, one of the reduction pathways, was one component of this NO_3^--N removal. A rapid gas-extraction technique was developed which facilitated direct ^{15}N-tracer assay by mass spectrometry. By application of this method to *in situ* measurements of denitrification rates, the natural site of significant activity was determined to be at the littoral sediment-water interface. Of the factors affecting denitrification examined, dissolved oxygen and NO_3^- concentration were those principally involved in controlling denitrification in the water column and in sediments. Thermal stratification which formed a sharp water density gradient in the thermocline restricted the downward movement of NO_3^- and thus deprived the oxygen-limiting upper hypolimnion and anoxic hypolimnion of the substrate for natural denitrification at these sites. During overturn when NO_3^- was available through NH_3 oxidation and circulation and, by means of the subsequent winter depletion of oxygen through CH_4 oxidation after freeze-up (Rudd 1976), denitrification in the water column would be expected to contribute significantly to NO_3^--N removal.

It was concluded that summer littoral denitrification was capable of removing 14% of the added NO_3^- annually

in Lake 227. In shallow lakes where the sediment surface area is large, littoral denitrification as a nitrogen sink would be more important. Since almost all the NO_3^- that entered the surface sediments was found to be denitrified, it implied that if NO_3^- was applied close to the sediment surface eutrophication would not be enhanced. Dinitrogen, the end product of denitrification, may also be biologically fixed by the indigenous N_2-fixers when there is a nitrogen deficiency. Hence, denitrification may also be responsible for the cycling of N_2 in aquatic systems. The seasonal variation of denitrification in relation to the trophic state of the lake requires further investigation in order to elaborate the annual N budget of that lake.

Phytoplankton assimilation of NO_3^- associated with summer blooms largely determined the ambient NO_3^- concentration in Lake 227. The uptake data showed that excreted organic nitrogen represented a significant portion of the NO_3^- originally assimilated and unless accounted for, could lead to a serious underestimation of NO_3^- uptake. It was concluded that corrections for excretion were necessary in NO_3^- uptake experiments of a few hours duration. The variation in NO_3^- uptake kinetics in relation to species composition needs further study. The environmental factors determining NO_3^--N dynamics as in plankton assimilation and excretion should also be studied in the future.

APPENDIX I

DISSIMILATORY NITRATE REDUCTASE

APPENDIX I

DISSIMILATORY NITRATE REDUCTASE

INTRODUCTION

Dissimilatory nitrate reductase (reduced-NAD: nitrate oxidoreductase, EC 1.6.6.1) catalyses the NADH-dependent reduction of nitrate to nitrite, a two-electron transfer process: $NADH + H^+ + NO_3^- \rightleftarrows NAD^+ + NO_2^- + H_2O$. It has been characterized as a membrane-bound metallo-flavoprotein functioning as a respiratory enzyme in facultative anaerobic bacteria under low oxygen tension or anaerobic conditions (Payne 1973). The enzyme is de-repressed by anoxia, and its synthesis is enhanced by the presence of nitrate. Electron transport to nitrate is also mediated by the respiratory chain involving cytochromes and, in bacteria, molybdenum is implicated. This energy-yielding electron transport system is coupled to phosphorylation although the P/NO_3^- ratio has been estimated only in a few organisms (John and Whatley 1970; Yamanaka *et al*. 1962). The respiratory enzyme differs from the assimilatory one in that its synthesis or activity is not repressed by ammonia or reduced organic nitrogen.

Although the respiratory nitrate reductase from the non-fermentative *Pseudomonas* had been studied (Fewson and Nicholas 1961; Radcliffe and Nicholas 1970), little is known about the true K_m of the enzyme. This section

summarizes some physical properties and steady-state kinetics of the partially purified enzyme from a denitrifying *Pseudomonas* species isolated from lake sediments.

METHODS AND MATERIALS

Cell Culture

Isolate C was chosen for enzyme studies. The organism was grown anaerobically at 28°C under helium in trypticase soy broth supplemented with 0.1% KNO_3. A series of three consecutive transfers were made at 18 h intervals before the culture was used as a 20% (v/v) inoculum for a 20 liter carboy containing 15 liters medium. The 18.75 liter batch culture was sparged with sterile helium for 10 min immediately after inoculation and then stoppered. The head space of the carboy was connected by tubing leading to the bottom of an open flask containing water. This provided for the release of accumulated gases during incubation. The culture was continuously stirred by a magnetic stirrer and incubated at 28°C for 24 h. After incubation, the cells were harvested by continuous centrifugation (Sharples), then washed twice in 0.05 M phosphate buffer, pH 7.2, containing 1 mM cysteine hydrochloride. Packed cells (about 1 g wet weight liter^{-1} yield) were stored at -76°C until the time of enzyme extraction.

Preparation of Crude Extract

To each gram of frozen packed cells, 6 ml of 0.5 M phosphate buffer-1 mM cysteine HCl, pH 7.2, were added. The cells were suspended by stirring and then passed through a French pressure cell twice at 18,000 psi. Unbroken cells and cell debris were removed by centrifugation at 17,300 x g for 5 min. The supernatant obtained was used as a crude enzyme preparation for further work. The entire procedure was carried out at 4°C.

Protein Determination

Protein contents of the enzyme preparation were estimated by the method of Lowry _et al_. (1951) using bovine serum albumin as standard.

Enzyme Assay

Nitrite formation formed the basis for the assay method. The 3 ml assay mix contained 0.5 μmole NADH, 1.0 μmole NO_3^- and 0.1 ml enzyme preparation in 0.05 M phosphate buffer containing 1 mM cysteine HCl, pH 7.2. The mixture minus enzyme was first incubated in a 30°C water bath for 2 min before starting the reaction by adding the enzyme. Reaction was allowed to proceed for 1 to 3 min.

In order to establish the optimum reaction time, 0.2-0.5 ml samples were drawn from the assay mix at various times following the addition of 0.05 ml of the enzyme for

the colorimetric determination of NO_2^- (Sawicki and Scaringelli 1971). The following controls were also included:

(A) complete assay mix minus NO_3^-;

(B) complete assay mix minus enzyme; and

(C) complete assay mix minus NADH.

The optimum reaction time for 0.05 ml of the partially purified enzyme was found to be 30 sec since the reaction velocity was constant until completion of the reaction at 60 sec. This reaction time was then used to determine the optimum enzyme concentration . As the reaction velocity was linear with enzyme concentration up to 0.1 ml enzyme, unless otherwise stated, all subsequent standard assays were carried out at 30°C with 0.05 ml enzyme, 0.5 μmole NADH and 1.0 μmole NO_3^- in a total volume of 3 ml in 0.05 M phosphate buffer-1 mM cysteine HCl, pH 7.2, for 30 sec after the addition of enzyme. The concentrations of NADH and NO_3^- used in this assay system were found to be at saturation levels for the enzyme.

Although more convenient, spectrophotometric assay by following NADH absorption at 340 nm was not employed because of strong NADH oxidase activity (found to be responsible for as high as 50% of the total oxidation of NADH in the presence of NO_3^- at times) in the enzyme preparation.

Enzyme units, U, were defined as the number of nanomoles (nmoles) of NO_2^- produced per assay per min, i.e.

$1 \times U = 1$ nmole NO_2^- formed $assay^{-1}$ min^{-1}.
Specific activity was expressed in terms of enzyme units per mg of Lowry protein in the assay mix.

Spectral Analysis

All spectral analyses of the enzyme preparations were performed using quartz cuvettes of 10 mm light path in a Shimadzu MPS-50L Spectrophotometer.

Chemicals

The biochemical reagents and inhibitors used in this part of the study were obtained from Sigma Chemical Co., St. Louis, MO, and prepared in distilled deionized water.

RESULTS

Demonstration of Enzyme Activity

Under the culture conditions in trypticase soy-KNO_3, isolate C had a 12 h log phase and a mean doubling time of 4.25 h. The culture was grown for 24 h before enzyme preparation since higher specific activity was found in 24 h cultures (i.e. at the beginning of stationary phase) than in 18 h cultures. From control assays it was known that the enzyme was NADH-specific and did not oxidize NADPH. Crude cell extracts were free of NO_2^- and NO_3^-. These results had been confirmed by spectrometric assays which also showed the absence of NADPH oxidase and NAD(P)H-NO_2^- oxidoreductase activities.

Purification

When the crude extract was subjected to ultracentrifugation at 100,000 x g for 1.5 h, nitrate reductase activity was found to associate with the pelleted membrane fraction only. A partial purification of 4.2 fold of the enzyme was achieved by a single adsorption step with calcium phosphate gel (Table XII). No further purification procedure was attempted.

Freeze-thaw Effect on Enzyme Activity

The effect of repeated freeze-thaw on nitrate reductase activity in fraction II was investigated. Figure 27 shows that about 50% of the activity was retained after the second freeze-thaw, and 95% of the activity was lost when the preparation was freeze-thawed for the fourth time. Hence, fraction II which had been freeze-thawed once was not refrozen for further use.

Temperature Stability

Nitrate reductase in crude extracts were tested for heat stability by holding aliquots of the same preparation at various temperatures ranging from 25°C to 55°C for 30 min. Highest activity was displayed by the aliquot held at 35°C (Fig. 28). The enzyme was unstable above 40°C.

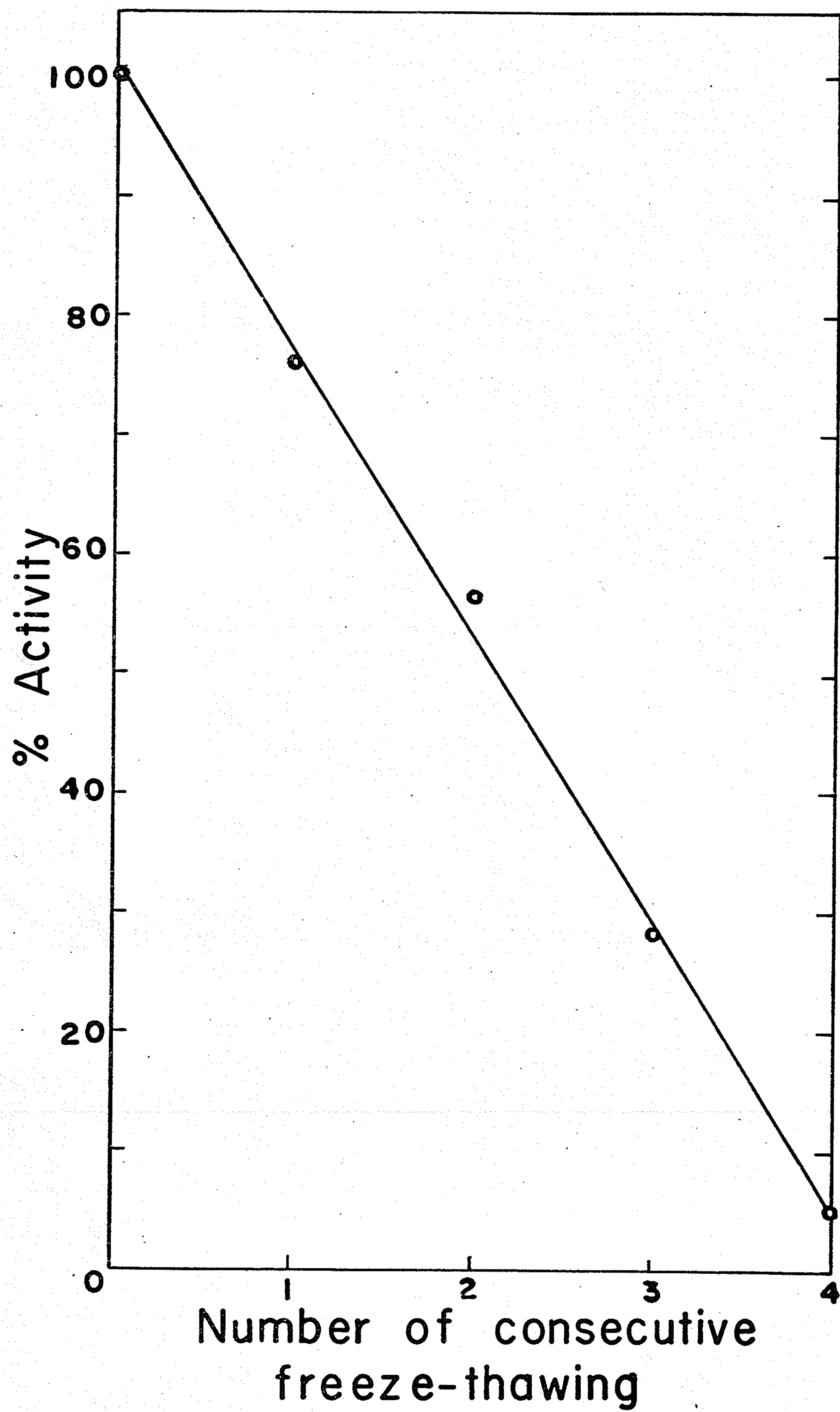
Table XII. Summary of the purification procedure[a] for nitrate reductase.

Fraction	Volume ml	Units ml^{-1}	Total Activity U	Protein Concn. $mg\ ml^{-1}$	Total Protein mg	Specific Activity $U\ mg^{-1}$	Yield %	Fold
Crude	60	1,794	107,640	23	1,380	78	100	1
I (100,000 x g pellet)	45	1,680	75,600	12	540	140	70	1.8
II ($CaPO_4$ gel, supernatant)[b]	45	1,647	74115	5	225	329	68	4.2

[a]The entire procedure was carried out at 4°C.

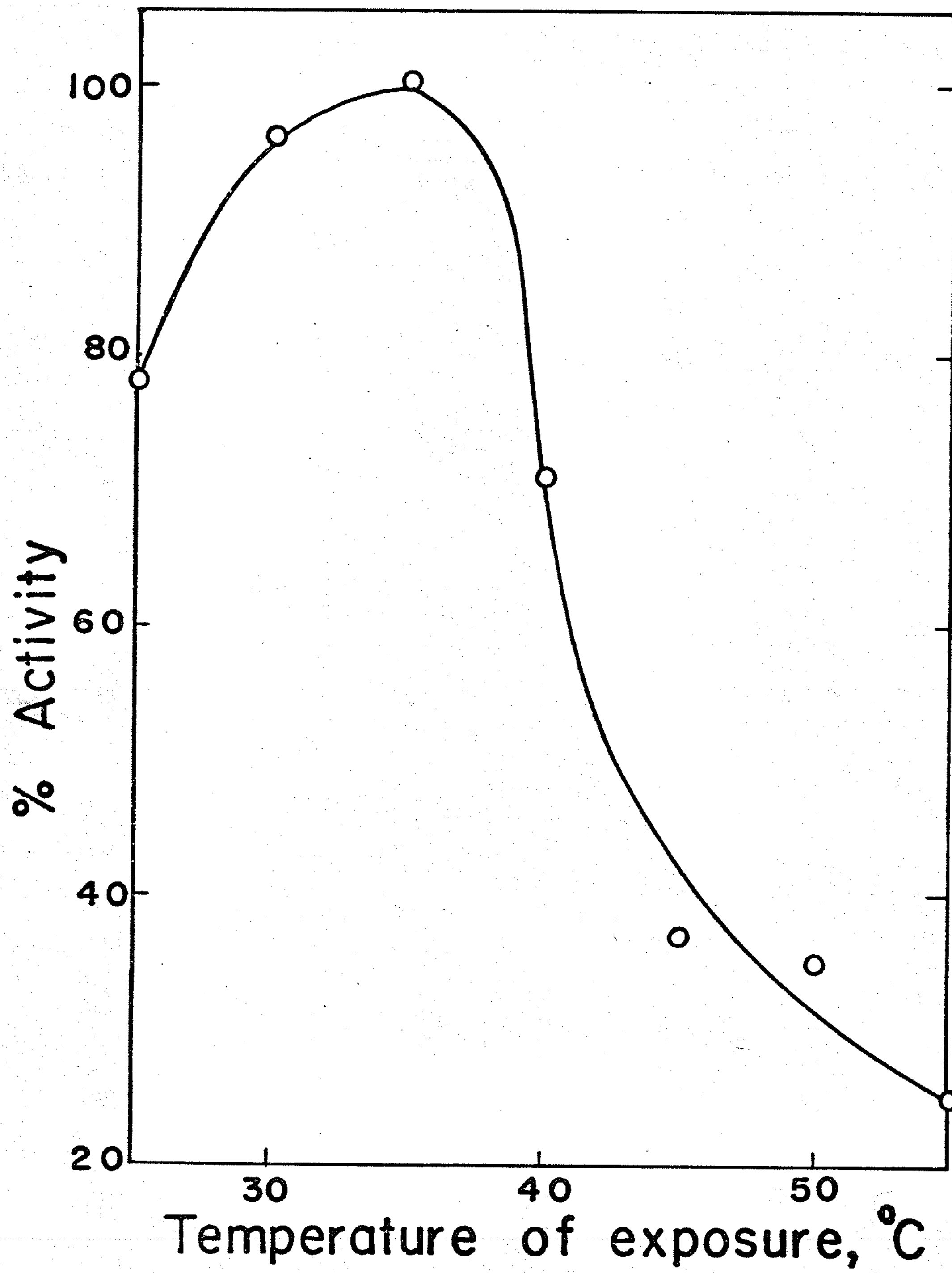
[b]The pellet from fraction I was redissolved in a minimum volume of 0.05 M phosphate buffer-1 mM cysteine HCl, pH 7.2, and assayed for Lowry protein. Ten mg of calcium phosphate gel was added to each mg of protein and the mixture was stirred for 10 min. The slurry was then centrifuged at 5,000 x g for 5 min and the supernatant kept. Proteins bound to the gel was eluted with an equal volume of 0.1 M phosphate buffer-1 mM cysteine HCl, pH 7.2. It was found that the enzyme did not bind to the calcium phosphate gel.

Fig. 27. Effect of repeated freeze-thawing on enzyme activity.



100
80
60
40
20
0
% Activity
1
2
3
4
Number of consecutive freeze-thawing

Fig. 28. Temperature stability of nitrate reductase. Aliquots of the same enzyme peparation were held at various temperatures ranging from 25° to 55°C for 30 min and then assayed for nitrate reductase activity.



100
80
60
40
20
% Activity
30
40
50
Temperature of exposure, °C

Effect of pH and Solubility of the Enzyme

The optimum pH for this nitrate reductase was 7.0 when the enzyme was assayed at pH values ranging from 5.0 to 9.0 (Fig. 29). The soluble fraction obtained after exposure to various pH's and readjusted to 7.0 demonstrated highest solubility of the enzyme also at pH7.0 (Fig. 29). At pH 8.0 and above, the enzyme was inactivated; at pH 5.0 and below it was totally precipitated and inactive.

Initial Velocity Kinetics

While a highly purified enzyme is desirable for kinetic studies, they can be carried out with impure preparations provided that no interfering reactions are present in the assay system. In the case of dissimilatory nitrate reductase which is a membrane-bound enzyme complex (Payne 1973), its natural form is more important to study than the product of a purification procedure which might alter its properties (Plowman 1972). Kinetic studies were performed with unfrozen fraction II of the enzyme preparation which was free of NADH oxidase activities. Initial velocity data and secondary replot data with varying NO_3^- concentrations and NADH as the fixed substrate at different concentrations are presented in Fig. 30. Figure 31 shows the reverse plots by rearranging NADH as the varying substrate and NO_3^- as the fixed substrate at different concentrations. Double-reciprocal plots of

Fig. 29. Effect of pH and enzyme solubility. Open circles, nitrate reductase activity at different pH values; closed circles, % activity in the soluble enzyme fraction after exposure to different pH values.

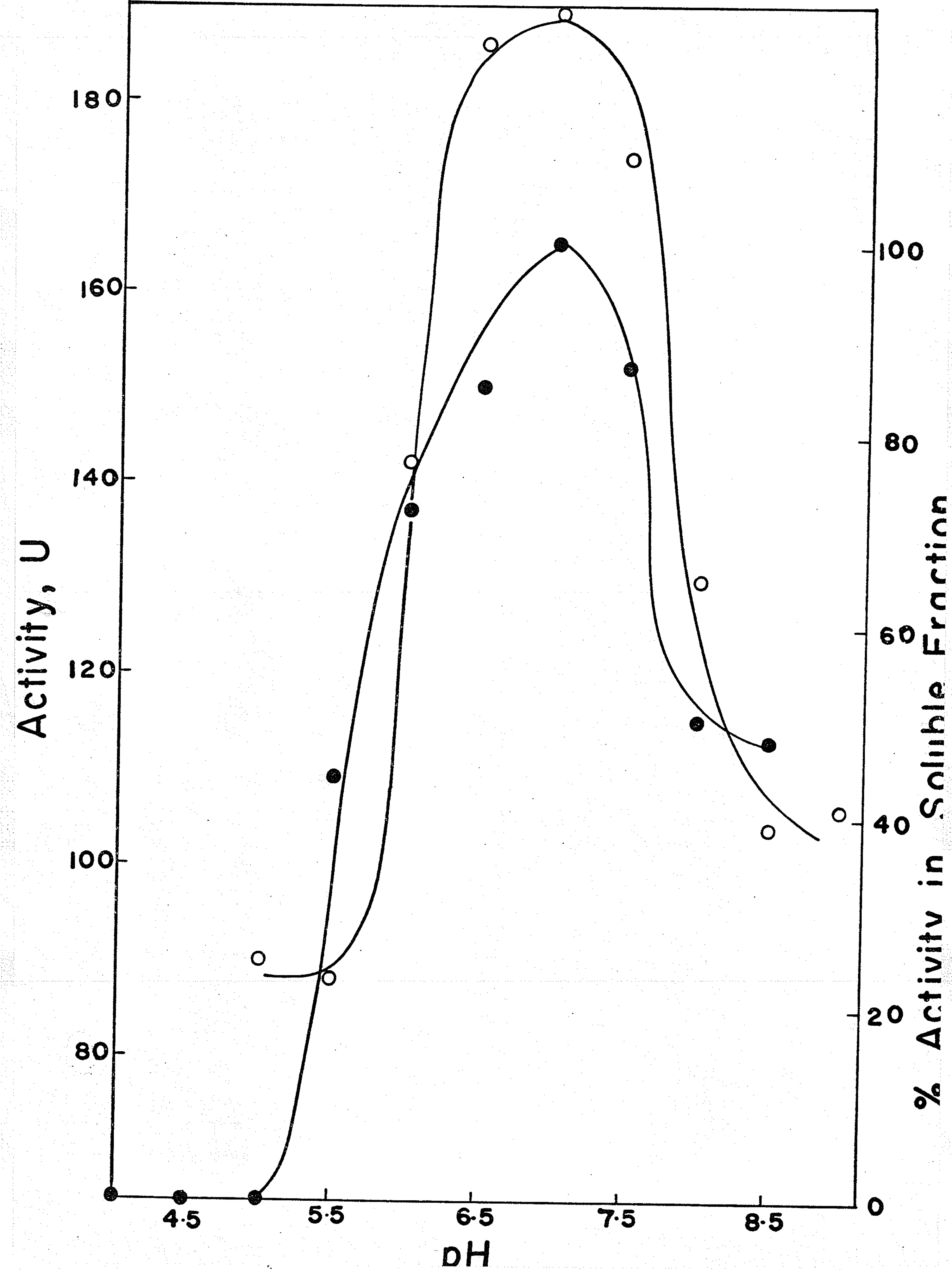
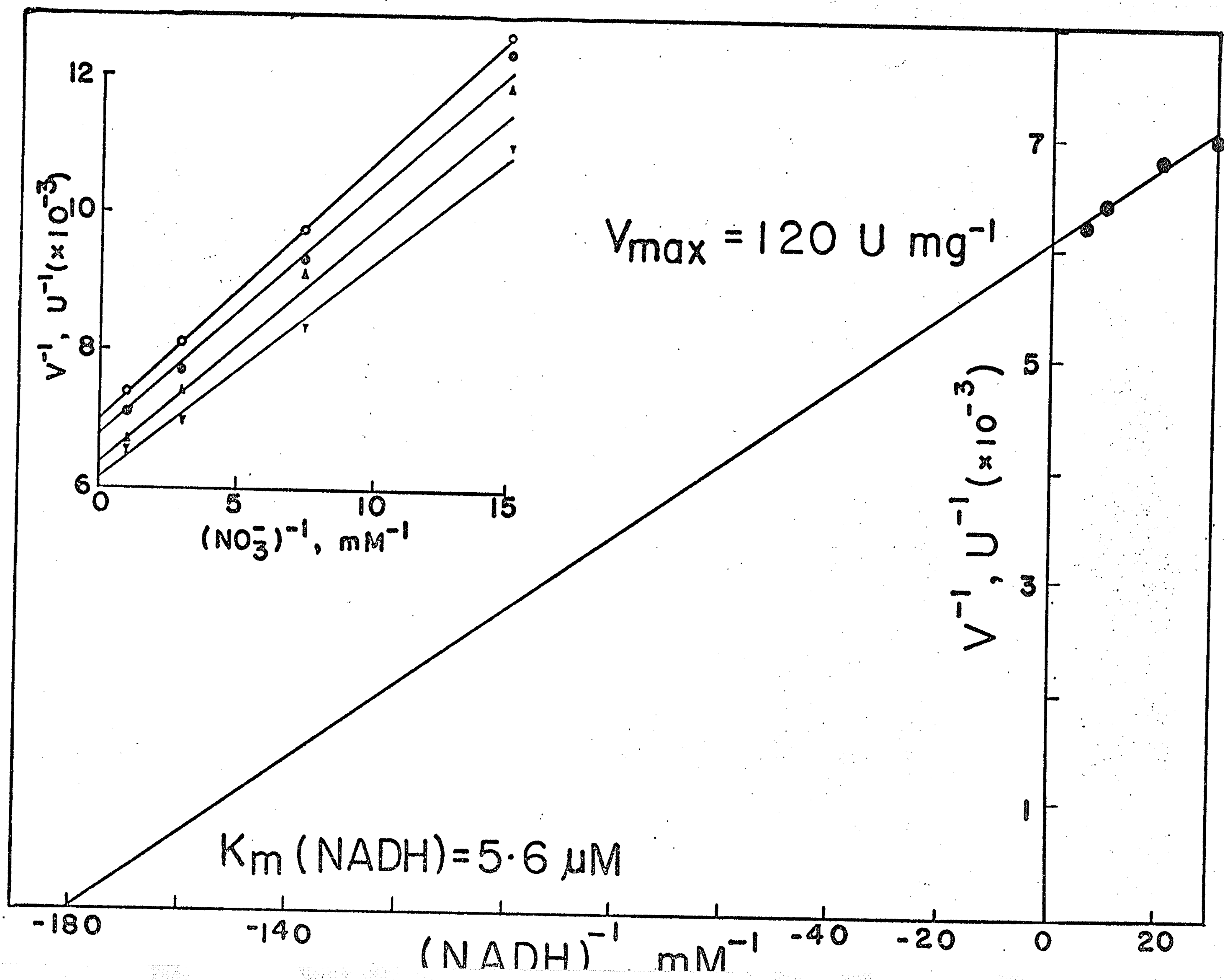
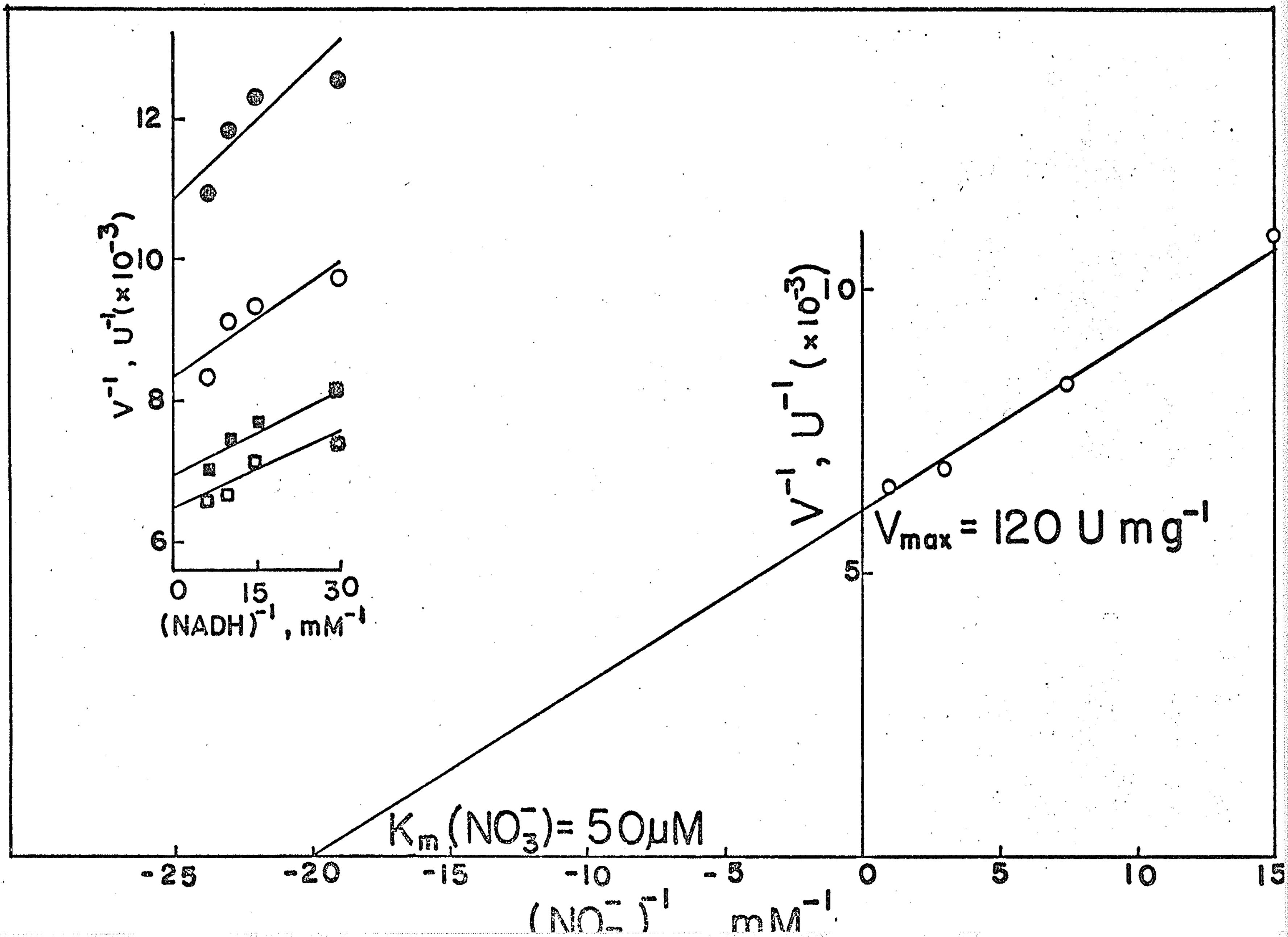

Activity, U
180
160
140
120
100
80
4.5
5.5
6.5
7.5
8.5
pH
100
80
60
40
20
0
% Activity in Soluble Fraction

Fig. 30. Determination of V_{max} and K_m for NADH by intercept replot from Lineweaver-Burk plots (inset) with varying NO_3^- concentration. Inset NADH concentrations: open circles, 0.033 mM; closed circles, 0.067 mM; 'up' triangles, 0.100 mM; 'down' triangles, 0.167 mM.



12
10
8
6
0
5
10
15
V^{-1}, U^{-1} ($\times 10^{-3}$)
$(NO_3^-)^{-1}$, mM^{-1}
V_{max} = 120 U mg^{-1}
K_m (NADH) = 5·6 µM
7
5
3
1
V^{-1}, U^{-1} ($\times 10^{-3}$)
-180
-140
-40
-20
0
20
$(NADH)^{-1}$ mM^{-1}

Fig. 31. Determination of V_{max} and K_m for NO_3^- by intercept replot from Lineweaver-Burk plots (inset) with varying NADH concentration. Inset NO_3^- concentrations: closed circles, 0.067 mM; open circles, 0.133 mM; closed squares, 0.333 mM; open squares, 0.100 mM.



12
10
8
6
0
15
30
V^{-1}, U^{-1} ($\times 10^{-3}$)
$(NADH)^{-1}$, mM^{-1}
10
5
V^{-1}, U^{-1} ($\times 10^{-3}$)
V_{max} = 120 U mg^{-1}
$K_m(NO_3^-)$ = 50μM
-25
-20
-15
-10
-5
0
5
10
15
$(NO_3^-)^{-1}$ mM^{-1}

initial velocity against substrate concentrations from either approach are shown as insets of Figs. 30 and 31 while the corresponding intercept replots are shown in the main figures. The maximum velocity thus determined is 120 U mg^{-1} while the true K_m value for NO_3^- is 0.05 mM and that for NADH is 0.0056 mM.

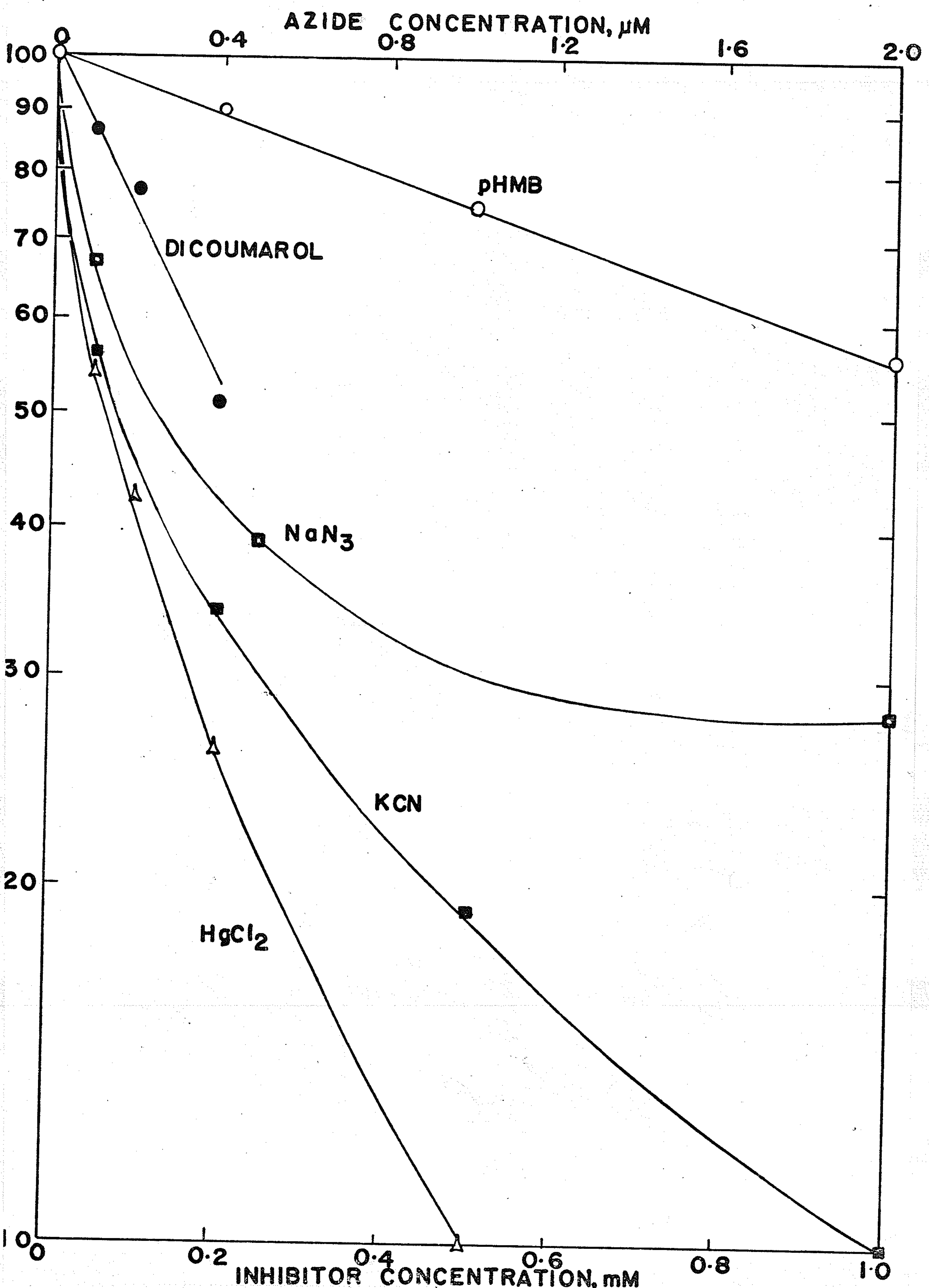
Inhibitors

Five inhibitors and their effective concentration ranges under standard assay conditions were studied (Fig. 32). In their increasing order of effectiveness, they were: p-hydroxymercuribenzoate, dicoumarol, KCN, $HgCl_2$, and sodium azide. The effective concentrationswere in the order of 1 mM except azide, which inhibited nitrate reductase at concentrations in the order of 2 μM. Such inhibition results suggested a requirement for sufhydryl groups, metals and the involvement of cytochromes in the enzyme system.

Spectral Properties

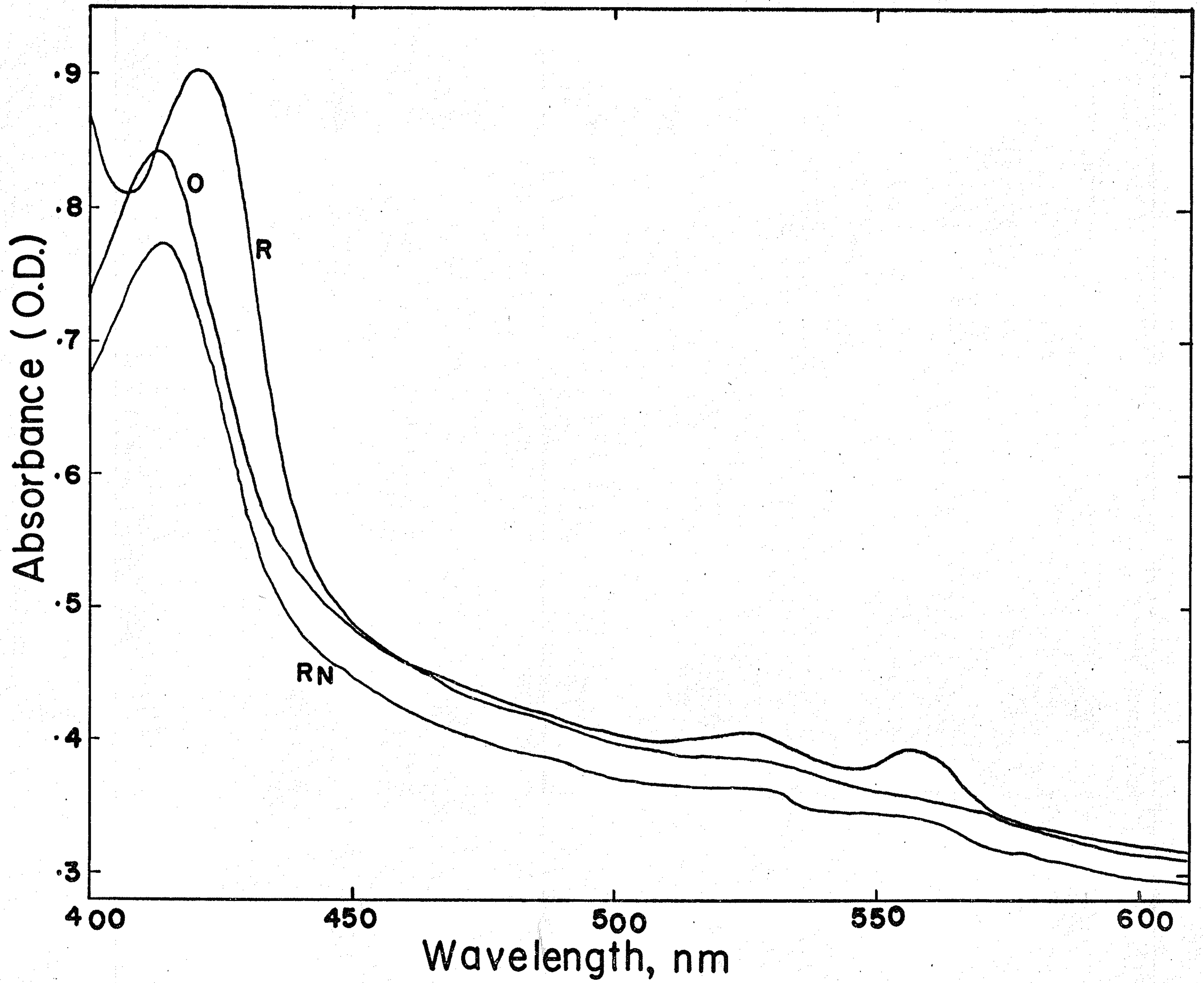
Fraction II of the enzyme preparation contained a c-type cytochrome that displayed a spectral absorption peak at 412 nm when oxidized and absorption maxima at 420 (γ), 522 (β), and 553 (α) when reduced with dithionite or NADH (Fig. 33). The absorption spectrum was very similar to that of cytochrome c_3 in Desulfovibrio and Escherichia coli (Kamen and Horio 1970). Nitrate prominently reoxidized the

Fig. 32. Inhibition of nitrate reductase under assay conditions.



AZIDE CONCENTRATION, μM
0
0·4
0·8
1·2
1·6
2·0
100
90
80
70
60
50
40
30
20
10
pHMB
DICOUMAROL
NaN3
KCN
HgCl2
0
0·2
0·4
0·6
0·8
1·0
INHIBITOR CONCENTRATION, mM

Fig. 33. Absorption spectra of nitrate reductase (Fraction II). O, oxidized form; R, reduced by (1.67 mM) NADH; RN, reoxidized by (8.3 mM) NO_3^-.



·9
·8
·7
·6
·5
·4
·3
Absorbance (O.D.)
O
R
RN
400
450
500
550
600
Wavelength, nm

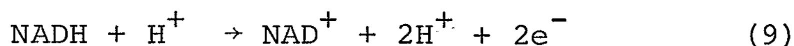
γ peak after reduction with NADH, the natural electron donor (Fig. 33). However, it did not reoxidize the cytochrome once the latter was reduced by dithionite.

DISCUSSION

The equilibrium constant, K'_{eq}, for the overall reaction catalysed by dissimilatory NADH-NO_3^- oxidoreductase (EC 1.6.6.1) can be estimated from thermodynamic data (Conn and Stumpf 1967; McCarty 1972). Nitrate reduction is considered to couple with NADH oxidation as represented by the following reactions:

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O \qquad (8)$$

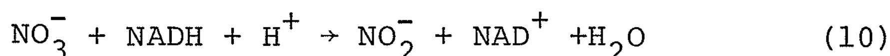
$$E'_o = 0.408 \text{ v (pH 7.0, 25}^oC)$$



$$NADH + H^+ \rightarrow NAD^+ + 2H^+ + 2e^- \qquad (9)$$

$$E'_o = -0.32 \text{ v (pH 7.0, 25}^oC)$$

The overall reaction is:



$$NO_3^- + NADH + H^+ \rightarrow NO_2^- + NAD^+ + H_2O \qquad (10)$$

$$\Delta E'_o = 0.728 \text{ v (pH 7.0, 25}^oC)$$

Assuming a homogeneous system in which thermodynamic considerations can be given to the steady-state oxidation-reduction reaction, the change in free energy, $\Delta F'$(pH7.0) will be -33,580 cal, and the K'_{eq} at pH 7.0 will be 10^{18}. Consequently, the reaction involving such a large K'_{eq} is virtually irreversible.

In the present study, it has been found that inclusion of NAD in the standard assay mix does not inhibit nitrate reductase. The non-inhibition by this product may be partly explained by the large K'_{eq} and partly by the small K_m determined for NADH. The high affinity of the enzyme for this natural electron donor (as indicated by its K_m of 0.0056 mM) may suggest that NAD does not bind to the enzyme. On the other hand, product inhibition by NO_2^- cannot be studied by the present assay method in which enzyme activity is determined by the formation of NO_2^-. Although NO_2^- does not reoxidize the c-type cytochrome present in the cell extract after reduction by NADH, its inhibition may not involve this electron carrier which has been implicated in the nitrate reductase complex. The enzyme mechanism cannot be elucidated until the pattern of inhibition kinetics is disclosed.

The NO_3^- reducing system in isolate C appears to be similar to that in *Pseudomonas aeruginosa* which has been shown to require iron in the form of cytochrome c and NADH as an effective electron donor (Fewson and Nicholas 1961).

APPENDIX II

A FORTRAN (WATFIV) Program used for Calculating Denitrification Products from Experimental Data

APPENDIX II

```
      $JOB  WATFIV
      C     ------------------------------------------------------------------
  1         REAL AI5/.0040/,SOLN2/.0143/,SOLN20/.624/,ATTN/400./,GCSTD/.00271/
  2         REAL NIOX/1.9/,VG/50./,VL/250./,VTZERO/0./,TEMP/23./
  3         REAL K,L,M,N2DP,N2DQ
  4         READ (5, 5) N
  5       5 FORMAT (I3)
  6         J=1
  7         DO 13 I=1, N
  8         WRITE (6, 51) J
  9      51 FORMAT ('1','NUMBER',I5)
      C ----------------------------------------------------------------------
      C **********ION VOLTAGES & RATIOS
 10         READ (5, 15) CM28,S28,CM29,S29,CM30,S30,CM32,S32
 11         READ (5, 16) CM40, S40
 12         READ (5, 25) P, Q, FC, TRITE
 13      15 FORMAT (8F9.2)
 14      16 FORMAT (2F9.2)
 15      25 FORMAT (4F10.2)
 16         WRITE (6, 52) CM28,S28,CM29,S29,CM30,S30,CM32,S32,CM40,S40
 17      52 FORMAT ('0', 'MASS SPEC DATA:',10F9.2)
 18         V28=(S28/12.)*CM28
 19         V29=(S29/12.)*CM29
 20         V30=(S30/12.)*CM30
 21         V32=(S32/12.)*CM32
 22         V40=(S40/12.)*CM40
 23         OTWO=V32/V28
 24         ARGON=V28/V40
 25         RIA=V32/V40
 26         CV28=V28-(NIOX*V32)
 27         R=V29/CV28
 28         R1=V30/(CV28+V29)
 29         ENRICH=(V29+2.*V30)/(2.*(CV28+V29+V30))

 30         WRITE (6, 1) V28, V29, V30, V32,V40
 31       1 FORMAT ('0','V28',F11.5,10X,'V29',F11.5,10X,'V30',F11.5,10X,'V32',
           1F11.5,10X,'V40',F11.5)
 32         WRITE (6, 10) ENRICH, R, R1, OTWO
 33      10 FORMAT ('_','N-15',F13.5,13X,'R',F16.7,13X,'R1',F15.7,13X,
           1'M32/M28',F10.5)
 34         WRITE (6, 53) ARGON, RIA, CV28
 35      53 FORMAT ('0', 'M28/M40',F10.3,5X,'M32/M40',F10.3,5X,'CV28',F10.3)
      C ----------------------------------------------------------------------
      C **********MOLEFRACTIONS OF ISOTOPIC N-TWO
 36         A=1./((1.+R)*(1.+R1))
 37         C=R1/(1.+R1)
 38         B=(1.-A-C)/2.
 39         WRITE (6, 11) A, B, C
 40      11 FORMAT ('0','A',F14.7,5X,'B',F14.7,5X,'C',F14.7)
```

```
       C ---------------------------------------------------------------------------
       C **********MOLEFRACTIONS OF TOTAL N-TWO FROM TRACER
       C **********(A) BASED ON CONSTANT N14 AND MECHANISM 1
  41         X15=.95*P/(P+Q)
  42         X14=1.-X15
  43         A14=1.-A15
  44         WRITE (6, 100) P, Q, X15, A15
  45     100 FORMAT ('_','P',F14.2,5X,'Q',F14.2,5X,'X15',F12.4,5X,'A15',F12.4)
  46         DA=(A-A14*A14)/(X14*X14-A14*A14)
  47         DB=(B-(A14*A15))/((X14*X15)-(A14*A15))
  48         DC=(C-A15*A15)/(X15*X15-A15*A15)
  49         DBAR=(DA+DC)/2.
  50         EXCH1=(VG+SOLN2*VL)/VG
  51         DCORR=DBAR*EXCH1
       C **********(B) BASED ON CONSTANT N14 AND MECHANISM 2
  52         D1A=(A-A14*A14)/(X14*A14-A14*A14)
  53         D1B=(2.*(B-A14*A15))/(2.*X14*X15-2.*A14*A15+A15*X14+X15*A14)
  54         D1C=(C-A15*A15)/(X15*A15-A15*A15)
  55         WRITE (6, 2) D1A, DA
  56       2 FORMAT ('0','D1A',F12.7,5X,'DA',F13.7)
  57         WRITE (6, 3) D1B, DB
  58       3 FORMAT (' ','D1B',F12.7,5X,'DB',F13.7)
  59         WRITE (6, 4) D1C, DC, DBAR
  60       4 FORMAT (' ','D1C',F12.7,5X,'DC',F13.7,5X,'DBAR',F11.7)
       C **********(C) BASED ON THE MEAN OF CHANGING N14 AND MECHANISM 1
  61         H=2.*A14*(1.-A14)-R*A14*A14
  62         K=(1.-A14)**2.-R1*(2.*A14*(1.-A14)+A14*A14)
  63         L=H*(R1+1.)+K*(R+2.)
  64         M=-2.*(H*(R1+1.)+K)
  65         PN14=(-M+SQRT(M*M-4.*L*H))/(2.*L)
  66         QN14=(-M-SQRT(M*M-4.*L*H))/(2.*L)
  67         DP=H/((R+2.)*PN14*PN14-2.*PN14+H)
  68         DPCORR=DP*EXCH1
  69         DQ=H/((R+2.)*QN14*QN14-2.*QN14+H)
  70         DQCORR=DQ*EXCH1
  71         WRITE (6,55) DP, DQ
  72      55 FORMAT ('0', 'DP',F13.7,5X,'DQ',F13.7)
  73         WRITE (6, 56) PN14, QN14
  74      56 FORMAT(' ', 'PN14',F12.5,5X,'QN14',F12.5)
       C ---------------------------------------------------------------------------
       C **********N-TWO PRODUCTION
  75         SUT=P+Q-FC
  76         G=341250.*(VTZERO+SOLN2*VL)/(TEMP+273.)
  77         EVOLN2=(DCORR*G)*1000./VL
  78         PCN2=EVOLN2/SUT*100.
  79         N2DP=(DPCORR*G)*1000./VL
  80         N2DQ=(DQCORR*G)*1000./VL
  81         DELTA=(((ENRICH/(1.-ENRICH))/(A15/A14))-1.)*1000.
  82         WRITE (6, 101) VG, VL, SOLN2, DCORR
  83     101 FORMAT ('0','VG',F13.1,5X,'VL',F13.1,5X,'ALFA N2',F8.4,5X,'DCORR',
            1F10.7)
```

```
 84            WRITE (6,110) VTZERO, TEMP,DPCORR,DQCORR
 85        110 FORMAT ('_','VTZERO',F9.1,5X,'TEMP',F11.1,5X,'DPCORR',F9.7,5X,
              1'DQCORR',F14.7)
 86            WRITE (6, 111) G, EVOLN2, PCN2, DELTA
 87        111 FORMAT ('0','G',F14.3,5X,'N2 EVOL',F8.2,5X,'% DENITN',F7.2,5X,
              1'DELTA',F10.2)
 88            WRITE (6, 112) N2DP, N2DQ
 89        112 FORMAT (' ', 'COMPARABLE VALUES', 2F15.2)
         C ----------------------------------------------------------------------
         C **********N-TWO-O PRODUCTION
 90            READ (5, 35) CMN2O, ATTN, GCSTD
 91         35 FORMAT (3F10.5)
 92            WRITE (6, 54) CMN2O, ATTN, GCSTD
 93         54 FORMAT ('0', 'GC DATA:', 3F10.4)
 94            EVN2O=12.5*CMN2O*GCSTD*ATTN*(VG+SOLN2O*VL)/VL
 95            PCN2O=EVN2O/(P+Q-FC)*100.
 96            WRITE (6, 1000) SOLN2O, EVN2O, PCN2O
 97       1000 FORMAT ('_', 'A_FA N2O',F7.3,5X,'N2O EVOL',F7.2,5X,'% N2O',F10.2)
         C ----------------------------------------------------------------------
         C **********CHEMICAL ANALYSIS
 98            PCNO2=TRITE/SUT*100.
 99            PCSUT=(SUT/(P+Q))*100.
100            WRITE (6, 1001) FC, TRITE, PCNO2
101       1001 FORMAT ('0','NO3- FC',F8.2,5X,'NITRITE',F8.2,5X,'% NO2-',F9.2)
102            WRITE (6, 1011) SUT, PCSUT
103       1011 FORMAT ('0','NITRATE UPTAKE',F21.2,5X,'% NITRATE UPTAKE',F19.2)
104            J=J+1
105         13 CONTINUE
         C ADDENDUM FOR OMISSION IN LAST RUN:
         C NUMBER 1  L227 4.5M NITRITE N-15 OCT 1/75 10 DEG C CONTROL WITH HGCL2
         C NUMBER 2 L227 4.75M OTHERWISE SAME AS NUMBER 1
106            STOP
107            END
```

$ENTRY

Example of Computer Output Data (Calculated Results):

```
MASS SPEC DATA:     6.50     10.00     7.70     0.10     5.90     0.01

                                       10.20     0.10     6.90     0.30
```

V28 5.41667 V29 0.06417 V30 0.00492

N-15 0.00695 V32 0.08500 V40 0.17250

RI 0.0009243 R 0.0122102

M32/M28 0.01569

M28/M40 31.401 M32/M40 0.493 CV28 5.255

A 0.9870260 B 0.0060253 C 0.0009234
P 500.00 Q 1689.00 X15 0.2170 A15 0.0040

DIA 0.0235222 DA 0.0131692
DIB 0.0074079 DB 0.0123024
DIC 1.0651090 DC 0.0192785 DBAR 0.0162239

DP 0.0098420 DQ 1378.7050000
PN14 0.69633 QN14 0.99600

VG 50.0 VL 250.0 ALFA N2 0.0143 DCORR 0.0173839
VTZERO 0.0 TEMP 23.0 DPCORR0.0105457 DOCORR 1477.2820000

G 4121.512 N2 EVOL 286.59 % DENITN 62.85 DELTA 742.49
COMPARABLE VALUES 173.86 24354520.00

GC DATA: 0.0000 400.0000 0.0032
ALFA N2O 0.624 N2O EVOL 0.00 % N2O 0.00

NO3- FC 1733.00 NITRITE 0.00 % NO2- 0.00

NITRATE UPTAKE 456.00 % NITRATE UPTAKE 20.83

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