DENITRIFICATION IN LAKE SEDIMENT AND SEWAGE SLUDGE

by

Ian Keith Nicholson

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ABSTRACT

Mass spectrometric analysis of atmospheres over lake sediment and flooded sewage sludge incubated with N-15 labeled NO_3^- was performed. The sequence of denitrification was NO_2^-, N_2O, and N_2 in lake sediment at 10C. However, added NO_3^- was reduced to NO_2^- and N_2 during incubation of sludge. Re^*, the electron acceptance rate by nitrogenous oxides during their reduction, was faster in the sludge. Addition of glucose to sediment greatly increased Re^* and reduced quantities of N_2O accumulated. The maximum of NO_2^- accumulation was greatly increased by the addition of lime to sediment. This retarded the rate of N_2O accumulation, but the effect on Re^* and the rates of NO_3^- loss and N_2 production was slight. Acidification of sludge decreased Re^* and the rate of NO_3^- loss. A lag period before N_2 production was evident, but no N_2O was detected.

In non-agitated sediment with relatively low concentrations [2.5 to 10 ppm] of added NO_3^--N, Re^* and the rate of NO_3^- loss were nearly first-order. However, the rates were almost zero-order when agitation ensured the homogeneity of the incubated sediment. Although the rates were first-order with 10 ppm added NO_3^--N in sludge, they were zero-order with higher concentrations [20 to 50 ppm]. These results indi-

cated that Re^* and the rate of NO_3^- loss were rate-limited by diffusion in non-agitated sediment. Re^* for sediment agitated with 10 ppm added NO_3^--N was approximately twice that of the non-agitated sediment. Trends in NO_3^- reduction, N_2O accumulation, and N_2 evolution with time in agitated sediment were similar to those generated by competitive Michaelis-Menten kinetics.

Lowering of the incubation temperature of sludge to 4C induced the accumulation of N_2O, which was not detected at a higher temperature [10C]. Also, Re^* and the rate of NO_3^- loss were less.

Although addition of sludge [20.7% $CaCO_3$ equivalent] to sediment at 10C increased NO_2^- accumulation, the rate of N_2O accumulation was not retarded and the rate of N_2 production was accelerated.

ACKNOWLEDGEMENTS

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Chapter I

INTRODUCTION

Nitrogen is the most important essential plant nutrient that affects the yield of an agricultural crop. In order to meet the food requirements of an ever-increasing world population, the use of nitrogenous fertilizers in crop production has also steadily increased. Large projected increases in fertilizer use have raised some concern about the possible hazardous effects of nitrogenous compounds on the environment. High nitrate levels in drinking water, the role of nitrate in lake eutrophication, and more recently, the detrimental effect of nitrogen oxides on the atmosphere are the major issues. Nitrogen applied to the soil in chemical fertilizers, animal manures, or digested municipal sewage sludge, as well as nitrogen from beneath feedlots, may be leached to groundwater or denitrified. In Canada, excluding Quebec, 80 percent of sewage sludge is dumped into lakes and rivers, while in Quebec most of the 150,000 tons annual production of untreated sewage sludge is discharged directly into the St. Lawrence River and its tributaries (Gagnon 1973).

Denitrification—the reduction of nitrate to gaseous products—is often mentioned in the discussion of the above

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issues. Losses of fertilizer-N applied to agricultural fields, sometimes in excess of 50 percent, are blamed on this process. On the other hand, denitrification in sewage treatment and waterways is promoted as a means of reducing nitrate-N concentration. Infant and ruminant methemoglobinemia, an impairment of oxygen transport by blood due to reaction of nitrite with hemoglobin, occurs when nitrate is ingested and reduced. The nitrate maximum in drinking water is set at 10 ppm nitrogen. Nitric and nitrous oxides, gaseous products of denitrification, may be involved in atmospheric ozone destruction (Crutzen 1976; Delwiche and Bryan 1976; Keeney 1978). Carcinogenic ultraviolet light is screened by the protective ozone layer in the upper atmosphere.

The ideal agricultural solution to denitrification is to minimize nitrate removal, while the ideal environmental solution is to maximize nitrate removal and minimize production of gaseous nitrogen oxides. Lake sediment as a site of denitrification is fairly well understood. However, the effect of nitrate concentration on the forms and quantities of the nitrogen oxides and molecular nitrogen has received limited attention. Work with denitrification in agricultural soils has shown a dependence of the ratio of the produced nitrogen oxides to produced nitrogen gas on the added nitrate concentration (Cho and Mills 1979; Cho and Sakdinan 1978). The purpose of this investigation is to study the denitrification process in flooded lake sediment and sewage

sludge samples, with respect to the removal of nitrate and the production of nitrogenous gases.

Chapter II

LITERATURE REVIEW

2.1   BIOLOGICAL REDUCTION OF NITROGEN OXIDES

Nitrate is reduced by microorganisms in the assimilation of nitrate-N and in anaerobic respiration. Reductase enzymes involved in the biological reduction of nitrogen oxides are flavoproteins and may contain molybdenum, iron, or other heavy metals which are alternately oxidized and reduced during electron transfer (Conn and Stumpf 1967; Lehninger 1970).

Nitrate assimilation occurs in both aerobic and anaerobic conditions and is the incorporation of inorganic-N into organic-N. However, less immobilization occurs in anaerobic conditions since microbial metabolism is less efficient and growth is slower (Bartholomew 1965). Most microorganisms preferentially assimilate ammonium over nitrate (Jansson et al. 1955). The presence of readily available organic matter promotes nitrogen assimilation (Bartholomew 1965). Assimilation can be written as a series of two-electron transfers:

$$NO_3^- \Rightarrow NO_2^- \Rightarrow [HNO] \Rightarrow NH_2OH \Rightarrow NH_4^+ \Rightarrow amino \ acids. \quad \{1\}$$
$$\ \ (5) \qquad (3) \qquad (1) \qquad (-1) \qquad (-3)$$
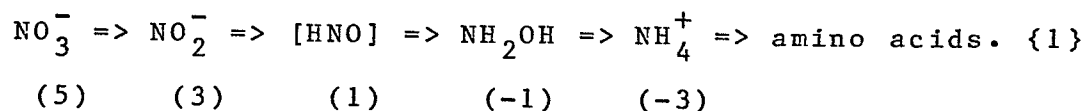
The numbers in parentheses denote the oxidation states. The

intermediates, nitrite, hydroxylamine, and ammonium, have been detected. The hypothetical intermediate [HNO] has not been detected, but is postulated to be hyponitrite (Campbell and Lees 1967).

In aerobic conditions, hydrogens donated by organic matter [represented by glucose] reduce oxygen to water according to:

$$C_6H_{12}O_6 + 6O_2 \Rightarrow 6CO_2 + 6H_2O. \qquad \{2\}$$
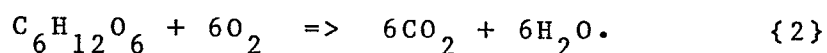
In the absence of oxygen, other compounds serve as terminal acceptors and are reduced.

The bacterial group containing the most genera able to reduce nitrate is comprised of the nitrate-respirers (Payne 1973). They reduce nitrate to nitrite by dissimilation in anoxic conditions.

Denitrification is the production of nitrogen or gaseous nitrogen oxides through the reduction of nitrate (Wijler and Delwiche 1954). Biological denitrification is carried out by facultative anaerobic bacteria in anoxic conditions. The complete reduction of nitrate to nitrogen, coupled with the complete oxidation of organic matter [represented by glucose], is written as:

$$C_6H_{12}O_6 + 4NO_3^- \Rightarrow 6CO_2 + 6H_2O + 2N_2. \qquad \{3\}$$
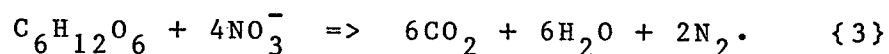
Payne (1976) lists the bacterial genera containing denitrifying bacteria. The number is small, but denitrifiers

are widely distributed in soil, sediments, and fresh and salt water. Many carry out other transformations involved in the decomposition of organic matter such as proteolysis and ammonification (Keeney 1973). The most numerous denitrifiers are <u>Pseudomonas</u> <u>sp</u>., <u>Achromobacter</u> <u>sp</u>. and <u>Bacillus</u> <u>sp</u>. (Alexander 1977). Different denitrifying species have different rates of gas production under similar environmental conditions (Valera and Alexander 1961). Organisms isolated from a waterlogged soil varied in their ability to reduce nitrate (Jordan <u>et</u> <u>al</u>. 1967).

The sequence of detectable products with most denitrifiers is nitrite, nitrous oxide, and nitrogen:

$$NO_3^- \Rightarrow NO_2^- \Rightarrow N_2O \Rightarrow N_2. \qquad \{4\}$$

$$(5) \qquad (3) \qquad (1) \qquad (0)$$
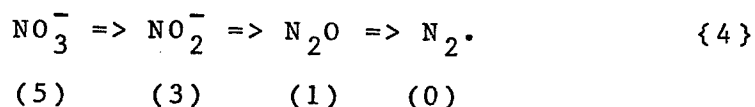
However, one species of Alcaligenes will reduce nitrite, but not nitrate, and some species or strains of Corynebacterium and Pseudomonas produce nitrous oxide as the terminal product (Payne 1976). Nitrous oxide is not normally detected during reduction of nitrite to nitrogen by <u>Pseudomonas</u> <u>aeru</u><u>ginosa</u>, but St. John and Hollocher (1977), using N-15 nitrite and an atmosphere of nitrous oxide, trapped tagged nitrous oxide and proved that it was a free, obligatory precursor of nitrogen in denitrification with this species.

Early studies investigating the sequence of denitrification and the gases produced in incubated soil amended with nitrate indicated that two pathways were operating. Nitric

oxide was the initial gaseous product from acid soil (Wijler and Delwiche 1954; Cady and Bartholomew 1960). It did not accumulate and was further reduced to nitrous oxide and nitrogen. Cooper and Smith (1963), working with seven near-neutral soils, found nitrite to be the first product with nitrous oxide and then nitrogen as subsequent gaseous products. No nitric oxide was detected. Broadbent and Clark (1965) concluded that since nitric oxide was only produced from acid soil, it was a product of the chemical decomposition of nitrite. However, nitrite production and the subsequent reduction of nitric oxide and nitrous oxide to nitrogen were biological. This was supported by the work of Bollag et al. (1973). Addition of nitrite resulted in nitric oxide production from both sterile and non-sterile acid soil [pH 5.0] and, to a much lesser extent, from sterile neutral soil. However, nitrous oxide and then nitrogen were produced from non-sterile neutral soil.

Most denitrifiers are heterotrophs, but some use energy sources other than carbon. The autotroph Thiobacillus denitrificans oxidizes sulphur compounds and Paracoccus denitrificans oxidizes hydrogen or organic compounds (Alexander 1977). Both of these bacteria utilize carbon dioxide as a source of carbon (Delwiche and Bryan 1976). Additions of sulfur to soil columns significantly increased denitrification and numbers of T. denitrificans (Mann et al. 1972).

Although the production of nitrogenous gases was first reported in 1886, it was not until 1946, when nitrous oxide was found in the atmosphere near the earth's surface, that large losses of nitrogen from agricultural soils were attributed to denitrification (Broadbent and Clark 1965). For this reason, much of the pioneering work on denitrification was done with soil samples. Early work with samples from aquatic systems indicated the occurrence of denitrification, but with nitrogen as the sole gaseous product (Goering and Dugdale 1966). Similar results were reported for lake 227 in the Experimental Lakes Area [ELA] in northwestern Ontario, although nitrous oxide was occasionally detected as a denitrification product (Chan 1977; Chan and Campbell 1975). The sequence of denitrification, equation {4} above, occurred in incubated sediment samples (Chen et al. 1972b; van Kessel 1978b). Nitrogen was produced at a much faster rate in lake water incubated over sediment (Goering and Dugdale 1966).

In eutrophic [nutrient-rich] lake water, numbers of denitrifying bacteria varied between 100 and 10,000 per ml, depending on the season and depth of sampling (Kuznetsov 1970). Numbers were reduced tenfold in oligotrophic [nutrient-poor] waters. During summer stagnation, denitrifiers were higher in the anoxic hypolimnion [bottom waters] than in the more aerobic epilimnion [surface waters]. Denitrifier density was high in eutrophic lake sediment with

counts of three million per gram, but was reduced to 10,000 per gram in oligotrophic sediment (Kuznetsov 1970). Chan (1977) concluded that the littoral sediment zone was a major site of denitrification in a eutrophic lake. Preliminary observations indicated that assimilatory and dissimilatory nitrate reductase levels were high in shallow reaches of a small lake receiving sewage effluent (Hall et al. 1978).

The main goal of sewage treatment is to lower the biological oxygen demand [BOD] of wastes by the microbial stabilization of readily available organic-C (Taber 1976). Modern activated sludge units combine nitrification and denitrification to reduce the high nitrogen content of domestic waste (Francis and Callahan 1975; Johnson 1968; McCarty and Haug 1971). Ammonia released by decomposition of urea and other organic-N is nitrified to nitrate in a stirred, heated reactor where optimum aeration and temperature prevail. In a consecutive anaerobic reactor, the produced nitrate is denitrified. In the treatment of wastewater containing 27 ppm total-N, the aerobic digestor removed 20% of the nitrogen, while the anaerobic unit removed 54 to 64% of the remainder (Francis and Callahan 1975). In total, 60 to 80% of the original nitrogen was removed.

Non-stirred, facultative sewage lagoons may be used for secondary or tertiary anaerobic waste stabilization. McCarty and Haug (1971) reported that an uncovered pond was unsatisfactory for efficient nitrate removal by denitrifica-

tion since algal growth produced oxygen, but a similar pond covered with large styrofoam blocks removed up to 90% of the initial 20 ppm nitrate-N in 10 days at 20C. They did not report whether or not harvesting of the algae increased the efficiency of nitrogen removal from the effluent.

Although studies on the gaseous products of denitrification in sewage treatment are scarce, it was found that nitrogen, but no nitrous oxide, was produced from laboratory activated sludge reactors (Johnson and Schroepfer 1964).

Work with pure cultures and cell-free extracts indicated that more than one enzyme was involved in denitrification. A resting-cell suspension of a denitrifier reduced nitrite to nitrous oxide, then nitrogen (Matsubara and Mori 1969). However, cell-free extracts reduced nitrite to both nitric and nitrous oxides, and nitric oxide to nitrous oxide alone (Matsubara and Iwasaki 1971). Separate fractions, each responsible for the reduction of nitrite, nitric oxide, and nitrous oxide, were isolated from crude extracts of the denitrifier _Pseudomonas perfectomarinus_ (Cox and Payne 1973; Payne _et al_. 1971). Payne (1973) proposed that the denitrification sequence was:

$$NO_3^- => NO_2^- => NO => N_2O => N_2. \quad \{5\}$$
$$(5) \quad\quad (3) \quad\quad (2) \quad\quad (1) \quad\quad (0)$$
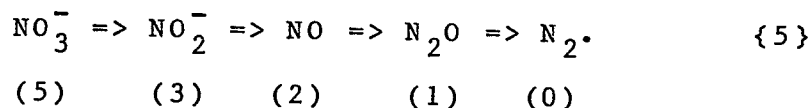
However, although intact cells and cell-free extracts of a denitrifier reduced added nitric oxide to nitrous oxide,

other results indicated that nitric oxide reductase had a high affinity for nitric oxide, which was not released to the gas phase, but immediately further reduced on the enzyme surface (Miyata et al. 1969). However, St. John and Hollocher (1977) failed to trap tagged nitric oxide in an atmosphere of unlabeled nitric oxide over a denitrifying culture of Pseudomonas aeruginosa, which was supplied with N-15 labeled nitrite. They concluded that nitrite and nitric oxide were reduced by separate pathways during denitrification in this species.

Nitrate reductase isolated from Pseudomonas aeruginosa contained molybdenum (Fewson and Nicholas 1961). Iron and molybdenum were involved in Paracoccus denitrificans dissimilatory nitrate reductase (Forget and Dervartanian 1972). Purified nitrite reductase probably contained copper (Walker and Nicholas 1961). A copper-containing enzyme catalyzed nitrous oxide formation from nitrite by a denitrifier (Suzuki and Iwasaki 1962).

2.2   FACTORS AFFECTING BIOLOGICAL DENITRIFICATION

The microbial process of denitrification, like other biological processes, is a function of environmental factors. These factors affect the growth and metabolism of both the denitrifiers and the general microbial population. Each exerts its own influence, but response to one factor may vary depending on the amount of stress applied by the others.

2.2.1   Oxygen concentration

Since denitrification is a respiratory function, the concentration of oxygen in the system is very important. Any condition which decreases the oxygen concentration or restricts oxygen supply promotes denitrification.

Denitrification has been reported in soil samples incubated under an air atmosphere (Broadbent and Stojanovic 1952). However, Bremner and Shaw (1958b) found no nitrate loss under similar aerobic conditions. Denitrification occurred when the oxygen level was less than 7%, by volume, in the atmosphere over soil amended with high levels of alfalfa (Cady and Bartholomew 1961). High biological activity created a demand for oxygen. An inverse relationship between denitrification and partial pressure of oxygen has been reported (Allison et al. 1960; Broadbent and Stojanovic 1952; Cady and Bartholomew 1961; Wijler and Delwiche 1954). With an aqueous medium, the dissolved oxygen concentration is more important than the atmospheric content (Focht and Chang 1975). Skerman and MacRae (1957) determined the threshold dissolved oxygen concentration, above which denitrification ceased in pure culture of Pseudomonas aeruginosa, to be 0.2 ppm.

Denitrification in soil increases with moisture content to above field capacity (Bailey and Beauchamp 1973b; Bremner and Shaw 1958a, 1958b; Mahendrappa and Smith 1967; Wijler

and Delwiche 1954). Pilot and Patrick (1972) determined critical moisture tensions above which increased air-filled porosity and aeration rapidly decreased denitrification. As soil texture became finer, the critical moisture tension increased since small water-filled pores and increased tortuosity restricted oxygen diffusion. The oxygen diffusion rate [ODR] is directly dependent on the temperature, the cross-sectional area of the pore space, and the concentration gradient and is slower in water than in air (Taylor and Ashcroft 1972).

Maximum denitrification occurred in fully saturated or flooded conditions (Bailey and Beauchamp 1973b; Bremner and Shaw 1958b; Cho and Sakdinan 1978). This could occur in soil after a heavy rain, irrigation, or during spring thaw [temperature permitting]. In a field study with a clay loam, the highest nitrous oxide concentrations occurred when the soil was waterlogged and oxygen levels were low (Dowdell and Smith 1974). Oxygen consumption during incubation of soil was greatly reduced by a 2 cm layer of stagnant water (Khdyer 1978). In a similar study, 5 mm of quiescent water above the soil surface restricted oxygen consumption to 40 percent of the original partial pressure while all of the added nitrate was reduced (Cho and Sakdinan 1978). Slight reduction in denitrification rate in flooded ditch sediment occurred when the dissolved oxygen concentration of the overlying water was raised from 0 to 2 ppm, but further increases, up to 9 ppm, had no effect (van Kessel 1977a).

Similar findings were reported for lake sediment (Terry and Nelson 1975) and flooded swamp and marsh soils (Engler et al. 1976).

2.2.2   pH

Acid conditions reduce the metabolic activity and growth of most microorganisms (Bollag et al. 1970; Paul and Victoria 1978). Variation in pH range and optimum was found with different denitrifying species isolated from soil and data suggested a greater selective effect of pH upon their abundance than that of the general microbial population (Valera and Alexander 1961).

Most studies with mixed soil populations have found an increase in denitrification with an increase in pH from acid conditions, and the optimum in neutral to slightly alkaline reaction (Bollag et al. 1970; Bollag et al. 1973; Bremner and Shaw 1958b; Cady and Bartholomew 1960; Cooper and Smith 1963; Khdyer 1978). Wijler and Delwiche (1954) found denitrification rates increased with pH up to pH 6.0, after which they became constant, in a soil not limited by organic matter. The rate of nitrate loss in calcareous lake sediment was much faster than in noncalcareous sediment (Chen et al. 1972a). On the other hand, in a study of virgin and cultivated Alberta soils amended with glucose and nitrate, no correlation between pH and denitrification rate was observed (Khan and Moore 1968).

In general, raising the pH of an acid soil by "liming"—with calcium carbonate, limestone, quicklime or slaked lime (Atkinson 1964)—increases the denitrification rate (Bremner and Shaw 1958b; Bollag et al. 1973; Cady and Bartholomew 1960; Cho and Sakdinan 1978; Khdyer 1978). Cho and Sakdinan (1978) noticed a slight increase in oxygen consumption by the general microbial population in limed, flooded soils. However, rates of nitrate loss were similar when a soil, initially at pH 5.9, was limed to pH 7.2 (Hauck and Melsted 1956).

A pH range for denitrification is difficult to assign (Alexander 1977). Denitrifiers were found in acid environments [pH 4.0], but more were found in soils above pH 6.0 and numbers were positively correlated with pH (Valera and Alexander 1961). Denitrification was rapid in soils with pH 8.0-8.6 (Bremner and Shaw 1958b; Wijler and Delwiche 1954). Gas evolution ceased above pH 9.0 with four out of five isolated denitrifiers (Valera and Alexander 1961). In acid conditions, no significant denitrification or growth occured below pH 6.0 with four isolates (Bollag et al. 1970). Denitrification losses were not significant below pH 5.0 (Bremner and Shaw 1958b; Valera and Alexander 1961). However, losses of nitrate up to 33 µg N/g soil-day occurred in incubated Keld soil at pH 5.4 and 35C (Khdyer 1978). Loss of nitrate by denitrification was measured in reservoir sediments at pH 5.6 (Terry and Nelson 1975). Biological

nitrate reduction occurred in flooded acid subsoils [pH 4.5] and topsoils [pH 5.0-6.0] (Gilliam and Gambrell 1978).

The pH of the environment also affects the production and further reduction, and therefore the accumulation, of denitrification intermediates. Little nitrite was detected in saturated acid subsoils during nitrate reduction (Gilliam and Gambrell 1978). Addition of lime resulted in nitrite accumulation three times greater than in acid soil (Cady and Bartholomew 1960). In naturally alkaline soils, nitrate reduction was rapid, but nitrite accumulated due to a lag in further reduction (Cooper and Smith 1963). However, accumulation of nitrite was less in limed Wellwood soil [pH 7.1] at field capacity, and was greater in the limed, flooded condition than in the respective unlimed treatments (Cho and Sakdinan 1978). Nitrite reduction increased with a pH increase from neutrality to pH 8.3 (Bollag et al. 1973). Nitrite accumulation varied in relation to pH with four isolates (Bollag et al. 1970). The maximum occurred at pH 6.0 with one [although pH 7.0 was optimal for growth and nitrate reduction] and above neutrality for the others.

Many researchers reported maximum nitrous oxide accumulation in acid conditions, below pH 6.0-7.0, and decreased accumulation as pH increased, which resulted in the predominance of molecular nitrogen (Bollag et al. 1973; Cho and Sakdinan 1978; Hauck and Melsted 1956; Khdyer 1978; Wijler

and Delwiche 1954). Nitrogen was the only nitrogenous gas formed during incubation of three flooded, slightly alkaline soils (Cho et al. 1979). During incubation of three flooded Manitoba soils of different natural pH, nitrogen was the major product from nitrate added to Morton soil [pH 7.8], while Wellwood [pH 6.3] and Keld [pH 5.4] soils produced nitrous oxide and then nitrogen (Khdyer 1978). Although the sequence of gas production was similar, nitrous oxide accumulated to a greater extent with the Keld soil. The accumulation of both nitrite and nitrous oxide was similar with respect to magnitude for different soils at the same natural pH, but the residence times varied with soil (Cooper and Smith 1963). Addition of calcium carbonate and 100 ppm nitrate-N to Wellwood soil at field capacity rendered nitrous oxide undetectable, whereas it attained a maximum of 40 µg N/g soil and persisted for 10 days in the unlimed treatment (Cho and Sakdinan 1978). However, liming of flooded Wellwood soil decreased nitrous oxide accumulation, but did not eliminate the gas. Similar results were reported for flooded Keld soil (Khdyer 1978). This cannot be explained by an increase in solubility of nitrous oxide in water since it decreases with an increase in pH (Focht and Chang 1975).

2.2.3 Temperature

Many workers have reported the temperature dependence of denitrification in soil (Bailey 1976; Bailey and Beauchamp 1973a; Bremner and Shaw 1958b; Cho et al. 1979; Cooper and Smith 1963; Gilliam and Gambrell 1978; Stanford et al. 1975a), pure cultures (Bollag et al. 1970), mixed cultures (Dawson and Murphy 1972), and sediments (van Kessel 1977a; Sain et al. 1977; Terry and Nelson 1975) within the biological temperature range. However, there is some disagreement about the precise range and optimum temperature. Both psychrophilic and thermophilic denitrifying bacteria, which have different optimum temperatures, are known (Delwiche and Bryan 1976). Little denitrification and poor growth at 10C, but optimum denitrification and growth at 30C, were reported for soil isolates (Bollag et al. 1970). Nitrate reduction was negligible at 2C and low at 10C, but rapidly increased up to 25C, and then was not significantly affected by further temperature increases (Bremner and Shaw 1958b). The latter authors reported a high optimum of 60C, but this was probably due to thermophilic Bacillus sp. (Focht and Chang 1975). According to Alexander (1977), the optimum temperature for denitrification is from 25 to 30C.

Low temperature denitrification is important in the temperate zone because nitrate can be lost during the colder part of the year (Alexander 1977; Cho et al. 1979; Cranston and Adams 1978; Dawson and Murphy 1972; Gilliam and Gambrell

1978). Reduction of added nitrate or nitrite and nitrogenous gas production in saturated soil ceased at 5C (Bailey 1976; Bailey and Beauchamp 1973a). However, denitrification was observed in the same soil at 5C when carbon was not limiting (Smid and Beauchamp 1976). Gilliam and Gambrell (1978) measured slow nitrate reduction at 5C in flooded acid soils which did not have added carbon. Although it had only one fifth the rate at 20C, denitrification at 5C by a mixed culture, dominated by P. denitrificans, was significant (Dawson and Murphy 1972). Denitrification occurred in lake water and sediments at 5C (Goering and Dugdale 1966). The rate of denitrification in lake and reservoir sediment increased with a rise in temperature from 5 to 23C and large losses of nitrate occurred at 10 to 15C (Terry and Nelson 1975). After a lag period, nitrate and nitrite were reduced at 4C in flooded ditch sediment (van Kessel 1977a). At 0C, denitrification was almost undetectable, but nitrate reduction was measured in the range 2 to 5C only after a long incubation (Stanford et al. 1975b). Three out of four isolates sampled from a river below a sewage lagoon effluent outfall during December and January were psychrophilic and grew at 0 and 4C (Cranston and Adams 1978). Extrapolation of rate versus temperature curves for denitrification, in six flooded alkaline subsoils over the temperature range 2 to 20C, gave a threshold temperature of 2.7C (Cho et al. 1979). In a similar manner, but with one

soil pre-incubated at various temperatures before nitrate addition, the threshold value was proposed to be near 0C (Smid and Beauchamp 1976).

The ratio of nitrous oxide to nitrogen in the evolved gas mixture was found to increase with decreasing temperature (Broadbent and Clark 1965; Martin and Focht 1977). In Keld soil [pH 5.4] at 15C, nitrous oxide was the only gaseous product after two weeks of incubation, but nitrogen was subsequently produced at 25 and 35C (Khdyer 1978). The graphs presented by Cooper and Smith (1963) and Khdyer (1978) illustrate the effect of temperature on sequential denitrification. With an increase in temperature, all rates of reduction and production in the sequence increased, products appeared earlier and accumulated after less lag. The residence time and time of maximum accumulation of the intermediate products decreased with an increase in temperature, but the magnitude of their maximum accumulation varied little with temperature.

The influence of temperature on the rate of a chemical reaction is exponential and is expressed by Arrhenius' equa-

tion (Barrow 1973; Dawson and Murphy 1972):

$$k = Ae^{-E/RT} \qquad \{6\}$$

where k = reaction rate,

    A = frequency factor,

    E = activation energy [cal/mol],

    R = universal gas constant [cal/mol-deg K],

    T = absolute temperature [deg K].

The plot of ln k versus 1/T generally yields a linear curve.

The rates of nitrogen production by flooded Morton soil over the the temperature range 15 to 35C obeyed Arrhenius' equation {6} (Khdyer 1978). A similar result was reported for nitrate reduction in mixed culture over the range 3 to 27C (Dawson and Murphy 1972). However, according to Focht and Chang (1975), the data of Bailey and Beauchamp (1973a) and Bremner and Shaw (1958b) have a sharp break in the curve below 10 to 15C. They proposed that this was due to temperature effects on solubility or diffusion, or to a differential influence of temperature on one of the sequential reactions. Cho et al. (1979) stated that observed deviation from Arrhenius' relation at low temperature was because biological activity ceases near 0C [273K], not at 0K. Another relationship expressing the dependency of the rate of bio-

logical reactions on temperature is (Focht and Chang 1975):

$$Q10 = K'/K'' \qquad \{7\}$$

where K' = rate at temperature, t = tC,

K'' = rate at temperature, t = (t-10)C.

Biological processes usually follow van't Hoff's temperature rule and double to triple their reaction rate for every 10C rise in temperature (Stevenson 1965). In the range 15 to 35C, Q10 for denitrification in soil was found to be approximately two (Cooper and Smith 1963; Khdyer 1978; Stanford et al. 1975a). Focht and Chang (1975) calculated Q10's from the data of various authors and most fell in the vicinity of two to three. However, the lower limit of the temperature range conforming to a Q10 of two was estimated to be 11C in waterlogged soils, and below this temperature, the denitrification rate declined rapidly, decreasing tenfold at 5C (Stanford et al. 1975a). On the other hand, a linear relationship existed for nitrate or nitrite reduction rates and temperature over the range 4 to 25C in flooded sediment (van Kessel 1977a). Nitrogen production rates for six flooded soils were found to be directly related to temperature over the range 2 to 20C (Cho et al. 1979).

2.2.4 Organic matter

The organic matter content of the environment affects denitrification in three ways. Organic matter serves as a reductant and donates electrons in both aerobic and anaero-

bic microbial respiration. It is also used in cell synthe-
sis during growth and maintenance. The third effect is not
as direct, but deals with the biological oxygen demand
[BOD]. A microbial population rapidly decomposing organic
matter has a high BOD and creates anaerobic conditions con-
ducive to denitrification.

Addition of plant material such as rice or wheat straw,
and pure compounds such as glucose, is known to increase
denitrification in waterlogged systems (Bowman and Focht
1974; Bremner and Shaw 1958a, 1958b; Burford and Bremner
1975; Engler and Patrick 1974; Gilliam and Gambrell 1978;
Smid and Beauchamp 1976; Terry and Nelson 1975; Wijler and
Delwiche 1954). Bremner and Shaw (1958a, 1958b) extensively
studied the effectiveness of organic nutrients in promoting
denitrification. The rate increased up to a maximum with
straw or glucose addition, then levelled off or decreased.
The type of added organic-C also affected denitrification.
The soluble compounds, citrate, glucose, mannitol, and suc-
rose, were of equal effectiveness. The order with insoluble
carbonaceous material was cellulose, straw, grass, sawdust,
and lignin. Although cellulose addition resulted in more
nitrate reduction, the initial lag in denitrification was
greater. Much more straw was required to give the same
increase in denitrification rate as a small amount of glu-
cose. Additions of fen soil, mountain or low-moor peat had
little effect on denitrification. Fresh straw was more sti-

mulatory than either water-extracted or composted wheat straw. Twice as much water-extracted straw as fresh straw gave the same increase in denitrification. The effect of composted straw addition on denitrification rates decreased with days of composting.

Little or no denitrification occurred in soils with an endogenous organic matter content less than 1% (Bremner and Shaw 1958a). In six flooded soils, nitrate disappeared most rapidly in the two soils containing the highest organic matter (MacRae et al. 1968). Sediment from a ditch, which received sewage treatment effluent, evolved nitrogen and carbon dioxide faster than field-drainage ditch sediment that was lower in organic matter (van Kessel 1977a, 1978b).

Water-soluble organic-C is readily metabolized by soil microorganisms. Rates of nitrate loss from 30 diverse soils correlated better with a soluble fraction of soil-C [r=0.88] than with total soil-C [r=0.83] (Stanford et al. 1975b). Similarly, rates of nitrous oxide and nitrogen evolution from 17 soils were very highly correlated [r=0.99] with water-soluble organic-C or mineralizable-C, and significantly correlated [r=0.77] with total organic-C (Burford and Bremner 1975). After 230 days of incubation, denitrification in ditch sediment ceased, but 53% of the initial organic matter remained and probably consisted of resistant humic substances (van Kessel 1978a). Pre-incubation of soil

at 30C, followed by incubation at various temperatures, resulted in less nitrate disappearance than with soils pre-incubated at lower temperatures (Smid and Beauchamp 1976). Optimal microbial activity in the pretreatment depleted readily available-C. Addition of 5,000 ppm [solid basis] glucose-C to lake sediments increased denitrification by 25 to 30% and it was concluded that native, available organic-C was enough for near maximum nitrate reduction (Terry and Nelson 1975).

With a large addition of alfalfa to waterlogged soil, production of nitrous oxide increased and the magnitude of maximum accumulation was greater (Wijler and Delwiche 1954). However, further reduction to nitrogen was also promoted. Addition of straw instead of alfalfa resulted in less denitrification, but the proportions of nitrous oxide to nitrogen varied little. Nitrous oxide accumulated less, but persisted twice as long in sediment with a lower organic matter content (van Kessel 1978b). The ratio of nitrous oxide to nitrogen was not well correlated with soil mineralizable-C or water-soluble organic-C (Burford and Bremner 1975).

The optimum C/N ratio for denitrification was 2:1 to 3:1 for glucose addition to soil, and 30:1 to 65:1 for straw addition (Bremner and Shaw 1958a). In sewage treatment, mg BOD/mg (nitrite-N + nitrate-N) of 2.3:1 ensured 100% denitrification, whether carbon was added as methanol, acetate, or chemically-treated raw sewage (Narkis et al. 1979).

2.2.5 Nitrate concentration

Conflicting reports concerning the effect of initial nitrate concentration on the rate of denitrification have been documented. The controversy is over which kinetic rate equation best fits nitrate reduction. A zero-order reaction rate is not dependent on the initial nitrate concentration, while that of a first-order reaction is. The zero-order disappearance of nitrate-N is:

$$\frac{-dN}{dt} = k \qquad \{8\}$$

which, upon separation and integration, yields:

$$N = N_0 - kt. \qquad \{9\}$$

The first-order disappearance of nitrate-N is:

$$\frac{-dN}{dt} = kN \qquad \{10\}$$

which, upon separation and integration, yields:

$$\ln N = \ln N_0 - kt \qquad \{11\}$$

where N = nitrate-N concentration at time $t=t$,

N_0 = nitrate-N concentration at time $t=0$,

k = reaction rate,

t = time (Daniels and Alberty 1975).

For zero-order reactions $\{9\}$, a plot of concentration versus time is linear and k has units of concentration per unit time. For first-order reactions $\{11\}$, a plot of ln concentration versus time yields a linear curve and k has units of reciprocal time. The half-life of a chemical reaction is

defined as the time required for half of the initial amount
of reactant to disappear. It is constant for a first-order
reaction and is expressed as:

$$t_{1/2} = \frac{0.693}{k} . \qquad \{12\}$$

Since the reactant disappears at a constant rate in a zero-
order reaction, the half-life of the reaction is concentra-
tion dependent and is expressed as:

$$t_{1/2} = \frac{N_0}{2k} . \qquad \{13\}$$

The rate of an enzyme-catalyzed reaction, when the sub-
strate is saturating, depends directly on the amount of
enzyme present (Conn and Stumpf 1967). However, with a
fixed enzyme concentration, an increase in substrate results
in an increase in reaction rate [Appendix A]. Further
increases in substrate concentration result in progressively
smaller rate increases until a maximum constant rate is
attained at high substrate concentration. At low substrate
concentrations, the reaction follows apparent first-order
kinetics, while at high substrate concentrations it seems to
follow zero-order kinetics. An enzyme-catalyzed reaction is

expressed by Michaelis-Menten kinetics as:

$$v = \frac{V_{max} S}{K_m + S} \qquad \{14\}$$

where v = denitrification rate,

V_{max} = maximum rate at saturating substrate

concentration,

S = substrate concentration,

K_m = Michaelis constant.

Bremner and Shaw (1958a) concluded that the percentage of added nitrate-N lost by incubation of saturated soil with different amounts of nitrate-N and sufficient glucose-C, was independent of the level of nitrate-N applied. This conclusion was interpreted as zero-order kinetics (Bowman and Focht 1974; Focht and Chang 1975; Phillips et al. 1978). However, if the percentage lost remains constant, the reduction is first-order. In flooded lake sediment, the rate of nitrate disappearance was a function of the initial amount of nitrate when 16 and 200 ppm nitrate-N were added (Chen et al. 1972b). However, nitrogen production rates were similar when 500 and 1000 ppm nitrate-N were added.

The rate of nitrogenous gas production was not affected by increases from 48 to 270 ppm nitrate-N [solid basis] added to alfalfa-amended soil at 20% moisture (Wijler and Delwiche 1954). Variation of initial nitrate-N concentration from 37.5 to 150 ppm in soil at field capacity with 1%

alfalfa, resulted in no change in rate of nitrate reduction (Cooper and Smith 1963). Similar findings resulted from additions of 50 to 200 ppm nitrate-N to soil at 30 and 67% [flooded] moisture (Cho and Sakdinan 1978). The initial rate of nitrate disappearance was independent of added nitrate-N concentration, over the range 100 to 200 ppm, with three flooded Manitoba soils of varying pH, organic matter content, and texture (Khdyer 1978). Nitrate reduction was zero-order with additions of 180 to 1,940 ppm nitrate-N [solid basis] to lake sediment (Terry and Nelson 1975).

Work with low concentrations [0.05 to 0.8 ppm] of nitrate-N added to water systems, indicated that denitrification rate increased initially, then increased progressively less and reached a maximum as nitrate-N was increased past 0.2 to 0.4 ppm (Chan 1977; Chan and Campbell 1975; Koike et al. 1972). A Lineweaver-Burk plot of reciprocal denitrification rates in marine sediment, versus reciprocal initial nitrate-N concentration, over the range 0.7 to 7 ppm, resulted in a linear curve, which is typical of Michaelis-Menten enzyme kinetics (Oren and Blackburn 1979). Denitrification rates in ditch sediment were dependent on nitrate-N concentration at low levels [5-100 ppm], but were independent at high levels [500 ppm] (van Kessel 1977a). Although rate versus nitrate-N concentration curves for two sediments approximated Michaelis-Menten kinetics, the maximum rate was much higher in the sediment containing twice as

much organic matter. At a constant high concentration of glucose-C [6,000 ppm], the rate of nitrate loss varied with initial nitrate-N concentration [50-750 ppm], then reached a maximum with further increases [1,000-3,000 ppm] in a fine sand at 100% moisture (Bowman and Focht 1974). These authors concluded that results of other workers were extremes of a Michaelis-Menten nitrate reduction curve. Futhermore, since a similar curve was obtained with varying glucose-C concentration at constant initial nitrate-N concentration, they proposed that denitrification followed dual substrate kinetics described by:

$$v = \frac{V_{max} CN}{(C + K_C)(N + K_N)} \qquad \{15\}$$

where v = denitrification rate,

V_{max} = maximum rate at saturating substrate concentration,

C = available carbon concentration,

N = nitrate-N concentration,

K_C = Michaelis constant for carbon,

K_N = Michaelis constant for nitrate-N.

If the carbon concentration is limiting, then $C/(C + K_C)$ does not approach unity and the rate is a function of both available carbon and nitrate-N concentration.

Variation in initial nitrate concentration of soil at field capacity affected the production, reduction, and accu-

mulation of denitrification intermediates, although the rate of nitrate disappearance was zero-order (Cho and Sakdinan 1978; Cooper and Smith 1963; Wijler and Delwiche 1954). Increased initial nitrate concentration increased the magnitude and time of maximum nitrite accumulation and nitrite persisted longer, but rates of nitrite production and reduction were zero-order (Cooper and Smith 1963). The magnitude and time of nitrous oxide maximum accumulation were dependent on initial nitrate level (Cho and Sakdinan 1978; Cooper and Smith 1963; Wijler and Delwiche 1954). The rates of nitrous oxide formation and disappearance were similar with 75 and 150 ppm nitrate-N additions, but were less at the 37.5 ppm level (Cooper and Smith 1963). The rate of nitrous oxide formation was independent of initial nitrate-N over the range 50 to 200 ppm, but the lengths of lag before nitrous oxide and nitrogen appearance were dependent on it (Cho and Sakdinan 1978). This dependence of lag in first appearance, especially at higher initial nitrate-N, was depicted in the graphs of Cooper and Smith (1963) too. Their data also indicated that the initial rate of molecular nitrogen formation was dependent on nitrate concentration. Nitrogen was the only gaseous product from denitrification in flooded Morton soil and its rate of production was independent of added nitrate concentration (Khdyer 1978). The lag period before nitrogen appearance was also independent of nitrate concentration. However, in two flooded soils,

which produced nitrous oxide as an intermediate, the lag period before nitrogen production increased with an increase in nitrate concentration. The initial nitrogen production rate decreased with increased nitrate concentration in a flooded soil which produced nitrous oxide as the first gaseous nitrogen product (Cho and Sakdinan 1978).

In 1956, Nommik observed that nitrous oxide was not further reduced until most of the added nitrate had been reduced (Broadbent and Clark 1965). Similar results were reported for denitrification in a flooded soil (Cho and Sakdinan 1978). Blackmer and Bremner (1978) concluded that the dependence of maximum nitrous oxide accumulation and persistence on initial nitrate was due to suppression of enzymatic reduction of nitrous oxide at low nitrate levels and inhibition at high levels. However, results from soil at field capacity showed further reduction of nitrous oxide in the presence of twice as much nitrate-N as nitrous oxide-N (Cho and Sakdinan 1978). Similar results were reported for three flooded soils at varying temperature and initial nitrate concentration (Khdyer 1978).

Cho and Sakdinan (1978) proposed that there was competition between nitrate and nitrous oxide as the electron acceptor in denitrification. A constant demand for electron acceptors, generated by a constant supply of available carbon and constant microbial respiration, was satisfied by any

one of nitrate, nitrite, or nitrous oxide. In their study, nitrite did not accumulate and was of minor importance. Nitrate was the major acceptor directly after addition, but became of less importance with time as nitrite, then nitrous oxide pools increased in size. A greater initial concentration of nitrate reduced the chance of nitrous oxide reduction to nitrogen. However, as the nitrate pool was reduced, there was more probability of nitrous oxide serving as an electron acceptor.

This hypothesis was subsequently mathematically formulated using competitive Michaelis-Menten kinetics [Appendix B] to describe the disappearance of nitrate, the formation and disappearance of denitrification intermediates, and the production of nitrogen (Cho and Mills 1979). The produced curves simulated the characteristics of experimental results reported by Cooper and Smith (1963) and Cho and Sakdinan (1978).

2.3 PROPERTIES OF THE WATER-SEDIMENT SYSTEM

When soil or sediment is flooded with water in a test tube or beaker, certain biological and physical processes result in visible chemical changes. These changes are qualitatively apparent in the colour of the sediment (Reddy et al. 1976; Russell 1973; Ponnamperuma 1972; van Kessel 1978a, 1978b). Most of the sediment is a dark gray or greenish-gray colour. At the sediment-water interface, there is usually a thin, light-coloured, reddish-brown zone

which may range in thickness from a few mm to 2-3 cm
(Patrick and Mahaptra 1968). The boundary between the two
zones may be sharply delimited by a very thin, reddish-brown
layer. In systems low in organic matter and open to the
atmosphere, the depth of the upper zone may increase with
time. The addition of readily-available organic matter usu-
ally decreases the depth of the light-coloured upper zone.

If the overlying floodwater contains oxygen, then the
surface sediment is supplied with oxygen by molecular diffu-
sion through the interstitial water (Ponnamperuma 1972).
The ODR is 10,000 times slower in water than in air and
decreases with decreasing temperature and concentration gra-
dient (Ponnamperuma 1972; Taylor and Ashcroft 1972). It is
dependent on the diffusion coefficient which is a character-
istic of the medium and the diffusing material (Bouldin
1968). The rate of oxygen supply is also influenced by the
rate of water percolation through the sediment (Patrick and
Mahaptra 1968). However, in most shallow water bodies, the
percolation rate is very slow (Patrick and Reddy 1976).
Disturbances, caused by the movement of aquatic organisms
and the release of gas bubbles from the sediment, influence
the distribution of oxygen in the surface sediment in a
minor way (van Kessel 1977a). In shallow waters, mixing
caused by wind action and by convection due to density gra-
dients is important in increasing oxygen solubility, and in
creating a more uniform oxygen concentration throughout the

water column (Bouldin 1968). During thermal stratification in summer, the hypolimnion of deep waters is usually oxygen deficient and the sediment is anaerobic to the surface (Bouldin 1968; Hargrave 1972). However, during spring and fall turnovers, when temperature-induced density changes cause water circulation, only the surface 1 cm of profundal sediment is oxygenated (Hargrave 1972).

The thickness of the oxidized zone is determined by the difference between the oxygen supply and consumption rates (Patrick and Mahaptra 1968). The rate of oxygen diffusion from the floodwater is usually much slower than its rate of reduction in the sediment. Where the demand for oxygen is greater than the supply, anaerobic conditions prevail. This demand is created by aerobic microbial respiration in the sediment and, to a much lesser extent, in the overlying water. Aerobes consume the oxygen diffusing through the surface layer and create an anaerobic environment in the underlying layers. There is a change in the microbial population with depth (Ponnamperuma 1972). Aerobes dominate the oxygenated surface layer, but as anoxia increases the proportion of facultative anaerobes increases. True or obligate anaerobes inhabit the deeper, completely anoxic sediments.

Respiration is the transfer of hydrogens [one hydrogen atom and one electron] from organic matter to a hydrogen

acceptor. The process can be viewed as a series of oxidation-reduction reactions (Yoshida 1975). The addition of readily available organic matter promotes microbial growth and provides an available source of hydrogen donors for respiration. Consequently, a greater demand for oxygen is generated and the depth of the oxidized zone decreases (Patrick and Mahaptra 1968; Engler and Patrick 1974). On the other hand, exhaustion of available hydrogen donors in the surface sediment permits the deeper penetration of oxygen to where donors are present (van Kessel 1978a). In the presence of oxygen, carbon is completely oxidized to carbon dioxide, and oxygen is reduced to water according to equation {2}. In the absence of oxygen, carbon is not immediately oxidized to carbon dioxide, but intermediates such as acetate, lactate, and butyrate are released (Bouldin 1968; Russell 1973). Oxidized ions replace oxygen as the terminal electron acceptor in microbial respiration. Nitrate, manganic, ferric, and sulfate ions occur in the light-coloured, oxidized layer, but their reduced counterparts, ammonium, manganous, ferrous, and sulfide ions respectively, predominate in the reduced, anaerobic zone. The dark gray or greenish-gray colour of the anaerobic layer results from the presence of ferrous compounds (Russell 1973).

The qualitative description of the oxidized and reduced zones in a flooded system can be quantified by measuring the redox potential. This value expresses the driving force

behind an oxidation-reduction reaction and is a measure of electron availability (Bohn 1971). Oxidizing conditions have a high redox potential, while reducing conditions have a low potential (Russell 1973). Redox potential is a function of temperature and pH (Patrick 1960). In sediments, the redox potential ranges from +700 to -300 mV (Patrick and Mahaptra 1968).

The biochemical and chemical reduction of oxidized ions during anaerobic respiration is sequential (Patrick and Deluane 1972; Patrick and Mahaptra 1968; Ponnamperuma 1972; Yoshida 1975). The most easily reduced ionic species is nitrate and it is reduced at a potential of about +200 mV (Bailey and Beauchamp 1973a). The remainder of the sequence is the manganous, ferrous, and sulfide ions. Each ion is reduced at a progressively lower redox potential and the reduction of a certain ionic species causes the redox potential to be stabilized at a new level (Engler et al. 1976). Usually one ionic species is not completely reduced before the reduction of the next in the sequence begins (Patrick and Mahaptra 1968).

Patrick and Deluane (1972) measured the vertical distribution of manganous, ferrous, and sulfide ions in a flooded soil and found a deeper apparent oxidized layer with lower redox potential. The depth of the apparent oxidized zone was a reflection of the difficulty of reduction. It was

deepest for sulfide since sulfate requires very strong reducing conditions for reduction. If ferric ions are abundant, they poise the system. (Patrick and Mahaptra 1968; Russell 1973). This means that the presence of oxidized iron prevents a rapid decline in redox potential. Iron and manganese remain in both the oxidized and reduced systems and provide a buffer against rapid changes in redox potential (Patrick and Mahaptra 1968). However, nitrate and sulfate are reduced to gases and leave the system.

Nitrate and manganic ions are readily reduced because the energy required for their reduction is low (Engler et al. 1976). The reduction of an ion poised at a lower potential releases less energy to the respiring microorganism resulting in slower growth (Yoshida 1975). The free energy change for aerobic respiration was determined to be -56.7 kcal, while for denitrification by Pseudomonas denitrificans it was -53.6 kcal, and for sulfate reduction by Desulfovibrio desulfuricans it was -9.6 kcal.

Bouldin (1968) presented five equations which modeled oxygen diffusion across the water-sediment interface. Two equations were based on steady-state models where only oxygen diffused. Three other equations described transient-state models where reductants such as ferrous and manganous ions were considered to be mobile, non-mobile, or both mobile and non-mobile. Experimental evaluation of these

models indicated that the one which included oxygen consumption by aerobic microbial respiration in the upper layer and oxidation of mobile and non-mobile reductants best described oxygen consumption in a flooded soil (Howeler and Bouldin 1971). Chemical oxidation of initially uniformly reduced, flooded soil cores accounted for about 50% of the total oxygen consumption.

Many of the changes occurring in a flooded system can be explained by diffusion of both ions and molecules. The oxidation of reduced ionic species in the upper aerobic layer creates concentration gradients and the mobile ions diffuse upwards in response to these gradients (Patrick and Deluane 1972). The sharp reddish-brown boundary between the aerobic and anaerobic zones is precipitated ferric oxide (Russell 1973). Organic intermediates and by-products of anaerobic metabolism also diffuse to the upper sediment layer where they are oxidized by the aerobic microflora (Yoshida 1975).

Ammonium was released during the decomposition of organic matter in flooded sediment (van Kessel 1977a, 1978b). Ammonification occurred in both aerobic and anaerobic environments (Bartholomew 1965). Some of the tagged nitrogen applied to a flooded soil as ammonium sulfate was recovered as nitrogen gas (Broadbent and Tusneem 1971). The vertical distribution of ammonium and nitrate in a flooded soil

amended with ammonium sulfate was investigated by Patrick
and Deluane (1972). Ammonium-N concentration increased [50
to 200 ppm] with sediment depth to about 15 to 20 mm, but
nitrate-N concentration decreased from almost 8 ppm to less
than 1 ppm over the same depth. A similar trend in nitrate
profile was reported for marine sediments and flooded soil
(Oren and Blackburn 1979; Reddy et al. 1978). It was pro-
posed that ammonium and nitrate diffused in opposite direc-
tions, up and down the soil column respectively, in response
to their concentration gradients (Patrick and Deluane 1972).
This was later verified using labeled ammonium-N (Patrick
and Reddy 1976). Ammonium diffused to the aerobic layer
where it was oxidized to nitrate by the nitrifiers, Nitroso-
monas sp. and Nitrobacter sp.—a process that requires oxy-
gen. The formed nitrate diffused to the anaerobic layer
where it was denitrified (Patrick and Tusneem 1972; Ponnam-
peruma 1972; Yoshida 1975). Nitrate, unlike ammonium, is
not strongly adsorbed to organic or clay micelles and is
relatively free to respond to a concentration gradient
(Engler et al. 1976). For this reason, the nitrification of
added ammonium resulted in comparatively low nitrate concen-
trations (Patrick and Deluane 1972; Patrick and Reddy 1976).

Little nitrogen loss occurred in flooded soils supplied
with ammonium sulfate and under an anaerobic atmosphere
(Patrick and Tusneem 1972; Patrick and Gotoh 1974).
Increases in the oxygen content of the incubation atmosphere

to 5 and 10% resulted in formation of a thin aerobic zone (Patrick and Gotoh 1974) However, the amount of nitrogen lost was substantial. Additional increases up to 20% and more [80%] increased the thickness of the aerobic layer, but did not increase the nitrogen loss appreciably. It was concluded that although low atmospheric oxygen content limited nitrification, ammonium diffusion was the major limiting factor in the nitrification-denitrification process when oxygen content was sufficient for nitrification.

Reddy et al. (1976) developed equations modeling the concentration of ammonium as a function of time and depth in a flooded soil column. Theoretical results compared well with experimental findings in the anaerobic layer, but underestimated ammonium oxidation, possibly because the thickness of the aerobic zone was assumed to be invariant with time.

Nitrate added to floodwater overlying sediment reduced to the surface, resulted in a lightening of the surface sediment colour (van Kessel 1978b). The light-coloured zone initially increased in depth to about 2 cm due to nitrate diffusion, and then decreased to 1 cm as the nitrate in the floodwater was completely depleted in 70 days. When nitrate-N in the floodwater was maintained at 25 ppm, the depth to which both oxygen and nitrate penetrated the sediment increased with time (van Kessel 1978a). Nitrate dif-

fused deeper than oxygen and the difference between the extent of oxygen and nitrate diffusion was taken as the zone of denitrification. Exhaustion of available electron donors in organic matter caused a gradual shift of both the aerobic-anaerobic boundary and the underlying denitrification zone. In a sediment containing less organic matter, the addition of nitrate resulted in the fading of initially black sediment to a depth of more than 5 cm (van Kessel 1978b). After 120 days, about half of the initial nitrate remained in the floodwater and the depth of the light-coloured layer did not decrease.

Although nitrate disappeared rapidly from floodwater incubated over soil, there was no loss of nitrate when the floodwater was incubated without soil (Engler et al. 1976). The rate of denitrification was dependent on the total sediment thickness up to 7 mm with ditch sediment high in organic matter, and up to 14 mm with sediment low in organic matter (van Kessel 1977a). Denitrification rate in stream sediment increased with sample depth up to 5 cm (Sain et al. 1977). Depths greater than 5 cm were considered to be infinite since nitrate disappearance rates in samples 5 and 10 cm deep were similar.

Studies in which nitrate was added to the floodwater without disturbing the sediment, showed nonlinear disappearance of nitrate with time (Engler and Patrick 1974; Engler

et al. 1976; van Kessel 1977a, 1977b, 1978b; Sain et al. 1977). Engler and Patrick (1974) added nitrate to floodwater over sterilized swamp soil and traced its nonlinear disappearance with time. Faster, though still nonlinear, nitrate disappearance occurred from floodwater over non-sterile soil. When nitrate was initially mixed throughout flooded soil, rather than just added to the floodwater, faster and initially more linear nitrate disappearance with time was found (Engler et al. 1976). However, data showed that as nitrate was depleted and its concentration became less uniform throughout the soil column, its disappearance with time became less linear (Engler and Patrick 1974; Engler et al. 1976; Khdyer 1978; MacRae et al. 1968; Stanford et al. 1975a, 1975b).

Addition of rice straw to flooded soil decreased the thickness of the aerobic zone and the distance over which nitrate had to diffuse to reach the site of denitrification (Engler and Patrick 1974; Reddy et al. 1978). Also, increased anaerobic microbial growth and respiration resulted in faster, more linear nitrate removal with time from the floodwater. The data of Stanford et al. (1975b) indicated that nitrate disappearance occurred to a greater extent and was more linear with time as the level of decomposable-C in the soil increased.

An increase in temperature resulted in an increased rate of nitrate depletion from floodwater over sediment (Sain et al. 1977). Although nitrate loss with time at the higher temperature was initially more linear, later it became more curvilinear than at the lower temperatures. This was due to the depletion of nitrate in the floodwater to a concentration where diffusion of nitrate limited the rate of denitrification in the sediment. A similar trend was evident in nitrate disappearance from flooded soils, especially at lower initial nitrate concentrations (Khdyer 1978).

Nitrate diffusion from the floodwater, through the oxidized sediment layer, and into the underlying anaerobic zone was found to be dependent on nitrate concentration [first-order] in the floodwater (Bouldin et al. 1974). Stanford et al. (1975b) emphasized that the denitrification rates they had determined for 30 different flooded soils were influenced by nitrate diffusion. Since the samples were not shaken during incubation, the rates resulted from a combination of nitrate diffusion and denitrification and were better described by first-order than by zero-order kinetics. When flooded soil samples were stirred during incubation, the disappearance of nitrate from the suspension was linear with time (Patrick 1960). Sain et al. (1977) realized the complications due to nitrate diffusion into stream sediment and did not attempt to determine the order of the denitrification rate, although quadratic functions

fitted their data best. They pointed out that even if denitrification was a zero-order reaction, nonlinear nitrate diffusion in the flooded condition would result in apparent first-order kinetics. Similar diffusion-limited, first-order denitrification rates resulted from the anaerobic incubation of flooded soil with a 0.5% rice straw amendment (Reddy et al. 1978). There was little difference in denitrification rate between 3 and 6 cm depths of floodwater with the same soil. However, when the soil was saturated with no floodwater standing above it, nitrate disappearance was faster and more linear with time, since the effects of nitrate diffusion were minimized. However, in one soil with no organic matter amendment, apparent first-order nitrate disappearance occurred in all treatments of floodwater depth, though again nitrate depletion was faster with no excess floodwater.

Phillips et al. (1978) derived an equation modeling the concentration of nitrate as a function of soil depth and time in a flooded column of soil where denitrification was occurring. Assumptions were that denitrification was a zero-order reaction, that the depth of soil was infinite, that significant denitrification did not occur in the floodwater, that the initial concentration of nitrate was uniform throughout the floodwater and the soil solution, and that the concentration of nitrate in the floodwater remained uniform after denitrification began in the sediment. Theoreti-

cal results indicated that increases in floodwater depth up to 3 cm increased the flux of nitrate from the floodwater into the underlying soil. When there was no standing layer of water, diffusion did not influence the order of denitrification or the apparent rate constant of denitrification. However, nitrate diffusion into the soil resulted in apparent first-order rate constants when there was a floodwater layer. Theoretical results of nitrate remaining in the system under 0 and 3 cm depths of floodwater agreed well with experimental values. A little underestimation with the 3 cm depth and much more with the 6 cm depth was probably because of denitrification occurring in the floodwater.

Chapter III

MATERIALS AND METHODS

3.1   SAMPLES

3.1.1   Lake Sediment

Geographic and bathymetric characteristics of the Experimental Lakes Area [ELA] and lake 227 in particular, were described by Brunskill and Schindler (1971) and Schindler et al. (1971), respectively. Lake 227 is a small, almost circular, perched basin lake. It does not have a significant groundwater outflow, but does have a single outlet stream. There are no inlet streams and most water inflow comes from the melting of snow and heavy rains. The surface area of the lake is 5.0 hectares, the mean depth is 4.4 m, and the maximum depth is 10 m.

Due to the protected nature of its location, mixing of the shallow water by wind action does not occur. In fact, complete mixing of water during spring turnover seldom occurs. During 1969, the hypolimnion was aerated only during fall turnover (Schindler et al. 1971). For the rest of the year, the oxygen content of the water below about 6 m was less than 2 ppm. The thermocline, or region of rapid water temperature decline between the epilimnion and hypolimnion, was quite shallow and occurred at the 3-4 m depth

during summer (Schindler 1971). At the 5 m depth, the water temperature reached a maximum of 7C in 1969. The temperature at this water depth was below 4C from near the end of October to the middle of May. The surface 2 m of water reached a maximum of 22C from the middle of July to the middle of August. In deeper lakes, the temperature of water between the 16 to 20 m depths did not vary much from 4 to 5C throughout the year.

According to Chan (1977), lake 227 was oligotrophic before 1969. However, weekly additions of nitrate and phosphorus during the ice-free period for six years resulted in eutrophication of the lake. Most of the added nutrients were taken up by the phytoplankton which sank to the sediment after death (Schindler et al. 1971).

Sediment was sampled from the 7 m depth of lake 227 on May 28, 1976 and stored in the dark at 4C in two, three litre, brown glass jars which were about three quarters full. When the jars were first opened, the sediment was transferred to 455 ml mason jars which were filled to the top and also stored in the dark at 4C.

3.1.2   Sewage Sludge

Liquid sewage sludge produced by an activated sludge process at the Winnipeg North End Treatment Plant is pumped to lagoons near the intersection of McPhillips Avenue and the

Perimeter Highway [Winnipeg, Manitoba] for tertiary treat-
ment (Mills 1973). The water is pumped out of the lagoons
in the fall and the frozen sludge is bulldozed out of the
cells in the winter. In the spring, the spread sludge thaws
and dries out.

Sewage sludge samples were taken from the surface 0-15 cm
depth of the land disposal area on June 6, 1977. The sludge
was air-dried, crushed to pass through a 2 mm sieve, and
stored at room temperature.

3.1.3 Physical and Chemical Properties

Some of the physical and chemical properties of the mate-
rial studied are presented in Table 1. The pH of water-
solid mixtures similar to those used in the incubations were
determined with a Beckman pH meter and Fisher glass-body
combination electrode. Organic-C was determined by the
Walkley-Black method [potassium dichromate and concentrated
sulfuric acid] as described by Allison (1965). Kjeldahl-N
was determined by the method described by Bremner (1965).
The calcium carbonate equivalent of sewage sludge was deter-
mined by the manometric method of Skinner et al. (1959).
The addition of 30% hydrochloric acid to dried and powdered
lake sediment resulted in no effervescence.

Initial nitrate-N and nitrite-N concentrations were
determined in the following manner. A 3:5 mixture of wet

TABLE 1

Some physical and chemical properties of lake sediment and
sewage sludge

	Lake Sediment	Sewage Sludge
pH	5.6	6.9
Organic-C [%]	26.6	20.6
Kjeldahl-N [%]	1.88	1.56
$CaCO_3$ equivalent [%]	none	20.7
H_2O [%]	-	6.00
H_2O [% wet basis][a]	94.8	-
NO_3^--N [ppm]	3.25	413.0
NO_2^--N [ppm]	0.94	1.43
NH_4^+-N [ppm]	103.0	54.8
Wet bulk density [g cm^{-3}]	1.04	1.45
Density [g cm^{-3}]	1.44	2.07
Volume [cm^3 g^{-1}]	0.69	0.48

[a]All other concentrations are expressed on an oven-dried
basis.

lake sediment and water was shaken for 30 minutes and then filtered through Watman No. 42 filter paper. A 1:10 mixture of air-dried sewage sludge and water was treated in a similar manner. Nitrate and nitrite concentrations were determined with a Technicon AutoAnalyser using a procedure similar to that of Kamphake et al. (1967). The nitrite concentration was determined in the absence of the reducing agent, hydrazine sulfate. Half an hour prior to the determination of nitrate concentration, nitrite present in the sample was reduced by the addition [3% by volume] of 3% sulfamic acid. After reduction by hydrazine sulfate, the nitrate in the sample was determined as nitrite.

Initial ammonium-N concentration was determined by the following procedure. A 1:1 mixture of wet lake sediment and 2N potassium chloride was shaken for 30 minutes and then filtered through Watman No. 42 filter paper. A similar procedure was followed for a 1:50 mixture of sewage sludge and 2N potassium chloride. Ammonium concentration was determined with an Orion ammonia electrode and a Fisher 520 digital pH/ion meter.

3.2 APPARATUS AND GENERAL PROCEDURE

Incubation tubes [Figure 1] of approximately 60 cm^3, internal volume, were made from taper standard [ℱ] 24/40 joints, 2 mm bore, high vacuum stopcocks, and a ℱ 10/18 cone [inner joints]. All stopcocks and joints were greased with
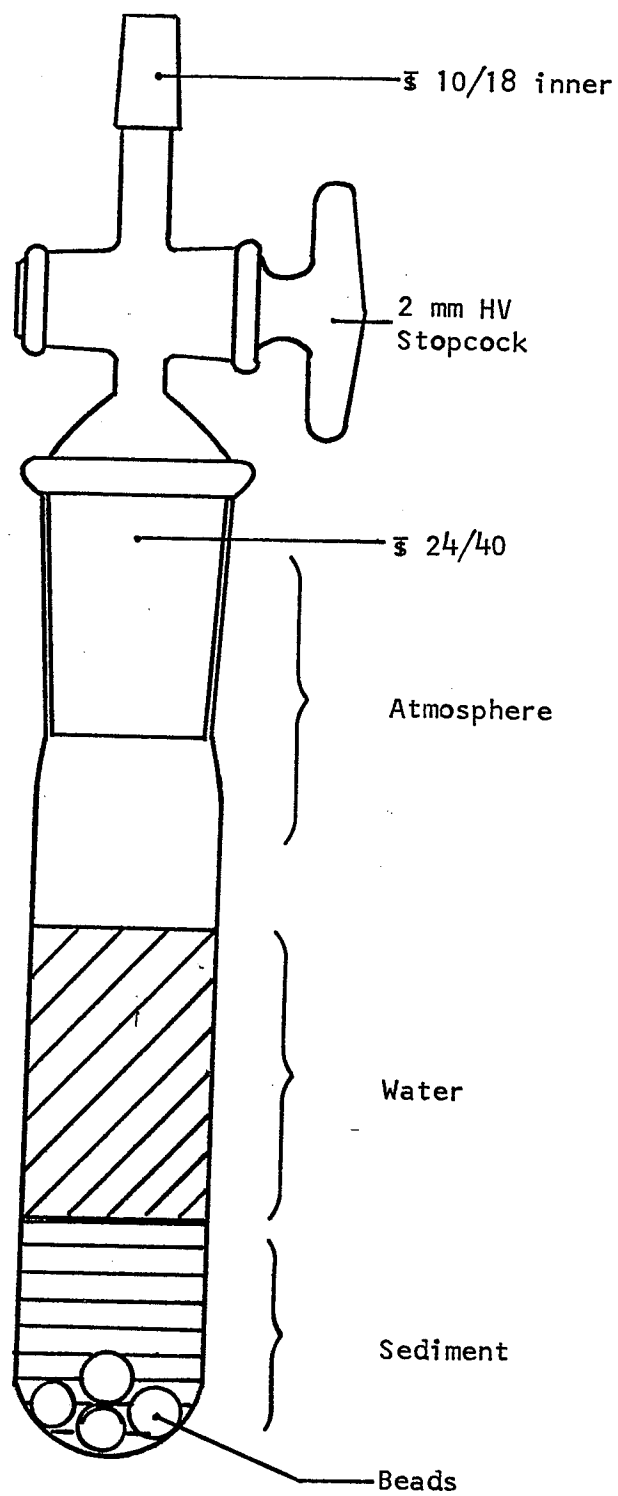
Figure 1:   Incubation tube

Corning high vacuum grease. Sets of tubes were adjusted to the same volume by the addition of glass beads [6 mm in diameter and 0.17 cm^3 in volume].

The samples were prepared for incubation in the following way. Lake sediment or sewage sludge was dispensed into an incubation tube by weight. Enough calcium nitrate solution [52.3% N-15] was added to give the desired total weight and added nitrate-N concentration. The samples were stirred to remove most of the air bubbles. Then the pressure of the incubation atmosphere was lowered to 650 mm Hg while the incubation tube and a mercury manometer were connected to a vacuum line [Figure 2, position C and position B, respectively, while the other position B was closed off]. The samples were then incubated in the vertical position and in the dark at the desired temperature.

Gas sample tubes [Figure 3], of about 50 cm^3 internal volume, were constructed from right angle 2 mm stopcocks and $ 10/18 socket [outer] joints. Two gas sample tubes were used to collect the evolved gases from each incubation tube. One gas sample tube contained about 5 g of potassium hydroxide as a water and carbon dioxide absorbant. The other contained 5 ml of concentrated sulfuric acid to absorb water only.

After the sample had been incubated for the required time, its atmosphere was sampled in the following way. A

McLeod Gauge

A   6 mm HV Stopcock
B $\bar{s}$ 10/18 inner – for gas containers
C $\bar{s}$ 10/18 outer – for incubation tube

To
Pump

Figure 2:   Vacuum line

2 mm HV Stopcock

$\bar{s}$ 10/18
outer

Concentrated H_2SO_4
or KOH pellets
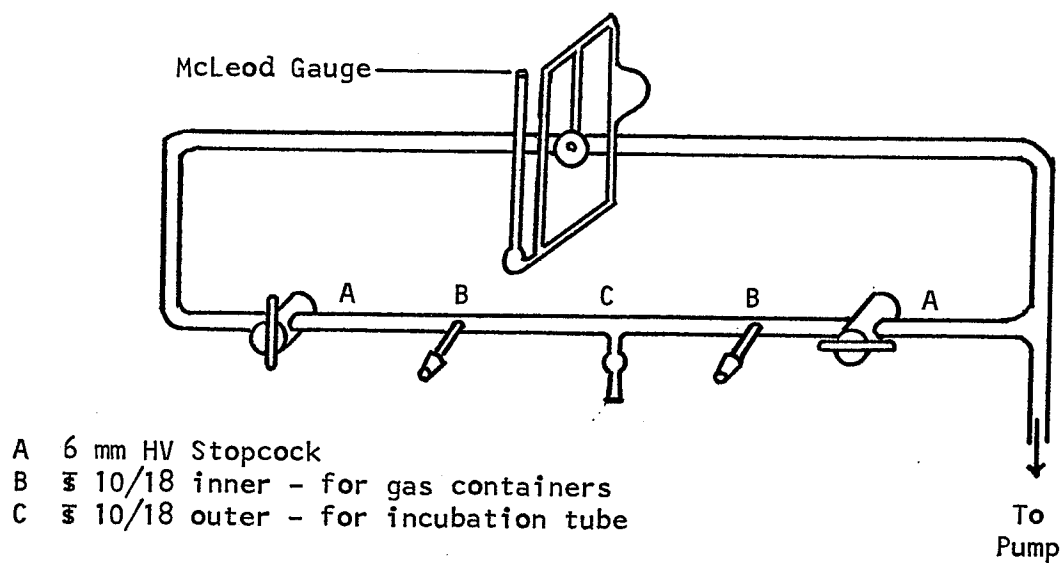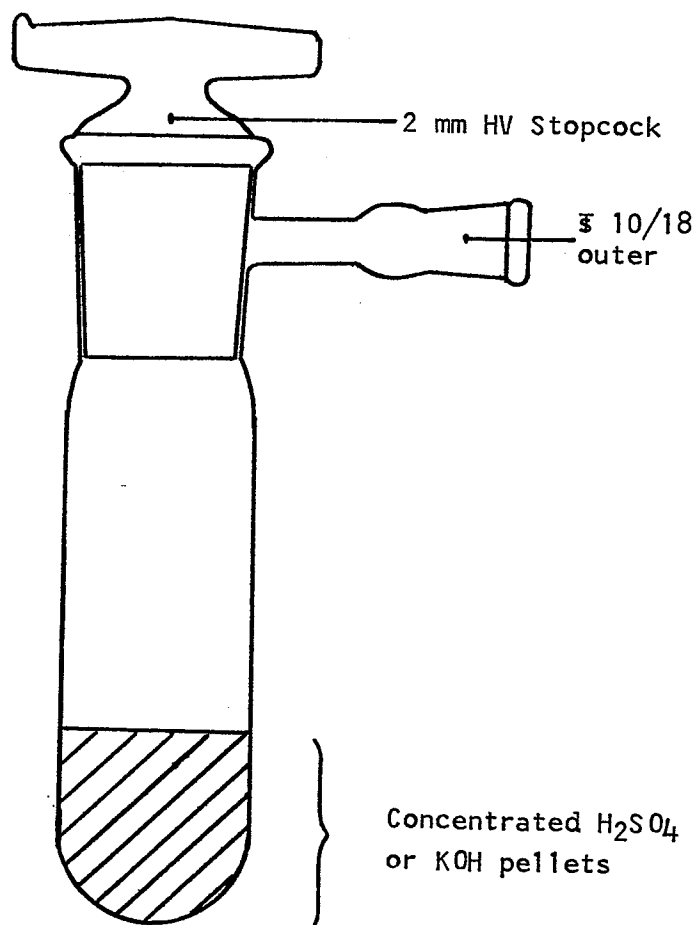
Figure 3:   Gas sample tube

pair of gas sample tubes, with their stopcocks open, were connected to positions B on the vacuum line. The sample was removed from the incubator and after 30 seconds, was shaken for 30 seconds and then connected to position C of the vacuum line. Stopcock A [on the right of Figure 2] was opened and the vacuum line and the gas sample tubes were evacuated with a Welch rotary vacuum pump. Stopcock A was closed when the McLeod guage showed the pressure to be near 5 Torr, and after two and one quarter minutes had elapsed since the retrieval of the sample from the incubator. Then the stopcock on the incubation tube was opened and the gas was allowed to expand into the evacuated gas sample tubes. After 15 seconds, the stopcocks on all tubes were closed. The gas sample tubes were vigorously shaken after removal from the vacuum line and were shaken periodically for an hour to ensure maximum absorbance of moisture. The sampled gas was introduced into a VG-Micromass 602C mass spectrometer [isotopic ratio] and scanned from atomic mass unit [amu] 28 to 46. Argon [amu 40] was used as an internal standard as described by Cho and Sakdinan (1978).

Unless otherwise stated, the entire contents of the incubation tube were transferred to an Erlenmyer flask and mechanically shaken for 30 minutes. After filtration of the suspension, nitrate and nitrite concentrations were determined as described previously.

3.3 CALCULATIONS

The method of calculating the partial pressures of the gases in the incubation atmosphere from the mass spectrometric peak heights was described by Cho and Sakdinan (1978). From the calculated partial pressures of nitrogen and nitrous oxide in the incubation atmosphere, the quantity of these gases dissolved in the water was calculated using Henry's Law constants. The weights of nitrogen, in the incubation atmosphere and in solution, were calculated and totaled for both nitrous oxide and nitrogen gas.

At any time during denitrification, the quantity of electrons that has been accepted can be calculated from the quantity of nitrogenous intermediates and products present in the system. The chosen sequence of intermediates [equation {4}] in the reduction of nitrate to nitrogen gas is:

$$NO_3^- => NO_2^- => N_2O => N_2. \qquad \{16\}$$
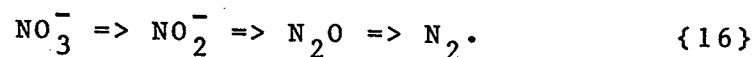
Each step in the sequence can be written as a redox reaction:

$$2[H^+ + NO_3^-] + 4[H^+ + e^-] => 2[H^+ + NO_2^-] + 2H_2O, \quad \{17\}$$

$$2[H^+ + NO_2^-] + 4[H^+ + e^-] => N_2O + 3H_2O, \qquad \{18\}$$

$$N_2O + 2[H^+ + e^-] => N_2 + H_2O. \qquad \{19\}$$
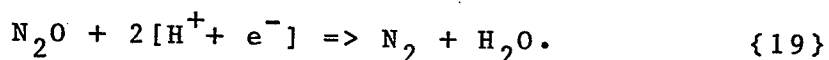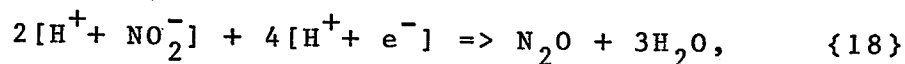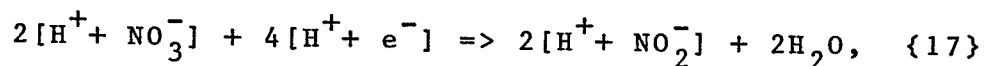
Note that equation {17} has been doubled for convenience. The sum of reactions {17} and {18} is the reduction of nitrate to nitrous oxide:

$$2[H^+ + NO_3^-] + 8[H^+ + e^-] \Rightarrow N_2O + 5H_2O. \qquad \{20\}$$
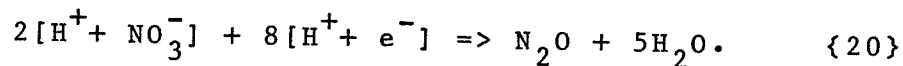
The sum of reactions {19} and {20} is the complete reduction of nitrate to nitrogen gas:

$$2[H^+ + NO_3^-] + 10[H^+ + e^-] \Rightarrow N_2 + 6H_2O. \qquad \{21\}$$
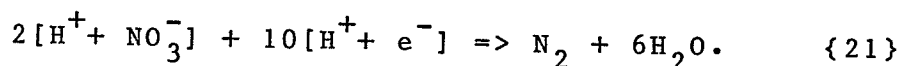
Equations {17} to {21} describe denitrification on a molar basis. According to equation {21}, ten moles of electrons are accepted by two moles of nitrate in the formation of one mole of nitrogen gas.

However, it is more convenient to express the reduction of nitrate to nitrite, nitrous oxide, or nitrogen gas on the basis of moles of N-atoms, since conservation of mass applies. Equations {17}, {20}, and {21} expressed on an atomic basis are, respectively:

$$[H^+ + NO_3^-] + 2[H^+ + e^-] \Rightarrow [H^+ + NO_2^-] + H_2O, \qquad \{22\}$$

$$[H^+ + NO_3^-] + 4[H^+ + e^-] \Rightarrow \tfrac{1}{2}N_2O + \tfrac{5}{2}H_2O, \qquad \{23\}$$

$$[H^+ + NO_3^-] + 5[H^+ + e^-] \Rightarrow \tfrac{1}{2}N_2 + 3H_2O. \qquad \{24\}$$
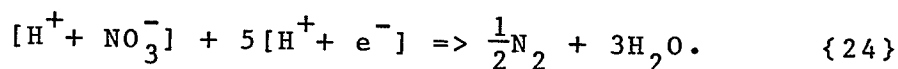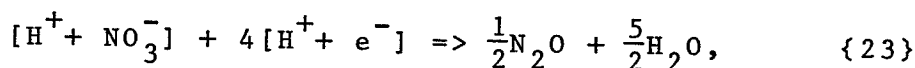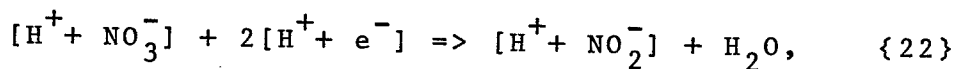
On reduction of one nitrate-N atom, two electrons are required to form one nitrite-N atom, four electrons to form one nitrous oxide-N atom, and five electrons to form one nitrogen-N atom.

Let Y = number of moles of N-atoms in NO_2^-,

Z = number of moles of N-atoms in N_2O,

G = number of moles of N-atoms in N_2.

Then, the number of moles of electrons accepted due to the formation of nitrite from nitrate, e_Y^-, is:

$$e_Y^- = 2Y. \qquad \{25a\}$$

Similarly, if nitrate is reduced as far as nitrous oxide, then the number of moles of electrons accepted, e_Z^-, is:

$$e_Z^- = 4Z \qquad \{25b\}$$

and, if nitrate is completely reduced to nitrogen gas, then the number of moles of electrons accepted, e_G^-, is:

$$e_G^- = 5G. \qquad \{25c\}$$

The total number of moles of electrons accepted, e_T^-, at any time, t, after the initiation of denitrification is:

$$e_T^- = e_Y^- + e_Z^- + e_G^- \qquad \{26a\}$$

$$= 2Y + 4Z + 5G. \qquad \{26b\}$$

Define Re as the rate of electron acceptance during denitrification expressed in moles of N-atoms. Then, differentiating equation $\{26b\}$ with respect to time yields:

$$\frac{d}{dt}[e_T^-] = Re = \frac{d}{dt}[2Y + 4Z + 5G]. \qquad \{27\}$$

However, at the termination of denitrification e_T^- will be five times greater than the number of moles of nitrogen gas N-atoms produced. For convenience in graphing of data, Re^* is defined as one fifth of Re, the electron acceptance rate. Similarly, e^*, expressed in ug N-equivalent, is defined as one fifth of e_T^-.

Therefore:

$$Re^* = \frac{d}{dt}[e^*] = \frac{d}{dt}[\frac{2}{5}Y + \frac{4}{5}Z + G], \qquad \{28\}$$

or:

$$Re^* = \frac{d}{dt}[\frac{2}{5}(NO_2^- - N) + \frac{4}{5}(N_2O - N) + (N_2 - N)]. \qquad \{29\}$$

Re^* can be determined from the slope of a graph of the quantity contained in the square brackets on the right of equation {29} versus time.

3.4 EXPERIMENTAL DESIGN

3.4.1 Experiment I: Denitrification in Lake Sediment and Sewage Sludge

Nitrate solution was added to the equivalent of 0.5 and 1.0 g oven-dried lake sediment to give a total weight of 29.5 and 30.0 g, respectively, and an added nitrate-N concentration[b] of 15 ppm. The equivalent of 1.0 g oven-dried sewage sludge and nitrate solution were mixed to give a final weight of 31.0 g and added nitrate-N concentration of 20 ppm. The samples were prepared as described in the

[b] All added compound concentrations are expressed on an incubation solution basis.

general procedure and incubated at 10C. Gas samples were taken and scanned on the mass spectrometer at intervals of one or two days for two to three weeks of incubation.

The incubation mixtures were extracted for determination of inorganic-N concentrations. About 10 g of the liquid suspension were extracted for nitrate and nitrite determinations with 30 ml of water. Ammonium was extracted from about 15 g of suspension with 30 ml of 2N potassium chloride. The resulting mixtures were shaken for 30 minutes and then filtered. The inorganic-N concentrations of the extracted liquid were determined by the methods described previously.

3.4.2 Experiment II: Effect of Addition of Glucose to Lake Sediment

Nitrate solution containing glucose was added to the equivalent of 1.0 g oven-dried lake sediment to give a total weight of 30.0 g, added glucose-C concentration of 600 ppm, and added nitrate-N concentration of 30 ppm. An additional set of samples was prepared, but the glucose amendment was omitted. All samples were incubated at 10C. Gas samples were taken and scanned at intervals over 12 days of incubation. The entire contents of the incubation tube were mechanically shaken and then filtered. Nitrate and nitrite levels were determined.

3.4.3   Experiment III: Effect of Change in pH

Nitrate solution containing lime[c] was added to lake sediment [0.5 g oven-dried equivalent] to give 2, 5, 10, and 20% lime on a total oven-dried basis. The liquid weight was 29.0 g and the added nitrate-N concentration was 15 ppm. Sewage sludge equivalent to 1.0 g oven-dried solid was mixed with 30.0 g nitrate solution containing 0.15 ml of 20% hydrochloric acid. The added nitrate-N concentration was 20 ppm. The samples were incubated at 10C and gas samples were taken and scanned over a two week period. The entire sample was shaken and filtered and nitrate and nitrite concentrations were determined. Similar samples were prepared, stoppered, and kept at room temperature and their pH was measured periodically.

3.4.4   Experiment IV: Effect of Nitrate Concentration

Nitrate solution was added to lake sediment, equivalent to 0.5 g oven-dried solid, to give a total weight of 29.5 g. The added nitrate-N concentrations were 2.5, 5, and 10 ppm. Similarly, nitrate solution was added to 1.0 g of sewage sludge [oven-dried basis] to give a total weight of 30.0 g and added nitrate-N concentrations of 10, 20, 30, 40, and 50 ppm. All samples were incubated at 10C for two to three weeks. Gas samples were taken at intervals during this period and scanned on the mass spectrometer. Nitrate and

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[c]Lime is defined as calcium carbonate and liming is defined as the addition of calcium carbonate.

nitrite concentrations were determined from the filtrate of the entire sample.

3.4.5 Experiment V: Effect of Low Temperature

Nitrate solution was added to the equivalent of 0.5 g oven-dried lake sediment to give a total weight of 29.5 g and added nitrate-N concentrations of 2.5 and 5 ppm.

The air atmosphere was replaced with an inert gas by evacuating and replenishing the incubation atmosphere three times with argon. This was done while the the incubation tube was connected to the vacuum line. The final pressure of the incubation atmosphere was adjusted to 650 mm Hg. A set of samples with 5 ppm added nitrate-N and an air atmosphere was also prepared. All samples were incubated at 4C for two weeks.

Nitrate solution was added to the equivalent of 1.0 g oven-dried sewage sludge to give a total weight of 30.0 g and added nitrate-N concentration of 20 ppm. The samples were incubated under an air atmosphere at 4C for 24 days.

Gas samples were taken and scanned throughout the incubation period and nitrate and nitrite concentrations were determined from the filtrate of the entire sample suspension.

3.4.6 Experiment VI: Effect of Sample Agitation on Denitrification in Lake Sediment

Nitrate solution was added to wet lake sediment [equivalent to 0.5 g oven-dried solid] to give a total weight of 29.5 g and added nitrate-N concentrations of 2.5, 5, and 10 ppm. The air atmosphere was replaced with argon as described in Experiment V.

The samples were not incubated in the vertical position, but were fastened to a rocking platform [Figure 4] in the horizontal position. The platform was rocked at two rpm by an electric motor through a connecting arm and gear assembly. This created enough movement in the sample to keep the solid matter in suspension.

Lake sediment was incubated in a similar manner at 5, 7.5, and 12.5C with 5 ppm added nitrate-N. Gas samples were taken daily for a week and scanned with the mass spectrometer. Nitrate and nitrite determinations were made on the filtrate of the entire sample suspension.

3.4.7 Experiment VII: Effect of Addition of Sewage Sludge to Lake Sediment

A 1:4 sewage sludge to water mixture was magnetically stirred for one hour. An amount of this suspension containing the equivalent of 0.5 g oven-dried solid was added to the equivalent of 0.5 g oven-dried lake sediment. Nitrate solution was added to bring the total weight to 30.0 g and
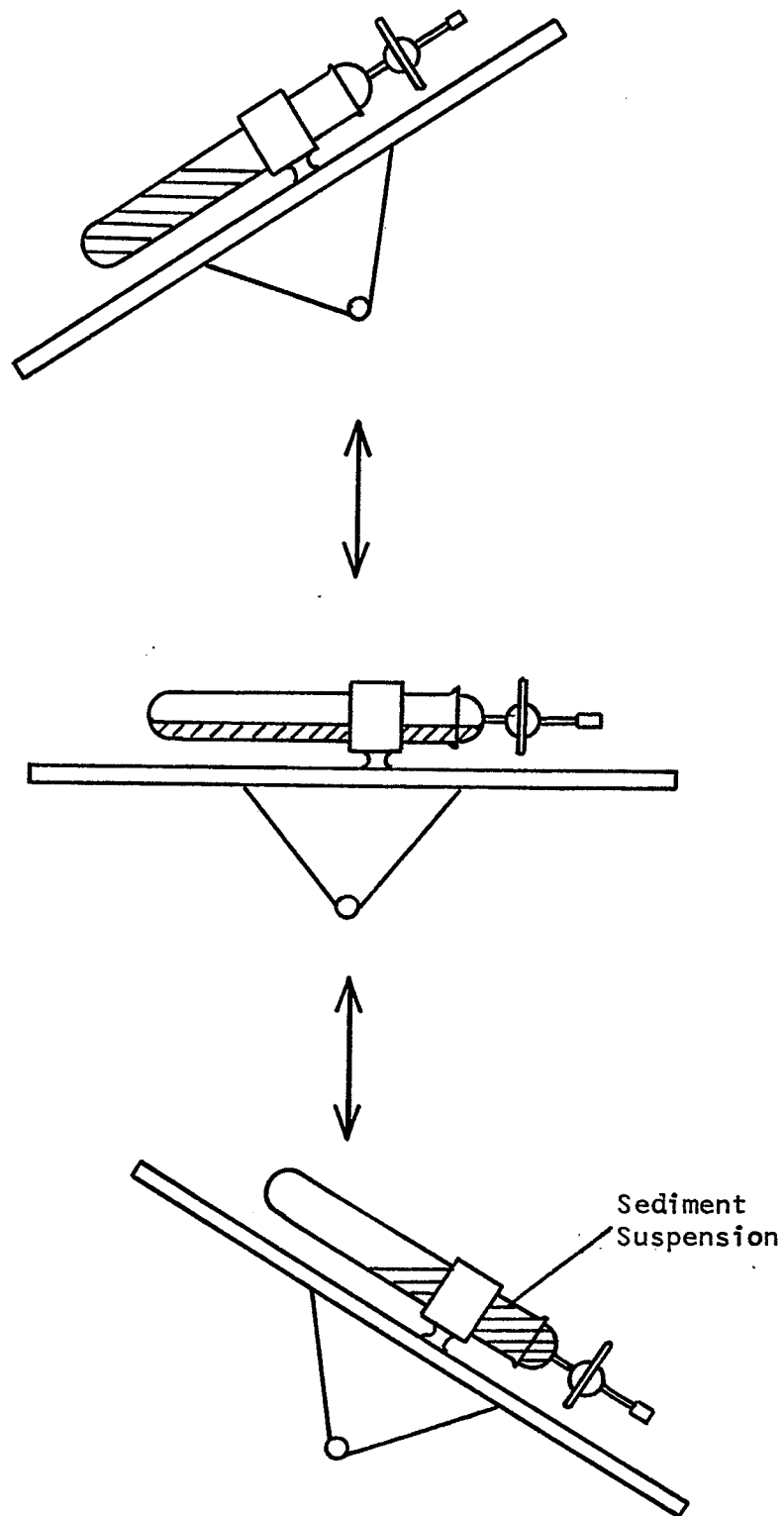
Figure 4:   Rocking platform apparatus

to give an added nitrate-N concentration of 50 ppm. The samples were incubated at 10C for three weeks. Gas samples were taken throughout the incubation period and nitrate and nitrite concentrations were determined for the filtrate of the entire sample suspension. The pH of a similar sample, kept covered at room temperature, was measured periodically.

Chapter IV

RESULTS AND DISCUSSION

4.1   EXPERIMENT I: DENITRIFICATION IN LAKE SEDIMENT AND
            SEWAGE SLUDGE

Mass spectrometric analysis of the gases evolved from
lake sediment [1.0 g solid] incubated at 10C with 15 ppm
added nitrate-N indicated that nitrous oxide and then nitro-
gen gas were produced as nitrate disappeared [Figure 5].
The evolution of these gases during denitrification in lake
sediment was previously observed (Chen et al. 1972b; van
Kessel 1978b). The rate of depletion of nitrate was ini-
tially rapid and almost constant for the first four to six
days of incubation, but then decreased and levelled out.
Nitrate disappearance with time over the incubation period
of two weeks was better described by first-order kinetics
[semi-log plot, $r^2 = 0.86$] than by zero-order kinetics
[$r^2 = 0.80$]. The first-order rate constant was 0.094 day^{-1}.
Although the analysis of nitrite in the sample solution was
performed, levels were below the limit of detection.

Nitrous oxide was detected in the sample atmosphere after
only one day of incubation. The concentration of this gas
increased to a maximum of 81.2 ug N_2O-N/g solid after 10
days of incubation and then decreased to 61.1 ug N_2O-N/g

Figure 5: Concentration of nitrate, nitrous oxide, and nitrogen gas during incubation of lake sediment [1.0 g solid] at 10C with 15 ppm added nitrate-N

solid after 14 days. Although small amounts of nitrogen gas were detected on the first and second days of incubation, it was not until the fourth day that a large increase in the concentration of this gas was measured. The value of e^*, one fifth of the total number of moles of electrons accepted by nitrogenous oxides, was calculated from the concentrations of nitrous oxide and nitrogen gas measured for each sampling day and plotted against time[d] [Figure 5]. Re^*, the slope of the regression line over the entire incubation period, was 15.7 ug N-equivalent/g solid-day $[r^2=0.83]$.

In the stored lake sediment, ammonium-N was the predominant inorganic-N compound and had a concentration of 103 ppm [Table 1]. This fact, as well as the low initial nitrate-N concentration of 3.25 ppm, indicated the reduced nature of stored lake sediment. However, after one day of incubation, the concentration of ammonium-N increased to 168 ug N/g solid and then gradually decreased to 104 ug N/g solid during the incubation period [Table 2]. The initial large increase may have been due to a microbial flush resulting from the disturbance of the sediment during sample preparation and from the change in temperature from 4 to 10C. The subsequent decrease in ammonium-N may have been the result of microbial immobilization or nitrification.

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[d]Although e^* has units of ug N-equivalent, it is expressed in ug N in all figures and tables.

However, analysis of the percentage of N-15 in the evolved nitrous oxide [Table 2] did not indicate any major dilution by unlabeled sediment nitrogen with increasing time of incubation.

TABLE 2

Concentration of ammonium, oxygen, and carbon dioxide and percentage of N-15 in nitrous oxide during incubation of lake sediment at 10C with 15 ppm added nitrate-N

Day	NH_4^+	N-15 in N_2O	O_2	CO_2
	[ug N/g solid]	%	% partial pressure	
1	168.0	48.77	20.2	0.58
2	159.0	50.65	20.1	0.86
4	136.0	51.21	19.9	0.91
6	130.0	50.51	19.2	–
8	130.0	48.66	18.3	1.53
10	119.0	49.90	18.2	1.40
12	110.0	49.80	18.2	1.36
14	104.0	48.33	17.8	1.70

The depths of supernatant water and sediment, when 1.0 g [solid] lake sediment settled after mixing with nitrate solution, were 12 and 54 mm, respectively. The layer of

water effectively prevented rapid oxygen transport from the sample atmosphere to the sediment. The partial pressure of oxygen dropped from 20.2% on the first day of incubation to 17.8% on the fourteenth [last] day [Table 2]. There was an initial large increase in carbon dioxide production during the first two days of incubation [Table 2]. It is not known why this occurred. However, there may have been an aerobic microbial flush due to increased aeration in the stirred sediment or to the sudden temperature increase from 4 to 10C.

Trends in nitrate disappearance and nitrogenous gas production, similar to those previously described for 1.0 g solid, were observed during the incubation of 0.5 g [solid] lake sediment at 10C with 15 ppm added nitrate-N [Figure 6]. The depths of free water and sediment in the 0.5 g [solid] lake sediment samples were 32 and 33 mm, respectively. Again, nitrate disappearance was initially rapid and nearly linear with respect to time, but the initial rate of nitrate depletion was less than that with 1.0 g solid. After the tenth day of incubation, the rate of nitrate removal decreased and levelled out, as with the 1.0 g samples. Nitrate disappearance with time over the entire incubation period was better described as a first-order reaction [$r^2=0.97$] with a rate constant of 0.085 day^{-1}.
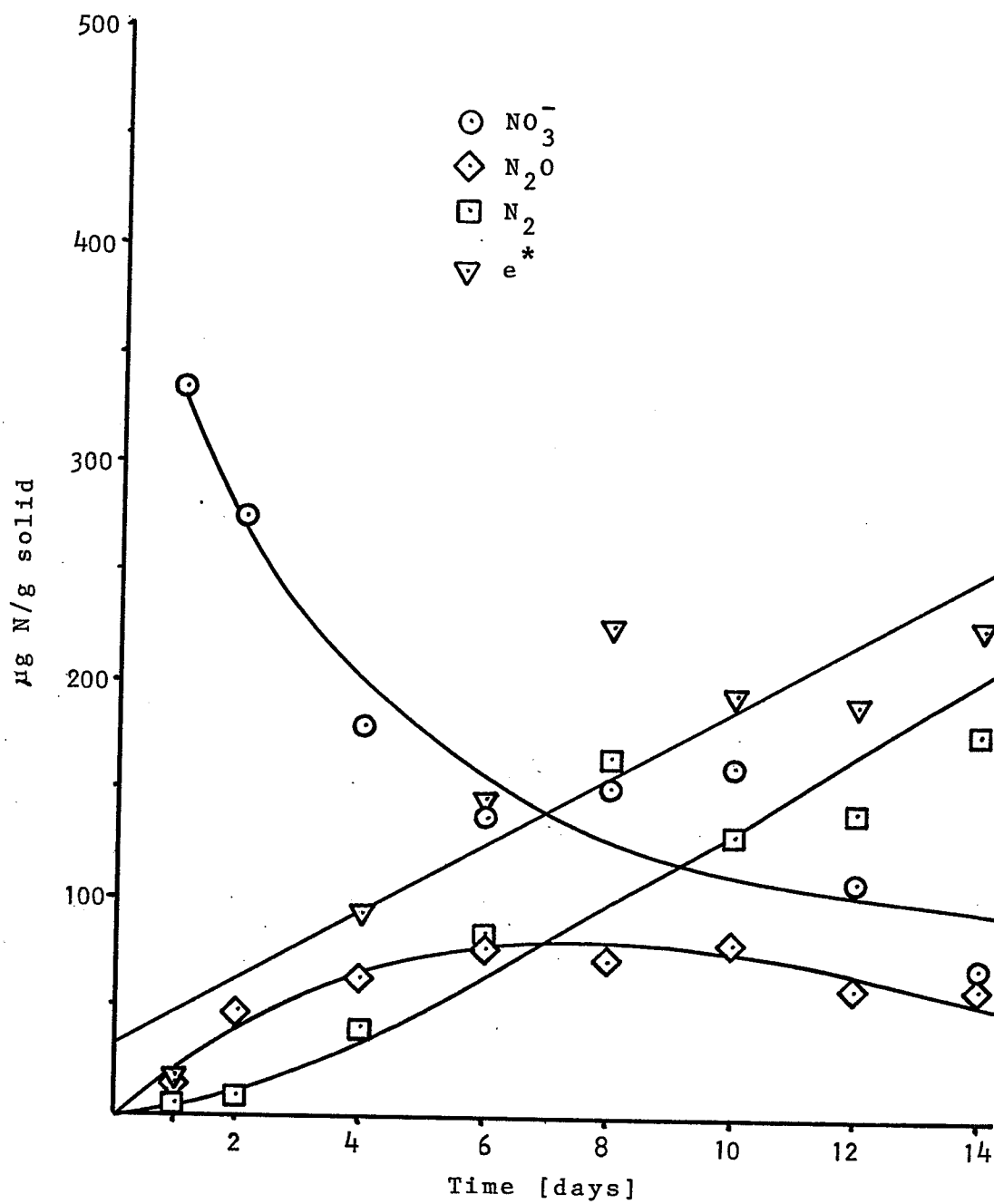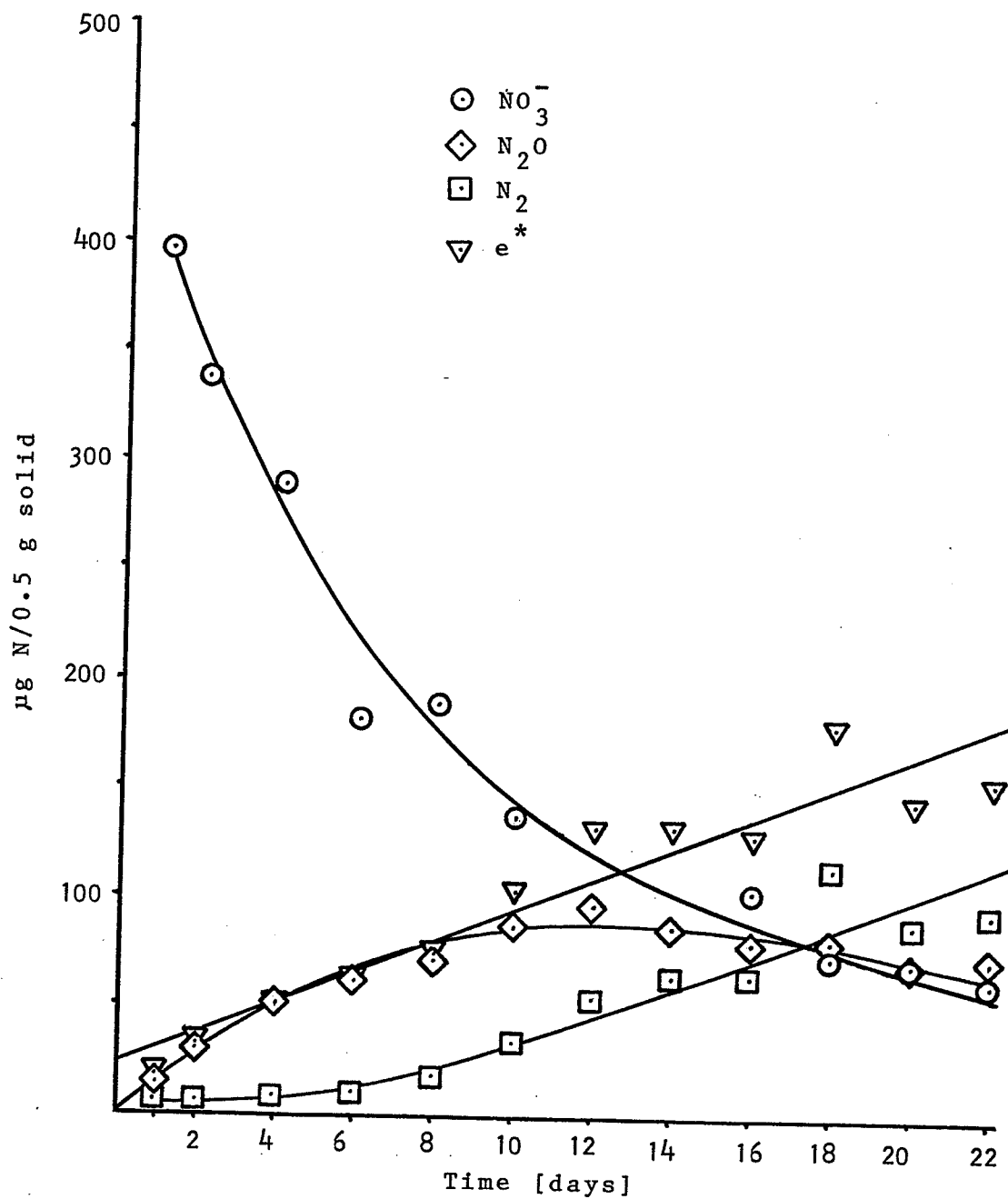
Figure 6:   Concentration of nitrate, nitrous oxide, and nitrogen gas during incubation of lake sediment [0.5 g solid] at 10C with 15 ppm added nitrate-N

Nitrous oxide was the first gaseous product detected and it was observed on the first day of incubation. A maximum of 97.4 ug $N_2O-N/0.5$ g solid was attained on the twelfth day of incubation. This was greater than the maximum of nitrous oxide evolved from 1.0 g [solid] lake sediment and occurred two days later. The initial rate of nitrous oxide production was similar over the first four days in both incubations, and was 14.5 ug N/g solid-day and 12.1 ug N/0.5 g solid-day. Nitrogen gas was detected in small amounts during the first eight days of incubation, but major production did not occur until the tenth day—six days later than with 1.0 g solid.

The values of e^*, calculated from the concentrations of nitrous oxide and nitrogen gas and expressed ug N-equivalent/0.5 g solid, were plotted against time for each sampling day [Figure 6]. Re^*, the slope of the regression line over the entire incubation period, was 7.06 ug N-equivalent/0.5 g solid-day or 14.1 ug N-equivalent/g solid-day. This value was slightly less than the Re^* of 15.7 ug N-equivalent/g solid-day previously calculated for 1.0 g [solid] lake sediment samples.

In both incubations with lake sediment, nitrous oxide was further reduced to nitrogen gas while relatively large amounts of nitrate were present in the sample. Complete inhibition of the reduction of nitrous oxide by nitrate, as suggested by Blackmer and Bremner (1978), was not apparent.

Major nitrogen gas production occurred before nitrous oxide accumulation reached a maximum.

The high initial nitrate-N concentration of 413 ppm indicated the oxidized nature of the sewage sludge [Table 1]. The sludge had a higher bulk density than the sediment [Table 1]. This resulted in depths of 59 and 12 mm for the liquid and sludge layers, respectively, when 1.0 g [solid] settled after mixing with nitrate solution in an incubation tube. Nitrate disappearance with time was linear [r^2=0.996] and better described by zero-order kinetics [Figure 7]. The slope of the regression line was -42.1 ug NO_3^--N/g solid-day. Although the determination for ammonium was performed, concentrations were too dilute for accurate measurement. The initial concentration of ammonium-N was 54.8 ppm [Table 1]. Nitrite-N concentration increased from an initial value of 1.43 ppm [Table 1] to a value of 10.5 ug N/g solid after 2 days of incubation and then declined to around 5 ug N/g solid after two weeks [Table 3].

There was no nitrous oxide observed in the atmosphere of the samples during the incubation period and nitrogen gas was the first nitrogenous gas detected. A similar finding from denitrification in laboratory activated sludge reactors was reported (Johnson and Schroepfer 1964). Nitrogen gas was observed after the first day of incubation and increased rapidly to 607 ug N/g solid after 16 days of incubation
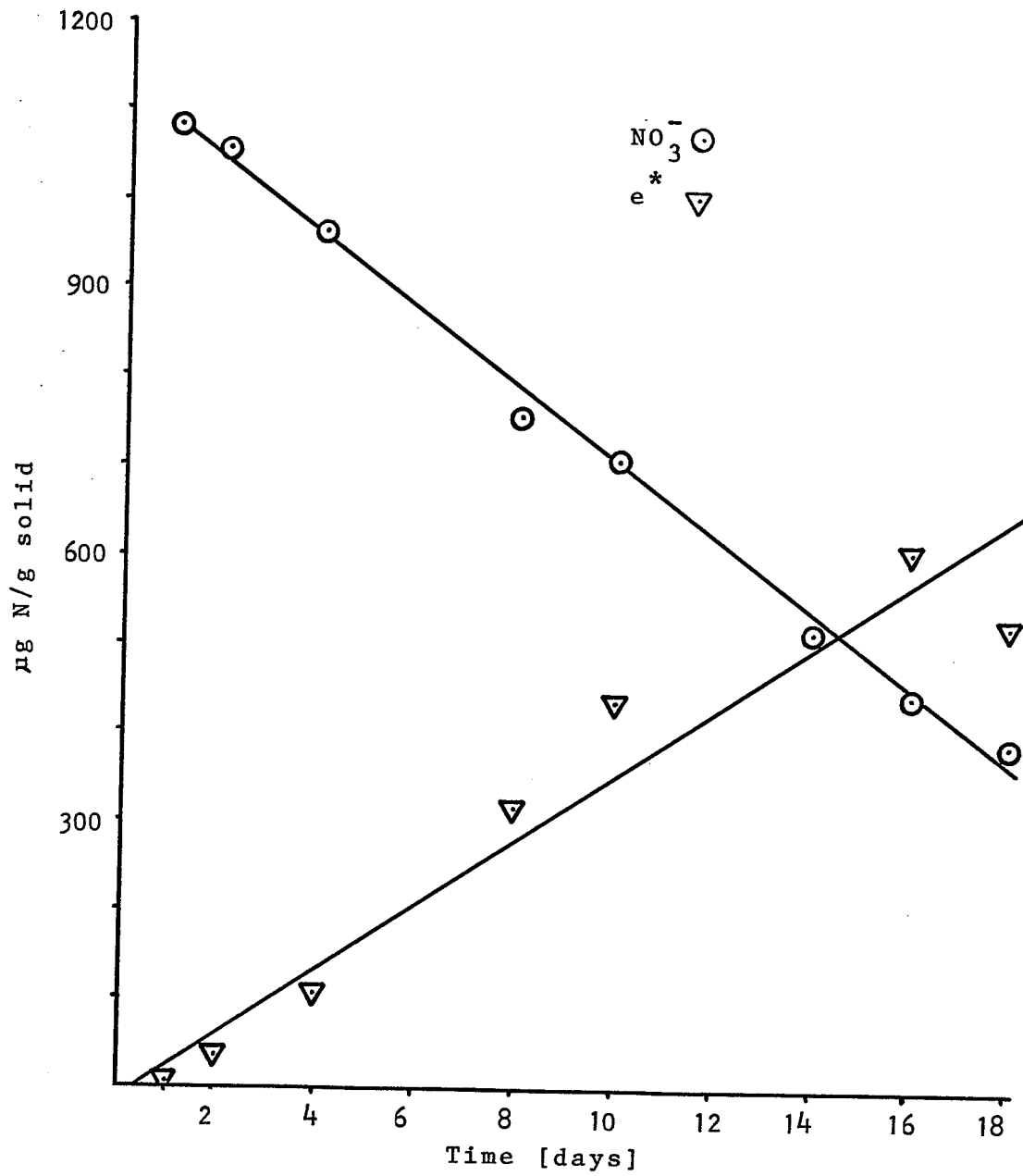
Figure 7: Concentration of nitrate during incubation of
sewage sludge at 10C with 20 ppm added nitrate-N

[Table 3]. The value of e^* was calculated from the nitrite and nitrogen gas concentrations on each sampling day and plotted in Figure 7. These values were very similar to those for nitrogen gas, especially after the second sampling day. Re^*, the slope of the regression line of e^* versus time over the entire incubation period, was 35.7 ug N-equivalent/g solid-day [r^2=0.94]. This was almost two and one half times the rate previously calculated for the same solid weight of lake sediment at the same incubation temperature, but with a lower nitrate concentration.

The partial pressures of oxygen and carbon dioxide in the sample atmospheres throughout the incubation period are presented in Table 3. Although the decrease in oxygen was similar to that observed with 1.0 g [solid] lake sediment [Table 2], the initial increase in carbon dioxide was relatively small. This may have been due to the difference in initial state between the two materials. Also, sewage sludge had a calcium carbonate equivalent content of 20.7% [Table 1], while carbonates were absent from lake sediment. The presence of carbonates in a flooded system increases the solubility of carbon dioxide, which precipitates as carbonate in response to the $CaCO_3$-H_2O-CO_2 equilibrium system [Ponnamperuma et al. 1966]. This system also maintains the pH of a flooded system near neutrality.

TABLE 3

Concentration of nitrite, oxygen, carbon dioxide, and
nitrogen gas during incubation of sewage sludge at 10C with
20 ppm added nitrate-N

Day	NO_2^-	N_2	O_2	CO_2
	[ug N/g solid]		% partial pressure	
1	4.80	4.56	20.8	0.06
2	10.5	34.1	20.4	0.17
4	8.40	103.0	20.1	0.26
8	7.50	313.0	19.3	0.55
10	6.30	431.0	19.3	0.63
14	5.52	508.0	18.1	0.92
16	5.03	607.0	16.4	1.09
18	4.80	514.0	17.9	0.93

Denitrification was faster in sewage sludge than in lake
sediment. Nitrate removal with time was zero-order with the
sludge and nitrogen gas was the only gaseous product. In
lake sediment, nitrate loss with time was first-order and
nitrous oxide was evolved as a precursor to nitrogen gas.

4.2 EXPERIMENT II: EFFECT OF ADDITION OF GLUCOSE TO LAKE SEDIMENT

Nitrate disappearance and nitrogenous gas production were followed during the incubation of 1.0 g [solid] lake sediment at 10C with 30 ppm added nitrate-N and without an additional energy source [Figure 8]. Trends were similar to those observed with the same weight of lake sediment incubated at 10C with 15 ppm added nitrate-N in Experiment I [Figure 5]. Although it did not level out during the incubation period, nitrate depletion with time was curvilinear and nearly linear in a semi-log plot [$r^2=0.98$]. The first-order rate constant was 0.094 day^{-1}, which was identical to that calculated for the rate of nitrate disappearance when the added nitrate-N concentration was 15 ppm.

Nitrite concentration was 0.753 ug N/g solid after one day of incubation, increased to 1.16 ug N/g solid by the fourth day, and then decreased below the limit of detection by the ninth day [Table 4].

Mass spectrometric analysis of the sample atmosphere after one day of incubation revealed small accumulations of nitrous oxide and nitrogen gas [Figure 8]. Nitrous oxide accumulation increased steadily with time to a maximum of 153 ug N/g solid after the twelfth [last] day of incubation. The rate of accumulation with time over the first nine days was 16.4 ug N_2O-N/g solid-day. This value was slightly greater than the rate of 14.5 ug N_2O-N/g solid-day

Figure 8: Concentration of nitrate, nitrous oxide, and nitrogen gas during incubation of lake sediment at 10C with 30 ppm added nitrate-N

TABLE 4

Concentration of nitrite, oxygen, and carbon dioxide during incubation of lake sediment at 10C with 30 ppm added nitrate-N and in the absence and presence of glucose

No glucose added

Day	NO_2^-	O_2	CO_2
	[ug N/g solid]	% partial pressure	
1	0.75	20.9	0.60
2	0.75	20.7	0.68
4	1.16	20.1	0.96
6	1.16	19.4	1.37
9	0	19.4	1.37
12	0	18.6	1.62

600 ppm glucose-C added

Day	NO_2^-	O_2	CO_2
	[ug N/g solid]	% partial pressure	
1	1.74	20.9	0.54
2	2.03	20.9	0.81
4	18.5	19.7	1.48
6	0	19.2	3.23
9	0	18.4	6.19
12	0	15.3	7.61

previously calculated for the similar incubation with 15 ppm added nitrate-N in Experiment I.

Nitrogen gas accumulation was low after the second day of incubation, but steadily increased to 106 ug N/g solid after the twelfth [last] day of incubation [Figure 8]. The trend observed in the incubation of 1.0 g [solid] lake sediment with 15 ppm added nitrate-N [Figure 5] showed a faster rate of nitrogen gas accumulation, earlier in the incubation, than with 30 ppm added nitrate-N.

The concentrations of nitrite, nitrous oxide, and nitrogen gas determined for each sampling day were used to calculate e^* values, which were plotted against time [Figure 8]. Re^* was calculated to be 21.2 ug N-equivalent/g solid-day over the entire incubation period. This value was greater than the Re^* of 15.7 ug N-equivalent/g solid-day previously calculated for the incubation of 1.0 g [solid] lake sediment with 15 ppm added nitrate-N [Figure 5].

The partial pressures of oxygen and carbon dioxide in the sample atmospheres during incubation are presented in Table 4. Comparison of these values with those obtained during the similar incubation with 15 ppm added nitrate-N [Table 2] revealed little difference as a result of the increase in added nitrate-N concentration. Again, the initial large increase in carbon dioxide partial pressure was evident.

When 600 ppm glucose-C was added to 1.0 g [solid] lake sediment at 10C with 30 ppm added nitrate-N, the shape of both the nitrate depletion versus time and nitrogenous gas production versus time curves changed dramatically [Figure 9]. This was a result of a large, available supply of carbon for microbial growth, as well as electron donors for respiration. However, nitrous oxide was still detected as an intermediate in denitrification. Nitrate disappearance with time greatly increased after a two day period when nitrate removal resembled that in the incubation without glucose. After nine days of incubation, only 8.69 ug NO_3^--N/g solid remained. In the incubation without glucose, 324 ug NO_3^--N/g solid remained after the same period of incubation [Figure 8].

Nitrite accumulated to a maximum of 18.5 ug N/g solid after the fourth day of incubation with glucose [Table 4]. Both nitrite accumulation and disappearance rates were greater with glucose present and the nitrite concentration was below detection after six days of incubation.

After the second day of incubation with glucose, large amounts of gas bubbles were observed throughout the sediment. As in the control, both nitrous oxide and nitrogen gas were detected after the first day of incubation [Figure 9]. Although the rate of nitrous oxide accumulation was much faster, the maximum amount of nitrous oxide measured was not as great as in the control incubation.
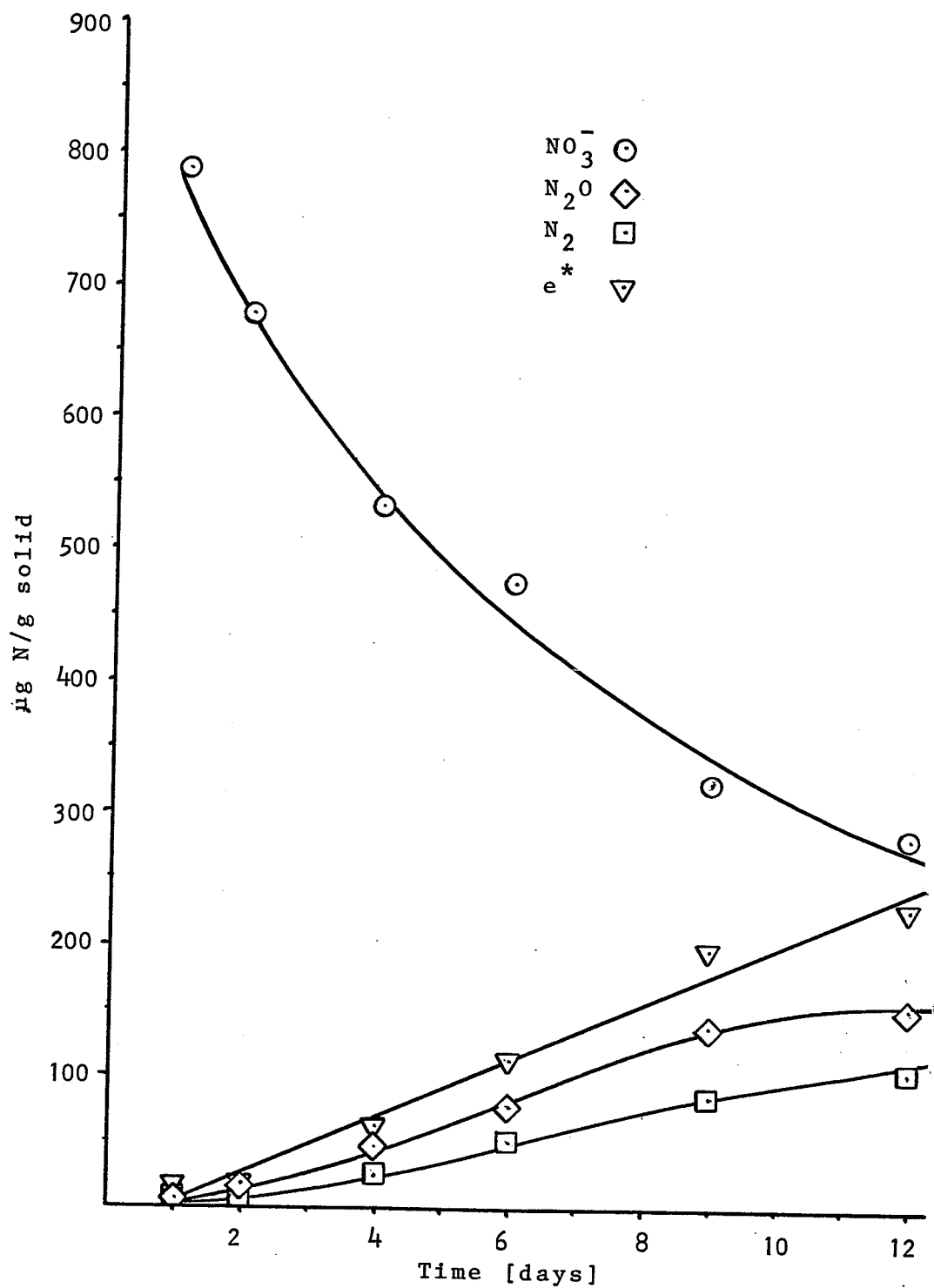
Figure 9: Concentration of nitrate, nitrous oxide, and nitrogen gas during incubation of lake sediment at 10C with 30 ppm added nitrate-N in the presence of glucose

After four days of incubation, a maximum of 63.0 ug N_2O-N/g solid was attained. Nitrous oxide was also reduced rapidly and 23.3 and 9.49 ug N_2O-N/g solid were measured after six and nine days of incubation, respectively.

After a lag of two days, nitrogen gas accumulation increased rapidly to a maximum of 362 ug N/g solid on the ninth day of incubation [Figure 9]. At this time, 375 of the 733 ug N/g solid [43%] measured after the first day of incubation were accounted for. In the control, 67% of the nitrogen determined on the first day was recovered at the end of incubation. Chen et al. (1972a) recovered from 5 to 10% of added nitrate-N as organic-N and ammonium-N in incubated lake sediments, but 37% of added nitrate-N was immobilized during the first four days of a field study. The decreased recovery of added nitrate-N in the presence of glucose probably resulted from increased assimilation of nitrogen due to stimulated growth of the microbial population.

The values of e^*, calculated for each sampling day, were plotted against time [Figure 9]. The slope of the regression line of e^* versus time over the first nine days of incubation was 54.5 ug N-equivalent/g solid-day. Since the line covered the period when nitrogen gas was accumulating, the slope was taken as Re^*. This value was about two and one half times greater than the Re^* of the control.

The decline in partial pressure of oxygen in the atmosphere of samples incubated with glucose did not initially differ from that observed when glucose was absent [Table 4]. However, after nine days, the partial pressure of oxygen in the atmosphere of samples incubated with glucose began to decline more rapidly than in the control. Carbon dioxide accumulation was slightly larger after two days of incubation with glucose, but much greater at the end of incubation due to increased microbial activity.

4.3   EXPERIMENT III: EFFECT OF CHANGE IN PH

The addition of lime to lake sediment resulted in an increase in pH [Table 5]. With 2% addition of lime, the pH stabilized near pH 7.0, while with 5%, 10%, and 20% additions it stabilized at slightly higher values [pH 7.2-7.4].

Nitrate depletion was curvilinear with time in all limed incubations of 0.5 g [solid] lake sediment at 10C with 15 ppm added nitrate-N. Graphically, nitrate removal with time for the 5% addition of lime [Figure 10] was very similar to that of untreated sediment [0.5 g solid] in Experiment I [Figure 6]. A semi-log plot of nitrate concentration versus time for 5% lime was nearly linear [r^2=0.93] and the first-order rate constant was 0.104 day^{-1}. This was greater than the rate constant of 0.085 day^{-1} previously calculated for sediment incubated without lime. However, there was an inverse relationship between amount of lime added and

TABLE 5

Change in pH resulting from the addition of different
amounts of lime to lake sediment

Day	2% lime	5% lime	10% lime	20% lime
0	6.3	6.6	6.6	6.9
1	6.8	7.0	7.0	7.1
2	7.0	7.2	7.0	7.1
3	7.0	7.2	7.2	7.2
5	7.1	7.3	7.3	7.3
8	7.0	7.2	7.3	7.4
16	7.0	7.3	7.4	7.4

nitrate removal rate. With 2% and 10% lime [Table 6] the
rates were 0.112 and 0.089 day^{-1}, respectively, while with
20% lime [Figure 11] the rate of nitrate depletion was
0.086 day^{-1}. The last two rates were slightly higher than
the rate in unlimed sediment.

Although the addition of small amounts of lime stimulated
nitrate reduction, this was not the case with nitrite
reduction. In all limed treatments, nitrite accumulated to
relatively large amounts [Figures 10 and 11, Table 6]. This
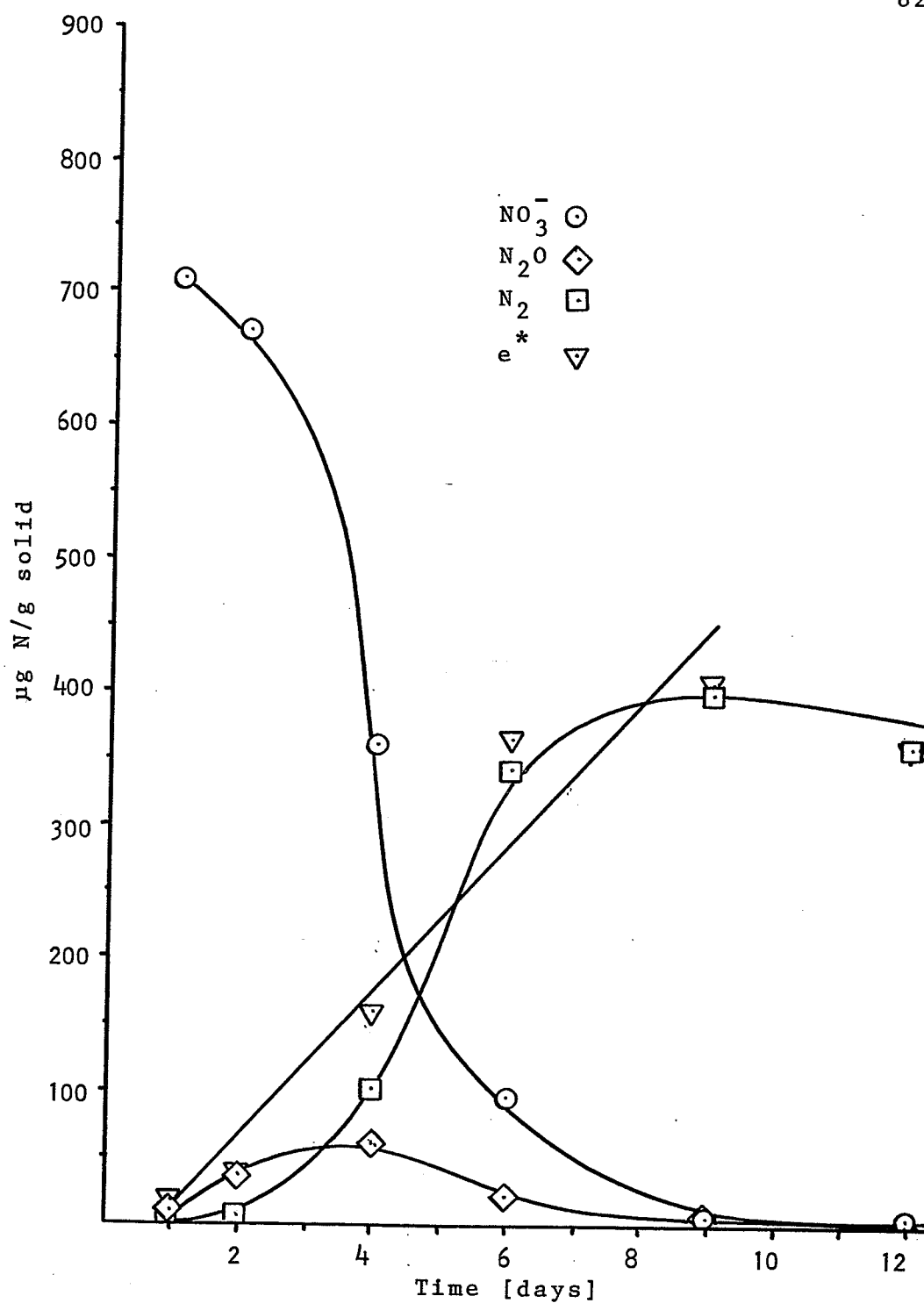was not previously encountered with unlimed lake sediment
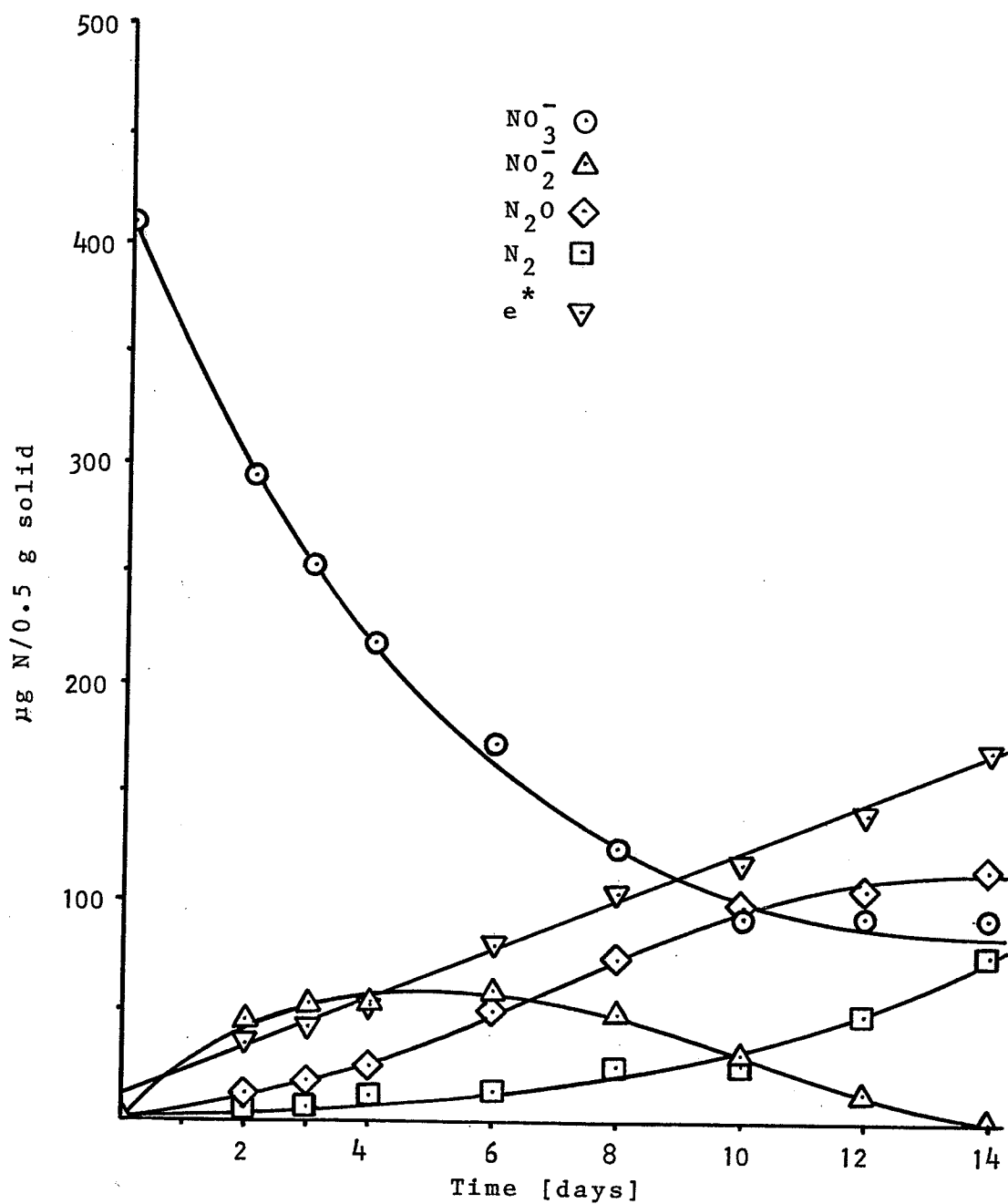
Figure 10: Concentration of nitrate, nitrite, nitrous oxide, and nitrogen gas during incubation of lake sediment at 10C with 15 ppm added nitrate-N and 5% lime

TABLE 6

Concentration of nitrate, nitrite, nitrous oxide, and nitrogen gas during incubation of limed lake sediment at 10C with 15 ppm added nitrate-N

Addition of 2% lime

Day	0	2	3	4	6	8	10	12	14
				[ug N/0.5 g solid]					
NO_3^-	424.0	308.0	267.0	208.0	154.0	137.0	109.0	98.8	74.4
NO_2^-	0.23	37.5	42.7	52.6	50.6	43.0	23.5	8.31	2.12
N_2O	–	10.6	20.2	25.8	42.2	84.6	109.0	116.0	119.0
N_2	–	4.3	6.01	14.3	16.5	22.6	34.0	47.5	81.6
e*	–	27.8	39.3	56.0	70.5	107.0	131.0	144.0	178.0

Addition of 10% lime

| Day	0	2	3	4	5	7	9	12	16
				[ug N/0.5 g solid]					
NO_3^-	424.0	286.0	259.0	215.0	196.0	129.0	119.0	96.5	94.5
NO_2^-	0.23	48.5	52.9	60.7	64.2	62.2	41.8	12.4	1.39
N_2O	–	12.2	19.9	25.9	35.0	65.3	101.0	96.9	113.0
N_2	–	4.29	6.85	11.4	9.58	28.3	26.9	41.3	112.0
e*	–	33.5	43.9	56.4	63.3	105.0	124.0	124.0	203.0
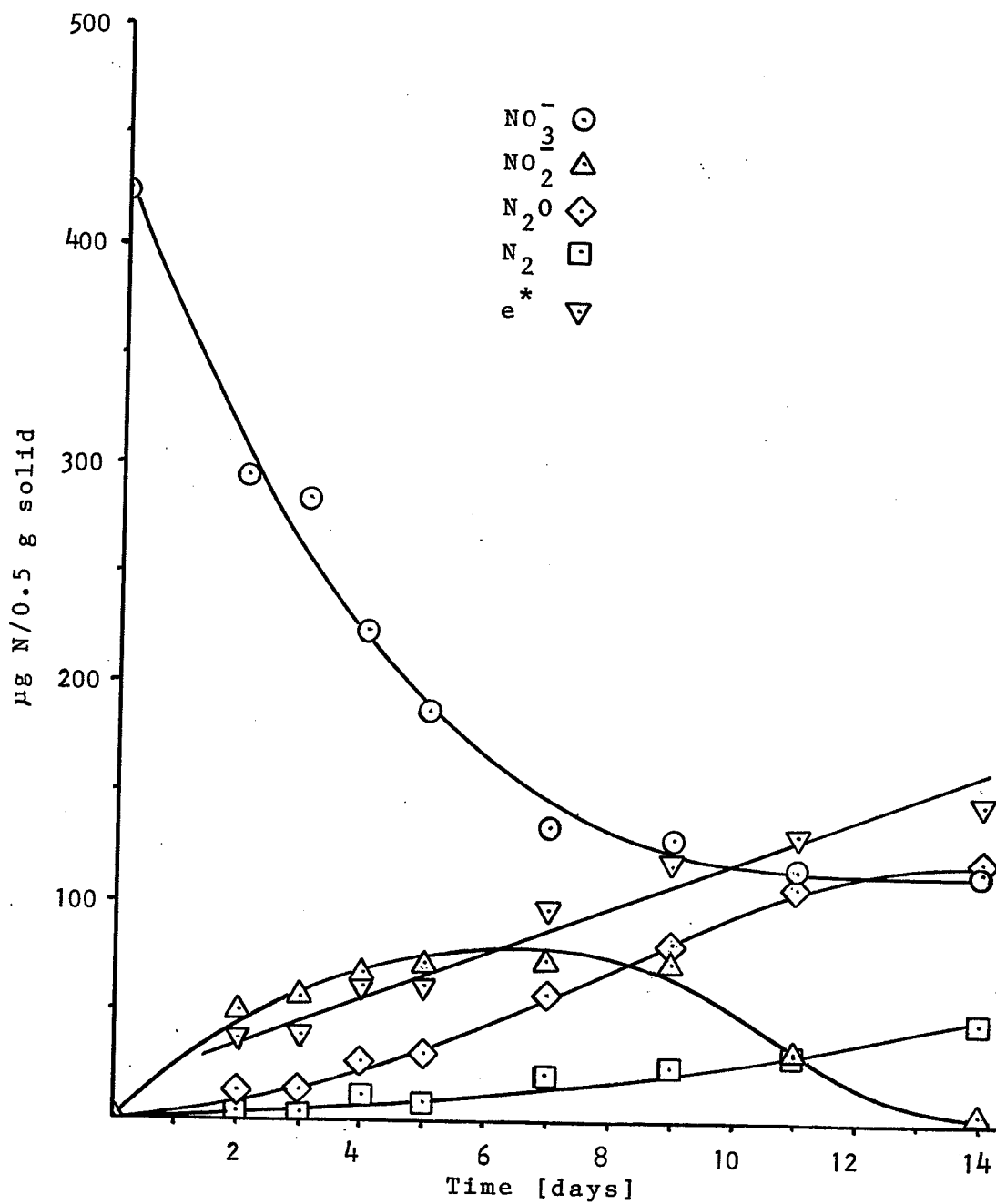
Figure 11: Concentration of nitrate, nitrite, nitrous oxide, and nitrogen gas during incubation of lake sediment at 10C with 15 ppm added nitrate-N and 20% lime

incubated at 10C in Experiment I [0.5 g and 1.0 g solid with 15 ppm added nitrate-N]. The maximum concentration of nitrite-N attained depended on the amount of lime added. For 2% addition, the nitrite maximum was 52.6 ug N/0.5 g solid; for 5%, 60.7 ug N/0.5 g solid; for 10%, 64.2 ug N/0.5 g solid; and for 20%, 72.9 ug N/0.5 g solid. There was a tendency toward dependence of time of nitrite maximum on level of lime added. Maximum nitrite accumulation occurred at four days of incubation with 2% lime, six days with 5% lime, five days with 10% lime, and seven days with 20% lime. However, the nitrite concentration was reduced to a low level after two weeks of incubation in all lime treatments.

The effect of lime on nitrous oxide evolution from sediment, apparent in Figures 10 and 11, was similar with all levels of addition. Due to the large accumulation of nitrite, the initial rate of nitrous oxide production was very slow. This resulted in a gently rising curve rather than the fast rising nitrous oxide accumulation curve observed with unlimed sediment [Figure 6]. Although the measured maximum [approximately 120 ug N_2O-N/0.5 g solid] occurred on the last day of incubation in every case, the data indicated that the actual maximum was being approached, since the rate of accumulation was decreasing [Figures 10 and 11, Table 6]. The maximum measured in unlimed sediment was 97.4 ug N_2O-N/0.5 g solid [Figure 6].

The evolution of nitrogen gas by sediment samples was not as greatly affected by addition of lime as were the production and reduction of its precursors. With 2 and 5% additions of lime [Table 6 and Figure 10, respectively], equal or larger amounts of nitrogen gas were measured on each sampling day when compared with unlimed sediment [Figure 6]. However, the amounts detected from samples taken after nine days of incubation with 10 and 20% additions of lime [Table 6 and Figure 11, respectively] were less than from unlimed sediment after a comparable time period. There was a tendency toward less nitrogen gas accumulation after 14 days of incubation with increased addition of lime.

The values of e^* for each lime treatment were calculated and plotted or tabulated against time [Figures 10 and 11, Table 6]. The slope of the regression line over the entire incubation period for each treatment was taken as Re^* for that treatment. For 2% addition of lime, Re^* was 12.3 ug N-equivalent/0.5 g solid-day; for 5%, 11.1 ug N-equivalent/0.5 g solid-day; for 10%, 11.4 ug N-equivalent/0.5 g solid-day; and for 20%, 9.97 ug N-equivalent/0.5 g solid-day. The Re^* previously calculated for unlimed sediment was 7.06 ug N-equivalent/0.5 g solid-day. Thus, there was a greater electron acceptance with time by nitrogenous oxides in sediment to which lime had been added. Also, there was a tendency toward greater stimulation of electron acceptance rate with smaller additions of lime.

Many researchers reported an increase in denitrification rate with an increase in pH resulting from the addition of lime to soil. However, Hauck and Melsted (1956) failed to measure an increase in nitrate loss when the reaction of soil was adjusted from pH 5.9 to pH 7.2 by the addition of lime. Cady and Bartholomew (1960) observed a very large increase in maximum accumulation of nitrite when lime was added to acid soil. An increase in the maximum of nitrite accumulation occurred in flooded soil which was limed (Cho and Sakdinan 1978). However, liming of soil increased the rate of nitrous oxide reduction, which caused less maximum accumulation of this gas and an increase in rate of nitrogen gas production (Bollag et al. 1973; Cho and Sakdinan 1978; Hauck and Melsted 1956; Khdyer 1978; Wijler and Delwiche 1954).

The pH of sewage sludge dropped rapidly after the addition of hydrochloric acid, but stabilized around pH 6.5 after three days [Table 7].

TABLE 7

Change in pH resulting from the addition of hydrochloric acid to sewage sludge

Day	0	1	2	3	4	6	9	16
pH	3.9	5.7	6.1	6.5	6.6	6.5	6.7	6.4

Nitrate disappeared slowly from 1.0 g [solid] sewage sludge samples acidified with hydrochloric acid and incubated at 10C with 20 ppm added nitrate-N [Table 8]. The rate of depletion was 20.2 ug NO_3^--N/g solid-day [$r^2=0.80$]. This was half of the nitrate depletion rate calculated for the same weight of non-acidified sludge incubated under similar conditions in Experiment I [Figure 7]. The nitrite concentration in the acidified sludge [Table 8] did not increase to the same extent as that in non-acidified sludge [Table 3]. In both incubations, nitrite concentration became constant at a relatively low level, but this occurred earlier and at a slightly lower value with the acidified sludge.

Mass spectrometric analysis detected nitrogen gas as the only gaseous denitrification product in the sample atmosphere above acidified sewage sludge. After two weeks of incubation, 300 ug N_2-N/g solid were measured [Table 8]. After the same period, non-acidified sludge produced 508 ug N_2-N/g solid [Table 3]. A four day lag period occurred in the nitrogen production from acidified sludge, while a one day lag was observed in the non-acidified treatment. The rate of nitrogen gas production after the lag was less in the former than in the latter.

Re^* was calculated as the slope of the regression line of e^* versus time over the two week incubation period

TABLE 8

Concentration of nitrate, nitrite, and nitrogen gas during
incubation of acidified sewage sludge at 10C with 20 ppm
added nitrate-N

Day	NO_3^-	NO_2^-	N_2	e*
		[ug N/g solid]		
0	983.0	6.89	–	–
1	983.0	6.29	6.14	8.66
2	935.0	4.50	9.09	10.9
4	935.0	4.80	41.0	42.9
6	923.0	3.30	88.0	89.3
8	797.0	2.61	175.0	176.0
10	857.0	3.00	159.0	160.0
12	809.0	3.90	222.0	224.0
14	665.0	4.00	300.0	302.0

$[r^2=0.96]$. It was found to be 22.2 ug N-equivalent/g solid-day, which was slightly less than two thirds of Re* calculated for the non-acidified sludge [Figure 7].

Although lowering of the pH of sludge samples resulted in slower rates of nitrate loss and electron acceptance during denitrification, nitrous oxide was not detected as an intermediate in the process.

4.4   EXPERIMENT IV: EFFECT OF NITRATE CONCENTRATION

Nitrate disappearance with time from 0.5 g [solid] sediment samples incubated at 10C with different concentrations of added nitrate was better described by first-order than by zero-order kinetics in all cases. However, the initial rate of nitrate depletion was fairly constant with time. The slopes of the regression lines of nitrate concentration with time [Figure 12], taken as first-order rate constants, exhibited a slight inverse dependence on added nitrate concentration [Table 9]. However, if nitrate loss with time were a true first-order reaction, then the rate constants would be independent of added nitrate concentration. The rate constant previously calculated for 15 ppm nitrate-N added to 0.5 g [solid] lake sediment, which was incubated at 10C in Experiment I, was 0.085 day^{-1}. Therefore, the rate of nitrate loss from lake sediment was a function of added nitrate concentration. Similar first-order denitrification rates were observed in flooded soil and were thought to result from a diffusion-limited process (Engler et al. 1976; Reddy et al. 1978). Nitrate diffusion from floodwater into sediment was dependent on nitrate concentration in the floodwater (Bouldin et al. 1974).

Nitrite accumulation was similar for all incubations and no dependence on concentration of added nitrate was discernible [Table 10]. There was a slight, initial

Figure 12: Concentration of nitrate during incubation of lake sediment at 10C with different amounts of added nitrate-N

TABLE 9

Rates of nitrate depletion and electron acceptance during
incubation of lake sediment at 10C with different amounts of
added nitrate-N

Added NO_3^--N	Rate of NO_3^- depletion	Rate of N_2 production	Re*
ppm	day^{-1}	[ug N/0.5 g solid-day]	
2.5	0.142	2.76	2.65
5	0.110	5.09	4.73
10	0.108	13.4	13.1

increase in accumulation of nitrite with time until a
maximum was reached within the first four days of
incubation. After this peak, the amount of nitrite present
in the sample solution decreased with time to a relatively
constant value. The concentration of nitrite-N was low in
all samples, relative to that measured for the other
components of denitrification.

Nitrous oxide was the first nitrogenous gas to accumulate
and was detected in all samples [Figure 13]. The trend in
its accumulation was similar in each incubation series. The
quantity of nitrous oxide rapidly increased with time to a
maximum and then quickly decreased. The rate of nitrous
oxide disappearance then slowed and a relatively constant,
steady-state accumulation was observed. The time of

TABLE 10

Concentration of nitrite during incubation of lake sediment
at 10C with varying concentration of added nitrate-N

Day	Added NO_3^--N		
	2.5 ppm	5 ppm	10 ppm
	[ug NO_2^--N/0.5 g solid]		
0	0.29	0.30	0.23
0.5	1.16	-	-
1	1.45	1.82	-
1.5	0.58	-	-
2	1.74	2.12	1.95
3	-	3.33	1.28
4	1.16	1.52	1.10
5	-	0.91	1.02
6	0.58	0.91	1.13
7	-	-	0.76
8	0.58	0.61	0.84
10	0.58	0.61	0.79
12	0.58	0.61	0.55
14	0.58	0.61	-
16	0.29	0.61	-
18	0.29	0.30	-

occurrence of the peak and the amount accumulated at the peak were directly dependent on the concentration of added nitrate-N. The maximum accumulation measured for 2.5 ppm added nitrate-N was 8.83 ug $N_2O-N/0.5$ g solid after one day of incubation; for 5 ppm, 28.6 ug $N_2O-N/0.5$ g solid after three days; and for 10 ppm, 71.3 ug $N_2O-N/0.5$ g solid after six days. The amount of nitrous oxide present during the steady-state stage was also directly dependent on the level of added nitrate-N. For 2.5 ppm added nitrate-N, the amount of nitrous oxide present during steady-state accumulation was approximately 4 ug N/0.5 g solid; for 5 ppm, it was approximately 15 ug N/0.5 g solid; and for 10 ppm, it was approximately 50 ug N/0.5 g solid. However, the initial rapid rate of production of nitrous oxide was very similar in every case and was approximately 10 ug N/0.5 g solid-day. In Experiment I, when the added nitrate-N concentration was 15 ppm [Figure 6], the maximum of 97.4 ug $N_2O-N/0.5$ g solid occurred after 12 days of incubation and the amount present during steady-state accumulation was approximately 75 ug $N_2O-N/0.5$ g solid. The initial rate of nitrous oxide accumulation was previously calculated to be 12.1 ug $N_2O-N/0.5$ g solid-day. These results were consistent with those observed in the present experiment.

The initial rate of nitrous oxide production was not a function of added nitrate concentration. However, time of maximum accumulation of nitrous oxide and the magnitude of the maximum were dependent on added nitrate concentration.
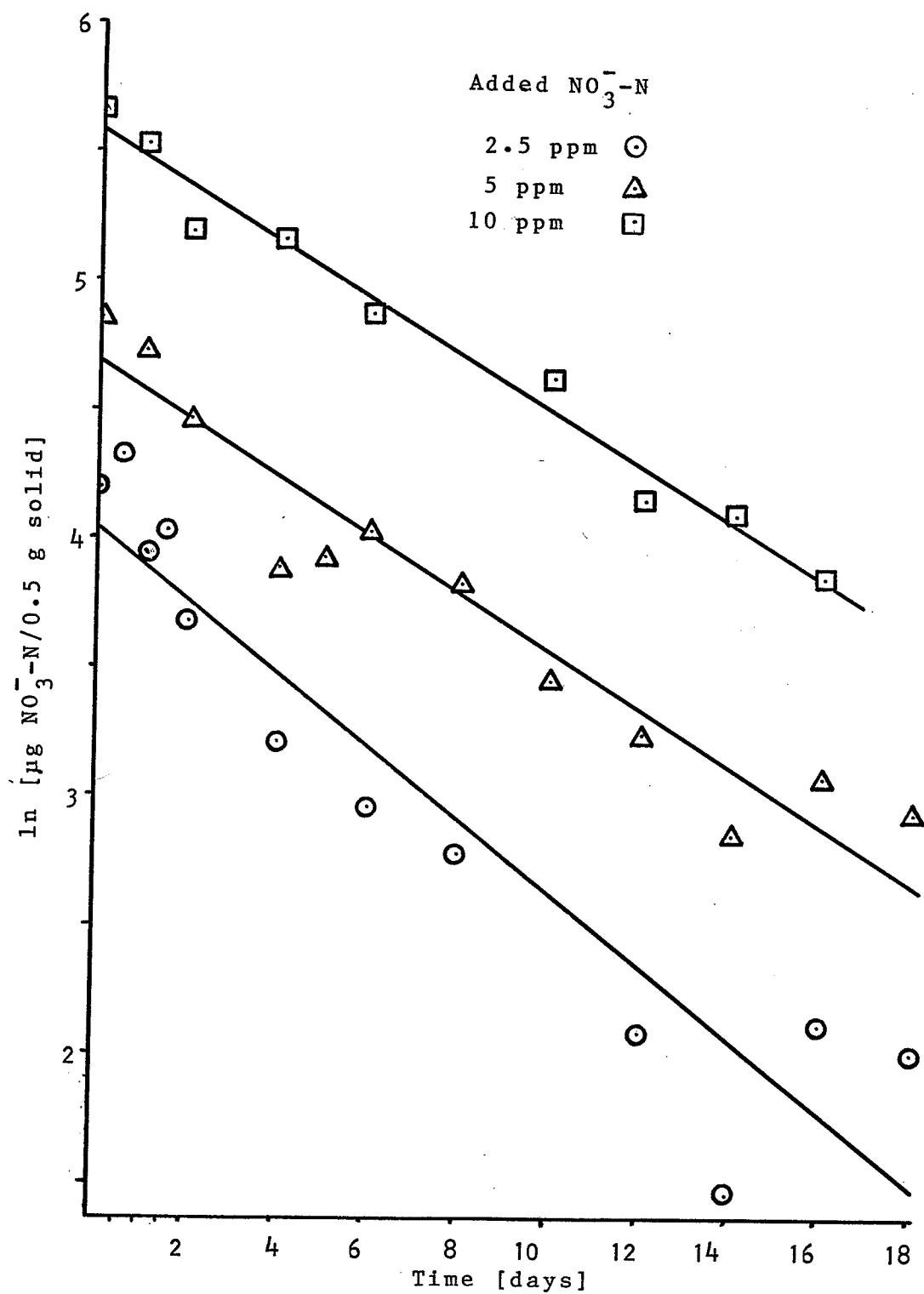
Figure 13:   Concentration of nitrous oxide during incubation
of lake sediment at 10C with different amounts
of added nitrate-N

Similar results were reported for nitrous oxide production from soil (Cho and Sakdinan 1978; Cooper and Smith 1963; Wijler and Delwiche 1954).

The amount of nitrogen gas measured on the first day of incubation was inversely dependent on the concentration of added nitrate-N [Figure 14]. This relationship was also observed in flooded soil when nitrous oxide accumulated as an intermediate in denitrification (Cho and Sakdinan 1978). For 2.5 ppm added nitrate-N, 2.65 ug $N_2O-N/0.5$ g solid were detected; for 5 ppm, 1.52 ug $N_2O-N/0.5$ g solid were detected; and for 10 ppm, 0.450 ug $N_2O-N/0.5$ g solid were detected. There was no lag apparent in the nitrogen gas production when the concentration of added nitrate-N was 2.5 or 5 ppm. However, a lag of four days was observed with 10 ppm added nitrate-N. During this period, less nitrogen gas was evolved than in the other incubations. Previously, a lag period of eight days was observed when the added nitrate-N concentration was 15 ppm [Experiment I]. The lag period before major production of nitrogen gas was also observed to be dependent on added nitrate concentration in two flooded soils (Khdyer 1978). The rates of substantial nitrogen gas production were directly dependent on the concentration of added nitrate-N [Table 9]. After 16 days of incubation, the amount of nitrogen gas produced by sediment, to which 2.5, 5, and 10 ppm nitrate-N were added, was 47.6, 79.2, and 169 ug N/0.5 g solid, respectively. A

similar dependence of Re^* on added nitrate-N concentration was observed [Table 9]. Re^* was 2.65 ug N-equivalent/0.5 g solid-day for 2.5 ppm added nitrate-N and 13.1 ug N-equivalent/0.5 g solid-day for 10 ppm. Re^* of 7.06 ug N-equivalent/0.5 g solid-day from Experiment I, when 15 ppm nitrate-N were added, was off the trend.

Nitrate disappearance with time was better described by first-order than by zero-order kinetics, when 1.0 g [solid] sewage sludge was incubated at 10C with 10 ppm added nitrate-N. The rate constant was 0.054 day^{-1}. Nitrate depletion rates were zero-order when the added nitrate-N concentration was incremented by 10 ppm from 10 ppm to 50 ppm. The rate of nitrate depletion was relatively constant for the different levels of addition of nitrate-N, except for 30 ppm [Table 11]. In flooded sediment columns with a solid depth of 1 to 2.5 cm, the bottom of the column was viewed as a barrier to diffusion (Sain et al. 1977). This was thought to cause a nearly uniform nitrate concentration throughout the shallow depth of sediment, which would minimize the effects of nitrate diffusion on denitrification.

The trend in nitrite accumulation with time varied little among the five levels of nitrate addition. It consisted of an initial increase in nitrite concentration to approximately 20 ug NO_2^--N/g solid on the second day of

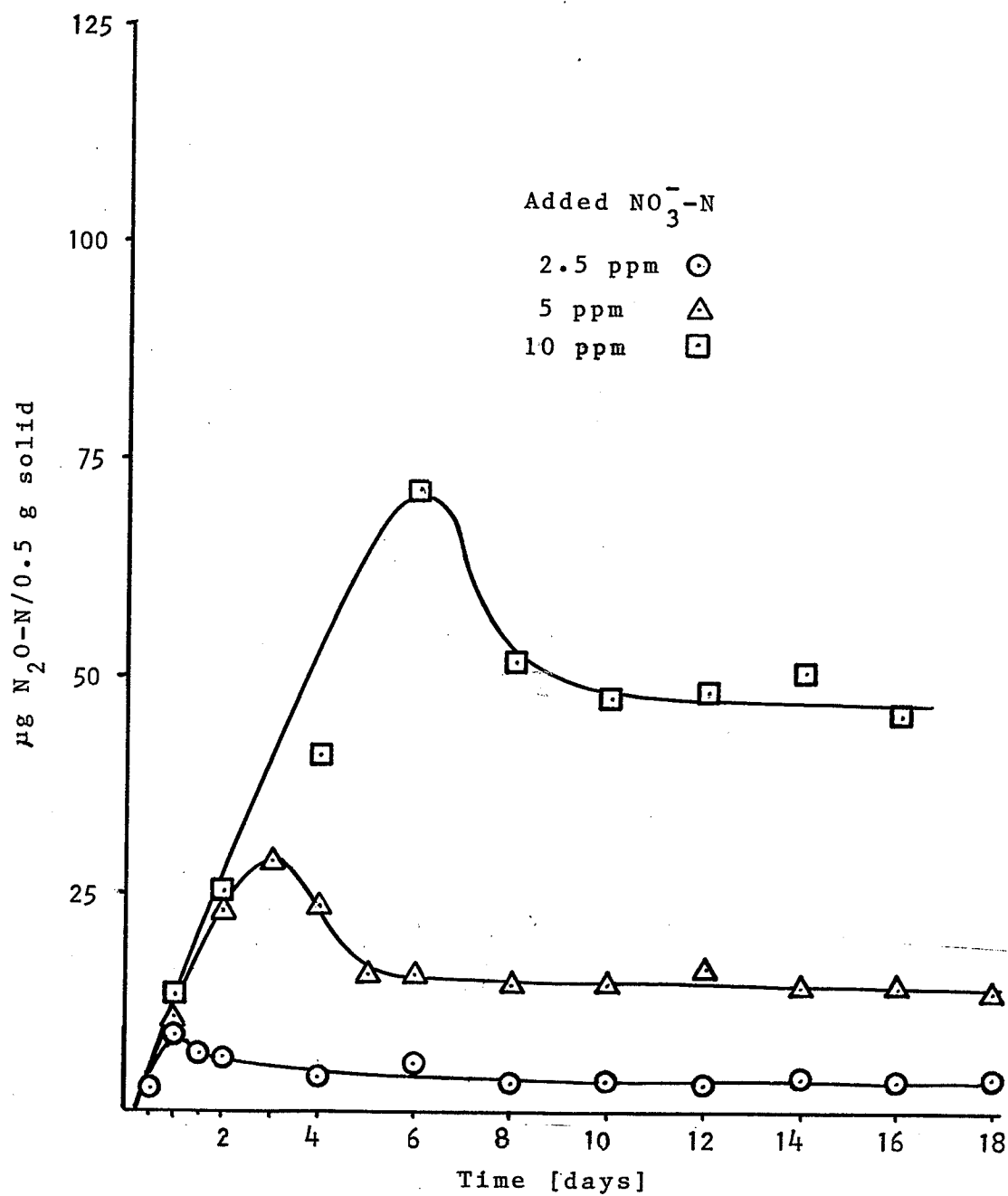Figure 14: Concentration of nitrogen gas during incubation of lake sediment at 10C with different amounts of added nitrate-N

TABLE 11

Rates of nitrate depletion and electron acceptance during incubation of sewage sludge at 10C with different amounts of added nitrate-N

Added NO_3^--N	10 ppm	20 ppm	30 ppm	40 ppm	50 ppm
		[ug N/g solid-day]			
Rate of NO_3^- depletion	-	41.2	24.0	36.1	37.2
Re*	25.6	35.6	35.2	45.1	42.8

incubation, then a gradual decrease with time to a steady-state value between 5 and 7 ug NO_2^--N/g solid after eight days of incubation.

Mass spectrometric analysis revealed evolution of nitrogen gas in all samples. No other nitrogenous gas was detected in the sample atmospheres. The amount of nitrogen gas produced on each sampling day during incubation of sludge with 10 ppm added nitrate-N was similar to that produced with 20 ppm added nitrate-N. In the same way, the quantity of nitrogen gas produced with 40 ppm added nitrate-N was similar to that produced with 50 ppm added nitrate-N. Nitrogen gas production with time during the incubation of sludge was sigmoidal for all added nitrate-N concentrations [Figure 15]. A slight dependence of rate of nitrogen gas

production on added nitrate-N concentration was evident when the latter was increased from 20 to 50 ppm. The initial lag in nitrogen gas production was similar in all treatments, but this was probably due to the use of air-dried sludge. The microbial population may have required some time to become established.

Although the rate of nitrate loss from sludge with 10 ppm added nitrate-N was first-order, it was zero-order with added nitrate-N concentrations of 20 to 50 ppm. A similar change in the order of denitrification rate with increasing concentration of nitrate was observed in lake sediment (van Kessel 1977a). Zero-order rates of nitrate depletion were observed in flooded soils (Cho and Sakdinan 1978; Khdyer 1978) and lake sediment (Terry and Nelson 1975). The lag before nitrogen gas production from sludge was similar with all concentrations of added nitrate. There was a slight increase in rate of nitrogen gas production with an increase of added nitrate-N concentration from 20 to 50 ppm, although the rate of nitrate disappearance was zero-order. However, the rate of a true zero-order reaction is not dependent on the concentration of reactant. When nitrogen gas was the sole gaseous product of denitrification in flooded soil, its rate of production and length of lag time before production were not functions of added nitrate concentration (Khdyer 1978).
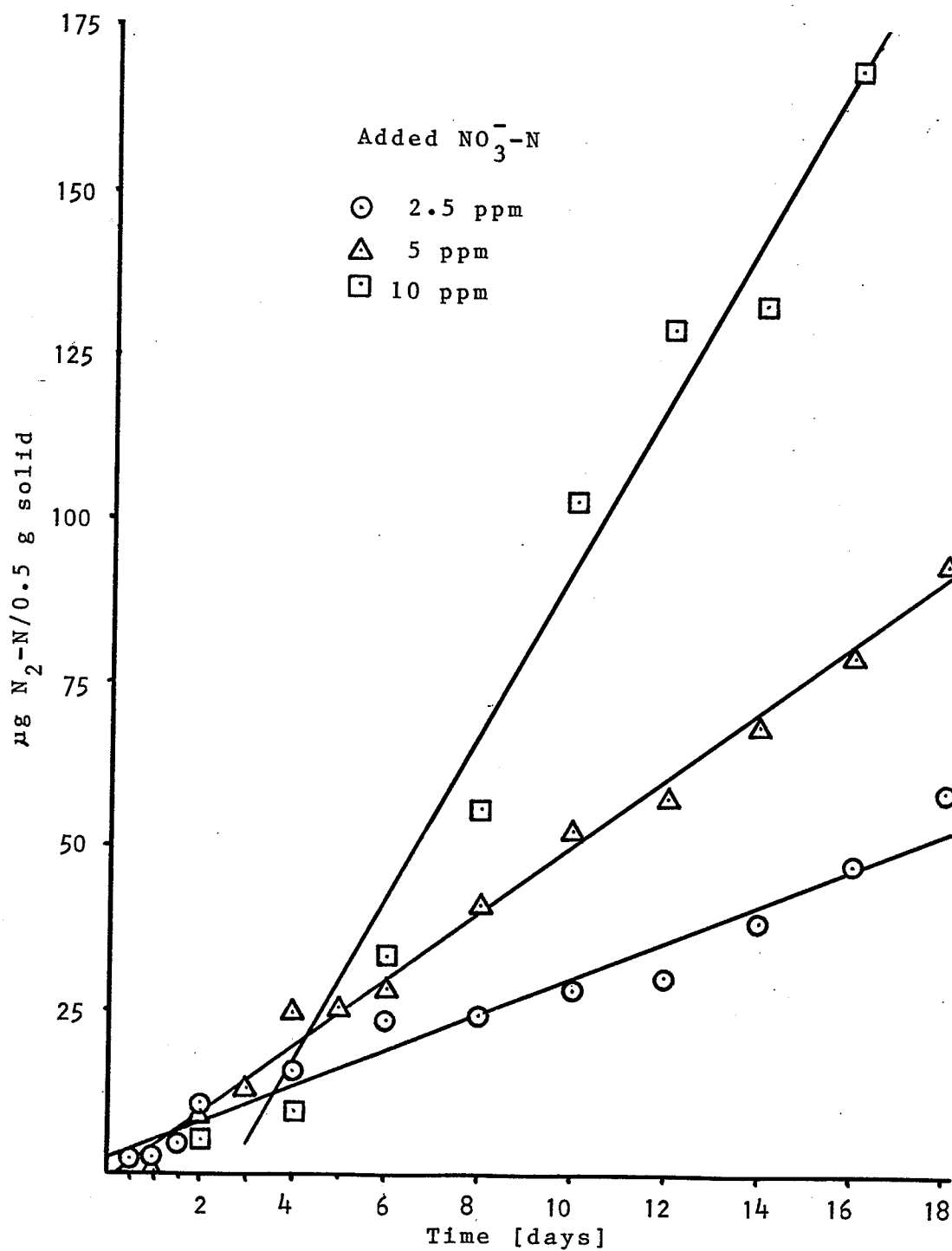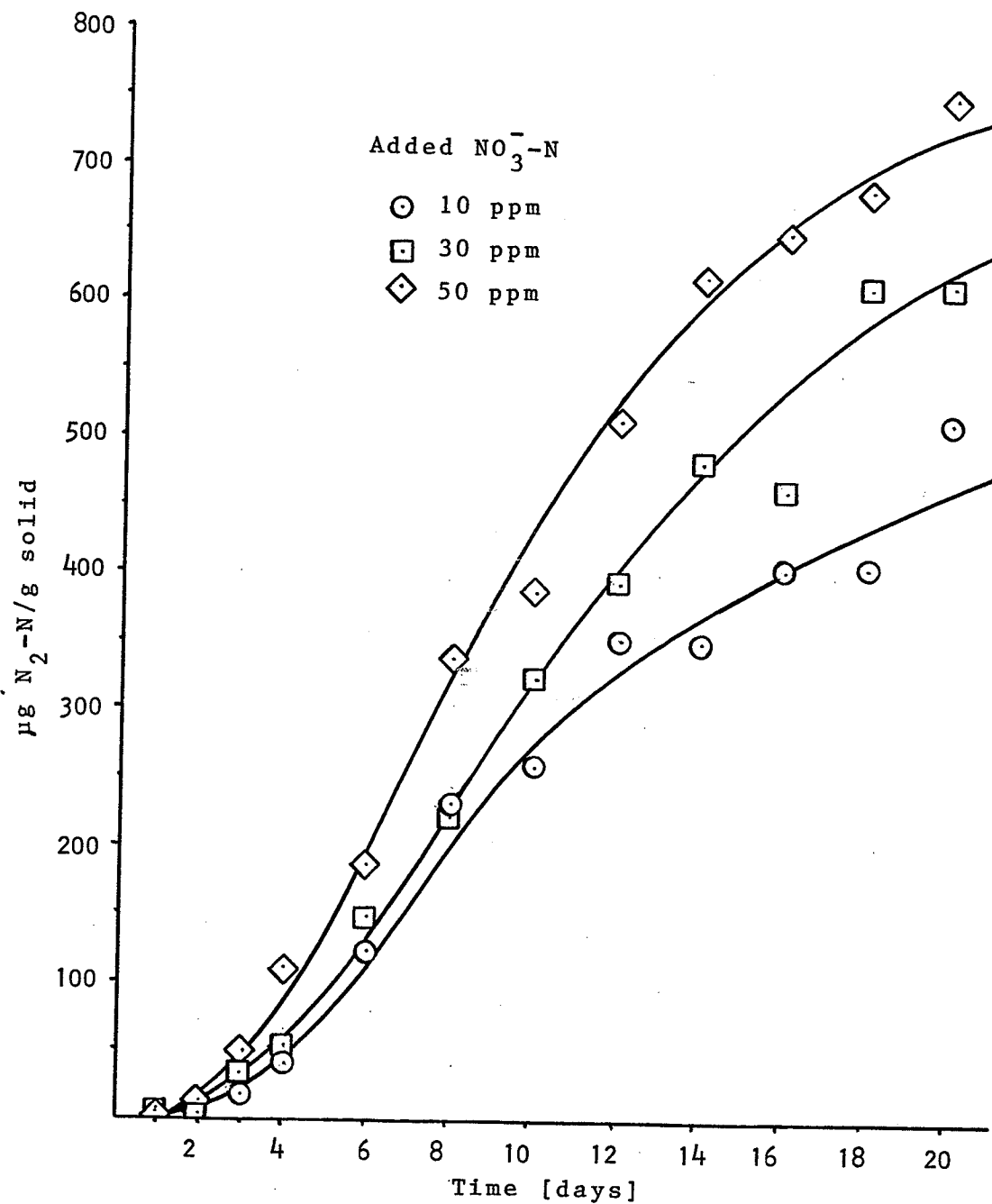
Figure 15: Concentration of nitrogen gas during incubation of sewage sludge at 10C with different amounts of added nitrate-N

Re^* was calculated from the regression line of e^* versus time for each incubation of sludge with a different level of added nitrate-N [Table 11]. In each incubation, the e^* value was very similar to the amount of nitrogen gas measured on the same day, due to the minor contribution of nitrite. Consequently, trends in Re^* were similar to those which occurred in nitrogen gas production with time. There was a tendency toward slight dependence of Re^* on added nitrate-N concentration. Re^* for 10 ppm added nitrate-N was much less than for the 20 ppm level. Re^* values for 20 and 30 ppm added nitrate-N treatments were almost the same, and Re^* values for 40 and 50 ppm added nitrate-N concentrations were similar.

Nitrate depletion with time in lake sediment was dependent on added nitrate concentration in the range of 2.5 to 10 ppm. Similarly, the rates of nitrogen gas production and e^* were first-order. With a range of 10 to 50 ppm added nitrate-N in sewage sludge, a first-order rate of nitrate loss occurred with the 10 ppm level. However, zero-order rates of nitrate removal were observed with the higher levels of nitrate addition, although the rates of nitrogen gas production and the Re^* values were slightly dependent on added nitrate concentration.

The depth of the lake sediment layer [0.5 g solid] was 33 mm, while the sludge layer [1.0 g solid] was only 12 mm

deep. The initial concentration of nitrate in the sludge samples was higher due to a greater endogenous concentration and a larger addition of labeled nitrate. It seems probable that the shallow solid depth and relatively high nitrate concentration effectively eliminated the effect of a diffusion-limited supply of nitrate from the supernatant to the sludge. Thus, a near zero-order rate of nitrate disappearance resulted from an almost uniform nitrate concentration.

4.5   EXPERIMENT V: EFFECT OF LOW TEMPERATURE

The rates of nitrate disappearance from 0.5 g [solid] lake sediment incubated at 4C with 2.5 and 5 ppm added nitrate-N were better described as first-order. Under an argon atmosphere, the rate constants for nitrate loss with time were 0.261 day^{-1} [r^2=0.97] and 0.150 day^{-1} [r^2=0.96], for 2.5 and 5 ppm added nitrate-N, respectively. When air was the incubation atmosphere, the rate constant for nitrate disappearance with time for 5 ppm added nitrate-N was 0.134 day^{-1} [r^2=0.95]. This indicated that the difference in composition of incubation atmosphere exerted virtually no effect on the rate of nitrate disappearance. Incubation of sediment under an air atmosphere at 4C with 2.5 ppm added nitrate-N was not attempted because of the possible errors in mass spectrometric determination of produced gases. The rate constants obtained at 4C were greater in magnitude than

the corresponding values obtained at 10C [Table 9] in Experiment IV: 0.261 day^{-1} versus 0.142 day^{-1} for 2.5 ppm added nitrate-N and 0.134 day^{-1} versus 0.110 day^{-1} for 5 ppm. It is not fully understood why the rate of nitrate disappearance was faster under colder conditions. However, as Cho and Sakdinan (1978) and Cho and Mills (1979) pointed out, the rate of nitrate disappearance under steady microbial activity may change depending on the presence or absence, and magnitude of accumulation of intermediate nitrogenous oxides.

The maxima of nitrite accumulation occurred on the first day of all incubations at 4C [2.5 ppm added nitrate-N, Figure 16 and 5 ppm, Table 12]. The amount of nitrite present during steady-state accumulation at 4C was greater than in incubations with the same concentrations of added nitrate at 10C in Experiment IV [Table 10].

Nitrous oxide and nitrogen gas were measured from mass spectrometric scans of the atmospheres over sediment incubated at 4C. No other nitrogenous gas was detected in samples incubated under an argon atmosphere. The initial rates of accumulation of nitrous oxide with time from lake sediment incubated under argon at 4C with 2.5 ppm [Figure 16] and 5 ppm added nitrate-N [Table 12] were similar. Nitrous oxide rapidly accumulated with time to a maximum and then quickly decreased until a relatively constant steady-state rate of accumulation was reached. The
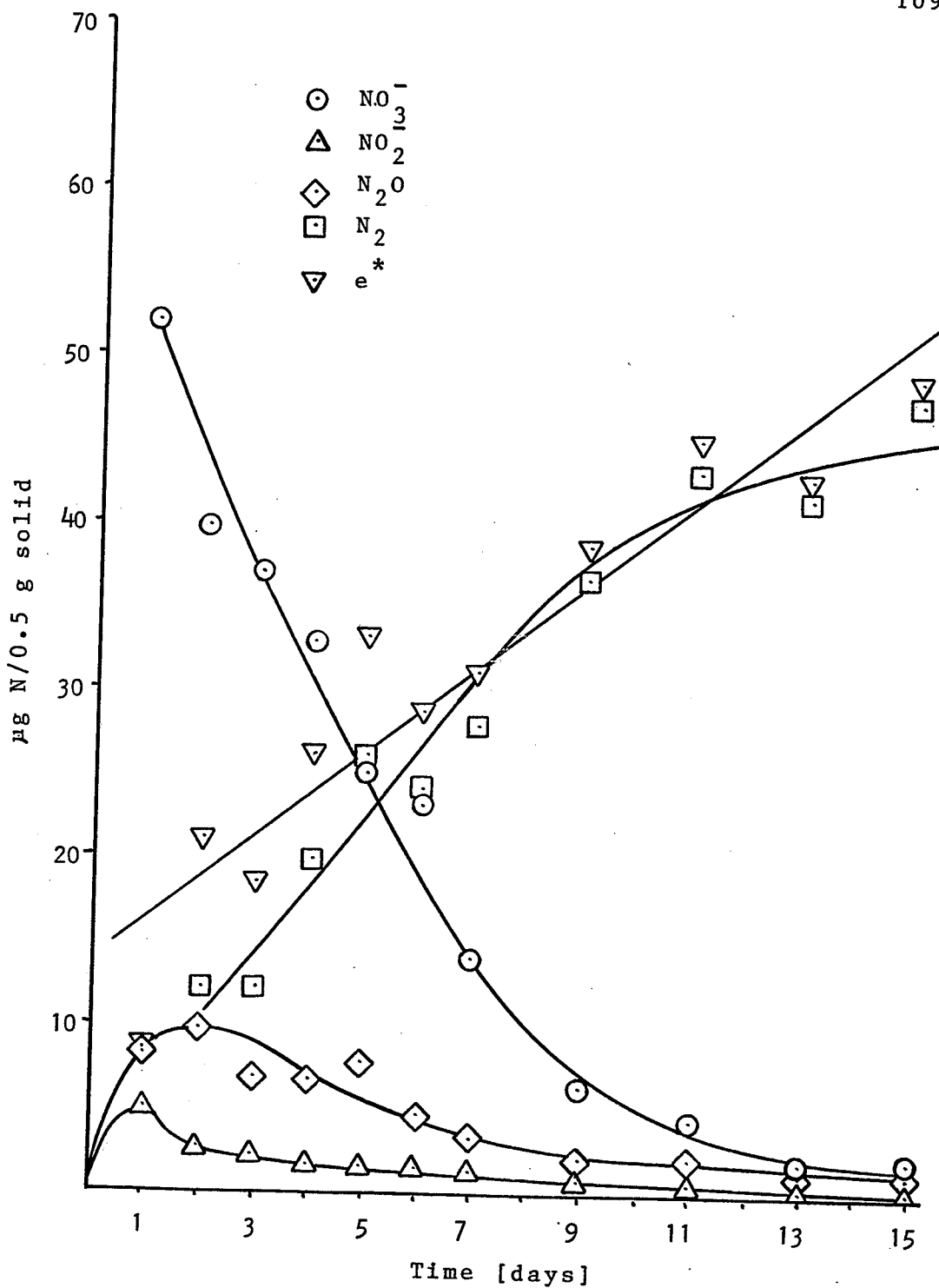
Figure 16: Concentration of nitrate, nitrite, nitrous oxide, and nitrogen gas during incubation of lake sediment at 4C with 2.5 ppm added nitrate-N under an argon atmosphere

TABLE 12

Concentration of nitrite, nitrous oxide, and nitrogen gas
during incubation of lake sediment at 4C with 5 ppm added
nitrate-N under air and argon atmospheres

	Air atmosphere				Argon atmosphere			
Day	NO_2^-	N_2O	N_2	e^*	NO_2^-	N_2O	N_2	e^*
				[ug N/0.5 g solid]				
1	2.88	15.9	3.40	17.3	3.63	8.52	0	7.74
2	2.50	26.1	4.43	26.3	3.63	19.0	9.61	26.3
3	1.63	38.3	12.1	43.4	2.96	24.1	10.2	30.7
4	1.98	27.0	13.6	36.0	2.01	27.0	16.5	38.9
5	-	-	-	-	2.30	25.5	19.4	40.7
6	1.54	24.6	27.8	48.1	1.86	21.5	20.9	38.8
7	-	-	-	-	1.42	23.4	36.7	56.0
8	1.54	29.0	35.2	59.0	-	-	-	-
9	-	-	-	-	1.13	19.0	45.2	60.9
10	1.08	20.4	45.7	62.5	-	-	-	-
11	-	-	-	-	0.79	17.5	49.4	63.7
12	1.28	23.6	60.5	79.9	-	-	-	-
13	-	-	-	-	0.64	13.9	60.2	71.6
14	-	-	-	-	0.52	14.0	80.4	91.8

maximum accumulation was greater and occurred later in the incubation with 5 ppm added nitrate-N. Also, the amount of nitrous oxide present during steady-state accumulation was greater. These trends were similar to those previously observed during incubation of lake sediment under an air atmosphere at 10C with varying amounts of nitrate [Figure 13]. The magnitude of the maximum of nitrous oxide accumulation, under an air atmosphere, was greater at 4C [38.3 ug N/0.5 g solid] than at 10C [28.6 ug N/0.5 g solid], but both maxima occurred on the third day of incubation. However, the initial rate of nitrous oxide accumulation and the amount of nitrous oxide present during the steady-state stage were both greater at 4C.

Nitrogen gas was not detected on the first day of incubation of sediment at 4C with 2.5 and 5 ppm added nitrate-N under an argon atmosphere, but relatively large amounts were measured on the second day [Figure 16]. The rate of production of nitrogen gas was greater with 5 ppm added nitrate-N, as was previously observed in Experiment IV [Figure 14]. A lag period of two days before major nitrogen gas production was observed in the incubation with 5 ppm added nitrate-N under an air atmosphere at 4C [Table 12]. In Experiment IV, there was no lag period observed before the production of nitrogen gas from sediment incubated at 10C with 5 ppm added nitrate-N [Figure 14]. Although similar amounts of nitrogen gas were measured on each

sampling day from the two incubations with 5 ppm added nitrate-N at 4C [Table 12], a slightly greater amount was measured from the incubation at 10C [Figure 14].

Re^* was calculated over the entire sampling period for each incubation. For the incubation of sediment under an air atmosphere at 4C, Re^* was 4.99 ug N-equivalent/0.5 g solid-day [r^2=0.94], while under an argon atmosphere it was 4.98 ug N-equivalent/0.5 g solid-day [r^2=0.94]. Re^* for the incubation under argon at 4C with 2.5 ppm was 2.52 ug N-equivalent/0.5 g solid-day [r^2=0.89]. In Experiment IV, where the incubation temperature was 10C, Re^* for sediment incubated with 5 ppm added nitrate-N under an air atmosphere was previously calculated to be 4.73 ug N-equivalent/0.5 g solid-day [r^2=0.97]. Re^* for a similar incubation with 2.5 ppm added nitrate-N was 2.65 ug N-equivalent/0.5 g solid-day [r^2=0.96]. Surprisingly, Re^* values for both 2.5 and 5 ppm added nitrate-N at 4 and 10C were almost equal. Denitrification in soil and sediment at low temperatures [5C or less] was previously known (Cho et al. 1979; Gilliam and Gambrell 1978; Goering and Dugdale 1966; Terry and Nelson 1975; van Kessel 1977a). However, the rate of a biological process generally decreases with a decrease in temperature. This was not the case in the present experiment and it is not fully known why the rate was unaffected by a change in temperature.

Nitrate depletion with time from 1.0 g [solid] sewage sludge incubated at 4C with 20 ppm added nitrate-N was a zero-order reaction with a rate of 19.3 ug N/g solid-day $[r^2=0.97]$. In Experiment I, when 1.0 g [solid] sludge was incubated at 10C with 20 ppm added nitrate-N, the nitrate removal rate was calculated to be 42.1 ug N/g solid-day [Figure 7].

Nitrite accumulated with time to a maximum of 32.0 ug N/g solid on the seventh day of incubation [Figure 17]. It then slowly decreased with time until a steady-state rate of accumulation was reached. The maximum of nitrite accumulation was much less and occurred on the third day of incubation during denitrification in sludge at 10C with 20 ppm added nitrate-N in Experiment I [Table 3]. Also, the amount of nitrite present during steady-state accumulation was less at 10C.

Mass spectrometric analysis revealed small amounts of nitrous oxide accumulated in the atmospheres over all sludge samples incubated at 4C [Figure 17]. This was not the case with sludge incubated at 10C in Experiments I and IV. Nitrous oxide slowly accumulated with time to a maximum of 20.4 ug N/g solid on the eighth day of incubation—a day after the maximum of nitrite accumulation. It then decreased until a steady-state rate of accumulation was reached after 12 days of incubation. The shape of the curve
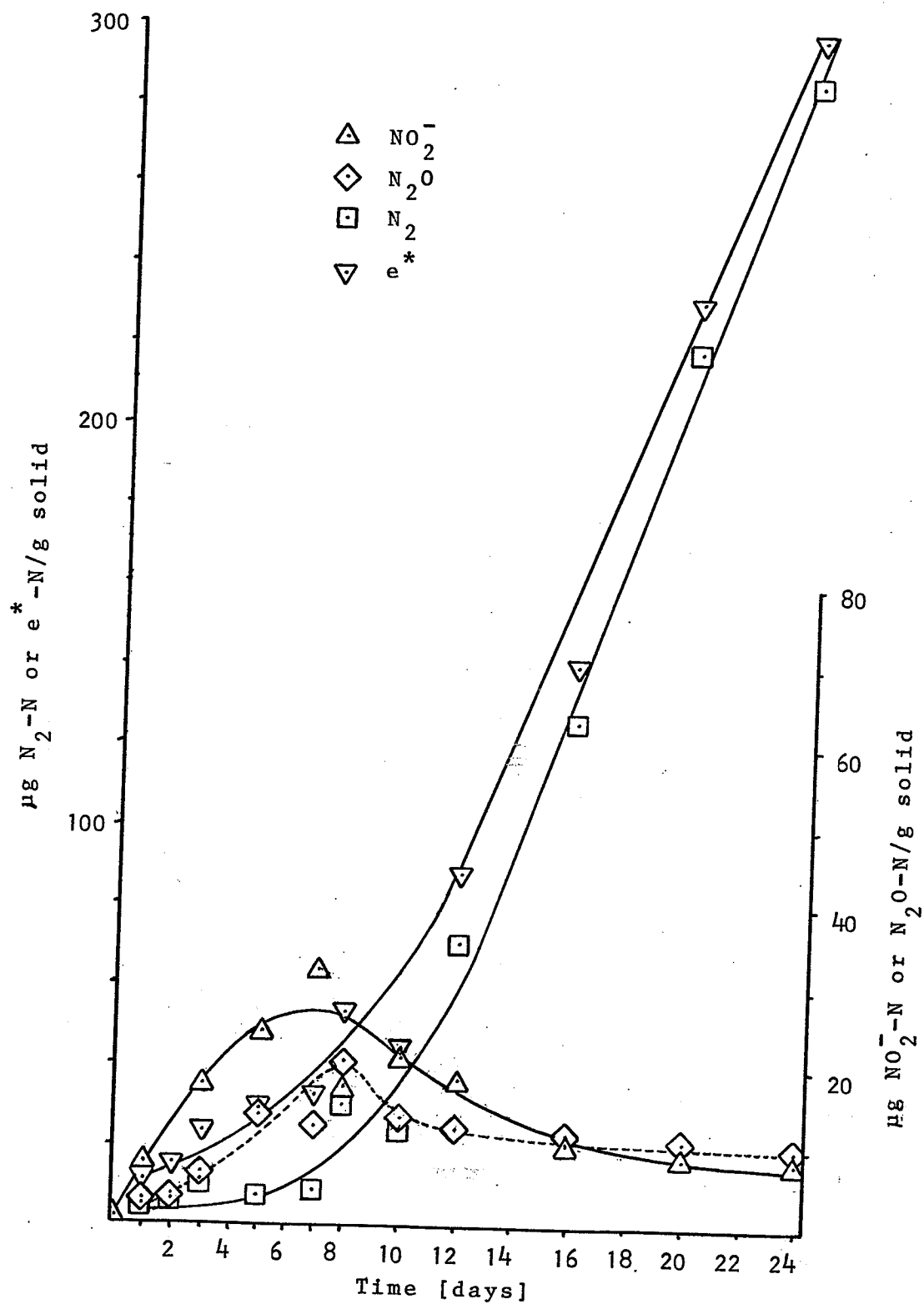
Figure 17: Concentration of nitrite, nitrous oxide, and nitrogen gas during incubation of sewage sludge at 4C with 20 ppm added nitrate-N

describing nitrous oxide accumulation with time was similar to the family of curves depicting nitrous oxide accumulation with time from lake sediment at 10C in Experiment IV [Figure 13]. During denitrification in sludge at 4C, the concentration of nitrous oxide was less than that of nitrite until the sixteenth day of incubation [Figure 17].

Small amounts of nitrogen gas were detected in the atmospheres over sludge samples incubated at 4C for the first seven days of incubation [Figure 17]. After this lag period, the rate of nitrogen gas production increased rapidly to approximately 20 ug N/g solid-day. There was little lag period before nitrogen gas was produced at a rate of approximately 40 ug N/g solid-day from sludge incubated at 10C with 20 ppm added nitrate-N in Experiment I.

Re^*, calculated over the entire incubation period, was 12.2 ug N-equivalent/g solid-day [$r^2=0.92$]. Re^*, previously calculated for sludge incubated at 10C [Figure 7], was 35.7 ug N-equivalent/g solid-day.

The rate of nitrate reduction and the rate of electron acceptance during denitrification in sewage sludge decreased with a decrease in temperature. Nitrite accumulated to a much greater maximum, later in the incubation, at 10C. Nitrous oxide accumulation was measured at 4C, although it was not detected at 10C. However, the accumulation of nitrous oxide was less than nitrite throughout most of the

incubation. Nitrogen gas was produced at a slower rate than at 10C and after a lag period of seven days. No lag was observed at 10C. These results indicated that nitrous oxide was an intermediate during denitrification in sewage sludge. A decrease in temperature decreased the rate of nitrous oxide reduction more than the rate of production. A similar result was observed with lake sediment, although the effect was not as great. Nitrogen gas was the first gaseous product detected during denitrification by Pseudomonas aeruginosa (St. John and Hollocher 1977). However, these authors used labeled nitrate and an atmosphere of unlabeled nitrous oxide to trap labeled nitrous oxide evolved during denitrification by this species.

4.6   EXPERIMENT VI: EFFECT OF SAMPLE AGITATION ON DENITRIFICATION IN LAKE SEDIMENT

Nitrate disappearance with time was observed from 0.5 g [solid] lake sediment samples incubated on a rocking platform [Figure 4] at 10C with different concentrations of added nitrate-N [Figure 18]. Nitrate disappearance was zero-order with respect to time [r^2=0.97-0.996] and the rate was similar in incubations with 5 and 10 ppm added nitrate-N [Table 13]. Similar zero-order rates of nitrate loss were obtained during denitrification of stirred soil suspensions (Patrick 1960). The rate calculated for 2.5 ppm added nitrate-N was the highest. This was possibly a result of error in determining the low concentrations of nitrate in

the sample solution, or due to the small number of determinations and the short time interval involved. In all incubations, nitrate was depleted to about 2 ug N/0.5 g solid. Dissimilar results were previously reported in Experiment IV for non-agitated sediment [0.5 g solid] incubated at the same temperature and with the same range of added nitrate concentrations. In these quiescent samples, nitrate disappearance was first-order with time [Figure 12]. Nitrate was not reduced to such low levels, although the incubation period was twice as long. Also, the initial constant rate of nitrate depletion was much less rapid than in the agitated samples.

Data indicated that the maximum of nitrite accumulation occurred very early in the incubations with agitated sediment. On the first day of incubation, 6.77 ug NO_2^--N/0.5 g solid were detected when the added nitrate-N was 2.5 ppm. After half a day of incubation, the amount of nitrite measured in sediment agitated with 10 ppm added nitrate-N was 4.21 ug N/0.5 g solid. However, nitrite concentration decreased rapidly with time after the maximum and was less than 0.5 ug N/0.5 g solid throughout most of the incubation period with every level of added nitrate-N.

Mass spectrometric analysis of the sample atmospheres over agitated sediment revealed the presence of nitrous oxide and nitrogen gas. These gases were present after half a day in each of the three incubation series. No other

Figure 18: Concentration of nitrate during incubation of agitated lake sediment at 10C with different amounts of added nitrate-N

TABLE 13

Rates of nitrate depletion and electron acceptance in
agitated lake sediment incubated at various temperatures
with different levels of added nitrate-N

Incubation Temperature	Added NO_3^--N	Rate of NO_3^- depletion	Re*
	ppm	[ug N/0.5 g solid-day]	
5C	5	24.7	10.6
7.5C	5	37.3	15.7
10C	2.5	46.4	23.9
	5	34.8	20.1
	10	35.7	26.9
12.5C	5	36.6	19.9

nitrogenous gas was detected in the sample atmospheres,
which were initially inert argon. Nitrous oxide
accumulation in samples agitated with 2.5 and 5 ppm added
nitrate-N increased rapidly with time to a maximum
[Figure 19]. Then it decreased very rapidly with time to
negligible amounts within two days of the time of maximum
accumulation. There was no steady-state rate of
accumulation of nitrous oxide, as previously described for
non-agitated sediment in Experiment IV [Figure 13]. The
curves describing nitrous oxide accumulation with time in

agitated samples [Figure 19] differed in shape from those describing the accumulation of nitrous oxide with time in non-agitated samples [Figure 13]. However, trends in the two families of curves were similar. The initial rate of nitrous oxide production was similar with the three levels of added nitrate-N. In the agitated sediment [Figure 19] it was approximately 25 ug $N_2O-N/0.5$ g solid-day and was about twice that previously calculated for the non-agitated samples. The maximum accumulation of nitrous oxide and the time of occurrence of this value were dependent on the concentration of added nitrate-N. In agitated sediment with 2.5 ppm added nitrate-N, the maximum accumulation of nitrous oxide was 30.4 ug N/0.5 g solid after the first day and a half of incubation; with 5 ppm, it was 63.3 ug N/0.5 g solid after the fourth day; and with 10 ppm, it was 96.4 ug N/0.5 g solid after the sixth day.

Nitrogen gas was produced without an initial lag period from sediment agitated with three levels of added nitrate-N [Figure 20]. Relatively large amounts of this gas were measured after half a day of incubation in each series. There was an inverse relationship between initial rate of nitrogen gas production and added nitrate-N concentration. The shape of the nitrogen gas production versus time curves was sigmoidal for 2.5 and 5 ppm added nitrate-N. Also, there was a tendency toward independence of maximum rate of nitrogen gas production and concentration of added nitrate-

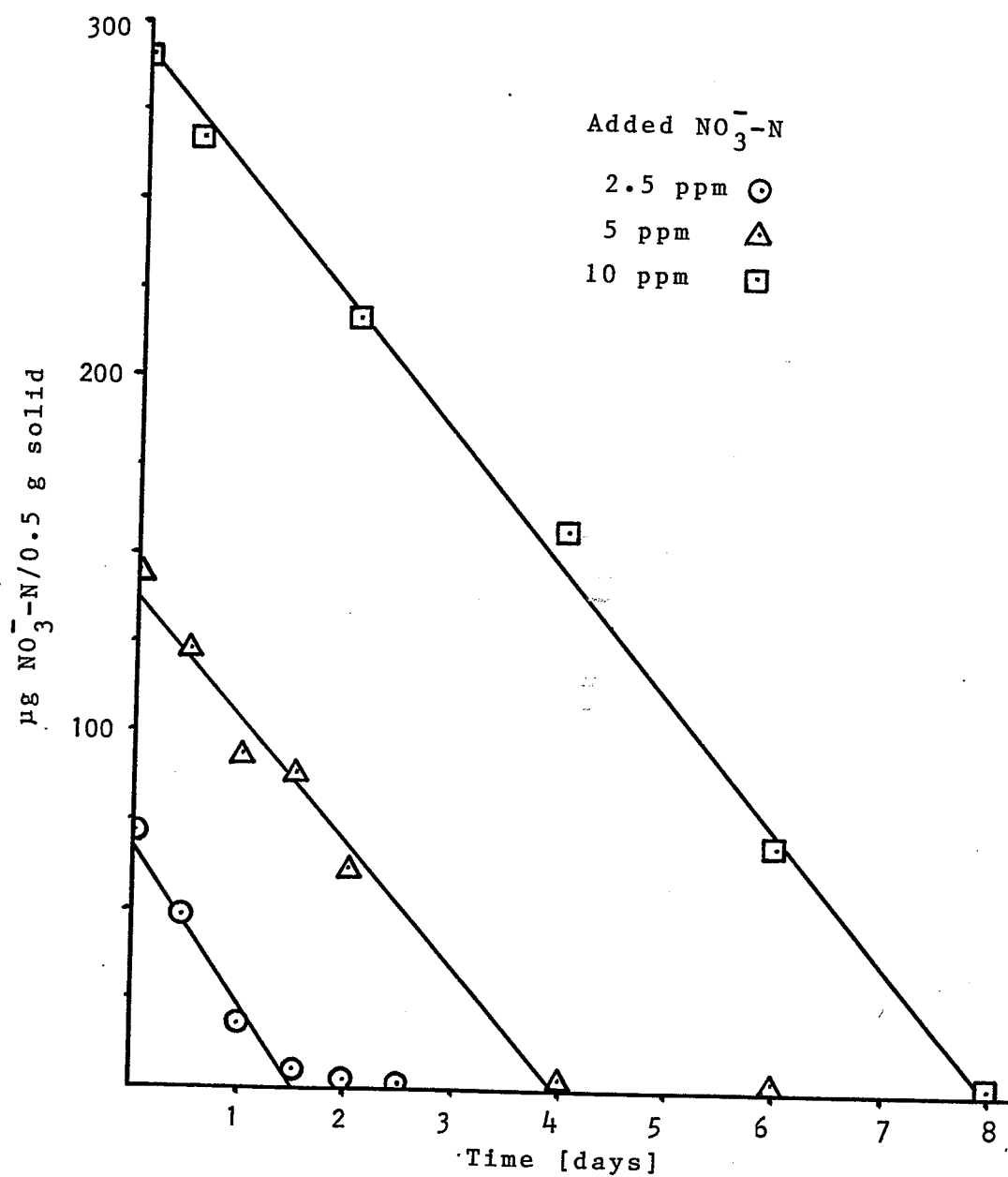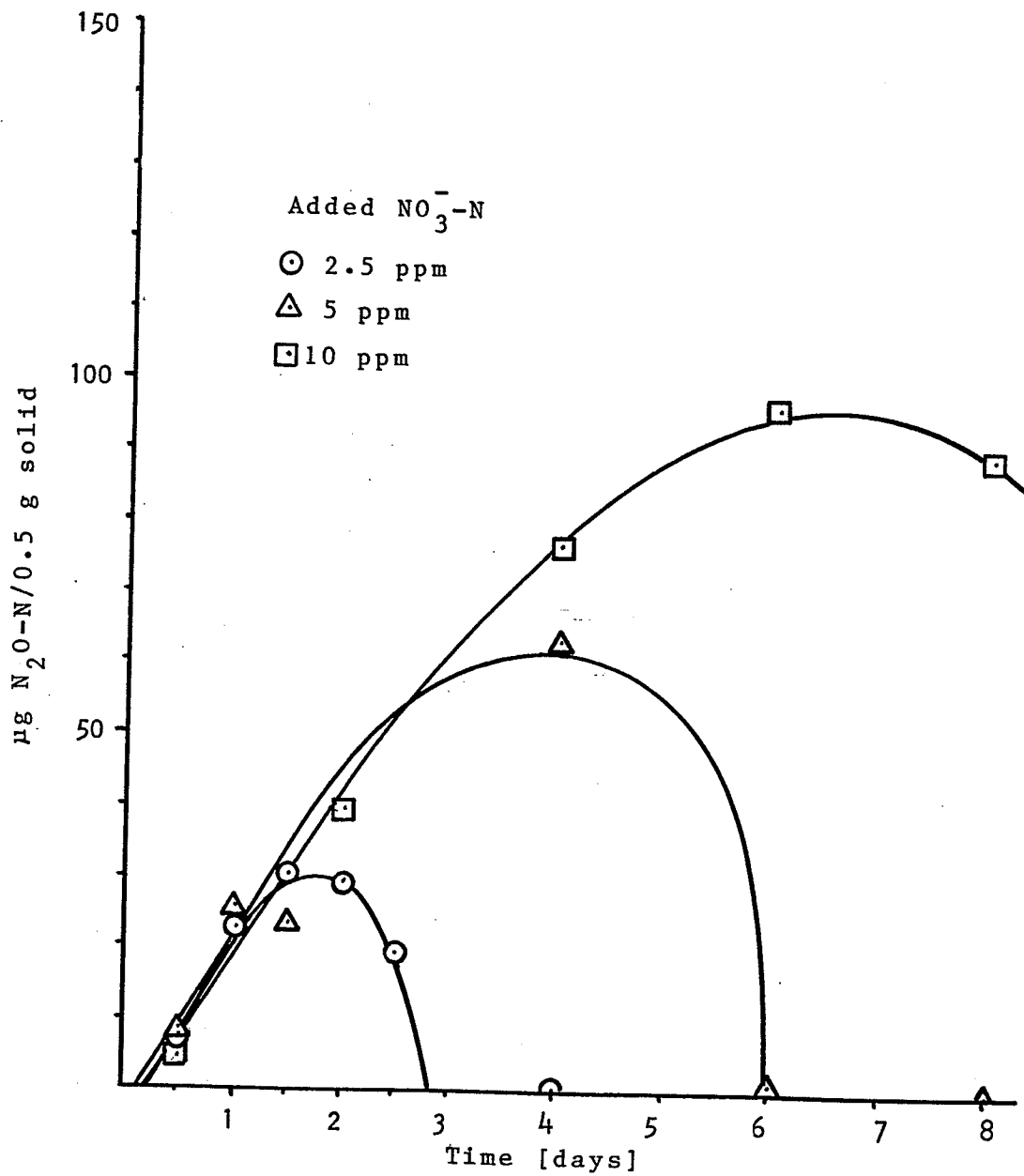Figure 19: Concentration of nitrous oxide during incubation of agitated lake sediment at 10C with different amounts of added nitrate-N

N. This was not the case with similar, but non-agitated, incubations of sediment in Experiment IV, in which the rate of nitrogen gas production depended on added nitrate-N concentration [Figure 14]. However, the amount of nitrogen gas measured on the first day of incubation with non-agitated sediment was inversely dependent on added nitrate-N concentration. Also, a lag of four days was observed with 10 ppm added nitrate-N, while no lag was apparent with 2.5 and 5 ppm. The rate of nitrogen gas production was less in all non-agitated incubations than in the agitated incubations.

The concentrations of nitrogenous compounds measured with time during the incubation of agitated sediment at 10C with 5 ppm added nitrate-N were graphed in a composite plot [Figure 21]. Points of note were the coincidence of the maximum of nitrous oxide accumulation and the maximum rate of nitrogen gas production with the disappearance of nitrate, and the coincidence of the maximum in nitrogen gas accumulation with the disappearance of nitrous oxide.

Re^* for each incubation at 10C was calculated from the slope of the regression line of e^* versus time $[r^2=0.90-0.99]$ over the period when nitrogen gas was accumulating [Table 13]. They were of similar magnitude and, unlike the Re^* values calculated for the similar but non-agitated incubations in Experiment IV [Table 9], were
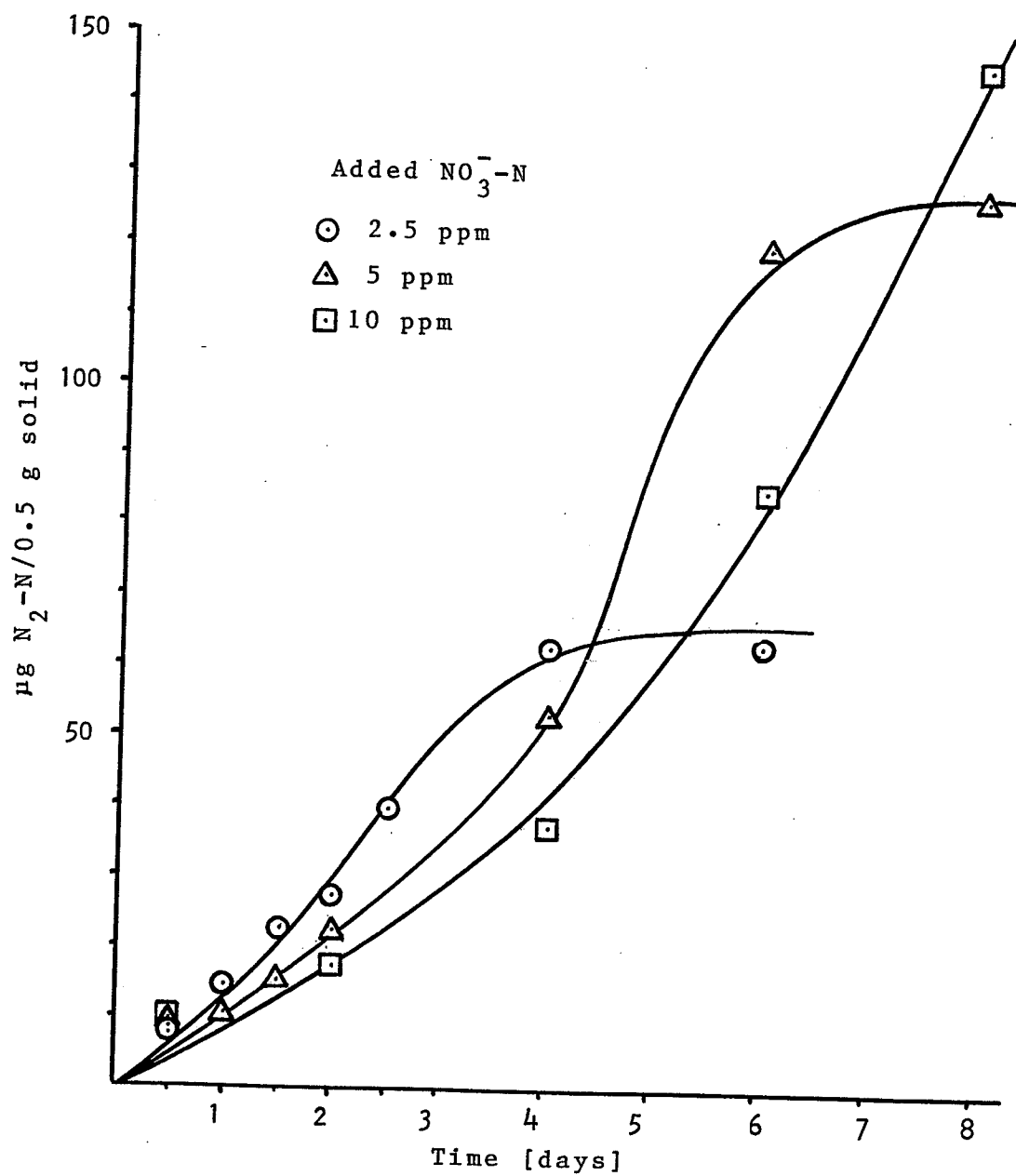
Figure 20: Concentration of nitrogen gas during incubation of agitated lake sediment at 10C with different amounts of added nitrate-N
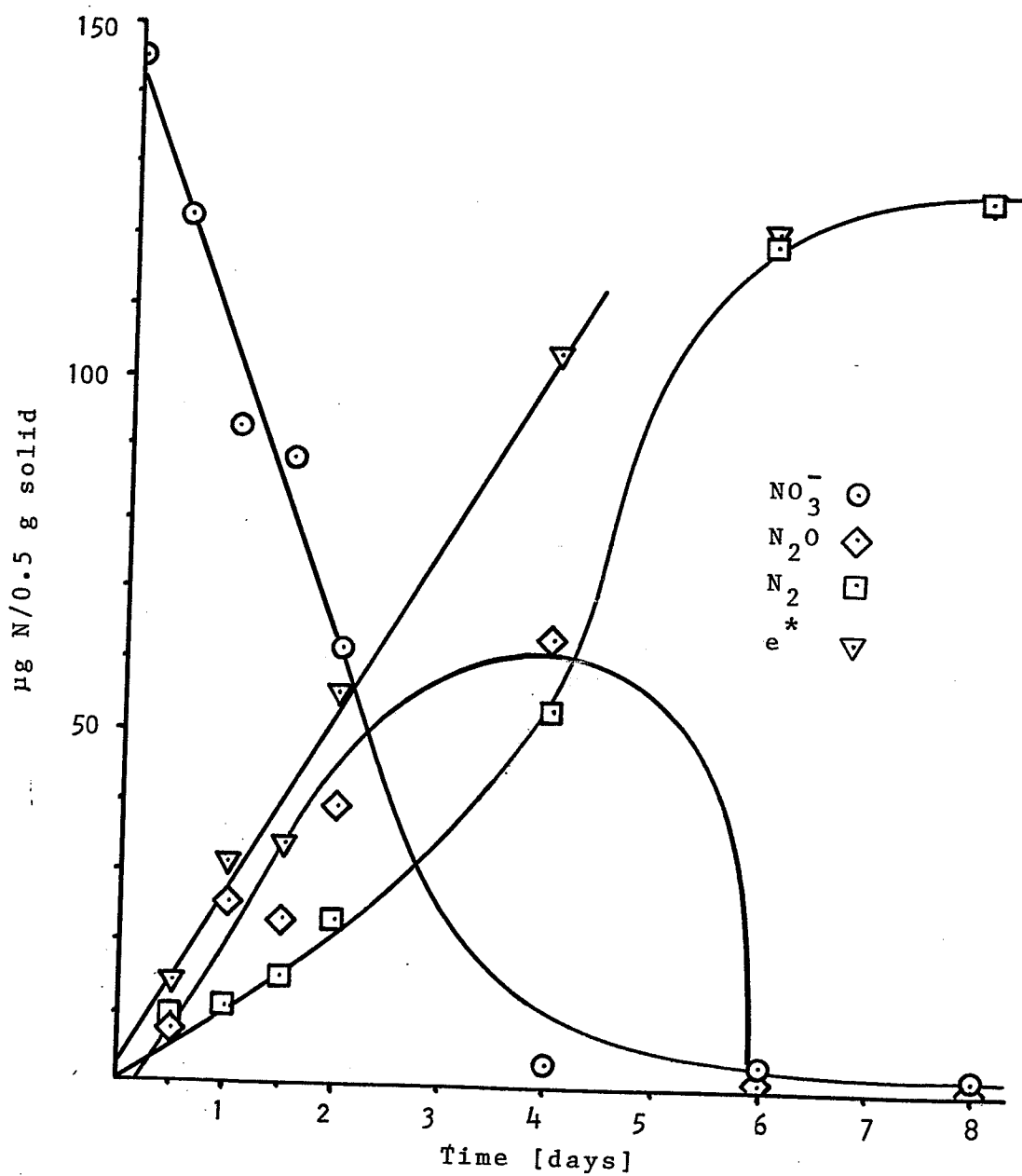
Figure 21: Concentration of nitrate, nitrous oxide, and nitrogen gas during incubation of agitated lake sediment at 10C with 5 ppm added nitrate-N

not dependent on concentration of added nitrate-N. Re^* for non-agitated sediment with 10 ppm added nitrate-N was about half of that calculated for the agitated incubations.

The rate of nitrate loss and Re^* were faster in the agitated sediment samples than in non-agitated samples. In the agitated condition, both rates were independent of added nitrate-N over the relatively low concentration range of 2.5 to 10 ppm. Due to agitation, the homogeneity of the sediment suspension was maintained during the incubation period. Effects of diffusion were minimized and electron donors and acceptors were both readily accessible to the denitrifiers. Under these conditions, denitrification rates were zero-order. However, the rates of nitrate loss and e^* were dependent on added nitrate-N concentration over the range of 2.5 to 10 ppm in non-agitated sediment [Experiment IV]. These results supported the hypothesis of apparent first-order denitrification rates caused by the limiting effects of nitrate diffusion on a zero-order process (Phillips et al. 1978; Reddy et al. 1978; Sain et al. 1977). This effect was more pronounced in lake sediment than in sewage sludge, due to the lower bulk density and greater solid depth of the sediment.

The shape of the curves describing nitrate disappearance, nitrous oxide accumulation, and nitrogen gas production with time [Figure 21] were remarkably similar to those generated

by the competitive Michaelis-Menten kinetics of Cho and Mills (1979). Of particular note was the shape of the nitrous oxide accumulation with time curve, which rose with time to a maximum and then very rapidly decreased to negligible amounts. Other similar characteristics were the constant, zero-order disappearance rate of nitrate and the inverse dependence of the initial rate of nitrogen gas production on added nitrate concentration. Trends in the nitrous oxide accumulation with time curves, common to the agitated and non-agitated incubations, were similar to those generated by the kinetic formulation when the concentration of nitrite was low. The initial rate of nitrous oxide accumulation was independent of nitrate concentration. However, the magnitude of the maximum accumulation and the time of occurrence of the maximum were dependent on added nitrate concentration.

Nitrate removal was zero-order with respect to time [r^2=0.98-0.99] in incubations of 0.5 g [solid] lake sediment agitated at temperatures ranging from 5 to 12.5C with 5 ppm added nitrate-N. The rate of nitrate depletion was similar [approximately 36 ug N/0.5 g solid-day] in the incubations at 7.5, 10, and 12.5C, but was less [24.7 ug N/0.5 g solid-day] at 5C [Table 13]. With non-agitated sediment samples in Experiment V, the rates of nitrate loss with 2.5 and 5 ppm added nitrate-N were greater at 4C than 10C.

The maxima of nitrite accumulation occurred on the first day of incubation at 5 and 7.5C and were 7.15 and 9.01 ug $NO_2^--N/0.5$ g solid, respectively. At 5C, nitrite concentration slowly decreased with time to less than 0.5 ug N/0.5 g solid by the sixth day of incubation. At 7.5C, the nitrite concentration reached this level by the fourth day. However, the trend in nitrite accumulation with time was different in agitated sediment incubated at 10 and 12.5C. A low maximum accumulation of approximately 2 ug $NO_2^--N/0.5$ g solid occurred on the first day of incubation. By the fourth day of incubation, the nitrite concentration decreased to less than 0.5 ug N/0.5 g solid.

Nitrous oxide and nitrogen gas were the only nitrogenous gases detected in the atmospheres of all samples agitated during incubation. The shape of the nitrous oxide accumulation versus time curves [Figure 22] was similar to that previously described for the family of curves in Figure 19. Nitrous oxide accumulation with time at 10 and 12.5C was represented by one line, since the amounts measured were similar. The initial rates of nitrous oxide accumulation at 5 and 7.5C showed a direct dependence on temperature. However, the initial rate of nitrous oxide accumulation at 10 and 12.5C was slightly less than at 7.5C. Similarly, the time of maximum accumulation of nitrous oxide was three days later at 5C than at 7.5C, but at 10 and 12.5C it occurred on the same [fourth] day as at 7.5C. However,

the amount of nitrous oxide present at maximum accumulation at each temperature [approximately 65 ug N/0.5 g solid] was independent of temperature. The time of disappearance of nitrous oxide from the sample atmosphere exhibited a direct dependence on temperature in the range of 5 to 10C. The initial rate of nitrous oxide accumulation, time of maximum accumulation, and residence time of nitrous oxide depended on temperature (Cooper and Smith 1963). However, the magnitude of maximum accumulation of nitrous oxide was relatively independent of temperature.

Nitrogen gas was detected in the sample atmospheres on the first day of incubation at all temperatures. Its production with time was sigmoidal, as in Figure 20, and the rate of production was slightly dependent on temperature, especially at 5C.

Re^* [r^2=0.91-0.96] for each incubation was calculated over the period of incubation when nitrogen gas was accumulating [Table 13]. There was a doubling of Re^* in the agitated samples with a temperature rise from 5 to 10C. Re^* at 7.5C was about half-way between the values calculated for 5 and 10C. However, Re^* values were almost identical at 10 and 12.5C. The Re^* of 10.6 ug N-equivalent/0.5 g solid-day for agitated sediment at 5C with 5 ppm added nitrate-N was approximately twice that previously calculated [4.98 ug N-equivalent/0.5 g solid-day] for non-agitated sediment under otherwise similar conditions in Experiment V.
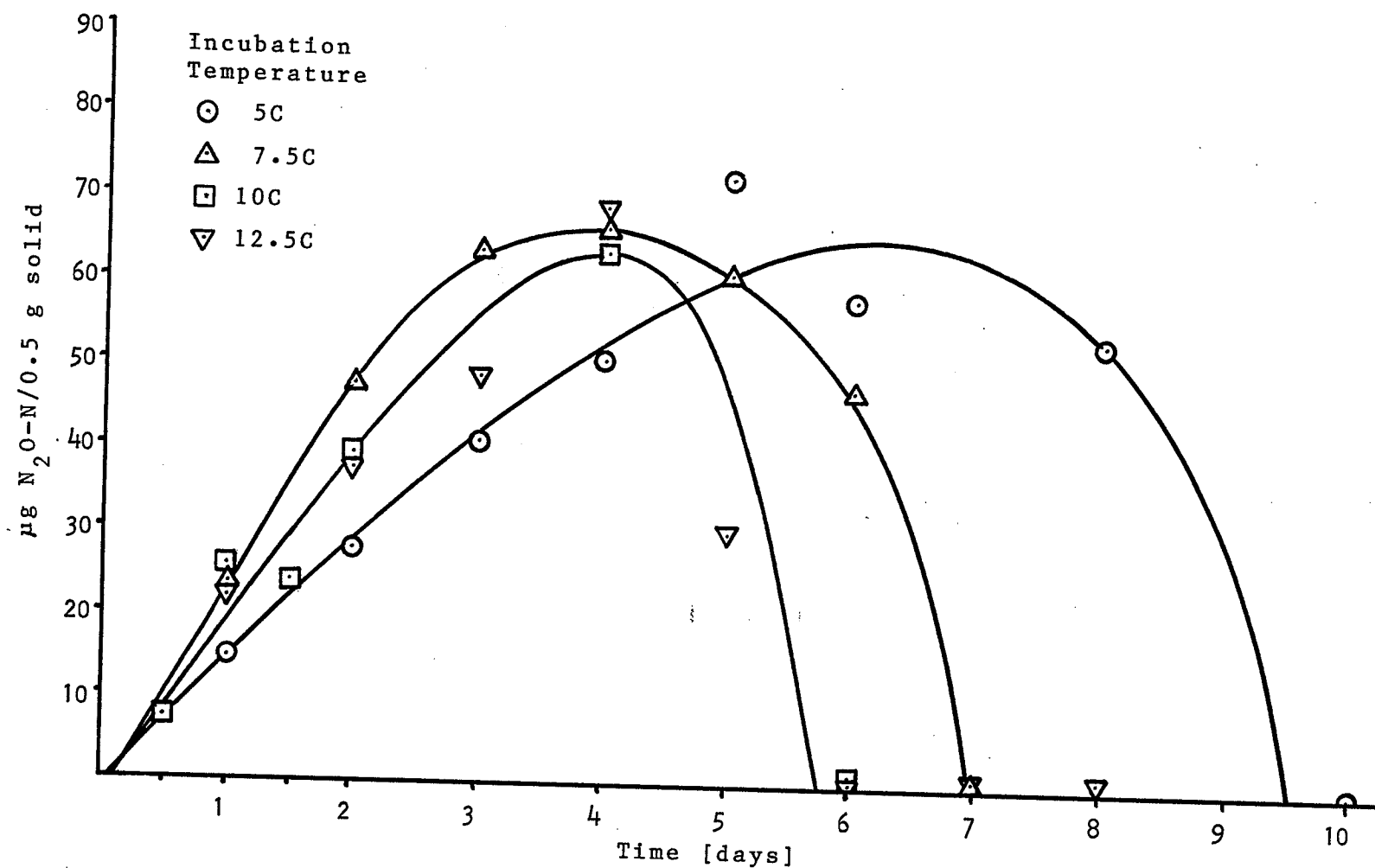
Figure 22: Concentration of nitrous oxide during incubation of agitated lake sediment at different temperatures with 5 ppm added nitrate-N

In the previous experiment, it was observed that Re* in non-agitated sediment was independent of incubation temperature. However, when sediment was agitated during incubation and there was no apparent diffusion-limited reaction, the usual dependence of reaction rate on temperature was observed. Thus, it is speculated that the nearly identical Re* values observed with both 2.5 and 5 ppm added nitrate-N at 4 and 10C under non-agitated conditions must be due to diffusion-limited phenomena.

4.7   EXPERIMENT VII: EFFECT OF ADDITION OF SEWAGE SLUDGE TO LAKE SEDIMENT

Nitrate disappeared with time from 0.5 g [solid] sewage sludge mixed with 0.5 g [solid] lake sediment and incubated at 10C with 50 ppm added nitrate-N [Table 14]. The rate of nitrate loss was slightly better described as zero-order $[r^2=0.96]$, with a constant of 45.7 ug NO_3^--N/g solid-day.

Nitrite accumulation with time in the sludge-sediment mixture was initially rapid and reached a maximum of 45.0 ug N/g solid on the sixth day of incubation [Table 14]. This trend was similar to that observed with nitrite accumulation with time in limed lake sediment in Experiment III [Figures 10 and 11, Table 6]. The pH of the sludge-sediment mixture increased from an initial pH 5.9 to pH 7.0 after one day. After nine days, a pH of 7.1 was measured. This trend was similar to the change in pH observed after addition of lime to lake sediment [Table 5].

TABLE 14

Concentration of nitrate, nitrite, nitrous oxide, and
nitrogen gas during incubation of sewage sludge combined
with lake sediment at 10C with 50 ppm added nitrate-N

Day	NO_3^-	NO_2^-	N_2O	N_2	e*
			[ug N/g solid]		
2	1828.0	31.4	25.2	5.90	38.6
3	1842.0	38.9	37.9	7.90	53.8
4	1610.0	44.5	44.3	28.8	82.0
6	1616.0	45.0	35.5	96.2	143.0
8	1552.0	33.4	30.6	142.0	180.0
10	1470.0	28.3	41.6	219.0	264.0
12	1470.0	13.9	37.7	328.0	364.0
14	1267.0	9.76	28.5	310.0	337.0
16	1101.0	6.31	16.8	389.0	405.0
18	1075.0	4.94	19.7	477.0	495.0
20	1011.0	4.42	16.9	508.0	523.0

Nitrous oxide and nitrogen gas were detected after two
days of incubation with the sludge-sediment mixture and were
the only nitrogenous gases observed during the incubation.
Nitrogen gas was the only gaseous denitrification product
detected during incubation of sludge at 10C in Experiments I
and IV. However, small amounts of nitrous oxide were

detected during incubation of sludge at 4C in Experiment V
[Figure 17].

The initial rate of nitrous oxide accumulation from the
sludge-sediment mixture was approximately 12 ug N/g solid-
day [Table 14], which was similar to that previously calcu-
lated for lake sediment [0.5 g solid] incubated at 10C with
concentrations of added nitrate-N ranging from 2.5 to 10 ppm
in Experiment IV [Figure 13]. A maximum of 44.3 ug N_2O-N/g
solid was attained on the fourth day of incubation with the
sludge-sediment mixture [Table 14]. After this point,
nitrous oxide accumulation decreased slowly with time and
was measured as 16.9 ug N/g solid after 20 days of incuba-
tion. This trend was different from the slow nitrous oxide
accumulation with time in limed lake sediment, previously
described in Experiment III. In Experiment IV, the maximum
of nitrous oxide accumulation from sediment increased in
magnitude and time of occurrence with an increase in added
nitrate concentration. The solid weight of incubated sedi-
ment was 0.5 g. The maximum accumulation of nitrous oxide
during incubation of the sludge-sediment mixture with 50 ppm
added nitrate-N was less and occurred earlier than during
the incubation of sediment with 10 ppm added nitrate-N. The
presence of sludge promoted the reduction of nitrous oxide
to nitrogen gas.

Although small amounts of nitrogen gas were measured after three days of incubation, major production of this gas with time did not occur until the fourth day of incubation [Table 14]. After this initial lag period, the rate of nitrogen gas production was fairly constant. Re*, calculated over the entire incubation period, was 27.8 ug N-equivalent/g solid-day [r^2=0.98].

Chapter V

SUMMARY AND CONCLUSIONS

Denitrification was studied during the incubation of lake sediment and flooded sewage sludge with N-15 labeled nitrate. Nitrate and nitrite concentrations in the sample solution were determined, and the quantities of nitrogenous gases in the sample atmospheres were measured from mass spectrometric scans. Re*, defined as one fifth of the electron acceptance rate during reduction of nitrogenous oxides, was calculated.

At 10C, the sequence of denitrification was different in the two materials. As nitrate was reduced, nitrite accumulated to a maximum and then decreased with time. Accumulation of nitrite was not major in either material, although amounts were greater in sludge. In lake sediment, nitrous oxide accumulated with time to a maximum and then decreased to a steady-state value as nitrogen gas was evolved. In sludge, nitrogen was the only nitrogenous gas detected. Re* for sludge incubated with 20 ppm added nitrate-N was two and one half times faster than for sediment with 15 ppm added nitrate-N.

Addition of glucose to sediment increased the rate of nitrate loss, the rates of nitrous oxide production and reduction, the rate of nitrogen gas production, and the Re^* value. Although nitrous oxide was still detected, its maximum accumulation and residence time were decreased.

There was an inverse relationship between amount of lime added to lake sediment and the nitrate removal rate. Nitrite accumulation was increased to a great extent and the magnitude of the maximum accumulation was dependent on amount of lime added. The rate of nitrous oxide accumulation was very slow. However, the effect on nitrogen gas production was slight. Although Re^* was inversely dependent on amount of lime added, the effect was also slight. Addition of acid to sludge decreased the rate of nitrate loss and the Re^* value. Nitrogen gas was produced after a four day lag period and was the only gaseous denitrification product.

In non-agitated lake sediment, rates of nitrate loss, nitrogen gas production, and e^* were dependent on added nitrate-N in the range of 2.5 to 10 ppm. The initial rate of nitrous oxide accumulation was independent of added nitrate concentration. However, the magnitude and time of occurrence of maximum accumulation, as well as the magnitude during steady-state accumulation, were dependent.

In sludge, rates of nitrate loss, nitrogen gas production, and e^* were dependent on added nitrate-N at the 10 ppm level. However, they were independent over the range of 20 to 50 ppm added nitrate-N.

In agitated sediment, rates of nitrate loss and e^* were independent of added nitrate-N in the range of 2.5 to 10 ppm. Denitrification was zero-order due to the homogeneity of nitrate concentration in the sample. First-order kinetics in non-agitated sediment resulted from denitrification being limited by nitrate diffusion. Zero-order kinetics in sludge resulted from the high bulk density of this material. The shallow solid layer, coupled with higher nitrate concentrations, promoted more complete penetration of nitrate into the sludge than into sediment.

Curves describing the decrease of nitrate and the accumulation of denitrification components with time in agitated sediment were very similar to those previously generated by a competitive Michaelis-Menten kinetic formulation. Trends in the nitrous oxide accumulation with time curves for the agitated and non-agtitaed incubations were similar. The initial rate of accumulation was independent of nitrate concentration, but the magnitude and time of occurrence were dependent. The initial rate of nitrogen gas production was inversely dependent on added nitrate concentration in the agitated sediment.

Nitrous oxide accumulation was measured over sludge at 4C, but not detected at 10C. However, nitrite accumulation was greater than nitrous oxide accumulation during most of the incubation. A decrease in rates of nitrate loss and e[*] occurred at 4C. However, in non-agitated lake sediment, a decrease in temperature had little effect on rates of nitrate loss and e[*]. The magnitude of nitrous oxide accumulation increased as the incubation temperature decreased. In agitated sediment, Re[*] increased with incubation temperature. However, the magnitude of the maximum of nitrous oxide accumulation was similar in every case and not a function of temperature.

Addition of sludge, which had a calcium carbonate equivalent of 20.7%, to sediment resulted in a large accumulation of nitrite. Although this was similar to the trend observed with nitrite accumulation in limed sediment, nitrous oxide accumulated just as rapidly with time as in the unlimed sediment. Reduction of nitrous oxide to nitrogen gas was accelerated by the presence of sludge.

It is likely that nitrous oxide is produced by lake sediment throughout the year in the natural system, especially if nitrate-N concentration is in the range of 2.5 to 10 ppm. Denitrification rates in natural sediment are probably first-order due to the limiting effect of nitrate diffusion from the overlying water into the sediment. Although the

addition of sewage sludge to sediment at 10C would increase
the nitrate concentration, it would probably promote the
reduction of nitrous oxide to nitrogen gas due to the higher
denitrifying capacity of the sludge.

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Appendix A

ENZYME KINETICS

According to Day $\underline{et}$ $\underline{al}$. (1978), for the enzyme reaction written as:

$$S + E \; \underset{K_1}{\overset{K_2}{\rightleftarrows}} \; E \cdot S \; \overset{K_3}{\longrightarrow} \; P + E \qquad \{A1\}$$
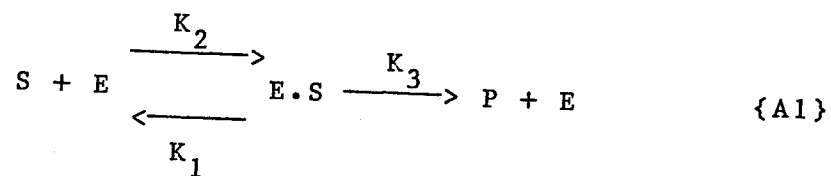
where S = substrate,

E = enzyme,

E·S = enzyme-substrate complex,

P = product,

K_i = rates of reaction, $\quad i = 1 \ldots 3$,

the rate of substrate disapearance is:
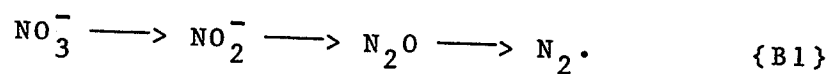
$$-\frac{dS}{dt} = K_3 \; \frac{E_t S}{K_m + S} \; , \qquad \{A2\}$$

where $K_m = \dfrac{K_2 + K_3}{K_1}$,
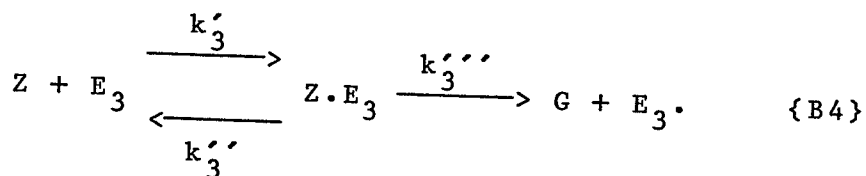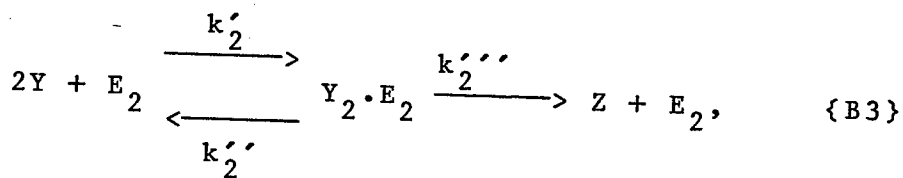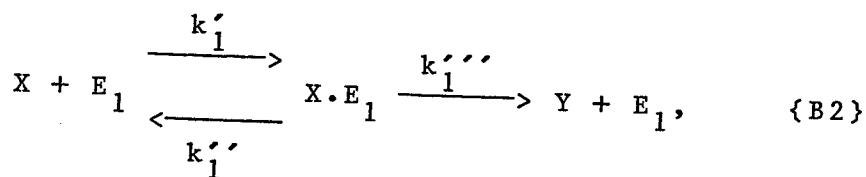
E_t = total enzyme concentration.

Appendix B

COMPETITIVE ENZYME KINETICS

The following pathway for denitrification will be used for the analysis:

$$NO_3^- \longrightarrow NO_2^- \longrightarrow N_2O \longrightarrow N_2. \qquad \{B1\}$$

Steady or constant microbial activity per unit weight of soil will be assumed in order to formulate a general form of enzyme kinetics for the above sequence $\{B1\}$. Nitrate, X, nitrite, Y, and nitrous oxide, Z, are assumed to combine with enzymes E_1, E_2, and E_3, respectively, in order to be further reduced to nitrogen gas, G, as follows:

$$X + E_1 \underset{k_1''}{\overset{k_1'}{\rightleftarrows}} X \cdot E_1 \xrightarrow{k_1'''} Y + E_1, \qquad \{B2\}$$

$$2Y + E_2 \underset{k_2''}{\overset{k_2'}{\rightleftarrows}} Y_2 \cdot E_2 \xrightarrow{k_2'''} Z + E_2, \qquad \{B3\}$$

$$Z + E_3 \underset{k_3''}{\overset{k_3'}{\rightleftarrows}} Z \cdot E_3 \xrightarrow{k_3'''} G + E_3. \qquad \{B4\}$$

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The rate equations are:

$$d(X)/dt = -k_1'(X)(E_1) + k_1''(X \cdot E_1), \qquad \{B5\}$$

$$d(Y)/dt = k_1'''(X \cdot E_1) + 2k_2''(Y_2 \cdot E_2) - 2k_2'(Y)^2(E_2), \quad \{B6\}$$

$$d(Z)/dt = k_2'''(Y_2 \cdot E_2) + k_3''(Z \cdot E_3) - k_3'(Z)(E_3), \quad \{B7\}$$

$$d(G)/dt = k_3'''(Z \cdot E_3), \qquad \{B8\}$$

where $X \cdot E_1$ = nitrate-enzyme complex,

$\qquad Y_2 \cdot E_2$ = nitrite-enzyme complex,

$\qquad Z \cdot E_3$ = nitrous oxide-enzyme complex,

$\qquad t$ = time.

Under steady-state conditions, the above equations [{B5} to {B8}] can be written as:

$$\frac{-d(X)}{dt} = \frac{k_1(X)R}{k_1(X) + k_2(Y)^2 + k_3(Z)}, \qquad \{B9\}$$

$$\frac{d(Y)}{dt} = \frac{[k_1(X) - 2k_2(Y)^2]R}{k_1(X) + k_2(Y)^2 + k_3(Z)}, \qquad \{B10\}$$

$$\frac{d(Z)}{dt} = \frac{[k_2(Y)^2 - k_3(Z)]R}{k_1(X) + k_2(Y)^2 + k_3(Z)}, \qquad \{B11\}$$

$$\frac{d(G)}{dt} = \frac{k_3(Z)R}{k_1(X) + k_2(Y)^2 + k_3(Z)}, \qquad \{B12\}$$

where $k_i = \dfrac{k_i' k_i'''(E_i)}{k_i'' + k_i'''}$, $\qquad i = 1 \ldots 3$,

$$R = k_1'''(X \cdot E_1) + k_2'''(Y_2 \cdot E_2) + k_3'''(Z \cdot E_3).$$

R is the total production rate of reduced nitrogenous compounds from their precursors.