DEVELOPMENT OF TECHNIQUES FOR THE ASSESSMENT OF DENITRIFICATION IN NEW ZEALAND SOILS

by

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ABSTRACT

New Zealand's agriculture is heavily dependent upon the symbiotic fixation of atmospheric nitrogen. Consequently little fertilizer nitrogen is used in pastoral farming. Nevertheless, N deficiency is widespread throughout New Zealand pastures, indicating that either the N supply is inadequate, or that significant losses are occurring from the system.

The contribution of denitrification to these losses is unknown, primarily because at present there is no suitable technique available for directly measuring denitrification losses in the field.

The major gaseous losses from the soil due to denitrification occur as nitrogen and nitrous oxide. Detection of nitrous oxide evolution is possible by several methods including gas chromatography, mass spectrometry and infra-red analysis. Several techniques have been proposed for direct N_2O measurements in the field. Measurement of the relatively small quantity of nitrogen evolved from soils in the presence of the atmospheric background has proved difficult for many years and is a significant barrier to the determination of N losses.

The objectives of this thesis were to:

1) develop a laboratory method to identify soils from which large denitrification losses may be expected,

2) develop a technique for direct measurement of denitrification losses in the field, and

3) gain some understanding of the significance and occurrence of the denitrification activity occurring in New Zealand soils.

A laboratory incubation technique was developed to measure the relative denitrification potential of soils. The denitrification potential is defined as the maximum rate of nitrate dissimilation under anaerobic conditions without addition of exogenous reductant.

The technique was based on the acetylene inhibition of nitrous oxide reductase.

Soils were amended with 250 μg NO_3-N.g^{-1} and incubated at 25°C and 100% water holding capacity for eight hours in an argon atmosphere containing 10% acetylene. The incubation atmosphere was sampled hourly for N_2O production. Six soils in duplicate can be assayed by one person in the course of a normal working day.

A widespread (approximately ten-fold) variation in denitrification potential was found between soils. Soils exhibiting the lowest denitrification potentials were also seen to exhibit low nitrification activities indicating that denitrification was limited by the availability of nitrate substrate. As the availability of nitrate substrate increased (increased nitrification activity), other factors influenced the denitrification potential and there was no relationship between denitrification and nitrification activity. Soils containing allophane generally exhibited high denitrification activities.

The denitrification potential also varied with stock rates, higher denitrification potentials being observed under high stocking rates on a given soil. This effect was attributed to the increased availability of C and N substrate through excreta return by the animals.

Denitrification potentials were also influenced by the type of crop. Soils under maize crops exhibited markedly lower denitrification potentials than adjacent soils in pasture. This effect was attributed to the depletion of available carbon by the maize crops.

The maximum denitrification potential in a soil always occurred in the 0-30 mm layer. This association of the denitrification potential with the rhizosphere could not wholly be explained on the basis of nutrient availability. Below 30 mm the denitrification

potential decreased markedly, even in the presence of adequate C and N substrate. Addition of further C and N substrate at depth did not stimulate increased denitrification activity.

Addition of cow's urine to a soil was not accompanied by an increase in the denitrification potential of that soil even though the C and N substrate levels increased many-fold. The soil was demonstrated to already be at its maximum denitrification potential *in situ* at the time of the experiment. After three weeks, a covered plot of the same soil exhibited a marked decrease (40%) in the denitrification potential. It appears that this decrease resulted from some enzymic inhibition or toxicity related to the addition of urine.

Storage techniques reported for soils used in denitrification studies vary widely. Soils are stored air dry at room temperature, at 4°C and frozen, for periods of a few days to several months. It was demonstrated in this work that the denitrification potential of a soil is significantly affected by even a few days' storage at 4°C. This effect was attributed to a rapid decline in available carbon.

Some soils demonstrated two linear phases of denitrification during an eight hour incubation. It was shown that the first phase was unaffected by chloramphenicol (a protein synthesis inhibitor) while the transition to the second phase did not occur in the presence of chloramphenicol.

The first phase was attributed to the activity of pre-existing enzymes (i.e. *in situ*) while the second phase was taken as an indication of the maximum denitrification potential of that soil due to stimulation of increased enzyme activity.

The maximum denitrification potential of a soil is relatively stable seasonally, decreasing markedly only during the peak summer months. This effect was attributed to the effect of low soil moisture on microbial processes. While the soil was denitrifying at the maximum

potential the denitrifying enzymes were in a state of full induction *in situ*.

Groundwater nitrate concentrations are high in shallow aquifers in the Waikato. This implies that denitrification in this region is limited more by available carbon than nitrate supply.

To facilitate the direct measurement of nitrogen evolution under field conditions a lysimeter was specially designed and constructed. The purpose of the lysimeter was to achieve and maintain a low nitrogen background within a soil core. By flushing the soil core with nitrogen-free gas, nitrogen concentrations between 2000 and 5000 ppm v/v could easily be achieved. A unique feature of this lysimeter was the ability to form an artificial barrier at the base of the soil core using a flow of nitrogen-free gas. The lysimeter walls were double skinned, permitting a flow of gas to exit through holes in the inner wall near the base. This barrier proved effective in preventing the inward diffusion of soil air during measurements of nitrogen evolution.

By introducing into the soil core a low concentration of nitrogen enriched in N-15, nitrogen evolution could be monitored using a high precision double beam Micromass 602c mass spectrometer.

By measuring the isotopic dilution of the N-15 spike, $\delta^{15}N$ changes representing rates of nitrogen evolution as low as 10 kg N/ha/yr could be measured.

Nitrous oxide evolution can be measured at the same time by switching the Micromass to the single beam mode.

Sample pre-treatment is kept to a minimum.

The lysimeter-^{15}N technique allows the first direct single sample measurement of nitrogen and nitrous oxide evolution in the field.

The lysimeter was also shown to be effective in achieving

and maintaining high acetylene concentrations in a soil core. This capability meant that field estimates of denitrification using only the evolution of nitrous oxide were also possible. The lysimeter offers an advantage over previous field acetylene techniques in that it does not suffer from the uneven acetylene distribution associated with the multi-point acetylene source technique. The lysimeter also allows the oxygen concentration within the soil to be closely controlled.

The contribution of Rhizobia to the leguminous fixation of atmospheric N is well known. It was demonstrated that free-living *Rhizobia japonicum* are capable of denitrification in soils under laboratory conditions. Even under moderate oxygen tensions (10% v/v), Rhizobia appear to remain as active denitrifiers. The question of the significance of Rhizobia denitrification in the field is raised in the light of previous results which indicated a marked preference for denitrification to occur in the rhizosphere, and the notable reduction of denitrification potential in soils of non-legumous crops.

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CHAPTER 1

INTRODUCTION

The annual production and use of nitrogenous fertilizers has increased dramatically over recent years. World consumption of fertilizer N increased from 38 to 48 million tonnes from 1974 to 1978 (FAO fertilizer yearbook, 1978). Consumption of nitrogenous fertilizer in Britain increased from 321,000 tonnes of N in 1959 to 1.1 million tonnes in 1979 (Chemistry and Agriculture, 1979). Seven million tonnes of nitrogen fertilizer were used in the USSR in 1975 and eleven million tonnes is the estimated consumption for 1980 (Makarov and Makarov, 1976).

This trend of increased fertilizer consumption will result in increased levels of nitrogen in soils and natural waters and concern is increasing for the potential adverse affects on environmental quality.

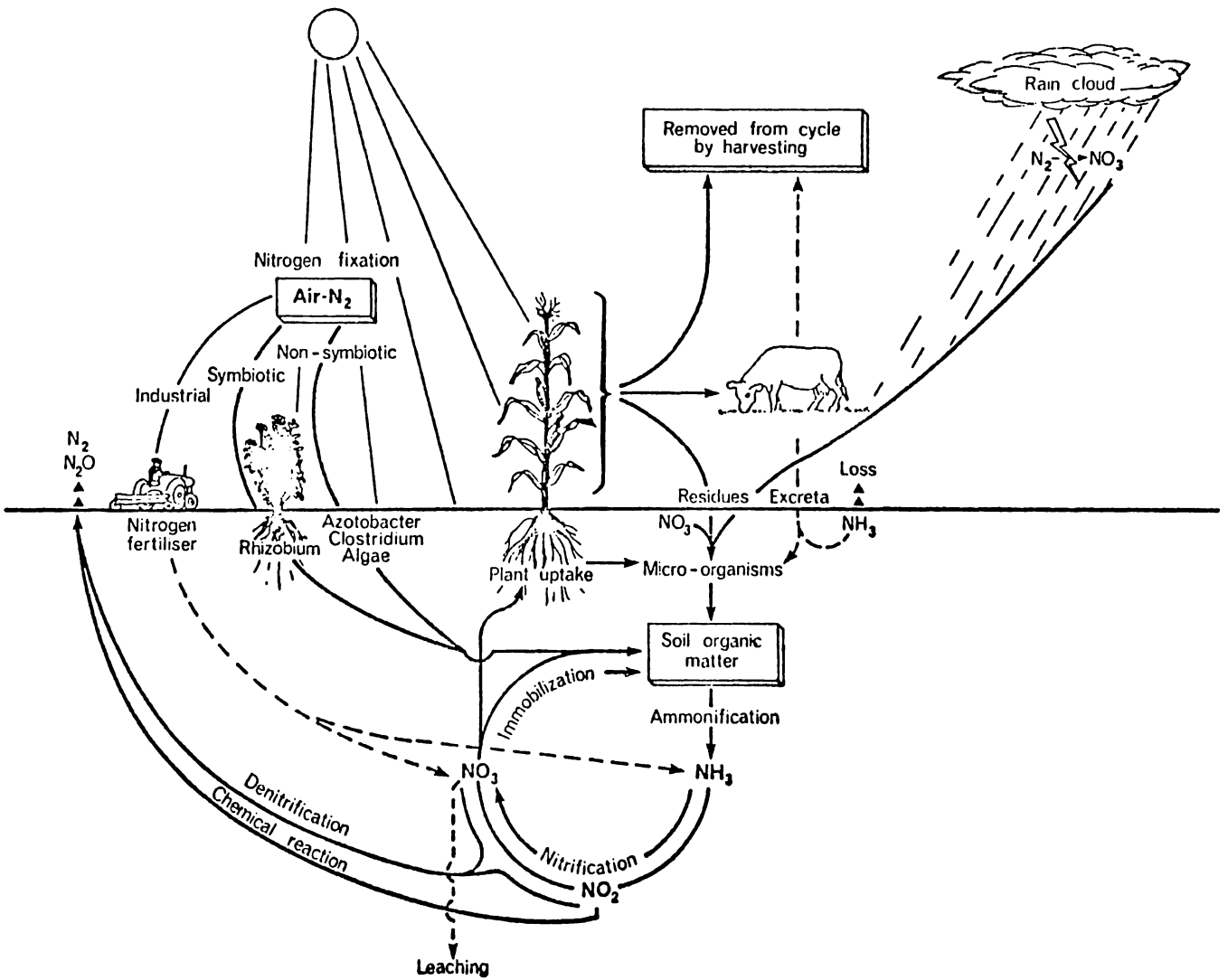
Recent estimates show that from 30 to 60% of the applied N-fertilizer may be lost from the system through denitrification (Makarov and Makarov, 1976). With the increasing cost and demand for N-fertilizers these losses are assuming greater significance. Although only a small part of the total nitrogen cycle in soils (fig 1.1), denitrification is one factor which may be expected to have a large influence on the conservation of nitrogen.

Denitrification is defined by the Soil Science Society of America (1962) as the biological reduction of nitrate or nitrite to gaseous nitrogen compounds. Such a definition excludes those nitrogenous gases derived from nitrification (Bremner and Blackmer, 1978) and chemo-denitrification.

Broadbent and Clark (1965) summarised the biological process described by the Soil Science Society of America as follows: Under aerobic conditions, the oxidation of an organic substrate (say glucose) by facultative anaerobic bacteria leads to the formation of CO_2 and H_2O

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \qquad 1.1$$

Figure 1.1 **Origin and distribution of Nitrogen in soil**



In the absence of oxygen but in the presence of nitrate, these bacteria are capable of a nitrate respiration which may be expressed as follows:

$$C_6H_{12}O_6 + 4NO_3^- \rightarrow 6CO_2 + 6H_2O + 2N_2 \qquad 1.2$$

The principal products of denitrification have been found to consist of dinitrogen and nitrous oxide.

Factors influencing denitrification include: i) availability of oxygen, ii) pH, iii) growth requirements, iv) temperature. The rate of denitrification is increased with temperature, pH, nitrate concentration, reductant (organic substrate) and decreased with aeration (Focht, 1978).

In spite of an ever increasing amount of research on denitrification, field estimates of denitrification losses are still restricted to nitrogen-deficit budgets. The actual contribution of denitrification to N losses remains doubtful, especially where errors in estimates of leaching losses and N immobilization are large. An *in situ* direct assessment of the denitrification products evolving from the soil has yet to be developed. Detection of the production of a relatively small quantity of N_2 in the presence of a high atmospheric background has proved difficult. However, the advent of sensitive isotope mass spectrometers and readily available supplies of N-15 enriched substrates have contributed greatly to data on N_2 field losses.

The N_2 and N_2O ratios have proved to be extremely variable, and consequently any estimate on the flux of a single denitrification product is an unreliable estimate of denitrification rates (Focht, 1978).

Recent attention to the quality of our environment has prompted an increased effort in the measurement of N_2O evolution from the soil. It is widely believed that N_2O poses a threat to the ozone layer (CAST, 1976; McKenney *et al*, 1978).

N_2O is prominent in the aeronomy of the stratosphere and is thought to be the dominant source of stratospheric nitrogen oxides (Crutzen, 1971).

$$O('D) + N_2O \rightarrow NO + NO \qquad 1.3$$

Subsequent chemical reactions of NO are believed to catalytically destroy O_3 in the middle and high stratosphere by the following reaction mechanism:

$$NO + O_3 \rightarrow NO_2 + O_2 \qquad 1.4$$

$$NO_2 + O \rightarrow NO + O_2 \qquad 1.5$$

$$O_3 + O \rightarrow 2O_2 \qquad 1.6$$

It is estimated that under normal circumstances reaction 1.6 consumes 50-70% as much ozone as would be necessary to maintain a steady state concentration (Moore and Moore, 1976, p189).

Studies have estimated the input of 2×10^8 tonne N_2O-N/yr to contribute to an ozone depletion of between 1.8 to 23% (Holdgate, 1979, p77). The wide variation in this estimation reflects the uncertainty in the values upon which calculations are based. Similarly high altitude jets are thought to contribute to approximately a 4% decrease in ozone content through the production of NO_x.

New Zealand's agriculture is largely based upon the symbiotic fixation of N by clovers and consequently we supply less N-fertilizer than our overseas counterparts. However, N deficiency in our pastures appears widespread (O'Connor and Cumberland, 1973; Steele, 1976). N loss to the groundwater systems also is significant. In the Waikato region of New Zealand, groundwater nitrate levels have been found to exceed World Health Organisation limits (11.3 mg NO_3-N/ml) many fold (Baber and Wilson, 1972). If used for human consumption this nitrate may produce methaemoglobinaemia - a condition affecting young children

where oxygen transport in the blood is restricted.

While the view expressed by the Soil Science Society of America (1962) - that denitrification is a detrimental phenomenon by way of dissimilating soil nitrogen - is widely held, there is a school of opinion which now rates denitrification as a desirable process. Terry and Tate (1980) have proposed denitrification as a means of removing excess nitrate from organic soils. Zablotowicz and Focht (1979) also subscribe to the view that denitrification is an agriculturally desirable characteristic. Their belief is that the denitrifying organisms exist in the soil not because of their denitrification capacity but because of some other property. These organisms turn to denitrification under conditions of oxygen stress as a means of ensuring organism survival until adequate oxygen is available for aerobic respiration to resume.

With increasing pressure both on N conservation and on maintaining environmental quality, it has become important to be able to assess denitrification losses *in situ*.

It was the object of this thesis to:

i) Develop an *in situ* field technique capable of measuring N_2 and N_2O evolution to 10 kg N/ha/yr,

ii) Investigate the denitrification activities in New Zealand soils to gain an understanding of the magnitude and occurrence of these nitrogenous losses.

CHAPTER 2

LITERATURE REVIEW

2.1.0 INTRODUCTION

The process of denitrification was first discovered in soil columns almost a century ago by Gayon and Dupetit (1886). Interest at this time primarily centred around the possible depletion of nitrogenous sources for food production. By 1927 the process of denitrification whereby nitrates and nitrites lose nitrogen in the form of gas through the agency of microorganisms was well known and studied in the laboratory (Waksman, 1927). Interest shifted towards conservation of nitrogen and maintaining a reasonable nitrogen level in the soil. As early as 1953 Arnold (1954) measured N_2O losses from soils and suggested that not only may these losses be important as far as fertilizer application is concerned, but that this N_2O evolution may in fact account for our atmospheric N_2O content.

Most recently, interest in denitrification has taken on the added dimension of possible pollution of the environment.

2.2.0 MECHANISMS OF DENITRIFICATION

2.2.1 Chemo-denitrification

The denitrification of most significance in soils has been attributed to biological origins but losses of N_2 and N_2O have been seen to occur through chemical processes (e.g. Clark, 1962; Bremner *et al*, 1980). Such losses are attributed to chemo-denitrification. Chemical reactions involving nitrous acid or nitrites are variously involved and give rise to various speculated pathways.

i) Under suitably acid conditions nitrous acid decomposes to yield NO according to the reaction:

$$3HNO_2 \rightarrow 2NO + HNO_3 + H_2O \qquad 2.1$$

$$2NO + O_2 \rightarrow 2NO_2 \qquad 2.2$$

This NO_2 may also react with water to form nitric acid:

$$3NO_2 + H_2O \rightarrow 2NO_3 + NO \qquad 2.3$$

$$2NO_2 + H_2O \rightarrow 2HNO_3 + HNO_2 \qquad 2.4$$

A pH of less than 5 was reported necessary by Allison and Doetsch (1951) for appreciable decomposition of nitrous acid. The rate and extent of decomposition were greatly accelerated with further increases in acidity. In neutral or alkaline soil, there would be little, if any nitric oxide formation. In aerated acid soils, where chemical oxidation of NO proceeds rapidly, it is unlikely that any considerable NO would escape to the atmosphere before oxidation and hydration to HNO_3 could occur (Allison and Doetsch, 1951; Broadbent and Clark, 1965; Allison, 1965).

Tyler and Broadbent (1960) and also Smith and Clark (1960) failed to detect NO in closed atmospheres of soil lots treated with nitrite. Cady and Bartholomew (1960, 1961) detected small amounts of NO during incubation studies, but coincident with the appearance of NO was the production of N_2O followed by the eventual disappearance of NO.

It was suggested (above) that NO is unlikely to escape from the soil due to rapid oxidation followed by rapid adsorption as NO_2. However the half life of oxidation is dependent on the NO concentration because it is a termolecular reaction (Leighton, 1961). At concentrations of 100 ppm or more, the half life for oxidation of NO to NO_2 can be one hour or less whereas at concentrations of <0.01 ppm the half life for oxidation may be of the order of 10^4 hours.

This variation in the oxidation rate by oxygen explains why low concentrations of NO may pass unoxidised from the soil to the atmosphere, and why some laboratory studies with high concentrations fail to identify NO. Galbally and Roy (1978) reported the first such measurements of NO field emissions using a chemiluminescent NO gas

detector. The measurements indicated that NO is continuously exhaled from soils including ungrazed, unfertilized grassland. The measured rates were equivalent to 0.2 to 0.3 kg N/ha/yr. On the basis of these calculations Galbally and Roy (1978) suggested an annual global emission of 10^7 tonnes NO-N/yr, and compared this to a global value of 2 to 3 x 10^8 tonnes N/yr fixed annually (Söderlund and Svensson, 1976). Galbally and Roy (1978) suggested that this NO loss is not permanent and the nitrogen is probably returned to the soil as nitrate in rain water. Thus NO exhalation may be a significant process in redistributing fixed nitrogen throughout the global biosphere.

ii) Reaction of nitrous acid with amino acids yields nitrogen according to the reaction:

$$RNH_2 + HNO_2 \rightarrow ROH + H_2O + N_2 \qquad 2.5$$

This reaction is commonly known as the Van Slyke reaction.

Work by Allison and Doetsch (1951) and Allison (1963) indicates that the Van Slyke mechanism is unlikely to occur to any significant extent under conditions commonly found in the soil. Conditions of pH 5 or less are required before the reaction can occur and such conditions are unfavourable for nitrite formation.

iii) Ammonia has also been shown to react with nitrous acid to yield nitrogen:

$$NH_3 + HNO_2 \rightarrow N_2 + 2H_2O \qquad 2.6$$

Van Slyke (1911) showed that compounds such as ammonia, urea and pyrimidines react accordingly with nitrous acid, but even slower than amino acids. Allison (1963) attributes the N_2 losses from this reaction to the decomposition of the rapidly formed intermediate ammonium nitrite:

$$NH_3^+ + HNO_2 \rightarrow NH_4NO_2 \rightarrow N_2 + 2H_2O \qquad 2.7$$

Data presented by Smith and Clark (1960) and Fowler and Kotwal (1924) suggest this mechanism is of little importance in chemo-denitrification. Fowler and Kotwal (1924) observed that neutral aqueous solutions of ammonium nitrite containing 30 ppm or less of NO_2^--N could be kept at room temperature for months without loss of nitrite.

(iv) Hydroxylamine (NH_2OH) is a well established intermediate in the oxidation of ammonium to nitrite by nitrifying organisms. Chemical decomposition of NH_2OH in soil has been shown to produce N_2O (Freney *et al*, 1979) and N_2 (Bremner *et al*, 1980). Production of N_2O via chemical decomposition of NH_2OH in soils greatly exceeded production of N_2O through chemical decomposition of nitrite (i.e. via chemo-denitrification) and so did N_2 production in most soils studied (Bremner *et al*, 1980).

2.2.2 Biological denitrification

The processes by which nitrate is utilized by microorganisms fall into two general classes:

i) nitrate assimilation or assimilatory nitrate reduction, and

ii) nitrate respiration or dissimilatory nitrate reduction.

Nitrate assimilation denotes the biological reduction of nitrate to the ammonia or amino level, with the products being used for the biosynthesis of nitrogen containing cellular constituents. The process of nitrate assimilation results in the nitrogen remaining in the soil as microbial protoplasm, and therefore the nitrogen is still potentially available to the plant. On this basis nitrate assimilation cannot be categorized as a mechanism for denitrification.

A singular characteristic of the process of denitrification in the classical sense is that nitrate or its reduction products serve as electron acceptors for the oxidation of some organic compound (or in a few inorganic compounds) as the primary energy-yielding reaction, upon which an organism is dependent for growth and development under

anaerobic conditions or under other conditions in which the denitrification process is either a preferred or at least a competitive metabolic sequence (Delwiche and Bryan, 1976).

2.3.0 DENITRIFYING MICROORGANISMS

Nitrate respiration is carried out by a variety of microorganisms in the absence of oxygen, and two distinct groups have been noted (Verhoeven, 1956). The first group includes those organisms which reduce nitrate to nitrite, and allow the product to accumulate. The second group, or true denitrifiers, completely reduce nitrate to nitrogen and nitric or nitrous oxides.

The ability to denitrify according to the requirements of the second group is restricted to comparatively few species although the number is still impressive (Payne, 1973). Most of these are facultative in the sense that under aerobic conditions they can use oxygen as an electron acceptor and many are capable of fermentative reactions in the absence of oxygen and nitrate. So far no fungi or actinomycetes have been implicated.

Gamble *et al* (1977) isolated two major groups of denitrifying microorganisms from soils which were representative of the *Pseudomonas fluoroscens* biotype II, and the genus *Alcaligenes*. Included among the denitrifiers are a number of autotrophic organisms. *Micrococcus denitrificans*, a facultative autotroph, will develop anaerobically while oxidising hydrogen at the expense of nitrate. *Thiobacillus denitrificans* oxidises sulphur while reducing nitrate in the absence of oxygen (Verhoeven, 1956).

A list of genera of chemosynthetic bacteria containing species reported to denitrify is found in table 2.1. Significantly, none of the numerically dominant denitrifiers isolated by Gamble *et al* (1977) resembled the most studied species; *Pseudomonas denitrificans*,

Pseudomonas perfectomarinus, and *Paracoccus denitrificans*.

Table 2.1 Genera of chemosynthetic bacteria containing species reported to denitrify

Achromobacter	*Moraxella*
Alcaligenes	*Paracoccus*
Bacillus	*Propionibacterium*
Chromobacterium	*Pseudomonas*
Corynebacterium	*Spirillum*
Cytophaga	*Thiobacillus*
Flavobacterium	*Vibrio*
Halobacterium	*Xanthomonas*
Hyphomicorbium	

(*Paracoccus* was formerly referred to as *Micrococcus*)

2.4.0 BIOCHEMISTRY OF DENITRIFICATION

2.4.1 Pathway intermediates

Although many studies have been done under a variety of experimental conditions, a clear pathway has not yet been elucidated, which probably reflects species differences more than anything else.

It has been established that the reduction sequence includes NO_2^-, N_2O, and N_2 in that order (Nommik, 1956; Cady and Bartholomew, 1960, 1961; Cooper and Smith, 1963). Other biochemical intermediates have been outlined including hyponitrite (Kluyver and Verhoeven, 1954), a nitroxy enzyme and an imidonitrate or nitramide (Delwiche, 1956).

According to Delwiche and Bryan (1976), debate still continues as to whether N_2O is an obligatory intermediate because of contradictory results.

Allen and van Niel (1952) and Pichinoty and D'Orano (1961)

indicated that nitrous oxide was not an intermediate in denitrification in *Pseudomonas stutzeri* and *Micrococcus denitrificans*. However, recent studies suggest that N_2O is indeed an intermediate. Resting cells of *Pseudomonas denitrificans* (Matsubara and Mori, 1968; Miyata *et al*, 1969), *Corynebacterium nephridii* (Renner and Becker, 1970) and *Thiobacillus denitrificans* form N_2O during NO_2^- and NO reduction. Also *Pseudomonas denitrificans*, *Pseudomonas stutzeri* and *Micrococcus denitrificans* can be grown anaerobically using N_2O as an electron acceptor in place of oxygen, nitrate, or nitrite (Matsubara, 1971). In addition an N_2O reducing system has been obtained in *Pseudomonas denitrificans* and *Alcaligenes faecalis* (Matsubara, 1971). These systems were induced by N_2O and repressed by O_2.

Also, work involving the use of C_2H_2 to inhibit reduction of N_2O by soil microorganisms suggests that N_2O is an obligatory precursor of the N_2 produced by denitrification in soils (Yoshinari *et al*, 1977).

Pure culture studies have also presented evidence in favour of NO as an intermediate in denitrification. For example the production and/or utilization of NO in the course of reduction of NO_3^- or NO_2^- has been reported for a variety of bacteria including *Alcaligenes faecalis* (Matsubara and Iwasaki, 1971), *Corynebacterium nephridii* (Renner and Becker, 1970) and *Paracoccus denitrificans*.

Furthermore a copper-containing nitrite reductase enzyme in *Pseudomonas denitrificans* (Miyata and Mori, 1969) and *Achromobacter cycloclastes* (Iwasaki and Matsubara, 1972) and a haemo-protein nitrite-reductase in *Alcaligenes faecalis* (Iwasaki and Matsubara, 1971) and *Paracoccus denitrificans* (Lam and Nicholas, 1969) catalyse NO production from NO_2^- in the presence of added electron donors.

2.4.2 Reduction of nitrate to nitrite

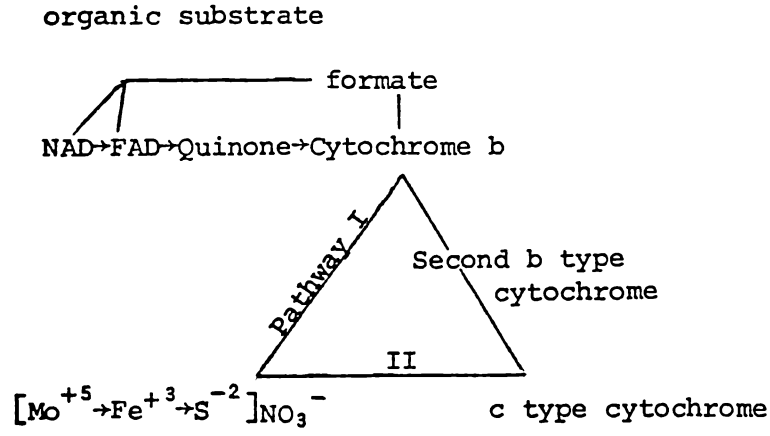
Dissimilatory or respiratory nitrate reductase is membrane

bound in *E. coli* *Pseudomonas stutzeri* (Kodama, 1970) and *Pseudomonas denitrificans* (Miyata, 1971) and normally distinct from that of the assimilatory nitrate reductase (Payne, 1973). Electron flow apparently proceeds from the cytochrome(s) to Mo onto Fe and then to nitrate.

Formate, lactate, pyruvate and NADH are effective donors for nitrate reduction in *E. coli* cells while NADH and formate are the most effective electron donors in particulate preparations from *E. coli* and *Pseudomonas denitrificans* (Radcliffe and Nicholas, 1970).

The presence of an active formate dehydrogenase system in cells grown anaerobically on nitrate and the much lower level of activity in cells grown on oxygen suggest that this system is an integral part of the nitrate respiration system and formate is of physiological importance as an electron donor for nitrate reduction in *E. coli* and *Pseudomonas denitrificans* (Radcliffe and Nicholas, 1970).

Payne (1973) presented the following scheme of electron transport:



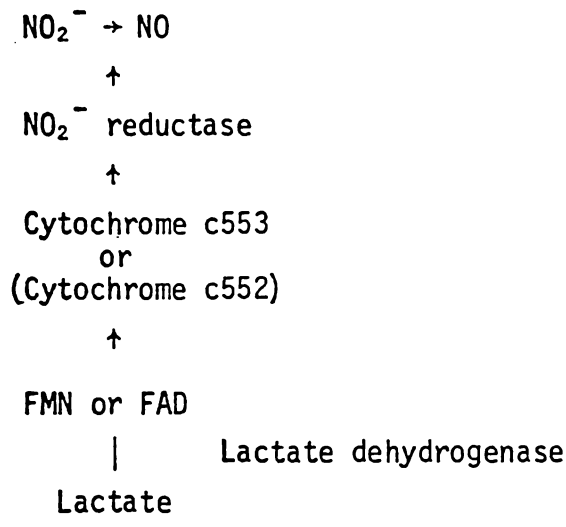
The branch point from the oxygen terminated electron transport occurs at the cytochrome b level. Most evidence is in support of pathway I (Payne, 1973) where electrons are transferred directly to nitrate reductase. Higher concentrations of c and b type cytochromes are found in cells of facultative bacteria when grown anaerobically with NO_3^- (Payne, 1973). However the increase in c-type cytochromes is

associated with higher nitrite reductase activity.

2.4.3 Nitrite reduction

Two types of nitrite reductase are found in facultative anaerobic bacteria. Both enzymes reduce nitrite stoichiometrically to nitric oxide (Radcliffe and Nicholas, 1968). The copper-containing enzyme catalyses the production of N_2O from nitrite and hydroxylamine, but there is no evidence for N_2O production in cells containing the haemo-protein nitrite reductase.

Miyata and Mori (1969) outlined the following electron transport system for nitrite reduction in *Pseudomonas denitrificans*:



Higher rates of nitrite reduction have been obtained with the addition of flavins to cell-free extracts from *Pseudomonas denitrificans* (Miyata and Mori, 1969). It is likely that flavins participate in electron transport between the soluble and particulate system in the reduction of nitrite in *Pseudomonas denitrificans* (Miyata and Mori, 1969). Radcliffe and Nicholas (1968) used NADH and NADPH as electron donors in nitrite reduction. These compounds were active only in the presence of a flavin carrier.

2.4.4 Nitrous oxide reduction

Little is known about N_2O reductase. It is located on the bacterial membrane in *Pseudomonas perfectomarinus* and contains metal co-factors (Payne, 1973). Azide, cyanide, 2-4 dinitrophenol (Matsubara and Mori, 1968) and C_2H_2 at low concentrations (Balderston *et al*, 1976; Yoshinari and Knowles, 1976) inhibit this enzyme. In *Pseudomonas denitrificans* the development of the N_2O-reductase system is repressed by oxygen and derepressed at low concentrations of oxygen in the absence of N_2O (Matsubara, 1970).

The system involved in N_2O reduction has not yet been evaluated. Matsubara (1971) found the amount of a soluble CO binding cytochrome c553 and a particulate bound cytochrome c552 to be appreciably higher in N_2O anaerobically grown cells than in NO_3^- anaerobically grown cells, and suggested that these cytochromes may be involved in the N_2O system.

2.4.5 Regulation of dissimilatory reductases

It is now generally accepted that dissimilatory nitrate reduction in most organisms becomes dominant only under anaerobic conditions. The mechanism by which oxygen affects nitrate reduction is not well known. However, Cox and Payne (1973) have shown that the synthesis of enzymes is controlled by oxygen repression rather than NO_3^- induction in *Pseudomonas perfectomarinus*. Repression is complete except for N_2O-reductase which is only partially repressed (Payne *et al*, 1971). Aerobically grown cells placed under anaerobic conditions exhibit a lag period before utilization of NO_3^- as an electron acceptor (Delwiche, 1959; Sacks and Barker, 1949). No lag has been reported in the utilization of oxygen by anaerobically grown cells. This phenomenon can be explained by oxygen repression of transcription of the NO_3-reduction enzymes. After the metabolite repressor is

removed, a finite time is required, presumably to transcribe and translate the genes involved. In *Pseudomonas perfectomarinus*, synthesis of all the denitrifying enzymes begins within 40 minutes after derepression (Payne and Riley, 1969; Riley, 1969).

The synthesis of NO_2^-, NO, and N_2O-reductases are also derepressed by anoxia or lowered oxygen tension. Nitrate does not appear to inhibit the synthesis of these enzymes in *Pseudomonas perfectomarinus*, but the activity of NO reductase is lowered in the presence of NO_3^- (Payne, 1973). On the other hand NO_3^- inhibits the synthesis and the activity of NO_2^--reductase in *Pseudomonas stutzeri* (Kodama, 1970) and NO reduction in *Pseudomonas denitrificans* (Miyata, 1971).

Nitric oxide has been suggested as an intermediate in the reduction of NO_3^- to N_2O (Delwiche, 1976) and the results of Kodama (1970) and Miyata (1971) concur with the findings of Blackmer and Bremner (1978) that high NO_3^- concentrations almost completely inhibit the anaerobic reduction of N_2O to N_2 by soil microorganisms. These results are in line with those of Cho and Sakdinan (1978) who concluded that higher NO_3^- concentrations favoured the accumulation of N_2O because of the competitive nature of NO_3^- and N_2O as electron acceptors. Focht and Verstraete (1977) noted that NO_3^- provides more energy per electron transfer than N_2O, which explains the observation that NO_3^- is the preferred electron acceptor (Koike and Hattori, 1975a).

Blackmer and Bremner (1979) also showed that if the denitrifying microorganisms have not had the opportunity to adapt for anaerobic reduction of N_2O to N_2, a small amount of NO_3^- has a stimulatory effect on their ability to perform this function.

2.4.6 Energy yield of denitrification

Phosphorylation specifically coupled to the reduction of NO_3^- to NO_2^- has been demonstrated in *E. coli*, *Paracoccus denitrificans* and *Pseudomonas denitrificans* (Koike and Hattori, 1975a). Phosphate esterification is also coupled to the reduction of NO_2^- to N_2O in *Paracoccus denitrificans*, *Pseudomonas denitrificans* and N_2O reduction to N_2 in *Pseudomonas denitrificans* (Koike and Hattori, 1975a).

The energy yield from denitrification is approximately 50% of that associated with oxygen respiration when considered on an electron basis (Koike and Hattori, 1975b). This value is similar to that of Rigaud *et al* (1973) and Ratcliffe *et al* (1980) who estimated NO_3^- respiration to yield about 40% of the energy gained from oxygen respiration by soybean bacteroids.

2.5.0 ASSESSING DENITRIFICATION

Many methods have been applied to assess rates of denitrification and after almost a century of work there is still no complete technique capable of assessing the total sum of the denitrification products in the field. For a comprehensive coverage of the techniques available the reader is referred to the review by Focht (1978) and the critiques following by Verstraete (1978) and Payne (1978), and the review by Makarov and Makarov (1976).

Focht (1978) divided the methods of assessment into five broad categories:

i) Indirect analyses (including available carbon, Eh, and bacterial number counts),

ii) disappearance of nitrate,

iii) ^{15}N tracer studies,

iv) ^{13}N tracer studies, and

v) evolution of N_2 and N_2O.

2.5.1 Indirect analyses

i) Available carbon

The supply of readily decomposable organic matter is a crucial factor for denitrification of nitrate in soils because it is well established that denitrification in waterlogged soils can be greatly promoted by addition of organic materials. Observations by Bremner and Shaw (1958) indicated that denitrification in soils is controlled largely by the supply of water-soluble or readily decomposable organic matter.

Available carbon is a difficult concept to define but has come to be accepted generally as that carbon extractable in aqueous soil solutions. The method of analysis of this extraction is described by Burford and Bremner (1975).

Stanford *et al* (1975c) found excellent correlations between water soluble carbohydrates which they termed 'glucose equivalents', and rates of denitrification. But Burford and Bremner (1975) calculated that the soluble organic carbon could supply only 76% of the total amount needed to reduce all of the NO_3^- to N_2. However the water soluble carbon was found to provide a good index ($r = 0.999$) of a soil's capacity for denitrification of NO_3^-. Such results indicate that the water soluble fraction of soil organic matter is particularly susceptible to decomposition.

ii) Redox Potential (Eh)

The redox potential is an indication of the reduction-oxidation potential, or potential difference, (mV) existing between two solutions. Measurement of Eh values in soils involves the insertion of a platinum tipped electrode. The Eh value reflects the degree of anoxia. It also takes into account the presence of Fe^{+3} and Mn^{+4} which are indicative of NO_3^- reduction because both are used as terminal electron acceptors (Ghoshal and Larsson, 1977; Focht, 1978).

The critical Eh for the NO_3^-/NO_2^- couple (i.e. where they are present in equivalent activities) is 421 mV at pH 7 and 25°C (Focht, 1978).

The Eh for reduction of N_2O to N_2 appears to be about 250 mV at pH 7 (Focht and Verstraete, 1977). Thus an Eh reading of 250 mV in soil would ensure that no NO_3^- would be present, yet very little N_2O would be reduced to N_2.

The Eh values for Fe^{+2} and Mn^{+2} are approximately 200 and 300 mV respectively. Ghoshal and Larsson (1977) demonstrated that Fe^{+2} and Mn^{+2} alone can stimulate the NO_3^- reduction process. It is their opinion that the biological and chemical reduction of NO_3^- may occur simultaneously in soil and that the chemical process may play a more prominent role in the deeper zones of the soil.

Meek *et al* (1969) showed a strong relationship between Eh values and soil water contents. Soils approaching saturation reflected the reduced oxygen status by a sharp decrease in redox potential and a significant increase in denitrification products.

Eh field measurements are susceptible however to many factors and usually yield somewhat variable results but can be used to make useful comparisons between soil systems (e.g. Meek *et al*, 1969; Knowles and Denike, 1974; Reddy and Patrick, 1975; and Flühler *et al*, 1976).

iii) Bacterial numbers.

The denitrifying bacteria are ubiquitous in soil and most respire aerobically in the presence of oxygen. Woldendorp (1963) found that when NO_3^- fertilizers were added, the most prominent denitrifying bacteria changed from *Bacillus* spp. to *Pseudomonas*, *Achromobacter* and *Bacillus macerans*. He concluded that the indigenous denitrifiers were present, not as a result of their denitrifying activities, but because of some other property they possessed. Consequently no relationship between the original number of denitrifying bacteria in the soil and the denitrifying capacity of that soil could be established.

The numbers and percentage of denitrifiers estimated in a sample also varies depending on the method of assessment. Enumeration of denitrifying bacteria is commonly based upon the most-probable number determination which is more tedious and less accurate than the standard plate count (Focht, 1978).

2.5.2 Disappearance of nitrate and nitrite

This technique has been very popular and provides some of the earliest quantitative data regarding denitrification. Upon assay the balance of the total NO_3^- present is assumed indicative of the rate of NO_3^- dissimilation (and assimilation). Assays for NO_3^- are relatively easy colourimetrically (Kamphake *et al*, 1967).

Using lysimeters, this technique has been extended to the field. Allison (1955) showed that 10-30% of the N balance was unaccounted for and attributed this loss to denitrification.

However, the errors incurred in such a technique can be large and a more correct measurement is considered to be the NO_3^-/Cl^- ratio. The mobilities are the same and the dilution effect similar. Therefore any change in the ratio is a result of biological processes involving NO_3^- as Cl^- is considered biologically inert. This technique does suffer from varying plant specificity regarding nutrient uptake.

Soil columns allow the opportunity to overcome this, and other such variables as run off and lateral dispersion. These types of models are useful for sorting out the important parameters that influence rate processes.

Incubation studies involving NO_3^- disappearance invariably lead to higher rates of denitrification than expected in the field. The system is often anaerobic, and the whole system is static, therefore such effects as leaching of the soil profile cannot be accounted for. The technique has value however by indicating the relative effect of

various parameters upon rates of denitrification and has found new favour with the introduction of inhibitors to the incubation vessel.

The question arises as to whether the loss of nitrate alone is sufficient criteria for assuming that most of the nitrate was reduced by dissimilatory and not assimilatory means. Under absolute anaerobic conditions without addition of exogenous carbon, Focht (1978) contends, on thermodynamic grounds, that assimilation losses are insignificant. Firstly more energy is required for the reduction of nitrate to NH_4^+ than to N_2. Secondly, the incorporation of one atom of N into bacterial biomass requires the incorporation of approximately 7 atoms of C, thus requiring a minimal C/N ratio of 7:1. Thirdly, the most efficient manner of generating the energy required for biosynthesis is through oxidative phosphorylation, which is best achieved by coupling the oxidation of carbonaceous substrate with nitrate (in lieu of oxygen) as the electron acceptor. Assimilation of nitrogen by fermentative, non-denitrifying organisms would be much less efficient and would involve considerably more carbonaceous substrate because of incomplete oxidation.

Notwithstanding this reasoning, Stanford *et al* (1975b) reported significant NH_4^+ production with concomitant NO_3^- disappearance in six widely differing soils under anaerobic conditions. Up to 30% of the added NO_3^- was converted to NH_4^+. Stanford *et al* (1975b) claimed their results seriously challenged the prevalent view that denitrification accounts for essentially all NO_3^- dissimilation in anaerobic soils. They presented a modified sequence of the denitrification pathway (equation 2.7).

$$2NO_3^- \rightarrow 2NO_2^- \rightarrow 2[HNO] \begin{cases} \xrightarrow[\text{Pathway 1}]{} H_2N_2O_2 \rightarrow N_2\ N_2O \\ \xrightarrow[\text{Pathway 2}]{} 2NH_2OH \rightarrow 2NH_4^+ \end{cases} \qquad 2.7$$

They suggested that assimilation of hydroxylamine (NH_2OH) and NH_4^+

by microbial cells be considered as an extension of pathway 2.

However, in the experimental system described by Stanford *et al*, varying amounts of glucose were added. The amount of assimilated NO_3^- was directly proportional to the amount of glucose added.

Focht (1978) concludes that NO_3^- disappearance from incubation vessels is an adequate criterion for denitrification only if an exogenous energy source is absent or if the addition of an exogenous energy source does not exceed the stoichiometric C/N ratio required for completion of the reaction to CO_2 and N_2. The system must be carbon limiting.

2.5.3 ^{15}N

The use of N isotopes has become more widespread as the isotope becomes more readily available cheaply, and as the accuracy of isotope mass spectrometers improves. Application of the isotope is usually in the form of $^{15}NO_3^-$ or $^{15}NH_4^+$ substrate. Generally the substrate is enriched in ^{15}N although ^{15}N depleted (^{14}N enriched) material has seen limited use (Broadbent and Carlton, 1978).

Analyses of results require mass spectrometric facilities, but high accuracies are possible. Double beam, dual inlet magnetic sector mass spectrometers are capable of determining differences in $^{15}N/^{14}N$ ratios to a standard deviation of ±0.05‰ (Steele, 1977). [Variations in isotopic ratios are generally expressed in parts per thousand as:

$$\delta^{15}N = \frac{^{15}N/^{14}N \text{ sample} - {}^{15}N/^{14}N \text{ standard}}{^{15}N/^{14}N \text{ standard}} \times 1000$$

$\delta^{15}N$ is sometimes written as $\Delta^{15}N$ where a difference between two δ values are reported. (Steele *et al*, 1978)] Quadrupole mass spectrometers are capable of determining isotopic ratios with much less sample due to increased sensitivity. However the standard deviation of

the isotopic determinations is in the order of 40‰ (Focht, 1978).

Mass spectrometers can be interfaced to gas chromatographs to give the added advantages of i)separating interfering peaks, ii) the mass spectrometer can be focused to resolve unseparated peaks.

Mass spectrometric isotope analyses do suffer from complex interferences however. For instance a m/e 29 peak could be due to $^{14}N^{15}N$, $^{13}C^{16}O$ or $^{12}C^{17}O$. CO_2 is commonly produced in a mass spectrometer by combustion of carbon deposited on the filament from previous organic analyses. This CO_2 is then capable of fragmenting in the ion chamber to produce CO of various isotopic combinations. Similarly N_2O (collected in soil gas samples) is capable of fragmenting to NO and N_2 producing the following major species: $^{14}N^{16}O^+$, $^{14}N^{14}N$ and $^{14}N^{15}N^+$, thus contributing to m/e peaks: 30, 29, and 28 (Hauck and Melsted, 1956). Most workers facing this problem have found it necessary to remove the N_2O from samples prior to analyses by passing the gas over hot copper (e.g. Hauck and Melsted, 1956; Schwartzbeck *et al*, 1961). Others have also found it necessary to remove CO_2 and O_2 by adsorption in KOH and alkaline pyrogallol respectively (e.g. Cho and Sakdinan, 1978). Oxygen is reputed to cause filament instability and may lead to the production of CO_2 and CO within the mass spectrometer. Paul (1973) found it possible to analyse quantitatively for N_2, CO_2, N_2O, NO, O_2 and Ar without prior sample treatment. However, sample accuracy was quoted as ±5% and minor isotopic contributions were not considered. For further discussion on the effects and problems of interference peaks on isotope analyses see section 8.6.3.

A further complication in the use of ^{15}N substrates for denitrification assessment can arise from isotope discrimination (or microbial fractionation) in soils. Blackmer and Bremner (1977a) showed the discrimination against ^{15}N during the $NO_3^- \rightarrow NO_2^-$ reaction can amount to 15 to 20 $\Delta^{15}N$ units with respect to the initial NO_3^--N. They

also showed that significant N isotope fractionation occurs during reduction of NO_2^- to N_2 and N_2O. The $\Delta^{15}N$ values measured agree with those of Wellman *et al* (1968) and Delwiche and Steyn (1970).

Such results invalidate the use of ^{15}N values in the assessment of the relative amounts of soil derived and fertilizer derived NO_3^- in surface waters as proposed by Kohl *et al* (1971). The variations in $\Delta^{15}N$ values found by Blackmer and Bremner (1977a) are twice the range of those observed by Kohl (1971) in an analysis of drainage effluent.

2.5.4 ^{13}N

^{13}N analyses have a limited application due to the short half life (10 minutes) of the isotope. Access to a cyclotron is necessary for utilization of this technique. ^{13}N is produced in the cyclotron by bombardment of ^{16}O (p, α). The high energy (1.08 meV) and low background of ^{13}N allow high sensitivity measurements to be made, although experiments rarely last longer than 15 minutes. Analyses can be made to the order of <1 ppb N/hr (Gersberg *et al*, 1976). Tiedje *et al* (1978) used ^{13}N assays to evaluate the C_2H_2 inhibition technique of denitrification assessment. $^{13}NO_3^-$ was added to a soil slurry and continuously stripped of product gases with helium. The flushed gases passed through a liquid N trap ($^{13}N_2O$) and then onto a molecular sieve trap immersed in liquid N ($^{13}N_2$). NaI crystals attached to photomultiplier tubes were employed as detectors and installed below each trap.

2.5.5 Evolution of nitrogenous gases

Direct fluxes of N_2 cannot be measured in the field without the use of isotopes due to the high background of N_2 in the soil atmosphere. Therefore measurement of N_2 production is largely confined to the laboratory where the ambient atmosphere is replaced with an inert gas,

usually argon or helium. Ar/O_2 growth chambers, as used by Stefanson and Greenland (1970) reduced the N_2 background to approximately 100 ppm v/v. These experiments however do suffer from leaks and diffusion of minute quantities of external air.

Infra-red may be used for analyses of N_2O (e.g. Hauck and Melsted, 1956; Schwartzbeck *et al*, 1961) and sensitivities better than ambient levels of N_2O can be expected (Denmead, 1979). However, infra-red analysis does require large sample volumes and can suffer from interferences.

Gas chromatography is widely used for both N_2O and N_2 analyses, mainly in conjunction with Porapak Q and molecular sieve 5A columns. Porapak Q is ideal for low molecular weight gases, but cannot separate O_2, N_2 and Ar. Molecular sieve 5A permits good separation of O_2, N_2 and Ar, but retains CO_2 and N_2O. No single column analyses have yet been devised, so multi-column analyses are common and varied. Graven (1959) did manage to separate O_2, N_2, CO, C_2H_6, N_2O and CO_2 on a single column of molecular sieve 5A by controlled heating to 400°C. However, the author found this technique difficult to duplicate and time consuming for routine analyses.

Detectors play a large part in gas chromatography with Thermal Conductivity Detectors (TCD) most commonly employed for incubation studies. Ultra-sonic and Electron Capture Detectors (ECD) are necessary for sub-ambient detection of N_2O.

Since N_2O has recently been accepted as an intermediate in the denitrification pathway the occurrence of N_2O in soils in concentrations above ambient can be taken as definitive proof that denitrification is or has been occurring. This has led to many attempts being devised to measure fluxes of N_2O from the soil in field conditions. The enigma of N_2O is that, kinetically, it represents a transient intermediate. Therefore a low flux of N_2O could indicate either a low

rate of N_2O production or alternatively, a higher rate of N_2O reduction. Therefore without knowing the rate of N_2O reduction, N_2O fluxes cannot be used to directly measure denitrification losses. However, such field measurements do yield valuable data as to the possible environmental consequences of N_2O release from the soil, and give clues to the influences controlling relative rates of N_2O/N_2 production.

Burford and Stefanson (1973), using Fick's law

$$\frac{dq}{dt} = -Da\frac{dc}{dz} \qquad 2.8$$

calculated the diffusive fluxes of N_2O and N_2 from $^{15}NO_3^-$ by the concentration gradients in soils. Fluxes of N_2 and N_2O have been estimated by several others using similar or slightly modified techniques (e.g. Rolston *et al*, 1976; Rolston, 1978). Such estimates however are subject to significant errors in values such as the concentration gradient $\frac{dc}{dz}$ and the effective diffusivity. Burford and Stefanson (1973) considered the most important error to be the estimate of air filled porosities. Rolston *et al* (1976) found the greatest problem in applying the diffusion technique to soil columns and in the field was the measurement of N_2O and N_2 concentration gradients near the soil surface. These errors could lead to a large error in the experimental determination of the field results. In this case the flux (F) of N_2 and N_2O diffusing out of the soil was calculated by:

$$F = -AD \int_0^{T_1} (dc/dx)\, dt \qquad 2.9$$

where A is the area of the columns or field plot, D is the apparent diffusion coefficient, dc/dx is the gaseous concentration gradient evaluated at $x = 0$, t is time and T_1 is the last day of sampling. Again there was considerable uncertainty in the estimation of D. The

values in duplicate soil cores ranged between 0.6 to 3.0 cm^2 hr^{-1} and the field measurement yielded values between 0.5 and 0.9 cm^2 hr^{-1}.

Another approach to the measurement of N_2O fluxes is found by placing soil covers on the surface and measuring the increasing N_2O concentration within the headspace. This approach has the disadvantage of possibly under estimating the true flux if the gradient between the zone of production and the surface is gradually decreasing due to the build up of N_2O in the chamber. For further discussion on this technique see section 7.3.0.

2.5.6 Nitrogen/Nitrous oxide ratios

Burford and Stefanson (1973) found the N_2/N_2O ratios to vary by 100 fold depending on the soil water content. Rolston *et al* (1976) observed N_2/N_2O flux ratios between 9 and 45 in soil columns and field plots also using $^{15}NO_3^-$.

Stefanson (1973) concluded that plant growth was a significant influence and consistently altered the amounts of N_2 and N_2O evolved.

Mahendrappa and Smith (1967) found that pH of the soils affected relative N_2O/N_2 compositions. In an alkaline soil, N_2 was observed while N_2O was still increasing. In acid soils, N_2 was not detected until N_2O production was at a maximum and had started declining. Keeney *et al* (1979) noted temperature effects on the relative proportions of N_2 and N_2O.

Focht and Stolzy (1979) in an experiment studying denitrification processes under organic amendments concluded the most important factor affecting N_2O concentrations was the NO_3^- concentration. Soil solutions containing greater than 16 µg NO_3-N/ml had much higher frequencies of high N_2O concentrations.

Blackmer and Bremner (1978) also observed that NO_3^- inhibits

N_2O reductase. They found the inhibitory effect of NO_3^- on N_2O reduction increased markedly with a decrease in pH. This observation agrees with that of Mahendrappa and Smith (1967) that a low pH favours the accumulation of N_2O.

Blackmer and Bremner (1979) also demonstrated that if the denitrifying microorganisms in soils have not had an opportunity to adapt for anaerobic reduction of N_2O to N_2, a small amount of NO_3^- has a stimulatory effect on their ability to do so, causing further confusion in the interpretation of N_2O/N_2 ratios. Firestone *et al* (1979) also noted that increased NO_3^- concentrations caused increased proportions of N_2O compared to N_2. Also, NO_2^- was found to exert a similar, but much stronger influence than NO_3^-.

Aeration influences the preferential utilization of NO_3^- and N_2O as the terminal electron acceptors. Nitrate appears to be the preferred electron acceptor with NO_3^- providing more energy per electron transferred than N_2O, (Koike and Hattori, 1975a). This observation accounts for the fact that considerably less N_2O is produced compared to N_2 as soils become less aerated (Arnold, 1954; Nommik, 1956).

In fact this observation is partly explained by considering changes in diffusion of gases due to increased wetness of soils. Diffusion of gases out of a soil pore system is hindered near zero percent air-filled pore space. Inward diffusion of oxygen will also be hindered, leading to increased anoxia. All these concepts are mutually inclusive since reduction of gaseous diffusion permits (i) a greater contact time of the dissolved N_2O with the bacteria, and (ii) a more rapid conversion and subsequent depletion of NO_3^-, which would otherwise be the preferred terminal electron acceptor.

With the discovery that C_2H_2 in small concentration effectively blocks N_2O reduction (Balderston *et al*, 1976; Yoshinari and Knowles, 1976), the possibility of a simple *in situ* technique of

field denitrification has occurred. This method could give misleading results if the concentration of N_2O within the sampling chamber was permitted to build up rapidly enough to reduce the N_2O flux. A successful attempt at using this technique was made by Ryden and co-workers (1979). For a further discussion on the problems of this technique, see sections 7.3.0, 7.3.2 and 7.6.0.

Garcia (1974) proposed that the disappearance of N_2O be considered as an index of denitrification. Soil cores are placed in incubation flasks and the atmosphere replaced with He, enriched in N_2O. Garcia (1974) claims that the rate of N_2O reduction observed during the first 6 hours represents the actual denitrification activity present in the soil. Apart from the usual drawbacks of complete anoxia and high levels of electron acceptor, this technique has the advantage of only measuring dissimilatory nitrogen transformations - unlike the NO_3^- disappearance technique.

2.6.0 SOURCES AND SINKS OF NITROUS OXIDE

As early as 1953 Arnold calculated the soils to be a source of atmospheric N_2O. Active denitrification in both the oceans (Goering and Cline, 1970) and sediments (Keeney *et al*, 1971) is known. Both N_2 and N_2O are demonstrated products of these environments. The super saturation of ocean waters to 100% or greater in N_2O (Hahn, 1972, p53-69) suggests that both denitrification and N fixation in the oceans are greater than had previously been assumed.

The interpretation of data on N_2O in the ocean is complicated by a strong temperature effect on its solubility and uncertainty of the extent to which present atmospheric concentrations represent a steady state and are not subject to long-term changes. The atmospheric N_2O concentration is an expression of the steady-state consequence of competitive biological reactions as modulated by atmospheric processes.

That portion of atmospheric N_2O that is readsorbed by the soil (and sea) is either further denitrified to N_2 or re-enters the assimilatory sequence. If N_2O is an obligatory intermediate in the reduction of NO_3^- to N_2 by soil microorganisms, an estimate of the average ratio of N_2O to N_2 in the gases evolved from soils through denitrification of NO_3^- indicates that about 94% of the N_2O produced by denitrification of NO_3^- in soils is reduced to N_2 (Council for Agricultural Science and Technology, 1976). However a small variation in the rate of N_2O reduction to N_2 could have a significant effect on this value. Blackmer and Bremner (1978) calculated that a 10% decrease in the rate of N_2O reduction could lead to a 157% increase in the amount of N_2O emitted.

Present estimates of the residence time of N_2O in the atmosphere are not consistent with current figures for nitrogen fixation and the overall atmospheric content of N_2O. Using an 8 year residence time it would be necessary to have an annual input of approximately 200×10^6 tonnes of N per annum as N_2O. Even a 75 year residence time would require about 25×10^6 tonnes. It appears some additional reaction will be necessary to explain N_2O production. The comparatively long residence time can be explained in terms of the activation energy required for bond disruption in the N_2O molecule (Delwiche and Bryan, 1976).

Cicerone *et al* (1978) measured atmospheric concentrations of N_2O and estimated a value of 329.5 ± 9.9 ppb (3σ). Schütz *et al* (1970) has observed considerable annual variation in N_2O concentrations, with data suggesting a global increase with time. Cicerone *et al* (1978) came to the conclusion that microbial denitrification is probably the major source of atmospheric N_2O. Soils were seen to act as both sources and sinks of N_2O but their net contribution to the N_2O distribution was deduced to be a source. Freney *et al* (1979) also found the soil to be

an important factor in maintaining the atmospheric N_2O concentration. The most important sink according to Cicerone *et al* (1978) is the stratospheric destruction of N_2O.

CHAPTER 3

DEVELOPMENT OF A TECHNIQUE FOR ASSESSMENT OF DENITRIFICATION POTENTIALS OF SOILS

3.1.0 INTRODUCTION

The denitrification potential of a soil is defined as the maximum rate at which nitrate will be dissimilated under an anaerobic atmosphere without addition of exogenous reductant (Focht, 1978). For many years denitrification potentials have been measured by laboratory anaerobic incubations (e.g. Cady and Bartholomew, 1960; Cooper and Smith, 1963; Bailey and Beauchamp, 1973), the objective of which is to induce a maximal rate of nitrate respiration since the synthesis of denitrifying enzymes is believed to be controlled by oxygen repression rather than nitrate induction (Cox and Payne, 1973). Although laboratory denitrification assays yield useful information concerning relative denitrification rates, they do not provide quantitative estimates of denitrification in the field.

Anaerobic incubation assays of denitrification potentials have received increased attention since acetylene was reported to block N_2O reductase (Yoshinari and Knowles, 1976; Balderston *et al*, 1976) owing to simplified analysis procedures. Previous incubation assays relied either on monitoring substrate disappearance or analysis of all gaseous products including nitrogen. Nitrate disappearance is an unsatisfactory measure of denitrification because of possible assimilation into the organic fraction (Stanford *et al*, 1975) and routine analyses of all gaseous products is difficult and time consuming.

The presence of 0.1 atm acetylene in the incubation atmosphere has been shown to achieve complete blockage of N_2O reductase in soils (Klemedtsson *et al*, 1977; Yoshinari *et al*, 1977) without affecting the denitrification rate (Ryden *et al*, 1979; Smith *et al*, 1978) thus permitting denitrification assays by monitoring N_2O production alone. The low concentration of N_2O in the atmosphere, accompanied by the ease with which it can be assayed by gas chromatography has led to a widespread acceptance of N_2O reductase inhibition for denitrification assays.

Rates of denitrification have been shown to increase with pH, nitrate concentration, reductant (organic substrate) concentration and decreased aeration (Focht, 1978). Factors such as pH and endogenous reductant vary between soils and affect denitrification potentials. It is important therefore that soil parameters which affect denitrification potentials are not amended prior to laboratory incubation since denitrification potentials measured in amended soils will reflect the changes induced in the soil and will not necessarily reflect potential denitrification rates of unamended soils.

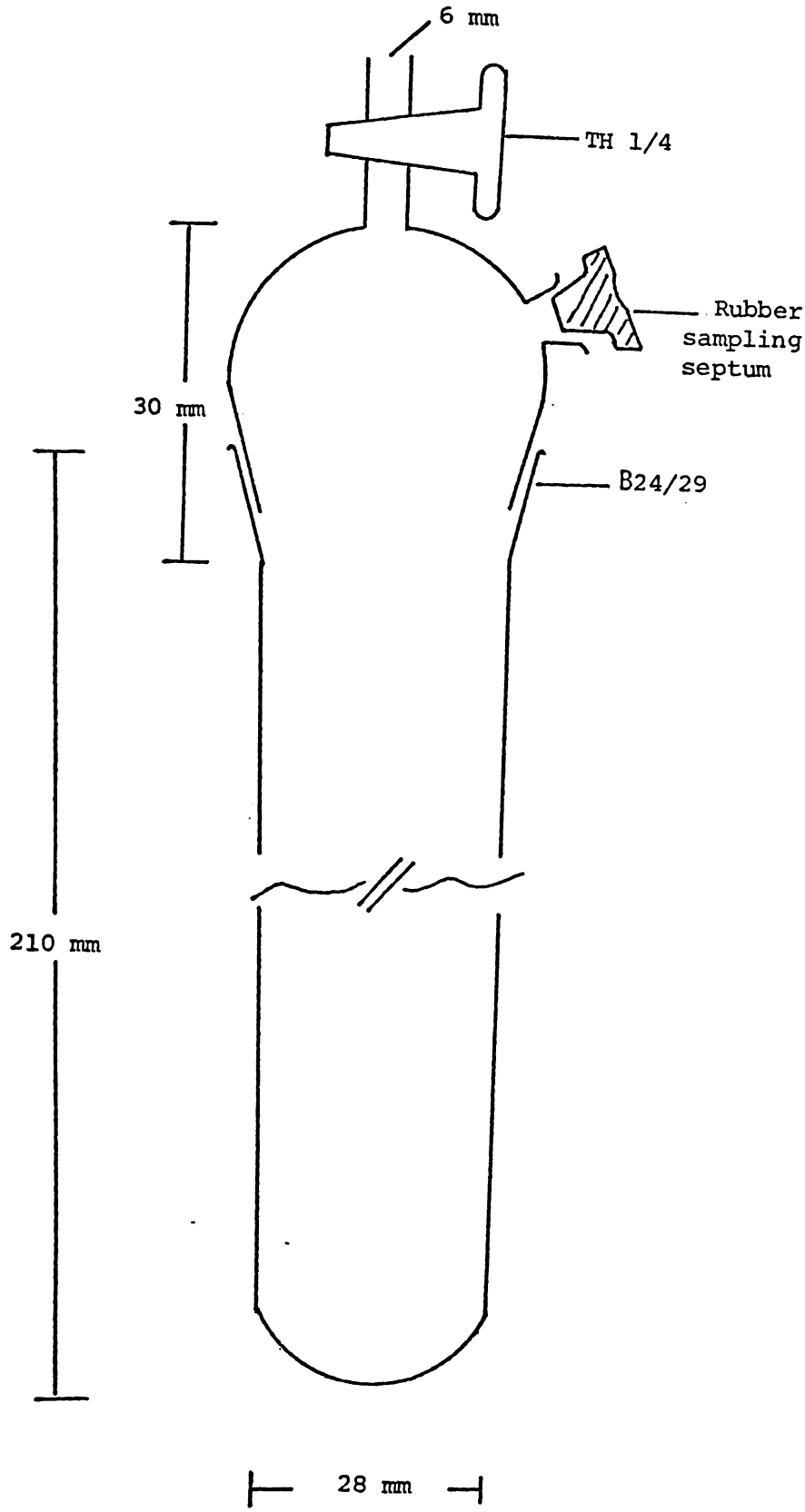
The objective of this chapter is to establish a technique capable of comparing relative denitrification potentials of soils under equivalent conditions.

3.2.0 EXPERIMENTAL MATERIALS AND METHODS

Soil cores (25 mm diameter, 0-50 mm depth) were collected from selected sites and transported to the laboratory in insulated boxes containing ice packs. Subsequent storage was at 4°C in sealed plastic bags. Samples were passed through a 4 mm sieve and moisture contents determined gravimetrically at 105°C.

Duplicate 20 g (105°C equivalent) samples were weighed into pyrex incubation tubes (28 mm diameter, 210 mm long) fitted with a cone giving a nominal volume of 100 ml (fig 3.1). The cone and stopcock were sealed with Apeizon L and M grease respectively. Whenever rigorous precautions were required against septum leakage (e.g. for monitoring N_2 production) the septum was further sealed with 732 RTV silicone rubber as described by Smid and Beauchamp (1974). Acetylene (0.1 atm) was added to the atmosphere of each tube and samples were stored overnight (~14 hours) at 4°C to facilitate complete diffusion of the acetylene into the soil crumbs.

Figure 3.1 Incubation vessel



Upon removal from the refrigerator incubation vessels were connected to a manifold using 6 mm cajon connectors. The oxygen level was reduced to less than 1000 ppm v/v by repeated evacuation with a rotary oil pump to 0.5 atm and refilling with He. Usually 5 or 6 cycles were required to achieve the desired oxygen content. During the last cycle, 10% C_2H_2 (v/v) is added via the manifold and the pressure amended to 1 atm with He. Incubations were conducted in high purity He (O_2 < 1 ppm) supplied by New Zealand Industrial Gases. Nitrate solution was added to each tube by syringe through the sampling septum with sufficient additionalwater to adjust each soil to the required water holding capacity. The incubation vessels were then placed in a water bath (35°C) for ten minutes to rapidly warm them from 4°C. Incubation continued at 25°C with regular sampling of the atmosphere for N_2O production.

The effect of the following parameters on denitrification under the conditions outlined were studied:

i) Water holding capacity

ii) Substrate (NO_3) levels

iii) Degree of anoxia

iv) Sample treatment and storage.

3.2.1 Gas chromatographic analyses

The gases of interest in this study are O_2, N_2, N_2O, CO_2 and C_2H_2. Numerous papers have described column separations for various mixtures of these gases (e.g. Bennett, 1967; Solomon, 1967; Burford, 1969; Van Cleemput, 1969; Bailey and Beauchamp, 1973; Blackmer *et al*, 1974; Ghoshal and Larsson, 1975). All of the reported analyses consist of either multiple columns (usually at differing temperatures) or achieve only partial separation of the total mixture on a single column.

While C_2H_2, CO_2 and N_2O can readily be separated on a Porapak Q column, separation of N_2 and O_2 from the carrier gas (He or Ar) remains impossible on the same column. Because of the complexity of the systems required to separate the above gases and the difficulty of manipulation of such systems, attempts were made to develop a rapid single column procedure which could be used for routine analyses.

A Varian 3700 gas chromatograph equipped with a Thermal Conductivity Detector (TCD) and sub-ambient temperature programme facilities down to -90°C was available.

The most promising technique of single column separation appeared to be a modification of the 3.3 m molecular sieve 5A column described by Graven (1959). Molecular sieve 5A is extensively used for separation of N_2 and O_2 but suffers from the disadvantage of irreversibly adsorbing CO_2 and N_2O. Graven (1959) however reports to have eluted CO_2 and N_2O from a molecular sieve 5A column using temperature programming up to 400°C. Complete elution took 25 minutes with CO_2 being the last peak eluted. The conditions described were not suitable for the present study because:

i) N_2O remained on the column for approximately twenty minutes, by which time tailing is evident;

ii) the Varian 3700 will not temperature programme to 400°C; and

iii) sample analyses required approximately one hour, including oven cooling between samples.

A 50 cm, 3 mm O.D. column was packed with Lindé molecular sieve 5A (30-60 mesh). While this column gave favourable O_2/N_2 separation, significant N_2O adsorption onto the column occurred.

A 3:2 mixture of molecular sieve 5A and Porapak Q (80-100 mesh) showed poor N_2O elution and a diminished N_2/O_2 separation.

Blackmer *et al* (1974) used a single column packing of Porapak Q

(50-80 mesh) and maintained one 5.5m column at 35°C and a 7.3m column at -78°C. These columns were linked in series through the two ports of the TCD detector.

Separation using a single 4.0m Porapak Q column (3 mm I.D. copper tubing) was attempted. Separation of N_2, O_2, Ar, CO_2 and N_2O was achieved using a He flow rate of 15cc/min. An initial temperature of -75°C was held for five minutes after sample injection. N_2 appeared, followed sixty seconds later by O_2. Ar followed twenty seconds later. Heating at 80°C/min to +50°C eluted CO_2 followed by N_2O within a further 4 minutes. Although this technique achieved the desired separations it suffered two disadvantages:

i) Cooling time between samples was inconvenient (approximately 10 minutes)

ii) Baseline drift during the temperature programme could become severe for samples requiring low attenuations.

For these reasons the single column separation was not developed further. Dual columns were installed in the gas chromatograph. The Porapak Q column was 3 m long and 2 mm I.D. The molecular sieve 5A column for separation of N_2/O_2 when required was packed with 30-60 mesh, I.D. was 2 mm and it was 1.0 m long. By using a He flow rate of 30cc/min on the Porapak column and 25cc/min on the molecular sieve column, N_2/O_2 and $N_2O/CO_2/C_2H_2/(Ar/O_2/N_2)$ separations could be achieved within 3-4 minutes using the appropriate columns at 40°C. A He regulator pressure of 35 psi gave an inlet pressure of 18 psi for the Porapak column and 10 psi for the molecular sieve column.

Using a filament temperature of 260°C (272 mA) and a detector temperature of 150°C a minimum N_2O detection level of ~ 8 ppm v/v was estimated for a 0.2 cc sample using a 1mv scale on the chart recorder. The N_2O peak was symmetrical and production was monitored by recording peak heights. Calibration curves for N_2O and N_2 standards in Argon

prepared by New Zealand Industrial Gases are presented in fig 3.2. A 2 ml Pierce pressure-lok (Precision Sampling Corp., Baton Rouge, La.) was used throughout the study for all gas chromatographic sampling and analyses.

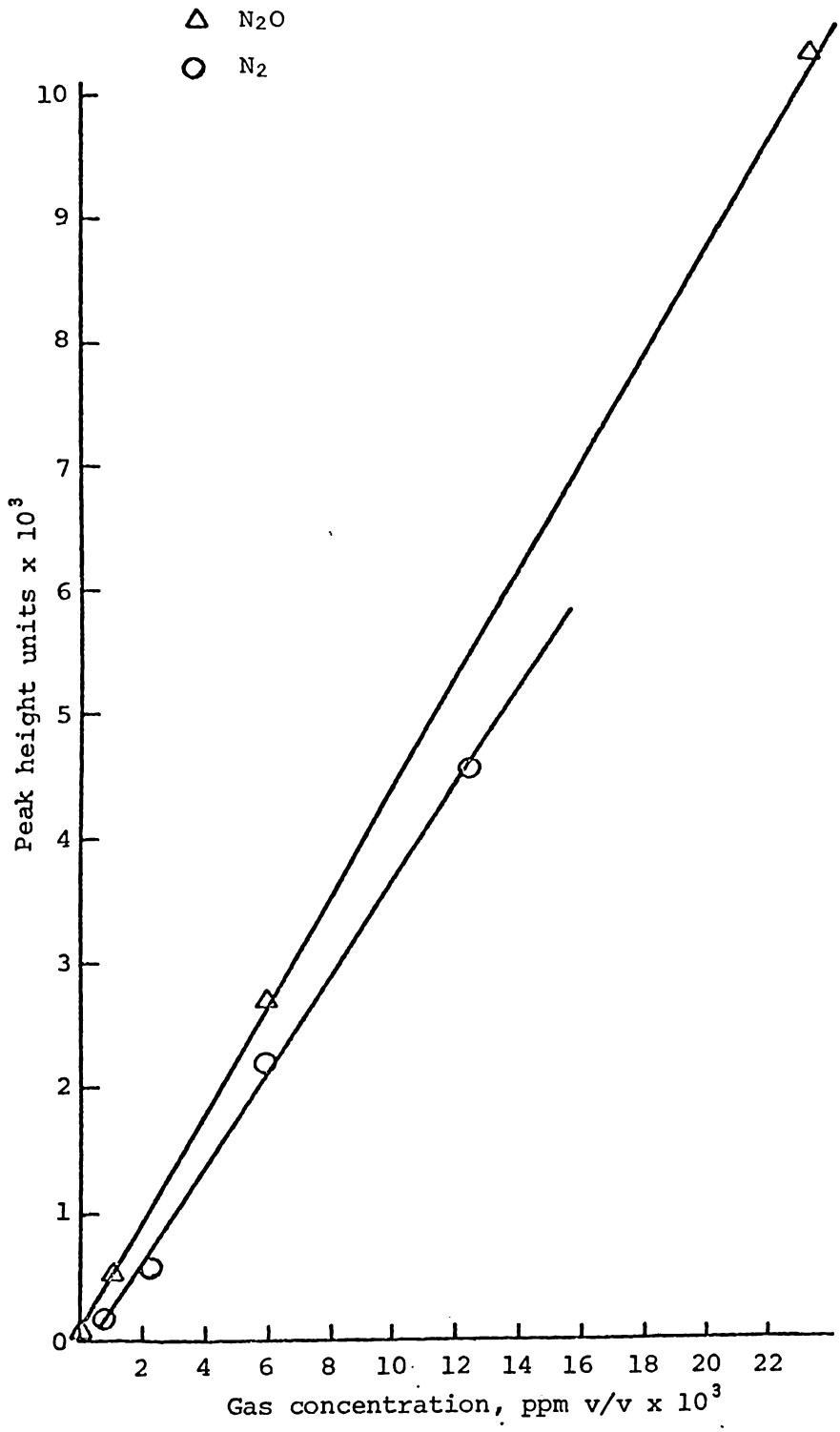
3.3.0 EFFECT OF WATER HOLDING CAPACITY

The level of soil moisture has been found to affect the rate of denitrification in soils (e.g. Mahendrappa and Smith, 1967; Stefanson, 1972a; Stefanson, 1972b; Craswell and Martin, 1974). Although it is generally accepted that the main influence of the soil moisture level is to control the rate of oxygen diffusion (Bremner and Shaw, 1958; Greenwood, 1962; Focht, 1978), moisture influences on rates of denitrification under anaerobic atmospheres have also been demonstrated (Cady and Bartholomew, 1960; McGarity, 1961; Mahendrappa and Smith, 1967). Cady and Bartholomew (1960) for example found that increasing the moisture content of a Norfolk sandy loam from 10 to 12.5 to 15% moisture gave increasingly faster rates of denitrification under anaerobic conditions.

The mechanisms by which moisture levels affect denitrification under anaerobic conditions appear uncertain. Mahendrappa and Smith (1967) proposed that some of the moisture effects were attributable to the distribution of organisms and/or N compounds. Part of the effect however was suggested to be due to a dilution effect on toxic products.

Although the quoted reports offer no explanation for this phenomenon, it is possibly due to the effective number of pore spaces enclosed by the soil volume. Assuming equivalent packing, a soil holding a greater quantity of water will contain effectively less vacant pore spaces. In accordance with Henry's Law, the nitrogenous gases produced will be found largely in the gas phase rather than

Figure 3.2 Gas chromatographic calibration for N_2O and N_2



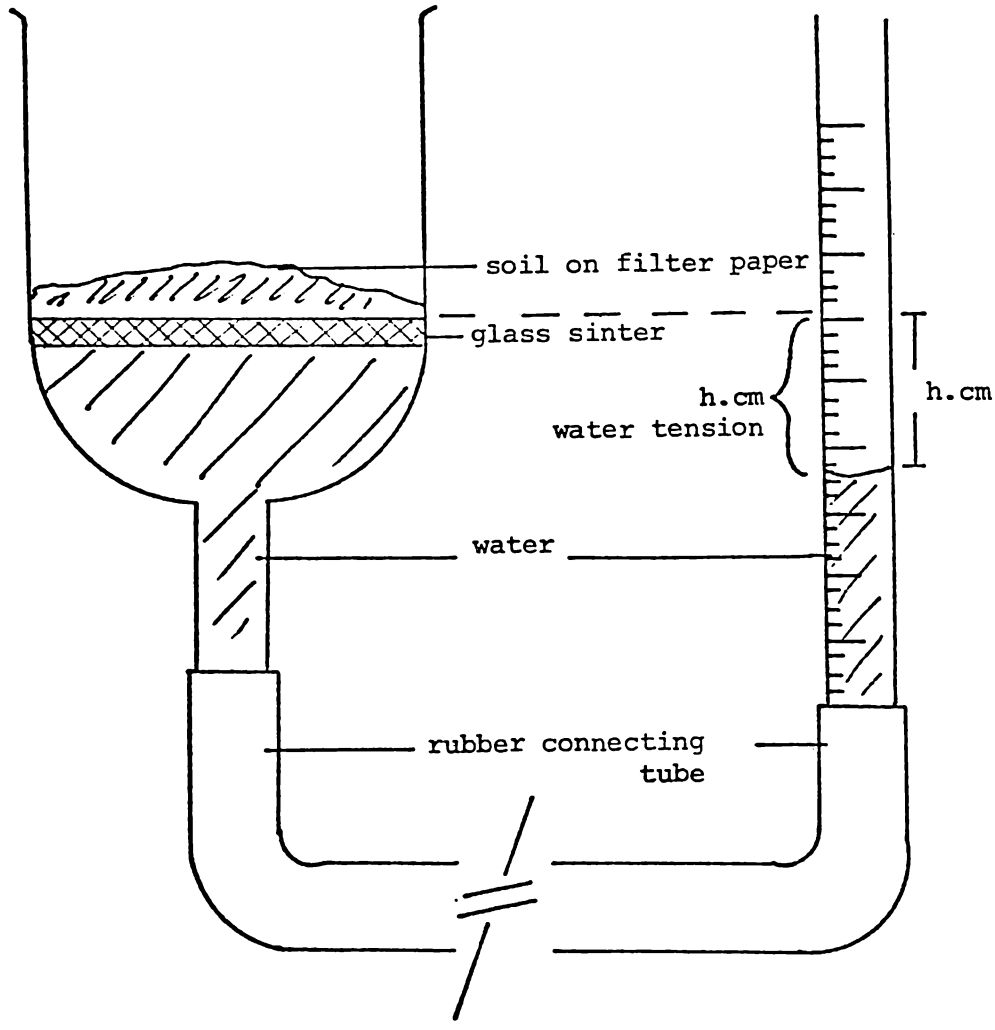
dissolved in the soil water. Because a concentration gradient must exist within the soil profile for these gases to diffuse to the headspace, these vacant pores within the profile will contain a higher concentration of gas than the headspace.

By adding more water to the soil the volume of pores with the high concentration of gases decreases, thus displacing more of the denitrification products into the headspace - causing an apparent increase in the rate of denitrification. Granted, increasing amounts of water will cause increasing quantities of gas to be adsorbed therein, but the gaseous distribution lies greatly in favour of the gas phase. This effect will be more noticeable when the ratio of gas filled to water filled pores is high. As the number of water filled pores increases, then so the concentration effect of the gas filled pores on the net effusion rate diminishes. Thus for moisture levels near the water holding capacity of a soil, the apparent influence of moisture on anaerobic denitrification rates will become less.

3.3.1 Experimental

The effect of moisture on the rate of denitrification was studied by incubating 20 g (105°C equivalent) of Waimate North clay loam amended with 250 µg NO_3-N.g^{-1} at 60, 80, 100, and 120 percent water holding capacity (W.H.C.). The W.H.C. was determined by a glass sinter tensiometer technique (see fig 3.3). Soils are placed on a filter paper over a porous glass sinter. Excess water is added to the soil and suction (water tension) is applied at the base of the sinter by lowering the connected column of water. 100% W.H.C. is determined at 0 cm tension.

Figure 3.3 Determination of Water Holding Capacity of a soil



3.3.2 Results and discussion

Corrections for the quantity of N_2O dissolved in the soil solution were made according to the procedure outlined in section 3.11.0. Moisture contents between 60 and 120% W.H.C. had little effect upon anaerobic denitrification rates as evidenced by the data presented in table 3.1.

Table 3.1 Denitrification rates of Waimate North clay loam incubated under He and 0.1 atm C_2H_2 at varying moisture levels.

% W.H.C.	µgm N_2O-N evolved over 8 hs incubation	r^2 of line of best fit
60	30.3	0.99
80	31.6	0.94
100	33.1	0.96
120	30.1	0.99

These results are in general agreement with the conclusion of Focht (1978, p465). He states that soil water per se does not directly affect microbial activity, except under extremely dry conditions where desiccation may occur. All samples were subsequently incubated at 100% W.H.C.

3.4.0 EFFECT OF SUBSTRATE CONCENTRATION

Nitrate concentration has been shown by many workers to influence the rate of denitrification (e.g. Bowman and Focht, 1974; Klemedtsson *et al*, 1977; Yoshinari *et al*, 1977; Cho and Mills, 1979). Michaelis-Menten kinetics apply to both C and N substrates (Bowman and Focht, 1974; Klemedtsson *et al*, 1977; Ryzhova, 1979). The Michaelis-Menten

equation

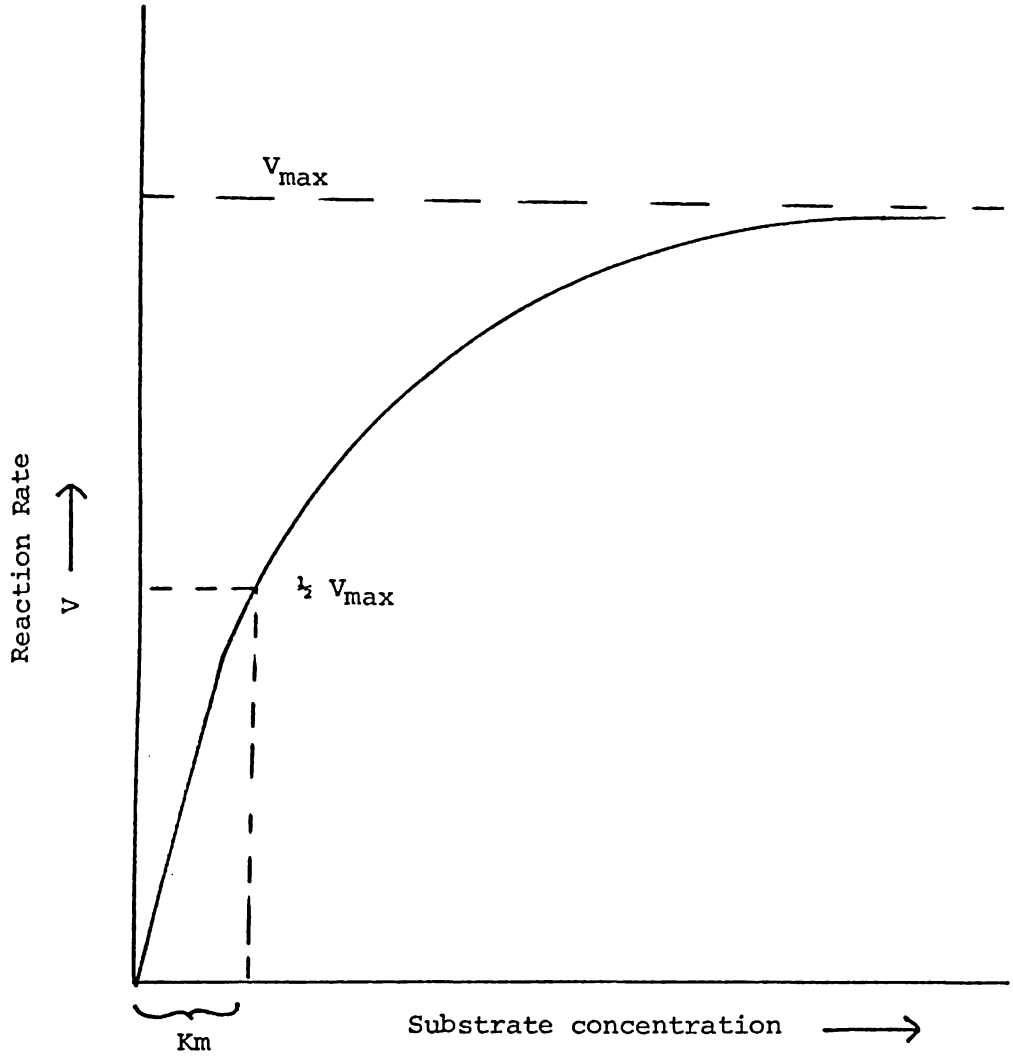
$$V = \frac{V_{max}\,[S]}{Km + [S]}$$

defines the quantitative relationship between the enzyme reaction rate V and substrate concentration S. A typical Michaelis-Menten plot is shown in fig 3.4. A value of substrate concentration, Km, is defined as the concentration at which the reaction is proceeding at half maximal velocity. Bowman and Focht (1974) demonstrated that within the Km region for nitrate concentration, denitrification rates of soils approximate first order kinetics with respect to nitrate concentration.

The initial rate of N_2O production during an acetylene incubation will be proportional to the concentration of nitrate within the Km concentration range. To attain maximum denitrification potentials it is necessary to elevate the nitrate substrate to levels well in excess of the Km values, at which point the denitrification rate becomes independent (zero order) of substrate concentration. Km values reported in the literature range from 0.7 (Yoshinari *et al*, 1977) to 48 μg NO_3-N.g^{-1} soil (Kohl *et al*, 1976). Addition of glucose increases the Km values. Yoshinari *et al* (1977) reported an increase from 0.7 to 6 μg NO_3-N.g^{-1} with the addition of glucose. Klemedtsson *et al* (1977) demonstrated the initial rate of denitrification to be independent of nitrate concentrations above 40 μg NO_3-N.g^{-1} while Bowman and Focht (1974) reported a value of 170 μg NO_3-N.g^{-1} using a glucose amendment. Yoshinari *et al* (1977) found the initial rate of denitrification of the St. Bernard sandy loam to be independent of nitrate concentrations above 2 μg NO_3-N.g^{-1}.

In overcoming the first order dependence of denitrification rates upon nitrate concentration during incubation assessments caution must apply. It is known that high levels of nitrate and to a lesser extent nitrite can greatly elevate the level of NO production to the detriment of N_2O production (Payne, 1978; p514).

Figure 3.4 Michaelis-Menten plot for enzyme kinetics



3.4.1 Experimental

The influence of nitrate concentration was determined by pre-incubating a Te Kowhai silt loam soil for 48 hours to reduce endogenous nitrate, and amending duplicate flasks with 0, 50, 100, 200, 300 and 500 µg NO_3-N.g^{-1}. N_2O production in the He/0.1 atm C_2H_2 atmosphere was monitored for the following 8 hours.

Denitrification rates were unaffected between concentrations of 50 and 500 µg NO_3-N.g^{-1} (table 3.2).

Table 3.2 Rate of denitrification of Te Kowhai silt loam incubated for 8 hs anaerobically with various nitrate amendments

Nitrate added (µg NO_3-N.g^{-1})	N_2O evolved (µg N_2O-N hr^{-1} $20g^{-1}$)	Linear regression coefficient r^2
0	0	0.99
50	8.1	0.99
100	7.3	0.96
200	7.4	0.94
300	8.1	0.99
500	9.2	0.98

The Km value for the Te Kowhai soil sampled appears to be below 50 µg NO_3-N.g^{-1}.

A Horotiu sandy silt loam soil was incubated under similar conditions but with lower concentrations of added NO_3-N (0, 12.5, 25, 50 and 125 µg NO_3-N.g^{-1}).

Results are shown in table 3.3.

Table 3.3 Rate of denitrification of Horotiu sandy silt incubated for 8 hs anaerobically with various nitrate amendments

Nitrate added ($\mu g\ NO_3\text{-}N.g^{-1}$)	N_2O evolved ($\mu g\ N_2O\text{-}N\ hr^{-1}\ 20g^{-1}$)	Linear regression coefficient r^2
0	1.8*	0.99
12.5	4.3	0.99
25.0	4.9	0.99
50.0	5.1	0.99
125.0	5.2	0.99

* not preincubated to reduce endogenous nitrate. N_2O evolution ceased at 100 mins.

The results in table 3.3 suggest that rates of denitrification in the Horotiu sandy silt are also independent of nitrate concentrations above 50 $\mu g\ NO_3\text{-}N.g^{-1}$. The results also offer the conclusion that Vo (control rate) is almost independent of substrate concentrations at 12.5 $\mu g\ NO_3\text{-}N.g^{-1}$. This would imply a Km value of the order of 2-4 $\mu g\ NO_3\text{-}N.g^{-1}$.

Based on the results of these two experiments a standard nitrate addition of 250 $\mu g\ NO_3\text{-}N.g^{-1}$ in the form of 2 ml of 1.78×10^{-1} M KNO_3 was made to each soil incubated.

Field capacities of New Zealand soils commonly range between 40 and 60 gm H_2O/100 gm oven dry soil (Gradwell, 1971; Gradwell, 1976). This will cause a variation in nitrate concentration of approximately 30 percent between soils of differing field capacities. The results in table 3.2 however clearly indicate that at 250 $\mu g\ NO_3\text{-}N.g^{-1}$, a 30 percent variation in nitrate concentration will not affect denitrification rates.

3.5.0 DEGREE OF ANOXIA

Oxygen is well known to be a potent inhibitor of nitrate respiration (Cady and Bartholomew, 1961; Focht, 1974; Flühler *et al*, 1979). Cady and Bartholomew (1961) for example, measured a 25 fold increase in denitrification products by lowering the oxygen content from 8 to 1% v/v. Since the desired effect of anaerobic incubations is to attain a maximal rate of nitrate respiration, it is essential to define the lowest level of pO_2 at which inhibition of denitrification is evident.

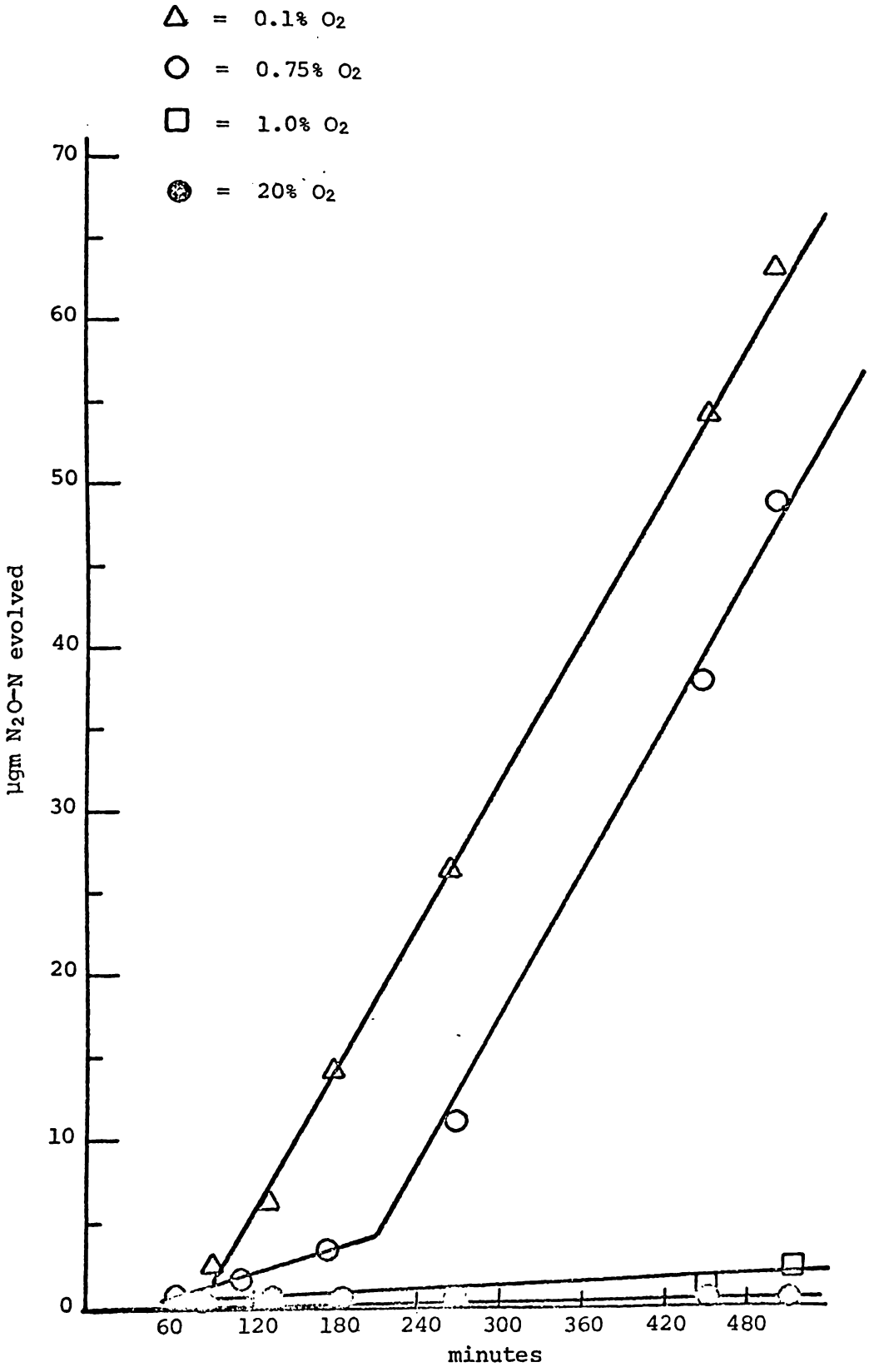
3.5.1 Experimental

Freshly sampled Te Kowhai silt loam was prepared as described in 3.2.0. Duplicate incubation tubes were evacuated to 0.5 atm and refilled with He until a variety of oxygen levels was achieved. The tubes were evacuated once more and 0.1 atm C_2H_2 added before returning the pressure to 1 atm with He. Each sample received a 250 µg NO_3-N.g^{-1} amendment. Results of the 8 hour incubations are presented in fig 3.5. Even at levels as low as 0.5% O_2 v/v an inhibitory effect is evident. The maximal rate of 8.8 µg N_2O-N.hr^{-1} achieved with 0.1% O_2 is comparable to rates subsequently achieved with regular assays of this soil when even less oxygen was present.

3.5.2 Discussion

Of particular note is the biphasic nature of the 0.5% O_2 incubation. This result is interpreted as being due to an initial temporary and partial inhibition of denitrification by O_2, the phase change occurring at the onset of full anaerobosis. The slightly increased gradient of the second phase (9.4 µg N_2O-N.hr^{-1}) compared to the 0.1% O_2 incubation may be due to the aerobic growth of denitrifying

Figure 3.5 Rates of denitrification under varying oxygen concentrations



microorganisms which occurred before depletion of oxygen reserves. A slight continued growth of denitrifiers in the presence of oxygen compared to a fully anaerobic soil could be expected to lead to slightly higher denitrification potentials upon attaining full anaerobosis. Due to the reduced efficiency of nitrate respiration (see chapter 2 and appendix 2) any growth induced by the addition of nitrate in an anaerobic situation would be slower than that in the presence of traces of oxygen.

If the incubation had been conducted for a longer duration, it is believed similar results would have occurred for the 1% and later, 18% O_2 as oxygen levels became further depleted. The sharp transition to the maximum denitrification rate (within approximately 60 minutes) suggests the microorganisms can switch from oxygen to nitrate respiration very rapidly. These results concur with the lag phase noted by Delwiche (1959) and Payne and Riley (1969). They observed that aerobically grown denitrifying cells placed under anaerobic conditions exhibit a lag period before utilization of nitrate. This lag time has been measured by Riley (1969) and found to be 40 minutes. Consequently, incubations in the presence of 0.1% O_2 or less normally do not demonstrate a lag phase as the first sampling is usually after 60 minutes. For a further discussion of this phenomenon see section 2.4.5.

Based on the results in fig 3.5 all incubations were conducted in an atmosphere of less than 0.1% O_2 v/v.

3.6.0 SAMPLE TREATMENT AND STORAGE

Methods of sample storage reported in the literature vary from air dry (e.g. Stefanson, 1974; Kohl *et al*, 1976; Klemedtsson *et al*, 1977; Guthrie and Duxbury, 1978; Blackmer and Bremner, 1979; Ryden *et al*,

1979) to freezing (McGarity, 1962). Storage times vary from days to several months.

It is now well documented that air dried samples yield increased rates of denitrification upon re-wetting (Myers and McGarity, 1971; Galsworthy and Burford, 1978; Patten *et al*, 1980). This increased rate is attributed to a release of available carbon during wetting and drying cycles resulting from physical breaking of bonds (Focht, 1978, p436). Available carbon has been seen to greatly influence potential rates of denitrification (Bowman and Focht, 1974; Burford and Bremner, 1975). Many microbial cells are killed by desiccation and only those with resistant propagules can survive long periods of drought. McLaren and Skujins (1968) reported a hundred fold decrease in the microbial population when a soil was stored dry for ten years. Focht (1978, p436) suggests excessive drying probably favours the survival of the gram-positive bacteria *Micrococcus* and *Bacillus*, which do not appear to be numerically significant in soil.

It is surprising in the light of the above evidence that air dried storage of soils is such a favoured technique for denitrification studies. It is doubtful that correlations of denitrification rates between soils so treated are valid, and certainly bear no resemblance to the *in situ* situation. Patten *et al* (1980) presented data showing that relative rates of denitrification between soils recently air dried bear no correlation to the relative rates of denitrification obtained after twenty weeks of air dry storage. Similarly the air dried rates bear no relationship to rates measured for fresh samples of the same soils.

Fewer studies have been reported where soils were stored at reduced temperatures (2-5°C) (e.g. Myers and McGarity, 1972; Smith *et al*, 1978; Firestone *et al*, 1979; Smith and Tiedje, 1979).

The effect of temperature on biochemical rate processes is well known to be exponential and adheres within limits to the classic Arrhenius rate equation at temperatures of 15°C and above (Focht, 1974; Stanford *et al*, 1975a). Between 2° and 10°C the change in the rate constant per degree change in temperature is proportionally larger than at higher temperatures, i.e. the denitrification process slows down markedly for every degree below 10°C. Storage of soils at reduced temperatures, while not destroying microbial activities, significantly reduces them. However, little attention has been directed towards the effect of duration of storage at reduced temperatures or subsequent rates of denitrification.

Many studies make no mention of storage times (Myers and McGarity, 1972; Firestone *et al*, 1979) while others compare denitrification potentials of soils stored for varying lengths of time (Smith and Tiedje, 1979), sometimes up to six months (Smith *et al*, 1978).

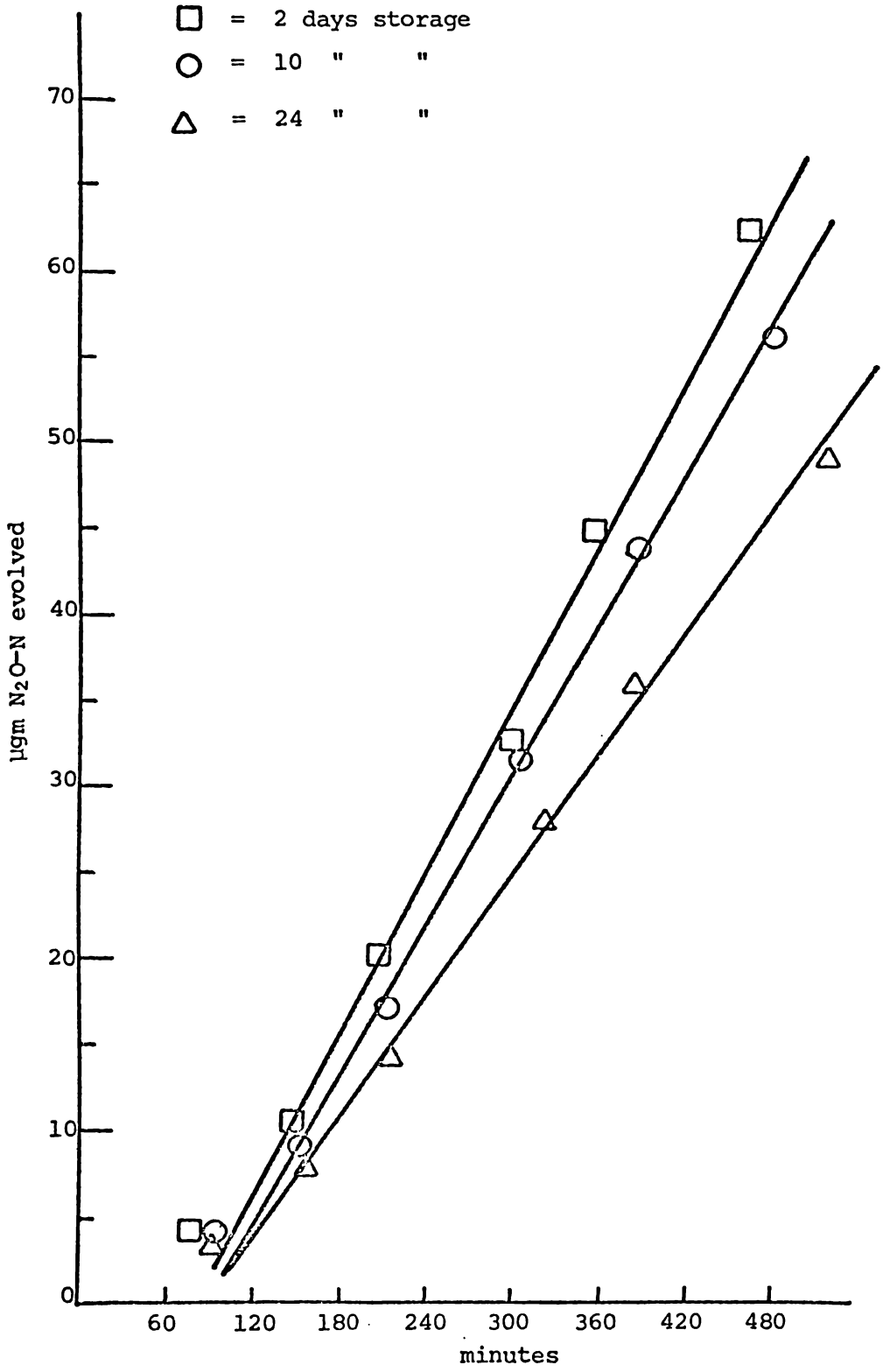
The effects on the denitrification potential of storing soils at 4°C were determined.

3.6.1 Experimental

Fresh 0-50 mm cores of the Te Kowhai silt loam soil were collected, sieved to 4 mm and stored in sealed plastic bags at 4°C. Samples were taken after various periods of storage for incubation analyses as described in 3.2.0, to monitor any changes in denitrification potential. The results are shown in fig 3.6.

The gradients of the incubation data (linear least squares fit) seen in fig 3.6 suggest the potential denitrification rates are decreasing with storage. Bremner and Shaw (1958) and Burford and Bremner (1975) demonstrated that the rate and extent of denitrification in soil under anaerobic conditions is controlled largely by the amount

Figure 3.6 Denitrification potentials after different periods of storage at 4°C



of organic matter readily available to denitrifying organisms.

Soil samples were regularly assayed for available carbon during storage using the wet dichromate oxidation technique described by Burford and Bremner (1975). The available carbon decreased to less than half the original value during the first ten days of storage (table 3.4) indicating that considerable microbial activity occurred at 4°C.

Table 3.4 Effect of storage at 4°C on the available carbon of the Te Kowhai silt loam

Days storage	Available Carbon ($\mu gm\ C.g^{-1}$)
0	85.2
2	60.6
10	30.9
17	24.3
24	5.2

The available carbon analyses typically included an error of $\pm 2\ \mu g.C.g^{-1}$.

Temperatures $\leq 4°C$ commonly occur in many Waikato soils during the winter season. During June 1980 the soil temperature measured at the Rukuhia field station at a depth of 10 cm reached 5°C or below 8 days out of 30. Burford and Stefanson (1973) found that denitrification losses increased during winter and concluded that the increased wetness of the soil influenced the rate of denitrification more than the reduced temperatures.

Since results in fig 3.6 and table 3.4 suggest considerable

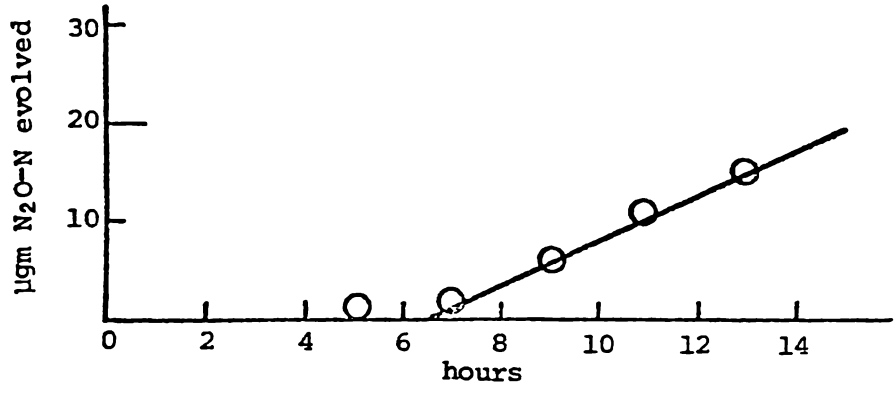
microbial activity at 4°C, fresh Te Kowhai soil was incubated in the manner described in section 3.2.0, but at 4°C. The results in fig 3.7 support the suggestion that denitrification is capable of proceeding at 4°C.

3.6.2 Discussion

Anaerobic incubation induces denitrification rates several orders of magnitude greater than that expected under aerobic storage. Results in table 3.4 however clearly show that under aerobic storage at 4°C, available carbon reserves could be almost exhausted after 30 days. After prolonged periods of storage at 4°C, when substrate becomes limited, it is reasonable to assume significant changes will occur within the microbial and enzyme balance of a soil. When bacteria are starved their proteins break down at an accelerated rate and provide a larger amino acid pool within the cells. This pool may be used to synthesise new enzymes even though no growth takes place (Gray and Williams, 1975, p27). A soil assayed in such a state is unlikely to yield data reflecting the true denitrification potential.

Because levels of microbial activity differ between soils, it is likely that soils will respond differently to storage at 4°C. Soils exhibiting high microbial activity could be expected to deplete the available carbon sources quickest. If denitrification potentials are to be compared between soils a standard short cold storage would minimise changes in nutrient and microbial status between soils. Ideally soils should be processed immediately but this is usually impractical and may lead to erroneous comparisons with soils stored for even a few days. For the purposes of this study, samples were transported after collection to the laboratory in insulated boxes containing ice. Those samples taken within a short distance of the

Figure 3.7 Denitrification rate under anaerobic atmosphere at 4°C



laboratory were simply placed in 4°C storage quickly after sampling. All denitrification assays were completed within 48 hours of collection.

3.7.0 INCUBATION TEMPERATURE

Rate processes are exponentially affected by temperature, and the standard Arrhenius equation can be used within moderate temperature limits (15-35°C) to describe the effect of temperature on the denitrification rate. Lower temperatures have a much more drastic effect on rate processes, common to all biochemical systems, and cannot be characterized by the Arrhenius equation.

Stanford *et al* (1975a demonstrated a two fold increase in denitrification rates for each 10°C rise between 15 and 35°C (i.e. a Q_{10} factor of 2). They calculated that a Q_{10} of 2 may extend down to 11°C. Below 5°C the rate of denitrification was seen to decline sharply. The temperature at which maximum rates of denitrification occur has been reported to be as high as 65°C (Nommik, 1956; Fillery, 1979). This has been attributed to the presence of thermophilic species of *Bacillus*. Since *Bacillus* are unlikely to be important at normal soil temperatures (Woldendorp, 1968; Vives and Paris, 1975) it was intended to avoid such temperature effects in choosing an incubation temperature.

All soils were incubated at 25°C.

3.8.0 DURATION OF INCUBATION

The presence of added N substrate, and warm incubation conditions may be favourable to inducing accelerated microbial growth during incubation assessments. It was hoped to avoid such influences on the measured denitrification potential by choosing an incubation period short enough that the response of the microbial population to these conditions was not significant.

An incubation was undertaken to measure long term effects and establish a safe minimum period for assessment of potential rates of denitrification.

3.8.1 Experimental

The Te Kowhai silt loam and Bruntwood sandy loam were both incubated (in duplicate) in the manner described in 3.2.0. Results are presented in fig 3.8. Both soils displayed a marked departure from the initial linear rates of N_2O evolution (see fig 3.9). The Te Kowhai soil displayed a logarithmic phase between approximately 12 to 40 hours. The Bruntwood soil developed a much less pronounced logarithmic phase between 13 and 30 hours (see fig 3.10). The results in figs 3.8 and 3.10 clearly invalidate any kinetic interpretation on N_2O evolution after approximately 12 hours. The two soils show markedly different logarithmic phases. The logarithmic phases were attributed to either microbial growth induced by favourable conditions, i.e. 25°C, and increased N substrate, or the induction of a more efficient enzyme for nitrate respiration.

It is interesting to note the failure of acetylene to inhibit N_2O reduction beyond 76 hours in the more active Te Kowhai soil. These results are in agreement with Yeomans and Beauchamp (1978) who found that acetylene would inhibit N_2O reductase for a maximum of 168 hours depending on the acetylene concentration. To explain these results they suggested a change in or growth of some microbial species which has the ability to reduce N_2O in the presence of acetylene. Such results invalidate the use of the acetylene inhibition technique for long term incubation studies on denitrification rates.

Soil incubations reported in this study were conducted for a maximum of 8 hours in the presence of acetylene.

Figure 3.8 Long term anaerobic incubation

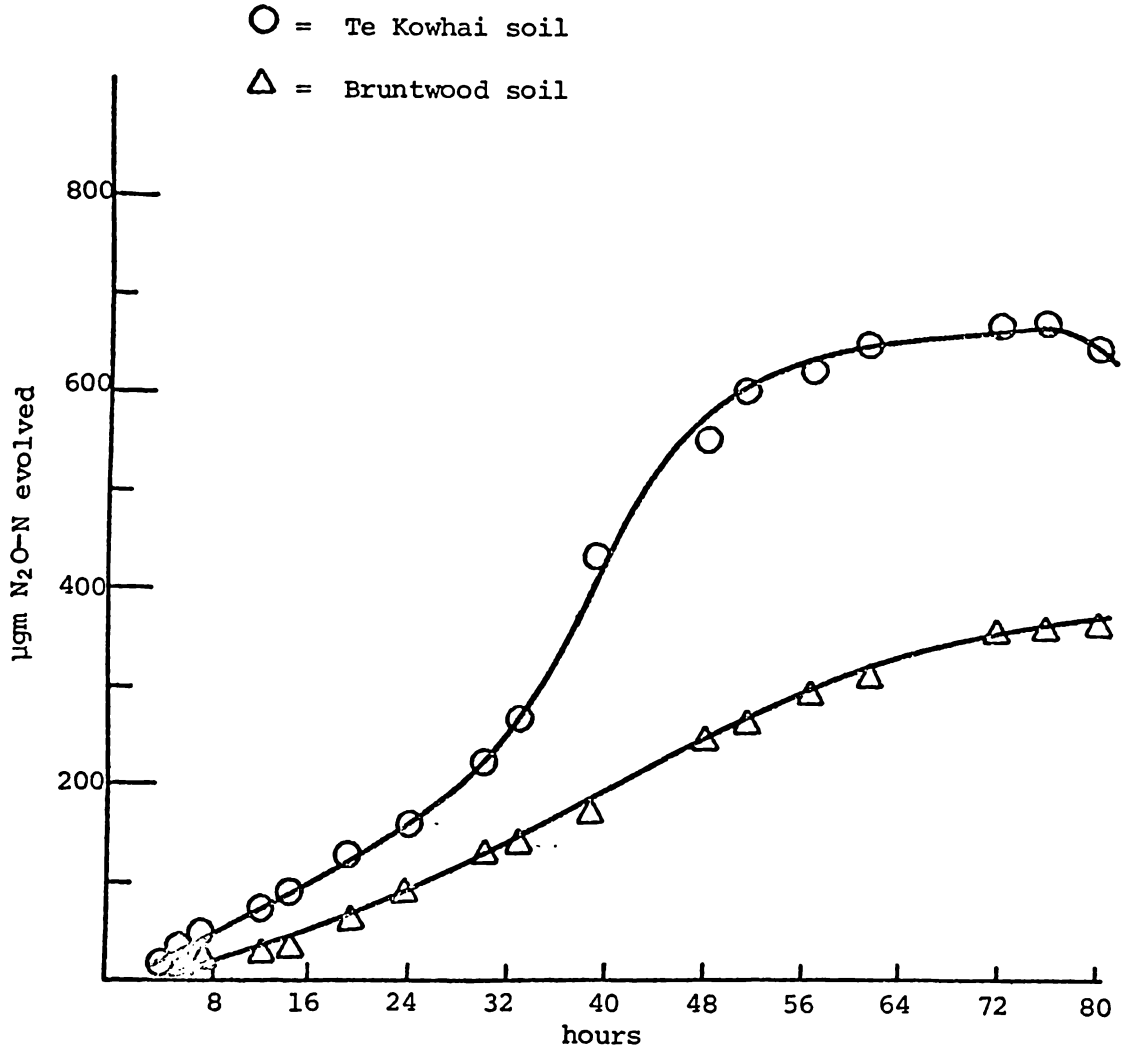


Figure 3.9 Initial rate of N_2O evolution

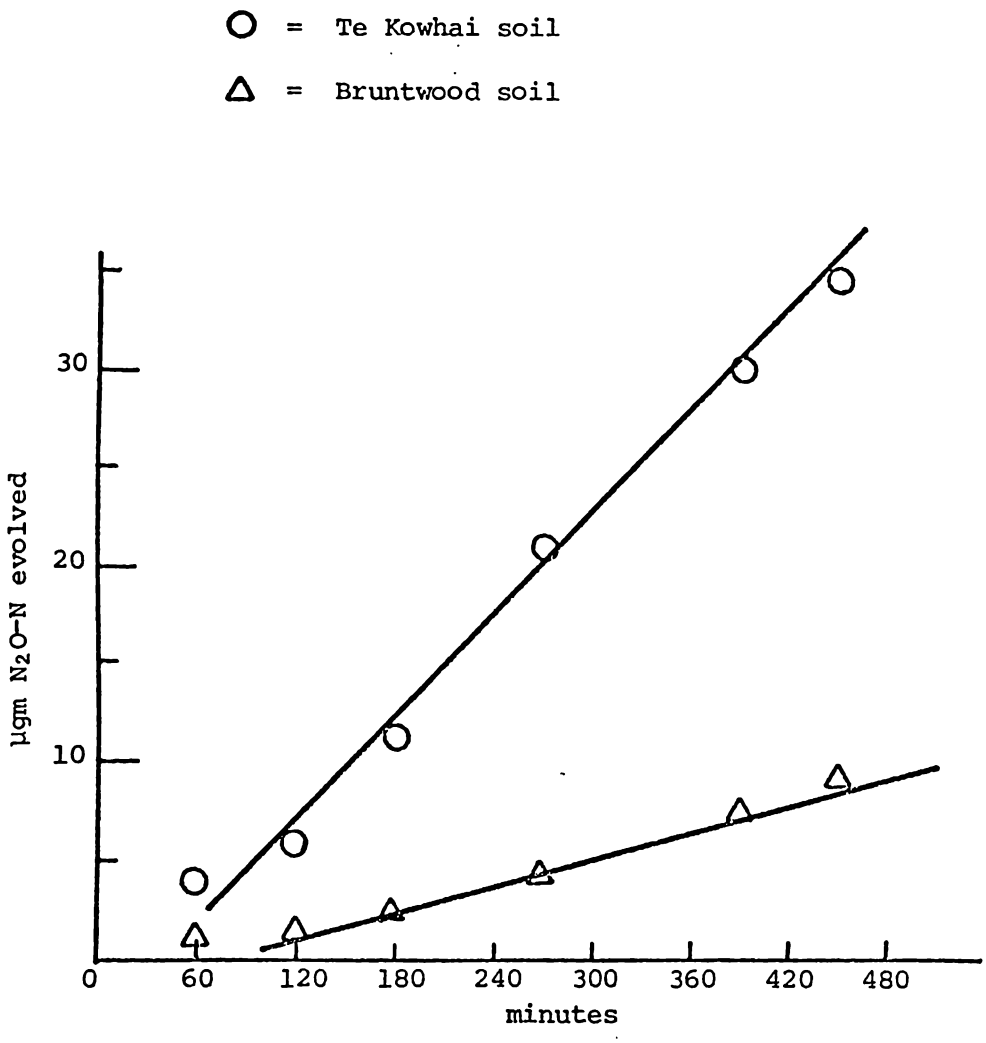
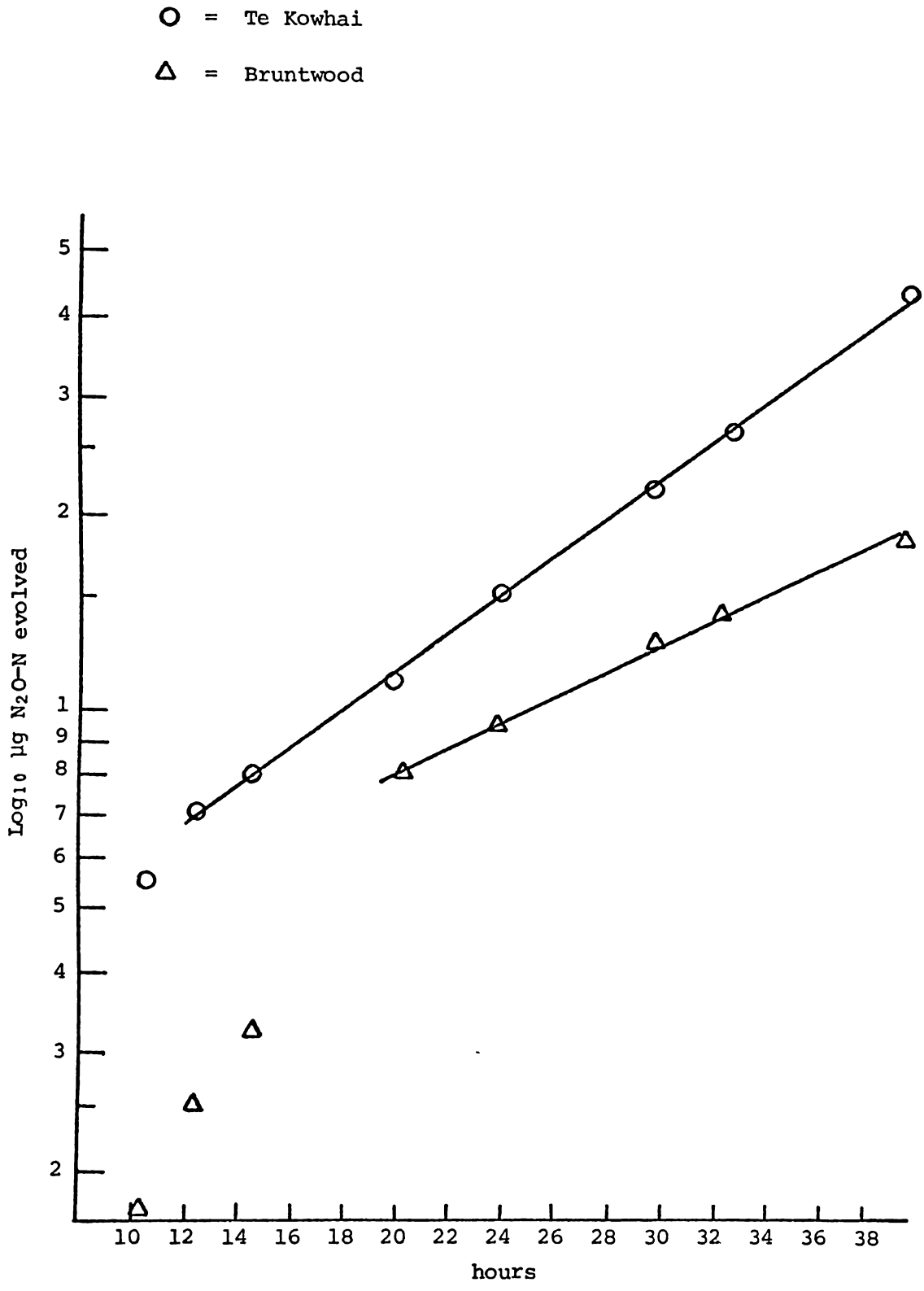


Figure 3.10 Logarithmic phase of N_2O evolution



3.9.0 INCUBATION ATMOSPHERE

Acetylene was found to serve as a useful internal standard during the course of analyses. Acetylene reduction has long been used as a measure of N-fixation, thus one may expect the levels of acetylene to decrease and ethylene to appear in the incubation atmosphere. Studies by Yoshinari *et al*, (1977) and Watanabe and Guzman (1980) however both noted that acetylene disappearance was inhibited by the presence of nitrate and while denitrification occurred, no ethylene was produced.

Fillery (1978) suggested a higher level of nitrate reduction might be expected where N_2O or NO are the dominant products since fewer electrons are utilized in the reduction of nitrate to NO and N_2O compared to N_2. This suggestion implies a higher rate of denitrification might be expected in the presence of acetylene. Studies by Smith *et al* (1978) and Ryden *et al* (1979) both demonstrate however that the rates of denitrification are unaffected by the presence of acetylene. Ryden *et al* (1979) also found the overall respiration of the incubation system, measured as CO_2 evolution, was unaffected by the presence of acetylene.

Most anaerobic studies of denitrification have been conducted under He or Ar atmospheres. Such studies are based upon the assumption that the activity of denitrifying organisms is not affected by the substitution of various inert gases. Work by Schriener *et al* (1962) questioned this assumption. Inert gases were shown to have an effect on the growth rate of the fungus *Neurospora crassa*, linearly related to the square root of the gases molecular weights. Growth was about 20 percent faster under He compared to N_2. More recently however, Blackmer and Bremner (1977a) demonstrated that denitrification by soil microorganisms is not significantly affected by the substitution of inert gases.

3.10.0 CARBON DIOXIDE EVOLUTION

It was noticed during incubations that soils with high rates of N_2O production also had high rates of CO_2 evolution. A correlation was sought between N_2O and CO_2 evolved using several soils with varying rates of denitrification.

3.10.1 Experimental

Rates of N_2O and CO_2 evolution for the various soils are plotted in figures 3.11 and 3.12. The appropriate equations derived from the lines of best fit are included. A correlation coefficient r^2 = 0.75 was obtained using linear regression analysis of the above results (see fig 3.13).

It can be seen in figures 3.11 and 3.12 that certain discrepancies occur. For example, the Hamilton clay loam demonstrates the highest rate of CO_2 evolution but only the second highest rate of N_2O evolution. For this reason, CO_2 evolution cannot be used as a sole criteria for determining denitrification activities.

Discrepancies between rates of N_2O/CO_2 evolution for various soils probably reflect the varying populations of soil microorganisms. A soil with a larger population of fermentative bacteria could well exhibit a higher rate of CO_2 evolution than a soil with a higher rate of denitrification but a small population of anaerobic fermenters.

While comparisons of CO_2 evolution between soil types may not be a valid indication of their comparative denitrification potentials, comparisons of CO_2 and N_2O evolution within the same soil may be valid if the soil microbial population remains relatively constant throughout the duration of the incubation.

To test this suggestion, a sample of Te Kowhai silt loam was incubated under the usual conditions outlined in section 3.2.0. A comparable sample was incubated with an additional amendment of glucose

Figure 3.11 Evolution rate of CO_2 during anaerobic incubation

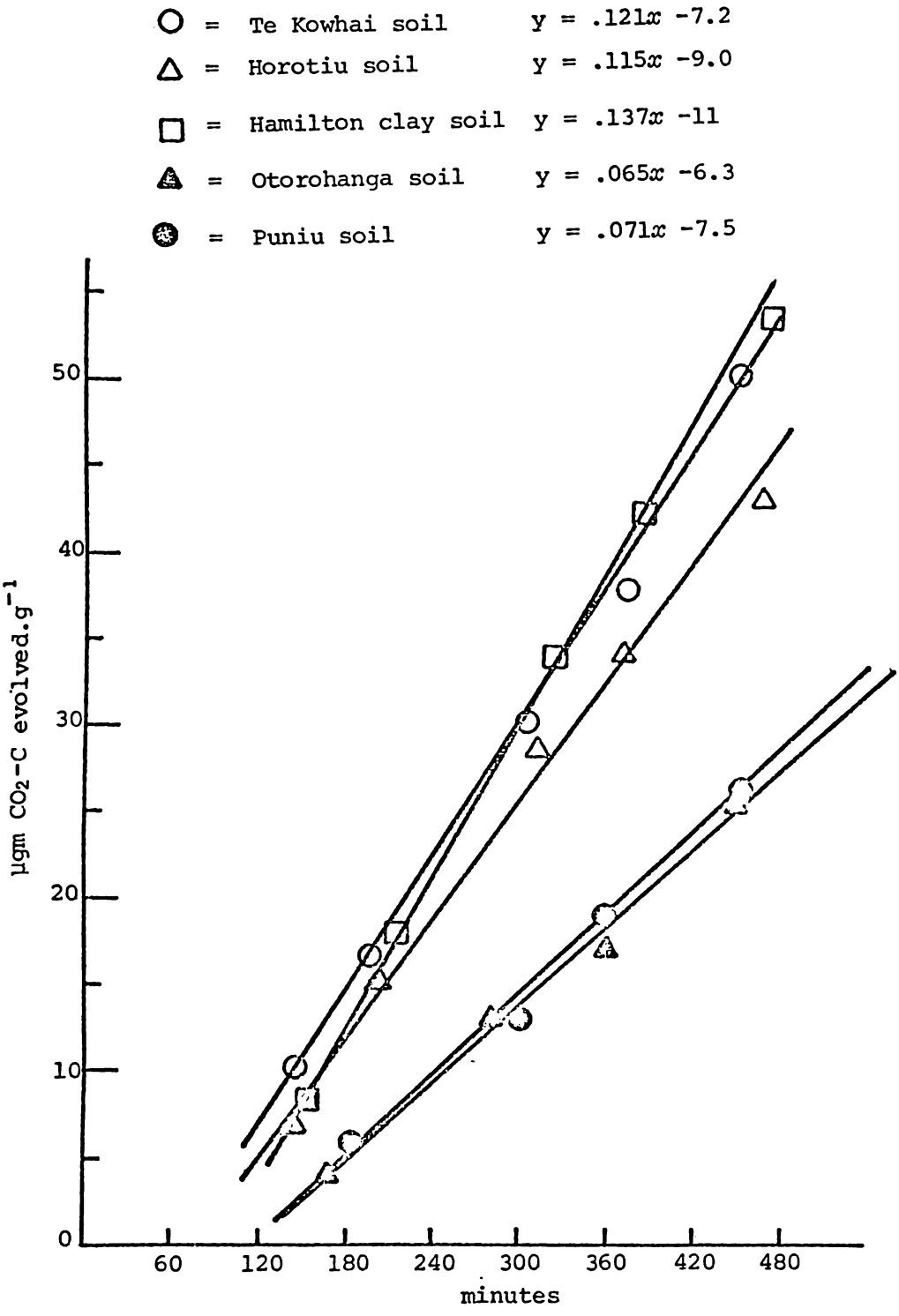


Figure 3.12 Evolution rate of N_2O

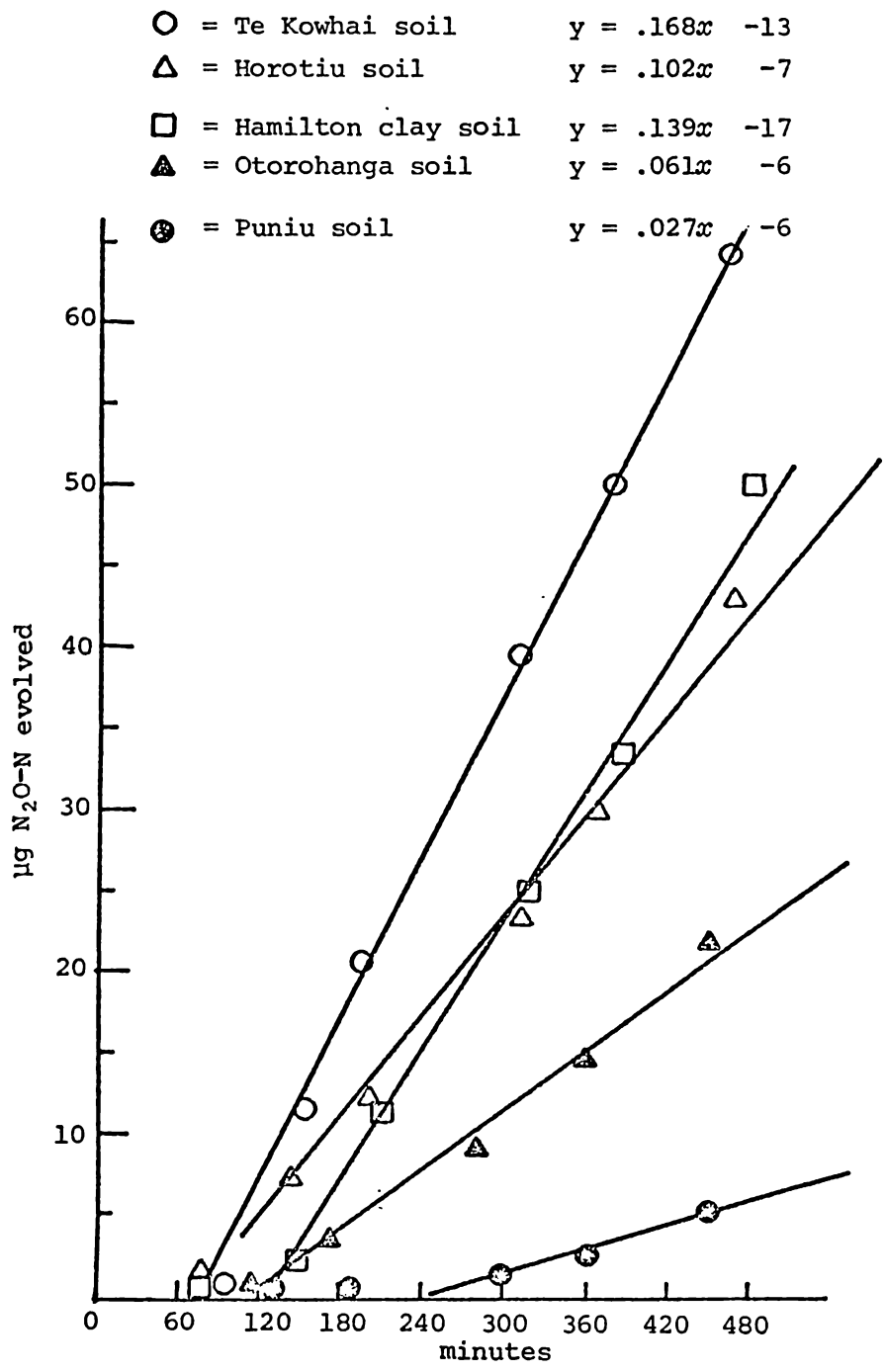
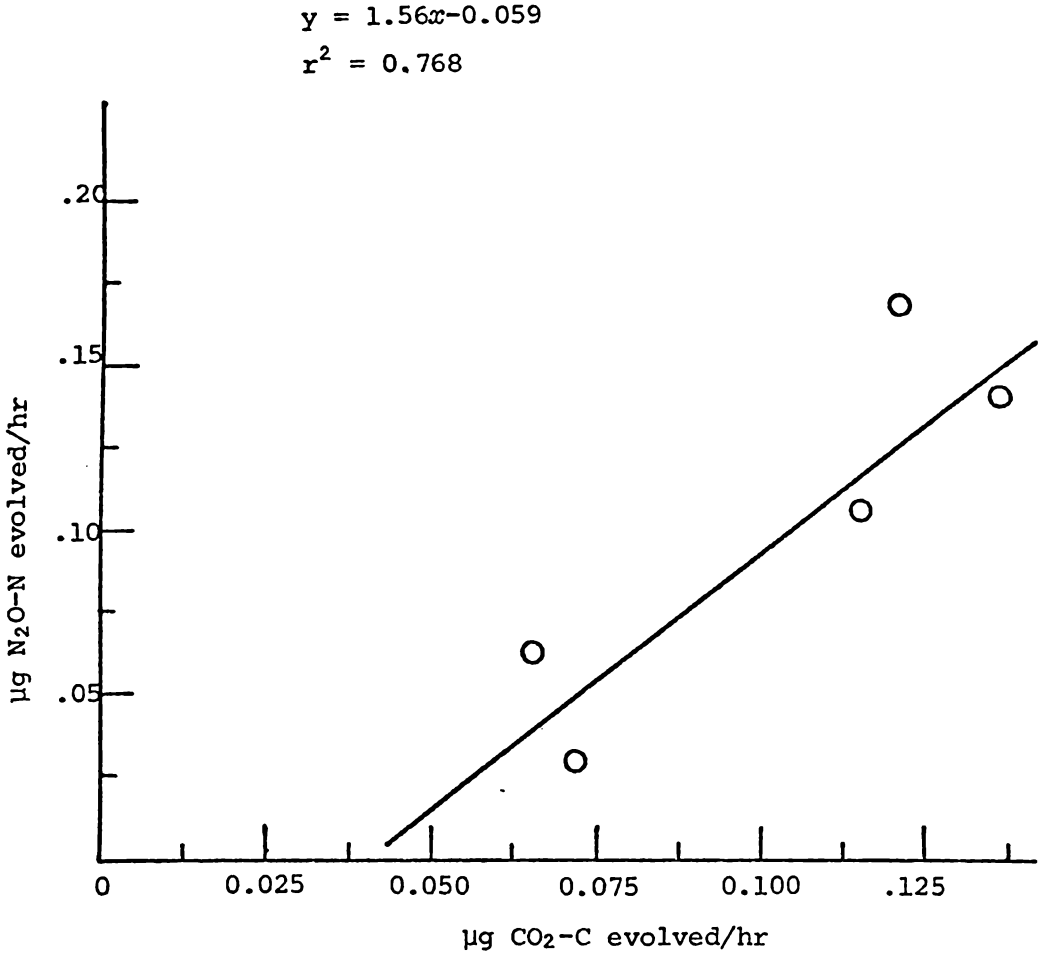


Figure 3.13 Relationship between CO_2 and N_2O evolution for various soils



(100 μg $C.g^{-1}$). The respective rates of N_2O evolved are displayed in fig 3.14. The increased rate of N_2O evolution with the glucose amendment suggests denitrification in the Te Kowhai soil at sampling was carbon limited. The rate of N_2O evolution increased by 32 percent (as measured by the gradients of lines of best fit) and was accompanied by a corresponding 28 percent increase in the rate of CO_2 evolution (see fig 3.15).

3.11.0 CALCULATION OF N_2O IN SOLUTION

Incubations were routinely conducted with soils at 100% W.H.C. The absolute amount of water present, therefore, varied by about 10 cc depending on the soil. In monitoring the N_2O produced, corrections for the N_2O dissolved in the soil solution must be considered.

The quantity of a gas in a headspace is related to the mole fraction in solution by Henry's law:

$$Pi = K\,\chi i \qquad 3.1$$

where: Pi = partial pressure of gaseous species i, χi = mole fraction in solution of species i, and K = Henry's constant.

K_{N_2O} at 25°C = 2.28×10^{-3} (Ramm, 1968; p19).

Thus the mole fraction of N_2O in solution

$$\chi i = \frac{n_{N_2O(\ell)}}{n_{N_2O(\ell)} + n_{H_2O}} = \frac{Pi}{K} \qquad 3.2$$

where: $n_{N_2O(\ell)}$ is the number of moles of N_2O dissolved in solution and n_{H_2O} is the number of moles of water

$$\therefore \quad \chi i \simeq \frac{n_{N_2O(\ell)}}{n_{H_2O}} \qquad 3.3$$

(Water being 55.5 M)

Figure 3.14 Denitrification rate of Te Kowhai soil with and without added glucose

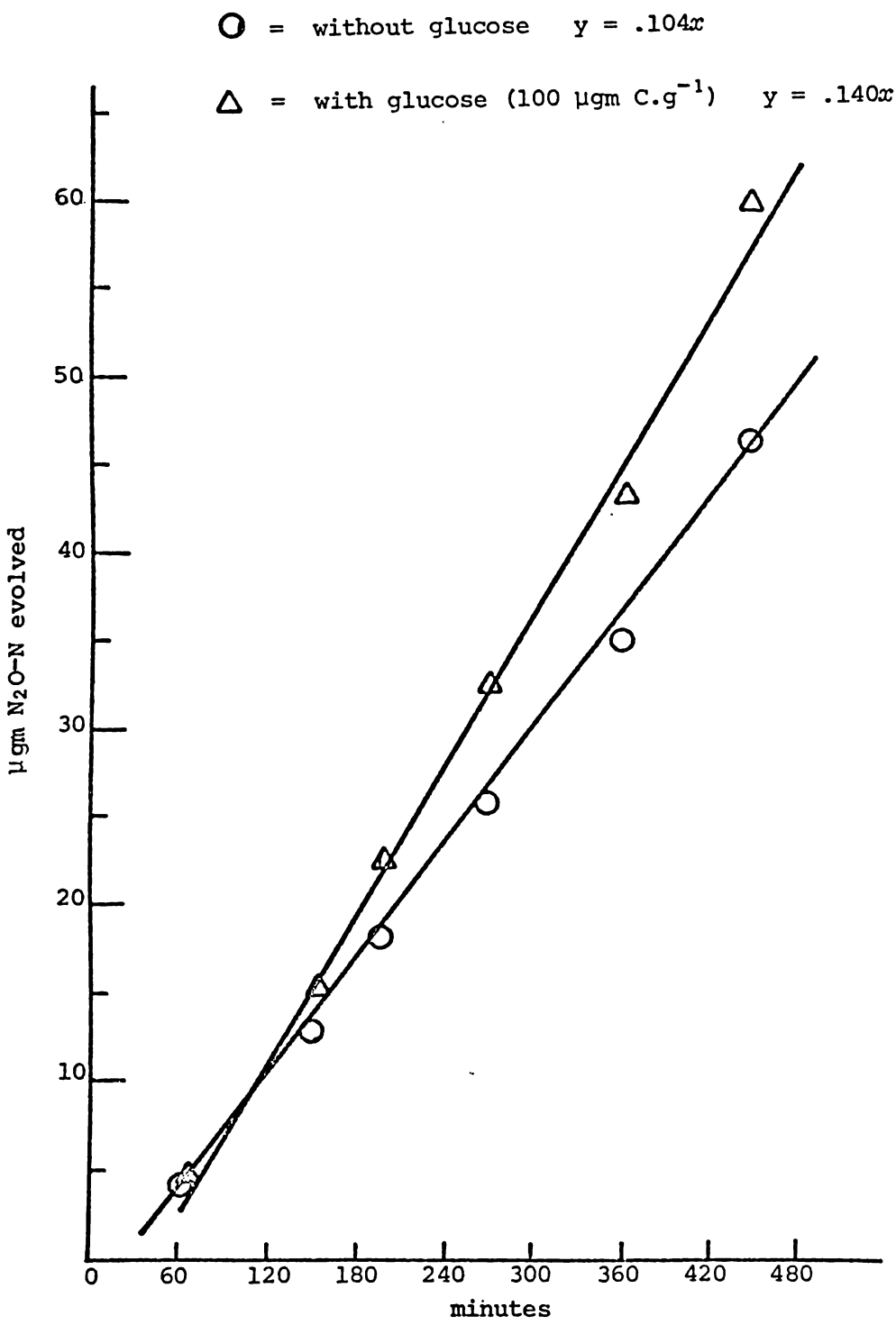
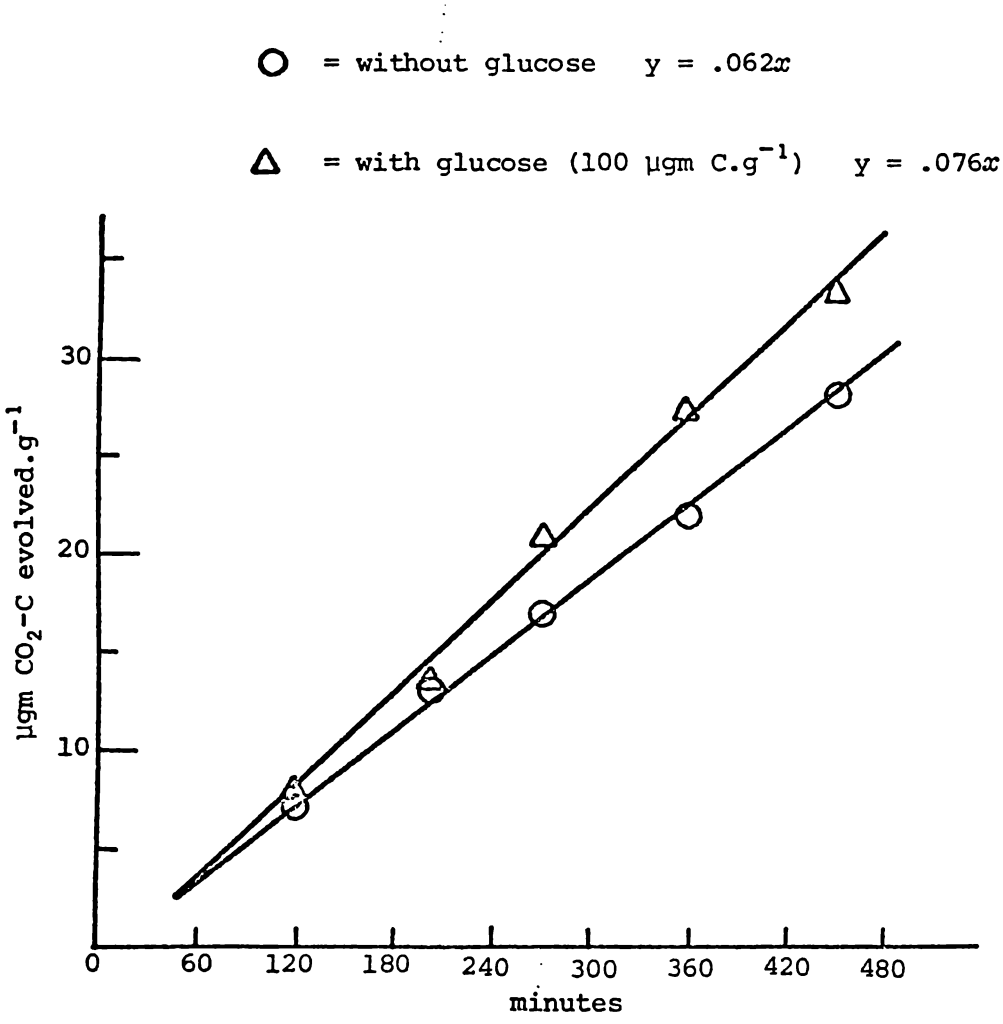


Figure 3.15 Evolution of CO_2 from Te Kowhai soil with and without added glucose



Then the number of moles of N_2O in solution

$$n_{N_2O(\ell)} = Xi\ n_{H_2O} = Xi\ \frac{V\ solution}{18} \qquad 3.4$$

Where V solution is the volume of water.

The number of moles of N_2O in the gas phase

$$n_{N_2O(g)} = \frac{Pi.\ V\ headspace}{24.5 \times 10^3} \qquad 3.5$$

Where $V_{headspace}$ = volume of headspace.

To calculate the percentage of N_2O in solution at any time:

$$\%\ of\ N_2O\ in\ solution = \frac{n_{N_2O(s)}}{n_{N_2O(g)}} \times 100 \qquad 3.6$$

$$= \frac{\frac{Xi\ V\ solution}{18}}{\frac{Pi\ V\ headspace}{24.5 \times 10^3}} \times 100 \qquad 3.7$$

$$\frac{Xi\ V\ solution}{18} \times \frac{24.5 \times 10^3}{Pi\ V\ headspace} \times 100 \qquad 3.8$$

But $\frac{Xi}{Pi} = \frac{1}{K}$,

$\therefore$ $\%\ N_2O$ in solution $= \frac{1}{K}\ \frac{V\ solution}{V\ headspace}\ \frac{24.5 \times 10^3}{18} \times 100$

$$= 0.56\ \frac{V\ solution}{V\ headspace} \times 100 \qquad 3.9$$

For a nominal headspace volume of 65 cc and a nominal solution volume of 15 ml, moderate variations in the water content (e.g. 5 ml) will cause only small (approximately 5%) variations in the percentage of N_2O in solution.

When soils fell within the above specifications, or when incubations of only one soil type are being conducted, no corrections for the N_2O in solution are necessary since the error introduced is within general experimental error (see section 3.16.0).

In the case of widely differing water contents, e.g. the experiment with moisture contents ranging between 80 and 120 percent W.H.C. (section 3.3.1) these corrections assume importance. It was only after such corrections had been taken into consideration that the rates of N_2O evolution displayed in table 3.1 were seen to be similar.

3.12.0 N_2O DIFFUSION

In order for the N_2O measured in the headspace to reflect the rate of N_2O production there must be no diffusion limitation between the N_2O production in the soil core and transport to the headspace. If the N_2O effusing into the headspace from the soil surface was diffusion limited, the N_2O production measured in the headspace would not assume linearity. It was concluded that where linearity was observed, N_2O diffusion was not a problem.

Some diffusion limitation will exist initially until the concentration gradient necessary to drive the transport of N_2O from the soil core into the headspace at a rate equal to its production is developed. As linearity is observed in most cases after 60 to 90 minutes, it was assumed that any diffusion limitations were overcome within this period.

N_2O diffusion into the headspace can be discussed in terms of the first law of diffusion:

$$J = -D \frac{dc}{dx}$$

where J = the flux of N_2O measured in the headspace, D = the diffusion coefficient of N_2O in the soil medium, and $\frac{dc}{dx}$ = the concentration gradient of N_2O over the soil core.

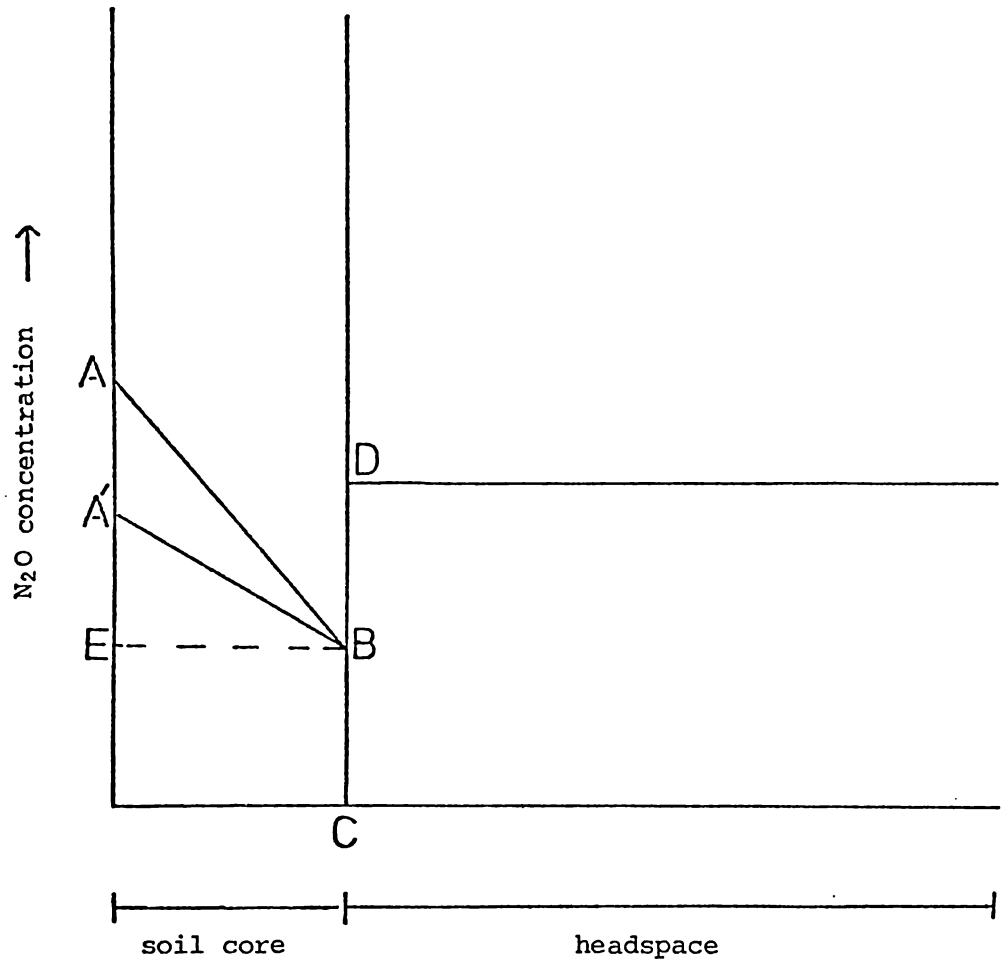
The concentration problem can be depicted by the model in fig 3.16 below. It is assumed the N_2O concentration gradient within the soil core is linear. At the steady state situation the rate of N_2O production equals the rate of N_2O effusion into the headspace.

AB represents the concentration gradient required to cause a flux of N_2O into the headspace equal to the rate of production (or denitrification). ABC represents that reservoir of N_2O dissolved in the soil solution. CD represents the concentration of N_2O in the headspace, and $\frac{CD}{DB}$ is going to approximate the phase distribution of N_2O between the headspace and soil solution, commonly referred to as Henry's K (or the Bunsen coefficient α) in the equilibrium situation.

Once the quantity ABC has been established within the soil core any further N_2O produced will cause an equal proportion of N_2O to be evolved into the headspace, thus establishing a steady state between N_2O production and N_2O evolution. A soil with a slower rate of denitrification will have a lower flux of N_2O and therefore a smaller concentration gradient (A'B).

The time lag before the onset of linearity should be similar. The ABC volume (and hence the lag time) is influenced by the volume of water present in the sample. The porosity, tortuosity, particle size and shape also affect the effective diffusion coefficient Dp (Currie, 1979) and these parameters are reflected by the gradient AB or the height AE.

Figure 3.16 Schematic model of diffusion limitations of N_2O produced in soil core



N_2O concentration is expressed as moles per total volume of each phase.

3.12.1 Experimental

To verify that N_2O production monitored in the headspace of the incubation vessel is not significantly affected by diffusion (i.e. there exists only a short lag time) the following experiment was designed.

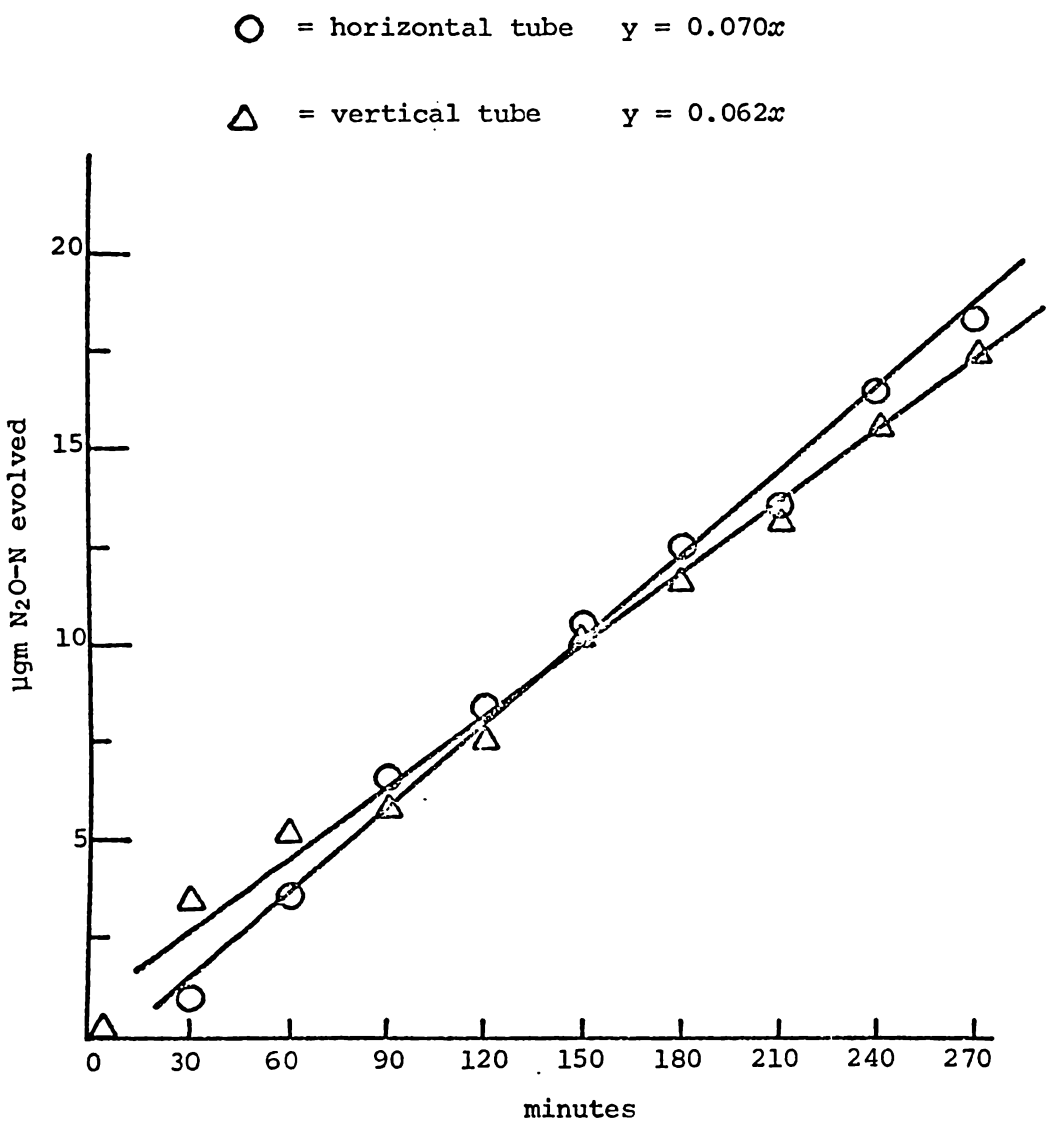
Four incubation tubes were packed with Wharekohe silt loam and prepared for incubation as described in section 3.2.0. Two of the tubes were laid on their sides and the soil evenly distributed along the tube, while the other two tubes remained in the vertical position during incubation. This had the effect of altering the N_2O diffusion path length (x) by a factor of 5. The horizontal tubes contained 15 mm of soil from the bottom to the headspace interface while the vertical tubes had a depth of 75 mm. In each case the quantity of soil remained identical and so did the headspace and moisture volumes, so the rates of N_2O production and effusion into the headspace should remain identical if no diffusion limitations occur. Only the geometry or path length x of the system has changed.

3.12.2 Results and Discussion

Results of the incubation are presented in fig 3.17. The data in fig 3.17 suggests that the rate of denitrification of the vertical plug of soil is less (by approximately 14%) than the horizontal.

Flux is defined as the number (N) of molecules crossing a unit area per time interval and may be expressed as $J = N/cm^2.sec^{-1}$. The surface areas of the two geometries of soil described above are calculated as 4.9 and 24.5 cm^2 for the vertical and horizontal tubes respectively. If the rate of effusion of N_2O into the headspace is to be the same in both cases, then the flux in the vertical tube must be 5 times greater due to the decreased surface area.

Figure 3.17 Rates of N_2O evolution from different depths of soil



Assuming the physical properties, water content and packing of the soils in their respective tubes are consistent, the only way the flux of the vertical tube can be five times greater is for the concentration gradient $\frac{dc}{dx}$ to be five times greater ($J = -D \frac{dc}{dx}$).

The five times greater concentration gradient must be maintained over a five fold greater distance, giving a net increase of twenty five times the N_2O in solution in the vertical tube.

This effect could be expected to cause a longer time lag before the onset of linear N_2O evolution. As both lines assume linearity within a short time, and the time lag difference is not noticeable, the indication is that the rate of N_2O evolution appears to be diffusion limited only for a short period.

The resultant lower rate of N_2O evolution by the vertical soil (14%) can only be explained by surface area effects. If either the concentration gradient $\frac{dc}{dx}$ or effective diffusion coefficient Dp are altered by changing the geometry of the soil packing, then the effect will only be to alter the lag time. Eventually a concentration gradient will be established such that the rate of N_2O production equals the rate of effusion into the headspace.

By altering the geometry however, the horizontal tube has acquired a five fold increased surface area. If we consider each soil (equivalently packed) to contain an equal number (x) of pores which are surrounded by saturated soil crumbs then the percentage of x which actually lie on the surface in direct contact with the headspace are going to increase in the horizontal tube by five fold. Consequently the number of pores in contact and equilibrium with the N_2O soil/water concentration will be less.

The Bunsen coefficient (α) for N_2O as previously seen in section 3.11.0 is 0.56 $\frac{cc\ N_2O(g)}{cc\ H_2O}$. Thus the distribution of N_2O at

equilibrium lies in favour of the gas phase. Therefore the gas phase represents a relative concentration of N_2O compared to the soil/water phase.

The soil/water N_2O content will increase in both tubes as N_2O is produced, but the number of enclosed pores over which this concentration gradient will be reflected will be less for the horizontal tube. Therefore for a given amount of N_2O produced, less will be bound within the soil/water phase at any given time. Consequently an increased proportion of the N_2O will be found within the headspace - giving rise to an apparent increase in the denitrification rate. It would require approximately 3% of the total pore volume in the vertical tube to be involved in this surface area effect to contribute to a 14% difference in the N_2O evolution into the headspace in the horizontal tube.

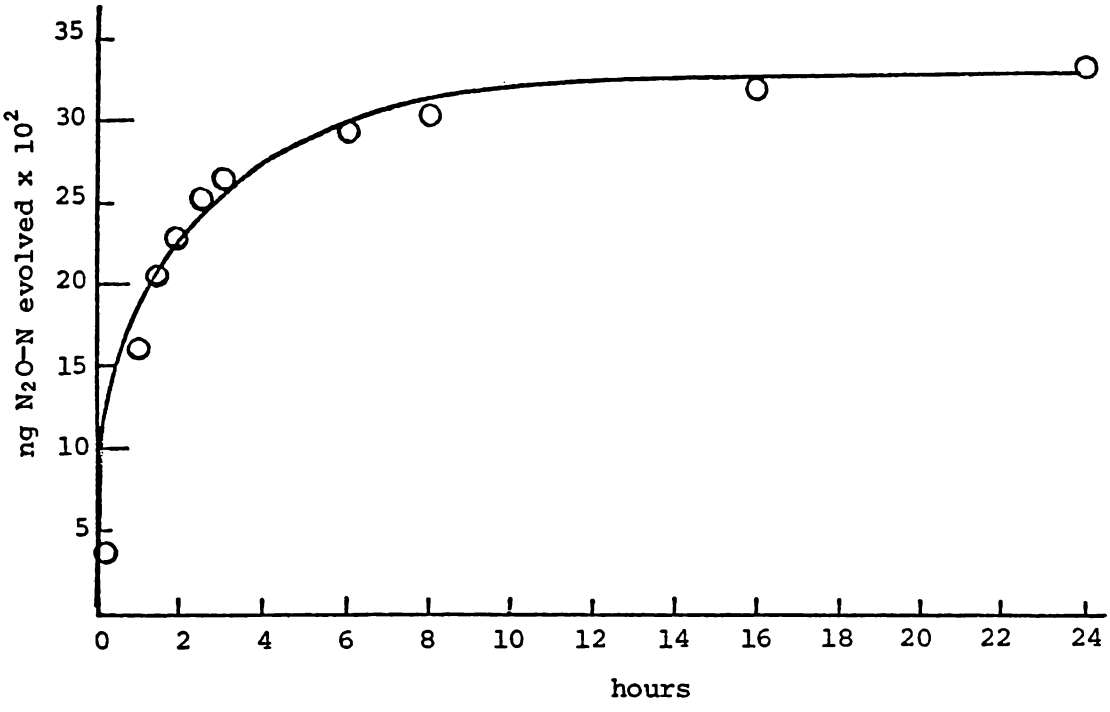
For samples of similar types of geometry, diffusion limitations are not going to be rate limiting, but only affect the time lag before the linearity of N_2O evolution reflects the rate of denitrification.

3.12.3 Diffusion limitations in calculating N_2O yields

Letey *et al* (1980) demonstrated that for saturated soil cores, only about 30 percent of the N_2O produced is evolved during denitrification. Several days were required after denitrification had ceased for the N_2O to reach equilibrium distribution. These results concur with a similar experiment conducted by the author (see fig 3.18).

Incubation tubes with 20 g autoclaved soil had a nominal 0.5 cc of N_2O added to the headspace. The tubes were left to equilibrate for two days. The headspace was then quickly flushed with

Figure 3.18 Diffusion of N_2O from soil into incubation headspace



helium and the N_2O evolution out of the soil core monitored.

This diffusion effect demonstrates why an N deficit is found during denitrification assays based on N_2O production *vs* nitrate additions (Letey *et al*, 1980). A valid N balance can only be obtained from such an experiment several days after the cessation of N_2O production - at which time the N_2O distribution between the gas/liquid phases can be adequately calculated using Henry's law.

It is interesting to note that the N_2O diffusion limitations occurring at the completion of N_2O production are of a significant duration, whereas the comparable limitations at the beginning of an incubation are insignificant. In the former situation the concentration gradient between the soil/water system and the gas phase is decreasing exponentially.

3.13.0 RATE OF NITRATE DISTRIBUTION

For the incubation assays to be valid comparisons of potential rates of denitrification there must be no N substrate limitations. Therefore it became important to know that mixing of the nitrate amendments was not rate limiting.

Klemedtsson *et al* (1977) suggested that discrepancies in their measured rates of denitrification on soils of low moisture contents may have been due to less efficient nitrate diffusion. However, Kohl *et al* (1976) concluded that nitrate diffusion from an overlying solution into the soil layer was not limiting the rate of denitrification.

The results of the concentration effect on rates of denitrification in section 3.4.1 suggest that nitrate transport is not a problem. The soil had been pre-incubated and controls showed no endogenous nitrate present. However, within 60 minutes of the

addition of nitrate, N_2O production was at full potential. Such indirect results were encouraging but not entirely definitive.

3.13.1 Experimental

A sample of Moanatuatua peat was incubated in the usual manner until N_2O production ceased. Fresh KNO_3 (2 ml) was added by syringe in the manner adopted for all incubation experiments.

The results (see fig 3.19) show that all endogenous nitrate was consumed within the first 60 minutes of incubation. Further nitrate was added at 270 minutes. By 360 minutes full denitrification potential had been achieved.

From these results it can be concluded that nitrate transport is not rate limiting and is in fact remarkably fast. The diffusion coefficients of electrolytes in aqueous solutions are in the order of $1 \times 10^{-5}/cm^2.sec^{-1}$ (C.R.C. Handbook F60, 1974). Therefore the transport process must be something faster than pure diffusion of ions in solution. It is suggested that a water potential caused by adding the solution to the surface of the soil may induce a flow process aiding the rate of nitrate transport.

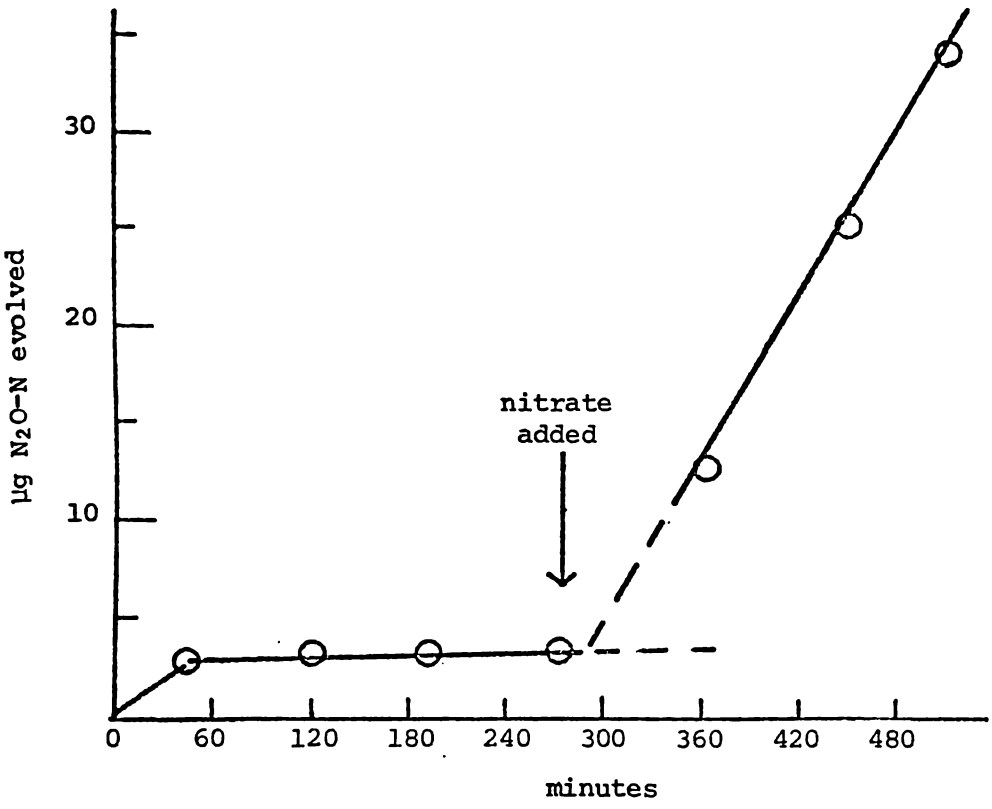
3.14.0 DEPTH OF SOIL CORES

All routine coring was to a depth of 50 mm unless otherwise required. For a discussion on the effect of depth on rates of denitrification see sections 4.3.2 and 5.5.0.

3.15.0 CRUMB SIZE

Sieving was required to maximise sample homogeneity. Two different size fractions (0-2 and 2-4 mm) were studied to measure any

Figure 3.19 Effect of nitrate addition on denitrification rate



effect particle size may have on the denitrification rate.

Results demonstrated that the potential rate of denitrification was not affected by sieving to different size fractions. Samples were subsequently passed through a 4 mm aperture sieve.

3.16.0 REPRODUCIBILITY OF ANALYSES

The replicates of Waimate North clay loam were prepared for incubation in the usual manner. Rates of denitrification were determined using the lines of best fit (least squares) analysis. The rates are listed in table 3.5 along with r^2 (correlation coefficient) values.

Table 3.5 Replicate rates of denitrification for Waimate North clay loam

Rate (μg N_2O-N evolved/hr)	Correlation Coefficient r^2
3.78	0.990
3.75	0.994
2.96	0.970
3.74	0.986
4.11	0.968
3.62	0.986
2.99	0.900
3.58	0.974
3.61	0.954
2.99	0.966

The mean was calculated at 3.51 μg N_2O-N/hr with a standard deviation (σ) of 0.40 μg N_2O-N/hr.

The errors represented by this standard deviation are going to vary in significance depending on the rate of denitrification. Random analytical errors such as sample injection volumes and peak height analyses will assume increasing importance as the denitrification rate decreases. Errors such as sample inhomogeneity will remain constant. Bearing these considerations in mind a standard error of ± 0.40 μg N_2O-N/hr was assumed for all measured rates of denitrification.

CHAPTER 4

COMPARISON OF DENITRIFICATION POTENTIALS

4.1.0 INTRODUCTION

Using the technique described in the preceeding chapter the denitrification potentials of a variety of North Island soils were measured and assessed with regard to the following conditions:

i) Soil types

ii) Depth in the soil profile

iii) Effect of agricultural practices

iv) Seasonal influences.

4.2.0 DENITRIFICATION POTENTIAL OF DIFFERENT SOIL TYPES

Soil is an independent heterogeneous dynamic environment which acquires properties in accordance with the forces which act upon it. These factors, be they parent material, climate, vegetation, or management practices, combine to impart a delicate balance of unique chemical and physical properties to each soil. Soil types from Northland to the South Waikato were assessed for differences in denitrification potentials.

4.2.1 Experimental

The denitrification potentials were assessed in the usual way described in section 3.2.0. Because it was desirable to process samples within 48 hours of collection (see section 3.6.1) the distance over which soils could be collected was by necessity somewhat restricted.

The soils were surveyed during September and October of 1979. Results are divided into two sections: i) Northland soils, ii) Waikato soils. The measured denitrification potentials of Northland soils are given in table 4.1 alongside their nitrification activities (INA values).

Table 4.1 Rates of denitrification of Northland soils

Soil	Rate of Denitrification (μg N_2O-N/hr)	INA (μg N_{ox}/hr/gm)
Hukerunui silt loam	6.49	0.53
Okaihau clay loam	5.31	1.57
Ohaewai silt loam	4.72	N.D.
Ruatangata friable clay	4.72	N.D.
Awarua clay	4.13	0.74
Waimate North clay loam	3.54	2.27
Kiripaka silt loam	3.39	0.18
Wharekohe silt loam	2.65	0.16, 0.07
Marua clay loam	0.88	<0.02

(N.D. = not determined)

4.2.2 Northland soils - Results and Discussion

The denitrification potentials of Northland soils are listed in table 4.1. Overall, the Northland soils demonstrate a moderately wide variation of denitrification potential compared to the highest measured potential (Te Kowhai silt loam at 9.71 μg N_2O-N/hr). Some rates however (Marua, Wharekohe and Kiripaka) are conspicuously low.

Steele (1980) studied several New Zealand soils for nitrification activity. Using a perfusion technique he was able to group soils according to their rates of NH_4^+ oxidation - expressed as Initial

Nitrification Activity (I.N.A.) Such an assessment was proposed as a means of comparison of the 'inherent' oxidation activities of the indigenous population of nitrifying organisms in different soils at the time of sampling. I.N.A. values of >2.0 μg N oxidised/g soil/hr were considered high.

Where the Northland I.N.A. values are extremely low (<0.18) there appears to be a significant correlation between the nitrification and denitrification activity. The Wharekohe, Kiripaka and Marua soils not only have the lowest I.N.A. values, but also exhibit the lowest denitrification potentials of the Northland soils.

At moderate I.N.A. values and above, denitrification potentials appear to become independent of the nitrification activity. As the I.N.A. value increases and nitrate becomes more available, other factors such as clay mineralogy, pH, available carbon and bacterial strain probably become more dominant influences. Differences in bacterial strains have been shown to affect the nitrifying capacities of soils (Fraps and Sterges, 1939; Pikous Kaya, 1940) and so might similarly affect denitrification potentials within different soils.

Steele in a study on the Wharekohe and Kiripaka soils (1980) found that the differences in the I.N.A. values were reflected in the NH_4^+-N : NO_3^--N ratios. The Wharekohe soil with a lower I.N.A. value had a NH_4^+-N : NO_3^--N ratio of 16:1 while the Kiripaka soil showed a ratio of 1:1. The increased proportion of NO_3^--N in the Kiripaka soil could well explain the increased denitrification potential of that soil compared to the Wharekohe.

Field trials on the Wharekohe and Kiripaka soils showed apparent N recoveries of 56% and 44% respectively, i.e. the Kiripaka soil did in fact demonstrate a larger N loss. However, such results do not take into account losses due to N immobilisation in the organic fraction.

4.2.3 Waikato soils - Results and Discussion

The Waikato soils exhibit a wider range of denitrification potentials compared to the Northland soils with a number of soils (Kaipaki, Horotiu, Hamilton Ash and Te Kowhai) exhibiting significantly higher denitrification potentials (table 4.2). It is noticeable that high denitrification potentials only occur in soils having a high I.N.A. value (table 4.2).

Table 4.2 Rates of denitrification of Waikato soils

Soil	Rate of Denitrification (μg N_2O-N/hr)	INA (μg N_{ox}/hr/gm)
Te Kowhai silt loam	9.7	2.0
Kaipaki peat loam	8.5	N.D.
Hamilton clay loam	7.8	N.D.
Horotiu sandy loam	7.4	2.26
Rukuhia peat	6.2	0.9 to 0.1
Otorohanga silt loam	3.8	N.D.
Bruntwood sandy loam	2.9	N.D.
Puniu silt loam	1.0	N.D.

(N.D. = not determined)

The lowest three potentials (Puniu, Bruntwood and Otorohanga) were all sampled from pastoral soils approximately 50 km south of Hamilton whereas the highest four potentials (Te Kowhai, Kaipaki,

Horotiu and Hamilton Ash) were all sampled on the Ruakura Research Centre experimental plots in Hamilton.

4.2.4 Allophane influence

It is notable that the two Waikato soils of lowest denitrification potential (Puniu and Marua) are both lacking in allophane. Allophane is an amorphous alumino-silicate clay with a characteristically large specific surface area. The allophane content of the other soils listed in tables 4.1 and 4.2 are variable and a correlation between the higher denitrification potentials and allophane content is not evident.

Cooper (1974) showed that bacterial metabolism was stimulated by the presence of allophane in pure culture laboratory experiments. This phenomenon was explained by the strong interaction between bacteria and allophane resulting in the bacteria being sorbed onto allophane and the nutrient rich environment provided by allophane.

A similar phenomenon was noted by Kunc and Stotzky (1974) whereby the rate of nitrification of a soil was enhanced by the addition of montmorillonite but not kaolinite, and the stimulation was proportional to the amount of clay added. The reason for this phenomenon was stated to be the greater cation exchange capacity of montmorillonite.

Baber (1977) and Steele (1977) both observed that higher I.N.A. values generally occur in allophanic soils.

4.3.0 VARIATION IN DENITRIFICATION POTENTIAL WITH DEPTH

Previous reports on the effect of depth on rates of denitrification have produced conflicting results. The depth at which denitrification is most likely to occur in a soil profile is dependent on several factors: available organic matter, a suitable denitrifying population, nitrate concentration, and anoxic zones. The first three

factors are almost always greatest near the surface. Myers and McGarity (1971) showed that in solodized solonetz profiles where soluble organic compounds accumulated, high denitrification rates were stimulated. Gambrell *et al* (1975) showed that only in the depths below plant roots (i.e. near the water table) were conditions sufficiently anoxic to effect denitrification. They calculated that more than sufficient soluble organic matter was leached with the nitrate to this zone to effect complete reduction of the nitrate. Jones (1974) using soil columns, measured no loss of NO_3-N to a depth of 7.5 cm, but between 7.5 and 10 cm considerable loss occurred which approached losses measured at 25 cm. Gilliam *et al* (1978) using columns of 3 different soils, found the maximum zone of denitrification to vary from the top 25 cm to the 60-90 cm zone.

Stefanson (1974) however showed reduced losses of fertilizer nitrogen with deep placement in the soil profile. Similarly, Starr *et al* (1974) and Rolston (1976) found that denitrification occurred close to the surface in the soils having a higher organic matter content. The soil poorer in organic matter required a greater depth to effect reduction of the oxygen concentration.

4.3.1 Experimental

Three soils (Te Kowhai silt loam, Horotiu sandy loam, and Rukuhia peat) were selected to compare the effect of depth on denitrification. Brief profile descriptions are included in table 4.3.

Table 4.3 Profile descriptions of Te Kowhai, Horotiu and Rukuhia peat soils

Te Kowhai silt loam	Horotiu sandy loam	Rukuhia peat
0-20 cm. A_1. Dark grey/brown silt loam; friable, indistinct boundary. 20-30 cm. Light grey, heavy silt loam. Weakly developed structure.	0-20 cm. A_1. Dark grey/brown fine sandy loam; friable, strongly developed crumb structure. Indistinct boundary. 20-40 cm. B_2. Brown sandy loam, moderately developed structure.	0-25 cm. Yellow brown to brown fibrous peat. Indistinct boundary. 25-50 cm. Very dark brown, weakly decomposed fibrous peat. Saturated.

Core samples were taken at depths of 0-3, 3-6 and 6-9 cm. These samples were prepared and incubated in the usual manner. Available carbon was assayed by the technique described by Burford and Bremner (1975). Results of the denitrification rates (table 4.4) are recorded as µg N_2O-N produced during 8 hours incubation.

Table 4.4 Potential rates of denitrification at varying depths

Soil	Sample Depth	Available Carbon (µg $C.g^{-1}$)	µg N_2O-N evolved
Te Kowhai silt loam	0-3 cm	23.6	69.32
	3-6 cm	4.8	0.73
	6-9 cm	7.3	0.0
Horotiu silt loam	0-3 cm	40.3	41.3
	3-6 cm	3.8	0.0
	6-9 cm	5.5	0.0
Rukuhia peat	0-3 cm	151.0	47.9
	3-6 cm	132.1	8.8
	6-9 cm	187.3	5.8

All soils sampled were under pasture and had been for many years. Each soil sampled in table 4.4 demonstrated a sharp decrease in denitrification activity below 3 cm. Below 3 cm the denitrifying population are severely limited by available carbon resources except in the case of the peat soil. If available carbon is the only factor causing the denitrification to be associated with the rhizosphere the denitrification potential of deeper samples could be expected to increase with increased available carbon.

Fresh cow's urine was applied to the Horotiu sandy loam and allowed to leach into the profile (see section 5.5.0). The plot was left for several weeks to allow the microbial population to respond to the increased substrate levels at depth. Core samples were then taken at 0-3 and 6-9 cm. Results are displayed in table 4.5.

Table 4.5 Denitrification of Horotiu soil after application of urine

Depth	Available Carbon ($\mu g\ C.g^{-1}$)	Nitrate Substrate ($\mu g\ N.g^{-1}$)	$\mu g\ N_2O$-N produced/8 hs
0-3 cm	166	500	42.7
6-9 cm	53	464	0

Nitrate levels were analysed at Ruakura Research Centre on a Technicon auto analyser by the method described by Kamphake *et al* (1967) after extraction with 2M KCl. In spite of very high levels of both C and N substrate at 6-9 cm, no increase in denitrification activity occurred.

4.3.2 Discussion

The results of tables 4.4 and 4.5 suggest a strong preference for denitrification to occur close to the rhizosphere in a pastoral soil. Even when substrate levels are increased at moderate depths, increased denitrification potentials are not recorded. In the case of the peat soil where abundant available carbon is present at the 9 cm depth, the denitrification activity still appears to be associated largely with the rhizosphere.

Yamaguchi *et al* (1962) and De Camargo (1974) show that under pasture the upper soil atmosphere is in rapid equilibration with the atmosphere and oxygen levels do not drop significantly. However, concentrations of gaseous oxygen in soil profiles are poor and misleading indications as to the anoxia status in microsites. Oxygen concentrations of 17% and higher are frequently observed in soil profiles where denitrification is known to occur (Meek *et al*, 1969; Stefanson, 1972a, b; Burford and Millington, 1968; Dowdell and Smith, 1974). Woldendorp (1962) postulated that increased denitrification activity within the rhizosphere was favoured due to root exudates (carbon source) and reduced oxygen concentrations from intense microbial respiration. Rolston *et al* (1976) measured a reduced oxygen level in the rhizosphere and which increased from 11% near the surface to 18% approximately 60 cm further down the profile. Woldendorp also suggested (1963) (and the results in table 4.5 support the view) that the indigenous denitrifying organisms are present not because of their denitrifying capabilities but because of some other property. Results of Katznelson *et al* (1956) support this conclusion by showing that denitrifying bacteria are approximately 90 times more numerous in the rhizosphere than in the soil.

The results in tables 4.4 and 4.5 suggest that N substrate leached beyond the top 3 cm zone should escape further dissimilation and be available to plants. It may be that moderate irrigation after application of N fertilizer will promote greater fertilizer uptake by plants rather than induce higher rates of denitrification in the soils studied.

4.4.0 EFFECT OF AGRICULTURAL PRACTICES ON DENITRIFICATION

Agricultural practices such as pasture or animal production each affect the soil in a distinctive way. The grazing animal has been shown to affect the nutrient status of New Zealand soils markedly (Steele, 1977, p156). Steele suggested that urine addition to the soil may be responsible for maintaining the large nitrifying population close to the soil surface

Cropping also is known to affect the nutrient status and physical properties of soils. Two New Zealand soils studied by Cotching *et al* (1979) both demonstrated changes reflecting the change from pasture to maize production. Both the Horotiu sandy loam and Puniu silty clay loam demonstrated a deterioration of soil structure and a decrease in soil aggregate stability. A reduction in total carbon in the 0-5 cm region was also noted.

Gradwell (1973) reported deterioration of soil structure on soil used for market gardening. Page and Willard (1946) reported that five years of continuous corn on the Nappanee silty clay loam resulted in a 25.4% reduction in aggregation and a 17.7% increase in bulk density when compared with continuous grass production.

The effect of maize and pasture production, and varying stock densities on denitrification potential in Waikato soils was studied.

4.4.1 Experimental

Soil cores to a depth of 50 mm were collected. The maize paddocks at the time of sampling (August, 1979) were lying fallow prior to seeding for next season's crop. Where possible the maize sites were chosen adjacent to the pasture site. Rates of denitrification were assayed using the technique described in section 3.2.0.

The measured denitrification potentials are presented in table 4.6.

Table 4.6 Potential rates of denitrification of soils under maize and pasture ($\mu g\ N_2O$-N evolved/hr^{-1})

Soil	Crop	Denitrification rate
Te Kowhai* silt loam	Pasture	9.71
	Maize	4.0
Hamilton clay loam	Pasture	9.05
	Maize	3.3
Otorohanga silt loam	Pasture	3.8
	Maize	0.0
Bruntwood sandy loam	Pasture	2.5
	Maize	0.7
Horotiu sandy loam	Pasture	8.4
	Maize	0.25
	Graveyard	5.2

* Maize and pasture soils were not sampled from adjacent paddocks.

All maize soils exhibited a lower denitrification potential than the corresponding pasture site. The soils under pasture which exhibited high denitrification potentials generally exhibited correspondingly higher potentials under maize. With harvesting, tillage and seeding on

an annual basis, maize soils undergo cyclic disturbances to the system. The trend recorded in table 4.6 may be indicative of the state of soil management and not a reflection of the maize crop. For instance it is possible the available carbon may increase significantly during the period where the maize stubble is ploughed back into the soil prior to seeding the next season's crop.

The Otorohanga pasture and maize sites were resampled six months later (February, 1980) when the maize crop was silking.

Table 4.7 Denitrification potential of Otorohanga soil under maize crop (February, 1980)

Soil	Crop	Denitrification rate (μg N_2O-N/hr)	Available Carbon (μg $C.g^{-1}$)
Otorohanga silt loam	Pasture	7.4	69.2
	Maize	0	0

The results (table 4.7) concur with the earlier results suggesting that maize soils consistently demonstrate lower denitrification potentials than their pastoral counterparts.

In view of the apparent influence of the available carbon level on denitrification potential (tables 4.4, 4.7) three peat soils were sampled. As the peat soils are not utilized for maize production, three soils of similar parent material (Rukuhia peat) but varying stock capacity were compared for denitrification potentials.

The Kaipaki peat was located on the property of the Ruakura Research Centre, Hamilton, and was subjected to a consistently high stock rate of grazing animals. The Rukuhia peat, site 1, was sampled from the Moanatuatua Research Station about 30 km S.E. of Hamilton. This peat

was under a rotational grazing experiment and subjected to high stock levels approximately one week in four. The Rukuhia peat, site 2, was located approximately 10 km S.W. of Hamilton (Collins Road), at a site not carrying any stock.

The results (table 4.8) indicate that even soils with high available carbon resources reflect their agricultural regime with respect to their denitrification potential. The Kaipaki peat with the highest stock rate exhibited the highest denitrification potential. All three peats had what could be considered high levels of available carbon compared to the surrounding soils of volcanic ash or alluvial origins.

Table 4.8 Denitrification potential of similar peat soils under varying stock rates

Soil	Denitrification Rate ($\mu g\ N_2O$-N/hr)	Available Carbon ($\mu g\ C.g^{-1}$)	Stock Rate
Kaipaki peaty loam*	7.5	123	High
Rukuhia peat (site 1)	6.0	110	Moderate
Rukuhia peat (site 2)	3.1	148	Nil

* This soil is formed on the Rukuhia peat but slightly more developed.

4.4.2 Discussion

The influence of available carbon on denitrification rates has been well demonstrated (Bowman and Focht, 1974; Burford and Bremner,

1975; Patten *et al*, 1980). This seems to be a likely explanation for the reduced potential found in soils under maize crops. Depletion of carbon under crops such as maize has been recorded by several workers. Kohl (1976) noticed that a soil under corn/soyabean crop rotation exhibited a lower denitrification rate than under pasture. The total C/N ratios were comparable and Kohl postulated that the differences in rates of denitrification must be due either to microbial activity variations or the available carbon levels. Stefanson (1972a) noticed lower denitrification rates under a crop soil compared to a pasture soil and attributed this to a decreased level of carbon. It is unlikely however that available carbon limited denitrification in the peat soils studied. These soils still demonstrated the effects of varying stock rates.

The Horotiu soil demonstrated a similar effect. Samples were taken from a site at the Ruakura Research Centre. Samples of the Horotiu silt loam were also taken from undisturbed areas of a local graveyard. This area, having not been subjected to agriculture, would serve as a comparison to the Horotiu soil under intensive grazing at the research centre. The pastoral Horotiu denitrification potential was significantly higher than that of the graveyard (see table 4.6). The only significant difference between these two soils is the influence of grazing animals on the pasture site.

Galbally and Roy (1978) noted that NO fluxes were higher from grazed than ungrazed pasture. The explanation for the observed increase in denitrification potential with increased stock rates is unlikely to be due to increased microbial levels. Davis *et al* (1980) recorded only a slight rise in the denitrifying population with large amendments of animal waste to soils.

High levels of stock are known to induce high localised

concentrations of nitrate with the return of urine to the soil. Under this urine spot concentrations of nitrate may well exceed 100 ppm along with concomitant increases in available carbon (see section 5.4.3). The higher nitrate levels may induce higher enzyme levels and activities - specifically of nitrate reductase. The stock effect may therefore be a response to increased nitrate reductase activity stimulated by high localised concentrations of both nitrate and available carbon. The stock rates appear to influence denitrification potentials and are a factor which cannot be ignored in comparing denitrification potentials. It is possible that the high denitrification potentials measured on the Ruakura sites are a reflection of the intensive agriculture rather than the soil character.

4.5.0 SEASONAL VARIATION OF DENITRIFICATION POTENTIAL

Denitrification rates are known to be affected by temperature, substrate concentration and aeration (or moisture content). Variations in the above parameters can be expected to occur seasonally.

The Te Kowhai soil was sampled regularly for 12 months from a plot on Ruakura No.5 Dairy to establish seasonal variations in denitrification potential.

4.5.1 Results and Discussion

A systematic shift occurred with time from a mono-gradient to a bi-phasic rate of denitrification (fig 4.1). During the summer months the rate of denitrification resolved into two distinct phases, gradually merging into one again with the onset of autumn.

Phase transitions during anaerobic incubations of the type conducted in this experiment have been noticed before by Smith *et al* (1978), Tiedje *et al* (1978) and Smith and Tiedje (1979). Using

Figure 4.1 Seasonal variations in the denitrification potential of the Te Kowhai silt loam

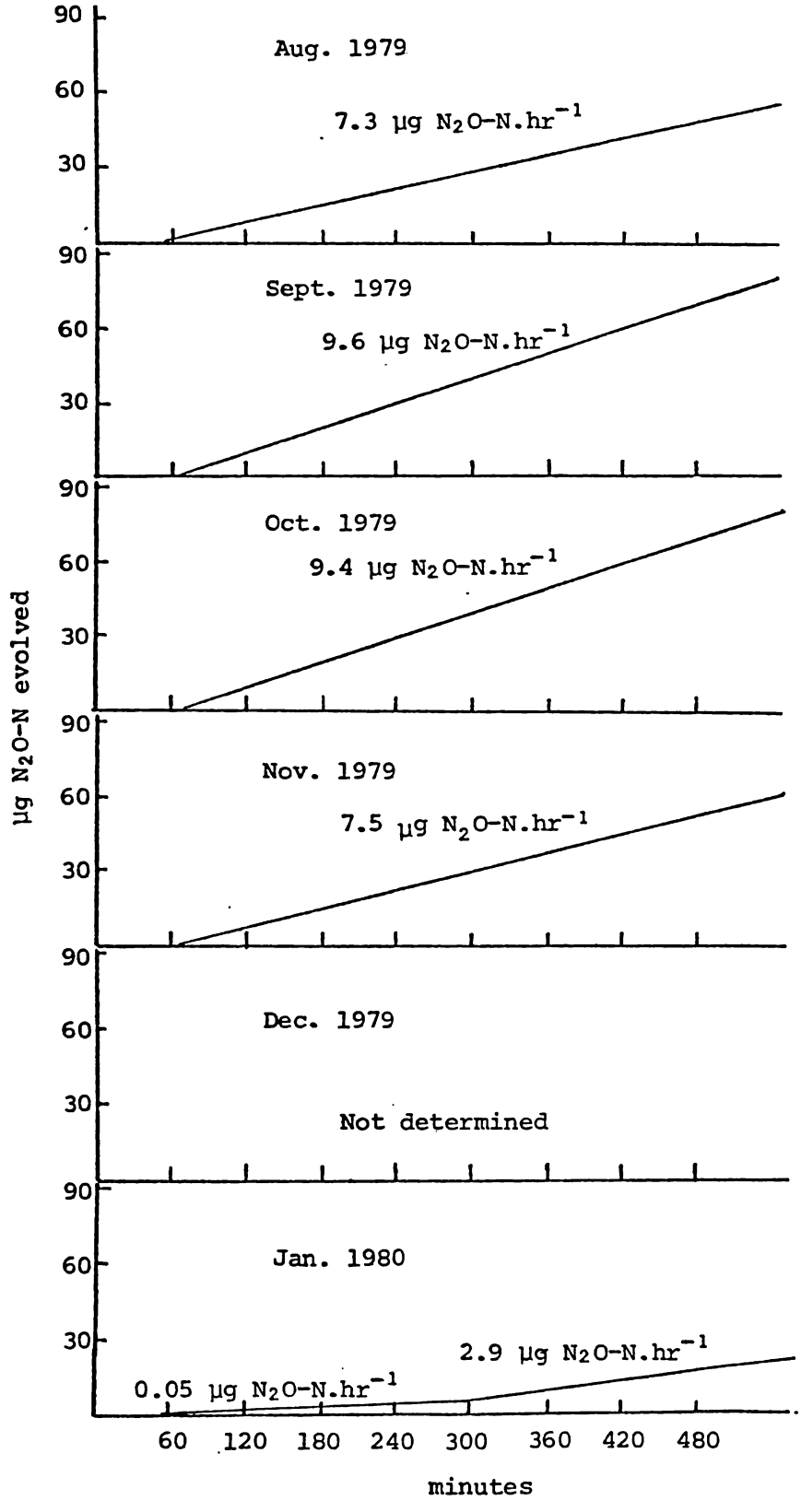
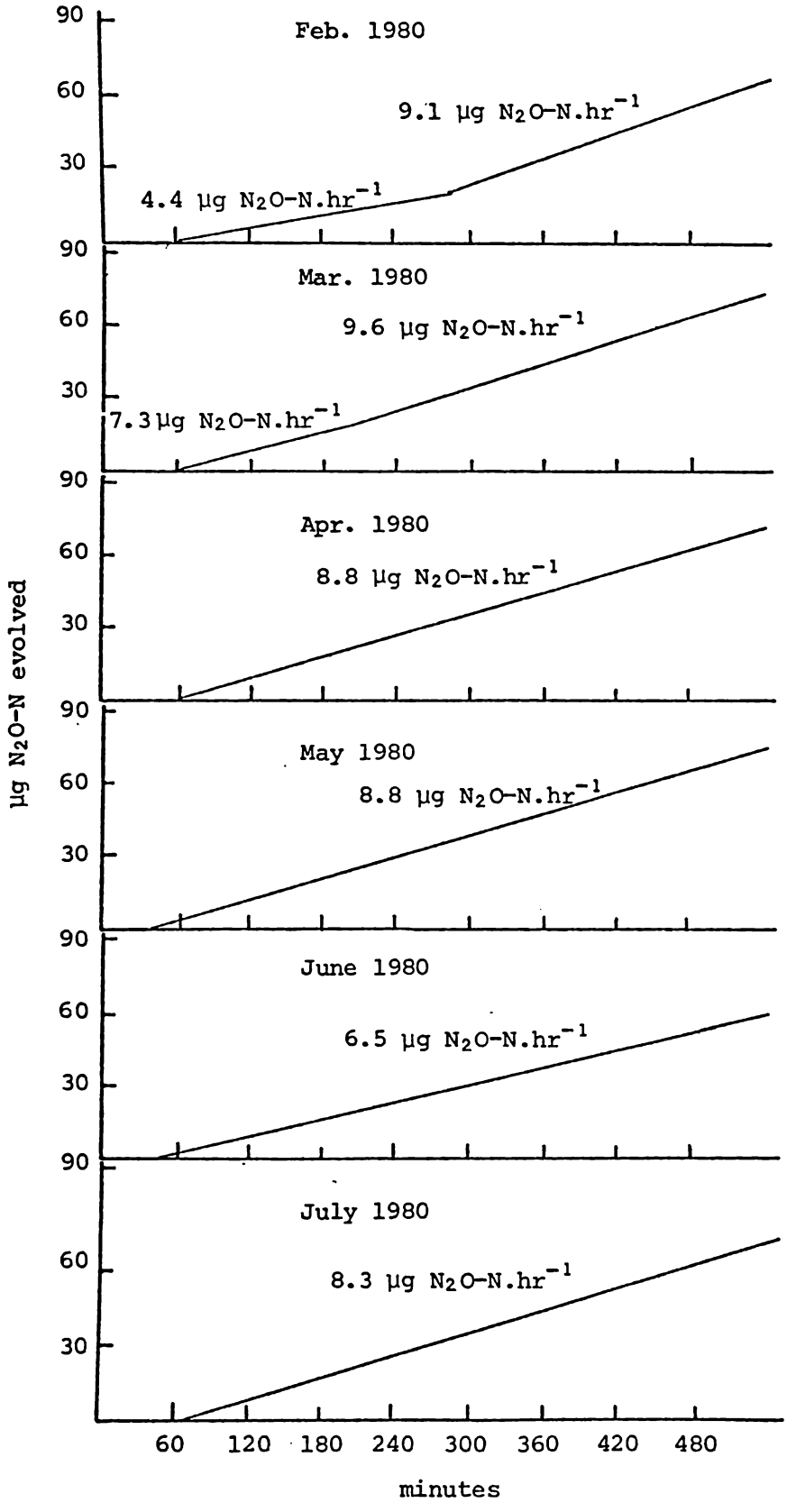


Figure 4.1 continued



chloramphenicol (a protein synthesis inhibitor) they showed that the initial phase (phase I) was unaffected by such an amendment, but the induction of phase II was inhibited in the presence of chloramphenicol. Phase I was attributed to the existence and activity of pre-existing denitrifying enzymes. The duration of the observed phase I was related to the time lag before synthesis of new denitrifying enzymes began. Comparable time lags of approximately 40 minutes for the onset of synthesis of denitrifying enzymes have been recorded in pure culture studies (see section 2.4.5). Phase I was also largely unaffected by the addition of glucose. It was postulated that the phase I (or initial) rate represented an assay of the cellular denitrifying enzyme activity *in situ* and reflects the conditions prevalent in the soil at the time of sampling.

The second linear phase (phase II) was attributed to the rate observed upon synthesis of additional new denitrifying enzymes in response to the incubation conditions. At this stage all denitrifiers are fully derepressed so each cell has attained its maximum capacity to denitrify. Phase II was attributed to be an indication of the 'denitrification potential' of the soil. At this point the denitrification rate can only increase further by an increase in the number of cells. Significant growth will only occur with a ready supply of available carbon. Without addition of carbon the phase II stage persisted for several days of incubation. With glucose additions, the phase II rate was sometimes seen to increase, and was always followed shortly after by a logarithmic rate increase. The results also suggested that numbers of denitrifiers remain constant in anaerobic conditions unless an exogenous carbon source is available.

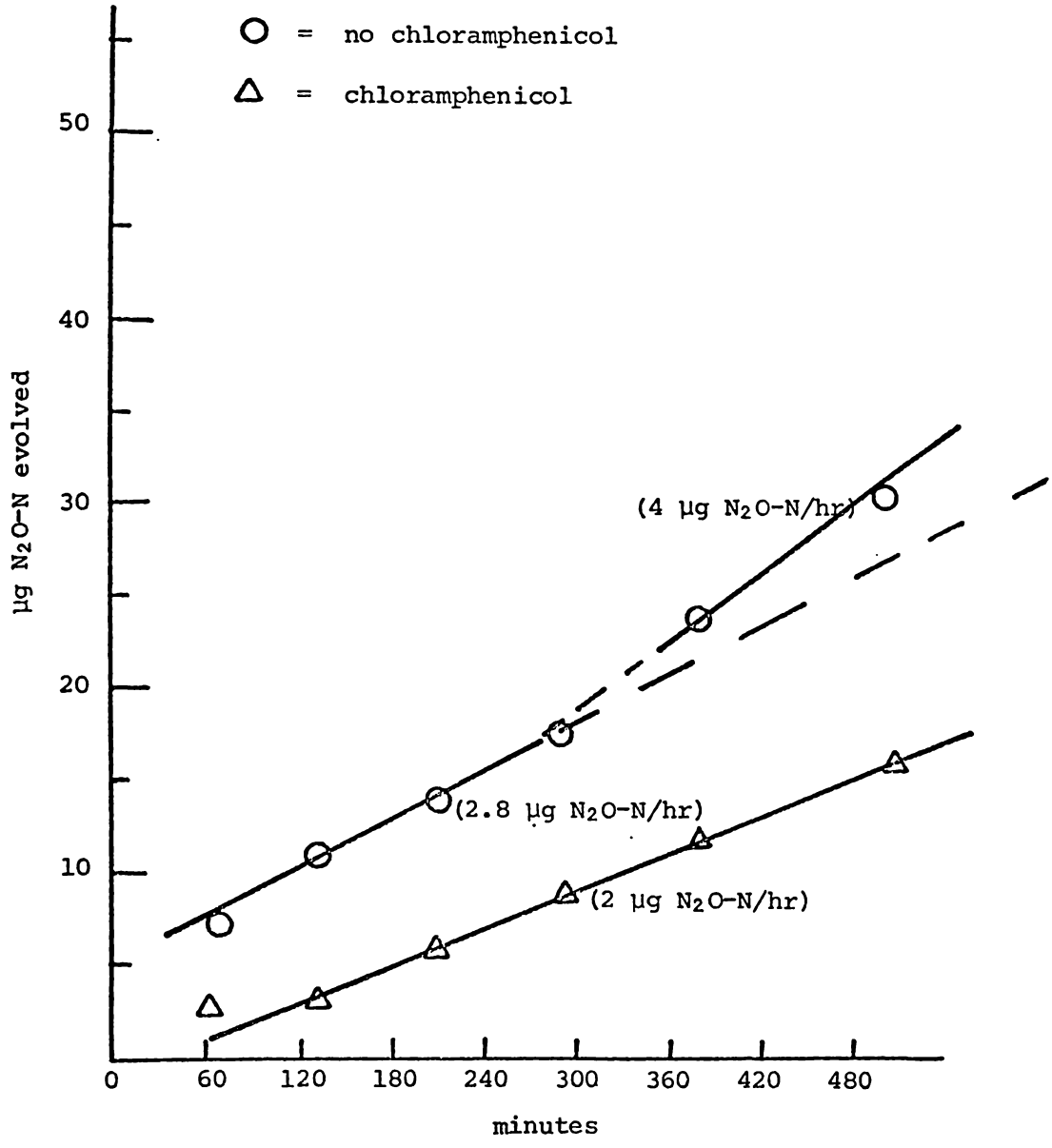
Using a similar technique to that described by Smith and Tiedje (1979) a sample of the Te Kowhai soil displaying the bi-phasic denitrification rate (March, 1980) was incubated with chloramphenicol.

The results (fig 4.2) clearly demonstrate the chloramphenicol effect on phase II. The experiment was repeated a few weeks later, after the Te Kowhai soil demonstrated no phase change during incubation. This time the denitrification rate was unaffected by the addition of chloramphenicol. The control sample of the Te Kowhai soil at this time also demonstrated no phase change during incubation. On the basis of this evidence it was concluded that a similar enzymic mechanism as outlined by Smith and Tiedje (1979) was operating in the Te Kowhai silt loam.

As there was no evidence of a phase change in the Te Kowhai soil sampled in April 1980 and the phase change recorded in March 1980 responded to chloramphenicol, it was concluded that at certain times of the year the Te Kowhai soil was in a natural state of full enzyme derepression. In fact the data in fig 4.1 suggests that the Te Kowhai soil is fully derepressed for some eight months of the year. Only during the peak of the summer is the soil in a state of reduced denitrifying enzymic activity. This observation concurs with the findings of Ryden and Lund (1980) who found the denitrification rate to remain relatively high throughout the year in vegetable crops.

After full derepression the enzyme level reaches a steady state maximum for a given cell population. This level is regulated by the constant degradation and resynthesis of the enzymes. Because chloramphenicol blocks protein synthesis, there will be a gradual decline in enzyme levels in the presence of chloramphenicol. The results of March 1980 did not display any decrease in the enzymic activity over the eight hour incubation period - suggesting negligible 're-synthesis' or degradation during this period. It is possible the re-synthesis time may be considerable (days or weeks) so assisting the explanation for the apparent consistency found in the pattern of denitrification potentials monitored during the year. Once full induction has occurred, and

Figure 4.2 Effect of chloramphenicol on phases of denitrification in Te Kowhai silt loam (10 g) (sampled March 1980)



providing significant C or N stress is not evident, it appears that enzymes may remain in a state of full derepression for some time after the event which caused the induction, returning only slowly to the low level of activity predominating before induction.

The results in figs 4.1 and 4.2 indicate that induction (or derepression) occurs much faster than the degradation of newly synthesised enzymes.

It will be noticed in fig 4.1 that as the end of summer approaches, the gradient of the phase I stage approaches that of the phase II stage, i.e. the degree of derepression is increasing with the approach of the winter climatic conditions. The ratio of the phase II gradient to the phase I gradient could be taken as a measure of the reduction of activity the denitrifying enzymes have undergone. When the phase II to phase I ratio equals unity (or the phase difference is zero) then the *in situ* denitrification activity is the same as the maximum denitrification potential (without addition of exogenous reductant). Smith and Tiedje (1979) showed that by irrigating a soil of 16% water content the phase I gradient (or rate) doubled with no noticeable change in the phase II gradient.

The denitrification potential has been shown to be dependent upon available carbon levels (e.g. Bowman and Focht, 1974). Smith and Tiedje (1978) found variable responses in the phase II gradients upon addition of glucose. This effect is probably because Michaelis-Menten kinetics have been shown to apply to both C and N substrates during denitrification. Those soils in which Smith and Tiedje found no glucose response in the denitrification potential probably already had their enzyme systems saturated with endogenous available carbon.

In June 1980 the phase II potential of the Te Kowhai soil was measured at 6.5 μg N_2O-N/hr compared to 8.8 μg N_2O-N/hr for the two

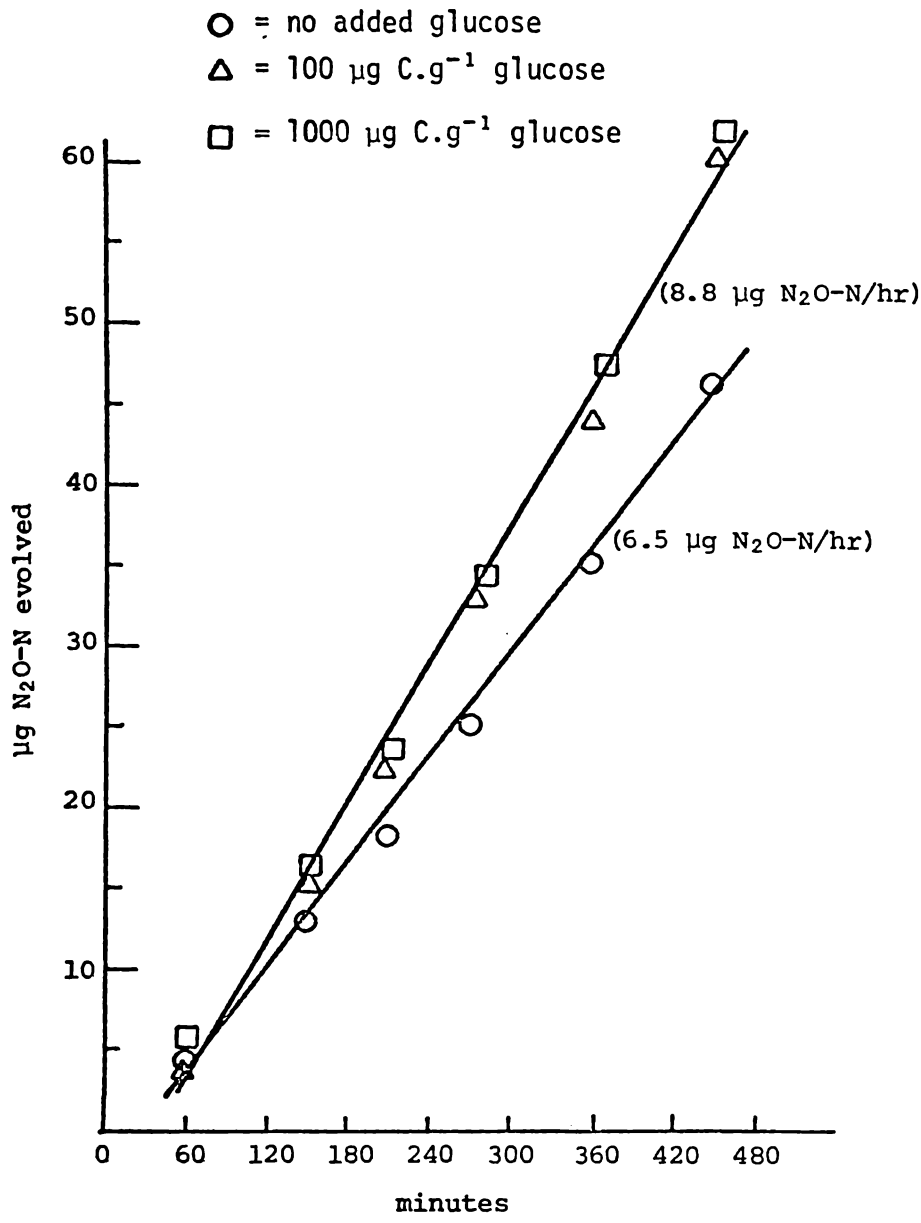
months previous. If this decrease in potential was in response to reduced available carbon, it may be expected that the potential should increase to the maximum recorded (~9 μg N_2O-N/hr) with the addition of glucose. An incubation was performed with added nitrate and glucose amendments of 0, 100 and 1000 μg $C.g^{-1}$. The results are displayed in fig 4.3. The subsequent increase in the denitrification potential with the glucose amendments confirms that the denitrification potential of the soil at this time was limited by available carbon. The potential recorded with both 100 and 1000 μg $C.g^{-1}$ glucose was similar suggesting that the denitrification was zero order with respect to the 100 μg $C.g^{-1}$ amendment. The 8.5 μg N_2O-N/hr recorded with the added glucose is comparable to the highest recorded potential for this soil (~9 μg N_2O-N/hr).

The available carbon content of the Te Kowhai soil measured at this time (~75 μg $C.g^{-1}$) was in excess of the usual value (~50-60 μg $C.g^{-1}$) at which levels higher denitrification potentials had previously been recorded. It is possible that the available carbon level alone is not sufficient to indicate carbon stress on denitrification rates. If there was increased competition among microorganisms for the available carbon denitrifiers may undergo carbon limitations even in the presence of a high concentration. It is also possible that the organisms may not have been able to respond to the increased carbon level because of reduced temperatures or some other limitation.

The results in fig 4.3 suggest that at certain times of the year, denitrification may not be limited by the availability of nitrate substrate, but by available carbon. This situation may be particularly true in the Waikato area where high soil nitrate values are common.

Baber (1977) found that 48% of the groundwater samples in the Horotiu/Te Kowhai soil area exceeded levels of 10 mg NO_3-N/ℓ. Samples

Figure 4.3 Denitrification potential of Te Kowhai soil (June 1980) with added glucose



as high as 50 to 60 mg NO_3-N/ℓ were recorded.

Ryden and Lund (1980) also suggested that denitrification in the field may often be controlled by available carbon rather than nitrate levels. They found the nitrate concentration only occasionally influenced the rate of denitrification and nitrate levels between 2 and 33 μg $N.g^{-1}$ recorded the same denitrification losses.

4.6.0 CONCLUSIONS

The denitrification potential was seen to vary approximately ten fold between soils. It is possible a correlation exists between I.N.A. values and denitrification potential in the case of those soils exhibiting low denitrification potentials. The three soils exhibiting the lowest I.N.A. values also showed the lowest denitrification potentials. Supply of N substrate is probably the limiting factor.

No correlation was evident between high I.N.A. values (>0.5) and the denitrification potential. Other factors such as clay mineralogy, pH, bacterial strain and available carbon probably become more dominant influences as the availability of nitrate is increased.

The results in the soils studied indicate the occurrence of most denitrification activities within the rhizosphere. This relationship is seen to continue even with the addition of both C and N substrate at depth. In all soils measured approximately 90% of the denitrification activity was confined to the top 3 cm.

The type of agricultural practice appears to exert a profound influence on the denitrification potential of a soil. Soils under maize crops exhibited markedly lower rates of denitrification compared to similar soils under pasture. Evidence suggests that lack of available carbon in the maize soils could be responsible.

Denitrification potentials appear to increase with increased stock rates. The three highest potentials measured were all on the Ruakura Research Station. Denitrification potentials measured on similar soil types carrying less or no stock exhibited lower activities.

Soils displayed two different denitrification potentials at certain times of the year. Chloramphenicol had no effect on the first potential while the second potential was inhibited in the presence of chloramphenicol. The first potential was attributed to the activity of *in situ* enzymes while the second potential was taken as an indication of the maximum denitrification potential of the soil.

The denitrification potential of the Te Kowhai soil was seen to reflect seasonal trends. For eight months of the year the soil was seen to be denitrifying at or near maximum potential.

The denitrification potential of soils in the Waikato region may often be controlled by the available carbon level rather than supply of nitrate.

CHAPTER 5

EFFECT OF URINE ON DENITRIFICATION POTENTIAL

5.1.0 INTRODUCTION

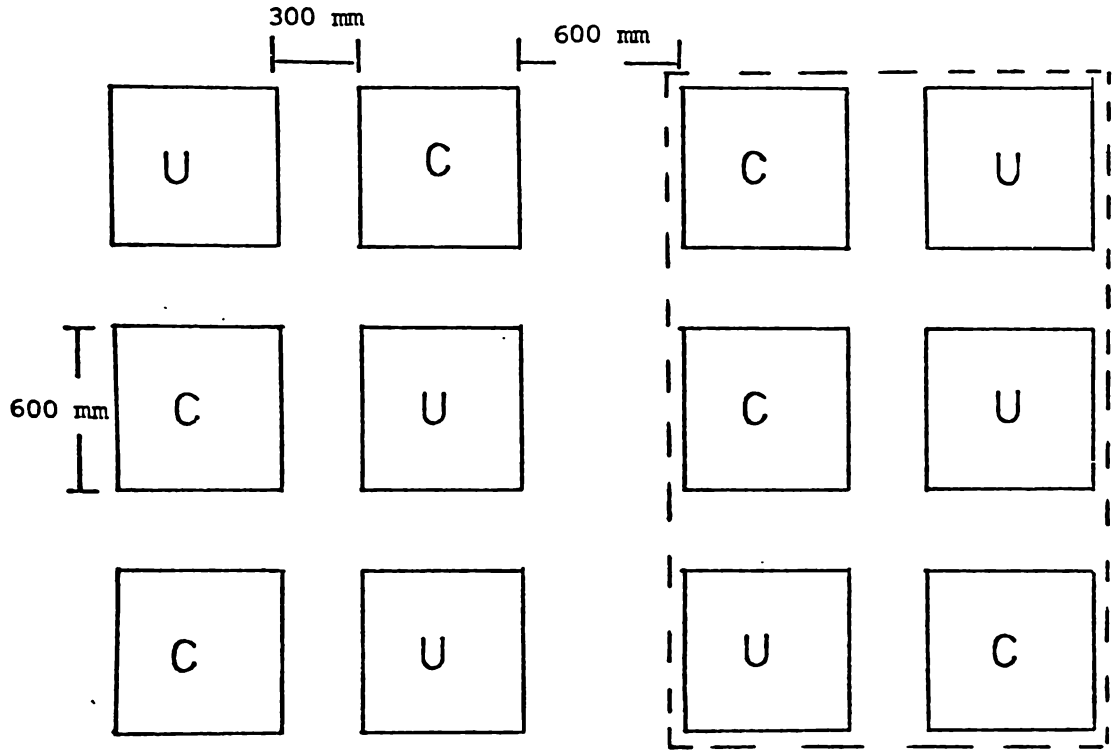
The grazing animal is an important part of the nitrogen cycle in that it ingests large quantities of nitrogen via its food supply, and excretes a large proportion as urea.

Beef cattle have been estimated to excrete up to 95% of their nitrogen intake while dairy cattle excrete about 75% (Walker *et al*, 1954). Up to 75% of this return has been estimated to be contained in the urine (Sears *et al*, 1946; Sears, 1950). If dairy cows ingest 12,000 kg dry matter /ha/yr containing an average 3.8% total nitrogen, the return of nitrogen in excreta would amount to 342 kg/ha. These urine returns will have the effect of providing highly concentrated pools of N substrate far in excess of what plants can utilize. The increases in N substrate will be accompanied by similar increases in available carbon which may be expected to affect microbial activity. The work described in this chapter was to determine if urine return to grazed pastures affected denitrification potentials.

5.2.0 EXPERIMENTAL

Twelve 60 cm x 60 cm field plots were marked out on Horotiu silt loam in two blocks of six. Plots were separated by a buffer zone of 30 cm and blocks by a buffer zone of 60 cm. The trial area had not been grazed by animals for approximately twelve months. Fresh cows' urine was applied evenly to three of the six plots in each block at a rate of 3.9ℓ/plot (2.2ℓ/0.2m^2) on May 1, 1980. One block was then covered with a raised clear PVC cover which overlapped each edge by approximately 30 cm, and sloped from 45 cm at the middle to 15 cm at the edge (fig 5.1). This was to determine whether the effect of urine on denitrification potentials differed when the urine was retained in the top soil or

Figure 5.1 Layout of urine spot trial



C = Control

U = Urine applied

= Covered plots

subjected to normal leaching. Five 0-5 cm cores were taken at weekly intervals from each plot for the duration of the experiment (approximately 7 weeks). On this basis 10% of each plot would have been removed in sampling by the completion of the experiment. The five cores of each plot were sieved, mixed and treated as an individual sample, giving triplicates of each treatment. Samples were assayed for denitrification potential by the incubation technique previously described in chapter 3. NH_4^+-N and NO_3-N were determined on 2M KCl extracts using an auto analyser (Kamphake *et al*, 1967).

5.3.0 RESULTS AND DISCUSSION

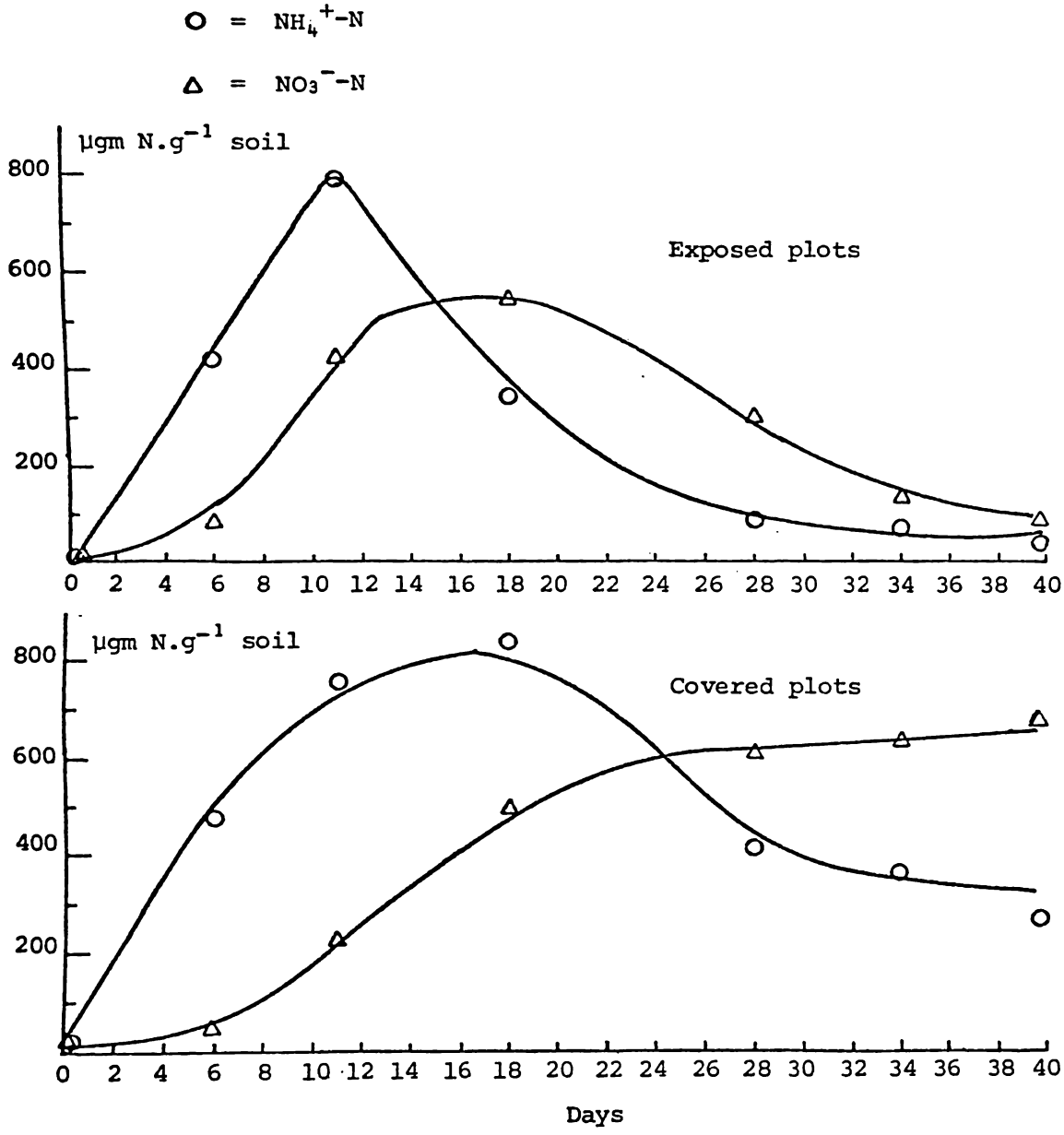
Mineral N concentrations during the trial are presented in fig 5.2. Differences between the covered and exposed plots are largely attributed to the leaching effects of rainfall. Rainfall data are presented in table 5.1.

Table 5.1 Rainfall data collected at Rukuhia field station

Weeks after commencing trial	Rainfall (mm)
1	0.0
2	4.2
3	25.6
4	9.3
5	41.1
6	26.7
7	5.5

Little rain fell during the first two weeks of the trial. Increases in

Figure 5.2 N- substrate levels in Horotiu silt loam after urine application



NH_4^+ in both blocks followed a similar pattern during this period indicating similar rates of urea hydrolyses. The NH_4^+ increases were followed by a NO_3^- increase of similar magnitude in both blocks. A time lag between the NH_4^+ and NO_3^- peaks of approximately 7 days was apparent. Rainfall during week 3 reduced NH_4^+ and NO_3^- levels in exposed plots which reached control concentrations after forty days. In the covered block NH_4^+ and NO_3^- concentrations remained high for the forty days. Changes in the denitrification potential were monitored by comparing the incubation potentials of the control and urine affected samples. Any change in the denitrification rate (or gradient of N_2O evolution) of a urine plot compared to a control plot could be interpreted as a response in denitrification activity, i.e.

$$\Delta \text{ denitrification potential} = \frac{dy}{dx} \text{ (urine plot)} - \frac{dy}{dx} \text{ (control plot)}$$

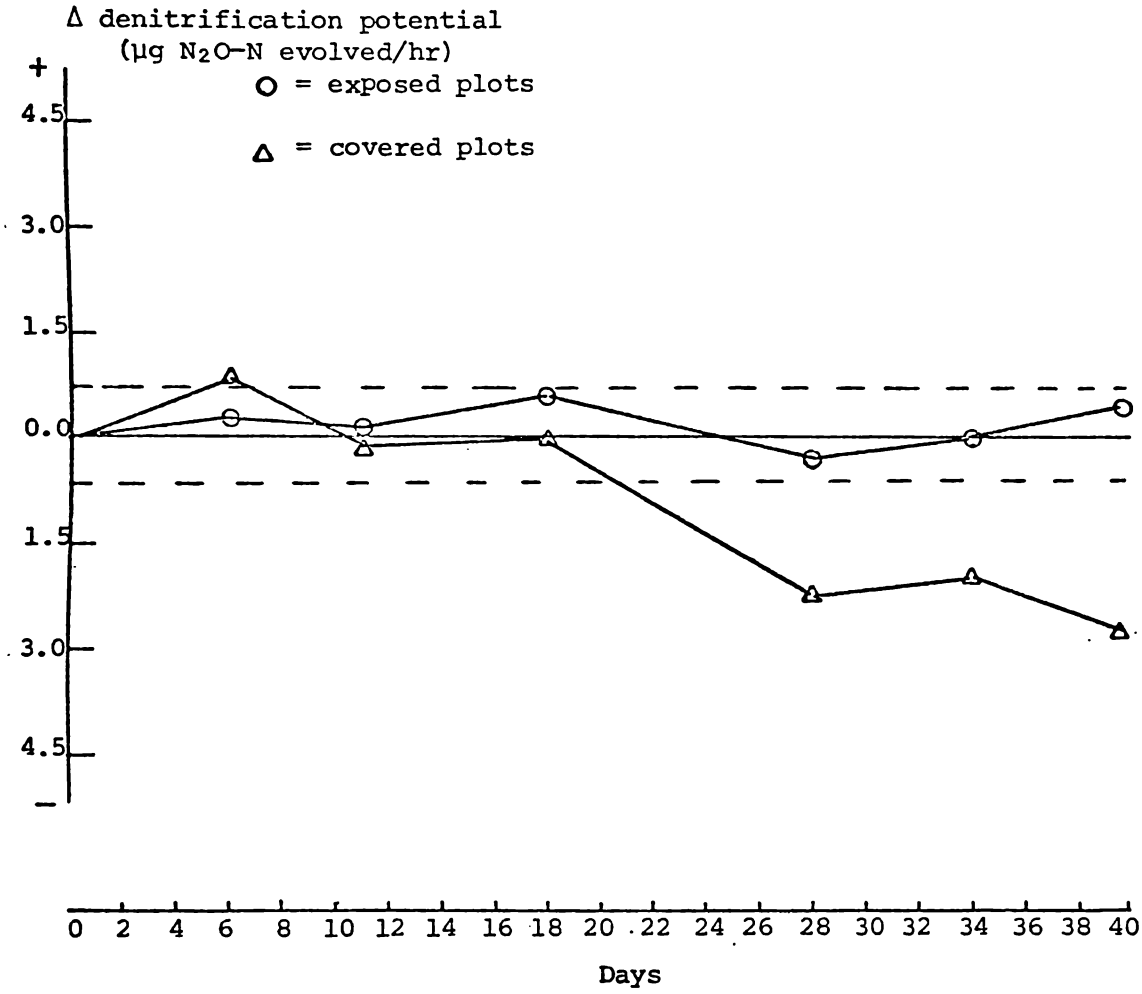
A summary of these results is presented in fig 5.3. Based on the standard deviation of analyses arrived at in section 3.16.0 ($\pm$ 0.40 µg N_2O-N/hr) a significant difference in the two gradients is represented by a change of:

$$\pm \sqrt{0.40^2 + 0.40^2} = \pm 0.56 \text{ µg } N_2O\text{-N/hr}$$

This region of uncertainty is represented by the dashed area in fig 5.3.

The results in fig 5.3 show that the denitrification potential in exposed urine plots was the same as the control plot. This can be explained by considering the enzymic activity of the Horotiu silt loam prior to the experiment. Incubations of the samples collected at the beginning of the trial displayed no phase change. Based on the evidence in section 4.5.1 this implies that the *in situ* status of this soil was in phase II, the denitrifying enzymes already being in a state

Figure 5.3 Change in denitrification potential with addition of urine



of full induction.

Bowman and Focht (1974) demonstrated Michaelis-Menten kinetics to apply during denitrification for both C and N substrates. Therefore, given the enzymic status described above, one would not expect an increased rate of denitrification with further addition of C and N substrates.

The results from the covered plot however require a different explanation. A transient positive response in denitrification potential is seen within the first week. Davis *et al* (1980) showed that soil denitrifying populations increased only slightly with amendments of 22*t*/ha/yr of animal waste. Amendments greater than 22*t*/ha/yr produced no further response in the soil denitrifying population. This slight increase in the numbers of denitrifiers is probably attributable to significantly increased pools of C and N substrate. The transient increase in denitrifying potential observed in the covered plot on day 6 may have a similar explanation.

The net effect of the urine addition seen in the covered plot appears to be a significant decrease (or inhibition) in denitrification potential approximately 21 days after application of the urine. A decrease of some 40% in denitrification potential continued for the duration of the experiment.

5.4.0 INHIBITION OF DENITRIFICATION

There are several explanations possible for the inhibition of denitrification which occurred in the covered plots.

5.4.1 Effect of pH

Denitrification rates have been shown to decrease with

decreasing pH (Bremner and Shaw, 1958; Jansson and Clark, 1952; Focht, 1978, p434). A decrease in the pH of urine plots could be expected to result from nitrification (Steele, 1977).

$$NH_4^+ + \tfrac{3}{2}\, O_2 \rightarrow NO_2^- + H_2O + 2H^+$$

$$NO_2^- + \tfrac{1}{2}\, O_2 \rightarrow NO_3^-$$

The change in the pH however was small, decreasing from 5.4 in the control plot to 5.0 in the covered urine plots.

5.4.2 Nitrite inhibition

Cooper and Smith (1963) suggested high nitrite levels may poison the denitrification system until some adaptation is made. Jansson and Clark (1952) reported that nitrite toxicity appeared to inhibit denitrification in acid substances. The only samples available for nitrite analyses at this stage of the study were those KCl extracts stored for previous NH_4^+ and NO_3^- analyses. The extracts had been stored at 4°C but were several weeks old when analysed for nitrite. Due to the instability of nitrite solutions it is unlikely these samples contained their original nitrite concentrations and an indication of nitrite at this time would imply higher levels existed previously. These results suggest the possibility of nitrite inhibition. Nitrite was determined colourimetrically by the N-(1-Napthyl)-ethylenediamine hydrochloride method as described by Hesse (1971, p201).

The KCl extract from the covered urine plot sampled at day 28 (with the maximal nitrate level of 600 µg $N.g^{-1}$) contained a nitrite concentration of 2.5 µg $N.g^{-1}$. The exposed urine plot with a corresponding nitrate maxima of 550 µg $N.g^{-1}$ at day 18 contained no detectable nitrite.

To measure the effect of nitrite on denitrification in the Horotiu silt loam fresh samples were collected. Six incubation tubes were filled with 20 g of soil and received amendments of 250 µg NO_3-N and 0, 10 and 50 µg NO_2-N of $NaNO_2$. The resultant rates of denitrification (gradients of the lines of best fit) displayed in table 5.2 indicate no nitrite inhibition.

Table 5.2 Rates of denitrification for nitrite incubation of Horotiu silt loam

Nitrite added (µg NO_2-N)	Line of best fit	µg N_2O-N evolved.hr^{-1}	r^2
0	y=0.108x-7	5.7	0.98
10	y=0.102x-5	5.5	0.97
50	y=0.103x-4	5.6	0.94

The differences in denitrification potential displayed in table 5.2 are within the 0.40 µg N/hr experimental error of incubation assays.

5.4.3 Available carbon resources

A decreased denitrification potential could occur if available carbon resources were depleted because of increased microbial activity. At day 40 the available C content was 141 and 240 µg $C.g^{-1}$.soil in the exposed and covered urine plots respectively. Both values are well in excess of the available carbon levels normally found in this soil (~40 µg $C.g^{-1}$).

The three samples described above were incubated in the usual manner with a glucose amendment of 100 µg $C.g^{-1}$.soil. The results support the suggestion that there was no carbon limitation on the

denitrification activities (fig 5.4).

5.4.4 Enzymic Inhibition

The decreased denitrification potential could result from an inhibition of nitrate reductase by either a urine decomposition product, or the accumulation of microbial excreta. Without leaching effects, material harmful to enzymes could increase to abnormally high levels and affect the function of specific enzymes. Premi and Cornfield (1969) noticed an inhibitory effect on nitrification activities with the addition of up to 102 lb/acre of sewage sludge to the soil. They discounted the possibility of enzymic toxicity due to large quantities of incorporate trace elements, and suggested that the inhibition was due to toxic organic materials.

To investigate the possibility of an inhibitory compound, equal weights (10 g) of soil collected from covered and exposed urine plots were mixed prior to incubation. The denitrification rates of these samples were established previously at 3.4 and 6.9 μg N_2O-N/hr respectively. If the reduced denitrification potential was not enzymic, one would expect an average denitrification rate of the mixed samples, i.e. approximately 5.1 μg N_2O-N/hr. Conversely, if the reduction in activity was a toxicity or enzymic inhibition, one could expect a rate similar to that of the covered urine plot, i.e. approximately 3.4 μg N_2O-N/hr. The resultant rate of the incubation was 3.8 μg N_2O-N/hr (fig 5.5). A glucose amendment of 100 μg $C.g^{-1}$ did not increase N_2O production. These results suggest the presence of an inhibitor of either nitrate or nitrite reductase.

5.5.0 SUBSTRATE EFFECT ON DEPTH OF DENITRIFICATION

It is conceivable that the high levels of C and N substrate

Figure 5.4 Denitrification potential of Horotiu silt loam (sampled 40 days after urine addition) with glucose amendment (100 µg $C.g^{-1}$)

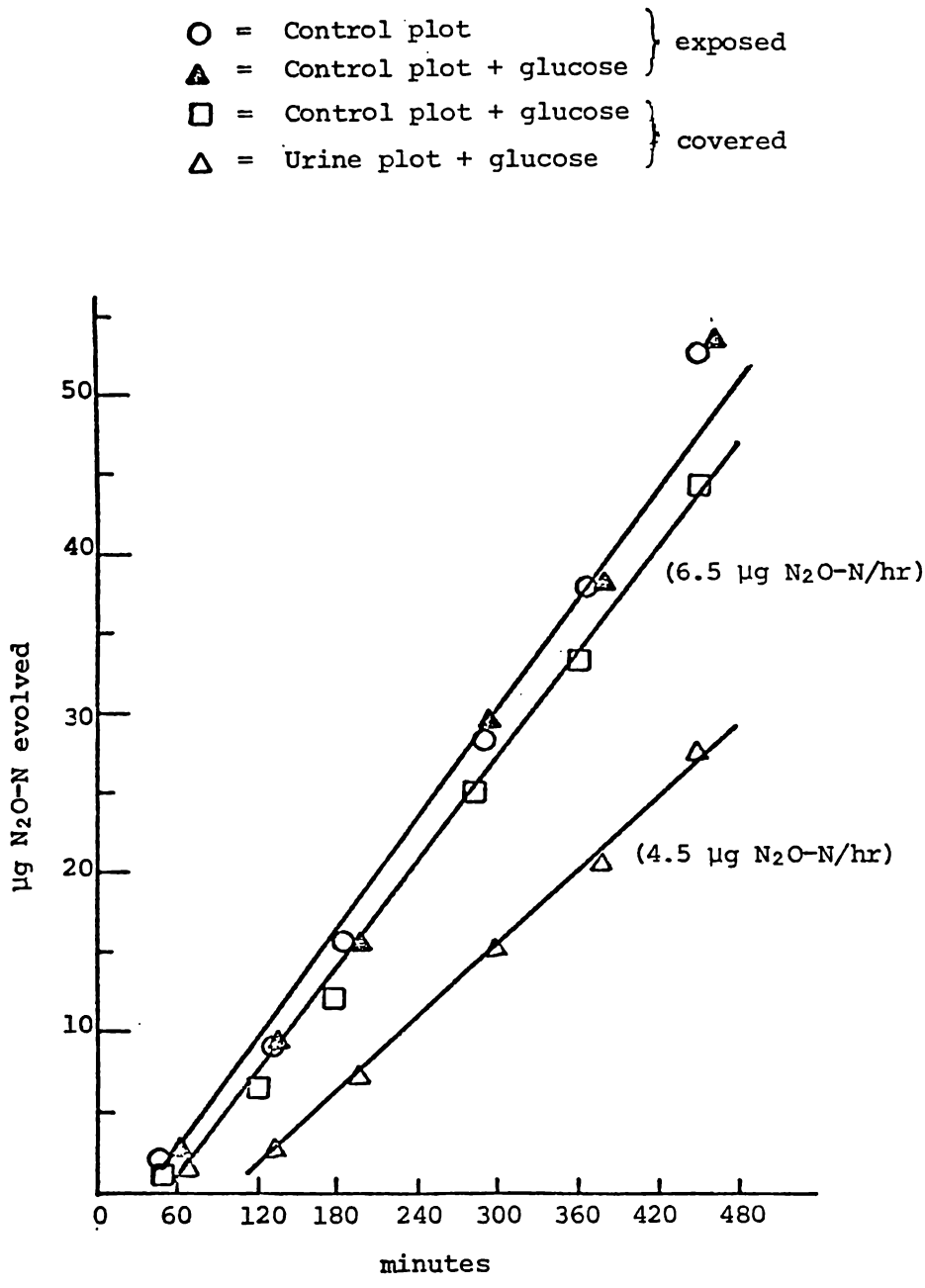
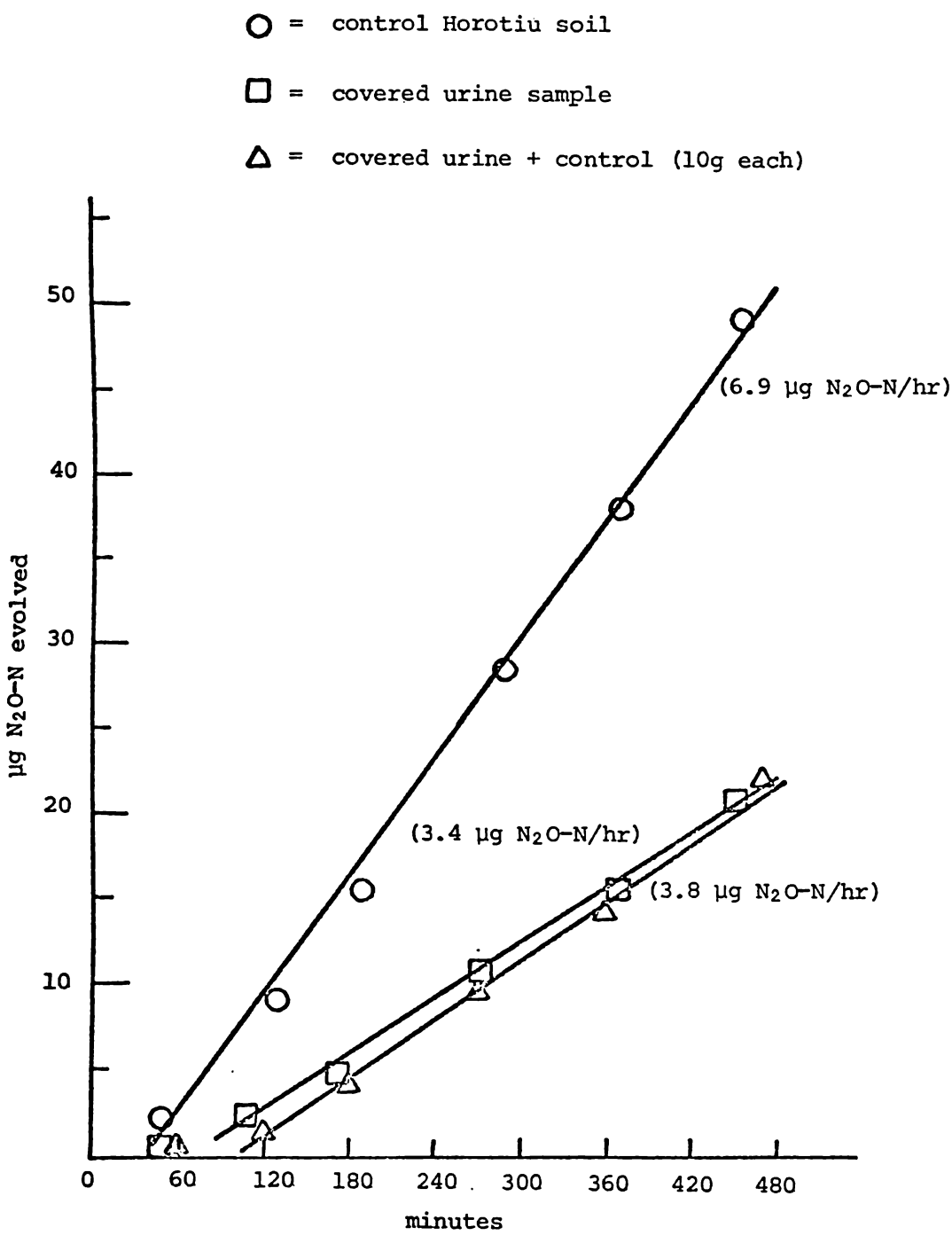


Figure 5.5 Denitrification potential of combined urine and control sample



produced in the top few centimetres of the soil profile during the urine field trials could cause an increase in substrate levels further down the profile. In view of the earlier results and discussion in section 4.2.1 which suggest denitrification occurs predominately in the 0-3 cm soil layer, substrate levels and associated denitrification activities in the urine experiment were studied at two soil depths. On day 50 the PVC screen was removed from the covered plot and ten days elapsed to allow leaching of C and N substrates into the profile. On day 60 core samples were taken at 0-3 and 6-9 cm depths in a urine plot and 0-5 cm at a control site. C and NO_3-N substrate levels increased significantly in the 6-9 cm layer (table 5.3).

Table 5.3 Available Carbon and Nitrate in Horotiu urine plot after 60 days

Depth	Available Carbon ($\mu g\ C.g^{-1}$)	Nitrate ($\mu g\ N.g^{-1}$)
0-3 cm	166	500
6-9 cm	53	464
0-5 cm (control)	48	5.8

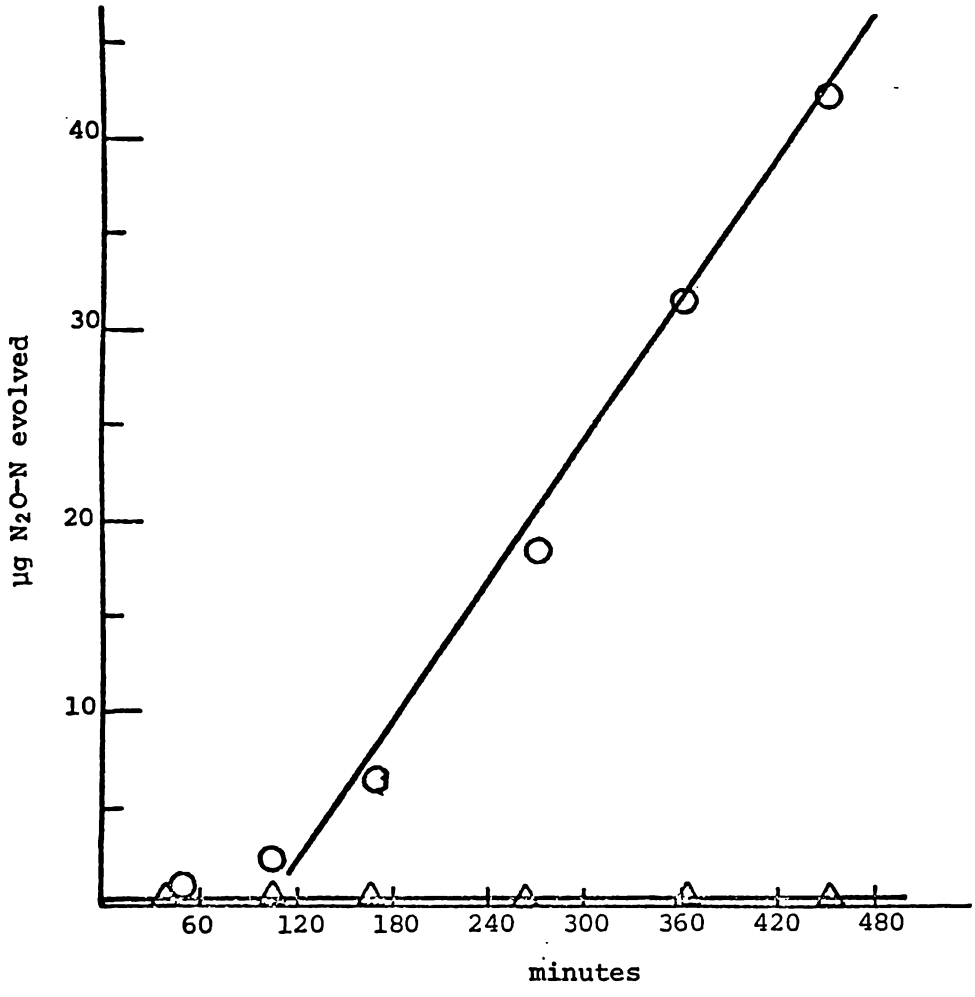
Previous available carbon measurements at a depth of 6-9 cm in the Horotiu soil yielded 5.5 $\mu g\ C.g^{-1}$ (see table 4.2). In view of the significant increase in C and N substrates, increased denitrification activities at depth could be expected.

Samples of the 0-5 cm control and 6-9 cm urine plot were incubated in the standard manner. The resultant rates of denitrification (see fig 5.6) indicate the preference of the denitrifying bacteria for

Figure 5.6 Denitrification potential of Horotiu silt loam with increased substrate at depth

○ = Horotiu silt loam (0-5 cm)

△ = Horotiu silt loam (6-9 cm)



the rhizosphere, even with abundant substrate at depth. Myers and McGarity (1972) measured denitrification activities in an undisturbed core of a B horizon of 30-45 cm depth. By adding glucose considerable denitrification activity was stimulated and it was hypothesised that these subsurface horizons could well sustain denitrification if sufficient carbon was leached through the soil profile. However, the actual measured rate of denitrification of this soil core was almost insignificant (0.2 µg N_2O-N/hr). The results of Myers and McGarity lead them to suggest that the presence of readily decomposable organic substrates greatly stimulates denitrification activities in B horizons of low activity. The results reported in this experiment do not support this view.

5.6.0 GENERAL DISCUSSION AND CONCLUSIONS

The nutrient effect of urine addition is seen to provide C and N substrate in concentrations far in excess of plant requirements. Leaching effects permitting, this nutrient effect lasts for approximately forty days.

Incubation studies showed the denitrifying population of this soil to be in a state of full enzyme induction at the time of the urine addition. Consequently no increase in the denitrification potential of the Horotiu silt loam was recorded during this period.

In the absence of rainfall and leaching, a decrease in the denitrification potential was recorded upon addition of urine. Results suggest that under these conditions an inhibitor of either nitrate or nitrite reductase is produced. Results also suggest that once nutrients have leached past the rhizosphere they remain relatively intact from further dissimilation.

Increased C and N nutrients at depth do not stimulate further denitrification activities.

CHAPTER 6

DEVELOPMENT OF THE LYSIMETER

6.1.0 INTRODUCTION

Prior to the commencement of this study the only direct measurement of denitrification in the field was restricted to assessment of N_2O evolution. These measurements were made using either infra-red (e.g. Arnold, 1954; Schwartzbeck *et al*, 1961) or gas chromatographic (e.g. Dowdell and Smith, 1974; Burford, 1976) techniques. Direct measurement of N_2 was technically impossible. Being produced in relatively minute quantities compared to the ambient soil atmosphere, assessment of N_2 production was impossible using conventional techniques.

The object of this study was to develop a technique using N-15 enriched gas to measure small changes in the N_2 concentration with an isotope mass spectrometer (see chapter 8). During the preliminary work of this investigation the acetylene inhibition of N_2O-reductase in soils was reported in the literature (Klemedtsson *et al*, 1977; Yoshinari *et al*, 1977). Because of the potential this discovery offered for measuring denitrification potentials *in situ*, an attempt was made to utilize this phenomenon in the field.

The first requirement was to develop a lysimeter capable of flushing the soil atmosphere as both the N-15 and C_2H_2 inhibition methods require the establishment of a particular gaseous mixture within a soil core and blockage of the inward diffusive flow of soil gases.

A base-flow system was designed to prevent the inward diffusive flux of soil gases by providing an artificial barrier of gas to the base of the lysimeter. To facilitate the introduction of the base-flow the lysimeter walls were double skinned. Near the base of the lysimeter, the inner double skin was perforated with a series of holes permitting the exit of the base-flow gas.

Sampling of the gases evolving from the soil surface was

intended to be via a probe installed through the top of the lysimeter.

6.2.0 CRITERIA FOR THE LYSIMETER

A lysimeter must be designed to create minimal disturbance when inserted into the soil. In particular two types of disturbances are of interest: i) changes in water content and void ratio (pore spaces, and ii) disturbance of the soil structure.

The degree of disturbance during insertion of the lysimeter is indicated by the extent of volume change in the soil core. Volume changes are commonly referred to as recovery ratios. A sample suffering from no expansion or compression would have 100% recovery.

Lysimeters most likely to cause the least disturbance fall within the category known as 'thin-walled samplers'. Thin-walled samplers have what is known as a minimum area ratio.

The annular area $Aw = \left[\frac{\pi}{4}(D^2w - D^2e)\right]$ (see fig 6.1) represents the amount of soil which is displaced when the sampler is forced into the ground.

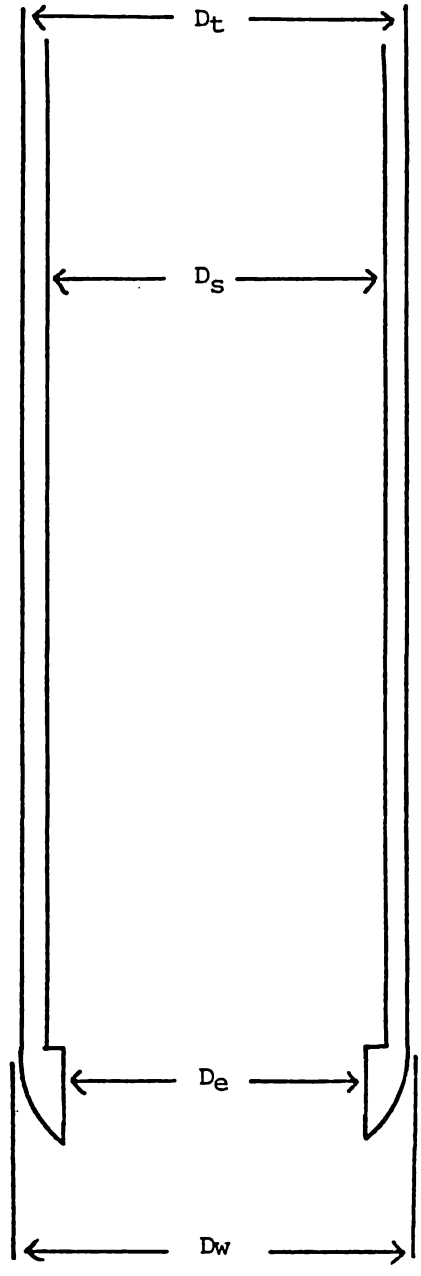
The ratio of Aw to the area $Ae = \left[\frac{\pi}{4} D^2e\right]$ enclosed by the cutting edge is known as the kerf or area ratio. The area ratio $Ca = \left[\frac{D^2w - D^2e}{D^2e}\right]$ is approximately equal to the ratio between the volume of displaced soil and the volume of sample.

Thin-walled samplers are defined as having a wall thickness less than 2.5% of the diameter corresponding to an area ratio of approximately 10% when the inside clearance of the cutting edge is not taken into consideration (Hvorslev, 1962).

The distortions of soil layers are very small for samplers with an area ratio between 10-15%.

Wall friction is one of the principal causes of sample disturbance. The outside diameter Dw is often made slightly larger

Figure 6.1 Parameters of a thin-walled sampler



than the outside diameter of the sampling tube Dt. This reduces outside wall friction. An outside clearance ratio $C_o = \frac{Dw - Dt}{Dt}$ of only a few percent may decrease the penetration resistance of the sampler. This feature was not included in the lysimeter design as its effectiveness varies greatly with the soil type.

The inside wall friction is particularly important as samples expand laterally once inside the corer. The penetration resistance offered by the sampler compresses the soil strata ahead of the sampler and deforms the strata so that an increased volume of soil is forced inside the tube. Once relieved of the vertical compression the cores expand and the frictional resistance offered by the inside wall plays an important role in recovering undisturbed samples. It also limits the depth to which samples may be cored.

The inside wall friction can be reduced by making the diameter of the cutting edge, De, slightly smaller than the inside diameter of the sampling tube Ds. The inside clearance ratio is expressed as $Ci = \left[= \frac{Ds - De}{De} \right]$. An inside clearance ratio of 1% is recommended, but varies slightly with the soil type.

The cutting edge on the tip of the lysimeter also influences recovery yield. Under a blunt tip an annular wedge of soil will form. This acts as a cutting wedge (with an effective diameter De = ½(Ds-Dt) and the theoretical inside clearance is therefore negative. Cutting tips with angles of ~15° have been shown effective in producing 100% recovery. Close to the cutting edge however, it is advisable to increase the angle to 20° or 30° to avoid an easily damaged feather edge.

6.3.0 CONSTRUCTION OF THE LYSIMETER

Stainless steel was chosen as the construction material because of its durability and the strength obtainable at the cutting

tip.

The larger the diameter of the lysimeter, the easier it is to build a double-walled system within the thin-walled sampler specifications. Too large a diameter could be expected to cause difficulties in the efficiency of the base-flow effect.

The largest diameter of thin-walled stainless tube readily available was 7.5 cm. The next closest tube size was 6.25 cm, thus forming an annulus 0.75 cm thick. The lysimeter constructed from these materials had an area ratio of 44% and gave recovery yields in the region of 150%.

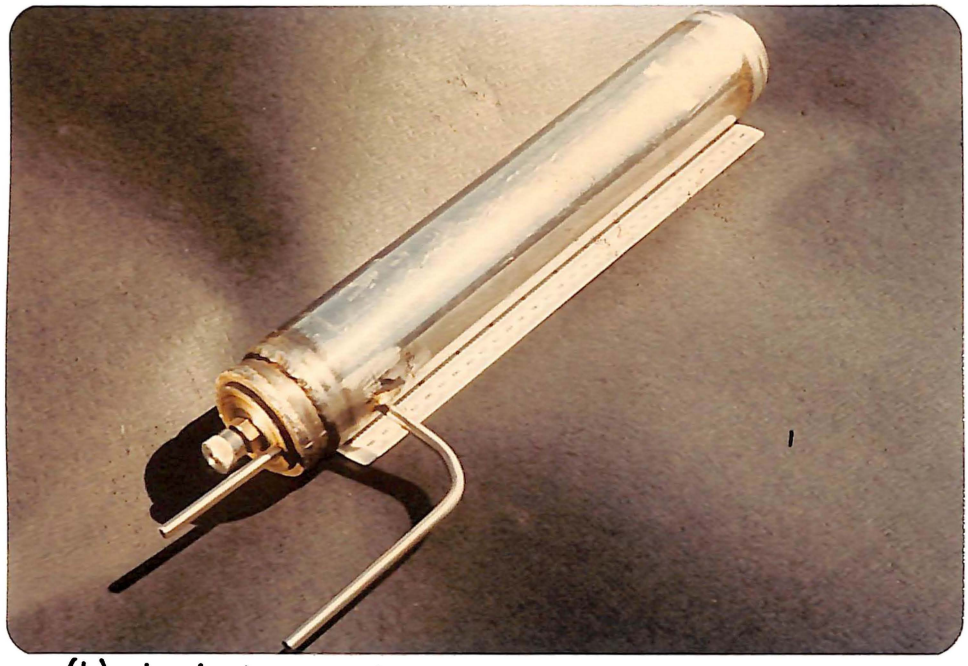
A second lysimeter was built using only 6.25 cm tube (see plate 6.1). The inside wall was fabricated from 6.25 cm tube with a small longitudinal section removed. The tube was then closed up and welded along the seam. It could be inserted as a press fit into the 6.25cm outer tube. To permit the gas flow between the walls to the base-flow holes, a scarf was cut around the outside circumference at the top of the inner tube directly opposite the gas entry port (see fig 6.2). A series of smaller grooves ran vertically down from this scarf to a similar scarf cut near the base of the tube. This inside skin had a series of 0.8 mm diameter holes drilled around the scarf 1 cm apart, 5.0 cm from the bottom edge of the lysimeter. When pressed into place, the base of the two tubes were welded together and then machined to produce a 20° taper at the tip. The tip edge was then rolled 0.63 mm inside to produce the necessary inside clearance.

The top of the lysimeter was sealed with a stainless steel plate machined to fit and welded in place. This plate had a machined lip around the circumference to take a steel cap upon which pressure was directed during insertion into the soil. A 6 mm cajon fitting was screwed into the centre of the plate for sampling attachments.

The overall length of the lysimeter was 35 cm.

Plate 6.1

(a) Lysimeter



(b) Lysimeter - end view showing base-flow holes

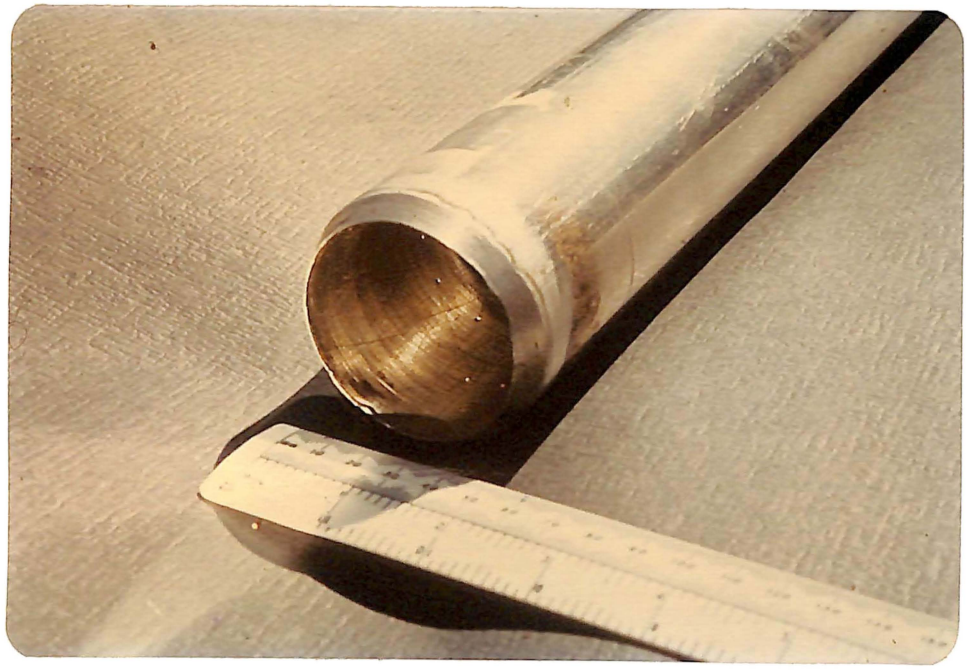
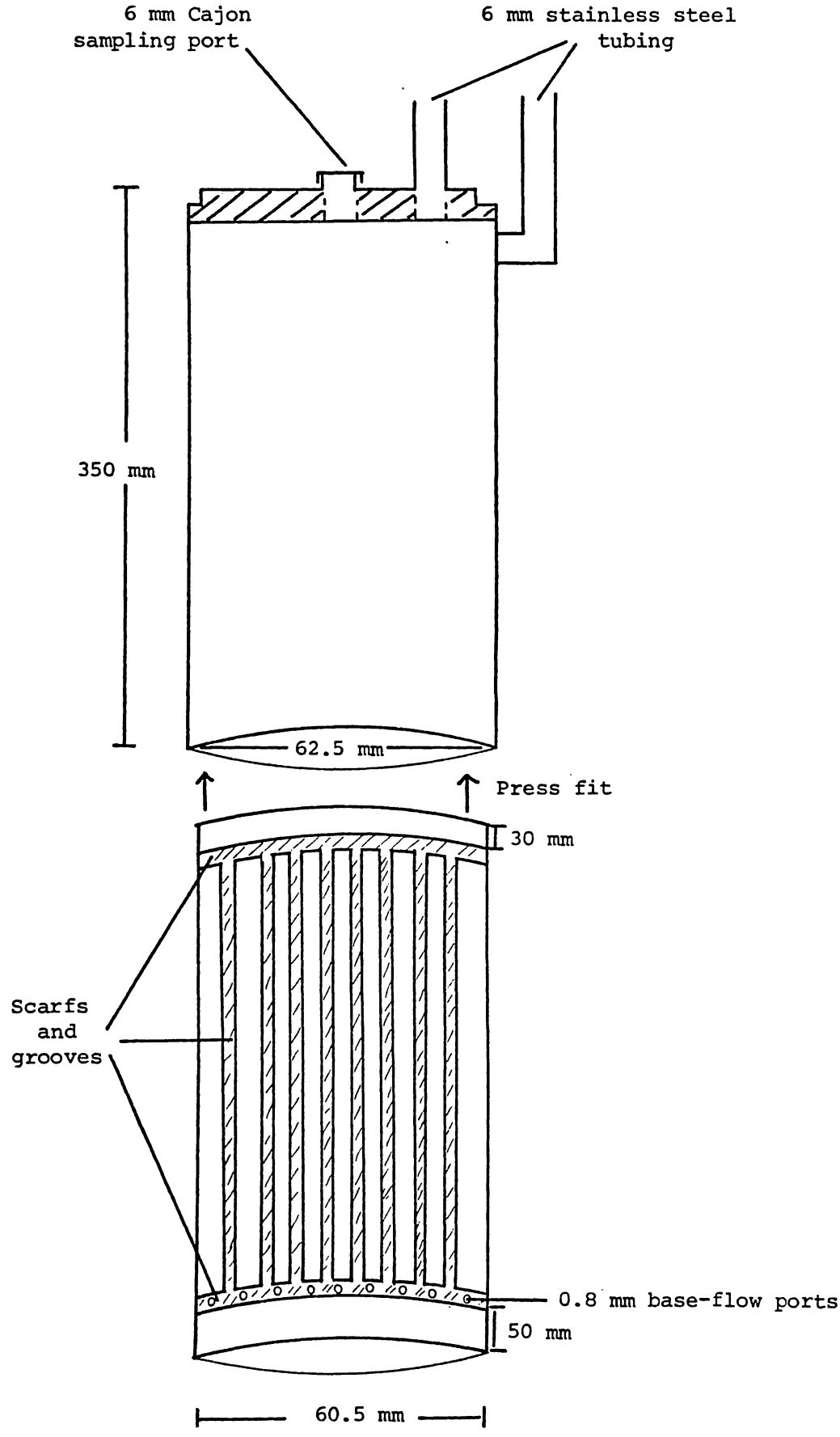


Figure 6.2 Lysimeter design



While the area ratio of this lysimeter (23%) still fell outside the area ratio specifications of 10-15%, it was found to perform satisfactorily on samples up to 35 cm in length providing the method of insertion was carefully controlled.

6.4.0 METHOD OF INSERTION

The initial method of insertion consisted of two augers, a bracing bar and hydraulic jack. The augers were inserted deep into the soil and the lysimeter gently tapped about 10 cm into the soil between the two augers. The hydraulic jack was placed on the cap fitting over the top of the lysimeter. The bracing bar rested on the jack and fitted between the augers (see fig 6.3). The lysimeter was then jacked manually into the soil against the bracing bar.

As the lysimeter penetrated further, the augers were inserted deeper. Insertion of the augers caused a considerable amount of difficulty and their performance varied widely depending on the soil type. In dry soils they were extremely difficult to insert, and in moist soils tended to rise as the penetration of the lysimeter increased.

This system was exchanged for one where the lysimeter is jacked into the soil against the weight of a vehicle. For this method, a platform was built (see fig 6.4) which allowed a wheel of the vehicle to rest upon a base plate while the lysimeter was jacked into the soil. This method was found to be satisfactory in all applications as long as vehicle access to the sampling site was possible.

The speed and continuity with which a lysimeter is forced into the soil has been shown to affect the recovery ratio (Hvorslev, 1962). Hammering will generally cause partial to serious lateral disturbance of a sample. While hammering practically eliminates the

Figure 6.3 Auger method of lysimeter insertion

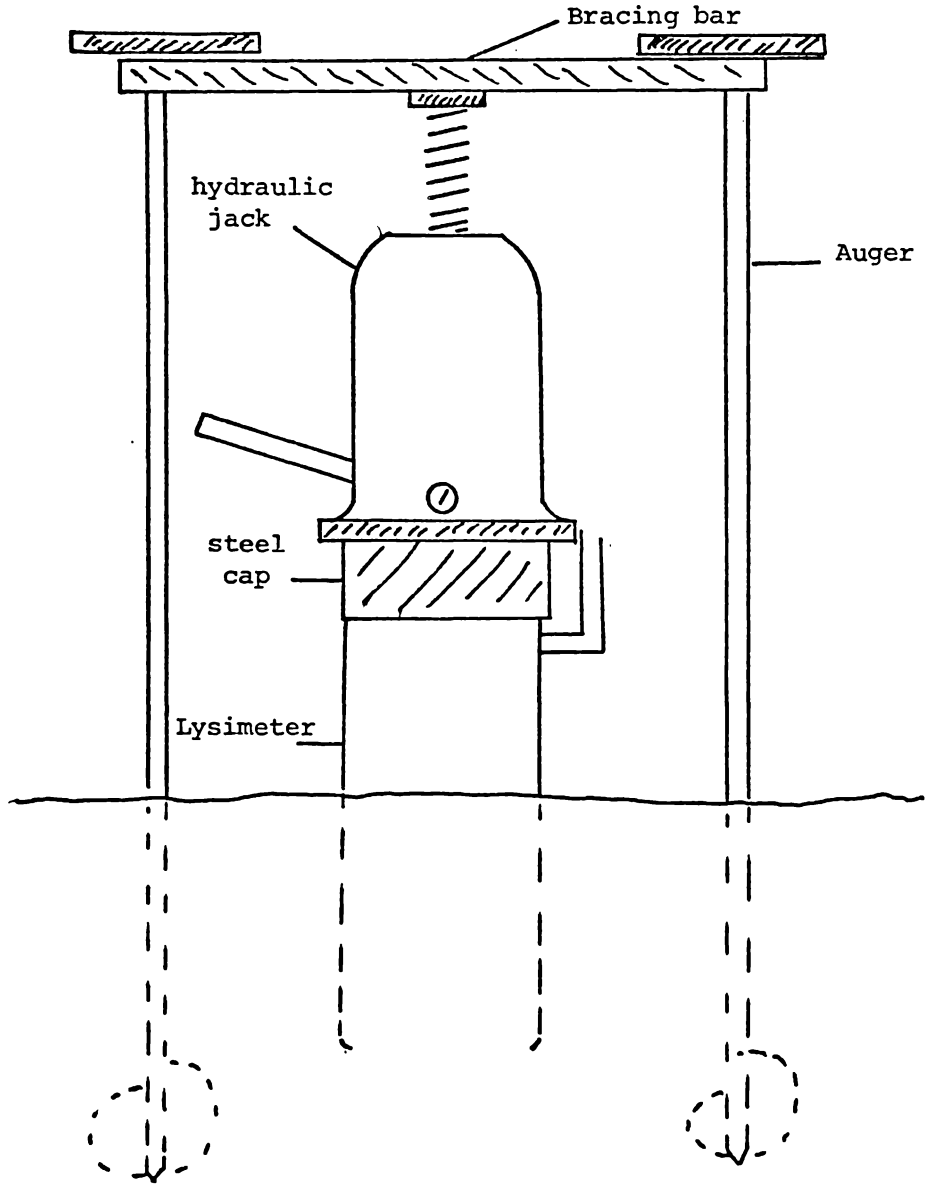
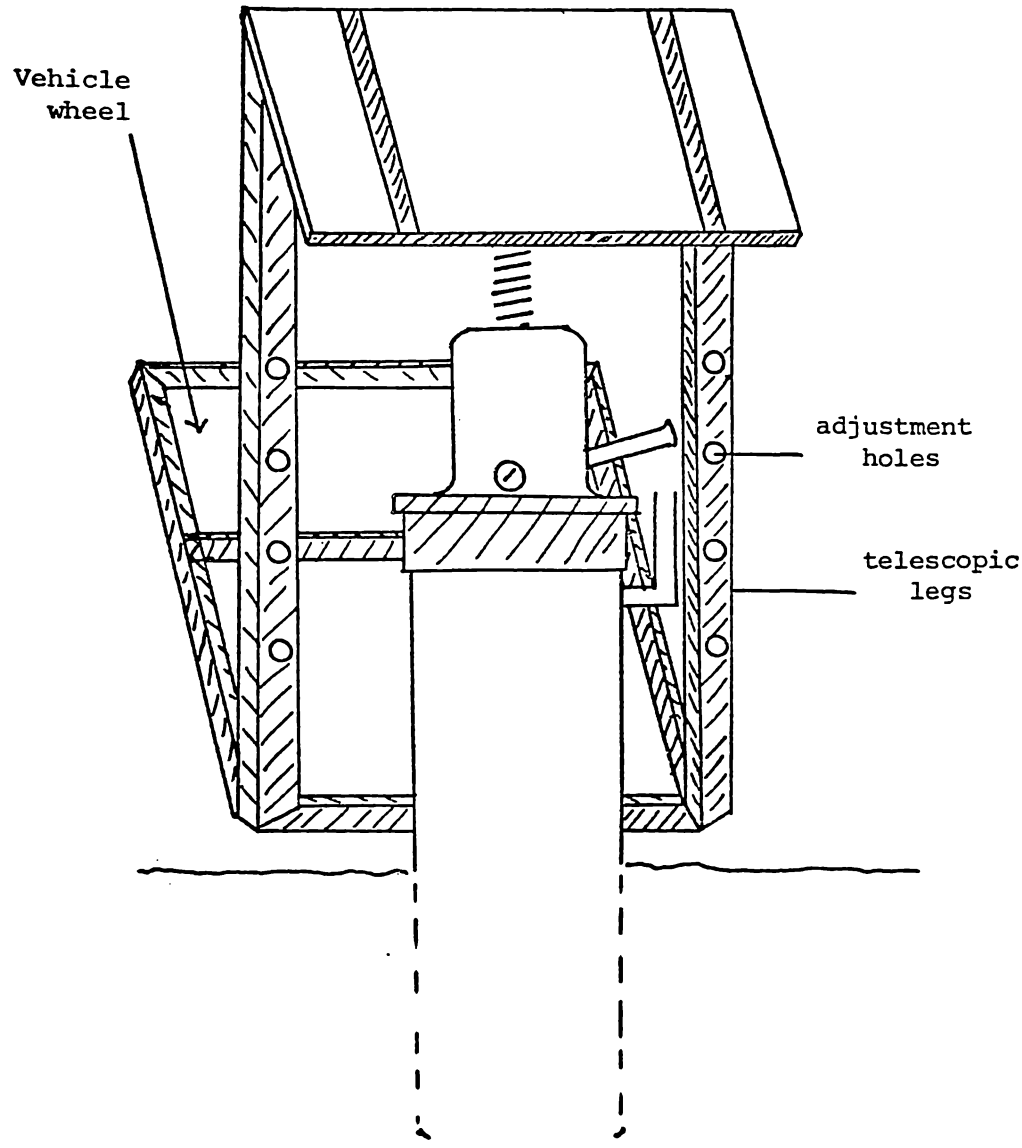


Figure 6.4 Lysimeter insertion using adjustable platform



entrance of excess soil during the first part of the drive even when the area ratio of the sampler is large, it also reduces the effectiveness of the inside clearance and tends to produce short and distorted samples of soils.

Slow jacking results in plastic deformations and volume changes. It promotes the entrance of excess soil, wall friction, and adhesion with the consequent increase of penetration resistance and distortion of soil layers. Satisfactory samples may be obtained with slow jacking provided thin-wall samplers are used.

Fast pushing, or an uninterrupted advance at 0.5 to 1.0 ft/sec produces longer and less disturbed samples than either hammering or slow jacking. Fast pushing is recommended for general use in obtaining undisturbed samples.

A single heavy blow may produce longer and even less disturbed samples than fast pushing. This method is usually employed using explosive charges.

The last two methods of lysimeter insertion were impractical for the project envisaged.

Hammering of the lysimeter was found to produce greater sample distortion than slow jacking. Slow jacking was found to be satisfactory under most conditions and only produced slight compression (approximately 110% recovery over 35 cm) under the most difficult conditions.

In order to facilitate good recovery ratios with the slow jacking method it was important to keep the lysimeter exactly vertical throughout the insertion. The lysimeter had a tendency to lean towards the vehicle under pressure and this was compensated for by placing a series of wooden blocks between the lysimeter, the jacking platform, and the vehicle's wheel. Such a system kept the entire mechanism upright throughout the insertion.

6.5.0 CORE FLUSHING EFFICIENCY

A preliminary experiment was conducted to test the efficiency of core flushing in reducing the nitrogen levels of the soil atmosphere. A tube of 7.5 cm diameter and 35 cm length (1 mm wall thickness) was driven 30 cm into the soil and retrieved with the core intact. The top of the tube was plugged with a large rubber stopper through which a 6 mm glass tube had been inserted in the centre. The bottom 5 cm of soil was then removed, and a similar plug and 6 mm tube inserted (fig 6.5). Each end of soil was covered by a layer of glass wool.

Argon was flushed through the core at 0.14 atm and 100 ml/min. The effluent gas was directed by a tube into a beaker of water to prevent back diffusion of air. During the flushing the effluent gas was periodically connected to a gas burette for oxygen determination (fig 6.6) using a standard alkaline pyrogallol technique as described by Vogel (1961, p1079).

6.5.1 Results and Discussion

Oxygen concentrations of the effluent argon gas were determined periodically and the results recorded in table 6.1

Table 6.1 Oxygen level in soil core during flushing with argon

Time (mins)	Oxygen concentration (v/v)
T_0	19.69% ± 0.12%
T_{10}	16.82
T_{20}	2.57
T_{30}	0.22

Figure 6.5 Preliminary core-flushing apparatus

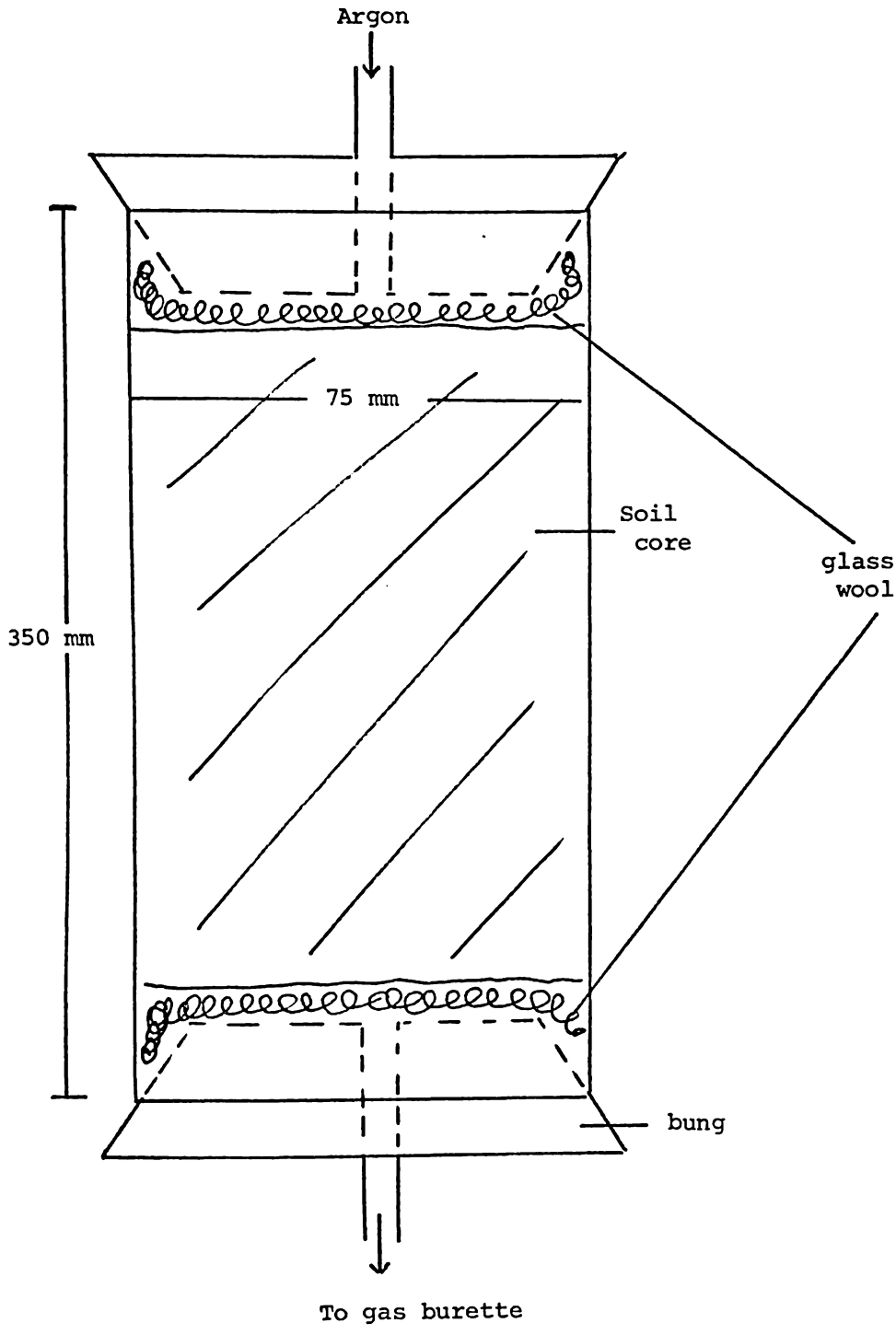
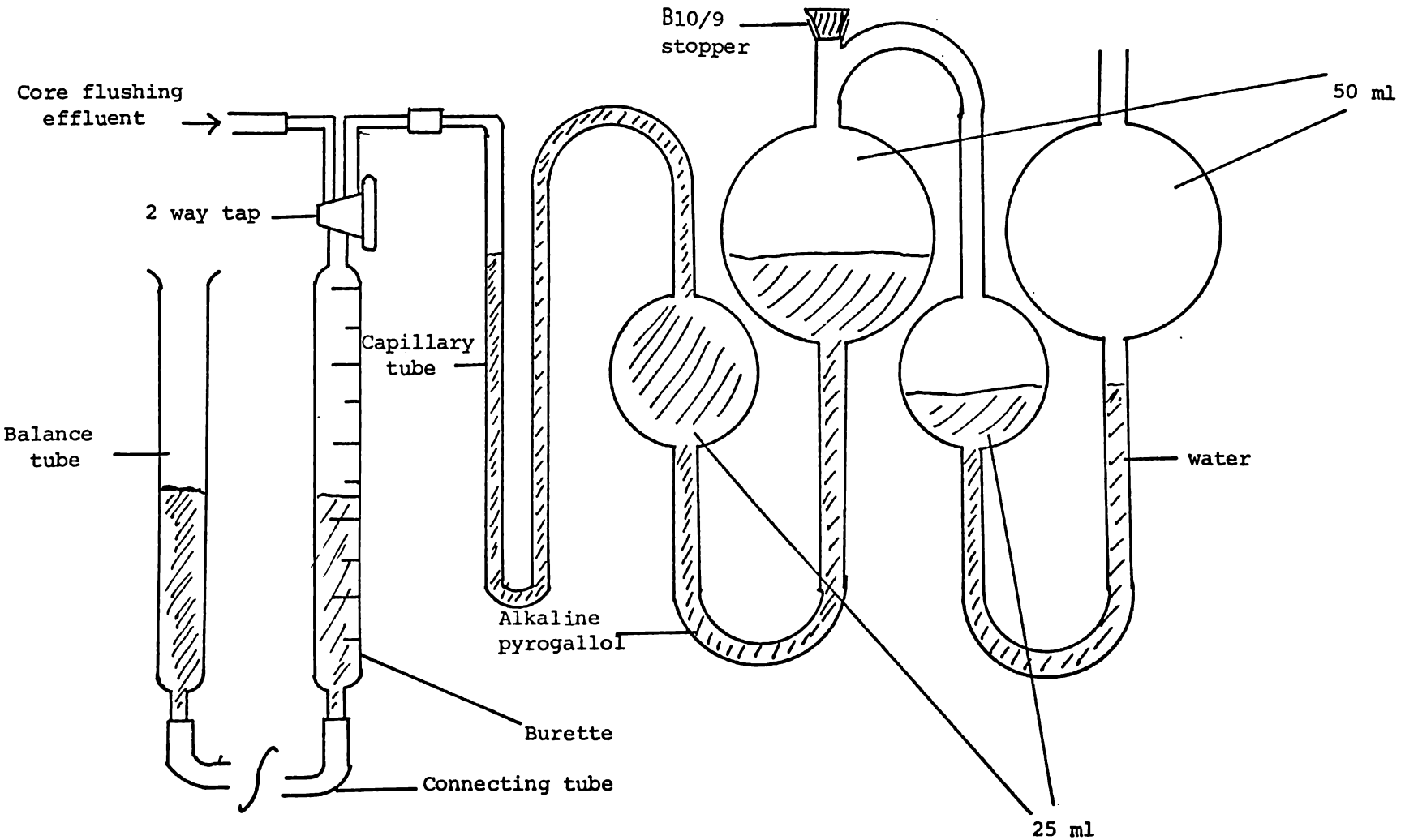


Figure 6.6 Gas burette apparatus for oxygen determinations



The results after 30 minutes of flushing represent a nominal 99% removal of oxygen from the soil. The same calculation applied to nitrogen would indicate a level of 0.8% or 8,000 ppm N_2 (v/v).

Subsequent flushing experiments using the Micromass mass spectrometer to measure N_2 in the soil cores *in situ* (see section 8.9) confirmed the effectiveness of this technique in establishing low N_2 backgrounds.

6.5.2 Flushing pressure

Flushing the soil core at excessive pressure could drive out the capillary and pore space water, creating an artificially high degree of aeration where a previously anaerobic situation may have existed. To minimise the disturbance to the soil core the flushing gas pressure must be kept to a minimum.

Macro-porosity is defined as those pores which can drain their water content under 50 cm water suction. This is equivalent to 0.05 atm pressure. Field capacity is defined as the water holding capacity under a tension of 0.2 atm (Soils of New Zealand, Part 3, 1968). Therefore, if the flushing pressure is kept below 0.05 atm water will only be displaced from the macro-pores which occupy a nominal 5-10% of the total pore space volume in New Zealand soils. Artificially drying a small percentage of the macro-pores on very wet soils will therefore represent a minimum disturbance to the soil core by the flushing gas. On any drier soils with a water tension greater than 0.05 atm, flushing at pressures less than 0.05 atm will have little effect on the moisture distribution within the soil pores.

Flushing pressures were maintained between 2 and 3 cm H_2O (0.002 - 0.003 atm). Such a pressure variation in the soil core is equivalent to atmospheric variations of the order of 3 mb - well within the order of everyday atmospheric variations. The pressure was

monitored by a U tube water manometer installed between the gas line and lysimeter (fig 7.4).

6.6.0 BASE-FLOW EFFICIENCY

A critical factor in the success of obtaining gas samples free of contamination is the effectiveness of the base-flow. The base-flow produces a downward flux of nitrogen-free gas from the base of the lysimeter to sweep away any N_2 or N_2O that may be diffusing into the lysimeter.

The effectiveness or success of the base-flow depends on the downward velocity (flow rate) of the carrier gas, and the rate of inward diffusion of the N_2 (or N_2O). For the base-flow to be successful the downward flow velocity must exceed the inward flow velocity (diffusion) of the nitrogen.

6.6.1 Calculated requirements for the base-flow

Assume a base-flow of 100 ml/min.

The inside area of the lysimeter is 28.7 cm^2. Therefore the average downward velocity of the carrier gas within the mouth of the lysimeter is 3.48 cm/min or 0.058 cm/sec. The inward diffusion velocity of N_2 can be estimated from the effective diffusion coefficient (Dp) of C_2H_2 in the soil. Ryden *et al* (1979) estimated the Dp value for C_2H_2 using the Millington and Quirk (1961) equation for gaseous diffusion under unsaturated conditions:

$$Dp = DoS\alpha^{2\cdot33}/(k + 1)S_T^2 \qquad 6.1$$

where Do = diffusion coefficient for C_2H_2 into air, and k = solubility coefficient for C_2H_2. The Do value for C_2H_2 (0.183 $cm^2.sec^{-1}$) was estimated from the diffusion coefficient of HCN (Jost, 1960).
S_T represents the total porosity of the soil while $S\alpha$ represents the

air filled porosities.

The rates of diffusion of gases vary inversely according to their molecular weights, i.e.

$$\frac{\sqrt{28}}{\sqrt{26}} = \frac{D_{C_2H_2}}{D_{N_2}}$$

Using a calculated value of 0.013 cm^3/cm^3 for the solubility of N_2 at 15°C (Ramm, 1968), and a molecular weight correction, values of Dp can be calculated for various porosities of soils.

For example:

$S_T = 0.50$
$S_\alpha = 0.05$ $\quad Dp = 6.4 \times 10^{-4}$ cm^2/sec

$S_T = 0.50$
$S_\alpha = 0.20$ $\quad Dp = 1.62 \times 10^{-2}$ cm^2/sec

$S_T = 0.40$
$S_\alpha = 0.20$ $\quad Dp = 2.53 \times 10^{-2}$ cm^2/sec

For a given total porosity the effective diffusion coefficient increases as the air filled porosities increase. For a given air filled porosity, the effective diffusion coefficient still increases as the total porosity decreases.

Taking the largest diffusion coefficient calculated above, find the inward flow velocity of the gas N_2 into the lysimeter.

$$\text{Flux } J = -D\frac{dc}{dx} \qquad 6.2$$

Assume the most pessimistic case where the base flow is only working within the confines of the lysimeter and is totally ineffective beyond the base. In this instance the concentration of N_2 at the mouth of the lysimeter will be that of the soil air, i.e. 800,000 ppm N_2 v/v or 3.26×10^{-5} $moles.cm^{-3}$. Assuming the base-flow is totally effective

at the point of the carrier gas entry, the concentration of N_2 at this point is zero. The concentration gradient $\frac{dc}{dx}$ is then defined as $\frac{3.26\times10^{-5} \text{ moles.cm}^{-3}}{5.0 \text{ cm}}$

$$J = \frac{-2.53\times10^{-2} \text{ cm}^2.\text{sec}^{-1} \times 3.26\times10^{-5} \text{ moles.cm}^{-3}}{5.0 \text{ cm}}$$

$$= 1.65\times10^{-7} \text{ moles cm}^{-2}.\text{sec}^{-1}$$

The effective inward flow velocity of the N_2 (v) is found by

$$\frac{J}{C} = v = \frac{1.65\times10^{-7} \text{ moles cm}^{-2}.\text{sec}^{-1}}{3.26\times10^{-5} \text{ moles.cm}^{-3}} = 0.506\times10^{-2} \text{ cm.sec}^{-1}$$

It was seen previously for a carrier gas flow of 100 ml/min, the downward flow velocity equalled 0.058 cm.sec^{-1}. The base-flow velocity in the most pessimistic case at 100 ml/min is approximately 10 times greater than that required.

It can also be noted from the equation

$$J = \frac{-dc}{dx} D$$

that the most important factor determining the effectiveness of the base-flow against the inward diffusion of N_2 is not the porosity of the soil, but the height at which the base-flow holes are placed within the lysimeter, i.e. the concentration gradient generated in the most pessimistic case.

The area of the lysimeter also affects the downward velocity of the carrier gas. By decreasing the surface area of the lysimeter, the effectiveness of the base-flow increases for a given flow rate because the downward flow velocity increases.

There are certain other assumptions inherent in these calculations. The first is that the velocity gradient of the carrier

gas is uniform across the diameter of the lysimeter. In fact this is unlikely to be the case. The velocity is likely to be the greatest nearest the exit holes, and least in the centre of the core. However, as we move down the core towards the mouth of the lysimeter, because of the pressure gradient generated by the unequal velocities, this velocity gradient would become more uniform.

The other assumption not considered in the calculations is that of turbulent mixing of gases. It is assumed that the gas flow between the source and lysimeter base is laminar in order to calculate the linear N_2 concentration gradient. If turbulence occurs, as would be likely, then the concentration gradient would become non-linear, probably in a fashion depicted by fig 6.7. This would reduce the inward diffusion path length for N_2.

However, these two above mentioned effects will be offset to some extent by the inadequacy of the assumption that the base-flow effectiveness ends at the mouth of the lysimeter. This is unlikely to be the case, and depending on the soil, the effectiveness is likely to extend outwards in a manner similar to that depicted in fig 6.8.

6.6.2 Determination of effectiveness of base-flow

The effectiveness of the base-flow was verified experimentally by placing a C_2H_2 source beneath the lysimeter. The sample probe (described in section 6.7.0) was inserted at 45° into the soil (Hamilton clay loam) to a depth of 10.6 cm (see fig 6.9). The lysimeter was placed at a distance 7.6 cm from the probe entry point, and inserted until it just touched the sample probe - a depth of 7.6 cm. This placed the C_2H_2 source 3.0 cm below the base of the lysimeter. The base-flow source holes were covered above by 2.6 cm of soil.

A base-flow of argon commenced at 100 ml/min and a source flow of C_2H_2 commenced at 25 ml/min, exiting at the tip of the probe.

Figure 6.7 Possible effect of turbulence on concentration gradient in lysimeter base-flow

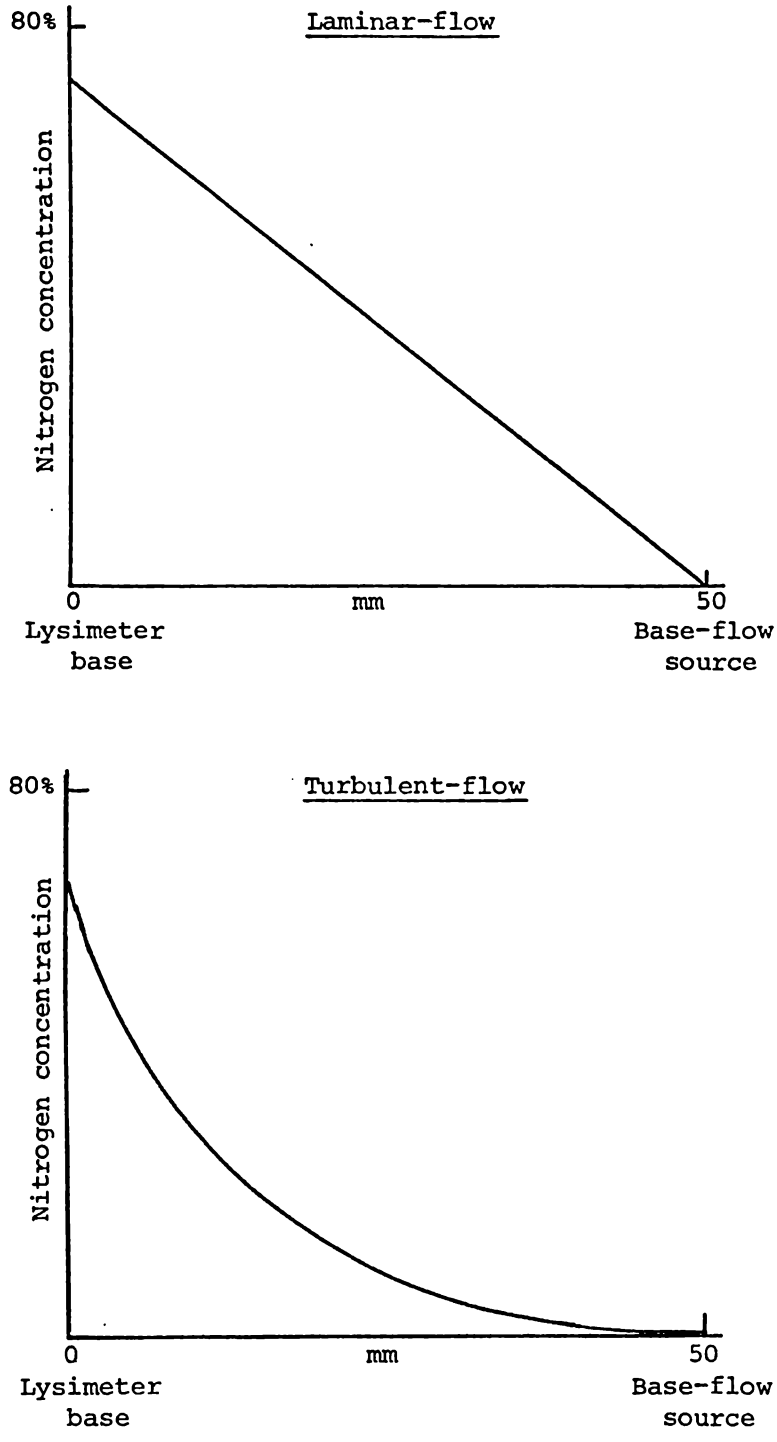


Figure 6.8 Likely base-flow pattern at the lysimeter base

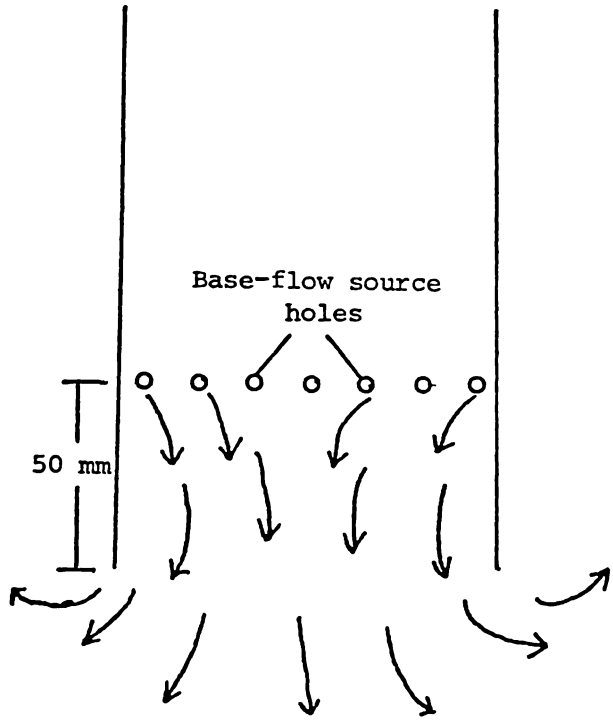
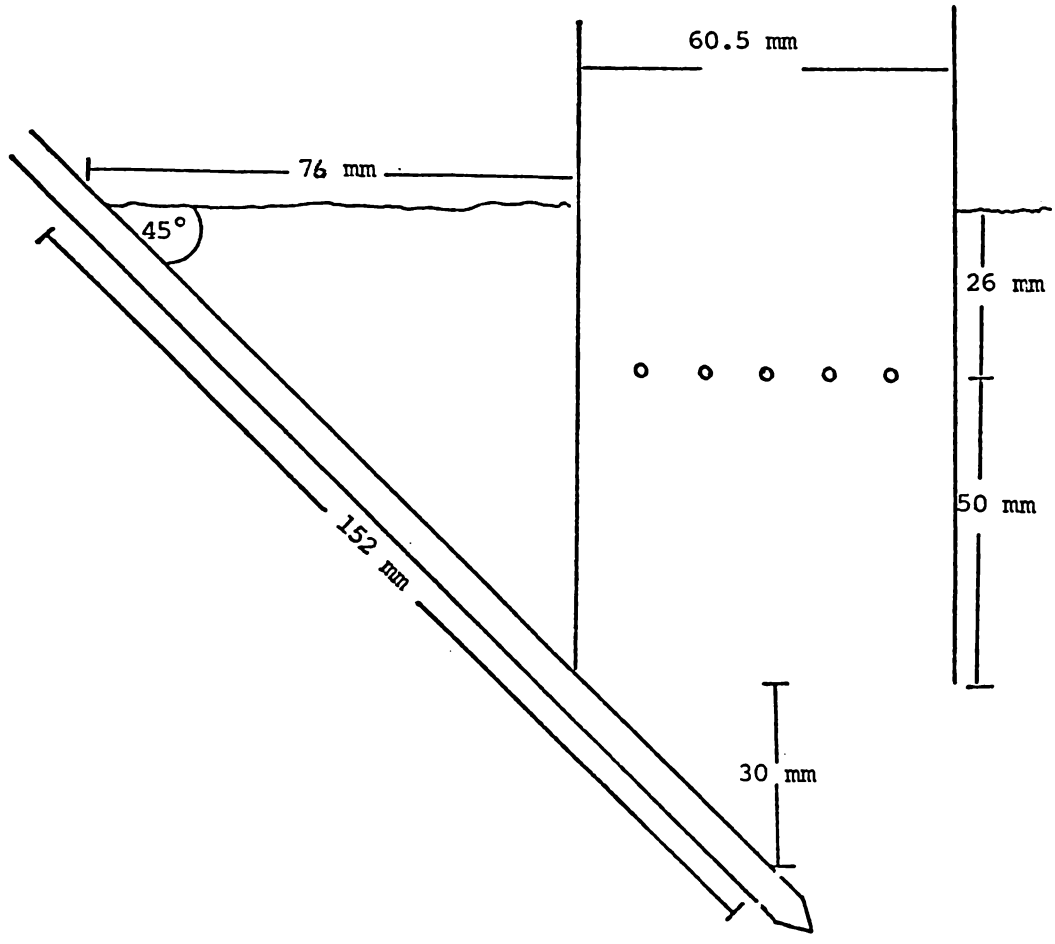


Figure 6.9 Lysimeter installation for testing base-flow effectiveness.
(Drawn to scale)



Headspace samples of the lysimeter atmosphere were taken regularly via a sampling septum. One ml samples were withdrawn using disposable syringes, the tips of which were immediately inserted into rubber bungs. Samples were assayed for C_2H_2 within 10 minutes of sampling using the gas chromatographic techniques described previously. The base-flow continued for 240 minutes and was then shut off while the C_2H_2 source continued. No C_2H_2 was detected in the headspace while the base-flow continued, but 60 minutes after ceasing the base-flow the headspace contained 1.1% C_2H_2 (table 6.2).

Table 6.2 Acetylene concentration in headspace

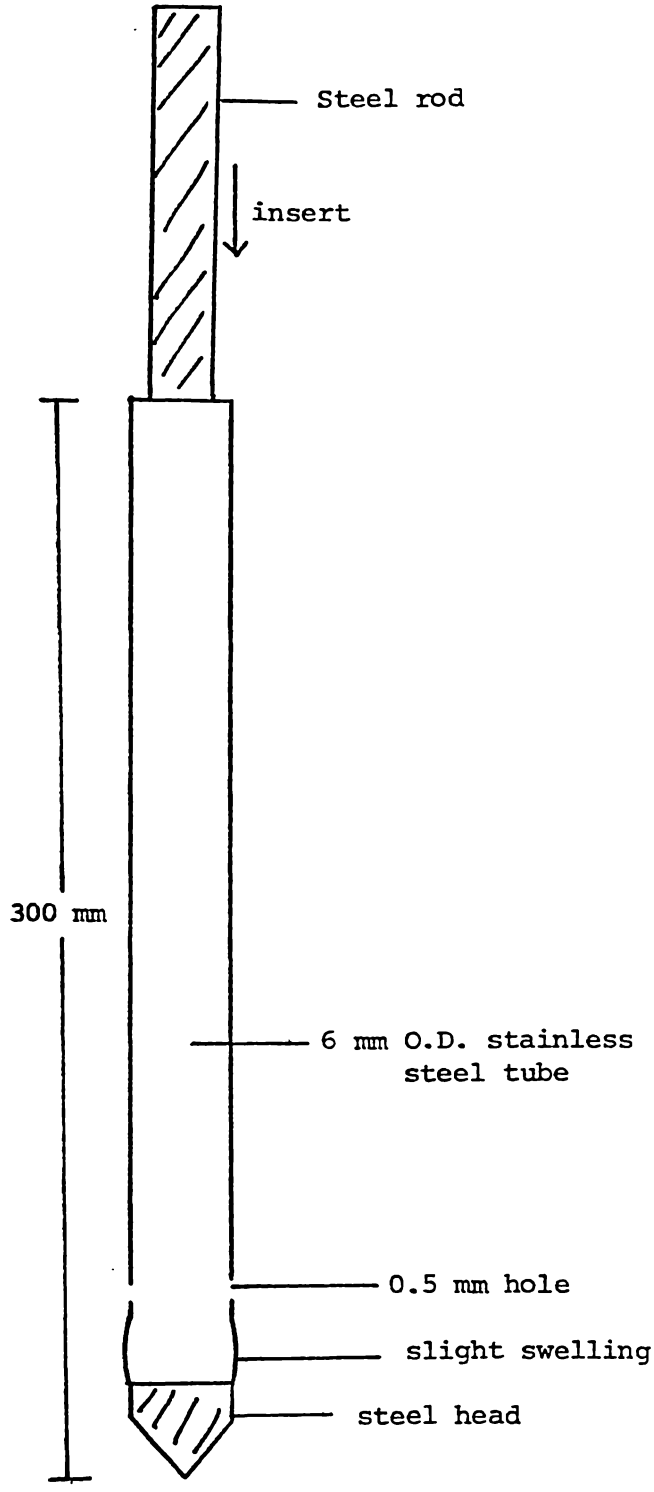
Time (mins)	C_2H_2 concentration (% v/v)
0	< D.L.
60	< D.L.
120	< D.L.
180	< D.L.
240	< D.L.
300	1.1

< D.L. = less than the minimum detection level of 8 ppm

6.7.0 GAS SAMPLING IN THE FIELD

A gas sample probe consisting of a 6 mm diameter stainless steel tube 30 cm long was developed for the lysimeter. A sharpened steel head was inserted and fixed permanently into one end (see fig 6.10). This end of the tube had a slight swelling to relieve outside wall friction and reduce the amount of soil entering the sample holes. Two sample holes (0.5 mm diameter) were drilled into the tube 0.5 cm above the swelling. The total internal volume of the system was minimised by inserting a steel rod of clearance fit into

Figure 6.10 Gas sample probe



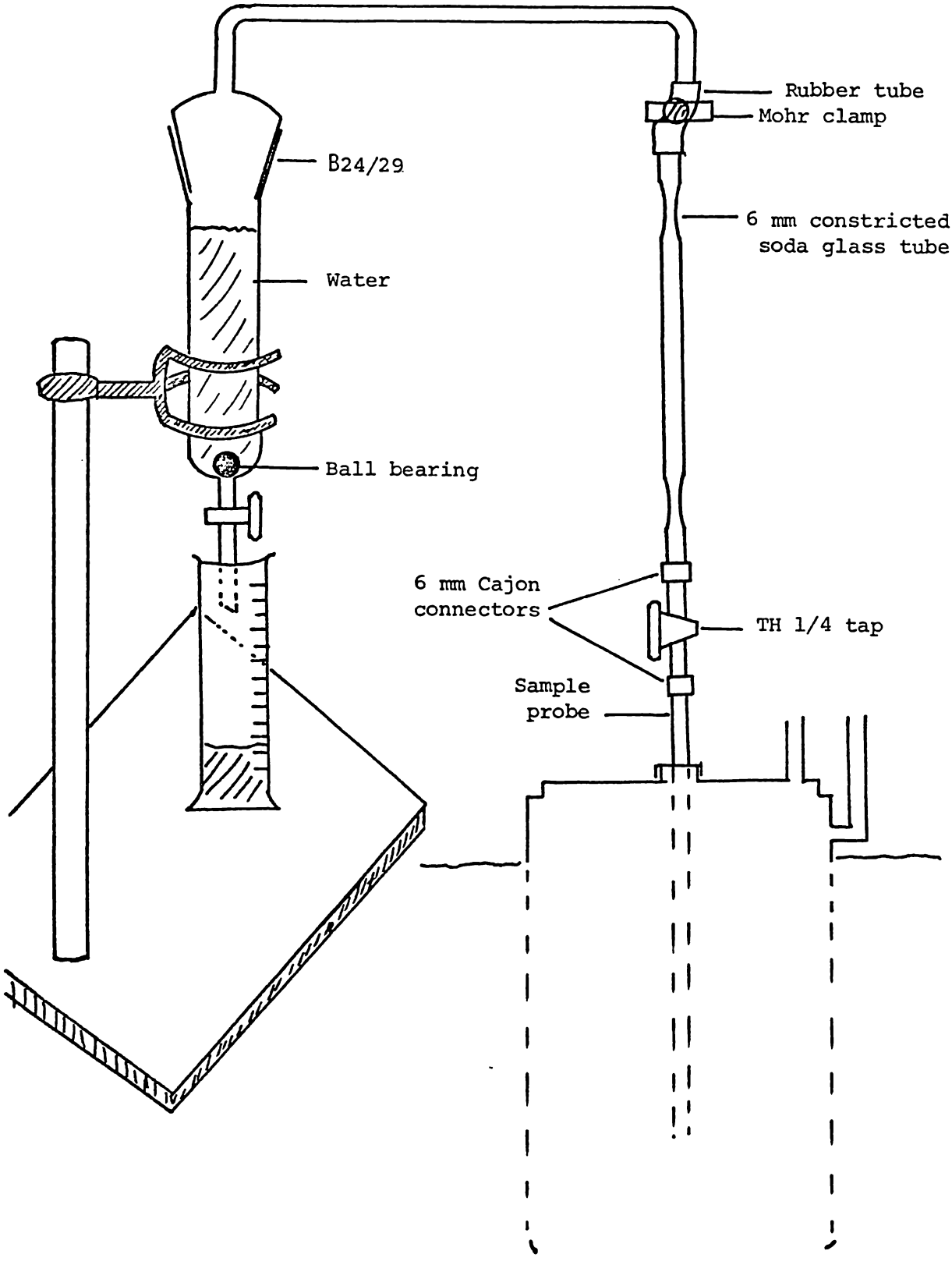
the sample tube. This sample probe was tested in a manner similar to that described in section 6.5.0. The probe was inserted through the centre of a rubber bung 20 cm into a soil core. The flushing argon entered through another 6 mm tube adjacent to the sample probe. At regular intervals during the flushing the gas burette drew nominal 10 ml aliquots from the sample probe by lowering the height of the balance tube (see fig 6.6) and opening the 2-way tap on the gas burette. Results obtained by the sample probe were similar to those described in table 6.1.

For field trials gas sampling was to be conducted in a similar manner to that described by De Camargo *et al* (1974). A 125 ml separating funnel filled with water was used as a suction device. A steel ball bearing (50 mm dia) was placed in the bottom of the funnel to assist in controlling a slow flow of water (fig 6.11). Cajon (6 mm) couplings were used for all joints except for a short piece of rubber tubing for the Mohr clamp. The constricted glass ampoules (approximately 2 ml internal volume) were formed from 6 mm soda glass because of its lower melting point. It was possible to seal off soda glass tubing in the field with a hand held propane gas torch.

Approximately five times the sample tube volume of the soil atmosphere was drawn slowly through the system before sealing off a sample. The volume of gas withdrawn was monitored by the quantity of water collected in the measuring cylinder. While the sample ampoule was being flushed, it was gently warmed with a propane flame to assist in degassing the inner walls of the glass tube.

Sealing of the ampoule had to be done under slightly reduced pressure or the expansion of the entrapped gas with the heat of the propane flame would burst the glass tube at the point of softening. A reduced pressure was maintained by closing the TH 1/4 tap and allowing the water to drip out of the separating funnel until

Figure 6.11 Field gas sampling apparatus



the pressure equalised. The Mohr clamp was closed and the constricted tube sealed off.

This sampling system functioned well in the field but had one serious drawback. Because of the nature of the low N_2 background in the flushed soil core, and the extremely small production of N_2 to be monitored,even small errors due to sample contamination could not be tolerated. A leak of even 0.02% N_2 into the sample ampoule would invalidate the results (as will be demonstrated in section 8.9.0).

It is suspected that the contamination was due to inefficient purging of the sample ampoule. To purge the ampoule rigorously before admitting the soil atmosphere a modification to the sampling system was designed (fig 6.12). The sample ampoule was alternately evacuated and refilled using the 500 cc reservoir flasks attached to the sampling system.

This system showed considerable promise except that the number of samples which could be collected by this technique was limited by the capacities of the reservoirs.

At this stage of development results from separate investigations (chapter 4.3.1) suggested that the area of interest and maximal rates of denitrification in the soils to be studied was in fact the upper few centimetres. The necessity for a sample probe diminished, allowing a new approach to the sampling problem.

By defining gaseous N losses from denitrification as those nitrogenous gases which actually evolved from the soil profile, one is able to measure denitrification by sampling the headspace enclosed by the lysimeter. The sample probe was replaced with a pyrex septum holder (fig 6.13). Using a silicone rubber vacutainer septum samples could be taken with a gas syringe. In order to avoid contamination of the lysimeter headspace by the small amount of nitrogen in the bore of the needle, a flushing system was designed using a small cylinder of

Figure 6.12 Modified gas sampling system

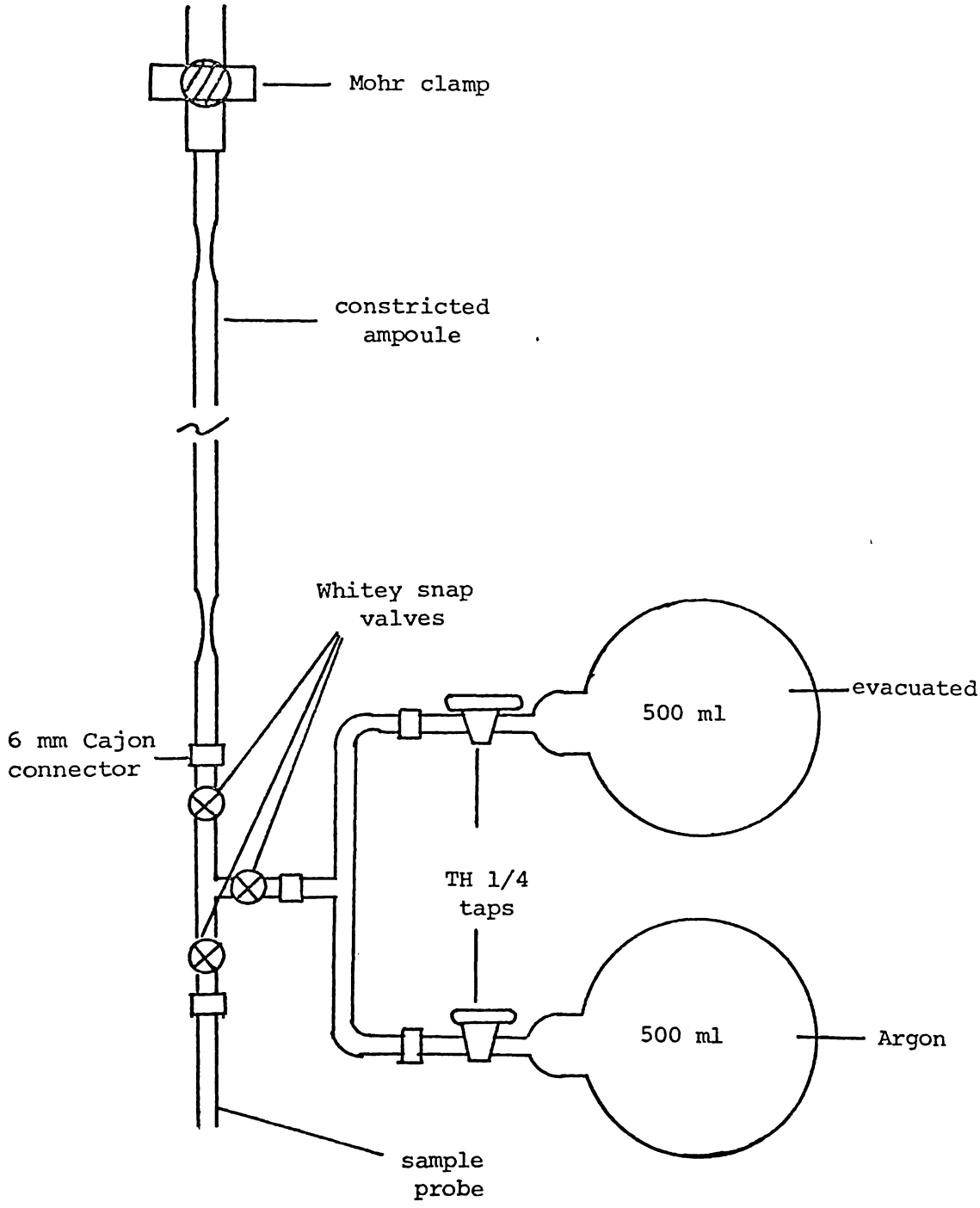
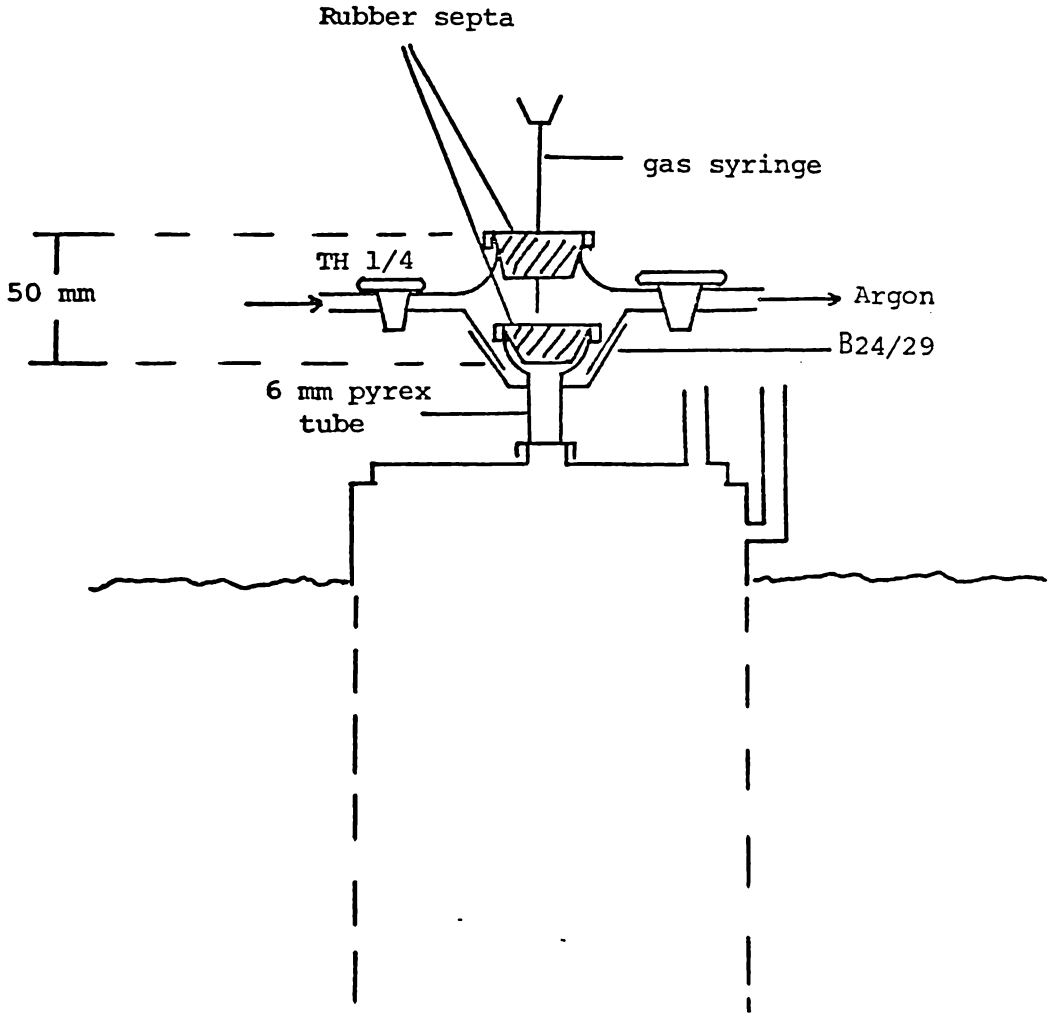


Figure 6.13 Sampling the lysimeter headspace with a gas syringe



argon.

The needle is inserted through the top septum and a flow of argon through the sampling device initiated. While the gas flow continues (50 ml/min) the syringe is purged several times, expelling any air contamination into the argon stream. The argon is then expelled from the syringe and the needle quickly pushed through the second septum into the lysimeter headspace. A 2 ml gas sample is withdrawn and upon removing the gas syringe the argon carrier gas is discontinued and the TH 1/4 taps closed. For a discussion of the evolution of gases into closed headspaces, see section 7.3.2.

This system was shown to produce samples of high integrity with N_2 levels no higher than the background of the flushing argon. However, the number of samples which can be taken is limited by the number of gas syringes available.

Samples were analysed by direct injection into the mass spectrometer. Upon inserting the needle through the injection septum, the bore space and inlet system was evacuated using the mass spectrometer vacuum system. When a suitable N_2 background was achieved the syringe valve was opened and the plunger depressed, releasing the sample into the mass spectrometer. Gas samples passed through a -80°C cold trap on the inlet system to remove traces of water.

6.8.0 TEMPERATURE EFFECT

Rates of denitrification are known to be temperature dependent. It is important that in field assessments of denitrification temperature effects induced by the lysimeter be kept to a minimum. It was noted in section 3.6.0 that the denitrification rate may double for a 10°C rise between 15° and 35°C. Small temperature rises between 15° and 35°C may significantly alter the rate of denitrification being monitored. Below 15°C the temperature effect is seen to be much greater.

Denmead *et al* (1979b) demonstrated a large diurnal flux variation in N_2O emissions from soils. A two-fold variation was noted over two days and closely corresponded to the daily variation in soil temperature of between 15° and 25°C. These results serve to indicate the importance of monitoring temperature effects induced in a soil profile by the installation of a lysimeter.

The influence of the lysimeter on soil core temperatures was investigated by using 0-50°C thermometers. One thermometer lay on the surface of a closely cropped pasture. Another was inserted 10 cm into the soil profile adjacent to the lysimeter while a third was inserted 10 cm into the soil core through the sampling port on the lysimeter. The temperatures were recorded throughout a sunny day which showed an air temperature minimum of 10.7° at 9 am and a maximum of 24.3°C at 2 pm.

Results are recorded in table 6.3.

Table 6.3 Influence of lysimeter on soil core temperatures

Thermometer position	Time				
	9.00 am	11.00 am	1.00 pm	3.0 pm	5.0 pm
Surface	18.0°C	22.5	22.5	26.0	20.0
Inside lysimeter	17.4	18.2	19.6	20.9	21.4
Adjacent to lysimeter	17.3	18.2	18.6	19.4	20.0

The greatest difference recorded between the lysimeter soil core and the adjacent soil was 1.5°C. The lysimeter core appeared to show a small time lag in warming to afternoon temperatures. However the temperature effects noticed were considered small.

CHAPTER 7

FIELD ASSESSMENT OF DENITRIFICATION USING THE ACETYLENE INHIBITION OF NITROUS OXIDE-REDUCTASE

7.1.0 INTRODUCTION

A major problem in assessing denitrification rates *in situ* has been the determination of N_2 evolution. The presence of a high background of N_2 makes the determination of the small amount of N_2 produced by denitrification difficult.

The acetylene inhibition of N_2O reductase enables denitrification products to be measured entirely as N_2O. This phenomenon has enabled a large number of denitrification assessments to be carried out in the laboratory but did not seem to offer a useful field technique.

Use of the acetylene technique for field studies suffers from three major drawbacks.

i) Acetylene has been shown to also inhibit nitrification.

ii) Difficulties in attaining uniformly high acetylene concentrations in the soil profile.

iii) The results attained yield no N_2:N_2O data.

As little as 0.1% C_2H_2(v/v) has been demonstrated to completely inhibit nitrification (Hynes and Knowles, 1978; Walter *et al*, 1979). Walter *et al* (1979) suggested that C_2H_2 should not be used for field techniques lasting longer than one day because of the anticipated effect the inhibition may have on respiratory rates in the soil - thus affecting the aerobic status of the soil. Acetylene has been shown to inhibit various processes for many bacteria and enzymes and as little as 10^{-5} atm brought complete inhibition of *Nitrosomonas europea* (Hynes and Knowles, 1978).

Both Hynes and Knowles, and Walter suggested that the use of the C_2H_2 blockage technique for the assay of denitrification in systems in which nitrification supplies the necessary substrate is invalid. Thus when nitrate is likely to become rate limiting, misleading data from *in situ* denitrification assessments using C_2H_2 may occur.

However, as was demonstrated earlier (section 4.5.1), in many soils in the Waikato region at least, high levels of nitrate

(>20 μg NO_3-N.g^{-1}) are common. Groundwater nitrate levels were found to exceed World Health Organisation limits of 10 mg NO_3-N/ℓ in so many areas that a health warning to families with young children was issued (Baber, 1977). The implication for these soils is that even if further nitrate supply was blocked, there may exist for a limited period, a time where the denitrification rate was independent of substrate limitations. An indication of this time span could be gauged by incubating soil samples by the method described in section 3.2.0 with and without added nitrate. The point at which the endogenous nitrate supply became rate limiting will become obvious by comparing the N_2O evolution rate of those samples without added nitrate.

The criticism of not being able to obtain N_2/N_2O data can be overcome by noting the N_2O evolution prior to the addition of C_2H_2 and comparing this to the subsequent N_2O evolution, as suggested by Focht (1978). The difference in the two values will reflect the N_2 production.

It has also been noted (Fillery, 1979) that blocking N_2O reductase may lead to an increased rate of denitrification. Since fewer electrons are utilized in the reduction of NO_3^- to N_2O compared to N_2, one might expect a higher level of nitrate reduction if the total products are expressed as N_2O. Ryden *et al* (1979) however demonstrated that the sum of the denitrification products was the same whether assessed with or without acetylene. The respiration rate as monitored by the CO_2 evolution also remained unaffected.

The third criticism, that of irregular C_2H_2 distribution in the soil, could well be overcome by using the lysimeter described in chapter 6. Acetylene concentrations as low as 0.1% are sufficient to cause complete inhibition of N_2O reductase (Ryden *et al*, 1979). This minimum C_2H_2 concentration however must be present within the soil crumb - the most likely anaerobic microsite of denitrification.

Different soil structures and crumb sizes will require different lengths of time to establish the required C_2H_2 concentration within the crumb.

Ryden *et al* (1979) produced a series of theoretical diffusion times for spherical crumbs of varying dimensions. Considering the C_2H_2 to emanate from a central core, they calculated diffusion values using the same mathematical treatment as applied to heat flow problems. The effective diffusion coefficient Dp was substituted for the thermal diffusivity k, and concentration c is substituted for temperature v. The time (t) required for C_2H_2 concentrations to reach a desired level in the macropores (1% v/v) at a radial distance r from the core can be determined from values for $\frac{kt}{r^2}$ (Fig 29, Carslaw and Jaeger, 1959). The value Dp for various total porosities (S_T) and air filled porosites ($S\alpha$) can be calculated from the Millington and Quirk (1961) equation for gaseous diffusion under unsaturated conditions:

$$Dp = Do\ S\alpha^{2\cdot33}/(k+1)S_T^2 \qquad 7.1$$

Do is the diffusion coefficient for C_2H_2 into air and k is the solubility coefficient for C_2H_2. Ryden *et al* (1979) used a Do value of 0.183 cm^2/sec derived from the accepted Do value of HCN, and the Bunsen coefficient was assumed to be approximately 1 at 20°C.

It was shown for various values of S_T and $S\alpha$ that the time required to establish a C_2H_2 concentration at a particular distance from the source increases as air-filled porosites decrease. The times calculated were reported to be overestimates as diffusion through the aqueous phase was ignored. They also demonstrated that for a given value of $S\alpha$, diffusion times increase with increasing total porosity S_T due to the solubility of C_2H_2.

Diffusion into saturated crumb systems has to be treated a little differently. This is analogous to heat flow into solid spheres of radius r with zero initial temperature at the centre. The value of the diffusion coefficient for C_2H_2 under saturated conditions (Dp) was

calculated from

$$Dp = Do\ S_T^{0.33} \qquad 7.2$$

where Do is the diffusion coefficient of C_2H_2 in water.

Using the above data the relationships calculated indicated that the time required to establish a certain concentration at the centre of a crumb increases as the external concentration and total porosity decrease.

Ryden *et al* (1979) demonstrated that C_2H_2 concentrations of approximately 0.1% can be established in the centre of saturated crumbs of diameter <0.5 cm within a few minutes (approximately five) of the external concentration reaching 0.2 to 1%.

For their field assessment, Ryden *et al* (1979) used a series of gas probes placed around the sampling chamber. Acetylene was supplied at rates ranging from 28 to 2.8 litres/hr. Concentrations measured in the pore spaces, while highly variable were in the order of 0.1% after fifteen minutes at a flow rate of 28 litres/hr. The observed C_2H_2 concentrations were consistent with the calculated relationships.

7.2.0 EXPERIMENTAL

7.2.1 Endogenous nitrate

To measure the duration which endogenous nitrate supplies might be expected to last, samples of the Te Kowhai and Horotiu soils were incubated anaerobically with and without added nitrate. The results (figs 7.1 and 7.2) indicate that endogenous nitrate levels are sufficient to sustain denitrification under anaerobic conditions for approximately 90 minutes.

Figure 7.1 Denitrification of Te Kowhai soil with and without added nitrate

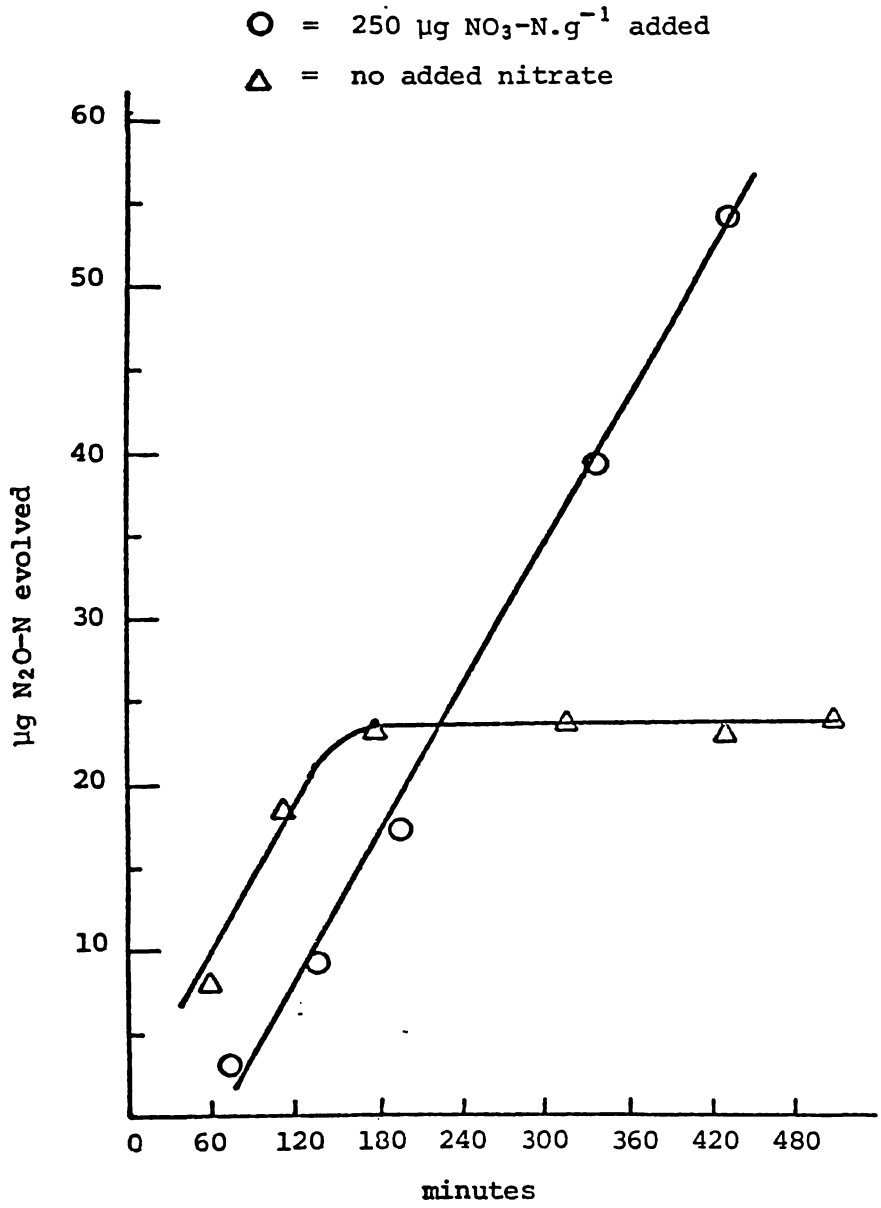
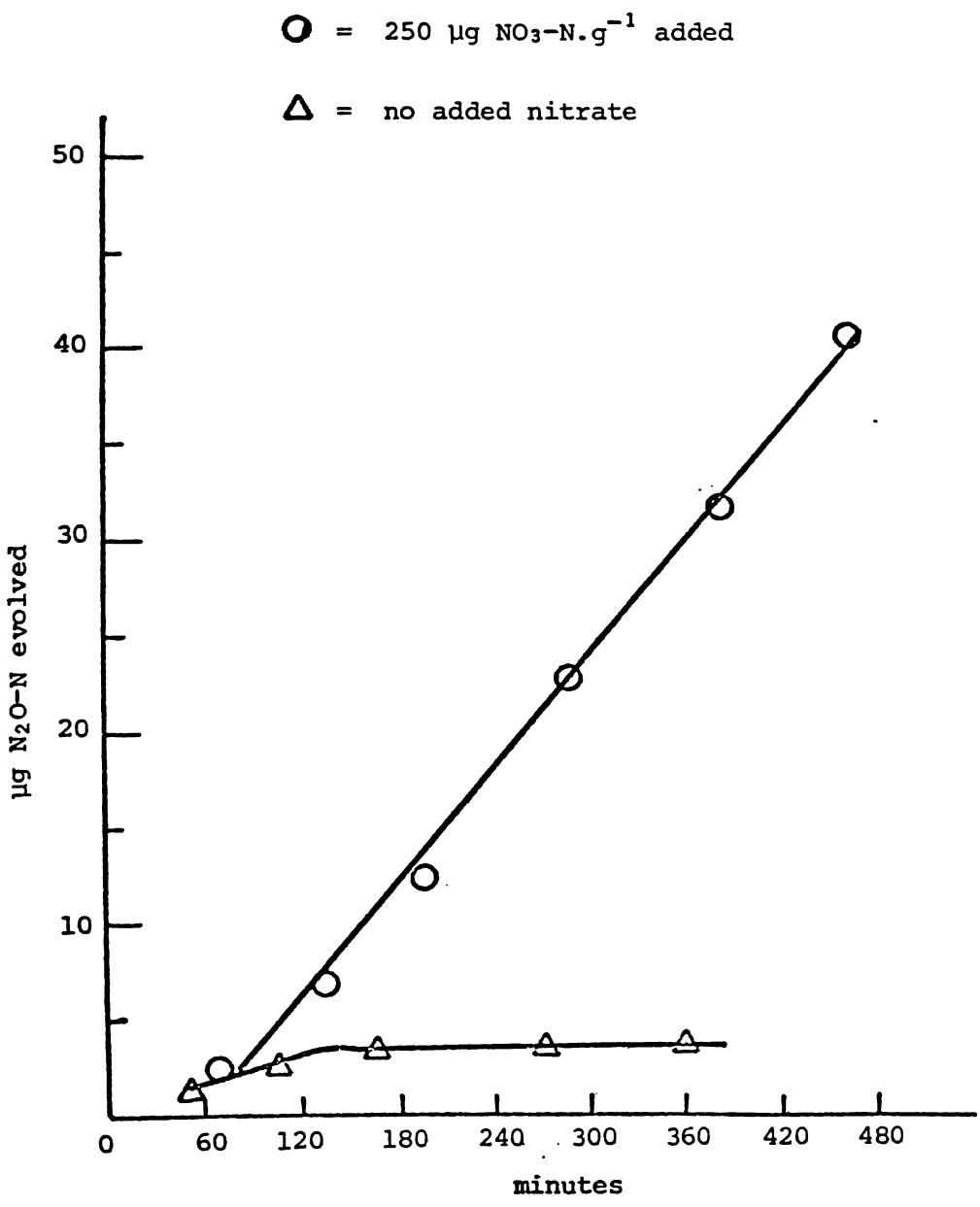


Figure 7.2 Denitrification of Horotiu soil with and without added nitrate



7.2.2 Acetylene flushing

The lysimeter was considered to afford excellent possibilities for rapidly establishing high C_2H_2 concentrations in a soil profile. By nature of its flushing technique, the area above the soil core could be considered a single point source of C_2H_2, overcoming some of the concentration gradient problems suffered by Ryden *et al* (1979) with a multitude of point sources at distance from the monitoring site. This assumption was seen to be borne out by measurements of the C_2H_2 concentration measured during a trial flushing in the Hamilton clay loam.

The lysimeter was inserted to a depth leaving a headspace height of 10 cm. The sample probe was inserted 10 cm into the soil profile. Gas samples of 1 cc were withdrawn through a septum using disposable syringes. The syringes were inserted into rubber bungs after sampling until assay using gas chromatographic methods described in section 3.2.1.

Core flushing with pure C_2H_2 began at 100 cc/min and gas samples were withdrawn at five minute intervals. The C_2H_2 concentration data at a 10 cm depth in the soil profile is shown in fig 7.3.

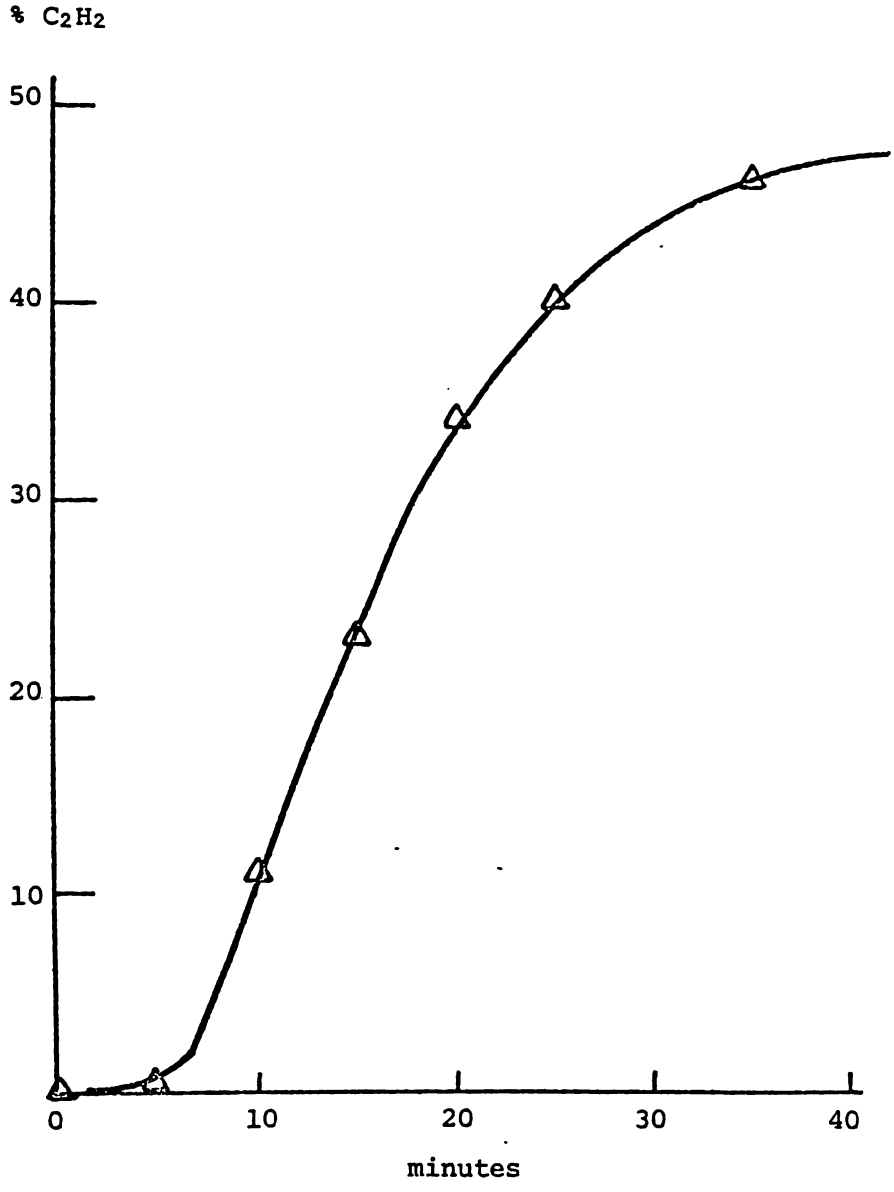
The results in fig 7.3 suggest the lysimeter is considerably more effective than the point source method used by Ryden *et al* (1979) in establishing high C_2H_2 concentrations quickly within a soil.

Ryden *et al* (1979) flushed with 100% C_2H_2 to inhibit N_2O reductase as quickly and effectively as possible. Using the lysimeter, this gas mixture would quickly induce anoxia and invalidate any *in situ* field measurements of rates of denitrification. The flushing gas used in the field experiments described in this chapter consisted of 80% C_2H_2 and 20% O_2.

7.2.3 Flushing system

Acetylene and oxygen were mixed in the gas lines prior to

Figure 7.3 Acetylene concentration established by flushing lysimeter soil core at 100 $ml.min^{-1}$



entering the lysimeter (see fig 7.4). Matheson check valves were installed on each line to prevent mixing of gases within the cylinders. A gas chromatographic analysis of the gases in each cylinder after two field trials showed no trace of foreign gases in either cylinder. The oxygen cylinder was a converted fire extinguisher of a nominal 1 litre volume. The C_2H_2 cylinder was a portable 'Presto' C_2H_2 cylinder also of a nominal one litre capacity.

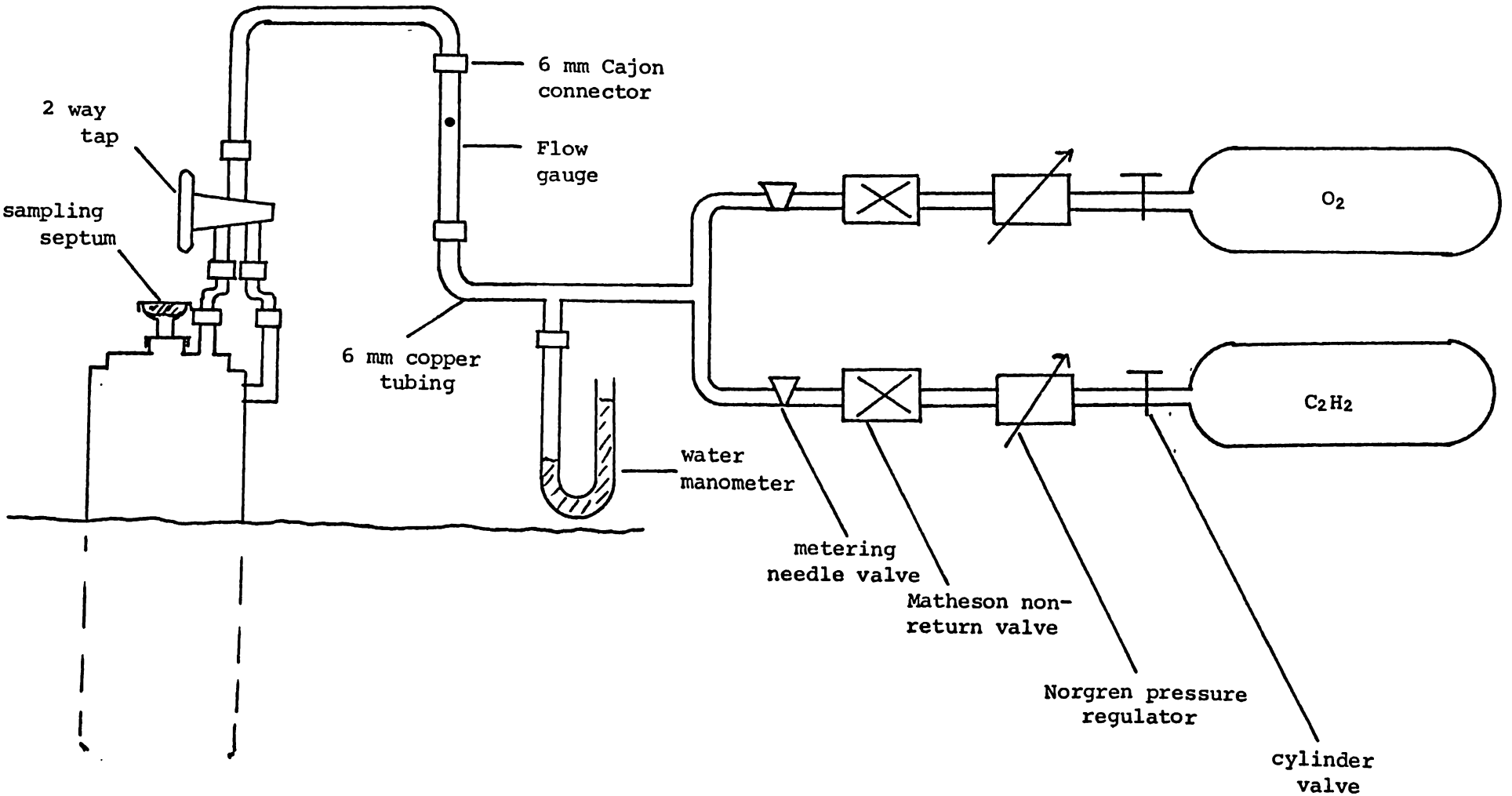
A standard flushing time of twenty minutes at 100 cc/min was adopted. The gas pressure at the lysimeter headspace was 2-3 cm H_2O (~2 mm Hg). Gas lines consisted of 6 mm copper or pyrex tubing with cajon connectors. The whole system could be installed by one person and flushing within one hour.

7.2.4 Results and Discussion

The results of section 7.2.1 demonstrate that there is sufficient nitrate reserve for denitrification to proceed unimpeded for approximately 1½ to 2 hours. The incubations were conducted in anaerobic atmospheres. Due to the powerful inhibition effect of oxygen it has been shown that rates of denitrification in anaerobic environments are of the order of a hundred or more times faster (Focht, 1978). If it can be demonstrated that in totally anaerobic incubation conditions (at 25°C) there is sufficient endogenous nitrate for denitrification to proceed unrestricted for two hours, then it is likely that sufficient nitrate is present to conduct an aerobic field assessment of a lesser duration. If during the field assessment nitrate did become rate limiting, this would be reflected by a decreasing evolution of N_2O.

It is interesting to note in figures 7.1 and 7.2 that denitrification appears to end abruptly with the exhaustion of nitrate substrate and does not show significant first order effects with the depletion of nitrate reserves. This may be related to the *in situ*

Figure 7.4 Layout of Acetylene flushing field apparatus



enzyme status at the time of sampling. Data in section 4.5.1 and the results of the nitrate amended graphs indicate that the nitrate reductase in these soils is already fully induced. The subsequent high rate of denitrification would minimise the duration of any first-order substrate effects.

7.3.0 GAS SAMPLING

The gas sampling system is very important to the success of field results obtained by the chamber sampling system. Chamber systems for measuring N_2O emission from soils basically consist of two types: i) the closed system and ii) the open system.

In the closed system the evolving gases accumulate within the headspace. Conversely, in the open system the headspace is continuously flushed and the product gases measured or trapped from the effluent.

In a study on chamber systems for measuring N_2O emission from soils, Denmead (1979) compared a closed system to an open system. N_2O analyses were conducted by linking an infra-red gas analyser in the flow-train of the sampled gas. The closed system consisted of a loop between the chamber air space and the gas analyser. In the open system, outside air was drawn continuously through the chamber space and its N_2O enrichment or depletion measured.

Both systems suffered from pressure deficits within the chamber common to cycling methods of gas sampling. Under the experimental conditions noted by Denmead (1979) a flow rate of 50 cc/min induced a pressure deficit of 1 cm H_2O. Calculations suggested a mass flow of N_2O from the soil induced by such a pressure deficit could be several times larger than the diffusive flow. This pressure deficit can be overcome by employing an internal baffle system.

Both the closed and open system share a common assumption. It is assumed that the rate of production of N_2O in the soil is not affected by the N_2O in the soil air.

Another problem associated with chamber systems is the equilibration of the N_2O diffusing from the soil with the chamber atmosphere. Nitrous oxide produced in the soil profile diffuses towards the atmosphere along a concentration gradient. The transfer process at the soil-air interface is characterized by the transfer coefficient H and defined by

$$F/\rho = H\ (Co-Ca) \qquad 7.3$$

F is the surface flux of N_2O, ρ is the density of N_2O, Co the concentration of N_2O just within the soil profile, and Ca is the N_2O concentration in the air above.

The transfer coefficient in the chamber will be different to that in the field. Thus when a chamber system is first operated, there will be a period in which the N_2O concentrations in the soil and chamber air adjust to their new equilibrium values. The magnitude of this effect was studied by Denmead (1979). The transfer coefficient varied with different flow rates. It was calculated for an open system that at a flow rate of 50 ml/sec the surface flux is 95% of its final value in eight minutes after placing a canopy into operation. At 17 ml/sec the adjustment to equilibrium would require eighty minutes. A higher flow rate of circulating gas is favourable, but the rate is also a compromise to the sensitivity of the N_2O measurement.

The closed system however also suffers a serious criticism. In a closed system a steady state of gaseous evolution is never attained. There is a continual change in the concentration of the chamber air, hence there must be continual readjustment of the soil profile. Denmead (1979) suggests that as the concentration in the chamber increases, readjustment of the soil profile will lead to an under estimation of the true field flux. However the closed system has been used successfully in studies of N_2O emission from a flooded field

(Denmead *et al*, 1979a). In this situation the transfer of N_2O to the chamber was by solution into a well mixed water layer free to move between the chamber and the outside, and by evolution at the air-water interface. The sampling chamber was situated with its bottom edge below the water but 5 cm above the soil surface, supported on two blocks so the water could move freely between the chamber and the flooded field.

Ryden *et al* (1978) used a variation of the open system to estimate N_2O fluxes in the field. A box with inlet and outlet ports was inserted into the soil. The enclosed air space was continuously swept with external air at a rate of 20 ℓ/hr (330 ml/min). Nitrous oxide evolved from the soil was adsorbed on molecular sieve 5A after passing through anhydrous calcium sulphate and Ascarite for removal of H_2O and CO_2 respectively. The molecular sieve 5A was contained in a screw cap vial fitted with a neoprene stopper. These vials could be exchanged on the flow line at any appropriate time. A 12 volt pump operated by a lead-acid battery provided the air flow.

Recovery of N_2O from the molecular sieve was based on a technique described by Dowdell and Cress (1974). The 20 g of molecular sieve was placed in a flask, sealed and evacuated. A measured quantity of water (50 ml) was admitted and the sample left to equilibrate. After a suitable equilibration time, the flask was returned to atmospheric pressure by admitting He. Samples of the headspace were analysed for N_2O and corrections for N_2O in solution were made. Corrections were also made for ambient N_2O drawn through with the air by passing a similar external flow through the Drierite and Ascarite traps and adsorbing the N_2O onto a similar molecular sieve trap. Providing flow rates, N_2O flux rates and sampling times were carefully calculated, Ryden *et al* (1978) found this system satisfactory for field work. The field operating conditions and analytical techniques

adopted (electron capture gas chromatography) permitted a detection limit of 0.16 to 0.24 g.N/ha/yr. Unlike Denmead (1979) they found that flow rates of 10 to 40 ℓ/hr through the sampling chamber had no effect on the measured N_2O evolution. Presumably this is due to the geometry of the soil cover. Ryden *et al* (1978) calculated from Poiseuille's equation for fluid flow through a cylinder pipe that the pressure deficit for the operating conditions used was not greater than 0.0048 mm H_2O. They also observed that small (four fold) accumulations of N_2O which developed within the cover using flow rates of 20 ℓ/hr had no observable effect on the rate of N_2O evolution.

This system offers advantages for field work in that it is simple to operate and the sampling system does not require rapid in-field analysis. Ryden *et al* (1978) demonstrated that samples could be stored for at least 28 days on molecular sieve before analysis without significant N_2O loss.

On the basis of the field applicability of the above technique it was decided to adopt a similar technique for the measurement of denitrification using the C_2H_2 inhibition in the field.

It was anticipated that at many sites vehicle access may be difficult. Also, a suitable 12 volt circulating pump was not available. Although some crude rubber diaphragm pumps designed for commercial applications were available, these were not tested. The field C_2H_2 assessment trials were developed initially as a preliminary to the $^{15}N_2$ assessment technique outlined in chapter 8. It was hoped to use the same field system for N_2 analyses. Any pumping system considered had to be free of the leak and diaphragm bleed problems associated with the type of pumps generally available. For these reasons the idea of a pumping system was abandoned and the flow through sampling system was based upon pressurised cylinders of gas.

7.3.1 Experimental

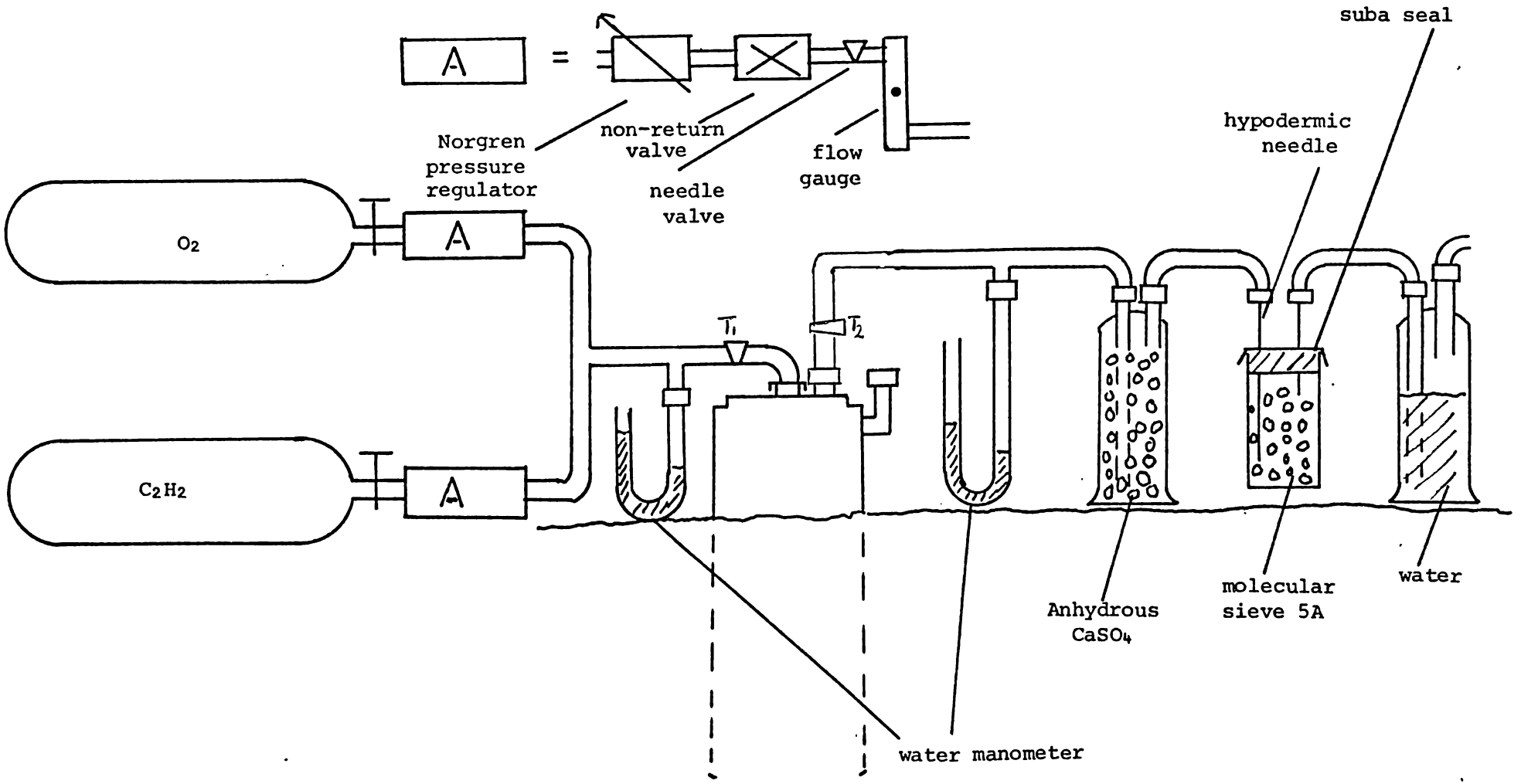
A system was designed and tested in the laboratory first (see figure 7.5).

The soil core was to be initially flushed with C_2H_2 and O_2. The headspace of the lysimeter was then to be continuously purged by opening T_2. By carefully monitoring flow rates and the pressures indicated by the manometers it was hoped to avoid any mass flow problems. The water bubbler (D) was expected to be modified in the field situation as the few cm H_2O pressure induced in the flow system with the water bubbler would have caused a significant downward mass flow of gas in the lysimeter unless exactly equalised by the base-flow pressure. It was expected to replace the bubbler with a length of wide bore capillary tubing in order to reduce the inward diffusion of air.

The molecular sieve trap (C) consisted of approximately 10 g of molecular sieve granules in a screw cap vial sealed with a suba seal. The molecular sieve was carefully prepared by heating at 110°C overnight under vacuum. Upon cooling, the flask containing the molecular sieve was filled to atmospheric pressure with argon. The molecular sieve was then transferred into vials and capped under an atmosphere of argon.

This sampling system appeared to have a problem. Flushing an acetylene/oxygen mixture through the trap at 15 cc/min produced only traces of C_2H_2 in the effluent gas while the molecular sieve trap became saturated with C_2H_2. The molecular sieve could be felt to warm up during this period. After approximately 40 minutes C_2H_2 was seen to appear in the effluent gas in the same concentration as the flushing gas. Presumably the molecular sieve trap was saturated with C_2H_2. This experiment was repeated with N_2O (2000 ppm) in the gas mixture to measure the effect of the C_2H_2 adsorption on the trapping capacity for N_2O. No N_2O appeared in the effluent even after the C_2H_2 had reached

Figure 7.5 Flow-through system for field assessment of denitrification using acetylene



saturation in the molecular sieve.

On the basis of these preliminary tests it was decided to change the molecular sieve vials every 30 minutes. Recovery of the N_2O was facilitated by adding 50 ml of water to the 10 g of molecular sieve in an evacuated 125 ml flask. However, upon adding the water the molecular sieve was seen to outgas vigorously and the enclosed system rapidly reached a dangerous overpressure. A strong smell of C_2H_2 evolved from the opened flask.

The experimental procedure described by Ryden *et al* (1979) used a similar sampling and recovery technique in the presence of C_2H_2 but did not involve the use of C_2H_2/O_2 as a carrier gas for trapping N_2O. While the enclosed headspace above the soil was swept with air, further C_2H_2 was admitted to the probes to maintain a high C_2H_2 concentration in the soil profile. Undoubtedly quantities of C_2H_2 would subsequently be swept onto the molecular sieve trap along with the N_2O. However Ryden *et al* make no mention of such difficulties with the recovery technique. Presumably the quantity of C_2H_2 trapped by their system was far less than that encountered by the technique using C_2H_2/O_2 as a carrier gas.

An obvious solution to this problem would have been to flush the headspace with a gas other than the C_2H_2/O_2 mixture. However, as seen in section 4.3.1, the top few centimetres of the soil profile is the most active zone of denitrification. It is also that part of the soil profile in most rapid exchange with the above atmosphere. Failure to maintain a high C_2H_2 concentration above this region would lead to reduced concentrations of C_2H_2 in the most crucial part of the soil profile.

The possibility of maintaining the C_2H_2 concentration by admitting further C_2H_2 to the soil via the base-flow of the lysimeter was also considered. This alternative was discarded for two reasons.

Firstly, in order to maintain a high C_2H_2 concentration in the top few centimetres this method would require a large input of C_2H_2. Otherwise the rate of removal of C_2H_2 from the upper few centimetres of the soil would far exceed the rate of diffusion of C_2H_2 from the base-flow. Secondly, in order to maintain such a rapid flux of C_2H_2 the base-flow C_2H_2 would have to be admitted under considerable pressure. Even an inlet pressure of a few cm.Hg would induce a pressure gradient in the profile between the base-flow and headspace. Such a gradient will inevitably induce a mass flow of gases towards the headspace and invalidate any N_2O flux measurements (Denmead, 1979).

The most likely solution to this problem seemed to be to remove the C_2H_2 from the molecular sieve prior to the N_2O determination. As N_2O is very strongly adsorbed to molecular sieve 5A (Graven, 1959), it was considered possible to bleed the C_2H_2 off leaving the N_2O.

Acetylene-saturated molecular sieve was left in an atmosphere of argon overnight. However, negligible C_2H_2 appeared to desorb. The same molecular sieve was placed in a 70°C oven overnight. Upon adding the molecular sieve to water, no sign of bubbles or C_2H_2 evolution was evident. The experiment was repeated with a known quantity of N_2O also adsorbed on the molecular sieve. No trace of either the C_2H_2 or N_2O could be detected upon adding water to the molecular sieve.

It is possible that with further experimentation a satisfactory sample treatment may have evolved. However, the technique was rapidly becoming cumbersome in terms of a routine field assay.

7.3.2 Static sampling chambers

Findlay and McKenney (1979) subsequently reported a very simple technique for field measurement of N_2O evolution. The sampling system consisted of single ring canopies of aluminium (approximately 15 cm diameter, 25 cm high, and 2 mm thick). The canopies were pressed

5-10 cm into the ground and sealed on top with 2 mm neoprene rubber sheets. Gas sampling commenced within sixty seconds of sealing the chamber. Gas samples were taken via a double ended needle into previously evacuated 20 cm^3 pyrex tubes. The needle remained open between sampling to allow equilibration of the chamber pressure and to minimise mass flow from the soil.

The total simplicity of this system made it highly desirable for field measurements. However criticisms have been levelled at such static chamber sampling systems. As pointed out previously (section 2.5.5) closed sampling systems suffer from artificially high build-ups of N_2O causing a readjustment of the N_2O concentration in the soil profile. Such an effect would under estimate the N_2O flux from a soil because build-up of N_2O in the headspace leads to a lowering of the N_2O concentration gradient in the soil profile.

Another criticism of the closed sampling system is that the rate of N_2O reduction in the soil closely follows first-order kinetics in the concentration ranges commonly encountered with this type of sampling (Garcia, 1974). This may lead to a further under estimation of N_2O fluxes as N_2O reduction may be expected to increase within the soil cover. However, in the presence of C_2H_2 this criticism can be negated.

Findlay and McKenney (1979) demonstrated that decreases in concentration gradients do not seriously affect fluxes of N_2O out of the soil if measurements were made over a short time. Although the accumulation of N_2O in a closed chamber is expected to be curvilinear with respect to time (Matthias *et al*, 1978), estimates based on short periods of time using Findlay and McKenney's data (1979) indicated the initial portion of the plot is nearly linear. Their results showed the N_2O concentration within the chamber to increase linearly for several hours to eventually reach levels often in excess of 10 ppm. Findlay and McKenney claim that N_2O fluxes can be calculated from N_2O

data using the initial linear portion of the plot. The decrease in concentration gradient beneath the canopy was not of serious magnitude during the first 5-8 hours. After this time the flux was seen to decrease corresponding to a downward curvilinear trend in N_2O concentration within the chamber.

Rolston *et al* (1978) applied a correction technique to allow for the decreasing flux of gases with the concomitant build-up of gases in the headspace. The correction was based on the steady state diffusion equation:

$$F = -D(\frac{dc}{dx}) \qquad 7.4$$

By assuming the gaseous concentration did not change between 0 and 2 cm (the shallowest sampling depth) and that the soil surface was in equilibrium with the canopy atmosphere, a solution to the above equation was given as:

$$Dp = -(VL)(At)^{-1} \ln [(C_2-Co/C_2(] \qquad 7.5$$

where V is the volume (cm^3) of the chamber, L is the depth of soil where the measurements were taken (2 cm), A is the surface area of the chamber, t is time, Co is the initial concentration in the chamber, and C_2 is the measured concentration at the 2 cm depth. The calculated Dp (effective diffusion coefficient) and measured concentration gradient at t=0 (assumed to be linear from 0 to 2 cm) were used to calculate the corrected flux.

This correction was found to over estimate the decrease in flux and resulted in a calculated flux greater than 100% of a test sample. This over estimation was shown to be influenced by poor mixing in the sample chamber causing a concentration gradient between x=0 and the ambient air.

Measurements of N_2O concentrations at the 2 cm depth in the field plots both before and after the cover had been in place for two

hours demonstrated only slight changes.

Using this technique Rolston *et al* (1979) compared the direct monitoring of N_2 and N_2O fluxes to deficit budget measurements of denitrification. The direct flux technique was found to under estimate the denitrification of 0-3 kg N/ha compared to the deficit technique for wet soils and 12-65 kg N/ha less for drier soils. These differences were attributed largely to the inability to monitor small gas fluxes over a long period of time.

Using the static chamber technique on several soil plots, Findlay and McKenney (1979) measured rates of N_2O evolution. All showed linearity within the period measured. Scatter which typically led to errors of ±5% in the rates was attributed to leakage around sample tube septums.

Matthias *et al* (1980) also used a closed chamber technique to estimate N_2O fluxes. Linearity was observed within the 30 minute sampling period.

McKenney *et al* (1978) present data showing N_2O evolution from soils to be linear over 90 minutes within a closed sampling chamber.

The sampling technique described by Findlay and McKenney (1979), in conjunction with the acetylene inhibition of N_2O reductase, promised to produce a viable field technique.

7.4.0 GAS CHROMATOGRAPHIC ANALYSIS OF N_2O

Gas samples of the lysimeter headspace were taken at regular intervals using 3 ml disposable syringes. Each syringe was flushed out several times in the headspace before withdrawing a sample and immediately plunged into a rubber stopper after removal from the septum. Samples were always analysed the following day on a Varian 3700 equipped with an electron capture detector. One ml samples were injected onto the same Porapak Q column described in section 3.2.1. Nitrogen carrier

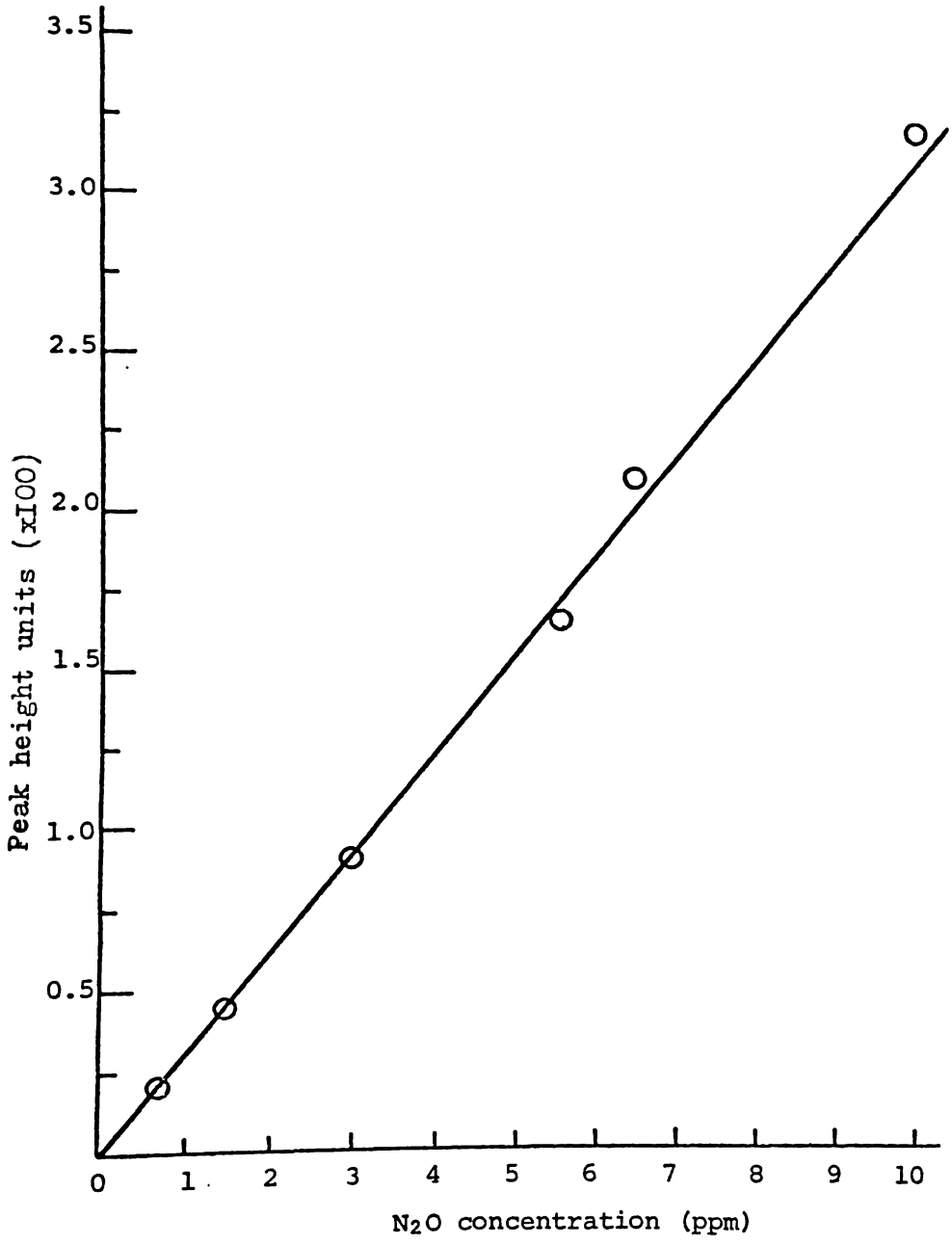
gas passed through a Varian gas purifier cartridge onto the column at 20 ml/min. The column was conditioned overnight at 150°C prior to use. With the ^{63}Ni electron capture detector temperature at 350°C the minimum N_2O detection limit was estimated to be 20 ppb. Complete sample separation was achieved in approximately 5½ minutes. The high concentration of C_2H_2 (80%) caused serious tailing and base-line drifting. Consequently between samples the column was purged at 120°C for 10 minutes and allowed to cool at 10°C/min to 35°C. Approximately 35-40 minutes elapsed between samples.

Two N_2O standards were made from a 1067 ppm N_2O NZIG standard. A measured aliquot of the standard was injected into flasks of known volume containing high purity helium, producing standards of 0.14 and 38.8 ppm N_2O. The N_2O content of air was calculated to be 305 ppb (i.e. approximately 10% lower than the nominally accepted value of 330 ppb (Cicerone *et al*, 1978). Providing the Porapak column was well conditioned, and using an Omniscribe recorder, peak heights were found to give consistent calibrations. Linearity of detector response was measured over a range of 0.5 to 10 ppm by injecting successively smaller aliquots of a nominal 10 ppm standard of N_2O in helium (see figure 7.6).

7.5.0 FIELD ASSESSMENT - RESULTS AND DISCUSSION

The Te Kowhai silt loam which had been sampled regularly as a reference site for laboratory studies was chosen to test the technique. The lysimeter was inserted leaving a headspace volume of 300 cm^3 (height 10 cm) in order to minimise the N_2O concentration effect. The headspace volume was a compromise between sample sensitivity and an anticipated rate of N_2O evolution. The soil core was flushed for 20 minutes with C_2H_2/O_2 at 100 ml/min and an inlet pressure of 20 mm H_2O. After 20 minutes the core flow ceased, a base-flow of 20 ml/min C_2H_2/O_2 began, and the first headspace sample was taken. The

Figure 7.6 Calibration of Electron Capture detector for N_2O



base-flow continued during the experiment in order to maintain the C_2H_2 concentration in the profile and to prevent inward diffusion of N_2O. The base-flow inlet pressure of 4 mm H_2O caused a slight positive pressure within the soil core. Sampling continued at regular intervals and results are displayed in table 7.1 and figure 7.7.

Table 7.1 N_2O evolved from C_2H_2 field trial on Te Kowhai silt loam

Sample time T (mins)	N_2O in headspace (ppm)	µg N_2O-N evolved
T_0	0.08	0.02
T_{30}	1.84	0.6
T_{60}	5.12	1.8
T_{90}	9.29	3.2
T_{120}	13.65	4.7
T_{150}	18.20	6.3
T_{210}	23.12	7.9

A typical error of analysis was calculated to be ±0.06 ppm

The tailing in the first 60 minutes (figure 7.7) can be interpreted as either:

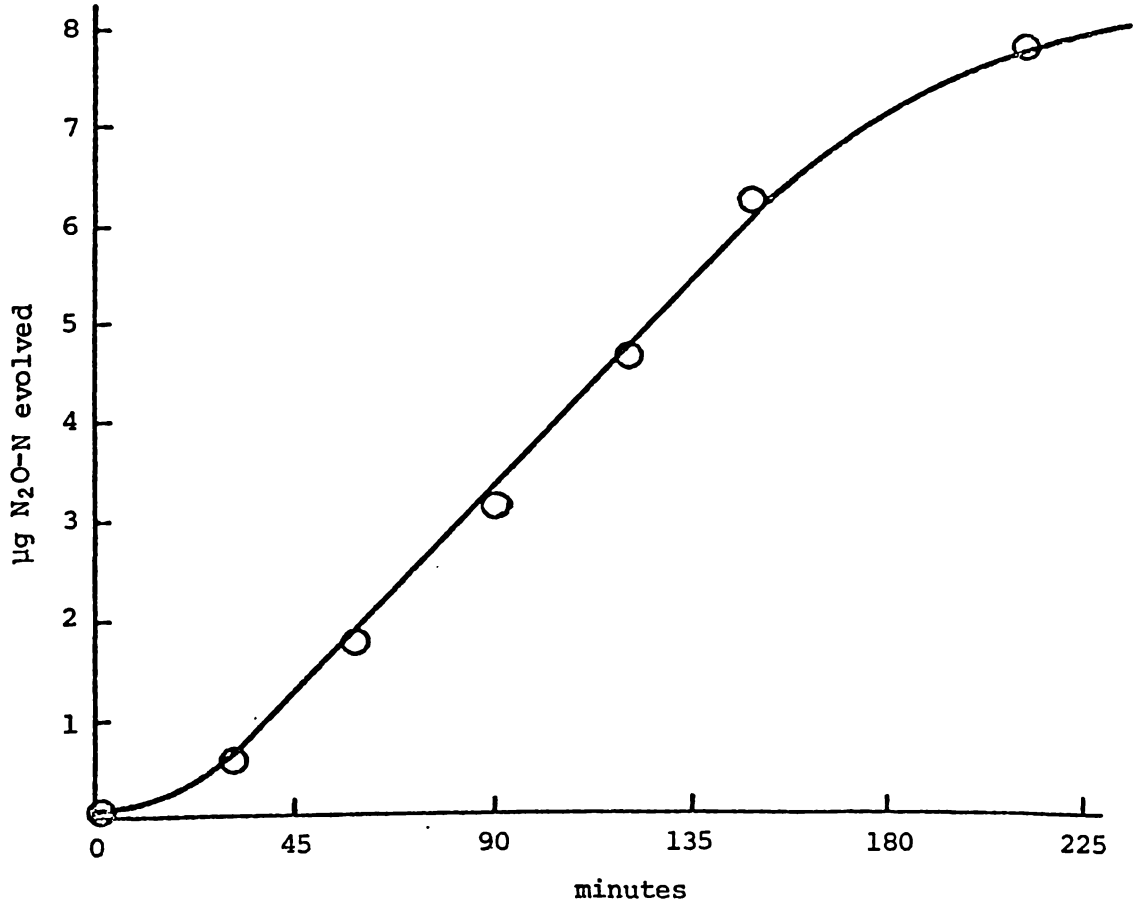
i) a stimulation of N_2O production resulting from the flushing of C_2H_2/O_2, or

ii) an increase in the rate of N_2O evolution.

The first alternative is unlikely as C_2H_2 has been shown to have no effect on the rate of denitrification, at least initially (sections 3.9.0 and 7.1.0), and the presence of oxygen can only inhibit denitrification.

Two factors could cause an increase in the rate of N_2O evolution

Figure 7.7 N_2O evolution from Acetylene field trial on Te Kowhai silt loam



(a) If sufficient C_2H_2 had not diffused into all actively denitrifying microsites before the first headspace samples were taken. In this case the rate of N_2O evolution would increase until complete inhibition of N_2O reductase had been effected. At this point the evolution of N_2O should become linear.

(b) Alternatively the tailing could represent a diffusion limitation on the rate of N_2O evolution. By flushing the soil core and establishing a high C_2H_2 concentration, the N_2O concentration within the soil profile would be reduced to near zero. Before evolution of N_2O at the soil surface can reflect the rate of production (i.e. assuming linearity) the concentration gradient within the soil profile must be re-established. Samples taken before this time would demonstrate a tailing effect similar to that seen in figure 7.7. The magnitude of the tailing effect can be calculated approximately using the diffusion equation (7.4)

$$F = -D\frac{dc}{dx}$$

where F = the flux of N_2O measured at the soil surface in the lysimeter, D is the diffusion coefficient of the soil and $\frac{dc}{dx}$ is the concentration gradient established such that the flux of N_2O equals the rate of N_2O production.

From table 7.1 the flux of N_2O during the period of linearity was ~3.1 μg N_2O-N/hr/lysimeter (28.6 cm^2). Using an assumed D value of 2 x 10^{-2} cm^2/sec (see section 6.6.1) the concentration gradient $\frac{dc}{dx}$ at the period of linearity can be calculated:

$$3.1\ \mu g\ N_2O\text{-}N/hr/28.6\ cm^2 = -2 \times 10^{-2}\ cm^2/sec \times \frac{\mu g\ N_2O\text{-}N/cm^3}{cm}$$

$$1.5 \times 10^{-3}\ \frac{\mu g\ N_2O\text{-}N}{cm^4} = \frac{-dc}{dx}\left(\frac{\mu g\ N_2O\text{-}N}{cm^4}\right) = \frac{\Delta c}{\Delta x}$$

Assuming the zone of N_2O production to extend over the top 3 cm (Δx):

$$4.5 \times 10^{-3} = -\Delta c \ (\mu g \ N_2O\text{-}N/cm^3).$$

The volume of soil enclosed by the production zone is ~85 cm^3, thus the quantity of N_2O produced to maintain the concentration gradient

$$= 4.5 \times 10^{-3} \times 85 \simeq 0.38 \ \mu g \ N_2O\text{-}N.$$

At a production rate of 3.1 µg N_2O-N/hr this represents a time lag of 0.12 hours or ~10 minutes.

This value is likely to be an underestimate as it was assumed in the calculations that no N_2O was lost from the soil while the concentration gradient was re-established. Therefore it is suggested that diffusion limitations are responsible for the tailing as the effect lasted for ~30 minutes.

The non-linearity after 150 minutes is likely to be due to the lowering of the concentration gradient between the soil profile and headspace to such an extent that the rate of N_2O evolution into the headspace decreases.

Linearity continued to a concentration of 18 ppm and the N_2O concentration in the headspace at 210 minutes was 23 ppm which is slightly higher than that recorded by Findlay and McKenney (1979).

Incubation studies conducted on the same soil (see figure 7.1) showed that nitrate was not likely to be a limitation to the denitrification rate measured over a short duration by the lysimeter. The linearity seen in N_2O evolution between 60 and 150 minutes (figure 7.7) also confirms that nitrate was not rate limiting at this period of the assay.

Based on the data in figure 7.7 an annual denitrification rate of 122 kg N/ha is calculated. This rate is however unlikely to occur consistently for 365 days of each year. Integrated over 12 months the loss is likely to be considerably reduced. Incubation data (figure 7.1) suggests the Te Kowhai soil was denitrifying at its maximum potential

in situ at the time of the lysimeter assessment. Therefore this value of 122 kg N/ha could be considered the maximum possible denitrification loss of N from the Te Kowhai soil annually.

Previous estimates (Steele, pers. comm.) on the N balance of the Te Kowhai soil are listed in table 7.2.

Table 7.2 N budget for Te Kowhai silt loam under grazing stock

Input (kg N/ha/yr)		Losses (kg N/ha/yr)	
N fixation	267	Animal products	66
Non-symbiotic fixation	14	Gaseous loss from dung	11
N in rain	3	Transfer to non-productive areas	46
		Gaseous loss from urine	17
		Leaching	110
	284		250

These figures suggest an annual denitrification loss in the order of 30-40 kg N/ha. The lysimeter data would support such an estimate.

Peat soils were considered a good contrast to the Te Kowhai silt loam. The Te Kowhai silt loam is known to possess high nitrification activity and subsequently has high nitrate concentrations within the soil. It was demonstrated earlier in this chapter that the Te Kowhai soil had sufficient endogenous nitrate reserves to provide valid *in situ* denitrification data using the C_2H_2 inhibition technique.

The Rukuhia peat has been shown to have a moderate to low INA value (section 4.2.3). Because of the very high water holding capacity and extreme porosity of peat soils significant leaching may be expected, and consequently, in line with low nitrification activities,

low nitrate reserves may be expected. If nitrate limitations were to occur due to the C_2H_2 inhibition of nitrification during the field denitrification assessment, peat soils were considered to be the likely site.

The method of assessment was as described for the Te Kowhai soils. The results (figure 7.8) offer several contrasts to those seen for the Te Kowhai soils. There is no time lag before the onset of full N_2O production - as evidenced by the immediate linearity of N_2O production. This most likely reflects the increased porosity of the peat soil and hence a reduced diffusion gradient within the profile. If the effective diffusion coefficient is increased due to the increased total porosity then the concentration gradient is similarly reduced if a flux equivalent to the rate of N_2O production is to be maintained.

The N_2O production is linear over a much shorter time span, and to a much lower level of N_2O within the headspace. This could be a reflection of either substrate or diffusion limitations. Due to the open porous structure of the peat, and the reduced concentration gradient, the reverse concentration gradient within the headspace can be expected to be much more significant for the peat soil compared to the Te Kowhai soil.

The substrate limitation was checked using the incubation technique described in section 7.2.0 with the following results (figure 7.9).

The incubation demonstrated that under anaerobic conditions endogenous nitrate substrate was totally exhausted after approximately 60 minutes. The quantity of N_2O evolved within the first sampling at 60 minutes is comparable in both the soils with and without added nitrate. The data suggests the endogenous nitrate supply lasted approximately 50-60 minutes.

While the incubation results demonstrate a smaller nitrate

Figure 7.8 N_2O evolution from acetylene field trial on Rukuhia peat

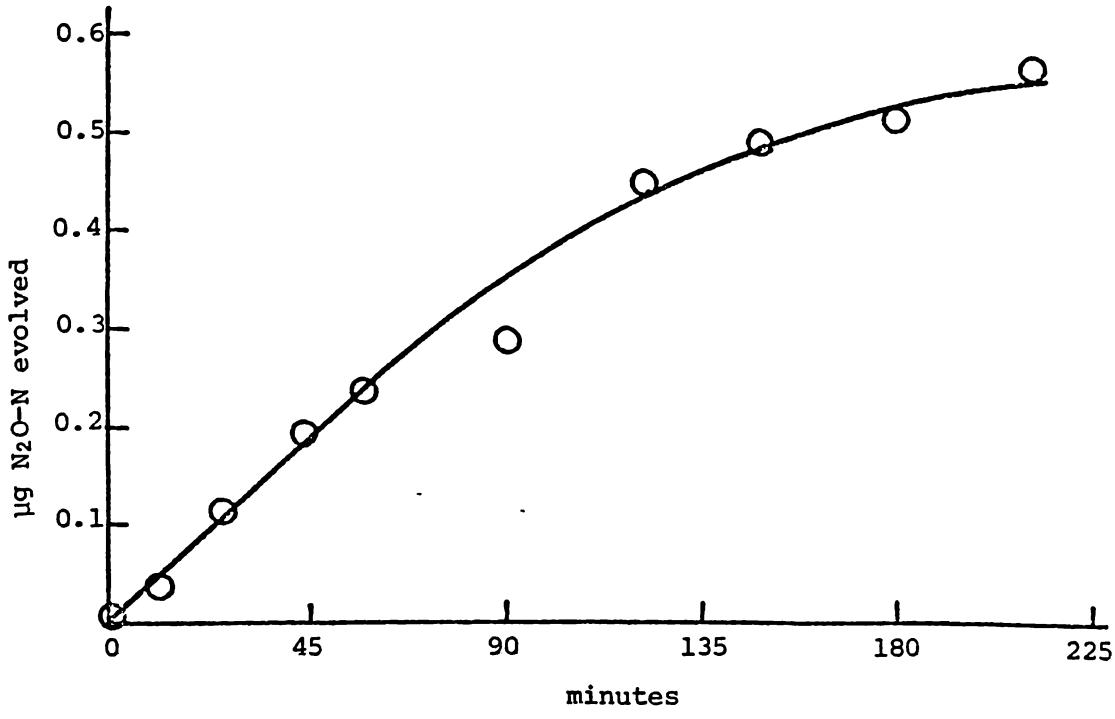
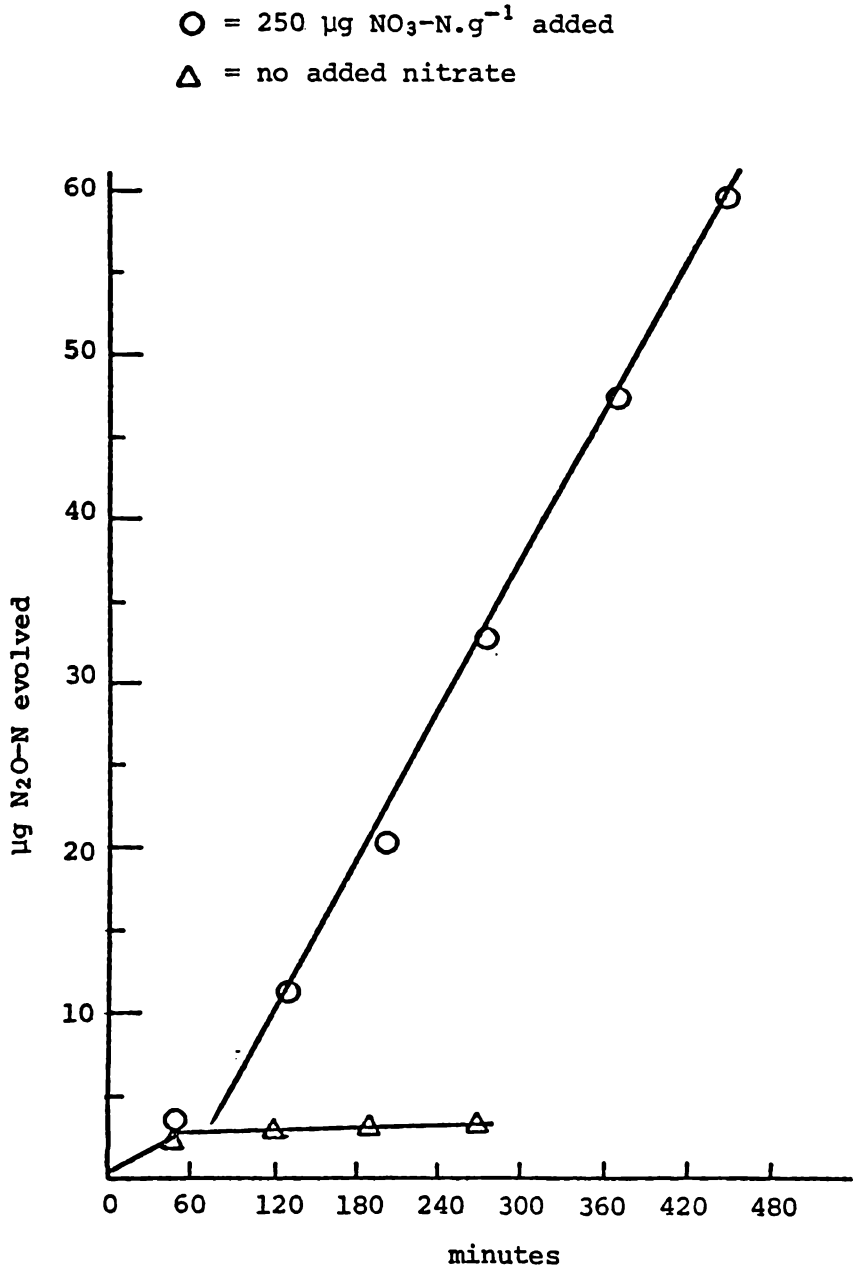


Figure 7.9 Incubation of Rukuhia peat with and without added nitrate



reserve compared to the Te Kowhai soil, the data in figure 7.9 demonstrates that nitrate would not be rate limiting within the first 60 minutes of the field assessment.

The N_2O yields confirm the belief that the peat soil had a lower endogenous nitrate level. The Te Kowhai soil yielded 19-25 µg N_2O-N while the peat only produced 3.5 µg N_2O-N during incubation.

The rate of denitrification calculated from figure 7.8 between 0 and 45 minutes is 10 kg N/ha/yr, if calculated on an annual basis at the same rate as measured. This rate is significantly lower than that of the Te Kowhai soil and also demonstrates the potential sensitivity of the technique.

The potential rate of N_2O production for the Rukuhia peat measured in the laboratory incubation (figure 7.9) is similar to that of the Te Kowhai. However, field measurements indicated rates of 10 and 120 kg N/ha/yr in the Rukuhia and Te Kowhai soils respectively.

It would appear that the rate measured on the Rukuhia peat in the field (10 kg N/ha/yr) was not the maximum potential. The biphasic rate of N_2O production of the Rukuhia peat continued some weeks after the Te Kowhai soil had achieved a state of full enzyme induction and a maximum denitrification potential. While the data in figure 7.9 does not demonstrate a distinct phase change, a time lag of between 60 and 90 minutes occurred before the onset of linearity in N_2O production indicating that the soil was not in a state of full enzyme induction.

The aerobic nature of the fibrous Rukuhia peat (see table 4.3) could be expected to result in an abnormally low phase I denitrification rate. However, with an abundance of available carbon (table 4.4) and in the presence of added nitrate (250 µg NO_3-N.g^{-1}) this soil could be expected to demonstrate a high denitrification potential in an anaerobic environment (as evidenced by figure 7.9).

Under drainage, peat soils are believed to undergo massive

mineralization. Nitrogen mineralization in histosols has been estimated to be as high as 1200 to 1400 kg N/ha/yr (Tate, 1976; Terry and Tate, 1980). Guthrie and Duxbury (1978) using columns of peat soil, demonstrated mineralization of up to 600 kg N/ha/yr without taking into account losses from denitrification.

Estimates suggest that losses by drainage water in these peat soils appear low in proportion to the rate of mineralization. Duxbury and Peverly (1978) measured losses rarely exceeding 100 kg N/ha/yr and Terry and Tate (1980) suggested losses of a similar magnitude.

The suggestion is therefore that peat soils are capable of removing large quantities of N annually via denitrification. Terry and Tate (1980) measured a denitrification capacity sufficient to denitrify all the mineralized N in the Everglades Histosols. Guthrie and Duxbury (1978) however, were only able to account for 9-18% of added nitrate as denitrification losses during column studies on histosols. It is interesting to note that a significant proportion of this loss (50-90%) was recovered as N_2O in leachate. Terry and Tate (1980) in spite of measuring a large denitrification capacity, did also notice a nitrate increase deep in the profile after rainfall. They concluded that at least a proportion of the nitrate present at the 60-70 cm depth resulted from leaching.

All the denitrification data recorded for the Rukuhia peat during the current investigation suggests a denitrification rate far below that required to remove 600 kg N/ha annually. The denitrification data suggests that either the rate of mineralization is not as high as in some other histosols or that leaching is a more significant loss of N in the Rukuhia peat than has been recorded for other histosols.

There is indeed evidence to suggest that rates of N mineralization for the Rukuhia peat may not be as high as reported for other histosols. Steele (1977) recorded INA values between 0.9 and 0.1

implying a moderate to low nitrifying activity in comparison to other soils. Terry and Tate (1980) and Guthrie and Duxbury (1978) reported pH values of 5.8 to 6.0 in the histosols of high N mineralization. Steele reported much less conducive pH values of around 5.0 in the Rukuhia peat.

It is also known that Rukuhia peat soils demonstrate N deficiencies during N response trials (Steele, pers. comm.) However, Guthrie and Duxbury (1978) also noted this phenomenon in histosols capable of mineralizing up to 600 kg N/ha/yr. Their explanation is that winter and spring leaching events remove much of the mineral N from the soil and the rate of mineralization, which is limited by low soil temperatures at the beginning of the growing season is insufficient to meet the early demands of the crop.

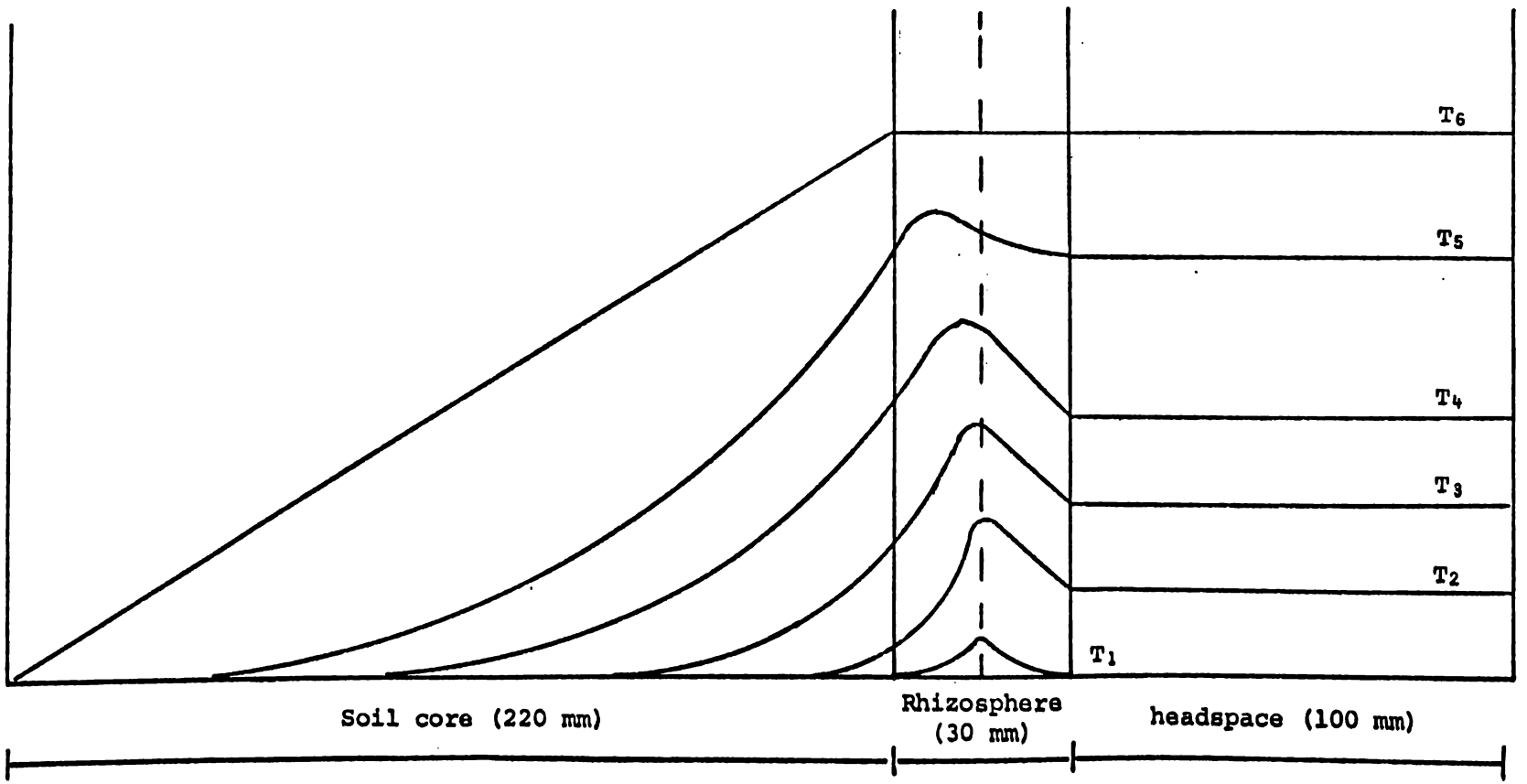
7.6.0 MODEL FOR N_2O FLUX DURING LYSIMETER ASSESSMENTS

The behaviour of the N_2O flux with respect to the changing N_2O concentration in the soil can be depicted by figure 7.10.

After purging the soil and headspace of N_2O (T_0) continuation of N_2O evolution within the 3 cm rhizosphere will re-establish a concentration profile (T_1). When the N_2O reaches the soil/headspace interface the concentration gradient will decrease rapidly as the N_2O escapes into the headspace. Therefore a "steady state" release of N_2O will rapidly develop (T_2). Diffusion of the N_2O into the soil profile is much slower because of the smaller diffusion coefficient and the greater diffusion path length. At some further time (T_3) the N_2O concentration within the zone of production and headspace will increase further. The flux into the headspace at this time is linear and the flux leakage into the soil profile is assumed to be negligible.

As the concentration within the headspace and rhizosphere increases, so does the concentration gradient between the rhizosphere

Figure 7.10 Schematic model for N_2O diffusion into an enclosed headspace and soil profile from a production source close to the surface



and the soil core. Effectively the 'site of N_2O production' moves down the profile a little in response to the increased concentration gradient created within the headspace and rhizosphere (T_4).

Production and effusion into the headspace continues until the concentration gradient between the headspace and soil core below the rhizosphere increases to the extent that the downward flux becomes significant. When this occurs the flux into the headspace decreases and the concentration gradient levels out (T_5). Non-linearity of N_2O evolution into the headspace is monitored at the point where this downward flux is significantly greater than the experimental error of N_2O measurement. Eventually a steady state situation is established where the total N_2O production is seen to move downward (T_6). At this point the headspace N_2O concentration is seen to reach the equilibrium value noticed by other workers (Galbally and Roy, 1978; Findlay and McKenney, 1979). The time taken from the deviation of linearity to the attainment of equilibrium reflects the time required to achieve a steady state situation in the soil core.

Both the rate of production and the effective diffusion coefficient of the soil affect the concentration gradient and therefore the time over which linearity is observed. The smaller the Dp and production rate, the longer the period of linearity.

The boundary condition where $C_{N_2O} = 0$ at the base of the lysimeter (figure 7.10) is fulfilled by the base-flow. This extends the period of linearity by preventing inward diffusion of soil N_2O.

The period of linearity can also be expected to be longer than the 30-90 minutes observed by surface chamber techniques (e.g. McKenney *et al*, 1978) due to the horizontal boundary conditions imposed by the lysimeter. Horizontal flux is zero therefore only vertical fluxes occur. In the surface chamber situation, once the N_2O concentration in the headspace increases, a small change in the

concentration gradient in the rhizosphere will allow N_2O to move horizontally and then out to the atmosphere.

7.7.0 CRITICISMS ACCOMPANYING THE LYSIMETER-ACETYLENE TECHNIQUE

i) *Profile disturbance*

Matthias *et al* (1980) measured lower rates of N_2O emission in chambers placed on the soil surface and sealed with a foam collar than in chambers inserted into the soil. Whether soil disturbances of this type increase the rate of N_2O evolution, or the entire rate of denitrification is unknown.

ii) *Estimation of chamber headspace volume*

The chamber headspace volume is considered to be defined by the soil surface. However, a certain volume of pores within the soil surface are in or very close to equilibrium with the headspace atmosphere. These pores must be considered as part of the chamber volume in calculating N_2O emission yields. This effect will vary depending on the porosity and effective diffusion coefficient for each soil. The error will be more significant in soils like Rukuhia peat where the porous nature of the peat contributes significantly to the headspace volume. This effect can be partially negated by increasing the headspace volume until soil porosity contributions become insignificant. The headspace volume in the case of the Rukuhia peat was increased from 300 to 400 cc. Such an effect is unlikely in most cases to extend further than one or two centimetres into the soil profile.

iii) *Estimation of N_2O evolution*

Matthias *et al* (1978) produced data showing that N_2 evolution into a headspace is curvilinear and thus straight line analyses under estimate the true N_2O flux. They calculated a series of curves of N_2O emission into closed chambers of various heights using the two

dimensional linear gas diffusion equation:

$$\frac{d}{dt} C(x,z,t) = D\left\{\frac{d^2}{dx^2} C(x,z,t) + \frac{d^2}{dz^2} C(x,z,t)\right\} + P \qquad 7.6$$

where $C(x,z,t)$ is the gas concentration (gm/cm^3) of the soil air at the horizontal and vertical position (x,z) and time (t). D is the constant gas diffusion coefficient (cm^2/sec) and P is the production or destruction rate ($gm/cm^3/sec$).

The boundary conditions selected for the closed chamber system designated the lower boundary at a constant value of 1200 ppb with an initial upper boundary of 300 ppb. These boundary conditions essentially simulate an upward flux from a source region that is constant over the measurement period.

Rates of N_2O emission were calculated for chamber heights between 5 and 30 cm. These curvilinear plots were then fitted with linear, quadratic and exponential regression equations to compare the best estimates of the N_2O flux. The linear regression was shown to under estimate N_2O fluxes by 55% for the smallest chamber and 10% for the largest chamber. The exponential regression was shown to fit the closest with only an 11% under estimation for the smallest chamber.

Several workers (e.g. McKenney *et al*, 1978; Findlay and McKenney, 1979) including Matthias *et al* (1980) have however demonstrated good fits of linear regressions to N_2O evolution data. Matthias *et al* reported r^2 values of 0.985 or better. The discrepancy between the linear correlations seen in the experimental data and the calculated predictions (Matthias *et al*, 1978) suggested an error in the assumptions of the predicted data.

Matthias *et al* (1978) made the assumption that there were no gas sources or sinks within the linear profile. In practise this is unlikely and the evidence (section 4.3.1) suggests the major N_2O source to be located very near the soil surface. This source close to

the surface would have the effect of diminishing the reverse concentration gradient contribution from the headspace as the source is so near the headspace itself. If the source was actually situated within the headspace then a diffusion limitation on the rate of N_2O evolution into the headspace would not exist.

Therefore the linear regression analysis of N_2O evolution data over a limited period is not likely to be a significant under estimation of the N_2O flux into a headspace in an undisturbed soil profile.

iv) *Effect of flushing*

The void space created within the lysimeter as a result of flushing constitutes a sink for N_2O subsequently produced. Thus the N_2O effusing from the soil surface is likely to under estimate the true flux of N_2O *in situ* while the sink in the soil profile fills up to its previous equilibrium level. During this period the N_2O evolution will appear non-linear. Eventually the equilibrium N_2O distribution will be achieved and the N_2O flux at the surface will become linear and representative of the rate of production.

The C_2H_2 flushing technique described by Ryden *et al* (1979) will also suffer from this criticism. Although the quoted C_2H_2 concentrations in the pores were low (~0.5-2.0%), flushing at 28 ℓ/hr must displace an equivalent amount of soil air from the profile. Therefore any C_2H_2 flushing technique in the soil profile is likely to suffer from such a criticism.

The magnitude of this effect can be lessened knowing that in those soils studied, most denitrification took place within the top few centimetres of the profile. Therefore the lysimeter need only be inserted to a depth of approximately 10 cm and subsequently the pore volume flushed below the denitrification zone is significantly reduced.

7.8.0 CONCLUSIONS

The static sampling system employed in conjunction with the lysimeter and acetylene inhibition technique offers a useful, simple *in situ* denitrification assessment. The lysimeter offers the advantage over previous C_2H_2 field techniques of establishing and maintaining a uniformly high C_2H_2 concentration within the soil profile easily and quickly. It also offers the opportunity of closely controlling the O_2 status of the soil profile. By monitoring the O_2 concentration of the soil air in the region of interest and flushing the soil core with a similar O_2 content, minimum disturbance to the aeration of the soil profile can be achieved. This is an advantage not available with current techniques. Similarly the lysimeter by accurately controlling the aeration of the soil core, allows the effect of small variations of the O_2 content upon denitrification rates to be studied.

Criticisms inherent in flushing a soil core and reducing the ambient levels of gases of interest can largely be overcome by inserting the lysimeter to a minimum depth (10 cm).

Due to the effectiveness of the base-flow in reducing inward diffusion of soil gases, longer periods of linearity of N_2O evolution should be possible compared to previous static chamber techniques.

Data collected by this technique suggests a maximum possible N loss by denitrification in the Te Kowhai soil of 120 kg N/ha/yr. This value supports previous estimates calculated by deficit budgets.

The denitrification rate of the Rukuhia peat appeared very low (~10 kg N/ha/yr). This value would be in accordance with the low levels of nitrification activity measured by others. It is suggested the low rate of denitrification measured in the field is a result of N substrate limitations and partial repression of denitrifying

enzymes. Incubation data suggests that the rate measured in the field was not the maximum denitrification potential.

The potential denitrification rate of the Rukuhia peat measured in the laboratory is similar to that of the Te Kowhai soil. This could be expected due to the presence of a high level of available carbon, and adequate N substrate in the form of nitrate additions.

Data suggests the rate of mineralization in the Rukuhia peat may not be as great as some histosols reported in the literature. This is possibly due to the pH of the Rukuhia peat being significantly lower than that of the histosols. Consequently the low measured rate of denitrification was attributed to low nitrate levels, and an aerobic profile. Leaching was considered to be the major method of N removal from the peat soil.

CHAPTER 8

DIRECT ASSESSMENT OF NITROGEN EVOLUTION

8.1.0 Measurement of N_2 production has been virtually impossible because of the extremely high atmospheric background (Hauck and Melstead, 1956; Galsworthy and Burford, 1978). The lysimeter described in chapter 6 was designed to reduce the N_2 background in a soil core by purging with an inert gas (Ar) containing the required O_2 concentration to maintain the existing O_2 concentration within the soil profile. A lowered N_2 background in the soil core would enable N_2 production from denitrification to be more easily assessed using isotopic dilution principles.

After a low N_2 background is established a spike of N-15 enriched N_2 is added to the flushing gas and the flow maintained until the isotopically enriched N_2 is established in the soil. The gas flow is then discontinued.

Nitrogen resulting from denitrification will alter the initial $^{15}N/^{14}N$ ratio in the soil atmosphere and the amount of N_2 produced is determined by measurement of the change in the $^{15}N/^{14}N$ ratio with time. The high precision obtainable using a Micromass 602c mass spectrometer will enable small changes in isotopic composition to be easily measured.

A base-flow of the same gas mixture containing the N-15 spike would hinder inward diffusion of soil air.

Preliminary calculations indicated that it should be possible to measure rates of N_2 evolution as low as 10 kg N/ha/yr.

8.2.0 PRINCIPLES OF MICROMASS OPERATION

The Micromass 602c is a precision mass spectrometer designed for the measurement of isotopic enrichment (or depletion) of gas samples relative to a reference gas. The ion beams of the major and minor isotopic components of the gas are collected simultaneously in twin Faraday bucket collectors. The current difference between the two collectors produces a current ratio which is repeatedly compared to

the current ratio of the reference gas of known isotopic composition.

Full details concerning the operation of the Micromass 602c are given in the Micromass 602c Instruction Manual.

While capable of measuring two ion beams simultaneously the Micromass is also able to function in the single beam scanning mode. This capacity is necessary for preliminary peaking up of the mass number to be measured.

8.3.0 QUANTITATIVE SINGLE BEAM MEASUREMENTS

Using the mass spectrometer in the single beam mode, it was hoped to quantitatively measure the other denitrification product of interest, i.e. N_2O. N_2O in the ion chamber of the mass spectrometer partially fragments to produce NO and N_2 (Friedel *et al*, 1953) and measurement of peaks m/e 30 (NO) or m/e 44 (N_2O) should permit an estimate of the N_2O content of samples.

A series of N_2O standards were prepared by injecting N_2O into flasks of Argon. The m/e 30 and 44 peaks were compared to a reference standard of 0.30% N_2O by switching manually between the sample and reference inlets. This technique compensates for instrumental variations during analysis. Single beam calibrations of m/e 30 and 44 for N_2O are shown in figures 8.1 (a) and (b). Peak heights (Amps) are expressed as the ratios of the $\frac{\text{Sample } N_2O \text{ peak height}}{\text{Reference } N_2O \text{ peak height}}$ in figure 8.1 (a).

Sample pressures were adjusted comparing the sample ^{36}Ar peak to the reference ^{36}Ar peak. There will be a small, negligible, error in this method equivalent to the partial pressure contribution of other gases produced during denitrification. If the product gases (e.g. CO_2, N_2O and N_2) in the sample amount to 1% of the composition then the pressure difference between the sample and reference gas will be 1%. Ion chamber pressures were equalised by adjusting the sample

Figure 8.1 (a) Mass spectrometer single-beam calibration for N_2O (in Argon)
Ion chamber pressure = $2 \times 10^{-8}\tau$

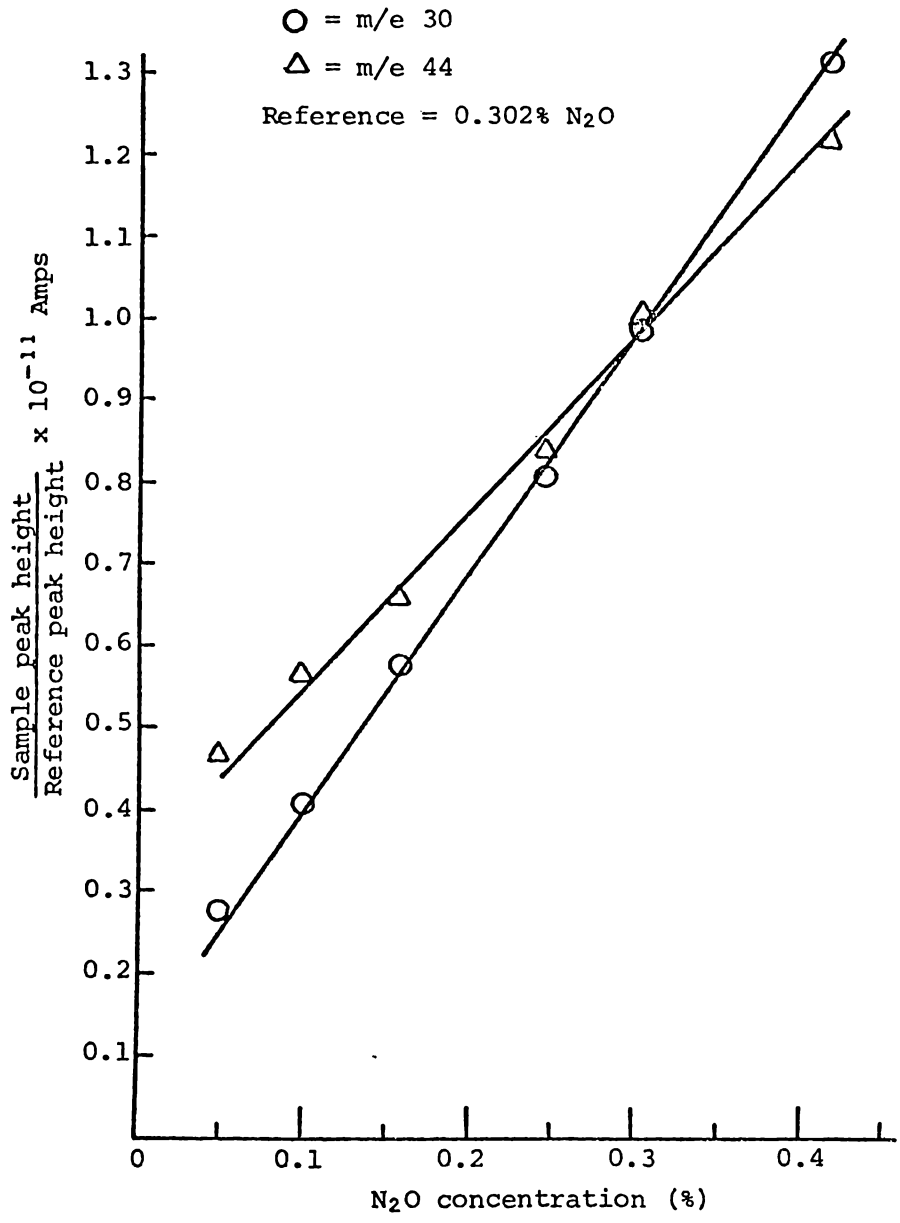
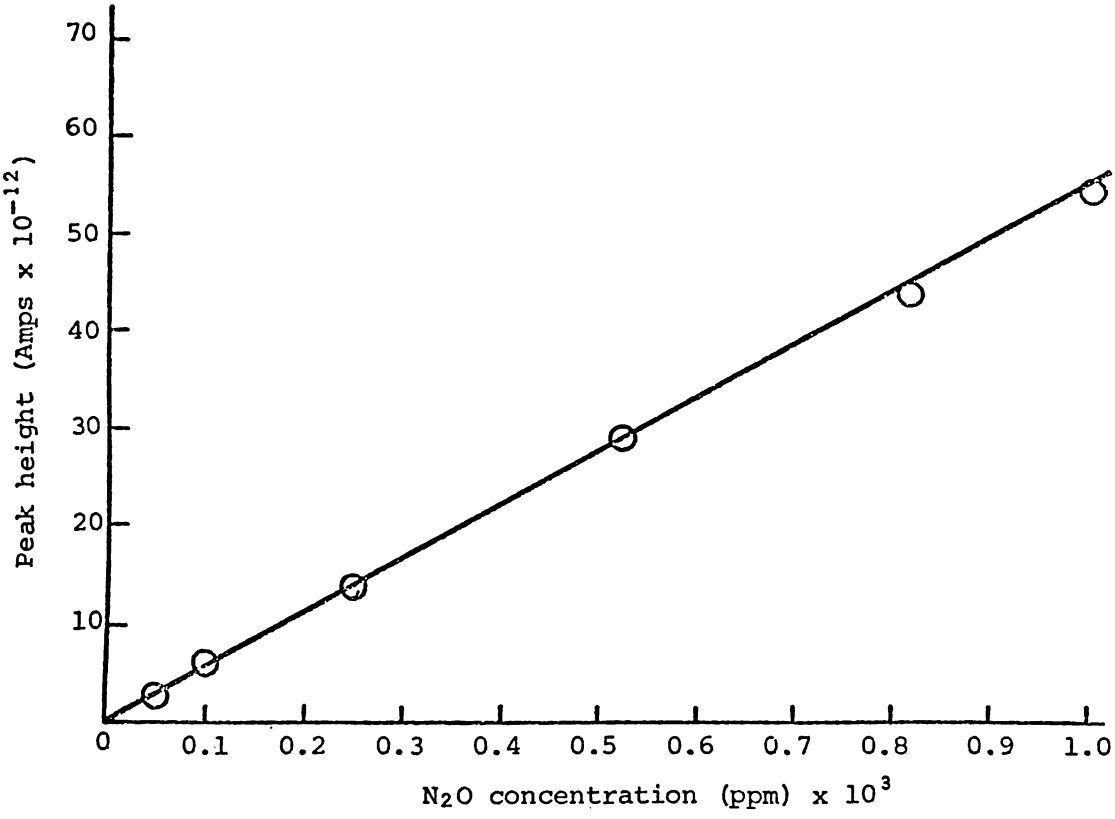


Figure 8.1 (b) Mass spectrometer single-beam calibration for N_2O (m/e 30)
Ion chamber pressure = $4 \times 10^{-8}\tau$



volume in the adjustable bellows.

The fragmentation pattern of N_2O i.e. m/e $\frac{44}{30}$ remained constant irrespective of the N_2O partial pressure. However, the fragmentation pattern was sensitive to pressure changes in the ion chamber (figure 8.2).

Fragmentation is greatest under higher ion chamber pressures ($10^{-7}\tau$) (figure 8.2.). A three fold increase in fragmentation occurred when the ion chamber pressure increased from $5 \times 10^{-9}\tau$ to $1 \times 10^{-7}\tau$. This variation is not a problem during analyses providing both the standard and reference gases are adjusted to the same ion chamber pressure, therefore inducing the same degree of fragmentation.

The other gas likely to be of interest in single beam analyses was CO_2. Calibration of the mass spectrometer for CO_2 using m/e 44 was conducted in a similar manner (figure 8.3). The CO_2/CO fragmentation pattern also demonstrated a pressure dependence.

8.3.1 N_2O/CO_2 Interferences

N_2O (a.m.u.44.013) has approximately the same mass as CO_2 (a.m.u.43.909). The Micromass was designed as a low resolution mass spectrometer. The low resolution feature allows broad, flat-topped peaks and assists stable ion beam ratio measurements. Because of this, the Micromass is not capable of resolving m/e values as close together as N_2O and CO_2 or N_2 and CO.

This overlapping of m/e values under a single peak causes interference problems through contributions from ion species other than the one of interest. As both CO_2 and N_2O contribute to the m/e 44, it is not possible to use the 44 peak as an indication of N_2O in the presence of CO_2. The NO peak (m/e 30) can however, be used to estimate N_2O (figure 8 (a)). If the fragmentation pattern for a particular ion chamber pressure is known, then the contribution of N_2O

Figure 8.2 Variation in N_2O:NO fragmentation pattern with ion chamber pressure

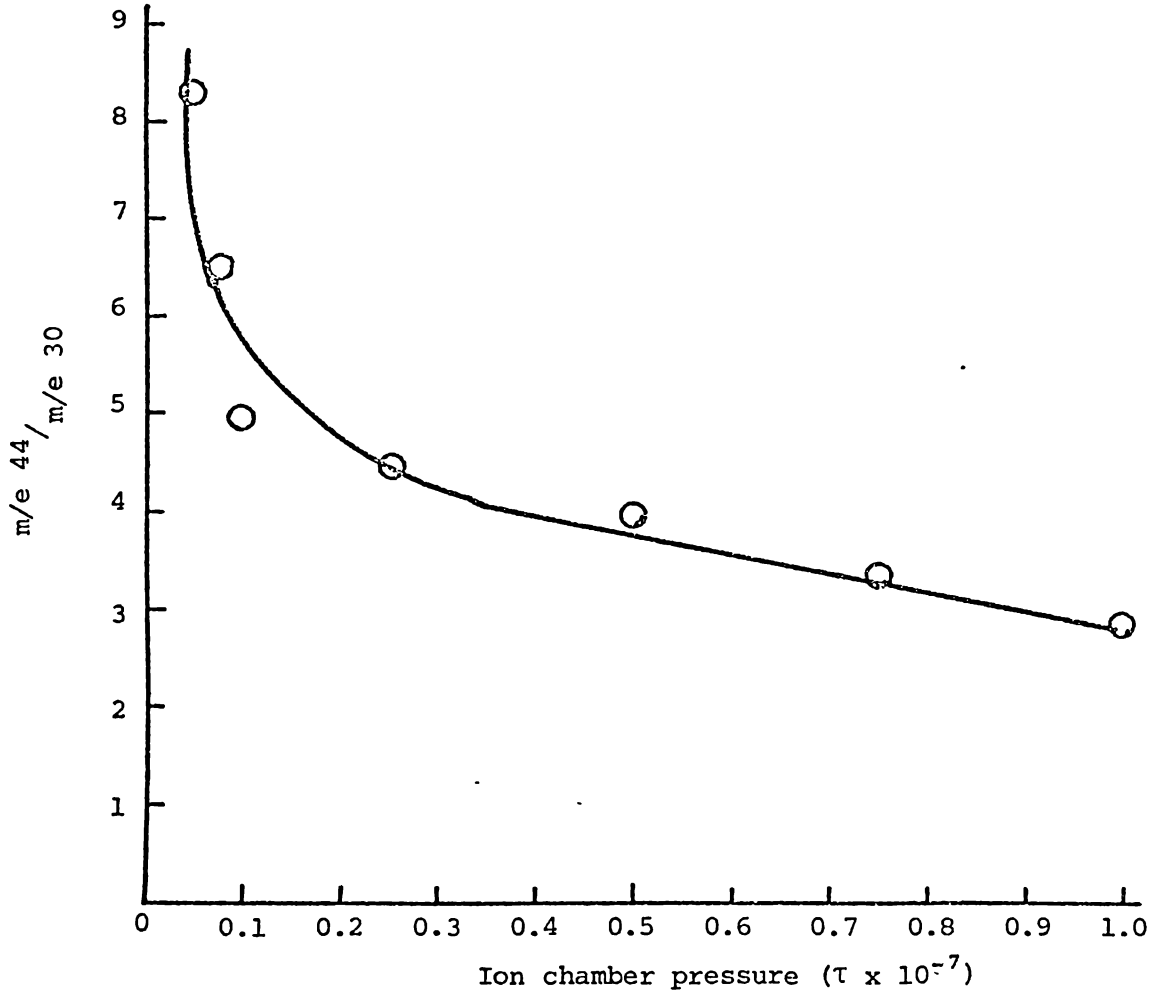
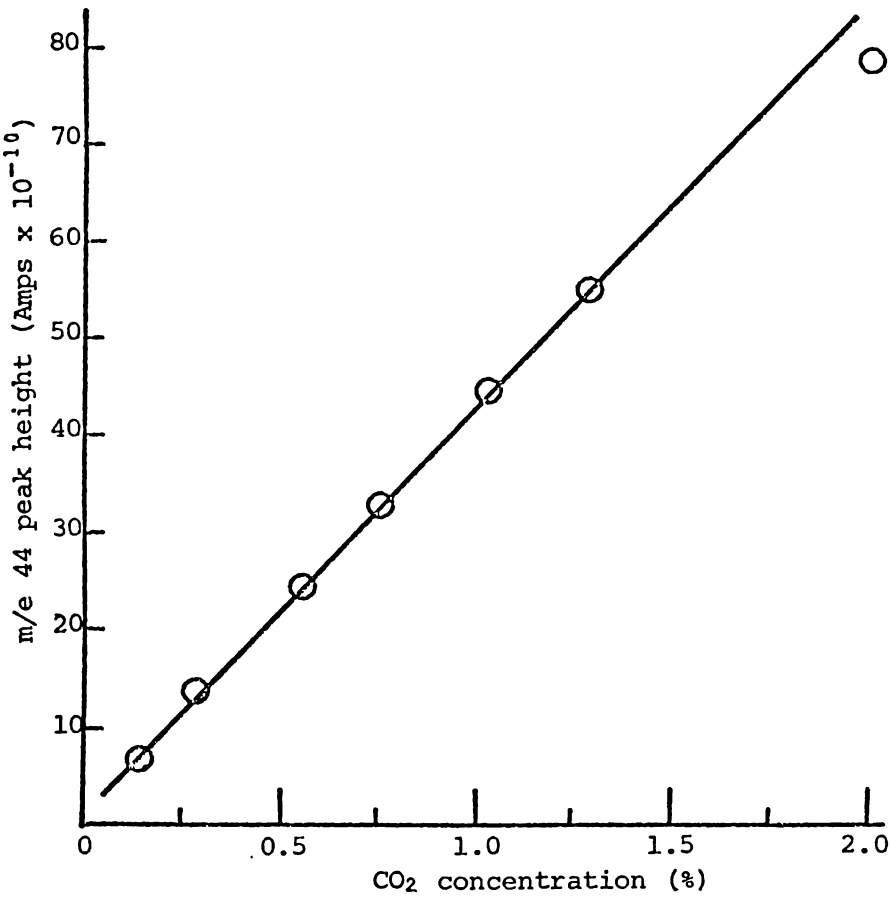


Figure 8.3 Mass spectrometer single-beam calibration of CO_2 (m/e 44) in Argon.
Ion chamber pressure = $1x10^{-7}$ τ



to the m/e 44 peak can be deduced.

CO_2 also contributes to a doubly ionized peak of m/e 22 (CO_2^{+2}). Measurement of this peak would allow direct assessment of the CO_2 content, simplifying analyses. Unfortunately the Micromass 602c is only able to scan as low as m/e 26 with the magnet supplied for double beam analyses of masses 29 and 28.

8.3.2 Standard deviation of single beam δ measurements

Reducing the N_2 concentration of the ^{15}N-N_2 gas could achieve an increase in sensitivity of the overall detection capability for N_2 evolution.

With N_2 concentrations of around 10,000 ppm, at ion chamber pressures of $10^{-6}\tau$, a maximum m/e 28 signal of approximately 20 x 10^{-9} Amps is obtained (varying with isotopic composition). As the Micromass was designed to analyse pure gas samples, the major beam facility is provided with limited amplification. It is not possible to increase the major beam (m/e 28) amplification beyond 10^{-9} Amps. Thus, while the minor beam aplification extends from 10^{-13} to 10^{-9} Amps, on the double beam mode, it is not possible to fully utilize this increased amplification; so double beam analyses are limited by the major beam amplification. For this reason the possibility of lowering the N_2 background even further, to say 1000 ppm with the subsequent $\delta^{15}N$ determination being made in the single beam mode was considered.

Analysis of the errors involved in these $\frac{29}{28}$ ratio measurements are analagous to comparing two peak heights (or areas) of a gas chromatographic trace as both the 29 and 28 peaks are scanned individually. The percentage error in the estimation of these peak areas will vary depending on the size of the peaks. To calculate an optimum error, assume both peaks, A and B are large, i.e. >90% full scale. An optimum error of 1% is normally associated with gas

chromatographic peak area analyses measured using an integrator.

From equation 8.1 (Appendix 1):

$$\delta^{15}N(^{\circ}/_{oo}) = \left[\frac{\left(\frac{29}{28}\right)\text{Sample} - \left(\frac{29}{28}\right)\text{Reference}}{\left(\frac{29}{28}\right)\text{Reference}} \right] \times 1000 \qquad 8.1$$

The error of the $\left(\frac{29}{28}\right)$ S or R estimate is calculated by $\sqrt{a^2+b^2}$ where a and b are the respective percentage errors of peaks A and B. Thus, a combined error of 1.4% is incurred in each $\frac{29}{28}$ calculation. The error in $\left(\frac{29}{28}\right)_S - \left(\frac{29}{28}\right)_R$ is calculated by $\sqrt{a^2+b^2}$ where a and b are the actual proportional errors of values A and B where A and B represent the $\left(\frac{29}{28}\right)_S$ and $\left(\frac{29}{28}\right)_R$ values respectively. Thus $\left(\frac{29}{28}\right)_S \pm 1.4\% - \left(\frac{29}{28}\right)_R \pm 1.4\%$ incurs a percentage error of approximately $\sqrt{(0.14)^2+(.014)^2} \times 100$ if both A and B are near full scale = 1.9%.

The final calculation of $\frac{[\ ]}{\left(\frac{29}{28}\right)_R}$ where [ ] is $\left(\frac{29}{28}\right)_S - \left(\frac{29}{28}\right)_R$ incurs an error of $\sqrt{(1.9)^2+(1.4)^2} = 2.36\%$.

This 2.36% error gives an error in δ values of 23.6‰. This is approximately 200 times the error (3δ) associated with a double beam measurement on a 10,000 ppm N_2 sample (see section 8.4.1).

The magnitude of the errors in single beam $\delta^{15}N$ estimates make such a method redundant when double beam facilities are available.

8.3.3 Correction for valve mixing

The reference/sample changeover is controlled by a solenoid operated sapphire ball. This ball seals off one gas port and simultaneously opens the other. It is necessary in some instances to make allowances for a leakage across the ball, known as valve-mixing.

By admitting a gas sample of 50 ppm N_2O at $4 \times 10^{-8}\tau$ and

changing the reference gas from 50 to 530 ppm N_2O, the m/e 30 peak of the sample increased by 20%. Thus valve-mixing becomes important when large concentration differences apply between sample and reference. An allowance can be made for this discrepancy by subtracting the contribution of the other peak or else a reference gas with a peak of similar magnitude can be selected to overcome this problem.

8.4.0 DOUBLE BEAM ^{15}N analyses

8.4.1 Standard deviation (σ) of double beam $\delta^{15}N$ measurements

The value of isotopic enrichment (or depletion) is normally expressed as the per mil ($^{\circ}/_{oo}$) deviation of a sample with respect to a reference, i.e.

$$\delta(^{\circ}/_{oo}) = \left(\frac{R_S - R_R}{R_R} \right) 1000 \qquad 8.2$$

where R_S is the isotopic ratio of the sample and R_R the isotopic ratio of the reference (Steele *et al*, 1978). (see appendix 1)

The standard deviation of $\delta^{15}N$ values will also affect the sensitivity of the technique. The best standard deviation for a sample of 12,400 ppm N_2 of $\frac{29}{28}$ ratio of 0.00700 (i.e. approximately natural abundance) was $\pm 0.04^{o}/_{oo}$.

This standard deviation is calculated from the formula:

$$\sigma(^{\circ}/_{oo}) = \frac{\sqrt{\frac{\Sigma(x_n - \bar{x})^2}{n-1}}}{R+\bar{r}} \times 10^3 \qquad 8.3$$

(see appendix 1) where R is the coarse ratio (R_c) and $\bar{r}$ is the average of the reference digital integrator results. These $\bar{r}$ values are x 10^{-4}, thus $R+\bar{r}$ for example becomes:

R_c	0.700
$+ \bar{r}$	63.72
$R_c + \bar{r}$	0.706372

It is apparent that $\bar{r}$ has only a small influence on $R + \bar{r}$ and that the $\sigma(‰)$ will vary directly with the magnitude of R_c. For large values of R_c (e.g. 0.999), σ is going to be the smallest possible value for a given number of measurements. For small values of R_c (e.g. 0.100), σ is larger, by a factor of 9.99 in fact.

The reason for this is that as the R_c value becomes smaller, so the trace on the chart recorder (see figure 8.6) (or the values represented by the digital integrator) represent a proportionally larger value. As the signal/noise ratio deteriorates, the errors become more significant.

So it is important in considering isotopic ratio measurements that the R_c be as large as possible. If both the sample and reference traces do not fall within the span of the chart recorder, then the R_c values must be different. To compensate, an adjustment using the offset ratio (R_o) must be used. If the R_c of the reference was 0.366 and the R_c of a sample was 0.416, then an R_o value of +.050 would be necessary in order that both traces coincided on the chart recorder page. This adjustment affects the σ value enormously. An error in the trace of 3 divisions (i.e. 3% full scale) on the chart yields a σ value of 135‰ compared to 0.8‰ at a coarse ratio of 0.366 if a R_o of +.050 is implemented. So it is essential that the coarse ratio of the sample be as close as possible to the reference coarse ratio, to avoid using any ratio offset.

For this reason it was planned to use the preceding sample as the next reference and so on.

8.5.0 Sensitivity of measurements of N_2 evolution

The dual inlet system allows direct and repetitive comparisons between sample and reference $\frac{29}{28}$ ratios. Steele (1977) was able to demonstrate a total error in N-15 determinations of less than ±0.5°/₀₀ using the Micromass 602c. This error included errors introduced during subsampling and chemical preparation of N_2 gas for analyses. The standard error for the Micromass measurement was in the range of ±0.05°/₀₀ (Steele, 1977, p287).

The overall sensitivity of the technique towards detection of N_2 evolved will depend on the N-15 enrichment of the N_2 gas. The N_2 evolved during microbial denitrification is essentially of atmospheric or natural N-15 abundance. Wellman *et al* (1968) and Delwiche and Steyn (1970) examined the isotope fractionation during denitrification by *Pseudomonas denitrificans* and reported β values of 1.02 and 1.017 respectively. The β value is defined as $\frac{Ra}{Rd}$ where Ra = $\frac{^{15}N}{^{14}N}$ in the atmosphere and Rd = $\frac{^{15}N}{^{14}N}$ in the N_2 produced by denitrification. These values are small enough to be ignored when compared to the high enrichment envisaged for the $^{15}N_2$ spike gas. Fractionations of this magnitude are only of significance in near natural abundance studies.

The natural abundance of N-15 in the atmosphere is 0.366 At% (Nier, 1950). Consequently, approximately 99% of the N_2 produced by denitrifying organisms will consist of m/e 28. Increasing the N-15 enrichment of the spike gas increases the sensitivity of the technique. By increasing the enrichment of the spike gas, the m/e 28 contribution becomes diminished, thereby increasing the proportion of m/e 28 contributed by microbial denitrification. There is however, a limit to the degree of enrichment the Micromass can analyse. It is a condition of the Micromass double beam facility that the signal from the minor isotope (m/e 29) does not exceed the magnitude of the signal from the major isotope, thus $I_{maj} > I_{min}$ (where I = ion current). The

$\frac{29}{28}$ signal ratio is initially balanced by the 3 digit coarse ratio control (R_c). Therefore if $I_{maj} > I_{min}$, the highest ratio signal that can be accepted is 0.999. Thus the m/e 29 peak $(^{14}N^{15}N)^+$ is 99.9% of the $(^{14}N^{14}N)^+$ peak, i.e.

$$\frac{29}{28} = 0.999.$$

The isotopic distribution of gaseous species $^{14}N^{14}N$, $^{15}N^{14}N$ and $^{15}N^{15}N$ are determined by the statistical values given by:

$$(p + q)^2 = p^2 + 2pq + q^2 \qquad 8.4$$

where p is the atom fraction of N-14 and q is the atom fraction of N-15, and p + q is equal to unity (Hauck and Bremner, 1976). Because of the extremely slow isotopic exchange:

$$^{14}N^{14}N + {}^{15}N^{15}N \rightleftharpoons 2\,{}^{14}N^{15}N \qquad 8.5$$

samples of N_2 gas with dissimilar distributions of isotopes will each retain their identity when mixed.

Therefore, for $R_c = 0.999$, $\frac{2pq}{p^2} = 0.999$

$$2pq = 0.999\ p^2$$

$$\text{therefore} \quad q = 0.499\ p$$

$$\text{as } p + q = 1$$

$$p + 0.499\ p = 1$$

$$p = 0.667$$

$$\text{or} \quad q = 0.333$$

Thus the highest $\frac{29}{28}$ ratio the Micromass can measure by double beam is 33.3 At% N-15. This value is approximate to the degree of preferential ionization of the $(^{14}N^{14}N)^+$ or $(^{14}N^{15}N)^+$ species in the mass spectrometer.

In fact the Micromass can cope with higher atom % N-15 enrichments, but only by considering the $\frac{30}{29}$ ratio. On a similar

basis to the calculations above, it can be shown that the maximum isotopic ratio to be measured by this procedure is 66.6 At% N-15.

However, the purpose of the calculations is to demonstrate the theoretical sensitivity of the N-15 technique in determining N_2 evolution from the soil. This N_2 evolved by denitrification from endogenous NO_3^- is approximately 0.366 At% N-15. The $\frac{29}{28}$ ratio in this instance is 0.00366, i.e. 99.634%of the N_2 evolved is in the $^{14}N^{14}N$ form. By using N-15 enrichments above 33.3 At% the technique becomes insensitive to the evolved N_2 because the $\frac{30}{29}$ ratio must be monitored. As 99.6% of the evolved N_2 consists of m/e 28, very little change will be noticed in the $\frac{30}{29}$ ratio.

To calculate the maximum sensitivity of the lysimeter technique using 33.3 At% N-15 enriched N_2, certain assumptions must be made:

i) Assume the lysimeter headspace volume is 500 cm^3,

ii) Assume the concentration of the enriched N_2 is 10,000 ppm.

If a minimum R_c value of 0.100 was accepted (any lower than this, the R_c value could be increased to near 0.999 by increasing the m/e 29 peak amplification by ten-fold), then based on the σ value of 0.04°/$_{oo}$ for a R_c value of 0.700 (section 8.4.1), a maximum σ of 0.28°/$_{oo}$ could be expected. Therefore a significant change in the isotopic ratio measured could be taken as being outside 3σ i.e. 1.12°/$_{oo}$ (4σ).

If the headspace gas mixture is to be the isotopic reference gas, then at the beginning of the experiment (time t=0), $R_{sample} = R_{reference}$ where $R = \frac{29}{28}$. At some later time t.,

$$\frac{29_s}{28_s} \text{ or } R_s = \frac{5\ cm^3 .2(0.33 \times 0.66) + x\ cm^3\ (0.0072)}{5\ cm^3\ (0.66)^2 + x\ cm^3\ (0.993)} \qquad 8.6$$

where x is the unknown volume of N_2 evolved due to denitrification. This gas will have a $\frac{29}{28}$ ratio of $\frac{0.0072}{0.993}$, therefore the new $\delta^{15}N$ (°/₀₀) at time t_1

$$= \left[\frac{R_S}{R_R} - 1\right] 1000$$

$$= \left[\frac{\frac{2.178 + x(0.0072)}{2.178 + x(0.993)}}{1} - 1\right] 1000$$

Let the significant change in the $\delta^{15}N$ value be 1.12°/₀₀.

Then:

$$1.12 = \left[\frac{2.178 + 0.0072x}{2.178 + 0.993x} - 1\right] 1000$$

$$x = 2.46 \times 10^{-3} \text{ cc } N_2.$$

Given the assumptions that the spike N_2 gas is of 4.7 At% (R_C=0.100) and 10,000 ppm; the headspace volume is 500 cm^3; and that the σ is ±0.28°/₀₀, then the minimum detectable quantity of N_2 evolved is 2.46×10^{-3} cm^3.

To detect 10 kg N-N_2/ha/yr requires a production of 3×10^{-4} cm^3 N_2/lysimeter/hr.

On the basis of the above calculations, the minimum detectable level of N_2 production is 61 kg N_2-N/ha/yr. If, however, the calculations are based on a minimum R_C value of 0.700 and a σ of ±0.04°/₀₀, then the sensitivity of the technique increases to 8.7 kg N_2-N/ha/yr. It can also be seen that the sensitivity can be proportionally increased by decreasing the headspace volume or the background N_2 concentration. Decreasing the headspace volume means that linearity of N_2 (and N_2O) evolution will occur over a reduced period. Decreasing the N_2 concentration to below 10,000 ppm will induce an increase in the σ value in the $\delta^{15}N$ analysis.

A 5,800 ppm sample of N_2 at $10^{-6}\tau$ ion chamber pressure yielded a σ of $\pm 0.082°/_{oo}$, i.e. no overall gain in sensitivity compared to a σ of $\pm .04°/_{oo}$ for the 12,400 ppm N_2 sample.

8.6.0 FACTORS AFFECTING DOUBLE BEAM $\delta^{15}N$ MEASUREMENTS

8.6.1 Partial pressure variations

The Micromass was originally designed to determine δ values of pure gases, i.e. the gas of interest was expected to be the sole species present. A condition of normal isotopic evaluations on the Micromass is that the major beam currents (I_{maj}) of both the sample and reference gas be set identical (thus equalising the pressures in the ion chamber).

In the case of the lysimeter experiment, using the T_o gas as a reference, small increases in the m/e 28 peak of the sample gas can be expected as N_2 is evolved. Although these changes will be small (in the order of a few ppm) it was necessary to know if the partial pressure of the species of interest affected the isotopic ratio measured.

Three samples of He were prepared with varying aliquots of air added and the $\frac{29}{28}$ ratios were determined (table 8.1).

Table 8.1 Effect of pN_2 on $\frac{29}{28}$ ratios

Press.	I_{maj} (m/e 28)	$R_c+\bar{r}\left(\frac{29}{28} \times \frac{I_{min}}{I_{maj}}\right)^*$
$5x10^{-7}\tau$	70 x 2 x 10^{-9}A	0.007275
	85	0.007275
	103	0.007275

* $\frac{I_{min}}{I_{maj}}$ = ratio of respective amplifier settings

The results indicate that even moderate changes in the partial pressure of N_2 (pN_2) will not affect the $\frac{29}{28}$ ratio. Therefore, even if the sample pN_2 is seen to deviate from the reference pN_2 it is not necessary to effect an equivalent imbalance in the ion chamber pressures to equalise the I_{maj}.

This result was fortunate because it was also demonstrated that variations in the ion chamber pressure cause variations in the $\frac{29}{28}$ ratio (see tables8.2 and 8.3).

Table 8.2 Effect of Ion chamber pressure on $\frac{29}{28}$ ratio of 5,800 ppm N_2 in Argon

Press. (τ)	R_c
1 $\times 10^{-6}$	0.700
7.5 x 10^{-7}	0.670
5.0 "	0.626
2.5 "	0.486
1.0 "	0.068

Table 8.3 Effect of Ion chamber pressure on $\frac{29}{28}$ ratio of 12,400 ppm N_2 in Argon

Press (τ)	R_c
1 x 10^{-6}	0.700
7.5 x 10^{-7}	0.686
5 x 10^{-7}	0.664
2.5 x 10^{-7}	0.614
1 x 10^{-7}	0.440

This effect is attributed to artificially induced preferential ionization of m/e 28 at lower ion chamber pressures. Although of a serious magnitude, it is noticeable that the $\frac{29}{28}$ ratio does not decrease so markedly for higher pN_2 values.

The results of tables 8.1, 8.2 and 8.3 demonstrate the importance of having the sample and reference ion chamber pressures equalised when measuring $\delta^{15}N$ values irrespective of any consequent variations in the m/e 28 peaks caused by varying partial pressures of N_2.

8.6.2 Effect of oxygen on $\delta^{15}N$ values

It is generally recommended that O_2 be removed from N_2 samples prior to isotopic analysis. The oxygen effect is attributed to: i) filament instability, and ii) effect of CO production (from residual carbon on the filament from previous organic analyses).

The removal of O_2 using alkaline pyrogallol solution (as described by Vogel, 1961, p1079) was attempted but found unsatisfactory. The pyrogallol solution introduced further peaks into the mass spectrum even though the pyrogallol was frozen prior to admitting the gas to the mass spectrometer. It is also suggested (Vogel) that pyrogallol may, in the course of removing O_2, produce quantities of CO. It would be advantageous to leave the O_2 in the samples if the errors or instabilities were not significant. To measure the effect of O_2 on $\delta^{15}N$ readings, $\delta^{15}N$ values of cylinder N_2 were determined with varying amounts of added O_2 (table 8.4).

Table 8.4 Effect of Oxygen on $\delta^{15}N$ determinations

$\delta^{15}N$ (°/oo)	pO_2
-0.07	0
+ 0.21	0.05
+ 0.46	0.13
+ 1.15	0.18
+ 1.69	0.24

The trend in the $\delta^{15}N$ values is actually opposite to what might be expected for increased CO production.

To overcome the O_2 effect on $\delta^{15}N$ values it was intended to make each sample measured the next reference for the following sample. This way the O_2 effect between consecutive samples will be similar as variations in the N_2/O_2 contents will be minimal.

8.6.3 Interferences of $\frac{29}{28}$ ratios

The presence of CO_2 and N_2O causes certain problems in the determination of $\frac{29}{28}$ ratios. CO_2 fragments produce a measurable quantity of CO (Friedel *et al*, 1953). The major contribution of this CO will be to the 28 peak. Allowances can be made for this contribution by measuring the fragmentation ratio and monitoring the m/e 44 peak. However, this is impossible in the presence of N_2O. The m/e 30 fragmentation peak of N_2O cannot be used to allow for the N_2O contribution to the m/e 44 peak because of the large contribution of highly enriched N_2 to m/e 30. Allowances for this contribution can be made, but only by using equation 8.4 in conjunction with a measured $\frac{29}{28}$ ratio. As just pointed out, this ratio cannot accurately be determined in the presence of CO_2.

It was intended to remove both CO_2 and N_2O from the initial determination of the $\frac{29}{28}$ ratio by admitting the gas to the mass spectrometer via a stainless steel trap (plate 8.1) immersed in liquid nitrogen. Once the $\frac{29}{28}$ ratio had been established the trap could be warmed and CO_2 and N_2O admitted and subsequently determined using the m/e 30 peak for N_2O and the m/e 44 peak for CO_2 (if so desired), with the appropriate corrections for contributions from interfering species.

Alternatively the sample could be collected and stored prior to analysis in the presence of carbsorb if the CO_2 content was not of interest. This would allow the N_2O determination to be carried out using the m/e 44 peak and, therefore do away with the need for any m/e 28 or m/e 30 peak corrections.

8.6.4 Ionization effect of carrier gas

The ionization effect of the noble carrier gas in gas chromatography ionization detectors is well known. The outer electrons of He, Ne and Ar when exposed to a flux of β particles are easily promoted from the ground state to an excited level. Collision of a gas molecule with a metastable noble gas atom will result in ionization by transfer of the energy of excitation from one species to the other. This will happen only provided the excitation energy of the noble gas is greater than the ionization potential of the compound.

Both He* and Ne* have sufficient energy to ionize all gases (see table 8.5) except He and Ne, while Ar* can ionize those gases with ionization potentials less than 11.5 eV.

Table 8.5 Ionization properties of gases

Gas	Ionization Potential (eV)	Electronic Excitation Energy (eV)
He	24.5	19.6
Ne	21.5	17.6
Ar	15.7	11.5
N_2	15.5	

This effect of increased ionization will be seen in the mass spectrometer as an increased sensitivity toward the peak of interest. Thus it is possible a He carrier gas may give an increased sensitivity towards $\delta^{15}N$ determinations at low N_2 concentrations.

Experimental results suggested better N_2 sensitivity in He compared to Ar, but possible low level contamination of these carrier gases ruled out definitive measurements.

8.7.0 CONCENTRATION TECHNIQUES

One possible method considered for increasing the accuracy of the $\delta^{15}N$ determinations was sample concentration.

Concentration of gas samples containing traces of CO_2 or N_2O is simple as these species can be frozen out of the gas stream and collected in a trap while the carrier gas is pumped to waste. Concentration of N_2 is more difficult due to its incondensibility.

The first concentration method considered was via gas chromatography. Ideally, by interfacing the gas chromatograph to the Micromass, and switching the gas flow into the mass spectrometer when the N_2 peak eluted, a sample concentration could be achieved. Gas chromatograph/mass spectrometer interfaced systems have been used successfully for several years in such a way (Focht, 1978).

However, such a system requires at least partial dedication of both instruments to one type of analysis. Limited instrumental facilities and the workload pressure on the Micromass did not permit such a reduction in the flexibility of operation.

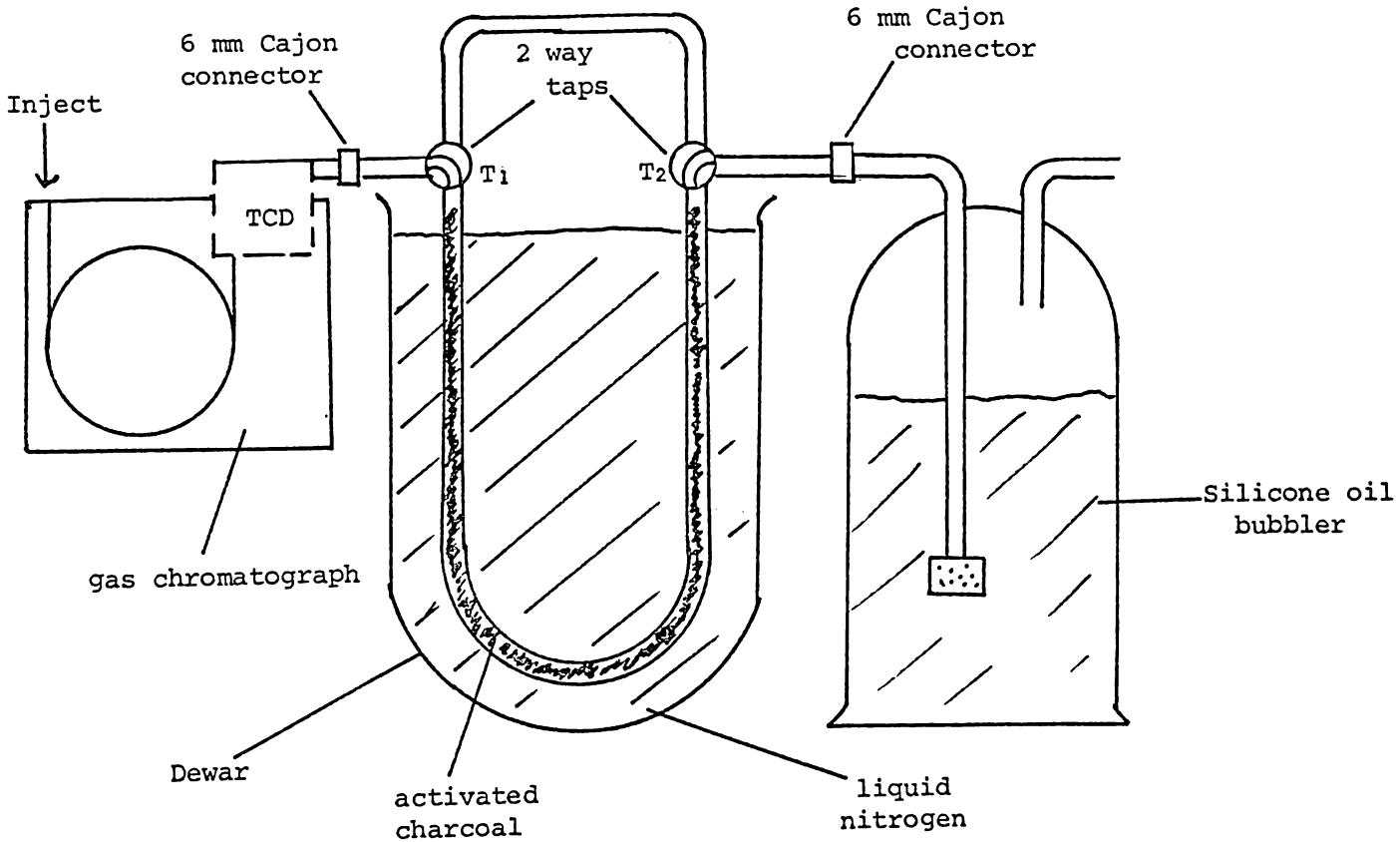
The concentration technique therefore centred around the gas chromatograph separation capability in conjunction with a sample trap. An activated charcoal column was attached to the exit port of the TCD detector of the gas chromatograph (figure 8.4). N_2 and $(Ar+O_2)$ can be separated using a molecular sieve 5A column. The first peak eluted is $(Ar+O_2)$. Using the analytical conditions described in chapter 3, the N_2 peak follows approximately 30 seconds behind the $(Ar+O_2)$ peak.

The charcoal trap was connected to the exit port of the TCD detector using a 6 mm cajon connector. The charcoal U trap had previously been evacuated at 300°C in a furnace and refilled to atmospheric pressure with He once cool. The two-way taps were lubricated with silicone grease because of its constant viscosity properties over a wide temperature range.

Once connected to the detector port the carrier gas flow passed through the by-pass bridge into the bubbler which prevented back diffusion of air. A dewar of liquid N_2 was placed around the charcoal column.

The sample of $(Ar+O_2)$ containing a trace of N_2 was then injected into the gas chromatograph and its progress monitored by the chart recorder attached to the gas chromatograph. Directing the gas flow through the bubbler caused some base line in stability at low attentuations (Att 2) due to small pressure fluctuations. However, it was only necessary to accurately determine the position of the $(Ar+O_2)$ peak. Once the massive $(Ar+O_2)$ peak appeared a timer was started and based upon the flow rate measured, the $(Ar+O_2)$ was allowed pass

Figure 8.4 Apparatus for trapping nitrogen eluted from gas chromatograph



through the by-pass into the bubbler. By switching T_1 at the appropriate time the effluent gas was drawn onto the charcoal column as a considerable vacuum existed while immersed in liquid N_2. Over-pressure of the charcoal column could be monitored by watching the recorder trace.

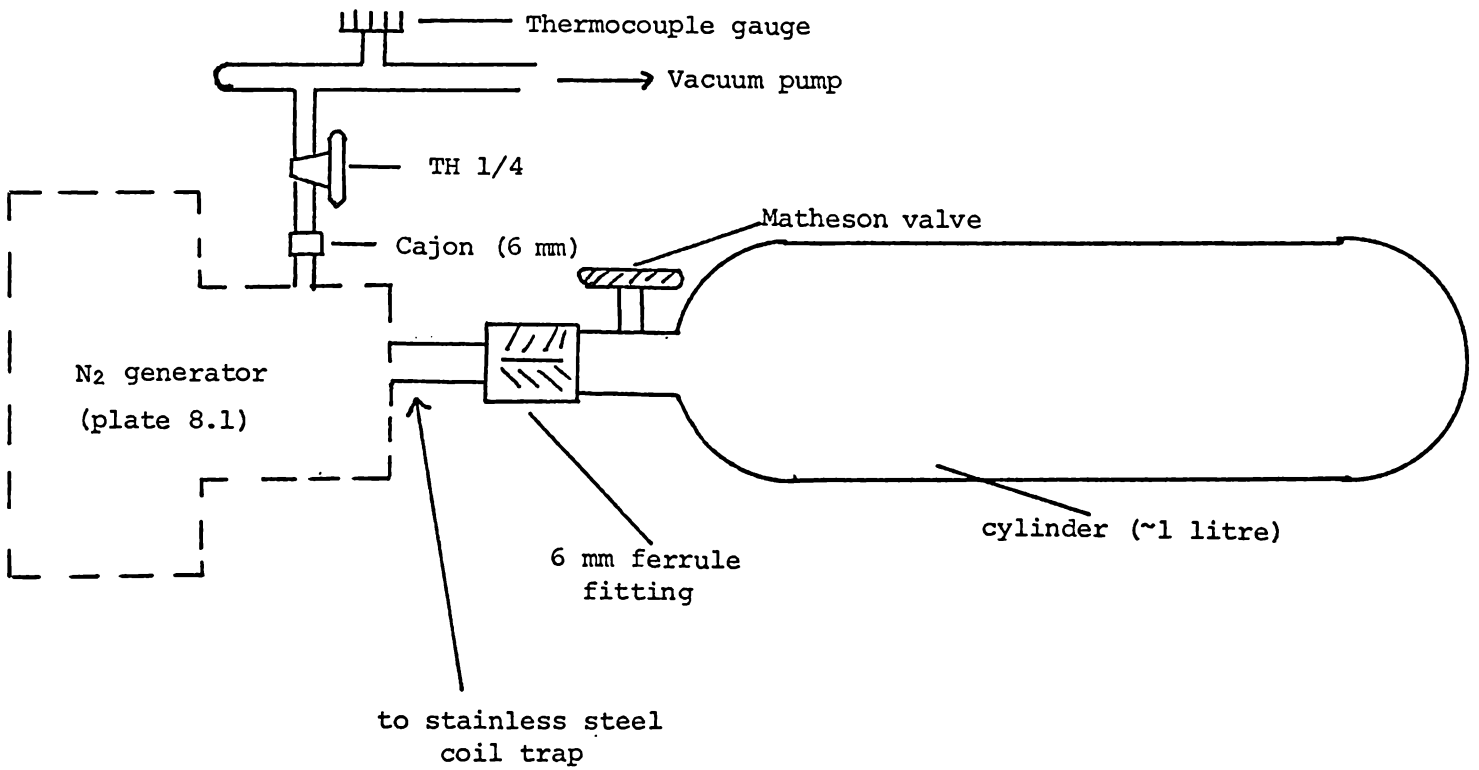
The column was then detached from the gas chromatograph and transferred to the mass spectrometer while still immersed in liquid N_2. Most of the He carrier gas was pumped off the column using the mass spectrometer vacuum system. The charcoal column was then isolated from the vacuum system by closing T_1 and allowed to warm in a 70°C water bath. The sample was subsequently drawn into the mass spectrometer sample chamber and the column detached, ready for reactivation.

The results suggested a significant sample concentration could be achieved (about 50 fold). However, this work was carried out before acceptable σ values were being obtained directly on the mass spectrometer by running the ion chamber at ten times ($10^{-6}\tau$) the recommended pressure and increasing the N_2 concentration from 5,000 to approximately 10,000 ppm. In view of the simplicity of the latter system and the reduced risk of sample contamination due to less handling, the concentration technique was not adopted.

8.8.0 PREPARATION OF $^{15}N_2/Ar/O_2$ GAS SAMPLES

A method had to be developed whereby cylinders of Ar/O_2 gas mixtures could be made containing low levels (~10,000 ppm) of N_2 enriched in N-15 (~33 At%). The system developed is depicted in figure 8.5. N_2 was generated using a modification of the lithium hypobromite technique described by Ross and Martin (1970). The 120 ml reservoir (see plate 8.1) contains air-free hypobromite solution under an atmosphere of He. A was unattached, B was connected to the gas cylinder inlet and C was connected to the vacuum line. Lithium

Figure 8.5 Vacuum apparatus for preparation of ^{15}N enriched nitrogen gas



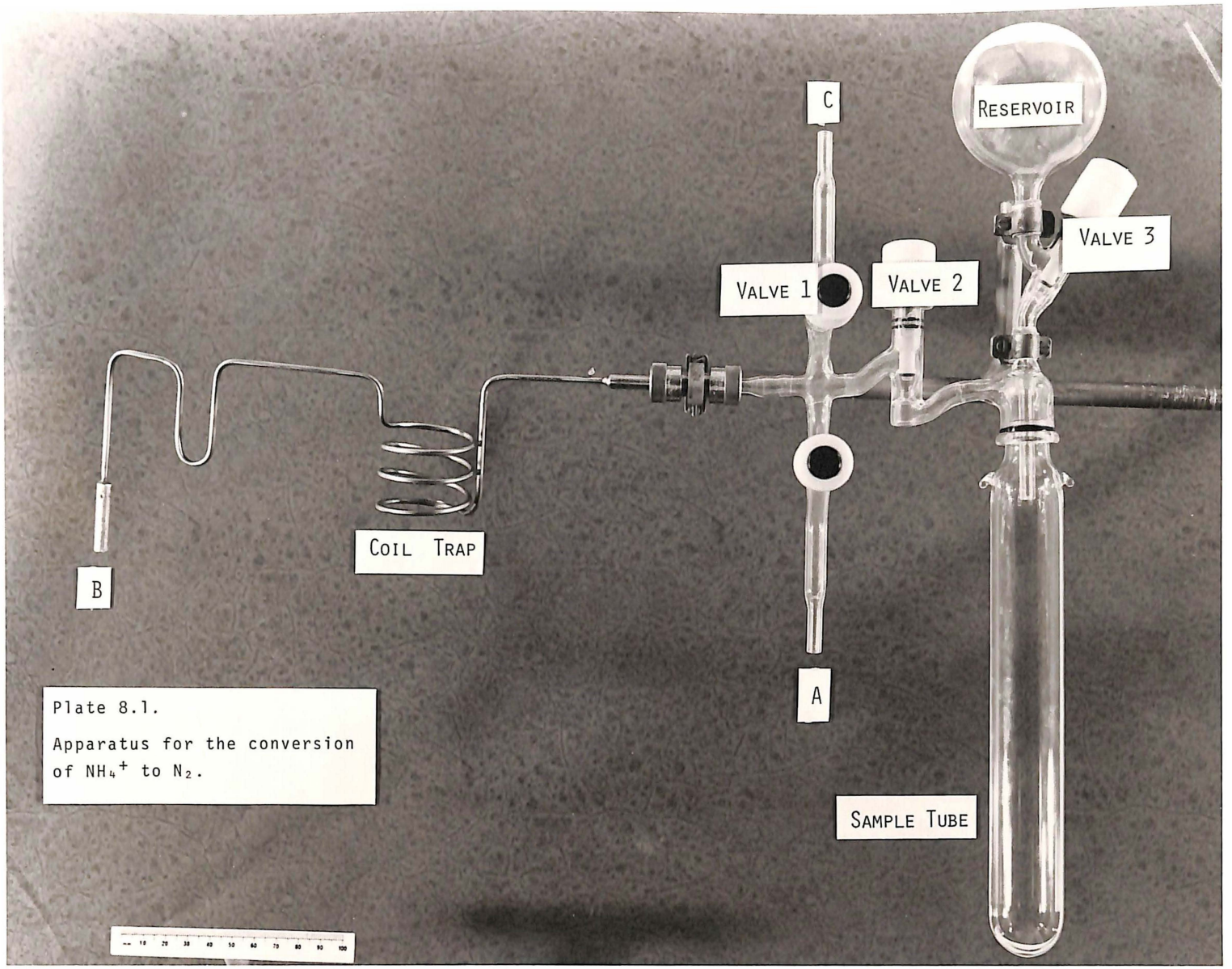


Plate 8.1.

Apparatus for the conversion of NH_4^+ to N_2.

hypobromite was used in preference to sodium hypobromite as the former is more stable (Steele, 1977). Lithium hypobromite solution was prepared by adding 2 ml of analytical reagent grade Br to 120 ml of cold (5°C), 12.5% w/v solution of analytical reagent grade lithium hydroxide and stirring until the Br dissolved. This solution was degassed after admission to the reservoir and placed under an atmosphere of He. N_2 is produced according to the reaction:

$$2NH_3 + 3LiOBr \rightarrow 3LiBr + 3H_2O + N_2$$

where LiOBr is added to $(NH_4)_2SO_4$.

A lightly greased 'O' ring was placed over the top of the sample tube which was attached to the 'U' socket by opening valves 1 and 2. After the conversion apparatus was evacuated valves 1 and 2 were closed. LiOBr was admitted to the sample tube by opening valve 3. The sample tube was not large enough to contain the entire volume of N_2 produced so the N_2 was regularly drawn into the evacuated cylinder by opening valve 2. The hypobromite solution was frozen before the N_2 transfer by placing an ethanol slush bath (-80°C) around the sample tube.

At the completion of the reaction the cylinder was removed from the vacuum line, connected to an Ar/O_2 cylinder and pressurised to a predetermined value to give the correct N_2 concentration.

Nitrogen gas of 33 At% N-15 was normally prepared using appropriate mixtures of 54 At% N-15 $(NH_4)_2SO_4$ and analytical reagent grade $(NH_4)_2SO_4$ which is very close to natural abundance in N-15.

Failure to achieve the exact calculated N-15 enrichment was attributed to N_2 contamination of the Ar/O_2 mixture. Only commercial grade Ar was available.

8.9.0 FIELD TRIALS

The first field trials using the $\delta^{15}N$ technique were conducted early in the experimental programme and served to point out several inadequacies in the experimental technique. Gas samples were taken in the sealed ampoules by the technique described in section 6.7.0.

Core flushing began at 200 ml/min with Ar/O_2 containing approximately 2,000 ppm N_2 of 4.2 atom% N-15. After 20 minutes of flushing the core-flow was discontinued and the base-flow commenced at 50 ml/min. Samples were withdrawn and sealed in soda glass tubes at appropriate times using the apparatus depicted in figure 6.11. Results indicated the problems inherent in double beam mass spectrometric analyses of samples of low N_2 concentration. Consequently, the only results that could be obtained from this experiment were obtained using the single beam mode. Variations in the isotopic ratio with time are shown in table 8.6.

Table 8.6 Results of a $\delta^{15}N$ field trial using sealed ampoules and single beam analysis. ($P = 5 \times 10^{-8}\tau$)

Time (mins)	m/e 28 (Amps)	m/e 29 (Amps)	$\frac{29}{28} \times 100$
0	21×10^{-9}	15.5×10^{-11}	0.74%
30	20.5×10^{-9}	14×10^{-11}	0.68%
90	27.5×10^{-10}	25.5×10^{-12}	0.93%
150	20.5×10^{-9}	14.5×10^{-11}	0.71%
210	44.5×10^{-10}	35.0×10^{-12}	0.79%

While inconsistent, these results illustrate two points. Firstly, the inadequacy of the ampoule sampling system is indicated by the large variation in the m/e 28 values. N_2 concentrations varied by

approximately ten-fold, suggesting gross contamination. Only those samples showing minimal contamination (e.g. m/e 28 = 27.5 x 10^{-10}) demonstrated any $\frac{29}{28}$ ratio above that of atmospheric N_2 $\left(\frac{29}{28} \text{ air} = 0.732\%\right)$.

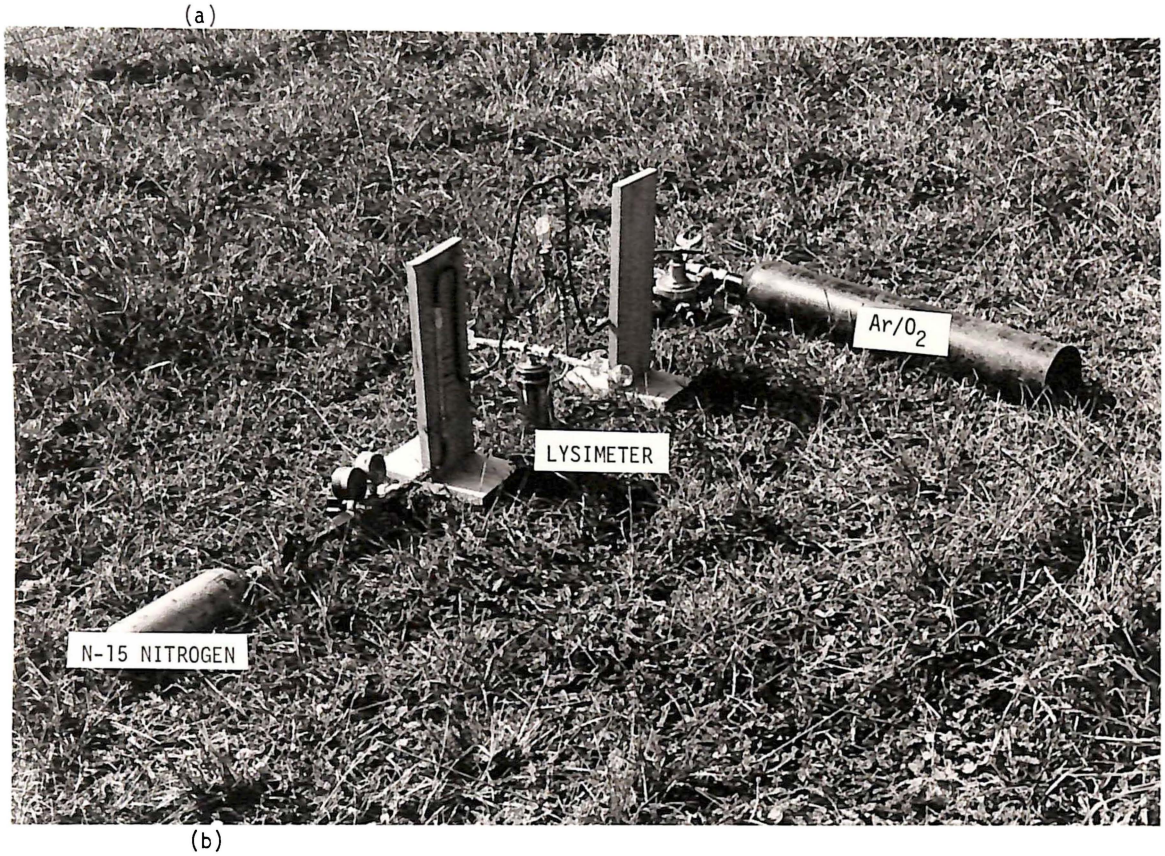
Secondly, the success of the flushing technique in reducing the N_2 level to the desired background is shown by the low N_2 levels recorded in those samples exhibiting minimal contamination. At an equivalent ion chamber pressure (5 x $10^{-8}\tau$), a sample of 12,400 ppm N_2 of approximately natural N-15 abundance gave a m/e 28 peak of 28 x 10^{-10} Amps, suggesting a core N_2 background close to the desired 10,000 ppm. Subsequent $\delta^{15}N$ field experiments used 25 cm^3 evacuated flasks attached to the sampling port of the lysimeter (see plate 8.2). 25 cm^3 sample volumes were necessary to effect an ion chamber pressure of $10^{-6}\tau$. As the withdrawal of such sample volumes was likely to cause a pressure deficit within the lysimeter, repeated sampling was not possible. An initial sample was withdrawn 10 minutes after cessation of the core-flow and used as a reference (Time = 0). After a further 60 minutes a second sample was taken for both the $\delta^{15}N$ and N_2O determinations. Samples were collected simply by opening the taps on the pre-evacuated flasks. The 60 minute sample flask also contained carbsorb to eliminate any CO_2 interferences of the N_2O determination. Therefore N_2O was determined by measuring m/e 44.

The flushing gas was He/O_2 with a spike (~1%) of Ar to allow the reference and sample pressures to be at exactly equal.

The results of such a trial are shown in figure 8.6 and table 8.7

Plate 8.2 FIELD APPARATUS FOR DIRECT DETERMINATION OF N_2 EVOLUTION

(a)



(b)

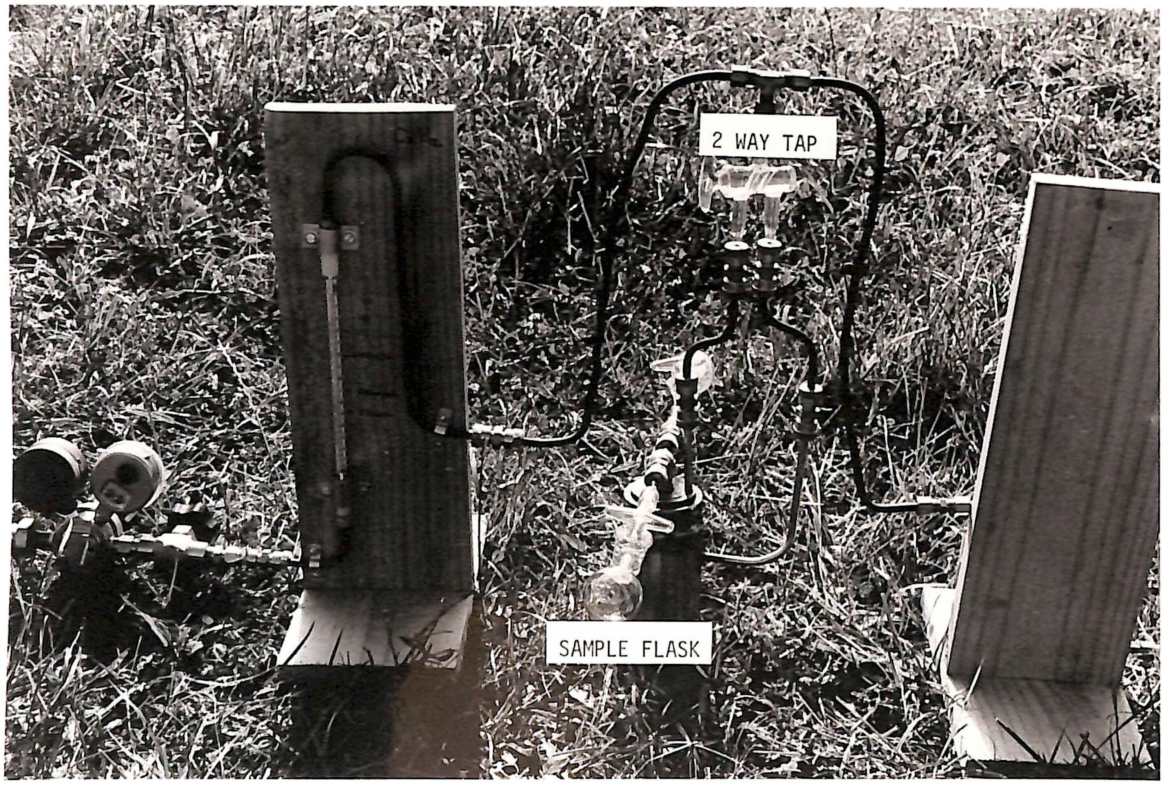


Figure 8.6 $\delta^{15}N$ trace of field experiment

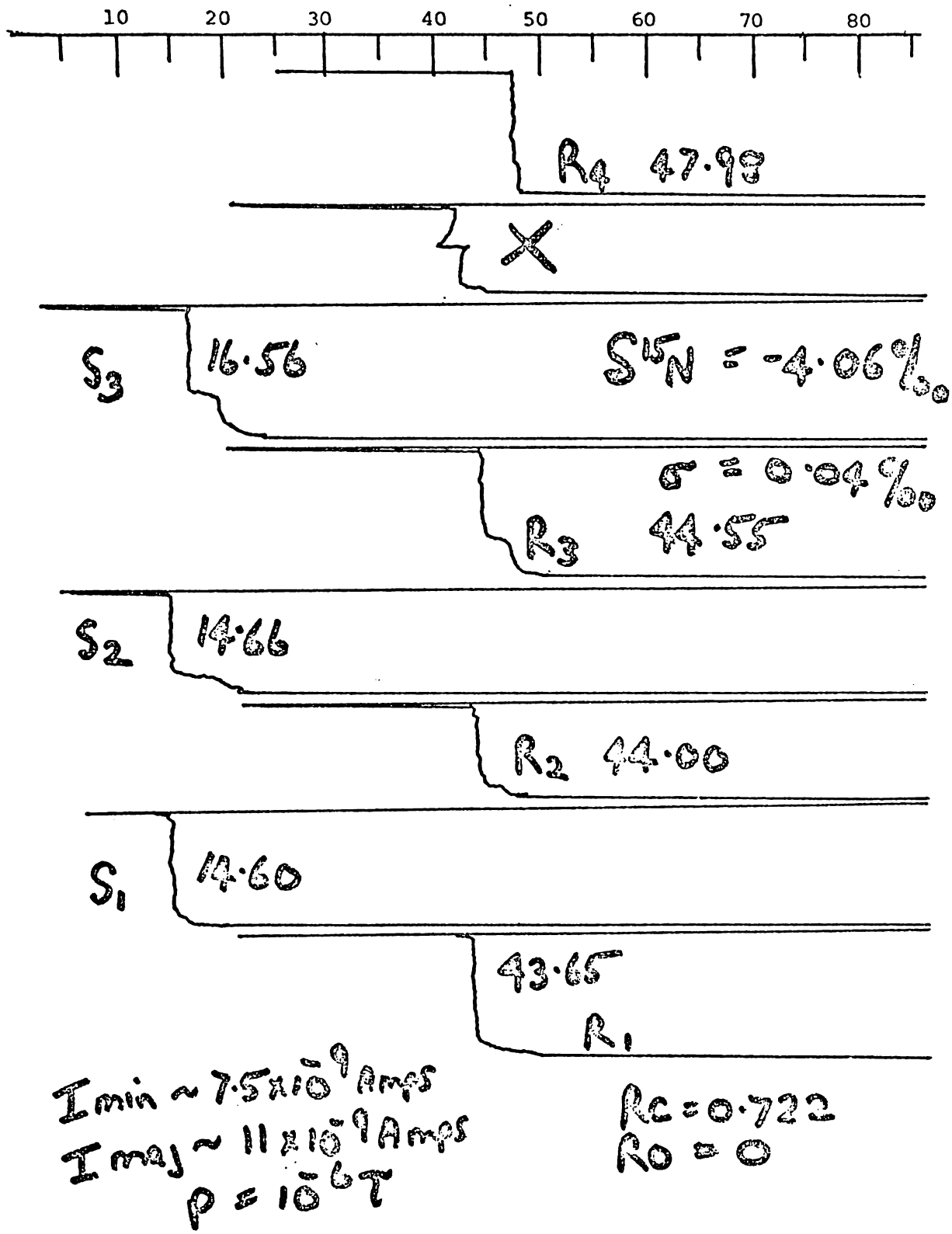


Table 8.7 Results of N-15 field trial using double beam $\delta^{15}N$ determination

$\delta^{15}N$	σ	N_2O (ppm)	N_2O emission (kg N_2O-N/ha/yr)	N_2 emission (kg N_2-N/ha/yr)
-4.06‰	.04‰	2.6	27	260

8.9.1 Criticisms of $\delta^{15}N$ technique for assessment of N_2 evolution

The results of this experiment indicate that such a technique is capable of measuring N_2 emissions from denitrification directly. The most serious drawback of this technique at its current stage of development is that the estimates are based upon a single $\delta^{15}N$ determination. There is also the underlying assumption that the evolution of N_2 and N_2O during the 1 hour sampling period was linear. However data produced by Findlay and McKenney (1979), Matthias *et al* (1980), McKenney *et al* (1978) and the results of section 7.5.0 suggest that such an assumption is likely to be valid.

Another serious criticism is that of diffusion of dissolved N_2 from wet soil crumbs, into the soil pores. A litre of water contains approximately 12 cm^3 N_2 and 5 cm^3 O_2 dissolved within it. If a soil core of a nominal 1 litre volume (lysimeter volume) and 50% porosity was near field capacity in moisture (40% H_2O w/w), the water content of the lysimeter would approach 200 cm^3. This water content would provide a reservoir of approximately 2.5 cm^3 of dissolved N_2 - approximately 500 times greater than the lowest hourly production rate to be measured.

It is important to know the contribution of the diffusion of this dissolved N_2 to the N_2 produced by denitrification. It is likely that N_2 diffusion from the soil crumbs is a contributing factor to the apparently high rate of N_2 evolution (260 kg N/ha/yr) measured.

It was initially anticipated that the rate of N_2 diffusion could be monitored by measuring the rate of diffusion of the accompanying ^{40}Ar into the headspace in the presence of a He/O_2 flushing gas. However, this Ar would have to be estimated using single beam techniques, the best error estimate for which could be assumed to be in the order of 1%. The N_2 evolution is measured by the $\delta^{15}N$ technique and has an estimated error of analysis of less than 0.1%. Thus the single beam analysis of an internal standard gas would allow a ten-fold contribution of diffusion N_2 to denitrification estimates without being evident in the ^{40}Ar results.

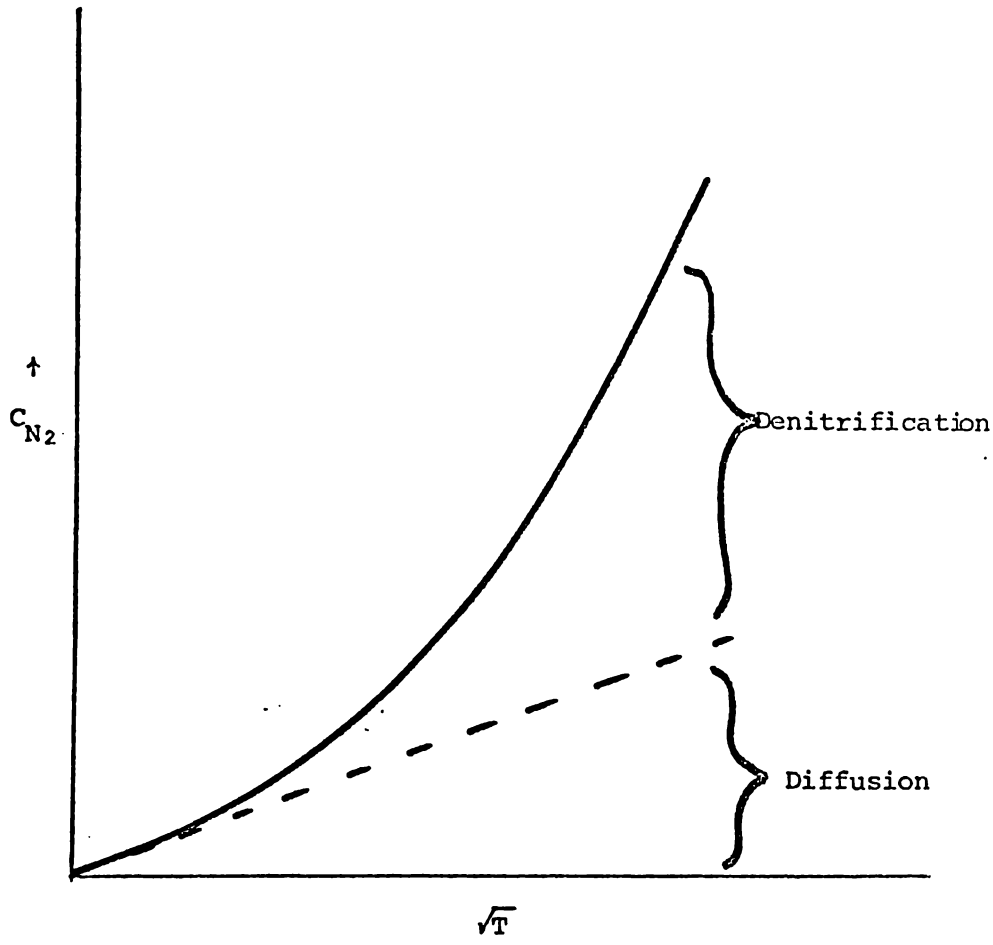
Another approach to the problem was considered. The atmospheric N_2 diffusing from the water in the soil crumb will show a diffusion limited evolution rate into the pore spaces. Conversely, the rate of N_2 evolution by denitrification is linear within short time periods. Consequently, the N_2 evolution monitored in the headspace will be a composite of a diffusion curve and a straight line. It was hoped that by separating the diffusion data from the composite curve, an estimate on the linear rate of N_2 evolution could be made.

Diffusion curves, being parabolic, yield straight lines on $\sqrt{\text{Time}}$ plots. Conversely, the kinetic data will assume a parabolic curve. The more significant the diffusion contribution to the N_2 data, the nearer the composite $\sqrt{\text{Time}}$ plot will be to linearity. The deviation from linearity will be indicative of the N_2 derived from denitrification (see figure 8.7).

However, it is mathematically impossible to resolve these two curves unless further data is available. The slope of the diffusion (dashed) line is impossible to predict unless either the rate of diffusion or the rate of denitrification is known.

The rate limiting step in the diffusion process while the flushing gas is sweeping the macropores will be the diffusion of N_2

Figure 8.7 Resolution of Diffusion and denitrification evolution of N_2



from inside the soil crumb into the macropore.

Ryden and co-workers (1979) calculated the time taken to establish an acetylene concentration of 0.1% inside a soil crumb (of different sizes) maintaining an outside (macropore) concentration of 2% acetylene. The method of calculation adopted centred around Carslaw and Jaeger's (1959) solution for the heat flow into a solid sphere with zero initial temperature (p233, Carslaw and Jaeger). Carslaw and Jaeger provide a figure plotting $\frac{v}{V}$ vs $\frac{r}{a}$ for a series of values of $K\frac{t}{a^2}$ for their solution to this problem where v = inside temperature, V = surface temperature, a = sphere radius, t = time taken for given values to occur, K = thermal diffusivity and r = distance from centre of sphere.

Temperature can be read for gas concentration and K can be replaced by Dp, the effective gas diffusion coefficient in the soil.

The estimated time T for a given concentration to occur within a sphere radius a with diffusivity Dp can be estimated from $T = \frac{Dp\ t}{a^2}$ where t = time taken to arrive at a given $\frac{v}{V}$ for a given $\frac{r}{a}$.

Ryden's data suggested that by maintaining a macropore gas concentration close to 1% then an inside concentration at the centre of a crumb of 2 mm diameter can be achieved within approximately 0.02 hours (1.2 mins) depending on the total porosity.

Unfortunately however, the reverse process, i.e. an initial concentration at the crumb centre C_o diffusing to the surface of concentration C (zero) is not analagous. This solution is found in Carslaw and Jaeger's treatment of radiation at the surface of a sphere where the medium temperature is kept at zero (p237).

The equation describing the temperature distribution within the sphere is:

$$\frac{dv}{dt} = K\left(\frac{d^2v}{dr^2} + \frac{2}{r}\frac{dv}{dr}\right) \text{ where } 0 \leq r < a$$

describing the boundary conditions as:

$$\frac{dv}{dr} + hv = 0 \text{ (at } r = a)$$

and the initial condition $v = f(r)$ at $t = 0$ (where $f(r)$ = initial N_2 concentration over whole sphere) Carslaw and Jaeger provide the solution:

$$v = \frac{2}{ar} \sum_{n=1}^{\infty} e^{-k\alpha^2 nt} \frac{a^2\alpha_n^2 + (ah-1)^2}{a^2\alpha_n^2 + ah(ah-1)} \sin\alpha_n r \int_0^a r'f(r') \times \sin\alpha_n r' dr'$$

where $\pm\alpha_n, n=1,2 \ldots$ are roots of $a\alpha\cot a\alpha + ah - 1 = 0$

This expression converges so rapidly that when sufficient time has passed the terms after n=1 can be neglected greatly simplifying the mathematical solution.

Thus, keeping only the first term:

$$v = \frac{2}{ar} e^{-k\alpha_1^2 t}\left[\frac{a^2\alpha_1^2 + (ah-1)^2}{a^2\alpha_1^2 + ah(ah-1)} \sin(\alpha_1 r)\right] \times \int_0^a r'f(r')\sin(\alpha_1 r')dr'$$

$$\sin\frac{(\alpha_1 r)}{r} \rightarrow \alpha_1 \text{ when } r \rightarrow 0$$

$$\therefore v(r=0,t) = \frac{2\alpha_1}{a} e^{-k\alpha_1^2 t}\left[\frac{a2\alpha_1^2 + (ah-1)^2}{a^2\alpha_1^2 + ah(ah-1)}\sin(\alpha_1 r)\right]\int_0^a r'f(r')\sin(\alpha_1 r')dr'$$

$$\int_0^a r'f(r')\sin(\alpha_1 r')dr' = \int_0^a r'\cos(\alpha_1 r')dr'$$

$$= \cos\int_0^a r'\sin(\alpha_1 r')dr'$$

Where Co = Initial gas concentration within sphere

$$= Co \left\{ \left[\frac{-Cos(\alpha_1 r')}{\alpha_1} r' \right]_0^9 + \frac{1}{\alpha_1} \int_0^a Cos\alpha_1 r' dr' \right\}$$

$$= Co \left\{ \frac{-Cos\alpha_1 a}{\alpha_1} + \frac{1}{\alpha_1} \frac{(Sin\alpha_1 a)}{\alpha_1} \right\}$$

$$= Co \left\{ \frac{Sin(\alpha_1 a)}{\alpha_1^2} - Cos \frac{(\alpha_1 a)}{\alpha_1} \right\}$$

$\therefore$ at the centre

$$v(t) = \frac{2\alpha_1}{a} e^{-k\alpha_1^2 t} \left\{ \frac{a^2\alpha_1^2 + (ah-1)^2}{a^2\alpha_1^2 + ah(ah-1)} \right\} \times Co \left\{ \frac{Sin(\alpha_1 a)}{\alpha_1^2} - \frac{Cos(\alpha_1 a)}{\alpha_1} \right\}$$

$$= Co \frac{2}{a} e^{-k\alpha_1^2 t} \left\{ \frac{a^2\alpha_1^2 + (ah-1)^2}{a^2\alpha_1^2 + ah(ah-1)} \right\} x \left\{ \frac{Sin(\alpha_1 a)}{\alpha_1} - Cos(\alpha_1 a) \right\}$$

Thus $$\frac{V(t)}{Co} = \frac{2}{a} \frac{a^2\alpha_1^2 + (ah-1)^2}{a^2\alpha_1^2 + ah(ah-1)} x \left\{ \frac{Sin(\alpha_1 a)}{\alpha_1} x Cos(\alpha_1 a) \right\} x e^{-k\alpha_1^2 t}$$

(we want t such that v(r=0,t) = 0.008 Co)

$$\frac{V(t)}{Co} = F(\alpha_1, a, h) e^{-k\alpha_1^2 t} \quad \text{say.}$$

$$\ln \left(\frac{V(t)}{Co} \right) = \ln F - k\alpha_1^2 t$$

$$\therefore \quad t = \frac{\ln F}{k\alpha_1^2} - \frac{\ln \left(\frac{V(t)}{Co} \right)}{k\alpha_1^2}$$

Unfortunately this solution is not as simple as that described by Ryden and co-workers because the time for the initial concentration inside the crumb to drop to some value depends on α_1, a, h, k. Values

for k and a can be found but α_1 and h remain unknown. The solution says that the heat (or gas) transfer is proportional to the temperature (concentration) difference (h) at the boundary.

If a value for h could be found then the time required for the diffusion process could be found. However the difficulties anticipated in measuring a gas concentration difference at the surface and inside a soil crumb in a stream of carrier gas suggest that a value for h is not easily found. This area of work requires further investigation.

8.10.0 SUGGESTIONS FOR FURTHER WORK

Further work on the ^{15}N-N_2 technique for measuring N_2 evolution from soils should include studies on:

i) The contribution of soil water nitrogen to the nitrogen evolving into the headspace,

ii) A method for taking several gas samples without causing pressure deficits within the lysimeter.

8.10.1 Allowances for soil-water nitrogen

It is possible that the contribution of dissolved nitrogen diffusing into the lysimeter headspace can be adequately controlled by the duration of the initial core flushing. If the dissolved N_2 diffuses out of the wet soil crumbs quickly enough, it may be possible to flush the core long enough to remove this contribution from the lysimeter. If on the other hand, the diffusion is very slow, it is possible that $\delta^{15}N$ determinations made within a short period may adequately reflect the denitrification contribution to the measured N_2 evolution.

8.10.2 Collecting multiple gas samples

If the sensitivity of the mass spectrometer could be significantly increased (i.e. by ten-fold) such that $\delta^{15}N$ determinations can be made on samples of lesser pN_2, then it would not be necessary to collect such large volumes of gas (25 cm^3). Alternatively, a concentration technique as described in section 8.7.0 could be adopted. Another possible approach would be to fill several sample flasks with high purity He. By connecting these flasks to the headspace, the denitrification product gases will diffuse into the sample flask - causing no pressure deficit.

Providing the dilution of the N_2 is not serious enough to affect the $\delta^{15}N$ determinations, this technique would appear the most feasible. A flushing system would be required whereby the dead space between the sample connector and the lysimeter could be rigorously purged before the connecting taps were opened. Allowances could be made for the subsequent dilution effect on the evolved N_2O.

CHAPTER 9

RHIZOBIAL DENITRIFICATION

9.0.0 INTRODUCTION

The Legume-Rhizobia symbiosis is well recognised as an effective means of providing the nitrogen requirements of pastures. However, recent studies have shown that under liquid culture conditions rhizobia are capable of using NO_3^- as the terminal electron acceptor for anaerobic respiration, resulting in the formation of N_2O (Daniel and Appleby, 1972; Daniel *et al*, 1980). Experiments were designed to determine if rhizobial denitrification could occur in soils.

9.1.0 MATERIALS AND METHODS

Three soils were examined in laboratory studies of rhizobial denitrification. They were the Marua clay loam (Yellow Brown earth), Otorohanga silt loam (Yellow Brown loam) and Te Kowhai silt loam (Yellow Brown loam). These soils were sterilized and then inoculated with various mixtures of rhizobia, nitrate and nutrients.

9.1.1 Methods of soil sterilization

Considerable difficulty was experienced throughout this study in obtaining sterile soil samples. Initial trials were conducted on soils sterilized by one 15 minute autoclaving at 121°C and 14 psi. The subsequent results (see tables 1 and 2, Appendix 2) were the most satisfactory of all attempted sterilizations. The difficulty with this technique is that after 6 or 7 days the control samples began to show microbial activity suggesting only partial sterilization. Samples subsequently were autoclaved twice under more stringent conditions, i.e. 125°C and 17 psi for 60 minutes each time. While this procedure significantly reduced the activity of controls, inoculation of the rhizobia cultures produced poor denitrification rates. Those soils with low cell numbers (i.e. $<10^5$ cell/g) often

failed to produce any detectable N_2O. This reduction in denitrification activity was attributed to poor growth characteristics of the rhizobia in this soil medium. It was concluded that the stringent autoclaving process was probably producing toxic organic compounds by way of pyrolysis of the soil organic matter (Gray and Williams, 1975; p64).

Other sterilization procedures were sought. Soils were sent to the Department of Nuclear Sciences (Wellington) for gamma (γ) irradiation (2 mega-rads). Once again, the control samples showed significant microbial activity evidenced by CO_2 and N_2O evolution. It is possible in this instance that the sterilization process was effective, and the soil was later contaminated. The sterilized soil was received poorly packaged.

Samples were sent to the Port Agriculture Department of the Ministry of Agriculture and Fisheries (Hamilton) for sterilization with ethylene oxide. Subsequent inoculation of these samples showed mixed results. The rhizobial activity was the most rapid seen in any inoculation with a lag time of less than 24 hours before the onset of N_2O production. All other methods of sterilization (especially autoclaving) produced significantly longer time lags during which period it is assumed many rhizobia cells died. The ethylene oxide treatment however showed variable results with regard to controls. It is possible that re-infection of samples could have occurred during subsequent handling and this technique bears further investigation.

Sterilization by methyl bromide was also attempted. Soil was weighed into 125 ml erlenmeyer flasks and placed inside a glove-box. A few mls of methyl bromide were poured into each flask and left to volatilise over several days. The glove-box was then flushed with N_2 (through a gas filter) several hours each day for ten days. While still in the glove-box, the flasks were capped with suba seals which had also been in the glove-box during the sterilization procedure.

Soils sterilized in this manner produced no N_2O or CO_2 upon inoculation. An assay for NO_3^- showed none had been consumed. Upon opening the flasks a presence of methyl bromide was evident. This method of sterilization bears further investigation as the control samples showed no signs of activity either. If a satisfactory technique could be developed for purging methyl bromide from the samples, this may be a viable method of sterilization for incubation experiments.

Tyndallisation was also attempted as a compromise to the deleterious effects of autoclaving. Samples were weighed into erlenmeyer flasks and stoppered with suba seals. Each suba seal was pierced with a hypodermic needle and the samples were warmed on a water bath to 75°C. After several hours at 75°C the flasks were allowed to cool to room temperature and left standing for 24 hours. This cycle of heating, cooling and standing was repeated for ten days. The results of this method were similar to those of the single autoclaving technique. Pyrolysis effects were evident. Controls showed significant activity and there were significant time lags (1-3 days) before the onset of N_2O production.

To summarise, both the ethylene oxide and methyl bromide techniques showed promise, as also did the γ sterilization. Due to the relative inaccessibility of the γ sterilization unit, further work, time permitting, would have pursued either the methyl bromide or ethylene oxide techniques. From a point of view of ease of manipulation, the ethylene oxide technique may be the more favoured method of sterilization.

9.2.0 HANDLING AND INCUBATION CONDITIONS

Rigorous precautions were taken to maintain the sterility of samples. All glassware, water and solutions were autoclaved for 15 minutes just prior to use. All sampling transfers were performed

in a laminar flow cabinet using sterilized (autoclaved) utensils. Each flask was then sealed with a previously autoclaved suba seal (wrapped in foil). Fresh disposable syringes were used for each inoculation, addition of water and nutrient solutions.

Each sample had sufficient water added to bring the soils to their respective 100% W.H.C. For those samples where less than ambient levels of oxygen were required, the atmosphere was replaced with 'high purity' helium. Flasks were attached to a glass manifold by a hypodermic needle on a glass 'luer-lock' fitting. The 6 mm luer-lock was packed tightly with glass wool (see figure 9.1) and had previously been sterilized by autoclaving. Helium was flushed into the flask (10 ml/min via the luer-lock. The effluent gas passed through another syringe needle attached to plastic tubing which vented into a beaker of water to prevent back diffusion of air.

The atmosphere was monitored for oxygen by the gas chromatographic technique described in section 3.2.1. and flushing continued until the desired O_2 level was achieved.

After this purging and each subsequent analysis sampling, the septum was resealed with a smear of clear 732 RTV silicone.

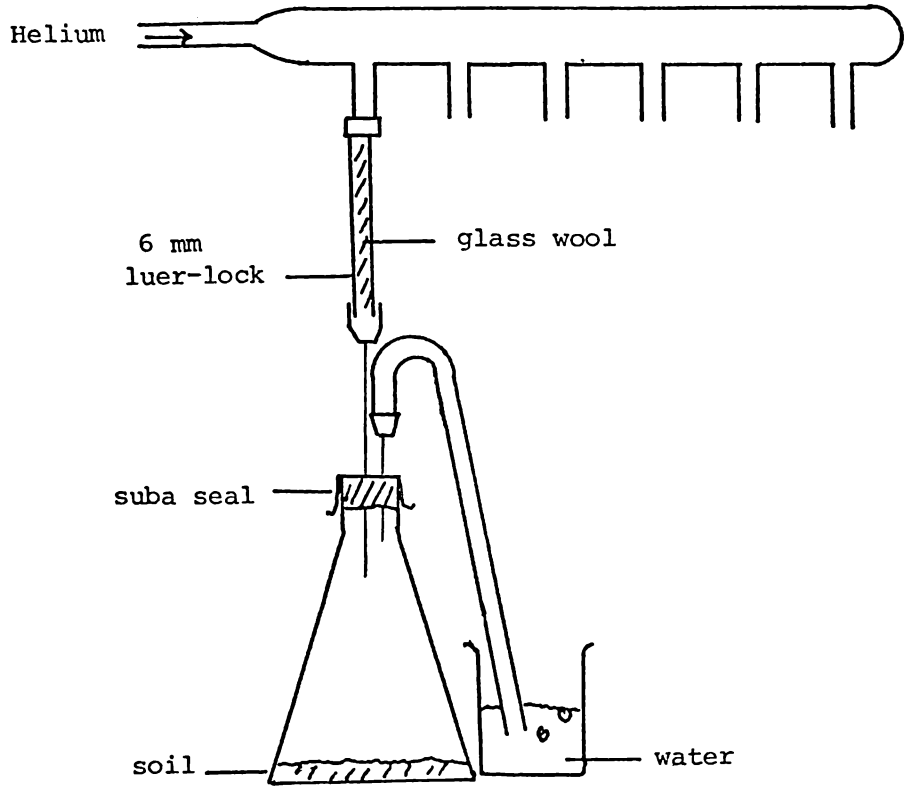
Samples were incubated at 25°C.

9.3.0 RESULTS AND DISCUSSION

The results and discussion are described in the accompanying published paper - see Appendix 2 - Denitrification by Rhizobia. A possible factor contributing to nitrogen losses from soils.

The experimental work reported under LABORATORY WORK ON RHIZOBIA IN SOILS (Appendix — 2) was conducted by the author.

Figure 9.1 Apparatus for exchanging flask atmospheres



CHAPTER 10

SUMMARY AND CONCLUSIONS

10.1.0 INCUBATION ASSESSMENT OF DENITRIFICATION POTENTIAL

A laboratory incubation technique, utilizing the acetylene inhibition of nitrous oxide reductase, was developed to provide a comparative measurement of denitrification potentials of New Zealand soils. The denitrification potential of a soil is defined as "the maximum rate of NO_3^- dissimilation under anaerobic conditions without addition of exogenous reductant". The technique developed provides a rapid method for identifying soils from which large denitrification losses are likely to occur, and will provide information on which to base the selection of soils for field studies. Using the technique in laboratory studies it was concluded that:

1) The rate of denitrification in soils incubated anaerobically is independent of the moisture content between 60 and 120% W.H.C.

2) Rates of denitrification during anaerobic incubations are independent of nitrate concentrations above 50 μg NO_3^- $N.g^{-1}$.

3) Denitrification rates of soils incubated anaerobically are extremely sensitive to traces of oxygen. The presence of 1% O_2 reduces the rate of denitrification to less than 0.01 of that observed in anaerobic conditions.

4) In the presence of small amounts of oxygen (~0.5% v/v) soils generally exhibit a biphasic denitrification rate. During the first phase oxygen inhibits full derepression of nitrate reductase until near anaerobic conditions occur. The second rate occurs after full anaerobosis is achieved and is commonly slightly higher than that seen in completely anaerobic soils. This increase is attributed to further growth of the denitrifying organisms during the initial aerobic phase.

5) Microbial activity which proceeds in soils stored at 4°C results in available carbon being depleted to approximately 40% of its original value during the first ten days after collection. Denitrification

activity also proceeds at 4°C under anaerobic conditions. Depletion of available carbon during storage results in a rapid decrease in denitrification potentials of soils with time during storage at 4°C.

6) During anaerobic incubation soils exhibit denitrification rates indicative of their maximum *in situ* denitrification potential. No logarithmic growth phase was noted in any soil studied during this period of incubation.

7) Linear biphasic rates of denitrification are frequently observed during the first eight hours of anaerobic incubation. The first phase reflects the activity of *in situ* denitrifying enzymes, while the second, higher, rate is a measure of the maximum denitrification potential of a soil.

8) The maximum denitrification potential of a Te Kowhai silt loam remains relatively constant throughout the year, decreasing only when very dry conditions occur during summer. The denitrifying enzymes in the Te Kowhai soil are fully derepressed during all but the summer months.

9) It appears that the denitrification potential in Waikato soils is often controlled by the available carbon supply rather than nitrate level.

10) A wide range (approximately ten-fold) of denitrification potentials exists between soil types. A relationship between the denitrification potential and the nitrification activity is evident in some soils. Soils exhibiting a low nitrification activity also demonstrate a low denitrification activity. As the nitrification activity increases, factors other than the supply of nitrate appear to influence the denitrification rate. Soils containing allophane generally exhibit higher denitrification potentials.

11) Denitrification potentials of soils are related to stocking rates, higher potentials occurring under high stock rates. This is attributed

to an increased availability of carbon and nitrate substrate.

12) The zone of maximal *in situ* denitrification is in the 0-3 cm layer of soils. Denitrification potential declines rapidly in the 3-6 cm depth. The intense concentration of denitrification activity in the top 3 cm of soil cannot wholly be attributed to the availability of nutrients. Denitrification activity is not stimulated below 3 cm by addition of available C and N.

13) Soils under maize crops exhibit markedly lower denitrification potentials than the corresponding soil under pasture. This effect is attributed to the depletion of available carbon reserves by the maize crops.

14) The denitrification potential of a Horotiu silt loam is not increased by the addition of urine even though urine increases available carbon and nitrate many-fold. Where leaching does not occur and urine remains in the top soil, denitrification potential is significantly decreased (40%). This is attributed to an inhibition of the denitrifying population either by a build-up of nitrite or the accumulation of some toxic waste product.

10.2.0 DIRECT FIELD ASSESSMENT OF N_2 EVOLUTION

A technique was developed which permits direct *in situ* assessment of denitrification. It comprises the following features:

1) A lysimeter which is capable of sealing off the base of the soil core *in situ* with a stream of gas. The base-flow is effective in preventing the inward diffusion of soil gases. The lysimeter also proved effective in purging the core of soil air. Nitrogen levels as low as 0.2% (v/v) can be readily achieved and maintained *in situ*.

2) These combined abilities allow the possibility of the first direct measurements of nitrogen evolution from the soil using N-15 enriched nitrogen gas. By measuring the rate of isotopic dilution of

a spike of N-15 enriched nitrogen, measurement of nitrogen evolution of the order of 10 kg N/ha/yr is potentially possible. This technique in conjunction with a Micromass 602c mass spectrometer is shown to be suitable for direct assessment of denitrification rates in the field providing the dissolved N_2 contribution can be accounted for.

3) Nitrous oxide emission can be calculated directly from the same gas sample.

4) Pre-treatment of samples is minimal.

5) The same lysimeter is also shown to be effective in achieving high acetylene concentrations within a soil core rapidly. Thus it is also possible to make denitrification estimates in the field by monitoring nitrous oxide production in the presence of acetylene.

6) Using the lysimeter-acetylene technique the *in situ* denitrification activity of Te Kowhai silt loam was measured. Calculations showed a maximal possible annual loss due to denitrification of 122 kg N/ha. This estimate supports previous deficit budget measurements of the N loss by denitrification.

7) Field measurements of denitrification in the Rukuhia peat suggest a low annual N loss and the data indicates leaching is likely to be the major source of N loss from this soil.

10.3.0 DENITRIFICATION IN SOILS BY RHIZOBIA

Using the incubation techniques described in this thesis, the denitrification capacity of free-living rhizobia was assessed in soil with the following conclusions:

1) Rhizobia were shown to be active denitrifiers in soils under laboratory conditions in cell numbers consistent with their *in situ* population.

2) Even in the presence of moderate oxygen tensions (10%), denitrification appeared to be uninhibited.

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APPENDICES

APPENDIX 1

CALCULATIONS FROM MICROMASS 602C MANUAL

The results may be set out in the form shown below:

Sample gas	-XYZ
Reference gas	-ABC (laboratory standard)
Ratio measured	-29/28
Peak height	-Reference major = Sample major 4.00 x 10^{-9} amps (10^{-8} amps full scale)
Ratio settings	-R(0.398 for example)
Ratio offset setting	-Ro (+0.01 for example)
Current ranges	-Ma = 10^{-8}A Mi = 10^{-10} (for example)

SAMPLE s	REFERENCE r	DIFFERENCE s - r	MEAN OF CONSECUTIVE READINGS	DEVIATION FROM MEAN	SQUARE OF DEVIATION FROM MEAN
s1					
	r1	s1 - r1 = A1	$\frac{A1 + A2}{2} = X1$	$X1 - \bar{X}$	$(X1 - \bar{X})^2$
		s2 - r1 = A2	$\frac{A2 + A3}{2} = X2$	$X2 - \bar{X}$	$(X2 - \bar{X})^2$
s2		s2 - r2 = A3	$\frac{A3 + A4}{2} = X3$	$X3 - \bar{X}$	$(X3 - \bar{X})^2$
	r2	s3 - r2 = A4	$\frac{A4 + A5}{2} = X4$	$X4 - \bar{X}$	$(X4 - \bar{X})^2$
s3		s3 - r3 = A5	$\frac{A5 + A6}{2} = X5$	$X5 - \bar{X}$	$(X5 - \bar{X})^2$
	r3	s4 - r3 = A6	$\frac{A6 + A7}{2} = X6$	$X6 - \bar{X}$	$(X6 - \bar{X})^2$
s4		s4 - r4 = A7	$\frac{A7 + A8}{2} = A7$	$X7 - \bar{X}$	$(X7 - \bar{X})^2$
	r4	s5 - r4 = A8	$\frac{A8 + A9}{2} = X8$	$X8 - \bar{X}$	$(X8 - \bar{X})^2$
s5		s5 - r5 = A9	$\frac{A9 + A10}{2} = X9$	$X9 - \bar{X}$	$(X9 - \bar{X})^2$
	r5	s6 - r5 = A10	$\frac{A10 + A11}{2} = X10$	$X10 - \bar{X}$	$(X10 - \bar{X})^2$
s6		s6 - r6 = A11			
	r6				

$$\frac{\Sigma r_n}{6} = \bar{r} \qquad \frac{\Sigma X_n}{6} = \bar{X} \qquad \Sigma(X_n - \bar{X})^2$$

From these figures the following can be calculated:

$$\delta = \frac{R_s - R_r}{R_r} \times 1000$$

$$\delta = \frac{(R + \bar{r} + Ro + \bar{X}) - (R + \bar{r})}{R + \bar{r}} \times \frac{(Mi/Ma)}{(Mi/Ma)} \times 1000$$

$$\delta = \frac{Ro + \bar{X}}{R + \bar{r}} \times 1000$$

Numerical definition of σ :

$$\sigma_1 = \sqrt{\frac{\Sigma(X_n - \bar{X})^2}{(n - 1)}}$$

$$\sigma_n = \sqrt{\frac{\Sigma(X_n - \bar{X})^2}{n\,(n - 1)}}$$

$$2\sigma = \frac{2\sigma_1}{\sqrt{n}}$$

σ expressed in °/₀₀ relative to reference $(R + \bar{r})$:

$$\sigma_1(°/_{00}) = \frac{\sqrt{\frac{\Sigma(X_n - \bar{X})^2}{(n - 1)}} \times 10^3}{R + \bar{r}}$$

$2\sigma_n$ expressed in °/₀₀ relative to reference $(R + \bar{r})$:

$$2\sigma_n(°/_{00}) = \frac{\sqrt{\frac{\Sigma(X_n - \bar{X})^2}{(n - 1)}} \times 10^3}{R + \bar{r}} = \frac{2\sigma_1(°/_{00})}{\sqrt{n}}$$

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