NITROGEN LOSS THROUGH DENITRIFICATION IN SOIL UNDER PASTURE IN NEW ZEALAND

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ABSTRACT

Denitrification is an important process in the N cycle that can affect the efficiency of use of soil nutrients and also the impact of agricultural activities on the wider environment. There have been few studies on the losses of N by denitrification from pasture soils. The current study was undertaken to investigate N loss through denitrification in a New Zealand pasture, and to examine relationships between denitrification and other environmental and soil factors. Denitrification was measured using the acetylene inhibition technique by incubating soil in a closed system.

A study on the effect of storage concluded that a soil's moisture status and the duration of storage can affect the denitrification activity, as measured by a short-term assay. This effect can operate by changing both denitrification enzyme activities and the availability of substrate.

Denitrification activities were greatest in the surface soil and generally decreased with depth in the soil profile. The decrease in denitrification activity with depth could be also attributed to both a decrease in enzyme activity and also decreasing availability of C and NO_3^--N.

High coefficients of variation (CV) and skewed distributions of denitrification rate were always observed in the field. The log-normal distribution generally provided a better fit than the normal distribution for denitrification rates measured in the field. The variance in denitrification rate changed temporally, and depended on the soil moisture content and the grazing pattern. Amendment of soil cores with NO_3^--N and soluble-C, either singly or together, substantially decreased the skewness of the frequency distribution of denitrification rates.

Denitrification rates varied according the location in the paddock. Highest rates were detected in the floor of a gully and in a gateway area.

Denitrification rates followed a marked seasonal pattern, with higher rates being measured

during the wet winter and lower rates during the dry summer. Higher denitrification rates were also observed during brief periods after rainfall events in the summer. An annual N loss of about 4.5 kg N ha^{-1} through denitrification was estimated in this dairy-farm paddock. Block grazing with cows at a high stocking rate increased the denitrification rate between 3 and 14 days after grazing under seasonally moist conditions. However, the total N loss through denitrification induced by grazing during that period was still very small, compared with the N returned by the grazing animals.

Correlation and multiple regression analyses revealed that relationships between single core measurements of denitrification rates and other edaphic factors in the field were poor for the combined data set. However better relationships between denitrification rate and NO_3^--N concentration in the individual soil cores existed at high soil moisture contents, and better relationships between denitrification rate and respiration rate existed at low soil moisture contents. Mean denitrification rates from individual dates were positively correlated to soil moisture content. Regression equations derived from the mean-value data for each sampling date improved the prediction of the observed denitrification rate, compared to those from the individual data sets. Soil moisture content and NO_3^--N concentration accounted for 51% of the observed variability in denitrification rate in the field.

Experiments conducted to obtain insights into factors regulating denitrification, by removing possible limitations to denitrification during the incubation, found that the addition of NO_3^--N solution to soil cores stimulated denitrification rates in all seasons. This result suggested that the NO_3^--N concentration, or more importantly, the accessibility of NO_3^--N to the denitrification sites in the pasture soil may have limited denitrification. Denitrification rates also increased when soluble-C was added to the soil cores, but the magnitude of the effect depended on other edaphic factors

A separate study demonstrated that the presence of acetylene during the denitrification measurement also inhibited the nitrification process, and consequently could affect the NO_3^--N availability for denitrification in the soil. However, this study also indicated that inhibition of nitrification by acetylene did not affect short-term measurement of denitrification rate.

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Nitrogen (N) loss through denitrification is of concern because of the potential impacts of some of the product gases on the environment and the possible economic significance of N losses from agricultural systems. Denitrification in soil has been a subject of study since last century. However, it was the availability of the isotope ^{15}N in the 1950's that allowed the denitrification process to be properly defined and the effect of environmental factors to be studied (reviewed by Myrold, 1991). Further development of a range of techniques from the mid 1970's has expanded our knowledge of denitrification and a general understanding of the process now exists (reviewed by Tiedje *et al.*, 1989). However, quantitative information on the magnitude of N loss through denitrification in agricultural, grassland, and forest soils is still limited, and the available data are extremely variable due to the wide range of conditions under which the data were collected. Therefore, reliable quantification of the process still remains a goal to be achieved.

New Zealand research has contributed a great deal to the understanding of N in pastoral agriculture. Although much of the earlier work was carried out in mown swards, recent attention has been given to N returns to the soil through dung and urine on grazed pastures, the transformations of this N in the soil, and the consequent potential for loss to the wide-

environment (reviewed by Ball and Tillman, 1994). However, it is notable that few published reports exist on denitrification in New Zealand pasture soils. This is a reflection of the lack of research on this aspect of the N cycle in New Zealand. Thus, the pursuit of an understanding of N loss through denitrification in New Zealand pastoral systems is the central focus of this study.

Most of the previous research on N cycling in grazed pastures has demonstrated the importance of the grazing animal in returning N ingested in the herbage to the soil in the forms of urine and dung (reviewed by Ball and Tillman, 1994). While the early research indicated that grazing animals had a beneficial role in nutrient cycling through the transfer of fertility in the form of excreta around the farm (Sears, 1950), this viewpoint has been modified. Research has shown that the return of N to the soil in the form of extremely concentrated urine spots can lead to greater losses than originally indicated, particularly on intensively managed, high fertility farms (Ball and Keeney, 1981). The fate of excretal N in pastures under New Zealand conditions has been investigated by a number of workers (e.g. Ball *et al.*, 1979; Carran *et al.*, 1982; Field and Ball, 1982; Field *et al.*, 1985; Ball and Field, 1987; Williams *et al.*, 1989; Brock *et al.*, 1990). In several of these studies, mass balance considerations indicated that significant amounts of N were unaccounted for. Loss of this N through denitrification is one possibility. Certainly, at first glance the potential for denitrification from pastures would appear to be high due to high levels of organic-C in the surface soil and high concentrations of nitrate-N (NO_3^--N) present in soil under urine and dung patches (Haynes and Williams, 1993). However, the extent of denitrification and the factors affecting it in New Zealand pastures requires further study.

Questions can also be raised about the effects of soil characteristics, such as soil moisture

content, temperature, soil NO_3^--N concentration, and soil microbial activity on the rate of denitrification in soil under pasture. The relationships between the rate of denitrification and many of these soil factors have been well demonstrated in laboratory studies (reviewed by Tiedje, 1988). Whether many of these relationships also apply in the field situation under pasture has not been clearly established. This highlights the need for research on denitrification under different field conditions throughout the year.

A further issue complicating the study of denitrification in the field is the marked spatial and temporal variability that has been observed (Ryden, 1983; Folorunso and Rolston, 1984). Although some information is available on the spatial and temporal variability in mineral N in pasture soils (White *et al.*, 1987), very little similar information on N loss through denitrification under grazed pastures is available. Better quantitative knowledge about the spatial and temporal variability of the process is required to improve the accuracy of field measurement of denitrification. To enable better quantification of denitrification, further consideration also needs to be given to defining the depth of the soil profile within which the most of denitrification occurs.

1.2 STRUCTURE OF THE THESIS

Given the background discussed above, the present study was designed to quantify the extent of denitrification occurring in the pasture of one paddock within an intensive dairy-farm and to gain information on the factors regulating the denitrification rate under field conditions.

Chapter 2 presents a literature review on denitrification and briefly outlines N losses in

pasture soils. In the subsequent chapters, results generated during this study are presented, discussed and compared to the findings of previous workers. The specific objectives of each of these chapters are described briefly below.

Prior to studies in the field, an assay for denitrification activity in pasture soils was established in laboratory. Optimum conditions were set for the assay, and the effects of storage of soils on denitrification activity were examined. These results are presented in Chapter 3. The assay provided a basic method for measurement of denitrification activity in subsequent field studies.

In the work presented in Chapter 4, the variability of denitrification activity with depth in soil was investigated under several field conditions. The effects of soil C and NO_3^--N, as well as rainfall events, on denitrification activity were also addressed. The appropriate sampling depth for denitrification studies from the pasture soil was deduced from these results.

The spatial variability of denitrification rate, together with the variability of other soil parameters was investigated (Chapter 5). The causes of such variability in grazed pasture were also examined.

In the following chapter (Chapter 6), the temporal variability of denitrification rate within several sampling sites is discussed. These data are then used to estimate annual N losses through denitrification in the study paddock.

The results from Chapters 5 and 6 indicated that although there was considerable temporal

and spatial variability in denitrification rate, the overall loss of N from the pasture through denitrification was low compared to other possible pathways of loss. The low observed rates of denitrification also prompted an examination of the effects of environmental and soil factors on denitrification rates, both temporally and spatially, in an attempt to identify the regulators controlling denitrification in the study paddock. The approach used in Chapter 6 was primarily statistical, with correlation sought between the observed denitrification rate and other concurrently-measured soil parameters.

It was recognised that redistribution of N in dung and urine due to animal grazing can have profound effects on N transformations in pastures. Given this, it was possible that the highly episodic nature of grazing events in intensive dairy-farming systems may results in large short-term losses of N by denitrification. These losses might not be detected by a regular sampling programme conducted independently of the grazing management. Accordingly, more intensive studies, described in Chapter 7, were designed to investigate animal grazing effects on denitrification in contrasting seasons.

The examination of the effects of environmental factors and gazing events on denitrification rates in the previous chapters was extended in Chapter 8 to include laboratory experiments in which soil cores from the field were amended in various ways to remove possible limitations to denitrification and the effect on denitrification rate was observed. By this mean, further insights into the factors regulating denitrification in pasture soils were obtained.

Results of the work to this stage had provided strong indicators that it was the rate of supply of NO_3^--N to the microsites and the total amount of NO_3^--N in the soil that regulated

denitrification. This raised questions as to link between nitrification and denitrification and also the effect that the acetylene used in denitrification assay may be having on nitrifying organisms, and hence, possibly, measured denitrification rate. Accordingly, the final section of this study involved a laboratory study investigating the effect of acetylene concentration and moisture content on denitrification rate (Chapter 9).

A synthesis and summary from the previous chapters are presented in Chapter 10.

CHAPTER 2

REVIEW OF LITERATURE

2.1 INTRODUCTION

Denitrification is the conversion of nitrate (NO_3^-) to the gaseous products nitrogen (N_2) and nitrous oxide (N_2O) and occurs worldwide in terrestrial, aquatic, and sedimentary ecosystems. Denitrification is an agriculturally-important process, since it can lead to losses of valuable nitrogen (N) from agricultural systems and thus decrease the efficiency of fertilizer use and reduce agricultural production. Apart from the agricultural interest in denitrification associated with the loss of N, there are increased concerns with respect to the environment. Denitrification can be both detrimental and beneficial to the environment. For example, one of the gaseous products from denitrification, N_2O, has possible deleterious effects on global warming (Wang *et al.*, 1976), and it also has a possible catalytic effect on the destruction of stratospheric ozone (Crutzen, 1981). In contrast, denitrification can be used as a means to remove N from wastewaters and minimize NO_3^- contamination of groundwater (Knowles, 1982).

A number of techniques and procedures to study the process of denitrification have been developed and consequently, considerable information on denitrification is now available. A number of comprehensive literature reviews on denitrification have been written from a variety of viewpoints. These include reviews by Firestone (1982), Fillery (1983),

Groffman *et al.* (1988), Stouthamer (1988), Tiedje (1988), Beauchamp *et al.* (1989), Nieder *et al.* (1989), Eichner (1990) and Aulakh *et al.* (1992). The nature of denitrification was firmly established a very long time ago, so there is also a wealth of older literature dealing with denitrification that should not be ignored (Nommik, 1956; Bremner and Shaw, 1958).

This review will summarize briefly current knowledge of the process of biological denitrification and comment on soil and environmental factors that affect denitrification. Some aspects of the methodology for measuring denitrification in soil will also be included.

2.2 BIOCHEMISTRY OF DENITRIFICATION

2.2.1 Microbiological basis of denitrification

Denitrification is the last step in the N cycle, where the fixed N is returned to the atmospheric pool of N_2. The definition of biological denitrification is the dissimilatory reduction of NO_3^- or nitrite (NO_2^-) by essentially anaerobic bacteria producing molecular N_2 or oxides of N when oxygen is limiting (Payne, 1981). Denitrification is carried out by respiratory denitrifiers that gain energy by coupling N-oxide reduction to electron transport phosphorylation (Tiedje, 1988). Denitrifying bacteria can be present in nearly all soils and are generally facultative aerobes (Tiedje, 1988). It is accepted that the main genera capable of denitrification in soil include *Pseudomonas, Bacillus, Alculigenes* and *Flavobacterium* (Payne, 1981; Firestone, 1982; Tiedje, 1988).

Many denitrifying bacteria are chemoheterotrophs, *i.e.* they can use NO_3^- as their primary electron acceptor for obtaining energy from organic compounds (heterotrophic

denitrification). In addition, some autotrophic organisms can obtain energy by using NO_3^- for oxidation of inorganic compounds, *e.g.* S^{2-}, Fe^{2+} (autotrophic denitrification). Autotrophic denitrification will not be discussed in this review. As facultative aerobes, denitrifiers can be considered as bacteria which prefer to use O_2 as their electron acceptor and which can use NO_3^- as a terminal acceptor of electrons only when O_2 is not available. Under conditions of limited O_2 availability, aerobic respiration can apparently provide the energy needed for synthesis of new enzymes required for NO_3^- reduction.

2.2.2 Process of denitrification

The general pathway of the reduction of NO_3^- during denitrification process may be represented as follows (Payne, 1981; Firestone, 1982):

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad (2.1)$$

The following stoichiometric equation for denitrification is often cited, with glucose as the C substrate:

$$5(CH_2O) + 4NO_3^- + 4H^+ = 2N_2 + 5CO_2 + 7H_2O \qquad (2.2)$$

The detail of mechanisms involved in denitrification can be found in several reviews (e.g. Firestone, 1982; Knowles, 1982). The general requirements for biological denitrification are: a) the presence of bacteria possessing the metabolic capacity, b) suitable electron donors such as organic-C compounds, c) anaerobic conditions or restricted O_2 availability and d) N oxides, NO_3^-, NO_2^-, NO, or N_2O as terminal electron acceptors. There has been

some doubt if NO is a true intermediate or a byproduct (Amundson and Davidson, 1990).

Although many soil bacteria are able to denitrify, denitrifying bacteria exhibit a variety of reduction pathways. Some bacteria produce only N_2, while others give a mixture of N_2O and N_2, and some only N_2O (Stouthamer, 1988). Normally in soils, N_2O and N_2 are produced in varying ratios, depending on the substrate, environmental conditions, the organisms involved, and also on the time elapsed since the onset of denitrifying activity (Sahrawat and Keeney, 1986; Arah and Smith, 1990). The rate of denitrification is usually rather low under the environmental conditions reported to favour production of N_2O relative to N_2.

The biological denitrification process is not solely responsible for the reduction of NO_3^- in soil. It is also subject to chemical reactions that lead to production of N_2 by nonenzymatic pathways under fully aerobic conditions (Paul and Clark, 1989). However, chemodenitrification is not considered further in the present review.

2.3 FACTORS REGULATING DENITRIFICATION IN SOILS

Environmental parameters which affect denitrification have been identified in laboratory studies (reviewed by Payne, 1981; Firestone, 1982; Knowles, 1982), and the relative importance of these parameters has been investigated in a number of field studies of agricultural, grassland, and forestry systems (Ryden, 1983; Rolston et al., 1984; Davidson and Swank, 1986; Aulakh et al., 1992). The diverse environmental factors affecting denitrification have been broadly divided into either proximate or distal regulators (Groffman et al., 1988; Tiedje, 1988). Proximate regulators are those that affect the

immediate environment of the bacterial cell, such as NO_3^--N concentrations, C levels, O_2 contents, pH and temperature. Distal regulators control the proximate regulators on a larger scale such as plant growth, agricultural practices, soil structure, rainfall and drainage pattern.

In general, denitrification appears to proceed in soils under a much broader range of conditions than would be predicted on the basis of the biochemistry of the process and the physiology of denitrifiers. Denitrification is promoted by high soil moisture conditions, neutral soil pH, high soil temperature, a low rate of O_2 diffusion as well as the presence of soluble organic matter and NO_3^--N. The qualitative roles of these determinants taken separately are well defined, but their interactions are poorly understood, and this makes difficult the prediction of actual fluxes of gaseous N from field soils. Many investigators have observed large spatial and temporal variability of denitrification rates from soils and it is believed that the presence of biological hotspots is the major reason for the large variability of denitrification in field environments. In the following sections, some of the factors that affect denitrification in soil ecosystems are briefly discussed.

2.3.1 Carbon availability

The importance of the supply of organic matter in denitrification has been recognised for a very long time (reviewed by Beauchamp *et al.*, 1989). The most abundant denitrifying bacteria in soil are heterotrophic and require a source of electrons and energy for the denitrification process. During denitrification, electrons are transferred from reduced, organic-C compounds to NO_3^- *via* an electron transport chain, resulting in the production of energy for the bacterial cell. Equation 2.2 shows a theoretical balance for this reaction.

Carbon compounds act as the main source for cell growth of denitrifying bacteria. Therefore, the supply of readily decomposable organic matter in soil is critical in controlling the rate of denitrification (Burford and Bremner, 1975; Stanford *et al.*, 1975a; Payne, 1981; Reddy *et al.*, 1982; Robertson and Tiedje, 1984; Myrold and Tiedje, 1985b). The presence of an ample C substrate can also result in rapid O_2 consumption and possible O_2 depletion, which may then also indirectly enhance the potential for denitrification (Firestone, 1982). Most studies have not identified whether the C was used by denitrifying bacteria directly or whether the C stimulated an increase in the population of soil bacteria.

The important relationship between soil organic-C and denitrification has been investigated in many studies. For example, Burford and Bremner (1975) showed that denitrification rates in soils were most highly correlated with water-soluble-C, but less well correlated with total C. A good correlation between water-extractable soil organic-C or mineralizable-C and denitrification rate in unamended soils has also been noted by Beauchamp *et al.* (1980) for well-drained soils, and deCatanzaro and Beauchamp (1985) found that mineralizable-C was a good predictor of denitrification rates provided there was enough available NO_3^--N in soils. The relationship between denitrification and C has also been expressed in mechanistic models (Grundmann and Rolston, 1987; Malhi *et al.*, 1990).

Denitrification is known to be stimulated by addition of organic materials such as plant residues and manure (Bowman and Focht, 1974; Smid and Beauchamp, 1976; Jacobson and Alexander, 1980; Aulakh *et al.*, 1984a; Christensen, 1985; Paul and Beauchamp, 1989). The effects of these additions are dependent on the quality of the organic materials added. More-readily available compounds, such as glucose, stimulate denitrification to a greater extent than more complex C compounds such as cellulose and lignin. It has also been

suggested that all soluble-C compounds are not equally available to denitrifiers (Paul *et al.*, 1989). In their study alfalfa-amended soil had a significantly higher rate of denitrification than soil amended with the same amount of straw. The data with alfalfa-amended soil suggested that denitrifiers used water-extractable-C materials produced by other organisms under anaerobic conditions (deCatanzaro and Beauchamp, 1985).

Furthermore, the accessibility of C supply to bacteria also has a strong regulatory effect on denitrification, especially in field conditions. Denitrification rate has been shown to be limited by the diffusion rate of organic compounds in some soils (Myrold and Tiedje, 1985a), and freezing and thawing of soil, or air-drying can increase denitrification by enhancing the amount of soil organic matter available to the denitrifiers (Patten *et al.*, 1980; Breitenbeck and Bremner, 1987). Organic-C content decreases with depth in most mineral soils. Thus although leaching of NO_3^--N into lower horizons is a common phenomenon in most agricultural soils, the availability of organic-C is usually one of the main factors limiting denitrification activity in subsoils (Weier and Doran, 1987; Parkin and Meisinger, 1989).

The availability of C has also been reported to influence the proportion of N_2O and N_2 produced. It is generally concluded that increasing C availability decreases the ratio of $N_2O:N_2$ (Smith and Tiedje, 1979a; Arah and Smith, 1990).

2.3.2 Nitrate concentration

There has been some debate about the effect of NO_3^--N on denitrification. A dependency of denitrification on NO_3^--N concentration has been observed (Bowman and Focht, 1974;

Stanford *et al.*, 1975a), and would be expected, since the availability of NO_3^--N for denitrifying bacteria is the first-step in biological denitrification. However, the rate of denitrification in soil has been found to be independent of NO_3^--N concentration in some studies (Bremner and Shaw, 1958; Smid and Beauchamp, 1976). It may be that at low NO_3^--N concentrations, denitrification kinetics in soils are first order, and the NO_3^--N concentration may be the rate limiting factor, but that when the endogenous NO_3^--N concentration exceeds a certain concentration, denitrification follows zero-order kinetics (Knowles, 1982).

The availability of NO_3^--N to denitrifying bacteria is dependent upon the rate of nitrification, the rate of N consumption by non-denitrifiers including plants and bacteria, and N leaching and diffusion rates through the soil (Tiedje, 1988). It has been often found that denitrification rate increases after NO_3^--N additions in the field (Ryden, 1983; Colbourn and Harper, 1987; Robertson *et al.*, 1987; Samson *et al.*, 1990), but there is usually no effect of increased NO_3^--N concentrations if organic-C is limited (Limmer and Steele, 1982; McCarty and Bremner, 1992).

Nitrate-N concentrations have been observed to influence the $N_2O:N_2$ ratio in the gaseous products of denitrification. NO_3^--N usually inhibits N_2O reduction to N_2 (Blackmer and Bremner, 1978). Therefore, at low NO_3^--N concentrations, N_2 is the predominant product and at high NO_3^--N concentrations, N_2O often predominates (Arah and Smith, 1990).

2.3.3 Soil aeration

The presence of O_2 causes a reversible inhibition of the bacterial enzymes involved in the

denitrification process (Ferguson, 1987), and O_2, competing with NO_3^-, also functions as a terminal electron acceptor (Firestone, 1982). Firestone *et al.* (1980) and Jorgensen *et al.* (1984) observed a sharp increase in denitrification activity at partial pressures below 0.2-0.3 Kpa O_2.

Any soil characteristic which influences either O_2 diffusion or consumption will affect the aerobic status of the soil and therefore the rate of denitrification. Oxygen diffusion into the soil is dependent on the number and size of pores in the soil and the way the soil particles are aggregated. Diffusion slows as pores become filled with water or damaged by physical disruption.

The rate of O_2 diffusion through water is 10^4 times less than through air. Hence increases in soil water content to levels that interfere with air diffusion progressively increase denitrification rate (Sexstone *et al.*, 1985). Most studies demonstrate a strong and positive correlation between soil water content and rate of denitrification (Groffman and Tiedje, 1991; Parsons *et al.*, 1991; Weier *et al.*, 1993). In field studies with several soils, Aulakh and Rennie (1985) showed that the rate of denitrification increased dramatically when soil volumetric water content exceeded a certain critical level between 40% and 50%.

Low soil O_2 contents resulting from soil compaction by heavy agricultural vehicles generally enhanced denitrification rate (Bakken *et al.*, 1987). Svensson *et al.* (1986) found that the soil casts produced by earthworms increased denitrification potential due to the higher O_2 demand which resulted in more anaerobic conditions. As indicated above, the addition of organic matter such as straw (Aulakh and Rennie, 1987) or manure (Guenzi *et al.*, 1978) to aerobic soil can increase denitrification and this was thought to be due in part

to a decrease in O_2 concentration caused by the consumption of O_2 by aerobic bacteria.

Denitrification can occur in soils under apparently aerobic conditions due to the presence of anaerobic microsites (Parkin, 1987). Localised areas of high energy supply can create a higher demand for O_2 by soil organisms and plant roots can have a similar effect.

By investigating the effect of O_2 on both N_2O and total gas production from denitrification in soil suspensions and in pure cultures, Firestone et al. (1980) concluded that the fraction of total gas production released as N_2O increased at elevated O_2 levels.

2.3.4 Soil pH

Soil pH is an important factor controlling the rate of denitrification. Most denitrifying bacteria grow best near neutrality (pH 6-8). But the pH range for denitrification is broad (Knowles, 1982). Denitrification slows in acid conditions (Nommik, 1956; Bremner and Shaw, 1958; Bryan, 1981), but denitrification can still occur at pH values as low as 3.5 and can account for significant N losses in naturally acid soils (Parkin et al., 1985a; Weier and Gilliam, 1986). It is thought that long-term acid conditions appear to select for denitrifier populations adapted to low-pH environments (Parkin et al., 1985a). The mechanism of pH control of denitrification is not clear. It has been speculated that indirect effects of low pH, such as C availability, may limit the size of denitrifier population in acid soils (Koskinen and Keeney, 1982; Fillery, 1983).

The degree of soil acidity also influences the $N_2O:N_2$ ratio in the gases produced. It has been observed that the proportion of N_2O increases as pH decreases, with N_2O frequently

appearing as the dominant product in acid soil (Christensen, 1985; Parkin *et al.*, 1985a). It has been suggested that the presence of increasing amounts of NO_2^- at lower pH levels may have been partly responsible for the increased mole fraction of N_2O (Koskinen and Keeney, 1982).

2.3.5 Temperature

Soil temperature affects denitrification directly in that microbial activity generally increases with increasing temperature up to a maximum temperature. Above the maximum temperature microbial activity declines. Denitrification can occur at temperatures between $0°C$ and $75°C$ (Knowles, 1982). Temperature correlation factors (Q_{10}) of 2 have been often reported (Reddy *et al.*, 1982; Dorland and Beauchamp, 1991).

Temperature also affects denitrification indirectly through the effect of temperature on both O_2 solubility and O_2 diffusion in water (Craswell, 1978). Temperature also affects a range of other biological process such as mineralisation and nitrification. Thus the overall effect of temperature on denitrification in soils may be very complex.

The studies by Powlson *et al.* (1988) and Malhi *et al.* (1990) indicate that denitrifying bacteria can adapt to soil temperature conditions. So the optimum temperature for denitrification could differ in different regions. Temperature is thought to be one of the main factors causing temporal fluctuations in denitrification rate (Ryden, 1983).

In some soils, temperature changes have been observed to affect the $N_2O:N_2$ ratio in the evolved gases. Decreasing soil temperature seems to cause an increasing proportion of

N_2O as the product of denitrification in laboratory incubations of soils (Keeney et al., 1979).

2.3.6 Plants

A number of studies have been made on the effect of plants on denitrification both in controlled laboratory and under field conditions. The mechanisms governing denitrification in the rhizosphere are highly complicated, since plants provide an input of degradable organic material to soil and remove NH_4^+-N and NO_3^--N. It has often been observed that growing roots have a stimulating effect on denitrification (Stefanson, 1972; Hailder et al., 1985; Scaglia et al., 1985; Klemedtsson et al., 1987a; Klemedtsson et al., 1987b; Lindau et al., 1990) and this can be attributed to the stimulation of bacterial respiration, by provision of C from root exudates, thereby creating anaerobic zones (Klemedtsson et al., 1987b). Other studies have shown that plant roots have neutral or even negative effects on denitrification (Smith and Tiedje, 1979b; Aulakh et al., 1983a; Hailder et al., 1985; Heinemeyer et al., 1988), and this may have been due to removal of NO_3^--N through uptake by plants (Smith and Tiedje, 1979b; Heinemeyer et al., 1988) and reduction of soil moisture content by transpiration (Bakken, 1988). Also O_2 availability may be increased near roots of aquatic plants in flooded soils (Prade and Trolldenier, 1990). Interpretation of these studies is difficult because the conflicting data were obtained from experiments with different plant species under widely different conditions. Prade and Trolldenier (1990) studied the relationship between K status and denitrification and found that K deficiency could stimulate denitrification on wheat roots, but not on rice roots.

High denitrification rates are found in soils where the plants have been cut or damaged, and

the roots remain in the soil (Beck and Christensen, 1987; Robertson *et al.*, 1987). It has been suggested that easily-available organic-C can leak out from the roots after the plants have been damaged. High N_2O emissions from grass-covered soils after the grass was cut were also demonstrated in a field study by Conrad *et al.* (1983). However, Hutchinson and Brams (1992) reported that emission of N_2O from pasture was not stimulated by clipping and removal of the grass. The study of Beck and Christensen (1987) also indicated that mature roots may supply more organic-C to the soil than young roots.

Reports on the influence of plants on the $N_2O:N_2$ ratio differ, and no clear trend can be found (Smith and Tiedje, 1979b; Klemedtsson *et al.*, 1987b).

2.3.7 Agricultural management practices

Permanent grassland develops a surface layer rich in organic material with potential for denitrification when fertilized or when urine and dung are deposited during grazing (Ryden, 1986). However, the difference in denitrification between arable lands and grasslands is not consistent. Agricultural management practices involving application of N-fertilizers, timing of irrigation, tillage technique, and use of nitrification inhibitors may influence denitrification to a very great extent. Individual field studies indicate that increasing N input results in increasing denitrification rate. This is so for all common N fertilizer types, including sewage sludge (Mosier *et al.*, 1982; Breitenbeck and Bremner, 1986; Duxbury and McConnaughey, 1986; Samson *et al.*, 1990; Bronson and Mosier, 1991; Ruz-Jerez, 1991). The extent of denitrification of fertilizer N can be considerable on clay or compacted soils in wet climates (Egginton and Smith, 1986; Hansen *et al.*, 1993), and after fertilizer application to grasslands (Ryden, 1981; Webster and Dowdell, 1982).

Greater rates of denitrification are usually observed with zero tillage compared to ploughed

soils (Rice and Smith, 1982; Aulakh *et al.*, 1984b; Linn and Doran, 1984; Staley *et al.*,

1990). This increase is related to increased soil organic matter and higher levels of

available-C in the upper part of the top soil, as well as to greater soil densities and

decreased soil aeration (Aulakh *et al.*, 1992). Moreover, no-till systems provide favourable

living conditions for denitrifying bacteria (Doran, 1980).

Microbial processes are affected by a variety of chemicals used in agriculture. Hence there

is some literature concerning the effect of agricultural chemicals, such as nitrification

inhibitors and pesticides, on denitrification (Walter *et al.*, 1979; Knowles, 1982; Goring and

Laskowski, 1982; Bremner and Yeomans, 1986). The magnitude of the effect depends on

the types and amounts of the agricultural chemicals applied.

2.4 EXPERIMENTAL CONSIDERATIONS IN THE STUDY OF DENITRIFICATION

The study of denitrification in soil is complicated by experimental difficulties. In this

section, a brief overview of the methods used in measuring denitrification is presented.

There is an extensive literature dealing with aspects of the methodology for measuring

denitrification in soils. This information is summarized in reviews by Ryden and Rolston

(1983), Smith (1987), Tiedje *et al.* (1989), Mosier (1990), Myrold (1991) and Aulakh *et al.*

(1992).

2.4.1 Outline of the methodology for measuring denitrification

Two basic approaches have been used to determine the extent of denitrification in soils.

The first one relies on the N balance. The second one involves the determination of the amount of nitrogenous gases produced in the soil. The N balance method involves the calculation of denitrification losses from the balance of N budget, accounting for plant uptake, leaching, ammonia volatilisation, and soil immobilisation. The major limitations of the N balance approach in estimating denitrification loss are that alternative pathways of gaseous loss exist and that errors in the estimation of the components of the balance can be cumulative.

It is only in the last few decades that denitrification has been widely measured by direct gas analysis. There are now several methods available that measure denitrification. The methods include:

- The use of acetylene (C_2H_2) to inhibit N_2O reduction to N_2 so that total denitrification N losses can be measured as N_2O; the approach has been facilitated by the development of gas collection systems and detectors for use in gas chromatography. This technique is the one that has been used most widely in studies on denitrification and it is discussed further in the following sections.

- The use of highly ^{15}N-labelled fertilizer to increase the isotope enrichment of the NO_3^- pool. The ^{15}N-labelled gases are then measured by mass spectrometry to quantify N_2 production due to denitrification against the large background of N_2 in ambient air. Use of ^{15}N in the measurement of denitrification has been reviewed by Myrold (1991). Direct measurement of ^{15}N-gaseous emission can be used only where substrate NO_3^--N for denitrification is added at a high level of ^{15}N enrichment and requires accumulation of evolved gases into a confined atmosphere. It is also

necessary to ensure that the ^{15}N-labelled NO_3^--N is evenly distributed through the native NO_3^--N pool in the soil. The cost of this method is high, and the measured rate of denitrification may be artificially high because of the addition of ^{15}N as NO_3^--N, particularly in soils where denitrification is limited by availability of NO_3^--N.

- The radioactive isotope, ^{13}N, has also been used for the measurement of nitrogenous gas production in short-term laboratory studies. Generally, the use of ^{13}N has been considered inappropriate due to its short half life (9.96 min) (Tiedje *et al.*, 1979), although Smith *et al.* (1978) successfully used ^{13}N to confirm that the C_2H_2 inhibition was an effective means of measuring denitrification rates in soil.

- A micrometeorological approach for measuring the trace gases. Micrometeorological methods are conceptually ideal for measuring trace gas emissions over large ecologically uniform areas, and the technique can reduce the spatial variability problems inherent in some other techniques. However, the techniques have not been extensively used to measure N_2O flux, because analytical methods that respond rapidly enough, or are sensitive enough to quantify N_2O are not available.

2.4.2 Acetylene inhibition method

Theoretical considerations. Denitrification assays based on blocking the reduction of N_2O to N_2 by C_2H_2 were demonstrated in pure culture in 1976 by Balderston *et al.* (1976), and tested in soils by Yoshinari *et al.* (1977). The accumulation of N_2O can be detected using a gas chromatograph equipped with an electron capture detector (Kaspar and Tiedje, 1980).

These contributions were major milestones in denitrification measurement and have led to an explosion of denitrification studies and an improved understanding of the process. The methods have already helped considerably to relate the rate of denitrification to soil and environmental conditions and will eventually enable the process to be mathematically modelled.

Laboratory studies have shown that N_2O is the sole gaseous product of denitrification in soils incubated in atmospheres containing 0.1-10% vv^{-1} C_2H_2, and the amount of N_2O (with C_2H_2) is equal to that of N_2O and N_2 (without C_2H_2) (Ryden *et al.*, 1979a). By comparing the C_2H_2 inhibition method with the ^{15}N method, Parkin *et al.* (1985b) concluded that denitrification rates from the C_2H_2 core method were not significantly different from the estimate by the ^{15}N technique. Even distribution of sufficient C_2H_2 through the soil is essential for the accurate measurement of denitrification rate, and 10% of C_2H_2 in the headspace volume is recommended (Tiedje, 1982).

Acetylene inhibition studies have been reviewed by Tiedje *et al.* (1989). The important advantages include the following:

- It has high sensitivity.

- Because the technique uses the natural NO_3^--N substrate pool, it determines the denitrification of all NO_3^--N irrespective of its source.

- A large number of samples can be assayed, and spatial and temporal variability of denitrification in the field can be analysed by using appropriate statistical techniques.

- It is a versatile technique suitable for laboratory, field, and remote-site studies.

- It is a relatively low cost method compared to ^{15}N or other techniques.

Areas of concern with the acetylene method have been also discussed by Tiedje *et al.*, (1989). These include:

- Inhibition of nitrification by C_2H_2 and effects on other processes such as sulphur cycling and methanogenesis.
- The potential decomposition of C_2H_2 by soil microorganisms. Metabolism of C_2H_2 could increase N_2O emission by providing an energy source for denitrification or it could reduce N_2O emission by decreasing the partial pressure of C_2H_2 below that required to inhibit N_2O reduction.
- The even dispersal of the C_2H_2 throughout the soil may be an important physical aspect that can lead to inaccurate results. The acetylene method may underestimate denitrification if the soil is very moist and/or heavily compacted thus limiting the diffusion of C_2H_2.

With sufficient care in application of C_2H_2, the method can be a useful approach to the direct measurement of denitrification in the field studies. However, as C_2H_2 also inhibits nitrification (Tiedje *et al.*, 1989) it might be expected that denitrification may be inhibited by a reduced supply of NO_3^--N. To avoid this potential shortage of NO_3^--N, the acetylene method should be applied for only short periods. Acetylene inhibition of nitrification in natural ecosystems can be solved using the gas-phase recirculation core method (Tiedje *et al.*, 1989). Measurement locations should also be changed frequently in order to avoid problems associated with microbial utilisation of C_2H_2.

Application of the technique in the field. There are two variants of the acetylene inhibition method that can be used in the field; one variant uses soil chambers, the other involves coring. In each case the method can be divided into two phases: the introduction of C_2H_2 into the soils, and the sampling and measurement of N_2O.

In the chamber technique, the experimental equipment for measurement of denitrification in the field consists essentially of three components: a chamber to confine the surface N_2O flux; a system to inject C_2H_2; and a system to take gas samples (Ryden *et al.*, 1979b). The method involves placing chambers over the soil surface and either measuring the accumulation of N_2O in the air space of the chamber or analysing N_2O in the exit air stream (Jury *et al.*, 1982). The main advantage of chamber methods is that they allow for in-field measurement of actual fluxes of N_2O from the soil to the atmosphere, and these methods cause minimal physical disturbance to the soil. Site spatial variability is undoubtedly the greatest problem in using chamber techniques to estimate N_2O flux from a field or ecosystem. Chambers also alter the immediate environment of the site of soil/atmosphere gas exchange by interfering with the natural air turbulence, changing temperature, altering gas concentrations, or altering solar radiation. Another problem with chambers occurs if gas concentrations become sufficiently high to inhibit diffusion of gases out of the soil. Using short times of flow through the chambers can minimize this effect (Christensen, 1983). Chambers come in two basic designs: closed and open, each with advantages for particular sites or objectives. Chambers with closed-loop air circulation or no forced air circulation and accumulation of N_2O under a sealed or closed cover box are closed chambers. Chambers with forced flow-through air circulation, and the trapping of the N_2O on a molecular sieve are open chambers.

Several approaches have been developed for introducing C_2H_2 into in-field chambers. These include:

- Radial diffusion of C_2H_2 from multiple C_2H_2-supply probes inserted into the soil to various depth (Ryden *et al.*, 1979b; Hallmark and Terry, 1985; McConnaughey and Duxbury, 1986).

- Saturating water with C_2H_2 or dissolving the desired volume of pure C_2H_2 into the water (Chan and Knowles, 1979; Terry *et al.*, 1986).

- Downward diffusion from an acetylene reservoir at the surface (Egginton and Smith, 1986).

Nitrous Oxide diffusing out from the soil under the cover box can been collected by molecular sieve entrapment in open chambers (Ryden *et al.*, 1979b) or by collection of N_2O by syringe from closed chambers (Webster and Dowdell, 1982). After collection the N_2O can be analysed by gas chromatography.

The core technique involves incubation of minimally-disturbed soil cores with C_2H_2 in the field. The detail process of the technique was described by Ryden *et al.* (1987). These workers found a very strong relationship between denitrification rates in cores versus chambers for well-drained soils over a wide range of denitrification rates. In very wet soils, they found that cores were superior to chambers due to the difficulty of introducing C_2H_2 into, and the slow diffusion of N_2O out of these soils, but the incubation method achieves rapid diffusion of C_2H_2 across the small radius of soil cores. An additional advantage of cores is that it is possible to run numerous incubations cheaply and quickly and to integrate the rates of denitrification. A further advantage is that the relatively short-term exposure

of soil to C_2H_2 overcomes the problem that the C_2H_2 may inhibit nitrification and therefore reduce the rate of denitrification.

The use of cores can be problematic since the coring process may disturb the soil environment, and create effects on denitrification rates that are difficult to interpret. The core method can cause underestimation of denitrification. The most likely cause of the underestimation is the removal of the core from the soil, which allows O_2 to enter the core and thus reduce the denitrification rate. Although the jar can be flushed with N_2 or Ar to reduce this risk, this in turn could create artificially low O_2 concentrations, thus increasing anaerobic conditions and the potential for denitrification.

Using the core method presupposes that denitrification occurs in the profile only to the depth of coring. So an assessment of the appropriate sampling depth is essential before application of this incubation system to field studies of denitrification. In grassland, soil is usually held together by a dense root system, but problems may arise with arable soils because the cores may not be stable. Parkin *et al.* (1984) and Tiedje *et al.* (1989) developed a flow-through soil core technique to rapidly measure the soil denitrification rate of field samples. This technique involves recirculating air, enriched in C_2H_2, through the macropores of a soil core in a closed loop and monitoring increases in N_2O concentration with time. With this method the distribution of C_2H_2 and removal of N_2O are enhanced, which allows the denitrification rate to be measured rapidly before the soil conditions have changed. A potential disadvantage of continuous gas flow through soil is that the aeration status of soil microsites may be altered such that the denitrification rate obtained does not accurately reflect the *in situ* rate.

Measurement of N_2O. Determination of N_2O concentrations in gas samples is central in studies of denitrification. Gas chromatography is the most-used method and permits convenient analysis of N_2O. The major character of the electron capture detector (ECD) is its great selectivity based on the electron absorption coefficients of the compounds which pass through the detector. Nitrous Oxide has been shown to have a high electron absorption coefficient at temperatures around 300°C (Kaspar and Tiedje, 1980). The gas chromatography system can provide a linear response to a large N_2O concentration range.

2.5 MEASUREMENT OF FIELD DENITRIFICATION RATES

2.5.1 Field denitrification rate

There are a limited number of measurements of denitrification in the field, and the rates of denitrification obtained vary from <0.01 to >100 kg N ha^{-1} yr^{-1} from different agricultural environments (Ryden, 1986; Bijay-Singh *et al.*, 1989; Ruz-Jerez, 1991; Aulakh *et al.*, 1992). Direct measurement of field denitrification using the previously discussed methods is complicated by the high temporal and spatial variability of denitrification rate (Folorunso and Rolston, 1984; Sexstone *et al.*, 1985). Large numbers of samples should be assayed, so that the spatial and temporal variability can be assessed and appropriate statistical analysis applied.

Temporal variability. Denitrification rates can exhibit large variations throughout a year, between months, from week to week, day to day and even within the day. The variations can be explained mainly by corresponding variations in soil temperature and water content (Ryden, 1986; Aulakh *et al.*, 1991). The general tendency is for much higher denitrification

rates to occur when soils are warm and wet than when they are cool and/or dry. Fertilizer application is also a reason for temporal variation in denitrification rate (Ryden, 1981; Aulakh et al., 1992). Plants differ in the timing of their uptake of N and thus influence the seasonal change of denitrification rate (Ryden, 1986). Animals grazing in pastures may also influence the temporal variation of denitrification rate (Ruz-Jerez, 1991).

Since denitrification rate varies temporally, realistic measurement programmes to estimate annual denitrification N loss should cover the whole year, and the method for denitrification measurement should reflect the rate over a 24 hour period.

Spatial variability. Soil denitrification is affected by soil physical, chemical, and microbiological processes and shows a high degree of spatial variability in the field. Coefficients of variability (CV) for denitrification rate from agricultural fields are commonly greater than 100% (Folorunso and Rolston, 1984; Aulakh and Rennie, 1985; Myrold, 1988; Robertson et al., 1988; Parsons et al., 1991).

The causes of high spatial variability of denitrification rate are not clear, but may be due to microscale variability of soil factors which regulate denitrification in the field. Parkin (1987) speculated that the heterogeneous distribution of particulate organic matter was responsible for the variability of denitrification rates in the field.

Denitrification rates in the field have been mostly observed as log-normally distributed (Folorunso and Rolston, 1984; Christensen et al., 1990a), although normal distributions of denitrification rate have also been observed (Groffman and Tiedje, 1989a). Statistical approaches have been proposed to estimate the mean and variance of denitrification rates,

when the data exhibit log-normal distributions (Parkin and Robinson, 1992). Mathematical models have also been developed to predict denitrification rates using log-normally distributed data (Arah and Smith, 1989).

2.5.2 Denitrification enzyme activity

Because the actual field denitrification rate is very dependent on environmental conditions at the time of measurement, some workers have suggested alternative procedures to indicate the likely potential for denitrification to occur in a soil. An example is denitrification enzyme activity (DEA) (Tiedje, 1982). Measurement of DEA is usually conducted under anaerobic conditions without limitation of NO_3^--N and C. The rates obtained by this method are therefore generally higher than rates of denitrification measured in the field. A strong correlation between DEA and annual rates of denitrification was found by Groffman and Tiedje (1989b). But Martin *et al.* (1988) concluded that DEA is better interpreted as an estimate of the biomass of denitrifying bacteria in soil rather than an index of actual denitrification rates.

A range of values of DEA in soil ecosystems have been measured (Limmer and Steele, 1982; Tiedje, 1988), but it is difficult to compare the DEA values measured in different environments, as there is considerable variation in the procedures used.

2.6 NITROGEN LOSS THROUGH DENITRIFICATION FROM PASTURE SOILS

Few studies have focused on denitrification in pasture systems. The rates of denitrification obtained from grasslands are quite variable and are affected by fertilization and other

management practices. The potential for N losses through denitrification from grassland soils may be high. In laboratory studies, potential denitrification rates from several grassland soils were found to be significantly higher than those from similar arable soils (Colboum *et al.*, 1984; Bijay-Singh *et al.*, 1989). These results are presumably due to the high levels of readily-oxidisable-C and the high biological activity in the surface soil under pasture. However, improved pasture soils often have greater porosity and enhanced infiltration and permeability due to the presence of the extensive, ramified root system. Therefore, the rate of denitrification from pasture soils is likely to be low because of the well-oxygenated nature of the soil. This is supported by the often very low denitrification rates from a permanent grassland measured by Bijay-Singh *et al.* (1989).

Responses of denitrification rates to fertilizer N are often high in pastures due to the limitation of native NO_3^--N and the high organic-C in soils (Webster and Dowdell, 1982; Christensen, 1983; Ryden, 1983). Ryden (1983) measured annual gaseous N losses through denitrification of 1.6, 11.1, and 29.1 kg N ha^{-1} from a grassland receiving 0, 250, and 500 kg N ha^{-1}, respectively, and observed that high rates of denitrification can occur for a short period after rainfall. Greater rates of denitrification from grazed pastures than cut swards were found (Ryden, 1986), and on an annual basis, Ryden (1986) estimated N losses of 40 and 20 kg N ha^{-1} yr^{-1} through denitrification from grazed and cut swards of ryegrass receiving 420 kg N ha^{-1} yr^{-1}. The reasons for those results may be the considerably higher contents of both soil NO_3^--N and soil water in urine- and dung- affected areas than in the remainder of the sward (Ryden, 1986).

2.7 SUMMARY AND FUTURE RESEARCH NEEDS

2.7.1 Summary

Biological denitrification is an important part of the total N cycle, and it converts and transfers a fixed form of N to the atmospheric pool of N_2. The significance of denitrification in N use efficiency in agricultural, grassland, and forest systems is well recognized. The significant implications of denitrification to environmental quality have also stimulated study over the past few decades. One of the products from denitrification, N_2O, has been implicated in both global warming and stratospheric ozone depletion. Ironically denitrification has also been used as a means to improve the efficiency of N removal from wastewaters and to protect water quality.

Studies on the ecology of denitrifying bacteria have enhanced our understanding of the denitrification process. Although denitrification is an anaerobic process, denitrifiers are generally facultative aerobes. The microorganisms can use NO_3^- as their electron acceptor for obtaining energy from organic or inorganic compounds when O_2 availability is limited.

Denitrification is regulated by a number of environmental and soil factors. The basic factors controlling denitrification in soil are C supply, NO_3^--N concentration, aeration status, pH, and temperature. Plant growth, agricultural management practices, as well as weather conditions, can also regulate denitrification in the field by affecting these basic factors that influence denitrification. The effect of individual parameters on denitrification is well established. However, there is a lack of understanding of the interaction of the many factors affecting denitrification in various soil environments.

There has been a substantial development in methodology and instrumentation for quantification of denitrification in recent years. Various methods of measurement are available. The most common one uses C_2H_2 as an enzyme inhibitor to block N_2O reduction to N_2. Denitrification can thus be measured as the amount of N_2O produced in soil treated with C_2H_2. This technique has been widely applied in the field using chambers and intact soil cores, and gives acceptable results, although the method presents some problems.

Research has now turned to the quantification of N loss through denitrification in field soils. The potential denitrification rate of a soil can be measured under non-limiting substrate and anaerobic conditions. The potential rates measured are generally higher than the actual rates of denitrification measured in the field, since the conditions in the field do not always favour denitrification. High temporal and spatial variation confound measurement of denitrification in the field, and reliable quantification of denitrification rates remains a goal to be achieved.

The rates of denitrification in pasture soils obtained from a limited number of measurements vary. Pasture soils have year-round root activity and hence O_2 demand, and the presence of active roots also stimulates soil microorganisms through exudation of C compounds. This favours denitrification, as does the presence of animals with their consequent effects on soil structure and localised high concentrations of available N. On the other hand, denitrification is unlikely to be a major N loss due to high porosity in improved pasture soils. Pasture management practices, such as fertilization, grazing management and irrigation may also have an impact on denitrification in pastures.

2.7.2 Future research needs

Given the current knowledge of denitrification, I consider the following research areas should be pursued.

- Little information is available on the ecology of denitrifiers. The denitrification enzyme activity (DEA) in soil is usually high, even in field soils in which the conditions (such as levels of C, NO_3^--N, and O_2) do not favour denitrification. Further work needs to establish how these bacteria can survive in such soils.

- Currently there are inadequacies in our understanding of the spatial and temporal variability in denitrification rates in the field. More information about the causes of variability in denitrification rates under various field conditions and management practices is required to enable better estimates of denitrification N loss on a landscape scale.

- One area of particular interest is the influence of animal grazing patterns on denitrification in grazed pastures. Little work has concentrated on N loss through denitrification from urine and dung patches, although urea hydrolysis, NH_3 volatilisation losses and nitrification have been extensively studied. Research is needed to quantify the extent of N losses through denitrification and to establish the factors affecting them. It is also necessary to understand the relationship and relative importance of denitrification compared with other mechanisms of N losses, such as N leaching and ammonia volatilisation, in grazed pastures.

- Research on the relationship between denitrification and management practices, such as fertilizer application, irrigation and tillage, is desirable to reduce N loss through denitrification. Information is also needed to minimize N loss through denitrification when plant residues and manures are used as nutrient sources.

CHAPTER 3

OPTIMIZING CONDITIONS FOR THE SHORT-TERM DENITRIFICATION ENZYME ASSAY AND EFFECTS OF SOIL STORAGE ON DENITRIFICATION ACTIVITY

3.1 INTRODUCTION

Denitrification rates under natural conditions are influenced by the size and potential activity of the existing population of soil denitrifying organisms and a range of environmental factors (Firestone, 1982). There have been two major approaches to assess the potential activity of the denitrifier population. The first is the denitrification potential or capacity measurement. The other is the short-term denitrification enzyme activity (DEA) assay.

Denitrification potential or capacity measurements have usually been conducted in soils with substrate additions, and with incubation under anaerobic and saturated conditions (Burford and Bremner, 1975; Breitenbeck and Bremner, 1987; Bijay-Singh *et al.*, 1988). Measurement of denitrification activity is made after 1 or more days' incubation of the soil in the assay. The method has yielded information concerning the effects of various factors upon denitrification (Firestone, 1982).

The short-term assay of DEA has been developed for measuring the activity of the

denitrifier population in soils when samples are collected (Smith and Tiedje, 1979a; Tiedje, 1982). Measurement of DEA is also usually conducted with non-limiting substrate and under anaerobic conditions. To reflect the existing denitrifying activity in the soil, however, the assay can only be performed for a few hours. The short-term assay is recommended for use in denitrification studies between different soils, since the measured DEA can reflect the environmental history of the site and offer the possibility of estimating field denitrification rates (Tiedje *et al.*, 1989). A wide range of DEA values has been recorded (Smith and Parsons 1985; Parkin, 1987; Martin *et al.*, 1988; Parsons *et al.*, 1991; Peterjohn, 1991), but it is difficult to compare the DEA values obtained by different workers with confidence because the assay conditions often vary. In our study the effect of experimental conditions on the estimation of DEA was investigated, with a view to recommending a standardized procedure for measuring DEA.

The DEA provides a "snap-shot" of the denitrifying potentials in the soil at the time of sampling. Therefore, the availability of nitrate (NO_3^-) and carbon (C) substrates should be optimal, and the growth of the organisms through fission should be avoided. The denitrification rate responds to NO_3^- concentration according to Michaelis-Menten kinetics (Firestone, 1982). Different amounts of NO_3^-, from 14 to 500 μg NO_3^--N g^1 soil, have been added in previous studies (e.g. Parkin, 1987; Martin *et al.*, 1988; Peterjohn, 1991). But very high NO_3^- concentrations may affect N_2O production (Renner and Becker, 1970; Lalisse-Grundmann *et al.*, 1988), so it is necessary to determine an optimum NO_3^- concentration for the assay that is neither rate-limiting nor inhibitory. The availability of C as an energy source for denitrifiers is also a critical factor governing the denitrification activity in soils (Bremner and Shaw, 1958; Bowman and Focht, 1974; Stanford *et al.*, 1975a; Beauchamp *et al.*, 1989). To ensure that denitrification is not limited by C, a

suitable amendment of soluble-C is required. Additions of C between 0 and 1800 μg soluble-C g^{-1} soil have been used in studies by Parkin (1987), Martin *et al*. (1988), and Schipper *et al*. (1993).

The incubation time for the assay has to be short enough to avoid measuring denitrification from new organisms, especially when soluble-C is added, but long enough to allow the products of denitrification to be measured accurately. The use of an antibiotic, chloramphenicol, has been suggested for use to inhibit protein synthesis and extend the measurable period for existing activity (Tiedje *et al*., 1989). However, chloramphenicol may have side-effects on the denitrification process (Smith and Tiedje, 1979a; Dendooven *et al*., 1994), and it should not be used if the period of DEA measurement is reasonably long.

Measurements should also be made on fresh soil, but soil samples have to be taken from the field to the laboratory and prepared for the assay, so a few days may sometimes elapse before DEA measurements are made. The key to successful storage of soils lies in maintaining denitrification activity at field levels. Drying of soils can sometimes increase their capacity for denitrification (Patten *et al*., 1980; Bijay-Singh *et al*., 1988), although Smith and Parsons (1985) observed that DEA decreased by about 20% in dried soils compared to fresh soils.

Few studies have been done to test the effect of temperature of storage of moist soil on the activity of denitrifying organisms. Soil microbial activity should be limited by reducing the temperature during storage. Stanford *et al*. (1975b), Ryden (1986) and Jordan (1989) estimated that denitrification should cease below 4-5°C, but Limmer and Steele (1982)

demonstrated that significant denitrification could occur at 4°C. They found that the potential denitrification activity of a fresh Te Kowhai silt loam stored at 4°C decreased over several days of storage, apparently in response to a decrease in available-C. In contrast, Breitenbeck and Bremner (1987) found that the denitrification activity of three field-moist Iowa soils did not change much over 30 days storage at 4°C.

The experiments were designed to determine the optimum incubation conditions and to assess the effect of soil pre-treatment on the short-term denitrification assay. Using two soils from permanent pasture, we tested the effects of added NO_3^- and soluble-C on denitrification activity and evaluated the changes in denitrification activity following different periods of storage at temperatures of 2 or 20°C.

3.2 MATERIALS AND METHODS

3.2.1 Soil sample preparation

Soil samples were taken from two sites at Palmerston North, New Zealand. The soil at the Massey University site was a poorly-drained yellow grey earth called Tokomaru silt loam; the soil at the AgResearch Institute site was a well-drained recent alluvial soil called Manawatu fine sandy loam. Some chemical and physical properties of both soils are given in Table 3.1.

Table 3.1 Chemical and physical properties of soils from the experimental sites

Soil	Depth (cm)	Total N (%)	Total C (%)	pH (H_2O)	Bulk density (Mg m^{-3})	CEC (cmol charge kg^{-1})
Tokomaru silt loam	0-5	0.38	4.8	5.5	1.1	22
	5-10	0.30	2.9	5.7	1.2	
Manawatu fine sandy loam	0-5	0.30	3.4	5.7	1.2	16
	5-10	0.15	1.5	6.2	1.2	

Samples of surface (0-5 cm) and subsurface (5-10 cm) soil were collected during the spring and summer of 1991. The field-moist samples were sieved (< 6 mm) in the laboratory immediately after sampling. The visible roots were removed and the samples from each depth were riffled several times to ensure homogeneity. Immediately after sample preparation, the soils were assayed for denitrification activity.

For the storage test, moist soil samples were kept in sealed plastic bags at temperatures of 2 and 20°C and assayed for denitrification activity after 5, 14, 28, and 50 days' storage. To test the effects of drying, the moist soil was air-dried on a sheet of plastic for 4 days. The air-dried samples were stored in paper bags at 20°C and assayed for denitrification activity 1 and 7 weeks after drying.

3.2.2 Procedure

The assay technique involved anaerobic incubation of soil samples in the presence of C_2H_2 to prevent conversion of N_2O to N_2 (Yoshinari *et al.*, 1977). N_2O is the sole gaseous product of denitrification in soils incubated in atmospheres containing 0.1-10% vv^{-1} C_2H_2, and the moles of N_2O produced (with C_2H_2) are equal to the moles of N_2O plus N_2 (without

C_2H_2) (Yoshinari *et al.*, 1977; Ryden *et al.*, 1979a). This procedure simplifies analytical procedures for denitrification assays, since the denitrification can be estimated by a single measurement of N_2O using a gas chromatograph. Denitrification activity was measured using the methods developed by Smith and Tiedje (1979a) and Tiedje (1982). Four replicate 20 g portions of soil were weighed into 125 ml Erlenmeyer incubation flasks fitted with suba-seals. In a test of the effect of C_2H_2 on N_2O emission, 50 µg NO_3^--N g^{-1} soil was added to the Tokomaru soil and the incubation was carried out with and without C_2H_2. Gas samples for analysis were collected at regular intervals up to 7 hours after the start of the incubation.

In the experiments to select the incubation conditions, the effect of NO_3^- on the assay was tested by adding KNO_3 solutions to the moist soil to give 0, 15, 25, 50, 100 and 150 µg NO_3^--N g^{-1} soil additional NO_3^-. To test the effect of soluble-C, glucose solutions were added to moist soil to give 0, 60, 120, 240 µg glucose-C g^{-1} soil in the Tokomaru soil, and 0, 55, 106 and 212 µg glucose-C g^{-1} soil in the Manawatu soil. KNO_3 solution was also added to all samples at the rate of 50 µg NO_3-N g^{-1} in the soluble-C tests. In the storage test, the soils were supplemented with KNO_3 solution (50 µg N g^{-1} soil) and/or glucose solution (300 µg C g^{-1} soil) immediately prior to the assay.

All soil samples in the incubation flasks were saturated (0% air-filled pore space) with deionised water (Limmer and Steele, 1982). The flasks were evacuated and flushed with pure N_2 gas three times and vented to atmospheric pressure. Headspace gas (12.5 ml) was removed from the flasks and replaced with C_2H_2 to give a final C_2H_2 concentration of about 10% vv^{-1}. The flasks were incubated at a controlled temperature (20°C) in the dark.

3.2.3 Analytical methods

Periodically, gas samples were collected from the flasks and transferred to evacuated 5 ml vials using double-ended needles. N_2O was measured using a Hewlett 5890 series II gas chromatograph equipped with a ^{63}Ni electron capture detector. The column and detector temperature were 70 and 300°C respectively. N_2O was separated on a 6 mm packed column at an Ar-CH$_4$ (10% v v^{-1} methane) carrier gas flow rate of 30 ml min^1 . Sample analysis was completed within 4 min. A standard concentration of N_2O in N_2 gas was used to calibrate the chromatograph for N_2O concentrations (ml l^{-1}). Given a gas density for N $_2$O at 20°C of 1.83×10^{-6} g N_2O ml^{-1}, the mass concentration of N_2O (g l^{-1}) in the headspace of an incubation flask could be calculated. Knowing the headspace volume, and allowing for N_2O dissolved in solution by using a Bunsen coefficient of 0.632 at 20°C (Tiedje, 1982), the mass of N_2O was calculated from:

$$N_2O\text{-}N\ (g) = 1.83 \times 0.636 \times 10^{-6} \times N_2O\ (ml\ l^{-1}) \times [Vol.\ of\ headspace(l.) + Vol.\ of\ soil\ solution\ (l.) \times 0.632] \quad (3.1)$$

The quantity of N denitrified was plotted against time for each incubation. The resulting slope divided by the mass of dry soil gave the denitrification activity.

The NO_3^- and moisture contents of the soil immediately before each incubation experiment were measured. Duplicate soil samples were extracted using $2M$ KCl solution at a soil:solution ratio of 1:5, and NO_3^--N in the extracts was analyzed by an autoanalyser method (Downes, 1978). Duplicate samples of moist soil were dried at 105°C for 24 hours to determine the gravimetric soil moisture content. Soil pH was measured in duplicate at a 1:2.5 soil-water ratio using a combined electrode pH meter. Statistical analyses were

performed using the Statistical Analysis System (SAS) (SAS Institute, 1985).

3.3 RESULTS AND DISCUSSION

3.3.1 Incubation conditions

Accumulation of N_2O. The rate of N_2O-N evolution for the Tokomaru silt loam incubated with 50 μg NO_3^--N g^{-1} soil in the presence and absence of C_2H_2 is shown in Figure 3.1. There was no significant difference in N_2O production during the first 5 h of incubation of the Tokomaru silt loam in the presence and absence of C_2H_2. However, N_2O did not accumulate in the absence of C_2H_2 for incubation periods longer than 5 h, suggesting it may have been reduced to N_2. The similarity between the two N_2O production curves suggests that N_2O was the only gas product from denitrification during short incubation times under anaerobic conditions. The transient accumulation of N_2O in the absence of C_2H_2 was probably due to differences in the kinetics of individual reactions in the denitrification process, notably the reduction of NO_2^- to N_2O, and the reduction of N_2O to N_2 (Betlach and Tiedje, 1981). Factors such as pH and NO_3^- concentration could affect these kinetic differences. For example, low pH is considered to favour the production of N_2O rather than N_2 (Christensen *et al.*, 1990a). Thus N_2O might be expected to accumulate during incubation of the Tokomaru soil, since the pH of the soil is around 5.5 (Table 3.1). The concentration of NO_3^- can also influence the end product of denitrification (Blackmer and Bremner, 1978; George and Antoine, 1982). It is also possible that differences between soils in the relative proportions of N_2 and N_2O produced depends on their populations of denitrifying organisms and the persistence of reduction enzymes.
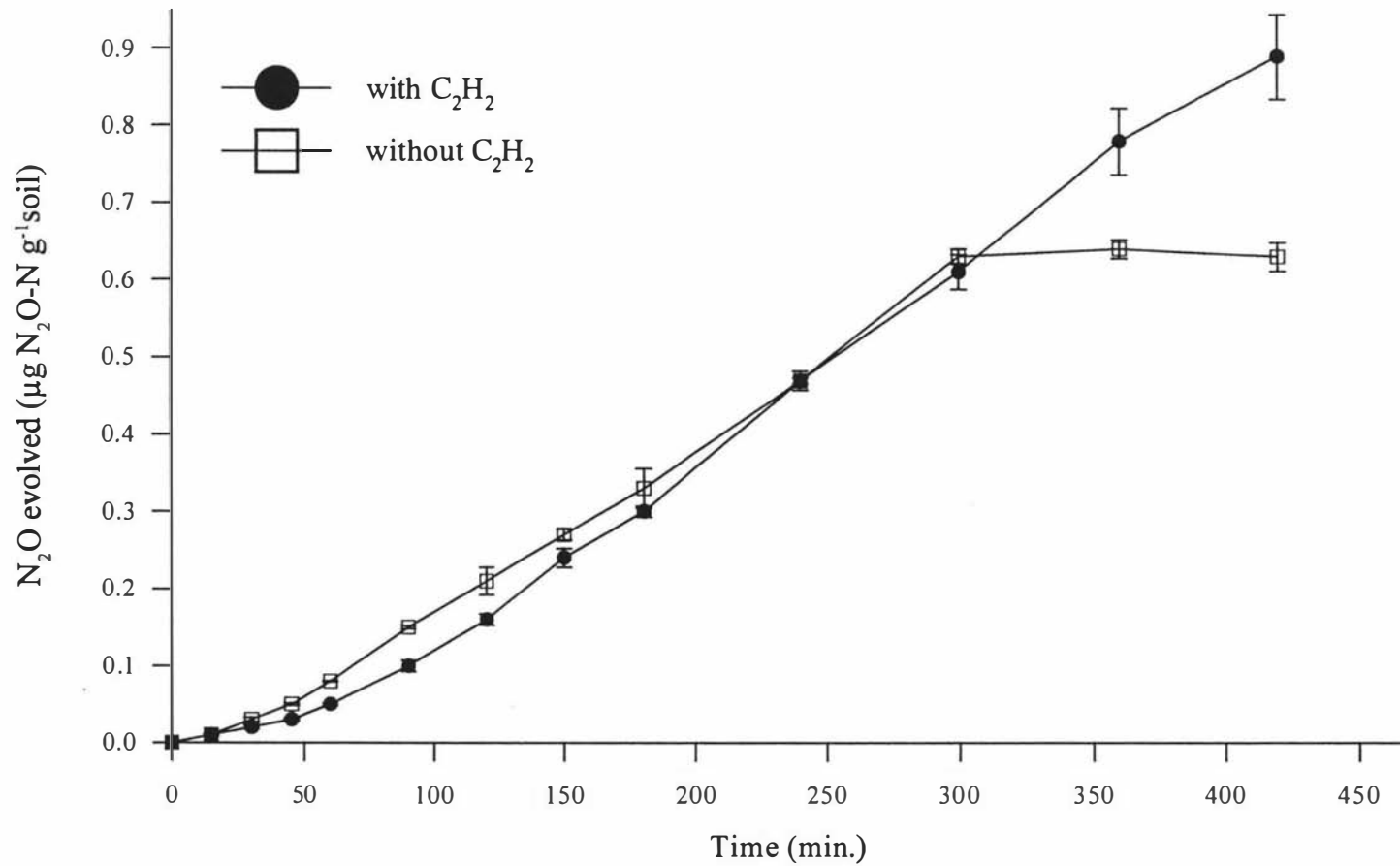
Figure 3.1 N_2O evolved during anaerobic incubation of Tokomaru silt loam (0-5 cm) after addition of NO_3^- (50 µg N g⁻¹soil) in the presence and absence of C_2H_2 (bars represent SD)

Incubation time. Figure 3.1 also shows that after an initial period of approximately 1 h, N_2O production in the presence of C_2H_2 from soils amended with 50 μg NO_3^--N g^{-1} soil occurred at a constant rate for at least 4 h. The switch-over from aerobic to anaerobic respiration by the facultative anaerobes appeared to be rapid and the organisms did not appear to multiply during this incubation period (Figure 3.1). When glucose as well as NO_3^- was added to the soil, the production of N_2O was still linear between 1 and 5 h of anaerobic incubation (Figure 3.2). In the surface soil there appeared to be a slight increase in denitrification rate after 5 h of incubation, perhaps suggesting that after 5 h the soil microorganisms may have begun to multiply in the presence of added glucose (Figure 3.2). The activity then would not represent the activity of the pre-existing organisms in the field, and the optimum incubation time with soluble-C for these soils should be less than 5 h. The duration of the incubation used by other workers in the short-term assay has ranged from 1 to 8 h (Smith and Tiedje, 1979a; Limmer and Steele, 1982; Martin *et al.*, 1988). Based on these results an incubation period of 5 h was chosen for the remainder of the study.

Effect of NO_3^- and soluble C. The rate of N_2O emission from surface samples (0-5 cm) of both the Tokomaru and Manawatu soils, initially containing 7.5 and 5.0 μg NO_3^--N g^{-1} soil, respectively, increased with added NO_3^- between 0 and 50 μg NO_3^--N g^{-1} soil (Figure 3.3a). However, the rate of N_2O emission decreased again at added NO_3^- concentrations of 100 and 150 μg NO_3^--N g^{-1} soil (Figure 3.3a), which indicates that high NO_3^- concentrations can have an inhibiting effect on denitrification. Nitrous oxide emissions in the subsurface soils (5-10 cm), that had initial NO_3^--N concentrations of 5.2 and 4.0 μg NO_3^--N g^{-1} soil in the Tokomaru and Manawatu soils, respectively, showed a similar response to that of surface soils to NO_3^- addition (Figure 3.3b). These results were obtained in soils which contained
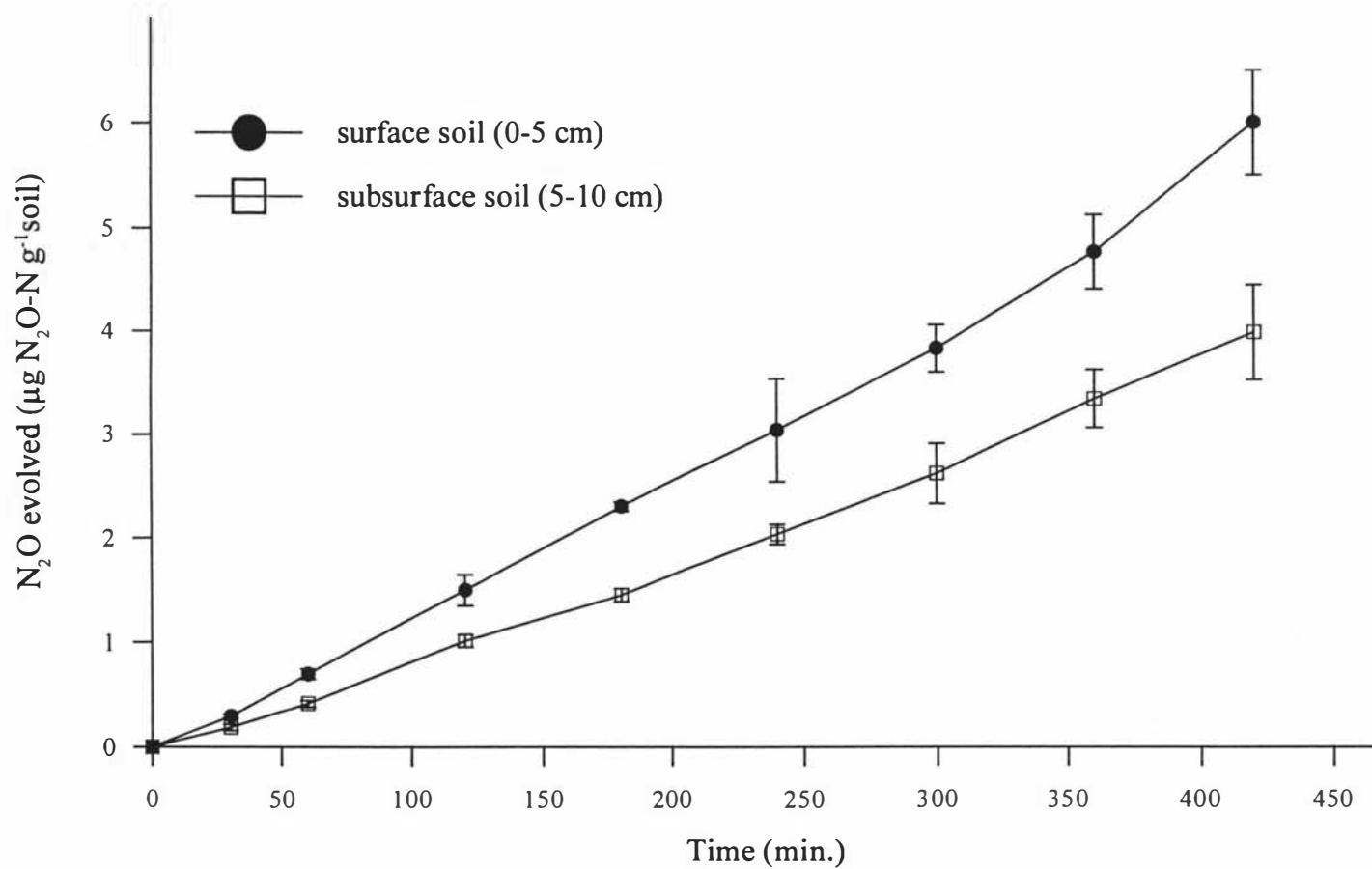
Figure 3.2 N_2O evolved during anaerobic incubation of Tokomaru silt loam after NO_3^- (50 µg N g^{-1}soil) and glucose (300 µg glucose-C g^{-1}soil) were added (bars represent SD)
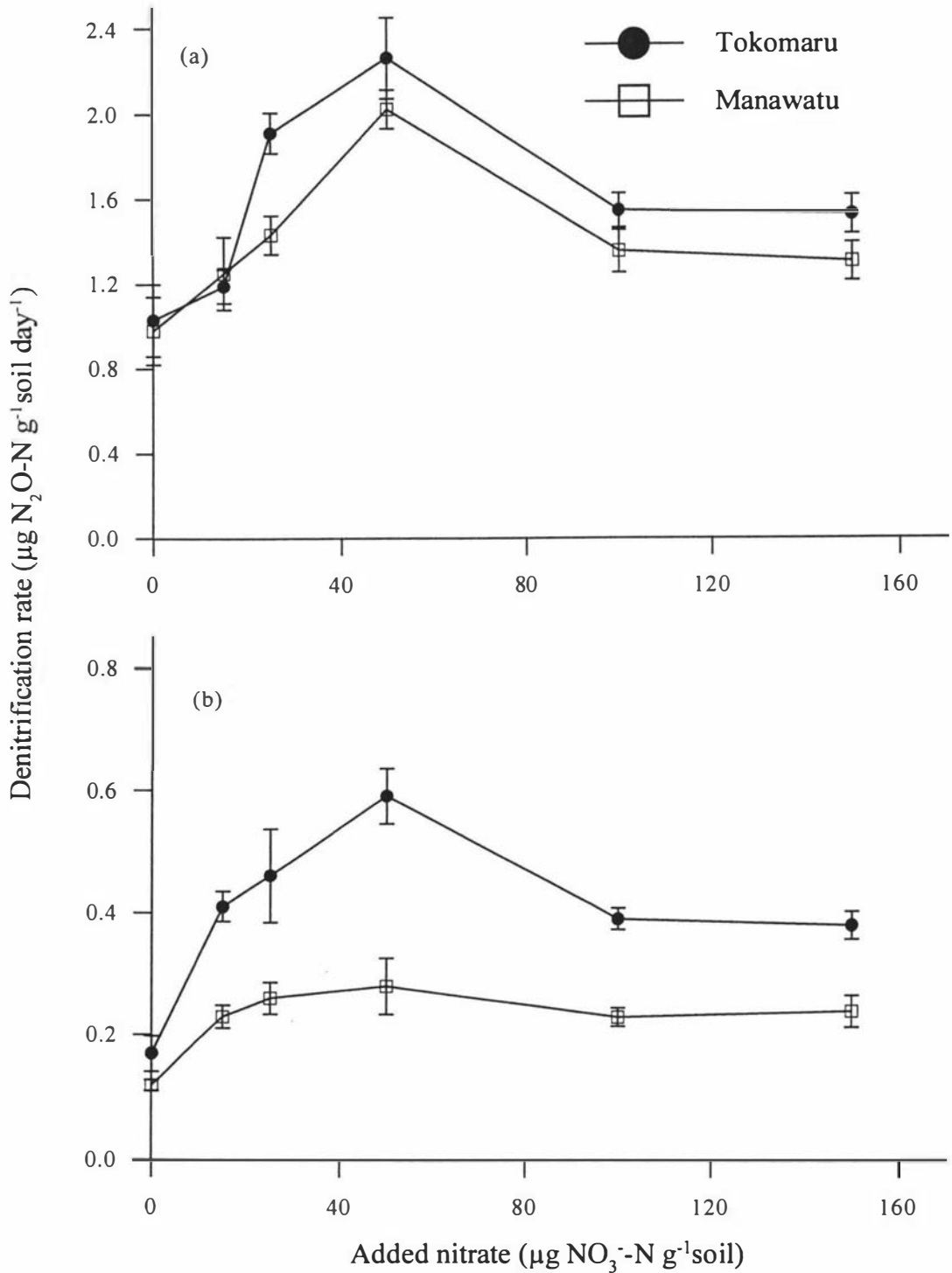
Figure 3.3 Effect of NO_3^- concentration on denitrification activity in the Tokomaru and Manawatu soils, (a) surface soil (0-5 cm) and (b) subsurface soil (5-10 cm) (bars represent SD)

only naturally-occurring amounts of organic C. It was possible therefore that at higher concentrations of available-C the NO_3^- concentrations corresponding to maximum denitrification may have increased (Limmer and Steele, 1982). However, a separate study (data not presented) indicated that high NO_3^- concentrations (>100 μg N g^{-1} soil) inhibited N_2O emission even when soluble-C had been added to the soil. The double reciprocal plots of the Michaelis-Menten kinetic analysis applied to the denitrification data of Figures 3.3a and b gave K_m values of 37.9, 25.1, 20.8 and 29.4 μg NO_3^--N g^{-1} soil in the Tokomaru and Manawatu surface and subsurface soils, respectively. These data demonstrated that the K_m values for denitrification were similar in the surface and subsurface soils, although a number of soil properties were different (Table 3.1). The K_m values in our study were similar to those found in other soils (Limmer and Steele, 1982; Malhi et al., 1990) but higher than those observed in pure cultures (Betlach and Tiedje, 1981; Myrold and Tiedje, 1985b). A limitation of NO_3^- diffusion to denitrifiers in soil aggregates could possibly be the reason for the higher K_m values in soil studies. According to the Michaelis-Menten relationship, the denitrification rate should follow first-order kinetics when NO_3^- concentrations are much less than the K_m value (Firestone, 1982), and the denitrification rate should then approach a maximum as the NO_3^- concentration is increased further (Limmer and Steele, 1982; Malhi et al., 1990; Schipper et al., 1993). However, studies by Renner and Becker (1970) and Lalisse-Grundmann et al. (1988) have also shown that the denitrification rate is inhibited at high NO_3^- concentrations, although a considerable range in the concentrations which are inhibitory has been observed. High NO_3^- concentrations are supposed to inhibit not only N_2O reductase activity (Blackmer and Bremner, 1978; Terry and Tate, 1980; Gaskell et al., 1981), but also NO^- reductase, NO_2^- reductase and NO_3^- reductase activities (Renner and Becker, 1970; Gaskell et al., 1981). Some believe that NO_2^- accumulation during anaerobic reduction of NO_3^- is responsible for the inhibiting

effect (Renner and Becker, 1970; Betlach and Tiedje, 1981), but it is not clear yet whether NO_3^- or NO_2^- is the effector. It is clear that concentrations of NO_3^- added to the soil should be optimized to avoid limitation or inhibition of denitrification in the assay. Based on these results, the optimum NO_3^- concentration for obtaining maximum production of N_2O in the short-term enzyme assay should be around 50 μg NO_3^--N g^{-1} soil.

The rate of denitrification increased with additions of soluble-C up to 106 and 212 μg glucose-C g^{-1} soil for the Manawatu surface and subsurface soils, and up to 240 and 300 μg glucose-C g^{-1} soil for the Tokomaru surface and subsurface soils (Figures 3.4a, b). No measurement of initial amounts of available-C was made on these soils. However, the substantial increase in N_2O emission observed after addition of glucose to all soils suggests that the initial concentrations were low. Since no NO_3^- limitation was present in the incubation system (NO_3^- was added), the addition of soluble-C to both the Manawatu and the Tokomaru soils had the effect of removing any potential limitation to denitrification imposed by a shortage of reducing power. The denitrification enzymes in the existing soil organisms could therefore function without restriction from substrate availability. Some workers have assessed DEA in soils without adding additional soluble-C (e.g. Smith and Tiedje, 1979a; Schipper *et al.*, 1993), while others have added amounts ranging from 70 to 1800 μg C g^{-1} soil (e.g. Smith and Parsons, 1985; Parkin, 1987; Martin *et al.*, 1988; Parsons *et al.*, 1991). Our results suggest that the DEA values obtained will be very dependent on the amount of C added. These results suggest that at least 300 μg glucose-C g^{-1} soil should be added to ensure that there is no C limitation and that the pre-existing enzyme activity in the soil can be fully expressed.
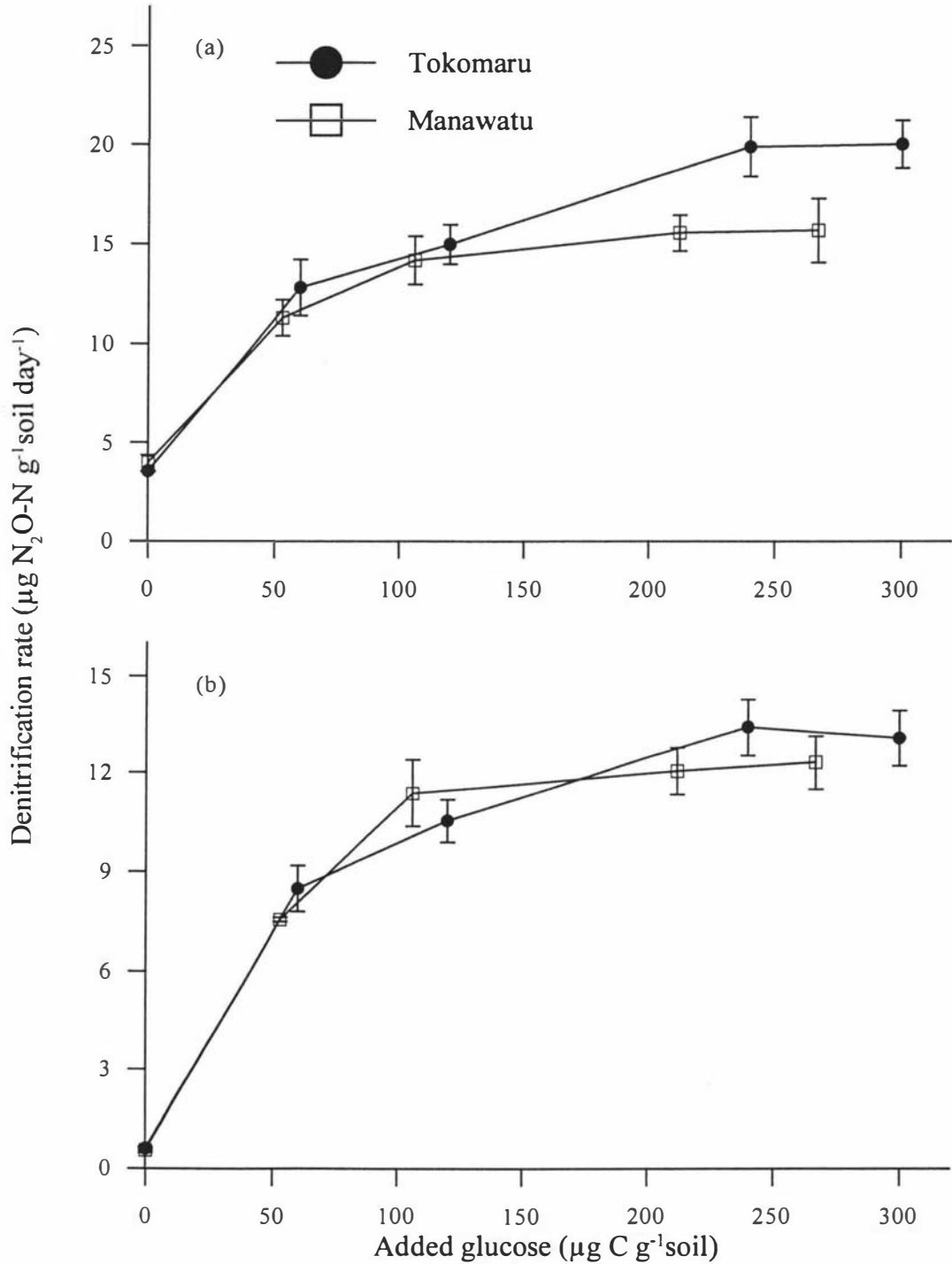
Figure 3.4 Effect of soluble-C on denitrification activity in the Tokomaru and Manawatu soils, (a) surface soil (0-5 cm) and (b) subsurface soil (5-10 cm) (bars represent SD)

3.3.2 Influence of soil sample storage

Moist soils. The results of storing moist soil samples are shown in Figures 3.5-3.8. Denitrification activity was measured in the Manawatu and Tokomaru soils without NO_3^- or glucose amendments to the incubation system, following storage periods up to 50 days at 2 and 20°C. After 14 days' storage at 2°C significant decreases in denitrification activity were found in both soils (Figures 3.5a, b). For storage at 20°C, there was a significant decrease in denitrification activity of Tokomaru soil after 5 days, but only a slight decrease in the Manawatu soil after the same time (Figures 3.5a, b). Since there was no additional NO_3^- and soluble-C in the incubation systems, these changes in denitrification activity may reflect changes in the available soil NO_3^- and C as well as the population of denitrifying organisms during storage. There was a slightly faster decline in denitrification activity during storage at 20°C compared with 2°C for the Manawatu soil, but a less consistent effect of temperature in the Tokomaru soil (Figures 3.5a, b). After 50 days' storage at 2 and 20°C, the residual denitrification activity in both soils was similar and only about 30% of the activity in fresh soil (Figures 3.5a, b).

Similar trends were found in the denitrification activity of the stored soil samples when additional NO_3^- (50 µg N g^{-1} soil) was added to the incubation systems (Figures 3.6a, b). The much higher denitrification rates presented in Figures 3.6a, b, compared to those in Figures 3.5a, b, clearly show that NO_3^- was a limiting factor for denitrification activity in these two soils, but the addition of NO_3^- did not change the pattern of decreasing denitrification activity during storage. The NO_3^- concentrations of these soils ranged from 4 to 10 µg N g^{-1} soil and 4 to 7 µg N g^{-1} soil for the Tokomaru and Manawatu, respectively and changed little during 50 days' storage.
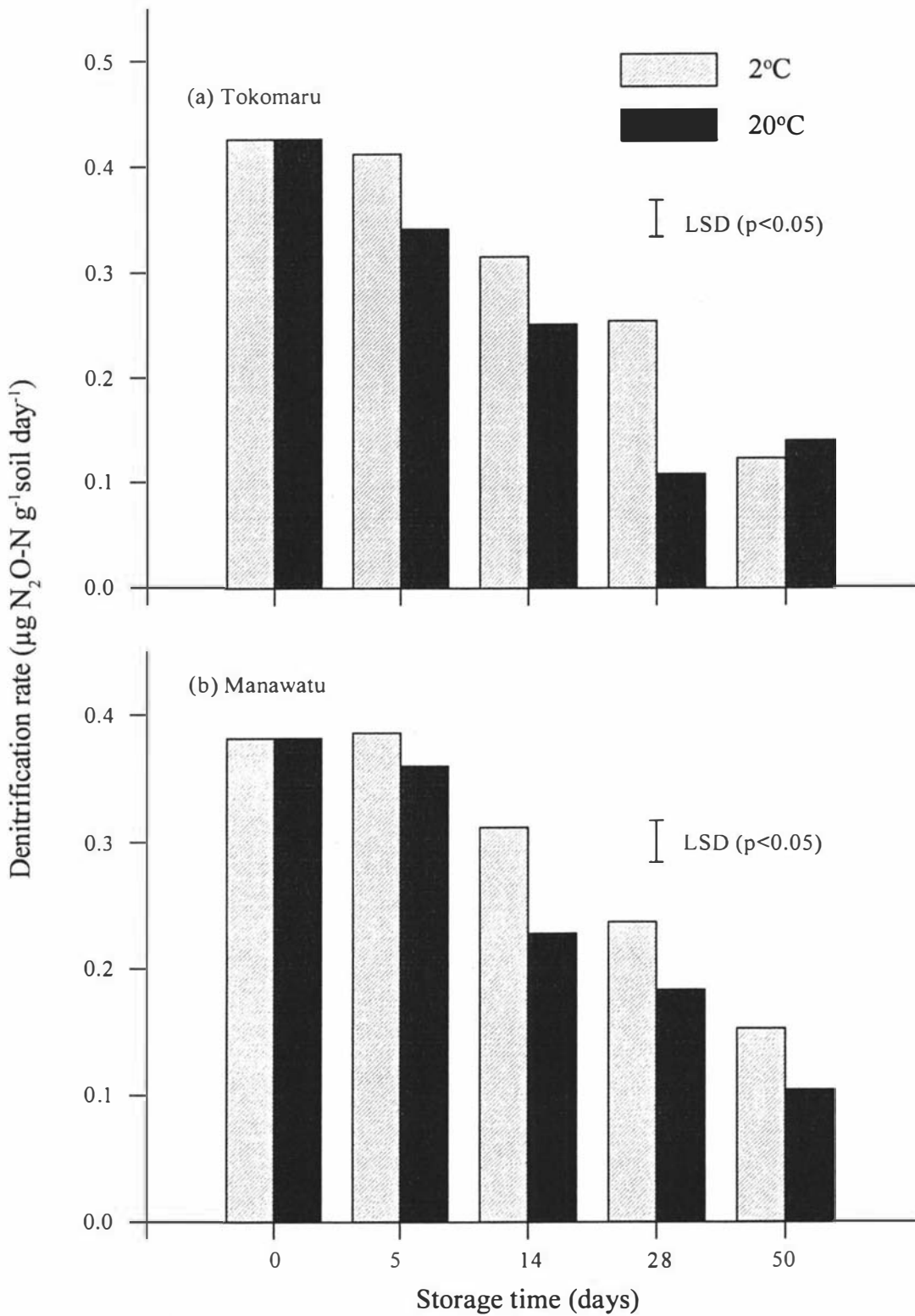
Figure 3.5 Change in denitrification activity of moist soil during storage at 2 and 20°C when assayed without NO_3^- and C addition, (a) Tokomaru soil and (b) Manawatu soil
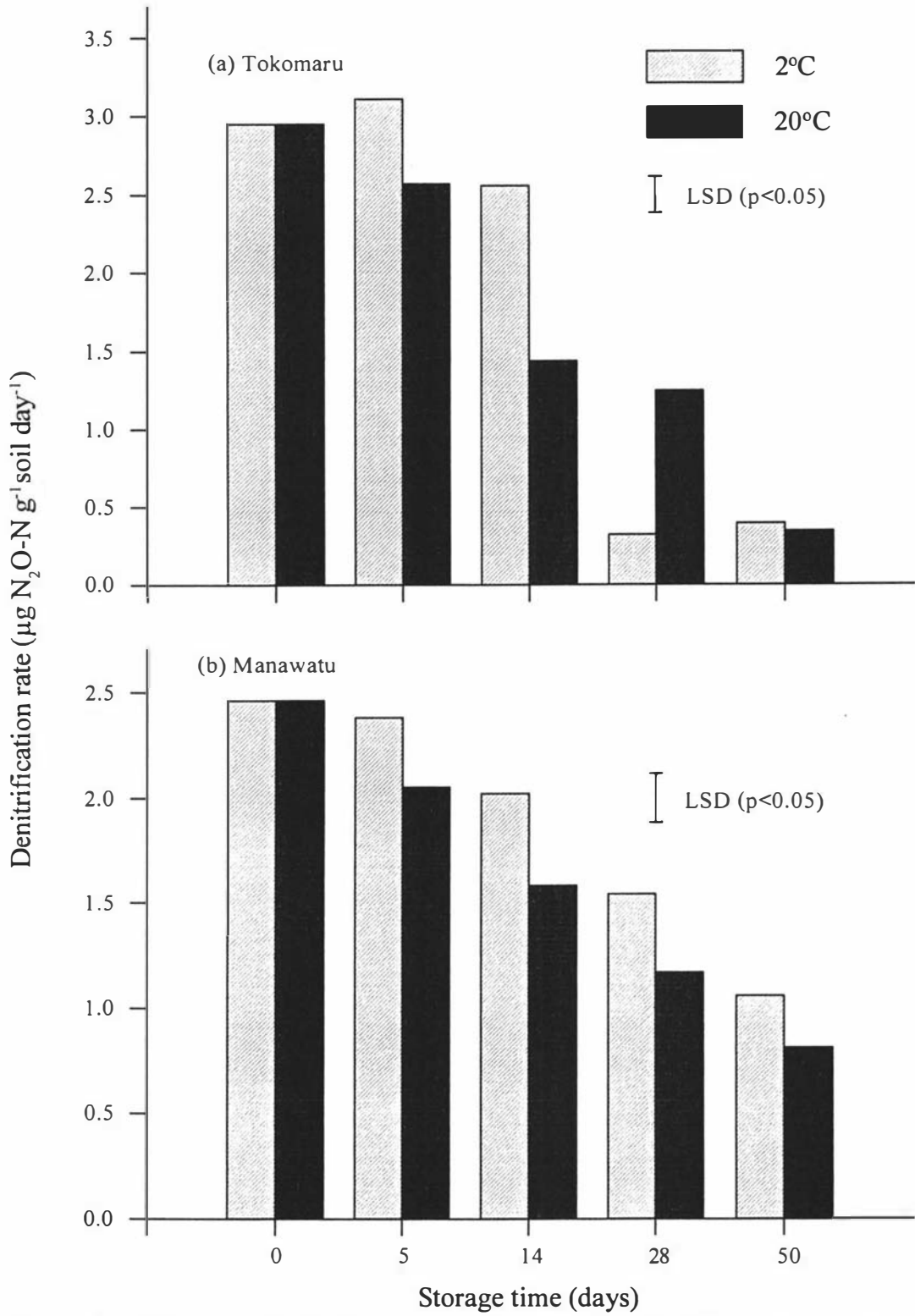
Figure 3.6 Change in denitrification activity of moist soil during storage at 2 and 20°C when assayed with NO_3^- addition, (a) Tokomaru soil and (b) Manawatu soil
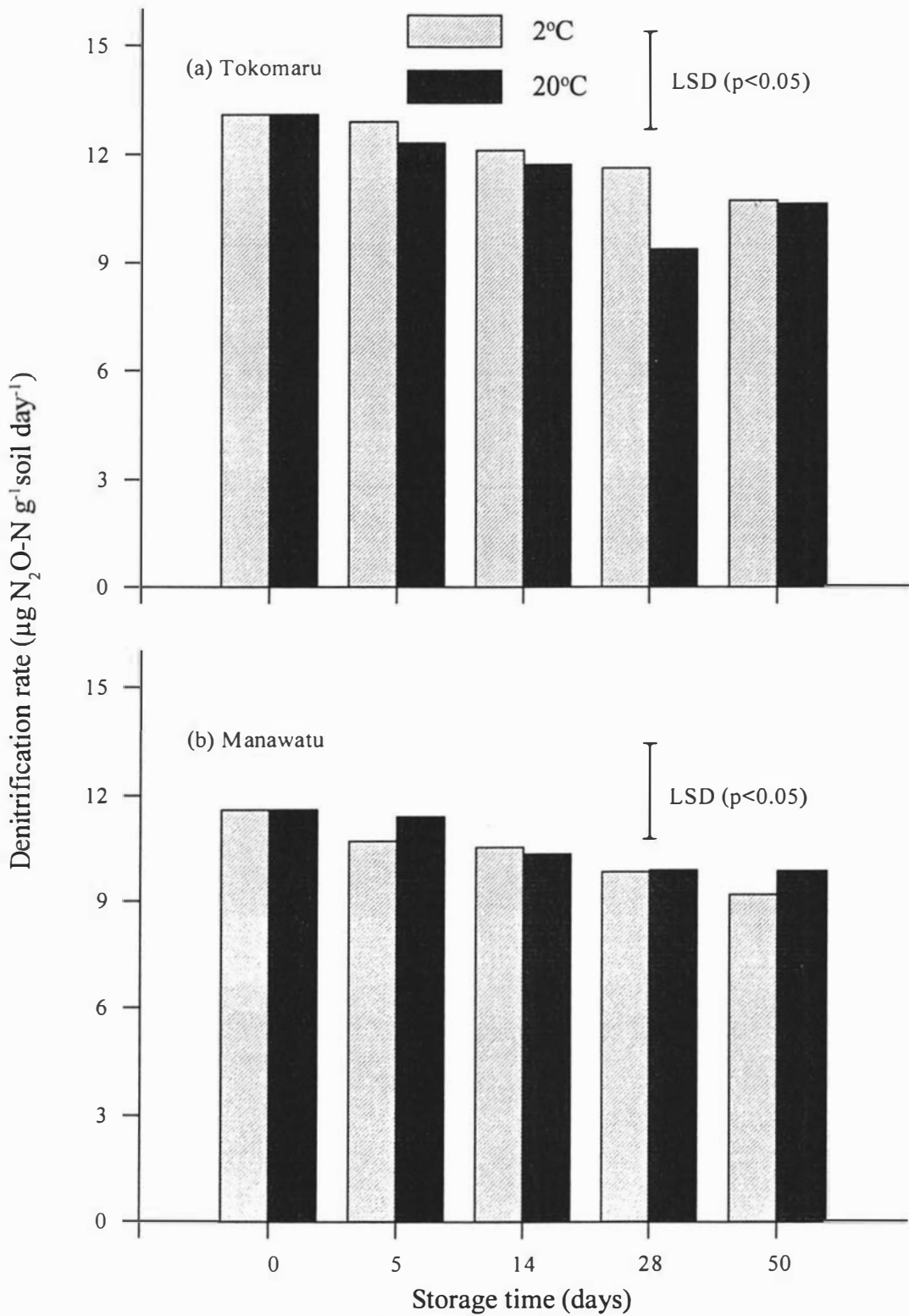
Figure 3.7 Change in denitrification activity of moist soil during storage at 2 and 20°C
when assayed with C addition, (a) Tokomaru soil and (b) Manawatu soil
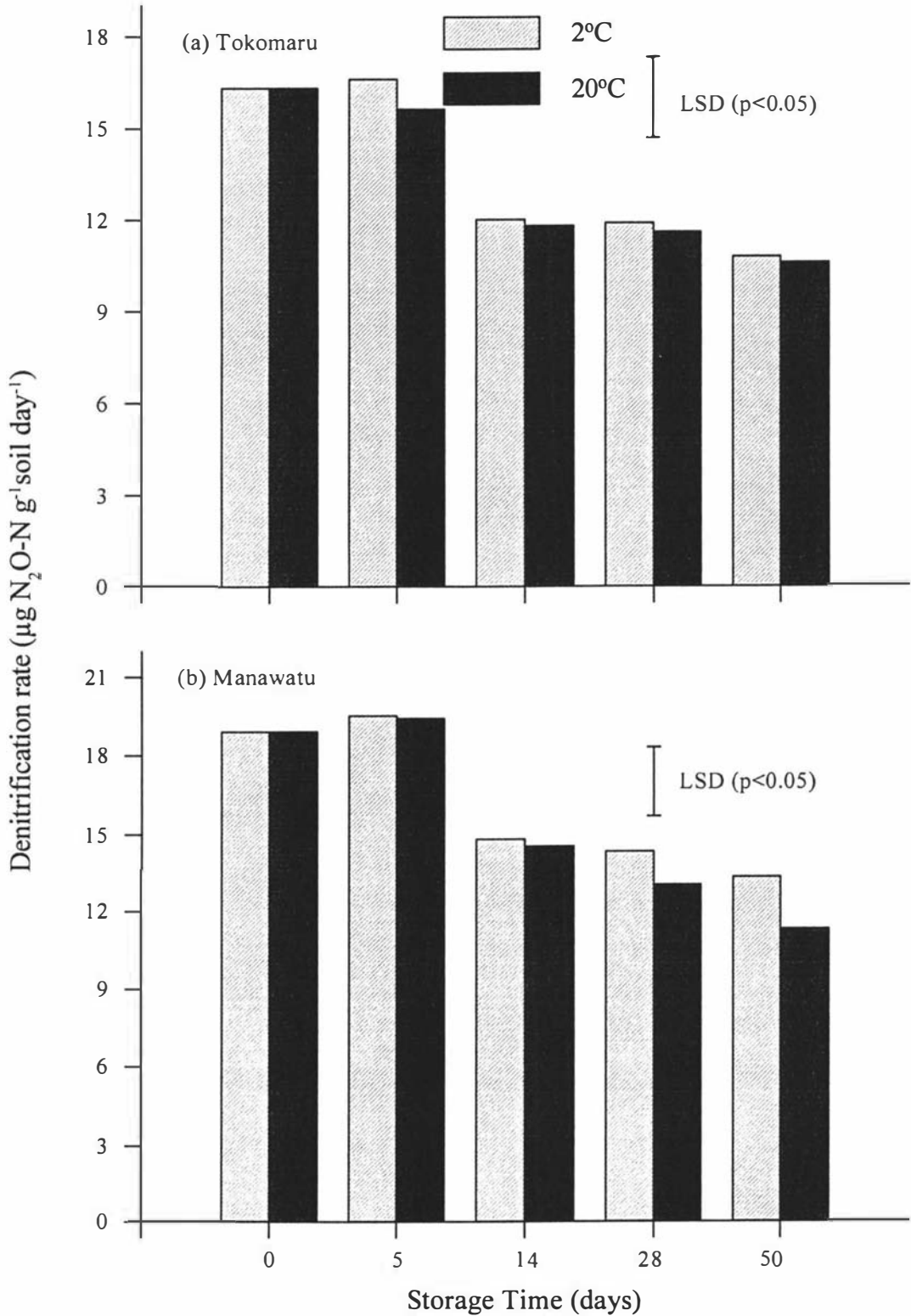
Figure 3.8 Change in denitrification activity of moist soil during storage at 2 and 20°C when assayed with NO_3^- and C addition, (a) Tokomaru soil and (b) Manawatu soil

When denitrification activity was measured in the presence of added glucose-C (but without added NO_3^-) there was no statistically-significant changes with time of storage, but there was a slight downward trend in denitrification activity with time of storage (Figures 3.7a, b). This result suggests that a decrease in available-C during storage accounts for much of the decrease in denitrification activity with time, a result similar to that of Limmer and Steele (1982) and Breitenbeck and Bremner (1987). Comparison of Figures 3.5a, b and 3.7a, b indicates that glucose amendment greatly increased the denitrification activity, confirming that soluble-C was a crucial limiting factor for denitrification in these soils.

When both glucose and NO_3^- were added to the incubation systems, there was a significant decrease in the denitrification activity of these two soils between 5 and 14 days of storage at both 2 and 20°C (Figures 3.8a, b). About 65% of the initial activity remained in the soils at both temperatures after 50 days of storage (Figures 3.8a, b). The decrease in denitrification activity with additions of both NO_3^- and glucose was not as marked as the decrease when NO_3^- only was added (Figures 3.6a, b), or without either NO_3^- or glucose additions in the assay (Figures 3.5a, b). These results suggest that the changes in denitrification activity during storage could be due to changes in the denitrifier community and in persistence of reduction enzymes, as well as to a decrease in available-C in the stored soil sample. If DEA is to be measured in a short-term incubation with glucose and NO_3^- additions, soil samples should not be stored longer than 5 days before assay, irrespective of the temperature during storage.

Air-dry soils. Air-drying the Tokomaru and Manawatu soils increased the denitrification activity compared to fresh moist soil whether the assay was performed without or with added NO_3^- (Figures 3.9a, b). The effect was greater for air-dry soil stored for one week
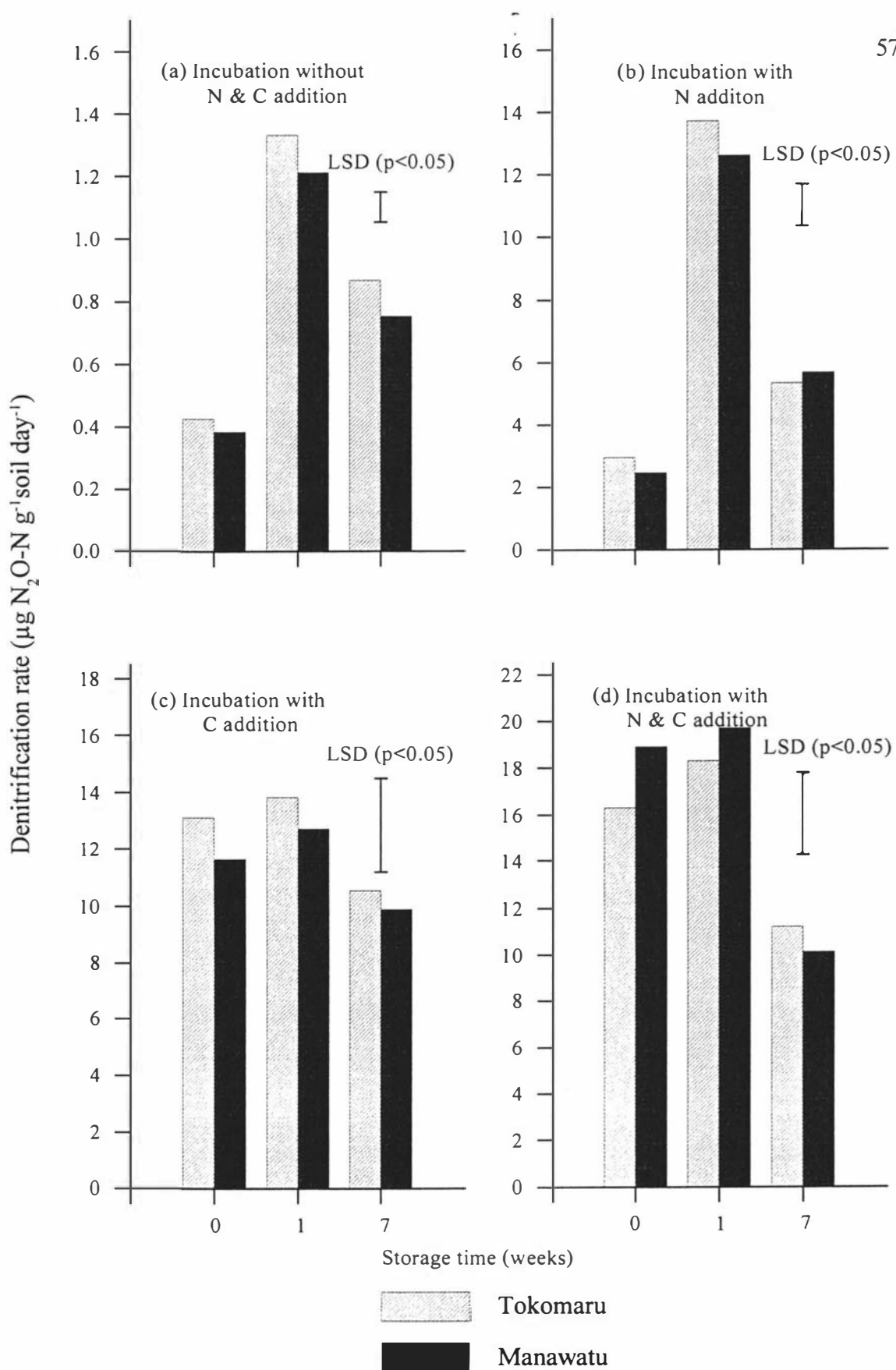
Figure 3.9 Change in denitrification activity following air-drying and storage of air-dry soil,
(a) assayed without NO_3^- and C addition, (b) assayed with NO_3^- addition,
(c) assayed with C addition and (d) assayed with NO_3^- and C addition

compared to 7 weeks. However, with addition of glucose in the assay, air-dry soils did not give significantly higher denitrification activities compared with the fresh moist soil samples (Figure 3.9c). Similar results were obtained with additions of both glucose and NO_3^- to the assays of dry soils stored for one week, but not for 7 weeks (Figure 3.9d). These results suggest that the increase in denitrification activity after drying was due to an increase in available-C (Bijay-Singh *et al.*, 1988). Soil drying could also kill some microorganisms, which could be another source of available-C (Agarwal *et al.*, 1971). Rewetting upon drying could also release physically stabilised organic material previously unavailable to denitrifiers. Figures 3.9a, b, c and d show, however, that the higher denitrification activity was not stable with longer-term storage of the air-dry soil, which suggests that drying and storage affects not only the available-C in the soil, but also the denitrifier population and persistence of reduction enzymes.

3.4 CONCLUSIONS

We conclude that a soil's moisture status (moist or air-dry) and the duration of storage can affect the denitrification activity, as measured by a short-term assay. Changes in available-C appear to be crucial, with an apparent increase in available-C occurring during air-drying and a possible decrease in available-C occurring during storage of moist soil (up to 7 weeks) and air-dry soil (between 1 and 7 weeks). The soil denitrifier population and persistence of reduction enzymes can also be affected by storage. Lowering the storage temperature from 20 to 2°C had little effect in preventing a decline in denitrification activity of moist soil samples.

We recommend that for DEA measured in the laboratory to reflect the potential activity of

microorganisms in the field soil, measurements should be made on fresh samples of moist soil, or moist samples stored for not longer than 5 days at a constant temperature 2°C and 20°C. To avoid NO_3^- and C limiting the denitrification activity, even in soils of high organic matter content, we recommend that 50 µg NO_3^--N and 300 µg glucose-C be added g^{-1} of soil. If this is done, assays performed on air-dry soil within one week of air-drying should give results comparable to the original moist soil. We also recommend that to measure the pre-existing enzyme activity in the soil, the incubation time for the assay be less than 5 hours.

CHAPTER 4

VARIABILITY IN DENITRIFICATION ACTIVITY

WITH SOIL DEPTH

4.1 INTRODUCTION

It is generally accepted that microbial activity is higher in the surface soil than at greater depth soil in the soil profile (e.g. Speir *et al.*, 1984; Higashida and Takao, 1985). The reason is that the organic and inorganic materials, that are released from plants and animals and provide the energy source for microorganisms, largely enter the soil at the surface. It would seem likely therefore that denitrification activity may also be higher in the upper layers of the soil profile. However, this assumption requires further validation so that the most appropriate depth of soil sampling can be assessed for further field studies to assess rates of denitrification.

The aims of the present study were to investigate the vertical variation in denitrification rate in soils under pasture in New Zealand and to identify those factors limiting denitrification rate at each soil depth.

4.2 MATERIALS AND METHODS

4.2.1 Soil sample preparation

The investigations were carried out during the early summer of 1991, the early winter of 1992 and the autumn of 1993. On each occasion soil samples (0-5, 5-10, 10-20 and 20-40 cm depths) were collected from random locations within the paddock and then bulked according to depth. At the first two sampling times (November, 1991 and May, 1992) samples were collected from two contrasting soils. The first soil was a poorly-drained silt loam (Tokomaru silt loam) and the second a well-drained sandy loam (Manawatu sandy loam). Detailed soil chemical and physical properties have been described in Chapter 3. Both sites were under a ryegrass/white clover pasture and were grazed by sheep.

The third sampling (March, 1993) was carried out at only one site. The soil was again the poorly-drained Tokomaru silt loam, but the location differed from the earlier samplings. On this occasion the ryegrass/white clover pasture was grazed by cattle and separate samples were collected four days apart, before and after heavy rainfall (28.5 mm).

Immediately after sample collection, soil samples were passed through a 6 mm sieve and stored at 2°C for a few days prior to laboratory assessment of denitrification activity (Chapter 3).

4.2.2 Laboratory measurement of denitrification activity

The basic laboratory procedure was as described previously in Chapter 3. Briefly, 20 g

portions of moist soil were weighed into 125 ml Erlenmeyer incubation flasks fitted with suba-seals. Four treatments were applied to these soils: 1) no substrate amendment; 2) amendment with N at 50 μg NO_3^--N g^{-1} soil; 3) amendment with C at 300 μg glucose-C g^{-1} soil; and 4) amendment with both N and C at 50 μg NO_3^--N and 300 μg glucose-C g^{-1} soil. These amendments were made in order to assess the substrate factors controlling denitrification activity in soil. Treatments were replicated four times. The soil samples in the incubation flasks were saturated with deionised water (no air-filled pore space) and the flasks were then evacuated and flushed with pure N_2 gas 3 times and vented to atmospheric pressure. Approximately 10% of headspace gas (12.5 ml) was removed from the flasks and replaced with C_2H_2. The flasks were placed at a controlled temperature (20°C) in the dark.

4.2.3 Denitrification measurement at field temperature and moisture

During the final investigation in March, 1993, when samples were collected 4 days apart before and after heavy rain (28.5 mm), additional samples were collected for incubation at field moisture and temperature, rather than the saturated, anaerobic laboratory conditions. Four replicates of 14 soil cores (2 cm diameter × 40 cm deep) were collected randomly from the paddock (2.5 ha), and sectioned and bulked according to depth (0-5, 5-10, 10-20 and 20-40 cm). Each replicate of 14 "intact" cores for each depth were placed in a 1.1 litre incubation vessel, and then sealed with a lid fitted with a rubber gasket and incorporating 1 terumo venoject rubber stopper. Approximately 10% of the volume of the air headspace was replaced with 60 ml of C_2H_2. There were four replicates for each treatment. Vessels were incubated for 24 hours on the ground in a shaded place close to the paddock. After 1 hour and also at the end of the 24 hour incubation, gas samples were transferred to evacuated 5 ml vials using double-ended needles.

4.2.4 Analytical methods

The procedures for N_2O analysis of gas samples using a gas chromatograph, and the denitrification activity calculation have been described previously in Chapter 3. Just before any incubation experiments, subsamples of soil were removed for gravimetric soil moisture determination, and for extraction of NO_3^--N with $2M$ KCl (Chapter 3).

The arithmetic means of replicate denitrification activities were calculated for the laboratory data. Analysis of variance was carried out to make statistical comparisons of the denitrification activities using the untransformed data. Due to the large spatial variation in denitrification rates among replicates in the field, the mean soil denitrification rates in the field before and after rainfall were calculated using the Uniform Variance Unbiased Estimators, assuming log-normally distributed sample population values (White *et al.*, 1987; Parkin and Robinson, 1992). The comparisons of the denitrification rates were carried out by testing the overlaps of upper and lower 95% confidence limits using the untransformed data (Parkin, 1993). Statistical analyses were performed using the Statistical Analysis System (SAS) (SAS Institute, 1985).

4.3 RESULTS

4.3.1 Denitrification activity measured in the laboratory in samples collected in the summer
       of 1991 and the winter of 1992

Soil gravimetric moisture contents were higher in both soil profiles in early winter (May) than in early summer (November) (Table 4.1). Measured denitrification activities under

anaerobic, saturated incubation conditions with different substrate amendments for both the Tokomaru silt loam and Manawatu sandy loam are shown in Figures 4.1 and 4.2 (note the log-scale for the x axis in the figures). The data indicate similar trends with depth and responses to C and N amendments in the two soil profiles. The denitrification activities had their maximal values in the surface soil (0-5 cm) and decreased with depth. In the deepest (20-40 cm) layers examined, the denitrification activities in the absence of added C and N in both soils were very low.

Table 4.1 Variation of gravimetric soil moisture content (% ww^{-1}) with depth at different sampling times in the Tokomaru and Manawatu soils

Soil	Depth (cm)	November 1991	May 1992	March 1993	
				Before rainfall	After rainfall
	0-5	33.4	43.4	28.3	48.8
Tokomaru silt loam	5-10	30.0	37.3	25.6	39.9
	10-20	26.6	30.5	23.6	31.3
	20-40	23.4	28.1	22.2	25.1
	0-5	31.4	39.3		
Manawatu fine sandy loam	5-10	27.3	34.1		
	10-20	23.5	28.2		
	20-40	19.1	22.6		

A seasonal effect was apparent with some samples collected in the early winter having denitrification activities in the absence of added C and N that were more than 10 times higher than in the corresponding samples collected in the early summer. This was the case for the 0-20 cm depth of the Tokomaru soil and the 0-10 cm depth of the Manawatu soil (Figures 4.1 and 4.2).

Denitrification rate (μg N_2O-N g^{-1}soil day^{-1})
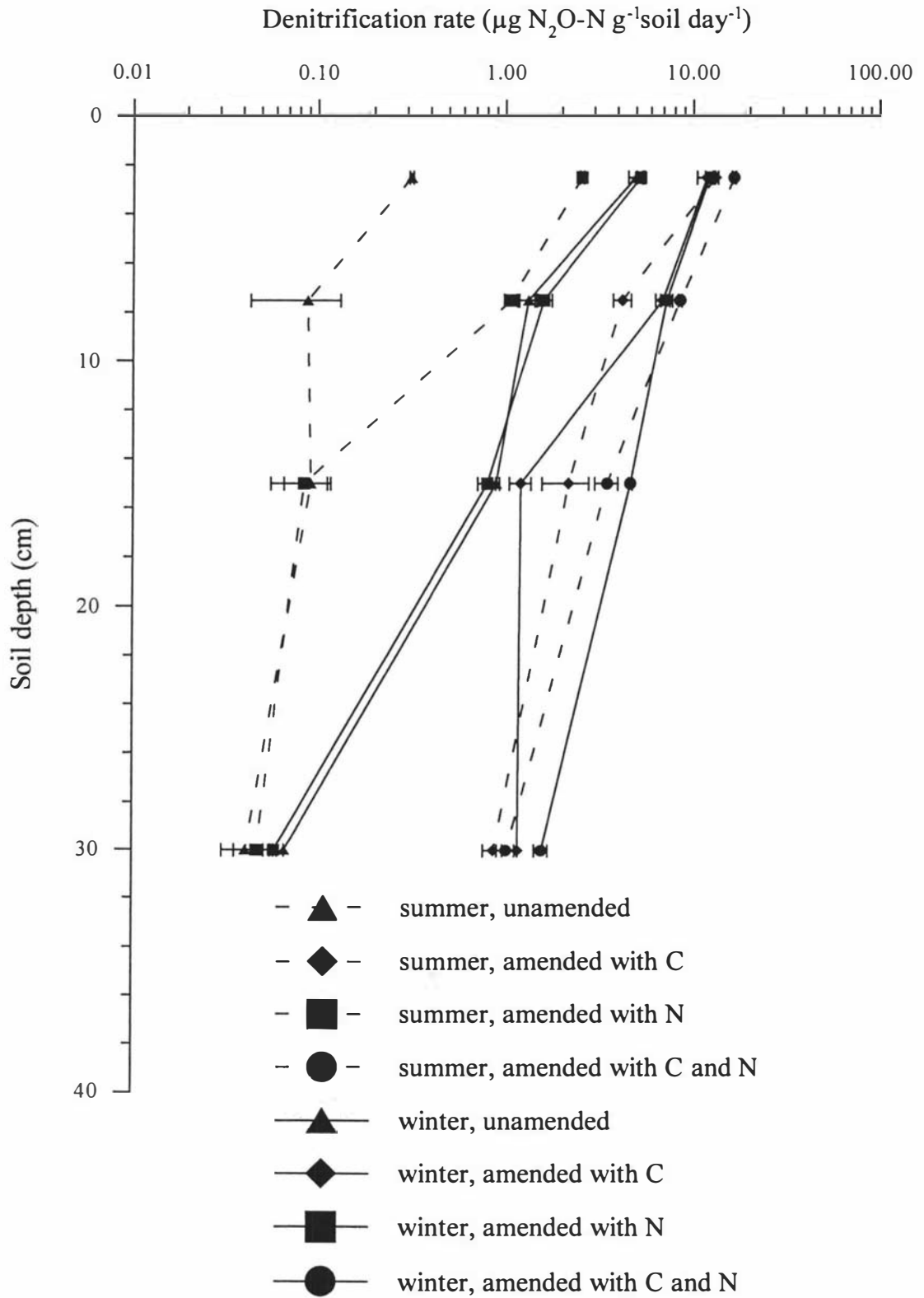
Figure 4.1 Denitrification activities under anaerobic condition in the Tokomaru soil (bars represent SD)
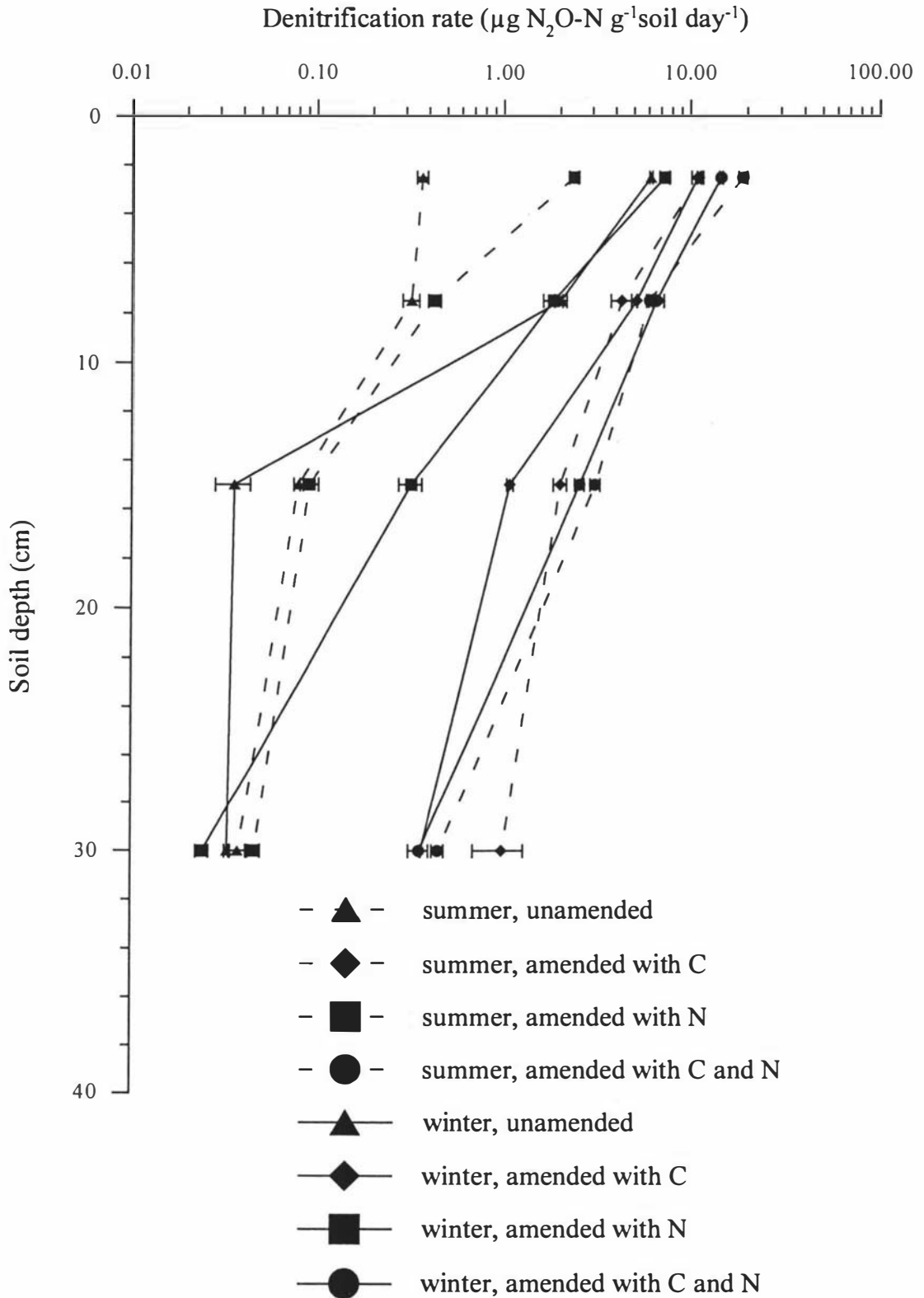
Figure 4.2 Denitrification activities under anaerobic condition in the Manawatu soil
(bars represent SD)

Addition of soluble-C greatly increased denitrification throughout the soil profile in both the early summer and the early winter (Figures 4.1 and 4.2). In the presence of added C there was little difference in denitrification activity under anaerobic incubation conditions between samples collected in the early summer and the early winter, and the decline in denitrification activity with depth was much less marked than when no C was added (Figures 4.1 and 4.2). These results suggest that soluble-C was a major limiting factor controlling denitrification activity in these soils. The similar denitrification activities under anaerobic incubation conditions in samples collected in the early summer and the early winter in the presence of added C may indicate similar denitrifier populations in the two seasons. The higher denitrification activity observed in surface soils in winter in the absence of added C probably indicates a higher amount of available-C in the soils at that time of the year.

Added NO_3^--N increased denitrification activities under anaerobic incubation conditions in the upper layers of the Tokomaru and the Manawatu soils in the early summer (Figures 4.1 and 4.2). In the early winter there was little effect of added NO_3^--N with the only significant increase in denitrification activity occurring in the 10-20 cm depth in the Manawatu soil (Figures 4.1 and 4.2). Therefore, NO_3^--N was not a major limiting factor for denitrification activity compared to soluble-C, when these two soils were incubated under anaerobic and saturated conditions in the laboratory. This was especially so in the early winter, when the NO_3^--N concentrations in the soil profiles were higher than in the early summer (Figure 4.3).

Soil NO$_3^-$ (μg NO$_3^-$-N g^{-1}soil)
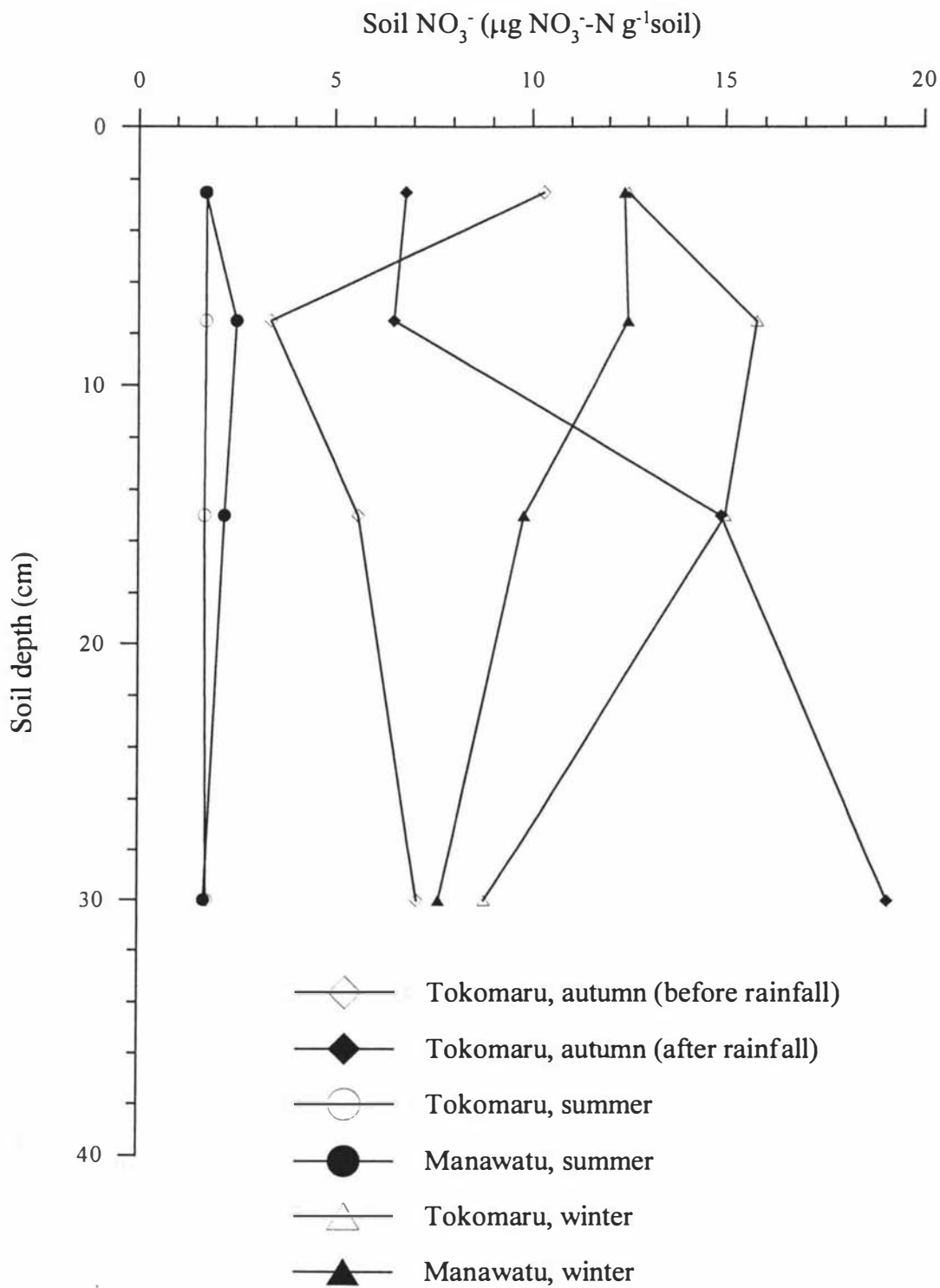
Figure 4.3 Nitrate concentrations in soil profiles of the Tokomaru and Manawatu soils
at three sampling times

Denitrification enzyme activities (defined as the denitrification rate measured under anaerobic, saturated conditions after amendment with both NO_3^--N and glucose-C) in the Tokomaru and the Manawatu surface soils (0-5 cm) were 16.5 and 19.1 µg N_2O-N g^{-1} soil day^{-1} in samples collected in the early summer. These activities were about 50 times greater than the activities when soils were not amended with C and N (Figures 4.1 and 4.2). In samples collected in the early winter, the denitrification enzyme activities in the Tokomaru and the Manawatu surface soils (0-5 cm) amended with C and N were 12.2 and 14.5 µg N_2O-N g^{-1} soil day^{-1}, respectively, and this was only about 2.5 times greater than the activity when no extra C and N were added (Figures 4.1 and 4.2). The study also revealed that denitrification enzyme activity decreased approximately exponentially with depth to 30 cm. Even though denitrification activities differed markedly between the amended and unamended samples, the rate of decrease in activity with depth was approximately the same in all cases (Figures 4.1 and 4.2).

4.3.2 Impact of rainfall on denitrification activity as measured in the laboratory

Laboratory denitrification activities in the absence of added C and N, measured under saturated, anaerobic conditions, were higher in samples collected before rainfall from the surface 10 cm than in samples collected from equivalent depths after rainfall (Figure 4.4). However, when NO_3^--N or glucose-C solutions were added to the surface (0-5 cm) soils, there was very little difference between the samples collected before and after rainfall (Figure 4.4). When NO_3^--N or glucose-C solution were added to the deeper samples (5-40 cm), the denitrification activities increased after the rainfall (Figure 4.4). This was particularly apparent in soils amended with NO_3^--N, and suggests that the rainfall may have transported soluble-C down the profile. This soluble-C could then stimulate denitrification
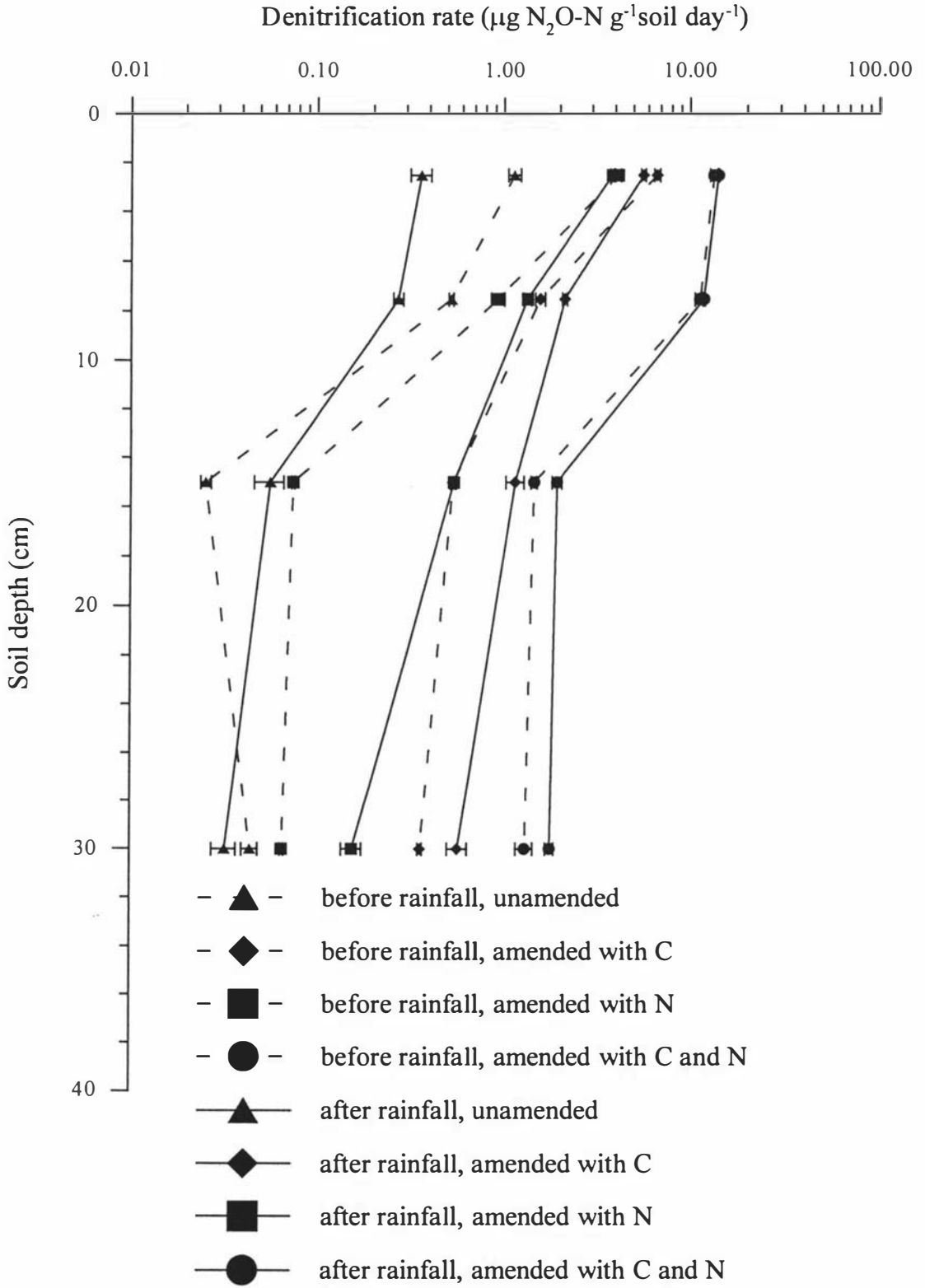
Figure 4.4 Denitrification activities under anaerobic condition in the Tokomaru soil
before and after heavy rain (bars represent SD)

when NO_3^--N was non-limiting. The maximum denitrification activities under anaerobic incubation were again observed where both NO_3^--N and glucose-C solutions were added to the soils.

4.3.3 Denitrification rates measured at field temperature and moisture before and after rainfall in the autumn of 1993

The effect of heavy rainfall (28.5 mm) on soil moisture at the autumn sampling in 1993 was very apparent, and the rain increased the soil moisture contents to at least 30 cm (Table 4.1). Denitrification rates, measured at field temperature and moisture contents, and in the natural gas atmosphere, increased significantly ($p < 0.05$) in the soil profiles after the rainfall (Figure 4.5), although the rate remained low compared to the potential rate under optimal conditions (Figure 4.4). The absolute magnitude of the increase in denitrification rate was larger in the surface soil than at depth, although the percentage increase in rate was much greater at depth than in the surface soil (Figure 4.5).

4.4 DISCUSSION

4.4.1 Decrease in denitrification activity with depth

The denitrification rate in the soils of the present study varied markedly with depth, regardless of the various treatments applied (Figures 4.1, 4.2, 4.4 and 4.5). Similar decreases in denitrification activity with depth have been observed in pasture soils (e.g. Limmer and Steele, 1983) and other soils (e.g. Cho *et al.*, 1979; Staley *et al.*, 1990; Ambus and Lowrance, 1991). It seems that the lower levels of denitrification activity at depth in

Denitrification rate (μg N$_2$O-N g^{-1}soil day^{-1})
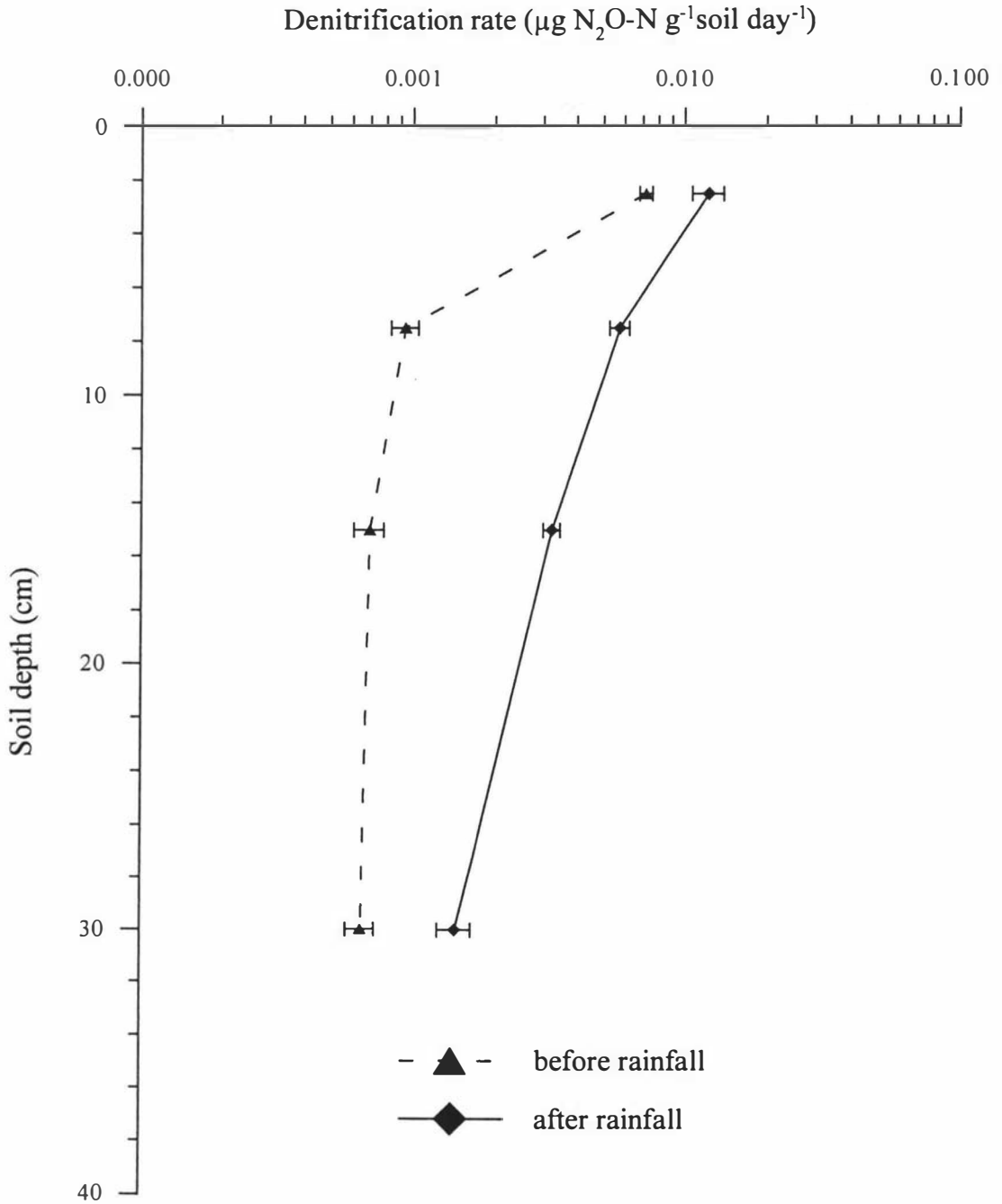
Figure 4.5 Denitrification rates in the Tokomaru soil under field conditions
before and after heavy rain (bars represent SD)

these studies might reflect a lower number of anaerobes in subsurface soils. This view is supported by the results of Parkin and Meisinger (1989). In their study, total viable bacteria and numbers of denitrifying bacteria were found to decrease exponentially with increasing soil depth in a well-drained silt loam. Our observations that denitrification enzyme activities following amendment with both N and C were still substantially lower at depth than in the surface soil also strongly suggest a decrease in the size of the denitrifier population with depth rather than solely a limitation of substrate.

Although the denitrifier population was lower at depth than in the surface soils, there was still appreciable denitrification enzyme activity at depth indicating that some living microorganisms, capable of denitrification, occur in the deeper layers of these pasture soils. An appreciable number of bacteria with denitrification capacity have also been found in deeper soil layers in some other studies. For example, Weier *et al.* (1991) found an increase in N_2O concentration with depth beneath a cultivated crop, and suggested that NO_3^--N may have been lost from the subsurface soil through denitrification. By measuring N_2O as an indication of the denitrification potential under anaerobic incubation, Lind and Eiland (1989) found that denitrifiers were present at all sampled depths down to 20 metres of sandy-clayey and sandy soil profiles.

4.4.2 Factors controlling denitrification activity in the soil profile

The results in our study indicate that denitrification activities were not markedly stimulated by the addition of NO_3^--N to subsurface soils, but the addition of glucose-C boosted the denitrification rate significantly in all the soil profile samples (Figures 4.1, 4.2, and 4.4). McCarty and Bremner (1992; 1993) found that lack of degradable organic material can be

the principal factor that restricts denitrification, especially in the subsurface soil. It is likely that a decline in available-C with depth in a soil limits the activities of the microbial population and that this is responsible for the decrease in denitrification activity. Any factor affecting the distribution of organic-C in the soil profile, such as inversion of the topsoil by ploughing, would be expected to have a substantial influence on denitrification rates at depth.

4.4.3 Denitrification activity in the soil profile affected by rainfall

The present study showed that denitrification rates in soil, measured at field temperature and moisture, increased throughout the soil profile after heavy rainfall (Figure 4.5). The increase in soil moisture content resulting from the rainfall may be one important cause of this increase, since the incubation temperatures of these two days before and after the rainfall were similar (13.3 vs 13.9°C). An increase in soil moisture leads to a decrease in soil aeration, and therefore denitrification in the soil will increase (Linn and Doran, 1984; Sexstone et al, 1985; Weier et $al.$, 1993). An increase in soil moisture content might also affect the diffusion of NO_3^--N and soluble-C in the soils, so that it would be much easier for NO_3^--N and soluble-C to move to the site of denitrification.

Heavy rainfall can also wash NO_3^--N, soluble-C, or even soil microorganisms from the surface soil down to depth, which may complicate the effect of the rainfall on denitrification in the soil profile. Incubation of the soils in the laboratory after saturation but without any substrate amendment resulted in relatively lower denitrification activities in surface soils, and relatively higher denitrification activities in the subsurface soils (10-20 cm) after rainfall compared to those before rainfall (Figure 4.4). This pattern of the

increase in denitrification activity after rainfall was also evident when the soils were amended with either NO_3^--N or glucose-C (Figure 4.4). The increase following rainfall was particularly large in the 10-20 cm depth after amendment with NO_3^--N. This suggests that both NO_3^- and, especially, soluble-C were washed down the profile by the rainfall. In the case of NO_3^- this suggestion was supported by measurements of soil NO_3^--N concentrations before and after the rainfall event (Figure 4.3). No measurements of available-C were made in this study but the circumstantial evidence for movement of soluble-C contrasts with observation of McCarty and Bremner (1992; 1993) that very little water-soluble organic-C could be leached into Iowa subsoils.

In our study, it seems that the heavy rainfall did not wash microorganisms from the surface soils down to depth, as the denitrification enzyme activities in soils amended with both NO_3^--N and glucose-C under anaerobic and saturated conditions were similar before and after the rainfall at each depth (Figure 4.4).

4.5 CONCLUSIONS

Results of the present study show that the denitrification activities in these pasture soils decreased with the depth. Although denitrification activity could be at least an order of magnitude higher in the surface 0-5 cm than between 20 and 40 cm, there were living microorganisms capable of denitrification in the deeper soil layers. Under favourite conditions of soil moisture, denitrification activity in both soils, especially at depth, was controlled largely by the supply of available-C and to a lesser extent by NO_3^--N. Denitrification enzyme activities in the soils after the addition of NO_3^--N and soluble-C under saturated, anaerobic conditions did not show marked temporal change between the

early summer and the early winter samples, nor before and after a rainfall event in the autumn. Heavy rainfall reduced the difference in the field denitrification rate between the surface and subsurface soils, which may have been due to leaching of NO_3^- and available-C from the surface to the subsurface soil.

CHAPTER 5

FREQUENCY DISTRIBUTION AND SPATIAL VARIABILITY OF DENITRIFICATION RATE

5.1 INTRODUCTION

Accurate estimates of nitrogen (N) loss through denitrification are required in both agricultural and environmental studies. However, it is difficult to obtain these accurate estimates because the denitrification rate in field soils generally exhibits a high spatial variability (e.g. Folorunso and Rolston, 1984; Parkin, 1987; Christensen *et al.*, 1990a), with coefficients of variation (CV) greater than 100% having been found frequently (e.g. Aulakh *et al.*, 1982; Christensen *et al*, 1990a; Parsons *et al.*, 1991). In addition to high variability, soil denitrification rates in the field are often not normally distributed, but exhibit skewed distributions. The recognition of the correct frequency distribution of denitrification rates is an important step in applying statistical techniques for characterising the variability, estimating denitrification N loss, and evaluating differences in denitrification under various natural conditions. Some studies have found that denitrification rates in field soils appear to be log-normally distributed (e.g. Folorunso and Rolston, 1984; Parkin *et al.*, 1987; Robertson and Tiedje, 1988; Svensson *et al.*, 1991), as do mineral N concentrations (White *et al.*, 1987; Starr *et al.*, 1992). A log-normal distribution of denitrification rates indicates that most samples of a given data set exhibit relatively low rates but a few samples have very high rates. In contrast, Kessel *et al.* (1993) reported that although a highly-skewed

distribution of denitrification rates was found, the distribution did not closely approximate a log-normal distribution.

Most frequency distributions of denitrification rates in field soils reported in the literature have been estimated from sets of data obtained from the field on a single sampling date. Only a few studies have involved sampling programmes extending over an appreciable period of time. Groffman and Tiedje (1989a) found that the frequency distribution of denitrification rates changed temporally and they observed that rates can be normally or log-normally distributed in forest soils in different seasons, due to differences in moisture content and available-C distribution. Patterns and frequency distributions of denitrification rates may also be influenced by topography (Groffman and Tiedje, 1989a). In their field study, Pennock et al. (1992) observed that the frequency distributions for denitrification rate were distinctly different among landform elements and, despite the absence of a log-normal distribution for the complete denitrification data set in the whole study area, the distribution within each landform element was log-normally distributed.

Studies have shown that the source of variability in natural denitrification rates is the patchy distribution of denitrifying "hot spots" in soil (Parkin, 1987; Christensen et al., 1990a). An analysis of the spatial dependence of the variability in a continuous cropping field, using a semivariogram, showed that denitrification exhibited a high degree of microscale variability (Parkin et al., 1987). Most of the variability in denitrification rate appeared to occur within a short distance (Parkin et al., 1987).

The sample variance of denitrification rate may be dependent on the sampling volume of soil from which individual observations are made. Use of a larger size of sample in

individual observations might be expected to give lower coefficients of variation (CV) due to the possible integration of the lower-scale variability. However, by analysing the log-transformed data of the denitrification rates, Parkin *et al.* (1987) found that sample size had little influence on the variability of the rates. However, the test of variances on the log-transformed data does not necessarily give the variances of the untransformed data (Parkin *et al.*, 1988).

A previous study has found that the variability of soil nitrate (NO_3^--N) concentration tended to decrease with increasing sample size, when it was estimated using the untransformed NO_3^--N concentration data and the UMVUE method (Uniform Minimum Variance Unbiased Estimators) (Parkin and Robinson, 1992). More studies on the influence of sample size on variability of denitrification rates are therefore required for the better design of soil sampling schemes in denitrification measurements.

Most of the studies on the spatial variability of denitrification rate discussed above have been conducted in cropping, forest, or natural systems. To our knowledge there have been no adequate studies of the frequency distribution and variance of denitrification rates in pastures. A previous study by White *et al.* (1987) has indicated that the intensity of animal grazing on pastures can affect the variance and skewness of mineral N concentration, although the frequency distributions of soil mineral N concentrations were log-normal at all the sampling times. To achieve an estimation of N loss through denitrification from pastures, more information is required about the variance and frequency distribution of denitrification rates.

The following three chapters report on a major study investigating the extent of

denitrification in a pasture. In planning the study, it was decided to choose a site that should provide favourable conditions for denitrification. It was felt that such a site might provide a useful "upper bound" to denitrification N losses from pasture.

Accordingly, the site chosen was located on an intensive dairy-farm on a poorly-drained soil. The combination of poor drainage and compaction from high stocking rates was expected to restrict aeration. The return of dung and urine should have ensured a ready supply of NO_3^--N and soluble-C.

Careful consideration was also given to the most appropriate scale for the measurement. Denitrification in pastures is affected by climate, soil properties and pasture management practices. On a dairy-farm, pasture management is largely organised on an individual paddock scale and so, although a single paddock may encompass a range of soil properties, it was decided to study denitrification N loss in a single dairy-farm paddock.

This chapter reports on the spatial variability and the frequency distribution of denitrification rate in different topographical areas in a pasture throughout a year. The variance and frequency distributions of other soil parameters are also included. The effect of sample size on the variance and distribution of denitrification rate will also be examined.

5.2 MATERIALS AND METHODS

5.2.1 Site description

The research was carried out using soil samples collected from Paddock 17 of the Massey

University No. 4 Dairy-farm, Palmerston North, New Zealand. The size of the paddock was about 2.5 ha. The paddock was under ryegrass/white clover pasture and was periodically grazed by cows at about 100 stock units ha^{-1}. During the period of study, the paddock received no N fertilizer. The soil at this site, the Tokomaru silt loam (Cowie, 1974), is classified as a Yellow Grey Earth (Taylor and Pohlen, 1968) or a Pallic Soil (Hewitt, 1992). It is a poorly-drained soil with wet conditions in winter, and relatively dry conditions in summer. The paddock was predominantly flat with a small gully running through it. Five contrasting sites were located within the paddock. These were a flat land area, north- and south-facing gully slopes, the gully bottom, and a fertile and compacted gateway area. Soil properties of the upper 7.5 cm of the profile are shown in Table 5.1.

Table 5.1 Major characteristics of the soils at 0-7.5 cm depth

Site		Gully bottom	N-facing slope	S-facing slope	Flat land area	gateway
pH (H$_2$O)		6.06	6.00	5.91	6.04	5.94
Texture	%sand	23.90	22.98	21.26	22.71	22.74
	%silt	63.79	62.39	63.08	67.32	62.83
	%clay	12.31	14.63	15.66	9.97	14.55
Bulk density (Mg m^{-3})		0.83	0.87	0.88	0.84	0.93
Total N (%)		0.47	0.36	0.37	0.42	0.48
Total P (%)		0.13	0.09	0.08	0.09	0.16
Total C (%)		5.16	4.80	4.91	5.22	5.50

5.2.2 Field denitrification measurement

Collection of samples. Within the study paddock samples were collected for measurement of denitrification rate on approximately 29 occasions between July 1992 and October 1993.

This total number of measurements comprised a number of separate studies investigating different aspects of denitrification. The results of these studies are reported later in this chapter and also in Chapters 6, 7 and 8.

Although the separate studies mentioned above were investigating different aspects of denitrification, on each sampling occasion a large number of replicate measurements were made. In this chapter all these data have been aggregated to provide information on the frequency distribution and spatial variability of denitrification rate in the paddock.

The details of each of the sampling programmes are described below. In the first three programmes denitrification measurements were made on individual soil cores, whereas the fourth programme, comparing grazed and ungrazed areas, involved composite samples of 14 cores.

- Topographical variation in denitrification. One sampling area (9×9 m) was usually selected within each of the gateway, S-facing and N-facing slope sites; and two sampling areas selected within each of the flat land and gully bottom sites. Denitrification measurements were made regularly at all the sites from July 1992 to October 1993, with more frequent sampling during warm, wet periods, particularly in the late summer of 1993. On most measurement occasions sixteen soil cores were taken from randomly selected area at each site. The sampling points in each sampling area were arranged at 3-m intervals over the 9×9-m.

- Identification of factors limiting denitrification rate. On another 7 occasions, samples were taken from the flat land site to study the separate effects of saturation,

substrate addition and anaerobic conditions on denitrification. (A detailed description of this study is presented in Chapter 8.) On each of these sampling dates 112 soil cores were collected randomly from the site.

- Spatial dependence of denitrification rate. A further 3 sampling exercises were carried out on the flat land site to study the spatial dependence of denitrification rate. Samples were taken on main and nested grids, which allow for greater number of lags to be tested within a given area (Bramley and White, 1991). On 20 April 1993, about a month after the previous grazing event, individual soil cores were taken from 115 points in the flat land site as shown in Figure 5.1. Sampling was also conducted on another two dates. The dates 20 July 1993 and 5 August 1993 were 6 days before and 10 days after an intensive grazing event, respectively. The sampling grids used on both occasions are illustrated in Figure 5.2. The paddock was grazed with cows at a high stocking rate (about 300 cows ha^{-1}). Soil samples for the study were taken from both the grazed site and a control area (an area from which cows had been excluded).

- Effect of animal grazing. As discussed in the previous section, the effect of intensive grazing on the spatial dependence of denitrification rate was investigated in the winter of 1993. On the same occasion 40 samples (each comprising 14 soil cores) were collected from both the grazed and ungrazed areas to estimate denitrification rates (Chapter 7).
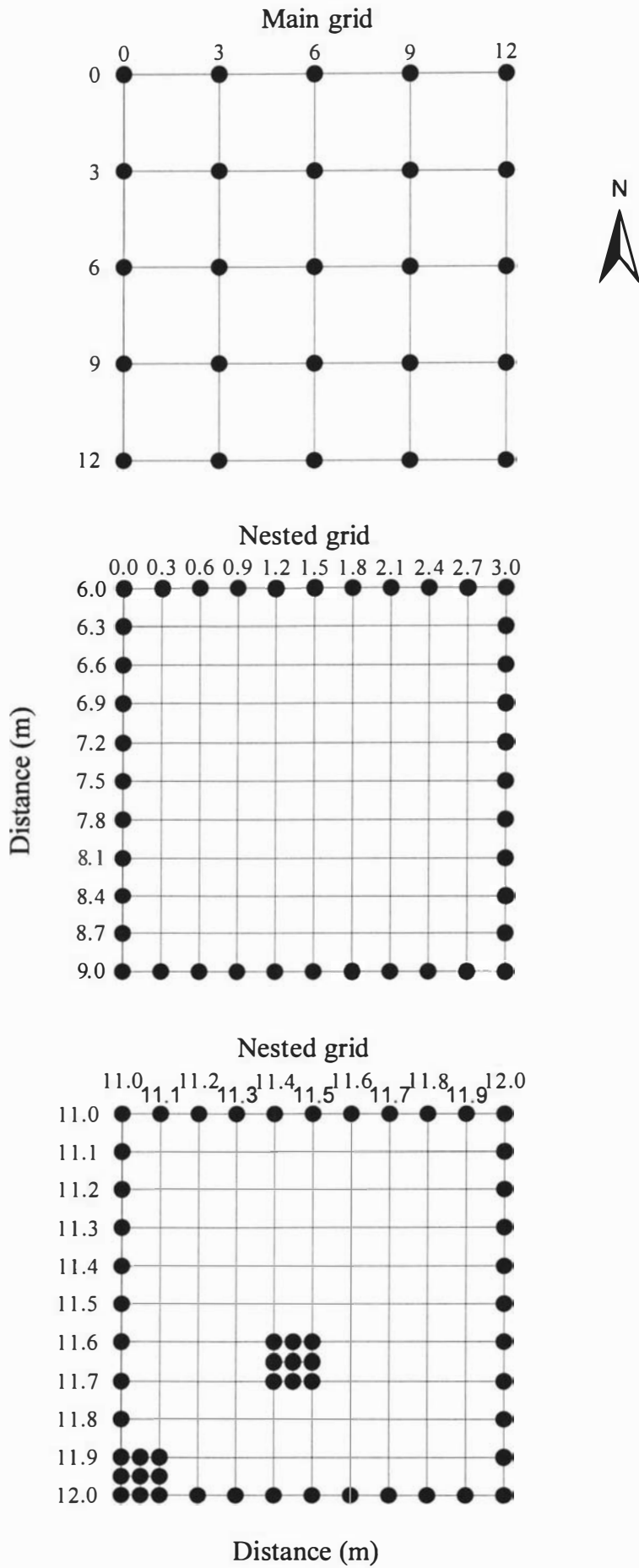
Figure 5.1 Sampling grid used for the analysis of spatial variability
in soil denitrfication rate on 20 April 1993
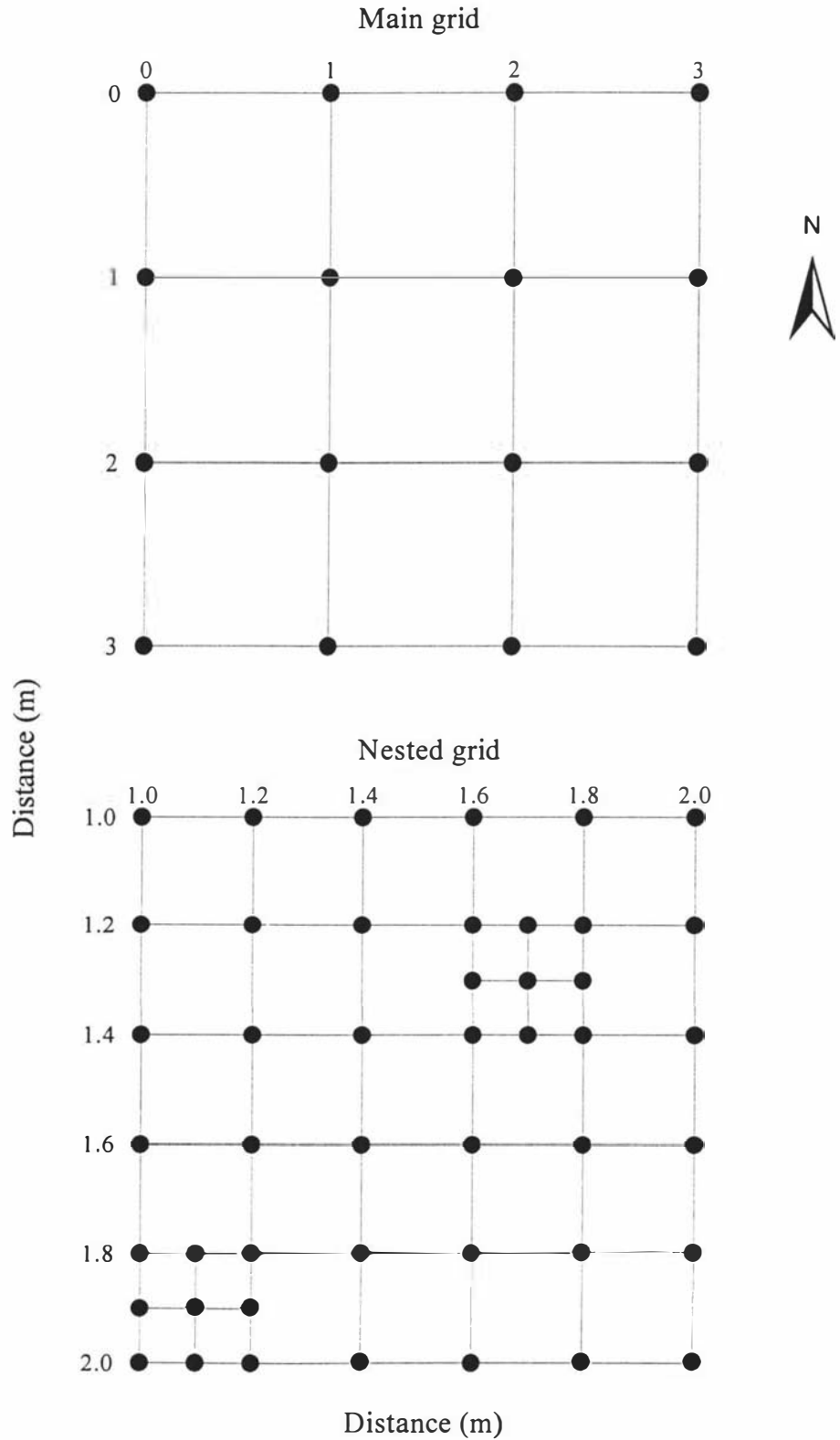
Figure 5.2 Sampling grid used for the analysis of spatial variability
in soil denitrification rate. Same types of grid were used
both in the control and the grazed sites on 5 August 1993,
as well as on 20 July 1993 before grazing

Measurement of denitrification rate. In the first 3 studies, the rate of denitrification was measured using the acetylene-inhibition technique (Yoshinari *et al.*, 1977), using the individual soil core incubation system under field conditions as described by Ryden *et al.* (1987). At each sampling date, soil cores were collected using a soil corer. A core was approximately 2 cm in diameter and 7.5 cm in length. Individual cores were transferred from corers into PVC tubes (2.5 cm in diameter by 15 cm in length). The tubes were closed at both ends with rubber septa. Six ml of air was withdrawn from the tube and the same amount of purified C_2H_2 (by passing industrial C_2H_2 gas through a high concentration of H_2SO_4) was injected into each tube using a syringe. The syringe was pumped several times to mix the C_2H_2 within the tube.

In the study investigating the effect of animal grazing on denitrification rate, glass jars (about 1100 ml in total volume) instead of PVC tubes were employed. Fourteen cores were placed into a jar, 60 ml of air was withdrawn and C_2H_2 was injected.

The tubes and jars were incubated for 24 hours on the ground in a shaded place close to the paddock. At 1 and 24 hours after the addition of C_2H_2, a sample of the headspace gases was collected in a 5 ml venoject evacuated test tube (Becton Dickinson Vacutainer Systems). A gas chromatograph equipped with a [63]Ni electron capture detector was used to measure the concentrations of N_2O in the samples. The details of the measurements and the calculations of the denitrification rates have been described in Chapter 3.

5.2.3 Soil moisture, mineral nitrogen and CO_2 measurement

Soil cores were brought to the laboratory after 24 hours of incubation and removed from

the tubes or jars. The individual soil samples from each of the tubes or jars were then bulked. Soil moisture was determined from the weight loss of subsamples dried overnight at 105°C. Soil NH_4^+ and NO_3^- (including NO_2^-) were extracted by shaking a 5 g soil sample with 20 ml of 2 M KCl for 60 min and filtering through Whatman No. 42 filter paper. Mineral N was determined colorimetrically on a Technicon Autoanalyser (Downes, 1978).

Carbon dioxide concentration was determined from the same gas samples as those used for N_2O analysis. No significant differences in CO_2 concentration had been observed between systems with and without added C_2H_2 during previous incubation tests. The same findings were made by Ryden (1982). Carbon dioxide was measured in a gas chromatograph equipped with a thermal conductivity detector.

5.2.4 Statistics

Coefficients of skewness were calculated to quantify departures from normality on both log-transformed and untransformed data (SAS Institute, 1982). The significance of the difference from zero of the coefficients of skewness was evaluated as described by Zar (1974). Frequency distributions of denitrification rates and log-transformed rates at each site or across the whole paddock, where applicable, on each sampling date were calculated by Wilk-Shapiro statistics to assess whether these rates were normally distributed (SAS Institute, 1982). The coefficients of variation (CV) were calculated using the UMVUE method for log-normally distributed data (Parkin and Robinson, 1992). Analyses of spatial variability were done using geostatistical methods (Webster and Oliver, 1990), and the semivariance $\gamma(h)$ was estimated for log-transformed values of denitrification rates of log-normally distributed data sets.

5.3 RESULTS AND DISCUSSION

5.3.1 Variation and frequency distribution of denitrification rate

Measurements of field denitrification rates by the acetylene inhibition and soil core incubation technique were made on 14, 13, 13, 29 and 13 occasions on gully, south-facing slope, north-facing slope, flat land and gateway sites, respectively, in the various separate studies from 7 July 1992 to 17 October 1993. The measured denitrification rates exhibited a high degree of skewness and a large spatial variation at all the sampling sites throughout the sampling period. Figure 5.3 gives some histograms of the frequency distributions of denitrification rates from selected sampling dates and sites.

The frequency distributions were positively skewed (p<0.01) for 71 of the total of 82 data sets for individual sites during the sampling period (Table 5.2). Positively skewed distributions of denitrification rates were also observed when, on each individual sampling dates, the data from all sites within the paddock were combined (Table 5.3).

Tests of the distribution confirmed that most of field denitrification rates measured by this technique were more closely approximated by the log-normal than the normal distribution, irrespective of sites and sampling dates (Tables 5.2 and 5.4). It was also observed that the values of the coefficients of skewness for log-transformed rates on most occasions were not significantly positive (Table 5.2). However, the distributions of denitrification rates in the whole paddock did not approximate a log-normal distribution for most of the sampling dates (Table 5.3). The CV of denitrification rates exceeded 100% in 50 of the total 82 data sets (Table 5.2). The CVs for the log-transformed rates were smaller than for the untransformed rates (Table 5.2).
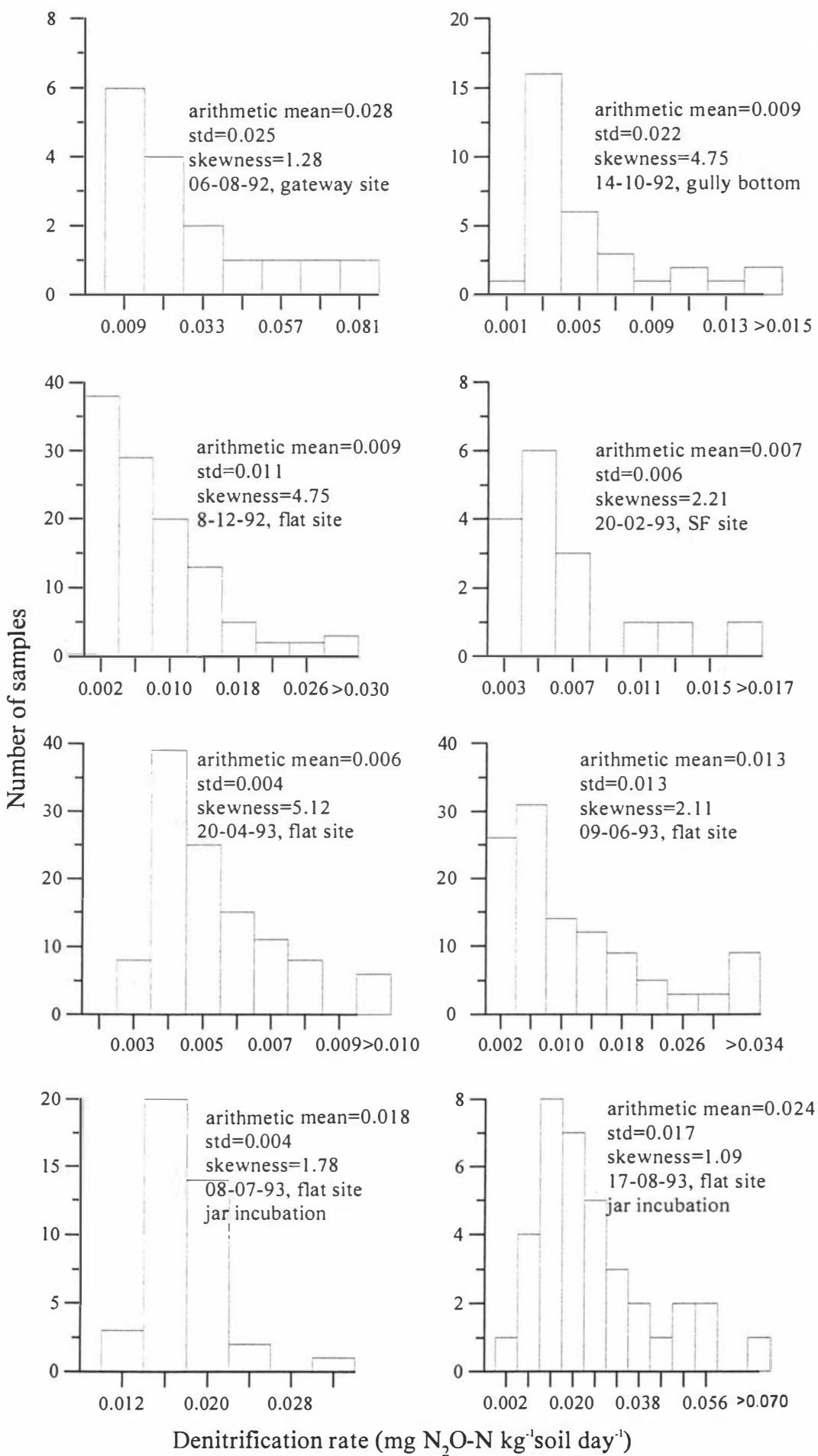
Figure 5.3 Histograms of denitrification rates at selected sampling dates and sites

Table 5.2 Statistical properties of some soil parameters measured on individual soil cores at all sampling sites and dates

Parameter	Log-transformed	Number of sampling events	Coefficient of skewness Range Min.	Max.	Sampling number (positive, p<0.01)	Distribution (number of sampling) Normal	Log-normal	Coefficient of variation (CV) (%) Range Min.	Max.	Sampling number (CV>100)
Denitrification	no	82	-0.25	7.0	71	6	76	20.9	337	50
(mg N_2O-N $kg^{-1}d^{-1}$)	yes	82	-1.2	3.1	27	82	0	5.09	39.2	0
NO_3^-	no	67	0.09	4.7	51	4	63	19.3	256	14
(mg NO_3^--N kg^{-1})	yes	67	-1.25	2.6	17	67	0	17.3	154	4
NH_4^+	no	15	-0.14	2.8	9	5	10	21.6	79.7	0
(mg NH_4^+-N kg^{-1})	yes	15	-3.7	1.0	1	15	0	6.81	34.1	0
CO_2	no	81	-0.92	2.3	20	57	24	9.80	59.4	0
(mg CO_2-C $kg^{-1}d^{-1}$)	yes	81	-2.7	0.97	0	81	0	3.55	26.0	0
Moisture	no	67	-0.91	3.2	11	57	10	3.10	26.8	0
(% ww^{-1})	yes	67	-1.1	2.3	7	67	0	0.79	4.98	0

Parameter	Log-transformed	Number of sampling events	Coefficient of skewness Range Min.	Max.	Sampling number (positive, p<0.01)	Distribution (number of sampling) Normal	Log-normal	Other
Denitrification	no	7	2.4	6.4	7	0	2	5
(mg N_2O-N $kg^{-1}d^{-1}$)	yes	7	-0.23	4.9	5	2	0	5
NO_3^-	no	7	0.19	3.8	6	0	3	4
(mg NO_3^--N kg^{-1})	yes	7	-0.06	2.7	3	4	0	3
NH_4^+	no	2	1.4	3.1	2	1	1	0
(mg NH_4^+-N kg^{-1})	yes	2	-0.12	4.0	0	1	1	0
CO_2	no	7	-0.46	2.3	2	5	1	1
(mg CO_2-C $kg^{-1}d^{-1}$)	yes	7	-0.55	0.39	0	6	0	1
Moisture	no	7	-0.33	1.5	1	5	2	0
(% ww^{-1})	yes	7	-0.35	0.48	0	7	0	0

Table 5.4 Summary of statistical characteristics of denitrification rates at each topographical site

Site	Number of sampling events	Coefficient of skewness		Distribution		Coefficient of variation (%)	
		Min.	Max.	Normal	Log-normal	Min.	Max.
Gully bottom	14	1.67	5.35	0	14	46.0	241
North slope	13	-0.16	3.91	2	11	26.4	133
South slope	13	0.32	3.96	1	12	34.3	297
Flat land	29	-0.25	6.95	3	26	20.9	337
Gateway	13	0.37	3.41	0	13	55.6	185

Large CVs and skewed distributions occur when most samples have low denitrification rates, and a few samples have very high rates. In the current study about 25% the soil cores contributed more than 50% of the total N loss through denitrification from all the soil cores on each sampling occasion. Large CVs and positively skewed distribution patterns of denitrification rates in field studies have been often reported (Folorunso and Rolston, 1984; Parkin, 1987; Robertson *et al.*, 1988; Christensen *et al.*, 1990a; Parsons *et al.*, 1991). Statistical tests have generally suggested that the log-normal distribution was most appropriate for describing the spatial variability in field-measured denitrification rates in agricultural soils, forest soils, and native vegetation sites (Folorunso and Rolston, 1984; Parkin, 1987; Myrold, 1988; Groffman and Tiedje, 1989a; Peterjohn and Schlesinger, 1991; Svensson *et al.*, 1991). These findings are perhaps not surprising, since the rate of denitrification in the field is controlled by a combination of several environmental and soil factors (such as O_2 availability, NO_3^- concentrations, available-C levels and denitrifier populations). The law of proportionate effects predicts that a random variable will exhibit a log-normal distribution when the factors controlling the variable are combined in multiplicative manner (Aitchison and Brown, 1957).

It is interesting, however, that denitrification rates aggregated across the whole study paddock did not often appear to be log-normally distributed (Table 5.3). This may reflect distinct differences in the background rate of denitrification in the different topographical areas. Similar results have been observed in a previous study of the distribution of denitrification rates associated with various landform elements (Pennock *et al.*, 1992).

Soil sample size may have a significant influence on the spatial variation. Our results indicate that the CV of denitrification rates with a larger soil sample size (14 soil cores) was

less than with smaller samples (individual soil cores). The distributions of denitrification rates obtained with larger samples were also less skewed than with individual soil cores (Figure 5.3). However, the distributions of denitrification rates obtained with the larger soil sample size were still log-normal. Similar results were also found by Parkin *et al.* (1987), who recognised that denitrification rates of larger cores were more evenly distributed than denitrification rates of smaller cores.

All these results can be related to the proposed existence of hot-spots of denitrification in soil (Parkin, 1987). The probability of smaller sized samples containing hot-spots is lower, hence the frequency distribution of denitrification rate will contain many low values with an occasional very high value. On the other hand, larger samples will have a higher chance of containing hot-spots of denitrification, but the effect of the high activity of the hot-spot on the measured rate of denitrification in the sample is diluted because of the large mass of low-activity soil. The frequency distributions of denitrification rate in the larger samples will contain few extreme values and therefore be less skewed.

5.3.2 Variation and frequency distributions of other soil variables

The concentrations of soil mineral N were variable, and they also appeared to be highly skewed and exhibited a log-normal distribution in most cases (Table 5.2). This agrees with previous studies (White *et al.*, 1987; Myrold, 1988; Bramley and White, 1991).

Carbon dioxide emission rates have also been found to be log-normally distributed (Focht *et al.*, 1979). However, in the present study soil CO_2 emission rates from core incubations did not often exhibit a high degree of spatial variation, and were fitted better by normal than

log-normal distributions on most sampling dates at most sites (Table 5.2). This may indicate that a high proportion of the measured CO_2 originated from the respiration of evenly-distributed soil C or possibly grass roots in this pasture.

Soil water content was relatively uniform and could be described by normal distributions on almost all sampling dates at all sites (Table 5.2). Similar results have been observed in a range of soil types (Myrold, 1988; Bramley and White, 1991; Pennock *et al.,* 1992). However, skewed distributions for soil moisture content were observed when the soil was relatively dry (Table 5.5). There may be a patchy distribution of moist soil under dry field conditions due to urine and dung deposits from dairy cattle.

5.3.3 Temporal patterns of spatial variability

Effect of grazing in winter. The pattern of variation in denitrification rates in this pasture showed a temporal dependence that was mostly influenced by grazing events and rainfall. After an intensive grazing event in winter, soil NO_3^--N concentration and denitrification rate increased (Table 5.6). The grazing also increased the skewness of the soil NO_3^--N concentration and denitrification rates (Table 5.6). These results suggest that the high skewness of the denitrification rates was probably due to the uneven distribution of soil NO_3^--N derived from animal excreta in the field.

Effect of rainfall in summer. Our study also showed that denitrification rates were low and the skewness of denitrification rates was also low when the soil was relatively dry in summer (Table 5.5). The rate of denitrification at this sampling time was likely to be limited by soil moisture conditions rather than unevenly-distributed soil NO_3^--N and C.

Table 5.5 Effect of rainfall on the variation of denitrification rate, soil nitrate concentration and soil moisture content

Date[a]	Site	Soil moisture		Soil nitrate		Denitrification	
		Content (% ww^{-1})	Skewness	Content (mg N kg^{-1})	Skewness	Rate (mg N kg^{-1}d^{-1})	Skewness
	Gully bottom	21.9	1.85	8.5	2.4	0.0046	1.8
	North slope	20.9	1.25	7.9	1.9	0.0048	1.7
19-2-93	South slope	25.6	-1.753	6.5	1.3	0.0047	2.4
	Flat land	20.0	2.4	7.5	2.1	0.0036	1.9
	gateway	22.2	-1.24	14.6	1.4	0.0084	1.1
	Gully bottom	37.2	1.185	5.2	2.8	0.0214	5.3
	North slope	33.6	1.16	1.3	2.2	0.0175	3.2
20-2-93	South slope	39.5	-1.75	0.18	1.6	0.00716	3.2
	Flat land	35.0	2.4	5.5	3.3	0.009	2.8
	gateway	40.4	-0.24	5.2	2.4	0.0863	3.4
	Gully bottom	39.5	-0.059	7.1	2.0	0.0161	3.2
	North slope	33.9	-0.410	3.3	1.6	0.0071	2.3
21-2-93	South slope	36.4	0.170	1.5	3.2	0.0104	0.73
	Flat land	36.1	0.161	0.92	3.5	0.0121	2.1
	gateway	40.2	0.186	1.6	1.3	0.031	0.96

(Table 5.5 continuing)

Date[a]	Site	Soil moisture		Soil nitrate		Denitrification	
		Content (% ww^{-1})	Skewness	Content (mg N kg^{-1})	Skewness	Rate (mg N kg^{-1}d^{-1})	Skewness
	Gully bottom	36.7	0.493	7.1	1.9	0.0234	2.6
	North slope	30.9	-0.338	1.2	1.6	0.0121	0.88
22-2-93	South slope	34.3	-0.484	1.2	2.0	0.0148	1.9
	Flat land	33.7	0.31	2.2	3.4	0.0122	4.0
	gateway	36.1	0.292	3.3	1.2	0.0177	2.6
	Gully bottom	30.6	-0.507	10.7	2.0	0.00701	3.9
	North slope	25.9	0.173	4.1	1.9	0.0047	2.9
10-3-93	South slope	30.9	-1.289	5.6	2.1	0.0065	0.76
	Flat land	28.8	3.22	6.8	3.2	0.0046	1.8
	gateway	31.0	-1.027	8.8	1.8	0.0056	0.37
	Gully bottom	48.9	1.39	6.7	2.0	0.017	3.6
	North slope	43.8	0.17	1.9	1.8	0.0081	2.6
14-3-93	South slope	44.4	0.22	2.1	2.1	0.0089	0.7
	Flat land	44.7	1.07	2.3	3.2	0.0067	2.8
	gateway	45.6	0.014	2.3	1.8	0.0085	2.5

[a] A rainfall (26 mm) started on 19 February 1993, stopped on 21 February. Another rainfall started on 12 March 1993.

Table 5.6 Effect of intensive grazing on denitrification and other soil parameters (sampled on 5 August 1993)

Parameter	Denitrification		Nitrate		Moisture	
	Rate $(mg\ N_2O\text{-}N\ kg^{-1}d^{-1})$	Coefficient of skewness	Concentration $(mg\ NO_3^-\text{-}N\ kg^{-1})$	Coefficient of skewness	Content $(\%\ ww^{-1})$	Coefficient of skewness
Control site	0.021	1.58	0.79	2.56	47.03	0.44
grazed site[a]	0.042	2.67	3.12	3.88	48.54	0.56

[a], 10 days after grazing event (stocking rate at 300 cows ha^{-1}).

After rainfall (26 mm), soil moisture content and denitrification rates increased and the skewness of denitrification rates also increased, but then tended to decrease again when the soil became very wet (Table 5.5). The coefficients of skewness for soil moisture content were large in relatively dry soil, and decreased rapidly when the soil became wet (Table 5.5). Although NO_3^--N concentration decreased rapidly, the skewed distribution remained after rainfall (Table 5.5).

It seems that rainfall increased the soil moisture content, so that patchily-distributed soil NO_3^--N became available to denitrifiers. Denitrification rates therefore increased as did the skewness of the distribution. A few days later after soil was saturated, soil moisture became less skewed, and hence a more uniform distribution of anaerobic sites was achieved in the soil, resulting in less spatial variability in denitrification rates (Table 5.5).

Effect of moisture content. As discussed above, high soil moisture contents are favourable to denitrification, and the variability of denitrification rate appeared to decrease because of a more even distribution of anaerobic sites in the soil. Christensen *et al*. (1990a) suggested that the distribution of denitrification rates appeared to be less strongly skewed on soil above field capacity compared with soil at field capacity. In our study, soil was over the field capacity for a period in winter time, but the variance and skewness of denitrification rate at this time did not tend to be less than the rest of the sampling dates in the study pasture (data not shown). Similar results were reported in a riparian soil by Ambus and Christensen (1993). It may be that the variability of NO_3^--N or available-C are more important factors controlling the spatial variability of denitrification, and soil anaerobiosis may not determine the skewed distribution of denitrification rate in this pasture when the soil is wet in winter.

5.3.4 Spatial dependence of denitrification

The semivariance $\gamma(h)$ at the flat land site was estimated by the equation (Webster and Oliver, 1990):

$$\gamma(h) = [1/2m(h)] \times \sum[Z(x_i) - Z(x_{i+h})]^2 \qquad (5.1)$$

where $m(h)$ is the number of pairs of points separated by lag h (h is a vector); $Z(x_i)$ and $Z(X_{i+h})$ represent the values of denitrification rate at two positions separated by lag h, and x_i denotes a pair of cartesian coordinates with $i = 1, 2, 3, \ldots\ldots, n$. Since denitrification rates were log-normally distributed, the rates were log-transformed prior to calculation of $\gamma(h)$. Values of $\gamma(h)$ were grouped into lag classes of the smallest separation distance for each grid. No evidence of anisotropy was detected from the variograms of NS and EW $\gamma(h)$ values: that is, the same degree of spatial variation occurred independent of direction at this flat land site. So values of $\gamma(h)$ were calculated for all directions (360°) by assumption of isotropy.

Inspection of the variograms for our regular denitrification measurement with grid sampling indicated pure nugget variation in denitrification rate in all sites and sampling dates (data not shown). Pure nugget variation implies no spatial dependence at the scale of sampling used, and that the minimum sampling distance (3 m) is larger than the range of spatial dependence. More detailed experiments using a nested sampling design (Figures 5.1 and 5.2) were conducted on 20 April 1993, 20 July 1993 and 5 August 1993. The latter two sampling occasions were before and after intensive grazing. Figures 5.4 and 5.5 show the variograms for denitrification rate in these studies. A spatial dependence of denitrification
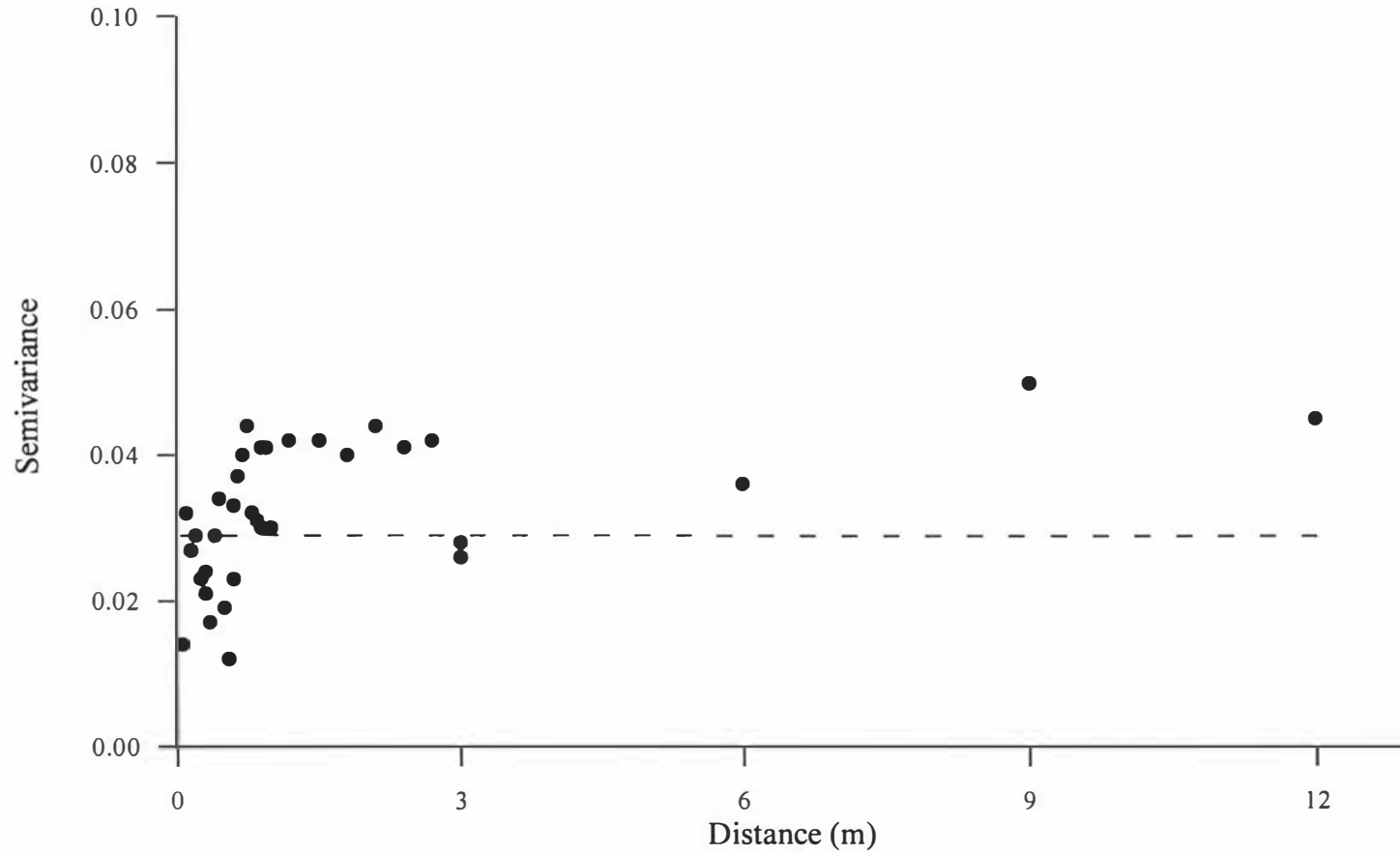
Figure 5.4 Analysis of spatial dependence in the variability of denitrification rate
(μg N_2O-N kg^{-1} soil day^{-1}, log-transformed data) (20 April 1993),
Dashed line indicates sample variance
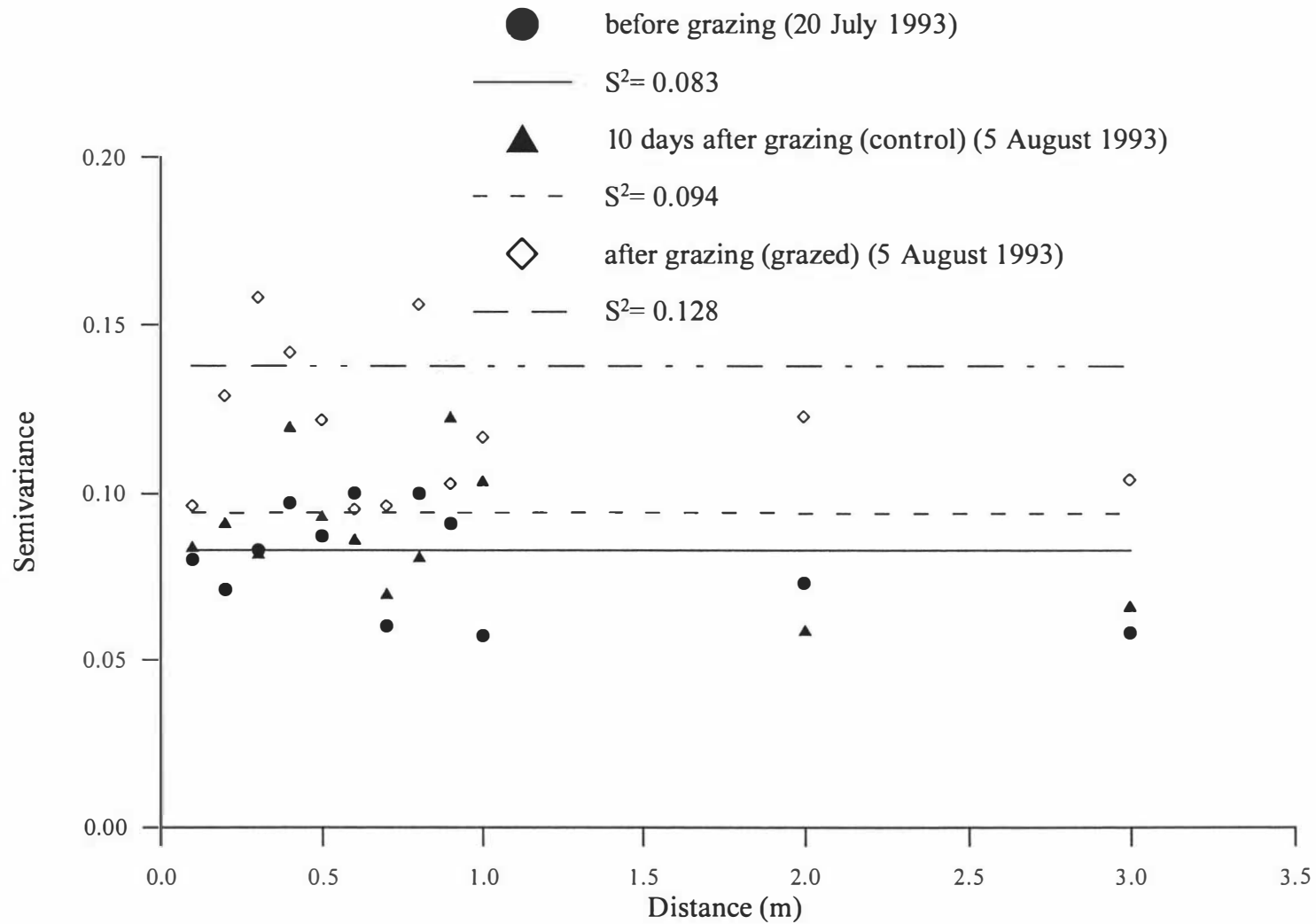
Figure 5.5   Analysis of spatial dependence in the variability of denitrification rate
(μg N$_2$O-N kg^{-1}soil day^{-1}, log-transformed data) before grazing and 10 days after grazing (Winter, 1993)

rate at distances less than 0.3 m was observed in the study area on 20 April 1993 (Figure 5.4). Beyond 0.3 m the variance had no relationship with the separating distance (Figure 5.4). However, in later samplings, before and after grazing, variograms (Figure 5.5) indicate no spatial dependence of denitrification beyond the minimum sampling distance of 0.1 m in both the grazed and control areas.

The literature contains only a few reports on the spatial analysis of denitrification rate (Folorunso and Rolston, 1984; Parkin *et al.*, 1987). Both of these studies also found denitrification variability to be predominantly short-range or spatially independent in field soil. The general lack of spatial structure in our study suggests that denitrification occurs at discrete positions, which is consistent with the hypothesis that the source of variability associated with the denitrification rates is the patchy distribution of denitrifying "hot-spots" in soil (Parkin, 1987).

5.4 CONCLUSIONS

Denitrification rates exhibited marked spatial variability in all sites in this pasture, with CV frequently being larger than 100%. The distribution of rates was generally skewed, irrespective of whether individual or bulked samples were being studied. A log-normal distribution was the mostly appropriate for describing the spatial variation among denitrification rates measured in the various topographical areas. However, the denitrification rates aggregated across the whole paddock were not generally log-normally distributed. These results may reflect the large scale variation in the rate of denitrification among the different topographical sites across the paddock, and implicate the spatial dependence of the rate of denitrification in the topographical scale.

Rainfall and animal grazing events affected the spatial variation of denitrification rates in this pasture. Rainfall in the warm, dry season increased the skewness of the denitrification rate initially, but then decreased as the soil became more uniformly wet. An intensive grazing event in winter increased the skewness of the frequency distribution of denitrification rates.

With knowledge of the frequency distribution of denitrification rates, estimates and comparisons of N losses through denitrification from different environments can be improved. Sichel's or Uniform Minimum Variance Unbiased Estimators (White *et al.*, 1987; Parkin *et al.*, 1988) have proved to be the most useful measure of the population mean and variance, when data are highly skewed and log-normally distributed.

Variograms showed short-range or no spatial dependence of denitrification rate beyond the minimum sample separation of 0.3 m in the flat land site throughout the sampling periods. Large nugget variances or random variations are likely to be a feature of soil denitrification rates in the field. For accurate estimations of field denitrification rate, the small-scale spatial variability requires that the soil sample volume should be as large as possible. This was also suggested for the measurement of mineral N in grassland soil by White *et al.* (1987). The lack of spatial dependence also indicates that it is not possible to predict denitrification rate for unsampled location using measured values at sampling locations.

CHAPTER 6

TEMPORAL VARIABILITY OF NITROGEN LOSS

THROUGH DENITRIFICATION

6.1 INTRODUCTION

With agricultural and environmental concerns, it is of interest to know annual nitrogen (N) loss through denitrification on an areal basis. There have been some measurements of field denitrification rates in different environments in some overseas countries, and reported losses of N through denitrification in agricultural ecosystems vary greatly, from 0.7 to >100 kg N ha^{-1} yr^{-1} (Ryden and Lund, 1980; Myrold, 1988; Bijay-Singh *et al.*, 1989; Weier *et al.*, 1991). However, there have been only a limited number of measurements of denitrification rate in New Zealand pastures (Ruz-Jerez *et al.*, 1994). More information about denitrification is needed to better characterise this process and to assess the contribution of denitrification in New Zealand grasslands to regional and global N cycling.

The spatial variability of denitrification rate in a study pasture has been reported in Chapter 5. In this chapter, the temporal pattern of denitrification rate and estimation of the annual losses of N by denitrification in the dairy-farm pasture are presented. This chapter also presents the relationships between denitrification rate and other edaphic factors using the extensive data set from this field study.

6.2 MATERIALS AND METHODS

6.2.1 Field sampling and variable analyses

Details of the study paddock, measurements of denitrification rate and characterisation of other edaphic variables have been presented in Chapter 5. Monthly data for soil temperature (10 cm depth at 0900h), rainfall and evaporation were obtained from the nearby meteorological station of AgResearch Grasslands. The data for soil temperature, rainfall and evaporation over the study period are shown in Figure 6.1.

6.2.2 Statistical analyses

Because the large spatial variation among replicates of denitrification rates and nitrate (NO_3^--N) concentrations in the paddock has been observed and the data are generally log-normal distributed (details in Chapter 5), the mean soil denitrification rate and NO_3^--N concentration were calculated using the Uniform Minimum Variance Unbiased Estimators (White *et al.*, 1987; Parkin and Robinson, 1992). Consequently, the comparisons of denitrification rates and NO_3^--N concentrations of the different sampling sites were carried out by testing the overlaps of upper and lower 95% confidence limits using the untransformed data (Parkin, 1993). Pearson correlation coefficients were calculated among the measured variables. Multiple regression models were run using the stepwise procedure. Previous tests indicated linear regression contributed to the best statistical fits in all cases, and other types of regressions did not improve the statistical fits. Significant levels for variable entry and variable stay were both set to 0.05. The log-transformed data for denitrification rate and NO_3^--N concentration were used as input variables in the
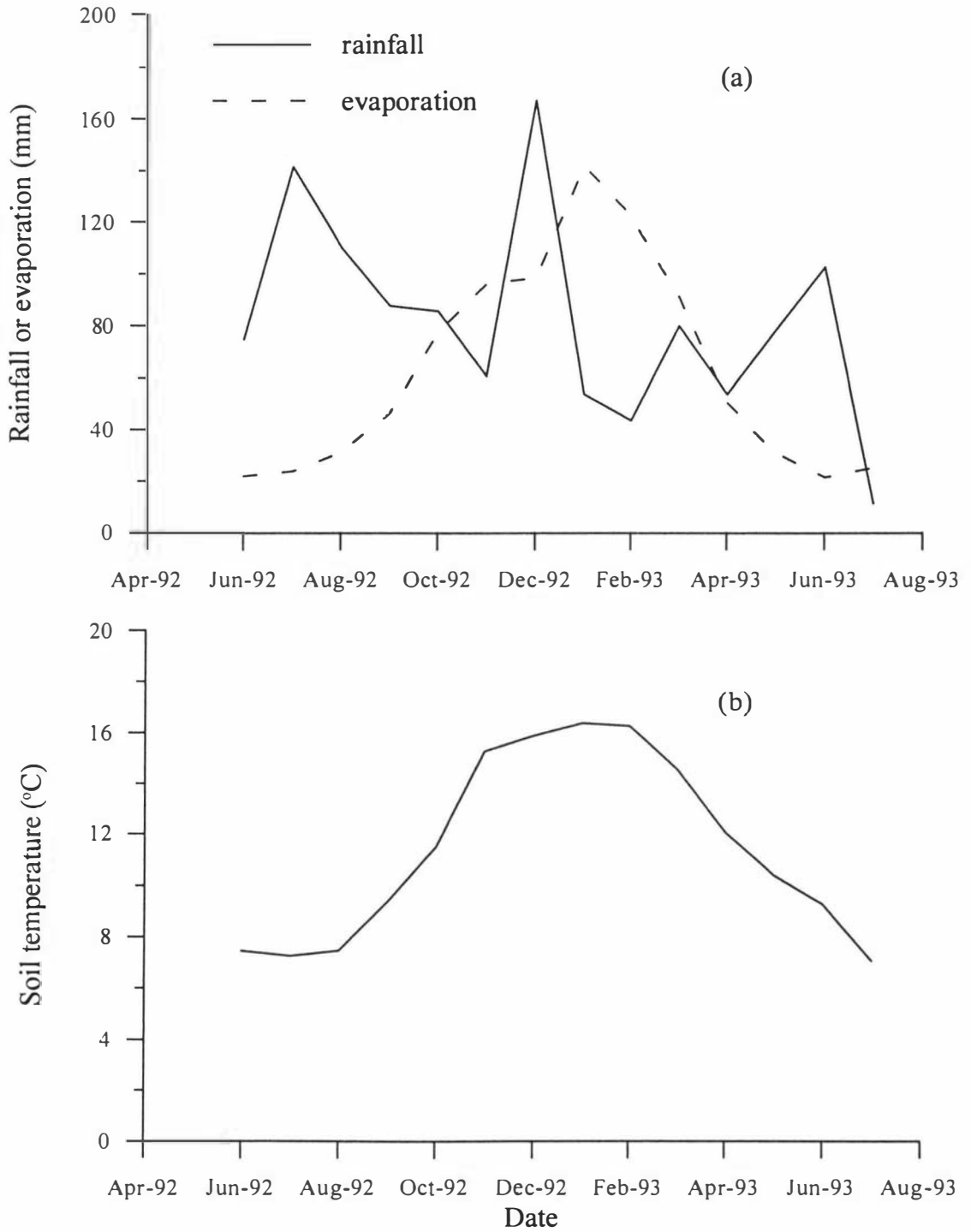
Figure 6.1 Monthly rainfall and evaporation (a), and mean soil temperature
(10 cm depth) (b) during the field denitrification study

correlations and multiple regressions. In the analysis of mean-data values, denitrification rate and NO_3^--N concentrations still needed log-transformation. All statistical analyses were done using SAS (SAS Institute, 1985).

6.3 RESULTS AND DISCUSSION

6.3.1 Temporal pattern of denitrification

The rates of denitrification from 7 July 1992 to 5 July 1993 at the gully bottom, North (N)-facing, South (S)-facing, flat and gateway sites in the study paddock are shown in Figure 6.2. Denitrification rates were highest in winter (May to August), followed by a decrease during spring (September to November). Denitrification rates were lowest in summer (December to February), and then increased during autumn (March to April). However, higher denitrification rates were observed for a short period after rainfall events in summer and autumn. When mean daily rates from the sampling dates were compared, the coefficients of variation (CV) in most study sites were about 50% (Table 6.1), although the temporal variation was noticeably higher in the flat site.

The season pattern in denitrification rate under field conditions observed in this study (Figure 6.2) was similar to that found by Ruz -Jerez (1991) on a freely-drained, fine sandy loam in the same locality. Both studies reveal that the highest N losses by denitrification occurred in winter and lowest during summer. Marked seasonal variation is a characteristic of denitrification in many soils and environments (e.g. Parsons *et al.*, 1991; Weier *et al.*, 1991). Changes in soil aeration, supply of NO_3^--N and availability of C under field conditions may all be implicated in seasonal variations of denitrification activity. The

dominant controlling factors affecting denitrification appear to vary temporally.

Table 6.1 Coefficients of variation (%) of measured parameters within sampling sites throughout the year

Site	Gully bottom	N-facing slope	S-facing slope	Flat site	Gateway
Denitrification ($mg\,N_2O$-$N\,kg^{-1}\,d^{-1}$)	51.80	48.73	53.35	71.89	49.27
Nitrate ($mg\,NO_3^-$-$N\,kg^{-1}$)	71.90	93.04	92.60	88.48	90.92
Respiration ($mg\,CO_2$-$C\,kg^{-1}\,d^{-1}$)	17.28	21.64	25.64	16.34	23.14
Moisture ($\%\,ww^{-1}$)	19.97	22.19	21.53	22.50	19.18

Peaks of denitrification occurred in late February and mid-March, after rainfall events in the present study (Figure 6.2). Formation of anaerobic sites by receipt of water from rainfall was probably a fundamental requisite for denitrification in those periods. The same response of soil denitrification to rainfall events has also been found by others (e.g. Ryden, 1983; Sexstone *et al.*, 1985; Goulding *et al.*, 1993). Increased denitrification rates following rainfall events can also be attributed to increased availability of C and NO_3^--N in soil. Drying and rewetting cycles in the soil may increase the availability of easily metabolizable organic matter and mineral N by stress of microbial biomass, therefore stimulating denitrification (Groffman and Tiedje, 1988). A high denitrification rate following rainfall is not always the case (Johnsson *et al.*, 1991). Decreased denitrification rates after repeated rainfall events have also been observed (Sexstone *et al.*, 1985). The absence of a denitrification response in these studies was probably due to depletion of substrates at the anaerobic sites by water, or uptake of substrate by growing plants after an earlier rainfall.
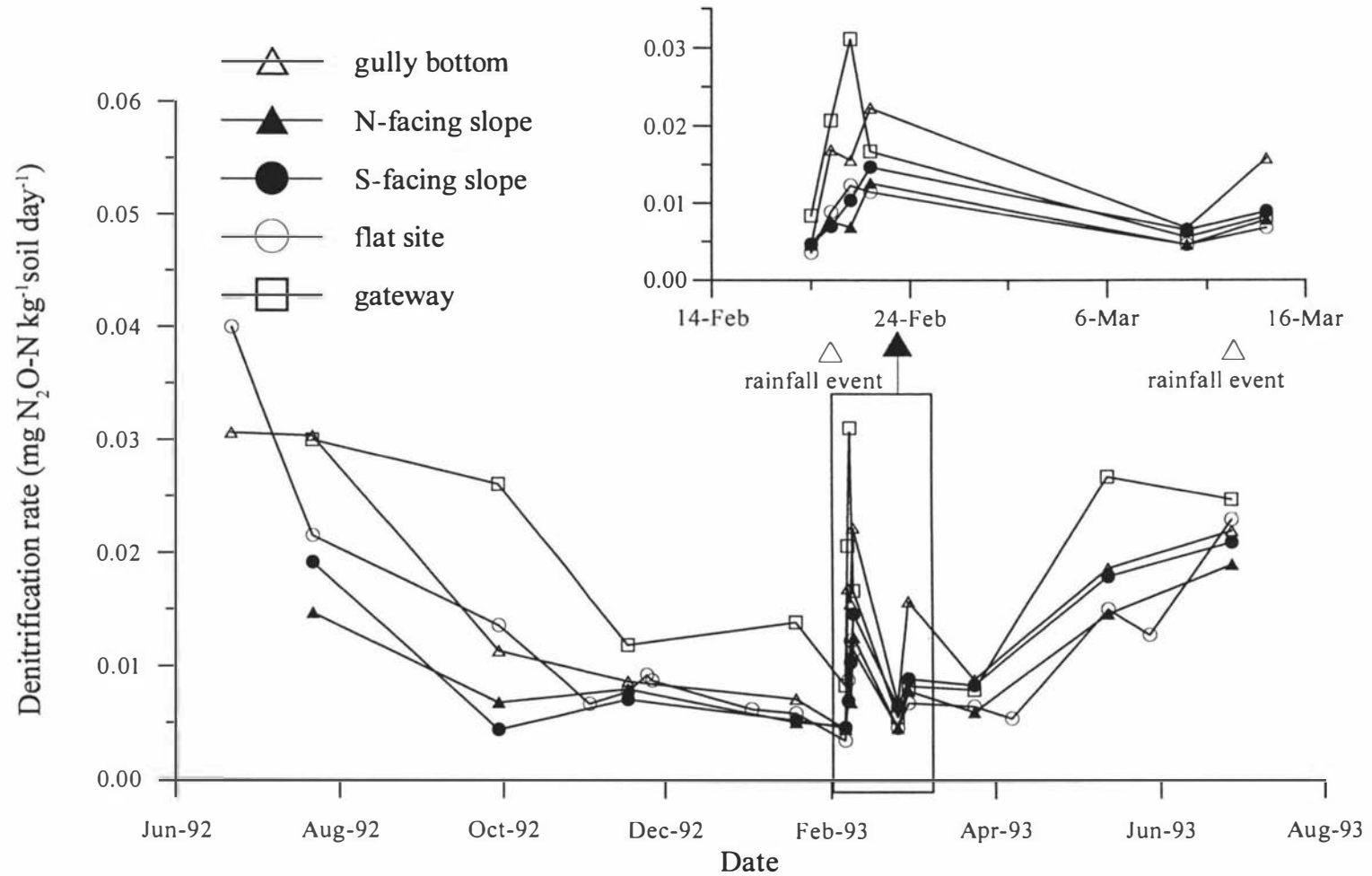
Figure 6.2 Temporal variation in the rate of denitrification

6.3.2 Temporal patterns of other edaphic parameters

Among the edaphic conditions, soil moisture content had a pattern similar to denitrification rate. The changes in soil moisture content during the denitrification study are shown in Figure 6.3. The coefficients of variation (CV) for soil moisture content among the sampling dates are summarised in Table 6.1. Changes in soil NO_3^--N concentration and respiration rate had opposite temporal patterns to denitrification rate (Figures 6.2 and 6.3). Significantly higher NO_3^--N concentrations were observed in summer and autumn (January to May) than in other seasons. The coefficients of variation (CV) for NO_3^--N concentration among the sampling dates were higher than those for denitrification rate in all the sampling sites (Table 6.1), indicating greater temporal variability for NO_3^--N concentration than for denitrification rate. High temporal variation in soil NO_3^--N concentration has also been found in cropping systems (e.g. Myrold, 1988). Soil respiration rate was high in summer and autumn, and low in winter. The coefficients of variation (CV) for the CO_2 emission rate among sampling dates were relatively low compared with that for NO_3^--N concentration or denitrification rate (Table 6.1).

6.3.3 Site differences in denitrification

There were differences in denitrification rate between sampling sites, but these were not always consistent (Figure 6.2). Soil near the gateway exhibited greater rates of denitrification than the other sites on most sampling dates through the year. When the denitrification rate was generally low during summer (December to February), no significant differences in the rates among the flat, slopes and gully bottom were found. However, from July to October the denitrification rates in both sloping sites were low
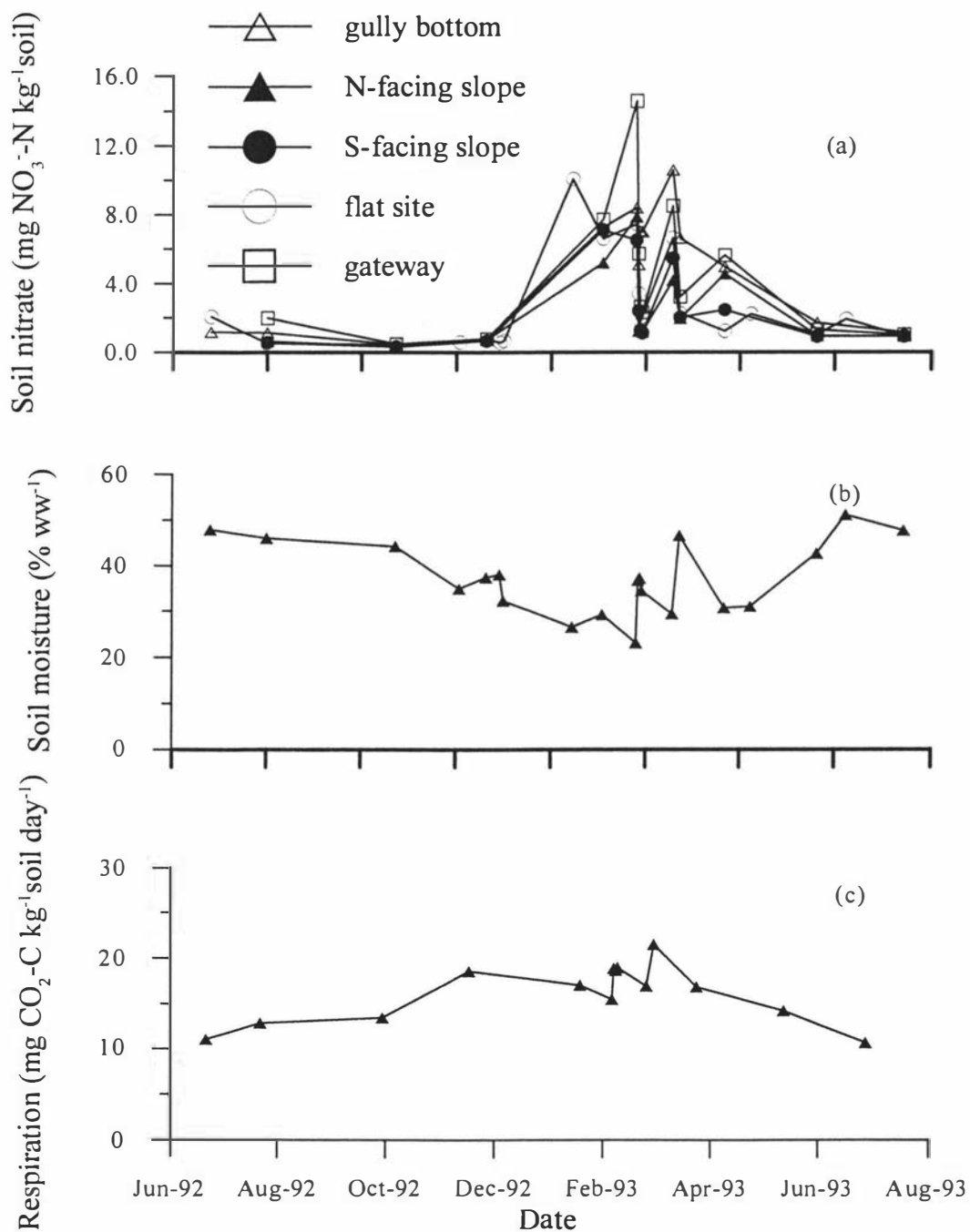
Figure 6.3 Seasonal variation in the soil nitrate concentration (a),
the soil moisture content (b), and the soil respiration rate (c),
(The soil moisture content and respiration rate are the averages over all sites)

compared to the rates at other sites. This observation emphasises the importance of animal effects on denitrification activity at various points in a pasture. More excreta from animals can be deposited in the paths of animal movement (Barrow, 1967), and on hill pasture the animals can transport significant quantities of nutrients to flat areas, because they tend to camp there (Saggar *et al.*, 1988). Loss of N by denitrification may thus be enhanced around gateways or in camp-site areas, due to more deposition of urine and dung.

A higher denitrification rate was observed at the gully bottom than in flat and slope sites after a rainfall event in February (Figure 6.2). A higher peak of denitrification rate was also observed at the gully bottom compared with the other sites after a rainfall event in March, but it was not as high as that in February (Figure 6.2). These different responses of denitrification to soil wetting in various areas of landscape could be a result of substrate redistribution. Substrates were most likely to accumulate at the gully bottom after rainfall events (Figure 6.3); presumably they were transported from slopes to the gully bottom in water.

6.3.4 Correlations and regressions between denitrification and other edaphic parameters

Factors related to denitrification in individual soil cores

Analyses of data obtained at individual times and at individual sites. Examination of the correlation-coefficient matrix and regression models revealed that at some individual sampling times and at some topographical sites, denitrification rates were closely related to one or more of the measured edaphic variables. However, these relationships were not consistent over time or between topographical sites (data not presented). For example,

denitrification rates and soil moisture contents were highly correlated (r=0.52, p<0.01) at the S-facing slope site, but not at all other study sites, on 6 August 1992. Denitrification rates were highly correlated to NO_3^--N concentrations at all sites (r between 0.37 and 0.47, p<0.01) on 14 October 1992, and in the grazed area (r=0.78, p<0.01) on 5 August 1993; but similar consistent correlations for all sites were not observed at other sampling dates. Occasionally, good correlations between denitrification rate and respiration rate were found in summer at some individual sampling sites. Stepwise regression models on individual dates at each site also indicated that no general relationship between denitrification and the measured variables could be established, and no single measured variable appeared to be a major contributor to the variation of denitrification rate at individual sites through all the sampling times. The inconsistencies of correlation and regression between denitrification rate and other measured edaphic properties on individual sampling dates or at the 5 sampling sites may indicate that different controlling factors on denitrification were at work at various times and at various locations in this study pasture.

Analyses of all combined data. When the data for all sampling times were combined for each site, denitrification rates were always weakly correlated to soil moisture content (Table 6.2). However, the correlations between denitrification rate and soil NO_3^--N concentration or respiration rate were not consistent across the five sites (Table 6.2). Stepwise multiple regression confirmed that denitrification rates in single soil cores can be partly predicted by soil water content in all five sites (Table 6.3). NO_3^--N concentrations and soil respiration rates were included in the regression equations for some of the sites (Table 6.3). The multiple regression models can only account for between 3% and 23% of the variation in denitrification rate at the five sites (Table 6.3).

Table 6.2 Pearson correlations between denitrification rate (mg N_2O-N kg^{-1} d^{-1}) and measured variables in individual cores[a]

Site	Gully bottom	N-facing slope	S-facing slope	Flat site	Gateway	Whole paddock
Number	448	208	208	564	208	1636
Nitrate (mg NO_3^--N kg^{-1})	0.040	-0.023	0.11	0.22**	-0.09	0.07**
Respiration (mg CO_2-C kg^{-1} d^{-1})	0.06	-0.077	0.34**	-0.052	0.20**	0.024
Moisture (% ww^{-1})	0.17**	0.34**	0.32**	0.25**	0.38**	0.28**

[a], Log-transformed data for denitrification rate and NO_3^--N concentration were used in correlation,
*, ** significant at p<0.05, 0.01, respectively.

When all data were combined from all the individual sampling sites in the paddock, denitrification rates were weakly, but significantly, correlated to soil moisture content (r=0.28, p<0.01) (Figure 6.4). Denitrification rates were also very weakly correlated with soil NO_3^--N concentration (r=0.07, p<0.01), but were not correlated at all with respiration rate (Table 6.2). A stepwise regression model, including moisture content and NO_3^--N concentration as independent variables, accounted for only about 8.4% of the variation in the denitrification rate (Table 6.3). The multiple regression analysis also suggested that soil respiration rate had little influence on denitrification rate (Table 6.3).

The correlations between denitrification rate and NO_3^--N concentration, or respiration rate, were improved, when the pooled data were partitioned according to soil moisture (Figures 6.5 and 6.6). The highest correlation coefficient between denitrification rate and NO_3^--N concentration was 0.38, when soil moisture content was over 45% (ww^{-1}) (about field capacity) (Figure 6.5). However, the highest correlation coefficient between denitrification rate and soil respiration rate was 0.44, when soil moisture content was less than 30% (ww^{-1})
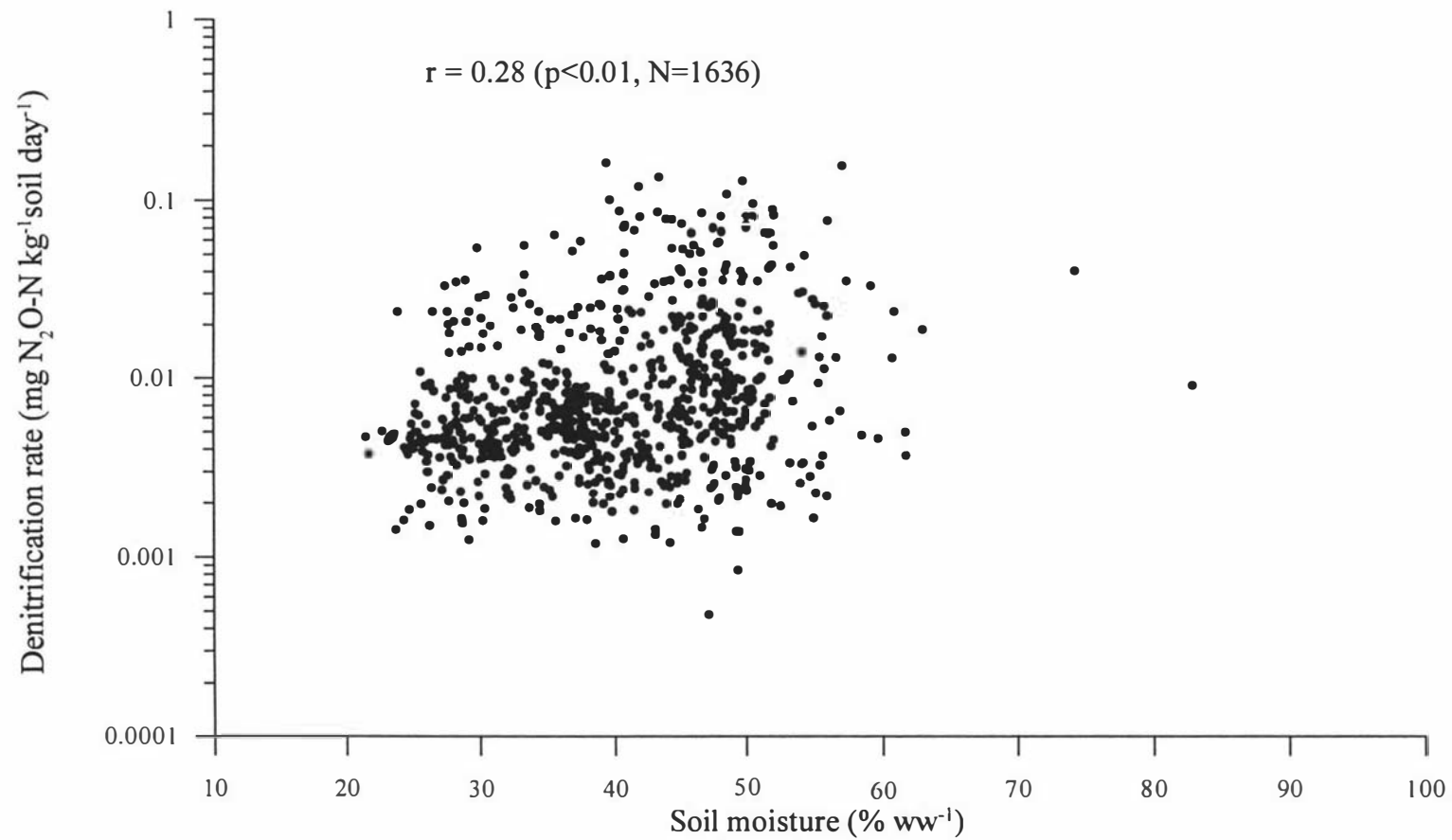
Figure 6.4 Relationship between denitrification rates and soil moisture contents
(based on pooled data)

Table 6.3 Stepwise regressions between denitrification rate (mg N_2O-N kg^{-1} d^{-1}) and measured variables in individual soil cores[a]

Site	Constant	Moisture (% ww^{-1})		Nitrate (mg NO_3^--N kg^{-1})		Respiration (mg CO_2-C $kg^{-1}d^{-1}$)		Model R^2
		Coefficient	Partial R^2	Coefficient	Partial R^2	Coefficient	Partial R^2	
Gully bottom	-2.55**	0.011**	0.029	0.15*	0.002			0.031
N-facing slope	-2.91**	0.023**	0.12					0.12
S-facing slope	-3.46**	0.022**	0.10	0.20**	0.012	0.012*	0.12	0.23
Flat	-2.77**	0.021**	0.061	0.43**	0.048			0.11
Gateway	-2.85**	0.017*	0.15			0.013*	0.040	0.19
Whole paddock	-2.82**	0.020**	0.079	0.28**	0.005			0.084

[a], Log-transformed data for denitrification rate and NO_3^--N concentration were used in stepwise regressions,
*, **, significant at $p < 0.05$, 0.01, respectively.
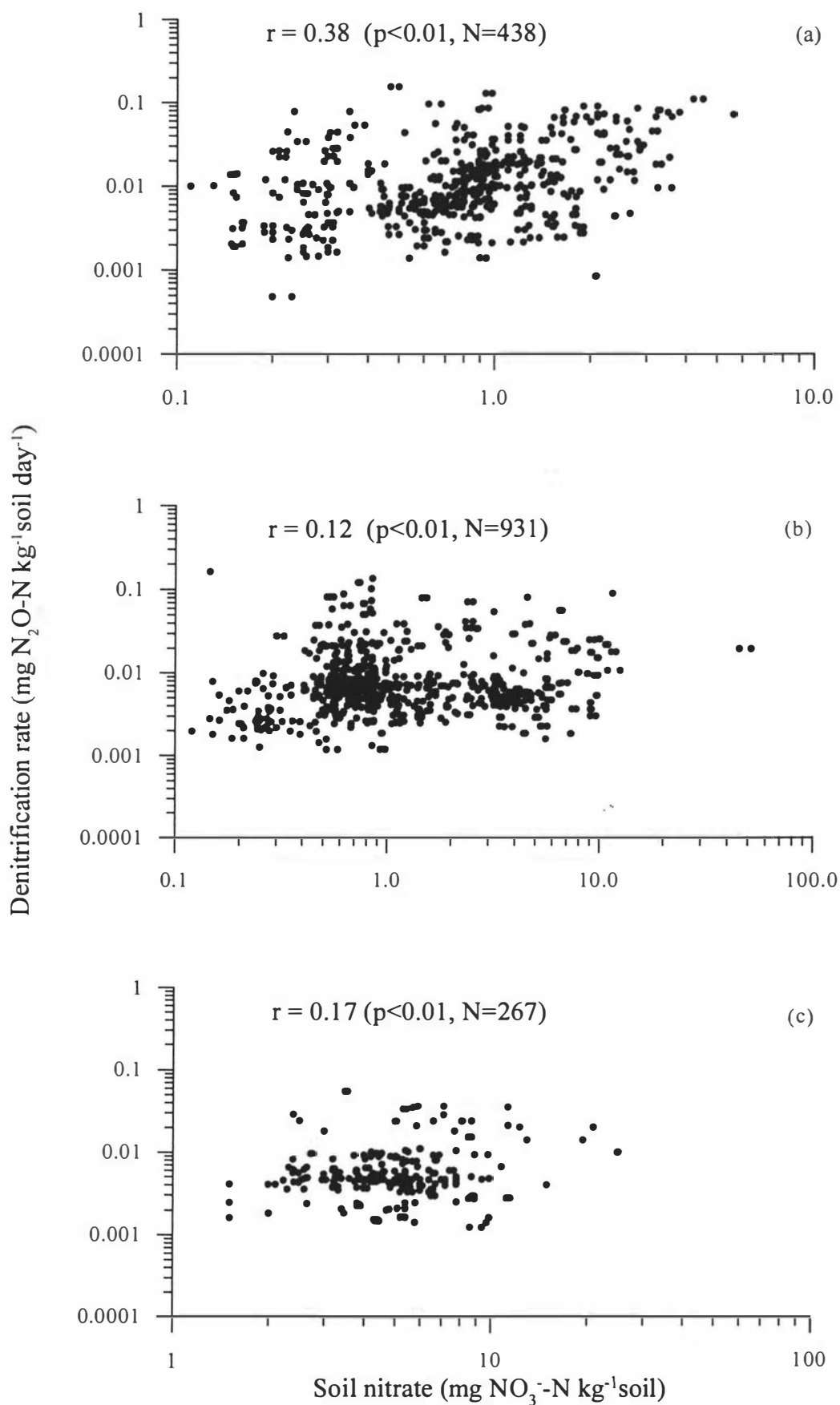
Figure 6.5 Relationship between denitrification rates and soil nitrate concentrations
(moisture ww^{-1} (a), >45%; (b), <45% and >30%; (c), <30%)
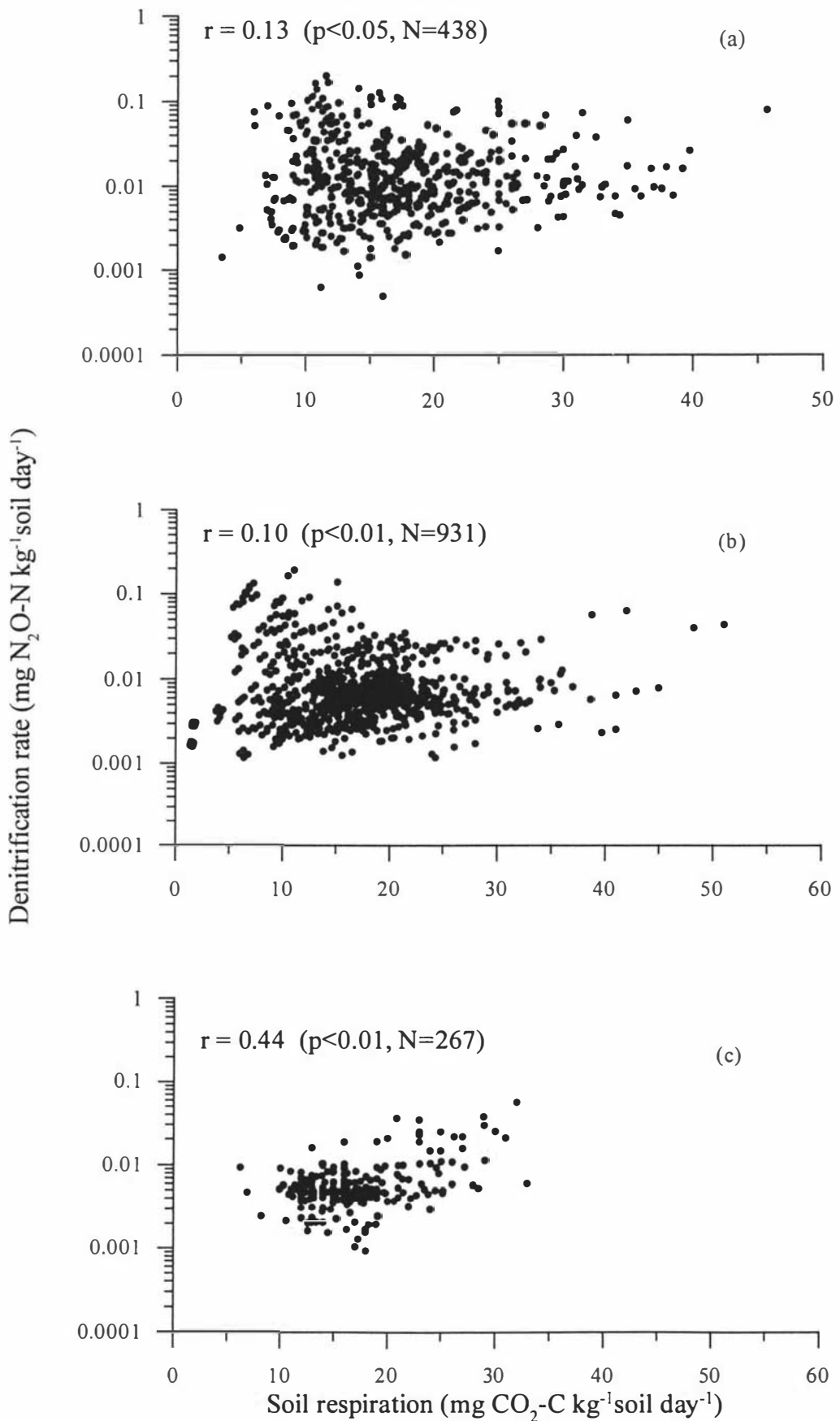
Figure 6.6 Relationship between denitrification rates and soil respiration rates
(moisture ww^{-1}(a), >45%; (b), <45% and >30%; (c), <30%)

(Figure 6.6). These results provide evidence for spatial and temporal interactions between soil NO_3^--N concentration and moisture content, soil respiration activity and moisture content, and their influence on soil denitrification.

When the entire data sets through the whole year were considered, the ability to predict denitrification rate from edaphic properties was low in either individual sites or the whole paddock (Table 6.3). The amount of variability accounted for by the regression models in our study was comparable with other studies conducted on different soils (e.g. Parsons *et al.*, 1991; Ambus and Christensen, 1993). It appears to be difficult to establish a regression model using soil properties measured on a single soil core to explain the small-scale variability of denitrification rate in fields. Improvements in predicability of variability in denitrification rate may rely on fully understanding the interaction between the primary factors (O_2, NO_3^-, C) that regulate denitrification, in the "hot spots" where denitrification can occur at a high rate. That the strength of correlation between denitrification rate and soil NO_3^--N concentration or soil respiration rate were dependent on soil moisture content (Figures 6.5 and 6.6) may suggest that the availability of NO_3^--N or the function of C can be affected by soil moisture. The distribution of "hot-spots" of denitrification regulated by soil factors which are spatially variable seems to be random, and apparently associated with grazing or rainfall events in this study pasture (Chapter 5). Regression models based on measurements of the soil properties in the individual soil cores may oversimplify the relationship between denitrification rate and regulatory factors in the "hot-spots".

Associations among means of measured variables. Correlation coefficients were computed between the mean values of denitrification rate and the other edaphic properties for each sampling dates for both the individual sites and the whole paddock. In all cases, closer

relationships between denitrification rate and soil moisture content were obtained (Table 6.4). Soil NO_3^--N concentration, soil respiration rate, and soil temperature appeared to be negatively correlated with denitrification rate, however, the significance of correlation varied among sites (Table 6.4). Data in Table 6.5 show that there were always negative relationships between soil temperature and moisture content, and positive relationships between soil temperature and NO_3^--N concentration, and soil respiration rate at all five sites and the whole paddock.

Table 6.4 Pearson correlations between denitrification rate (mg N_2O-N kg^{-1} d^{-1}) and measured variables using means from individual dates[a]

Site	Gully bottom	N-facing slope	S-facing slope	Flat site	Gateway	Whole paddock
Number	14	13	13	15	13	68
Nitrate (mg NO_3^--N kg^{-1})	-0.41	-0.64*	-0.44	-0.48	-0.61*	-0.33**
Respiration (mg CO_2-C kg^{-1} d^{-1})	-0.33	-0.49	-0.33	-0.58*	-0.44	-0.41*
Moisture (% ww^{-1})	0.82**	0.74**	0.59*	0.80**	0.60*	0.67**
Temperature (°C)	-0.57*	-0.60*	0.37	-0.75**	-0.63*	-0.57**

[a], Log-transformed data for denitrification rate and NO_3^--N concentration were used in correlation,
*, ** significant at p<0.05, 0.01, respectively.

Multiple regression models to predict denitrification rate from the mean values of measured variables at each sampling visit were also developed for both individual sites and the whole paddock. Stepwise multiple regression showed that denitrification rate was best predicted by soil moisture content alone at each site (Table 6.6). The models for individual sites accounted for between 34% and 67% of the variation in denitrification rate (Table 6.6).

When data from the whole paddock were considered at each sampling date, the predicability was similar to that in the individual sites (Table 6.6). Soil moisture contents and NO_3^--N concentrations were the dominant variables explaining 51% of the variation of denitrification rate. Other measured variables were not satisfactory predictors for denitrification rate in this case (Table 6.6). The data in Figure 6.7 demonstrate the increase in denitrification rate with increasing soil moisture when mean values were used.

Table 6.5 Pearson correlations between soil temperature and other measured variables using means from individual dates[a]

Site	Gully bottom	N-facing slope	S-facing slope	Flat site	Gateway	Whole paddock
Number	14	13	13	15	13	68
Nitrate (mg NO_3^--N kg^{-1})	0.79[**]	0.63[*]	0.61[*]	0.32	0.63[*]	0.55[*]
Respiration (mg CO_2-C kg^{-1} d^{-1})	0.87[**]	0.84[**]	0.84[**]	0.87[**]	0.84[**]	0.85[**]
Moisture (% ww^{-1})	-0.73[**]	-0.69[**]	-0.69[**]	-0.70[**]	-0.70[**]	-0.71[**]

[a], Log-transformed data for NO_3^--N concentration were used in correlation,
[*], [**] significant at p<0.05, 0.01, respectively.

The regression equations derived using the mean values are better predictors of the observed mean denitrification rate than the regression equations derived using the entire set of measurements (Tables 6.3 and 6.6). This suggests that the measured characteristics of the bulk soil are better predictors of long-term temporal variability than of small-scale variability. Several other studies have also demonstrated an improvement in predicability of denitrification rate by using mean values of measured parameters (e.g. Groffman and Tiedje, 1989b; Parsons *et al.*, 1991; Ambus and Christensen, 1993). The amounts of temporal variability accounted for by the regression models in the present study were not

high, but comparable with the results obtained by other workers (Robertson and Tiedje, 1984; Parsons *et al.*, 1991; Bergstrom and Beauchamp, 1993; Schipper *et al.*, 1993).

Table 6.6 Stepwise regressions between denitrification rate (mg N_2O-N kg^{-1} d^{-1}) and measured variables using means from individual dates[a]

Site	Regression model[b]	Partial R^2		Model R^2
		Moisture (X_1) (% ww^{-1})	Nitrate (X_2) (mg N kg^{-1})	
Gully bottom	$Y = -2.87 + 0.026\ X_1$	0.67		0.67
N-facing slope	$Y = -2.80 + 0.019\ X_1$	0.55		0.55
S-facing slope	$Y = -2.70 + 0.018\ X_1$	0.34		0.34
Flat	$Y = -3.00 + 0.025\ X_1$	0.64		0.64
Gateway	$Y = -2.55 + 0.020\ X_1$	0.36		0.36
Whole paddock	$Y = -2.81 + 0.022\ X_1 + 0.25\ X_2$	0.45	0.06	0.51

[a], Log-transformed data for denitrification rate and NO_3^--N concentration were used in stepwise regressions,
[b], Y denitrification rate.

Table 6.7 Estimated annual nitrogen loss through denitrification from the study paddock

Site	Gully bottom	N-facing slope	S-facing slope	Flat site	Gateway	Whole paddock
Relative area (%)	10	5	5	77	3	100
Nitrogen loss (kg N ha^{-1} yr^{-1})	4.70	3.56	3.70	4.54	5.80	4.50
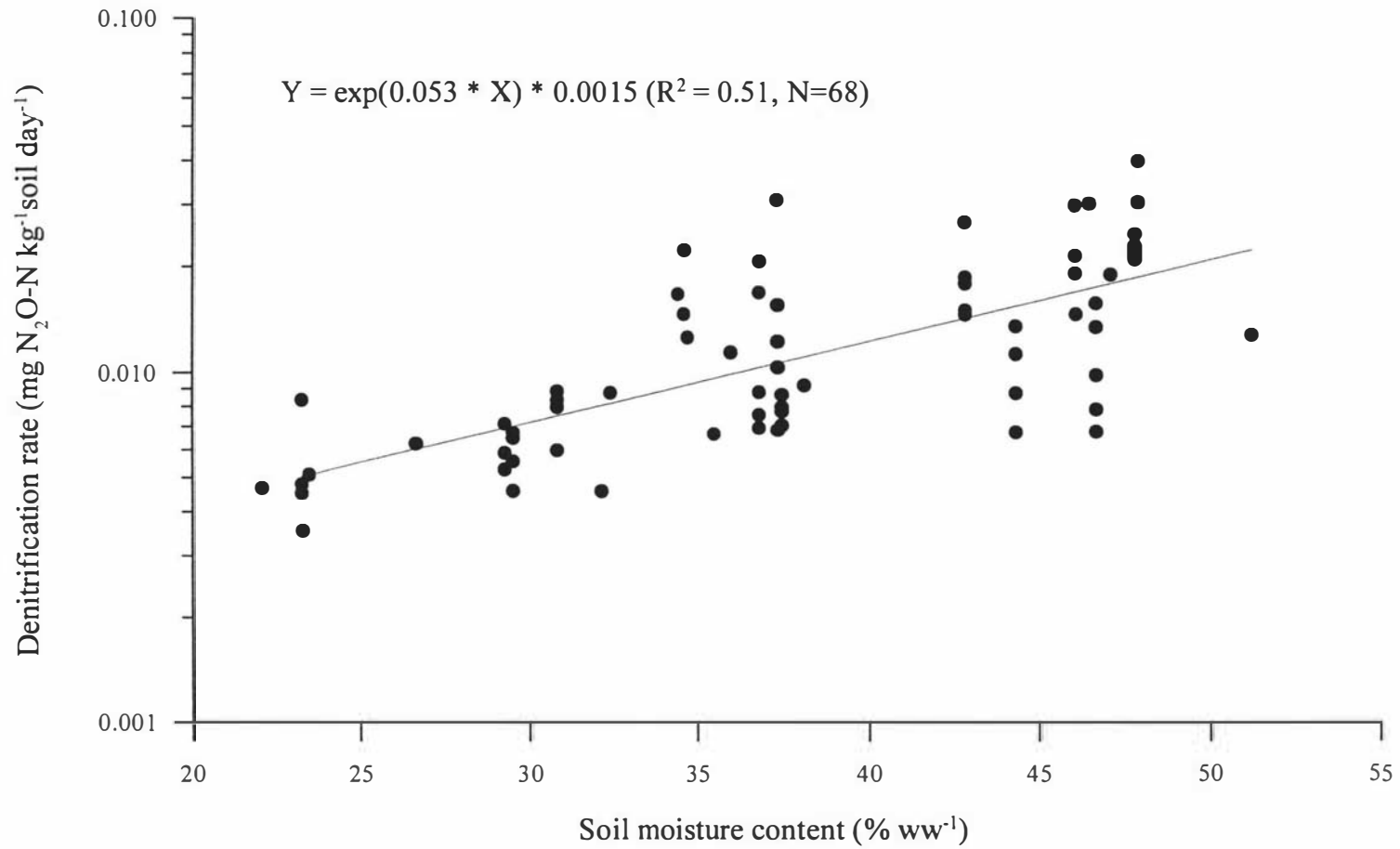
Figure 6.7 Relationship between denitrification rates and soil moisture contents (based on mean data)

$$Y = \exp(0.053 * X) * 0.0015 \ (R^2 = 0.51, \ N=68)$$

6.3.5 Annual nitrogen loss by denitrification

Denitrification rates measured on soil cores were expressed on an areal basis, using bulk density values (Table 5.1), and were interpolated over the period between sampling dates to estimate annual N loss by denitrification. Cumulative annual N losses monitored in this study were 4.70, 3.56, 3.70, 4.54 and 5.80 kg N ha^{-1} at the gully bottom, N-facing slope, S-facing slope, flat and gateway sites, respectively (Table 6.7). These data indicate greater annual N loss from gateway and flat sites than from the slope sites. The overall estimate was 4.5 kg N ha^{-1} yr^{-1} for the study pasture by using weighted averages for the losses at the different sites in this paddock. This annual N loss of 4.5 kg N ha^{-1} through denitrification from the pasture field in the current study is of the same order as the estimated N loss found by Ruz-Jerez (1991) in a pasture without N fertilizer on a freely-drained soil. The N loss through denitrification does not appear to be important in terms of N balances for the pasture.

6.4 GENERAL DISCUSSION

6.4.1 Denitrification associated with soil moisture

Soil aeration with denitrification. A relatively high denitrification rate was observed in winter in the current study (Figure 6.2), although soil temperature was low (Figure 6.1). The active denitrification in winter appears to have been associated mainly with high soil moisture contents. Due to frequent rainfall and low evaporation (Figure 6.1), the moisture content in soil can readily reach levels greater than "field capacity" in this poorly-drained soil in winter (Figure 6.3). Most soil pores would then be filled with water and oxygen

diffusion through water is considerably slower than through air. It has long been recognised that O_2 concentrations can affect both synthesis and activity of denitrification enzyme (Firestone, 1982; Knowles, 1982; Stouthamer, 1988). Therefore, soil denitrification rate may be increased by an increase in the number of anaerobic sites in the soil in winter. The obvious factor limiting denitrification activity in summer was low moisture contents in the soil (Figure 6.3). Previous field studies have demonstrated that the rate of denitrification often remains negligible during dry periods, but then increases dramatically when soil water content exceeds a certain critical level (e.g. Aulakh and Rennie, 1985).

Substrate mobility with denitrification. Soil water can also influence substrate mobility in soil, which can control the availability of substrate to denitrifying microorganisms (Groffman *et al.*, 1988). High soil water contents provide an optimum medium for diffusion, and therefore, NO_3^- originating from nitrification can more easily move to anaerobic denitrification sites within the soil. It has been suggested that nitrification and denitrification can occur simultaneously on opposite sides of an aerobic-anaerobic interface (Knowles, 1978). So NO_3^--N could be denitrified rapidly, and would not accumulate in a soil with high moisture content. High soil moisture contents may also increase the accessibility of C to denitrifying organisms, since the study by Myrold and Tiedje (1985a) showed that denitrification could be limited by the diffusion rate of organic compounds in some soils. The low diffusion rate of NO_3^- or organic-C to denitrification sites in the relatively dry soil may have been another important factor limiting denitrification in summer.

Relationships between denitrification rate and soil moisture content. From the discussion above, it is not surprising to see that in most cases, denitrification rate was more positively related to soil moisture content than to any other measured variables, and the correlations were relatively strong in the study pasture both using data from individual soil cores and the mean data from individual dates (Tables 6.2 and 6.4). Significant relationships between denitrification rate and soil moisture content have also been observed in other field studies (e.g. Aulakh *et al.*, 1983a and b; Mosier *et al.*, 1986; Myrold, 1988; Jarvis *et al.*, 1991; Parsons *et al.*, 1991; Weier *et al.*, 1993), and substantial fractions of the variation in denitrification rate have been found to be attributable to variation in soil moisture content (Klemedtsson *et al.*, 1991; Ambus and Christensen, 1993; Bergstrom and Beaucamp, 1993). In contrast only weak relationships between denitrification and soil moisture have been found in some studies (e.g. Hixson *et al.*, 1990).

Many studies of denitrification have used water-filled porespace (% WFP) rather than gravimetric moisture content (e.g. Groffman and Tiedje, 1989b; Weier *et al.*, 1993), since it may more accurately describe the availability of anaerobic sites in soil, and may better account for the variation of denitrification rate. However, bulk densities of individual core samples were not measured in our study, and the WFP assessed by the common bulk density did not improve the prediction of variation of denitrification rate, when compared with simple gravimetric water contents. Bulk densities of the individual samples may have varied considerably both spatially and temporally in the paddock. Better relationships between denitrification rate and soil moisture content could perhaps have been found if the WFP were considered using the bulk densities in individual samples.

6.4.2 Denitrification associated with soil nitrate

Good relationships between denitrification rate and soil NO_3^--N concentration were not often observed (Tables 6.2 and 6.4), and the NO_3^--N concentration was poor predictor of denitrification rate both temporally and spatially in the present study (Tables 6.3 and 6.6). It was difficult to detect the availability of NO_3^--N in "hot-spots" of denitrification by using the bulk concentration of NO_3^--N in samples. Further to this, denitrification rate can be insensitive to variations in soil NO_3^--N concentration, when other factors, such as soil moisture content and C supply, were limiting. Poor relationships between soil NO_3^--N concentration and denitrification rate have also been found in other studies (Aulakh *et al.*, 1983a; Kroeze *et al.*, 1989; Ambus and Christensen, 1993; Bergstrom and Beauchamp, 1993). However, significant relationships between denitrification and soil NO_3^--N, or significant response to NO_3^--N addition, have been observed in some field studies (Roberston and Tiedje, 1984; Davidson and Swank, 1986; Samson *et al.*, 1990; Ambus and Lowrance, 1991; Schipper *et al.,* 1993).

Denitrification rate could be limited by the diffusion of NO_3^--N to denitrification sites when soil was relatively dry in summer, and possibly limited by the low concentration of NO_3^--N when soil was relatively wet in winter in the present study. Weak correlations between denitrification rate and NO_3^--N concentration were found at low soil water contents (Figure 6.5). This may indicate the NO_3^--N concentration was not a major limiting factor for denitrification when soil moisture was low. NO_3^--N may become a more important limiting factor for denitrification when the potential rate of denitrification was higher at high soil moisture contents (Figure 6.5). The results perhaps suggest that at a given concentration of NO_3^--N, NO_3^--N availability for denitrification may be dependent on soil

moisture conditions. This is in accordance with results obtained by Jarvis *et al.* (1991), Ambus and Christensen (1993) and Kessel *et al.* (1993). Myrold and Tiedje (1985a) showed NO_3^--N diffusion into the anaerobic zone within aggregates is limited by low bulk soil NO_3^--N concentration. A previous field study has also shown that diffusion can limit NO_3^--N availability to denitrification even at a high concentration of soil NO_3^--N (Ryden, 1983).

6.4.3 Denitrification associated with soil respiration

Previous studies have shown increases in denitrification rate associated with increases in soil respiration or C addition (Robertson and Tiedje, 1984; Lowrance and Smittle, 1988; Myrold, 1988; Parsons *et al.*, 1991). However, CO_2 production was generally an unsatisfactory parameter to explain the variations of denitrification in the present study (Tables 6.3 and 6.6). Keeney *et al.* (1985) and Klemedtsson *et al.* (1991) similarly could not find relationships between denitrification rate and C supply in soils. As discussed above, the effects of C availability for the denitrification at the microsites may have been "hidden" in the whole-core variation of CO_2 emission rate. The correlation between soil respiration and denitrification rate in this study was also possibly confounded by other soil microbial processes from which CO_2 is produced (Reddy *et al.*, 1982). The relationships between denitrification rate and CO_2 production in the present study tended to be tied to soil moisture content (Figure 6.6). This difference in correlations between denitrification and CO_2 at low or high soil moisture content was possibly caused by the role of C on denitrification. The role of C in relatively dry soil may involve O_2 consumption by respiration, so producing anaerobic sites for stimulating denitrification (Parkin, 1987). The fact that there would be plenty of anaerobic zones and lower concentration of NO_3^--N in

wet soils would explain why soil C was not an important regulatory factor for denitrification in winter.

6.4.4 Denitrification associated with soil temperature

The rate of denitrification can undoubtedly be limited by low temperatures in winter. However, the mean soil temperature (Figure 6.1) in winter in the present study was always above the critical temperature for denitrification, as the lowest temperature at which field denitrification can occur is 5°C (Ryden, 1986). The effect of temperature on denitrification in the natural environment is complicated by other factors. In the present study, high denitrification rates at relatively low soil temperatures in winter were probably caused by an opposing, temporal relationship between temperature and water content in soils (Table 6.5). It also appears that denitrifying bacteria adapt to soil temperature in different regions. The study of Powlson *et al.* (1988) showed that denitrifying bacteria from temperate soils reduced NO_3^--N at a lower optimum temperature than did bacterial populations from sub-tropical soils.

The temperature effect on denitrification is also possibly affected by simultaneous changes in plant growth in the field. The relatively high denitrification rate observed during winter was probably also due to limited uptake of available NO_3^- from soil by pasture, since the growth of grass slowed as daylight and temperature decreased in winter. On the other hand, the low denitrification rates in summer were probably also closely tied to the activity of plant roots, since water and NO_3^--N uptake by rapidly-growing pasture may be substantial. This process would markedly decrease the availability of NO_3^--N to denitrifying microorganisms, particularly in the rhizospheric zones, where the availability of C may be

high.

6.4.5 Nitrogen loss through denitrification from agricultural systems

From the available data, it can be generally concluded that the losses of N from field denitrification are lower in grassland than arable land, although the potential for N loss by denitrification from improved pasture is thought to be higher due to higher levels of organic C and greater biological activity. The expected C-induced rhizosphere effect on denitrification in pasture may be counter-balanced to a large extent in soils under pasture by rapid uptake of NO_3^--N (Ryden, 1983). This was supported by the conclusions of a study by Smith and Tiedje (1979b). The improved soil structure and soil porosity associated with improved pastures can also be associated with low denitrification, because of the increased soil aeration. In contrast, soil structural deterioration often occurs in arable cropping systems. Most N in dung and urine returned by grazing animals to pastures is present as organic forms, and is thus protected from denitrification until it has undergone mineralization and nitrification. However, N in some fertilizers used in cropping systems is present as inorganic N, and denitrification N loss could occur soon after fertilizer application. Thus the amount of N loss through denitrification in cropping systems may be larger because of greater N inputs, less developed root systems and generally poorer soil structure.

As the study area is in a temperate region, high soil water conditions in winter necessary for the denitrification process are associated with low temperatures. During this period, soil NO_3^--N supply for high denitrification rate is also restricted. Therefore, denitrification rate is limited. Soil temperature increases in summer, but little N loss by denitrification can

occur because of low soil moisture content and rapid uptake of N by pasture. Most of the soil mineral N in this paddock was associated with excreta from the grazing animals. So the effects of animal grazing on denitrification could be significant. This direct effect of grazing on denitrification in the paddock, which was complicated by weather patterns, is investigated in the next chapter (Chapter 7). Overall, shortage of soil NO_3^--N and low soil water contents during most times of the year are considered to be the main causes for the small loss of N by denitrification from this pasture.

6.5 CONCLUSIONS

Denitrification rates in this dairy-farm pasture were highest in winter and lowest during summer. However, high denitrification rates did occur for brief periods after rainfall events in summer or early autumn.

Soil moisture appeared to be the most consistent factor related to denitrification in the study paddock. Soil NO_3^--N concentrations appeared to be more closely related to denitrification rates when soil moisture contents were high. At low soil moisture contents, diffusion of NO_3^--N to denitrification sites may be more limited by soil moisture than by the concentration of NO_3^--N. The relationship between respiration rate and denitrification rate suggests that in dry soils O_2 consumption by respiration is necessary to produce anaerobic sites which can then stimulate denitrification.

About 4.5 kg N ha^{-1} annual N loss by denitrification was estimated. Low soil moisture content was the primary factor limiting denitrification during summer. Relatively low annual N loss by denitrification was probably also caused by lack of available NO_3^--N for

denitrification. Denitrification cannot be regarded as a major pathway for loss of N from this pasture.

CHAPTER 7

EFFECT OF GRAZING EVENTS ON DENITRIFICATION DURING TWO CONTRASTING SEASONS

7.1 INTRODUCTION

In the previous chapter (Chapter 6), seasonal patterns of denitrification in a pasture were reported. The results suggest that soil moisture content, as affected by rainfall and evaporation rates, is a key factor influencing the pattern of denitrification rate in the study area. Another important factor affecting denitrification *in vivo* is thought to be the grazing animal.

Most of the herbage nitrogen (N) ingested by animals is returned to the soil in urine and dung (Whitehead, 1970; During, 1972). The quantities of N in these urine and dung patches (from 30 to 100 g N m^{-2}) exceed the plant requirements (Ball, 1979; Steele, 1982; Ball and Ryden, 1984). Therefore, the high N contents of urine and dung deposited during grazing offer the potential for considerable N loss. These losses from dung and urine patches have been confirmed by a number of workers (e.g. Ball *et al.*, 1979; Carran *et al.*, 1982; Sherlock and Goh, 1984; Sugimoto and Ball, 1989).

The substantial N outgoings may be caused through NH_3 volatilization, leaching and denitrification. Using a mass balance approach, Carran *et al.* (1982) observed that some

30-40% of the urine-N remained accounted for in their study, and suggested that denitrification could be the principal mechanism of N loss for that unaccounted N. By applying ^{15}N-labelled synthetic urine to soil cores in controlled environmental chambers, Clough *et al.* (1994) observed that denitrification was the major pathway for N gaseous loss from urine patches in their experiment. A study on dynamics of N in pastures has also indicated that higher rates of denitrification could occur soon after grazing events (Ruz-Jerez, 1991).

Although this evidence suggests that an increase in N loss through denitrification may occur due to animal grazing, there has been no direct measurement of the effect of animal grazing on the rate of denitrification in the field.

Due to the slow growth of grass in the winter and also the requirement to restrict feed intake, block-grazing systems are often used on dairy-farms in New Zealand. This is a relatively intensive grazing pattern with large number of cows being confined to a small break of the paddock for a day, and then being shifted to the next break. Less intensive grazing patterns usually occur in the dry summer season. The objective of the study reported in this chapter was to determine the temporal changes in denitrification rate in relation to grazing events in a pasture, under seasonally moist and dry conditions.

7.2 MATERIALS AND METHODS

The characteristics of study site and details of the soil have been given in Chapter 5. The study paddock had been under a ryegrass/white clover pasture for several years, and had been grazed regularly by cows. Pasture production can be limited by wet conditions and

low temperature in winter, and by drying in summer.

7.2.1 Experimental design

The assessments of grazing effect on denitrification rate were carried out in a moist, cool winter and a dry, warm summer, to provide contrasting seasonal conditions. In the first experiment during winter, two areas in the paddock were excluded from grazing to act as controls. The paddock was separated into eight breaks, and sequentially block-grazed with cows at a high stocking rate (about 300 cows ha^{-1}). Soil samples for the study were taken from all eight breaks and the two control areas. All breaks and control areas were sampled at each measurement. The second study was undertaken on an adjacent paddock during summer. The pasture was rotationally grazed with cows at a relatively low stocking rate (about 40 cows ha^{-1}). The soils for this second study were sampled from both grazed and control areas (areas from which cows were excluded). Measurements were continued until differences in denitrification rate were no longer apparent among grazed breaks and the control in the winter experiment, and for a period of four weeks in the summer study.

7.2.2 Measurement of denitrification and respiration rates

The rate of denitrification was measured using the acetylene inhibition technique (Yoshinari *et al.*, 1977), by incubating the minimally disturbed soil cores in a closed system under field conditions. Soil cores were collected randomly from each break or area. Fourteen cores (2 cm diameter; 7.5 cm deep) were placed in a glass jar (about 1100 ml in total volume), then sealed with a lid fitted with a septum stopper. Air (60 ml) was withdrawn and replaced with purified acetylene to produce a concentration of about 10% acetylene (vv^{-1})

in the remaining air spaces of each jar. Each jar was then incubated for 24 hours on the ground, in shade, near the experimental areas. There were 4 replicates from each break or area. The details for gas sampling and analyses of gas samples using the gas chromatograph for N_2O and CO_2 have been given in Chapters 3 and 5.

In order to see whether grazing had any effect on denitrification enzyme activity, bulk soil samples were collected only from one of the breaks (Break No. 5) and the control area in the winter, and from the grazed and control area in the summer experiment, at the same sampling times as for denitrification rate measurements. The denitrification enzyme activity of the soil was determined using a method developed by Smith and Tiedje (1979a). The description of this method is in Chapter 3. In this study, a 20 g fresh soil sample was placed into a 125 ml flask. Fifty μg NO_3^--N g^{-1} soil and 300 μg glucose-C g^{-1} soil were added to the soil. The soil was brought to saturated condition, and the air inside the flask was flushed out with pure N_2 gas. This was repeated several times and the flask was filled with oxygen-free N_2 gas. Headspace gas of 12.5 ml was replaced with purified acetylene. Flasks were placed in the dark and the incubations were conducted at 20°C. Gas samples from the headspace were collected into 5 ml Decton Dickinsen vacutainers using double-ended needles, 1 and 5 hours after incubation began. The concentrations of N_2O in the gas samples were determined using a gas chromatograph (Chapter 3). The incubations for measurement of denitrification enzyme activity were replicated 4 times.

7.2.3 Analyses of other soil properties

Soil moisture contents and mineral N (NH_4^+-N and NO_3^--N) concentrations were measured as described in Chapter 3. The pH of soil was measured in H_2O (1:2.5) after overnight

equilibration, using a combined electrode pH meter.

7.2.4 Climatic information

Daily data for soil temperature (10 cm depth at 0900h), rainfall and evaporation records were obtained from the nearby meteorological station of AgResearch Grasslands. Soil temperatures at a depth of 10 cm varied between 5.7 and 9.6°C for all the sampling dates in the first experiment in winter (Figure 7.1a). Precipitation was relatively low, with 62.4 mm rainfall recorded over the study period (8 July to 17 August). In the second experiment during January and February 1994, soil temperatures were relatively constant at 15-20°C (Figure 7.1b). Only 4.6 mm of rainfall was observed, on 24 January 1994, shortly after the experiment started.

7.2.5 Statistical analyses

Since marked spatial variation in denitrification rate and soil NO_3^--N concentration among the replicates was observed, and the data were generally found to be log-normally distributed (Chapter 5), the mean values were calculated using the Uniform Minimum Variance Unbiased Estimator (White *et al.*, 1987; Parkin and Robinson, 1992). The UMVUE was also used for variance calculation (Parkin and Robinson, 1992). Differences in the means of these variables among treatments were determined in the test by comparing overlap of upper and lower 95% confidence limits (Parkin, 1993).
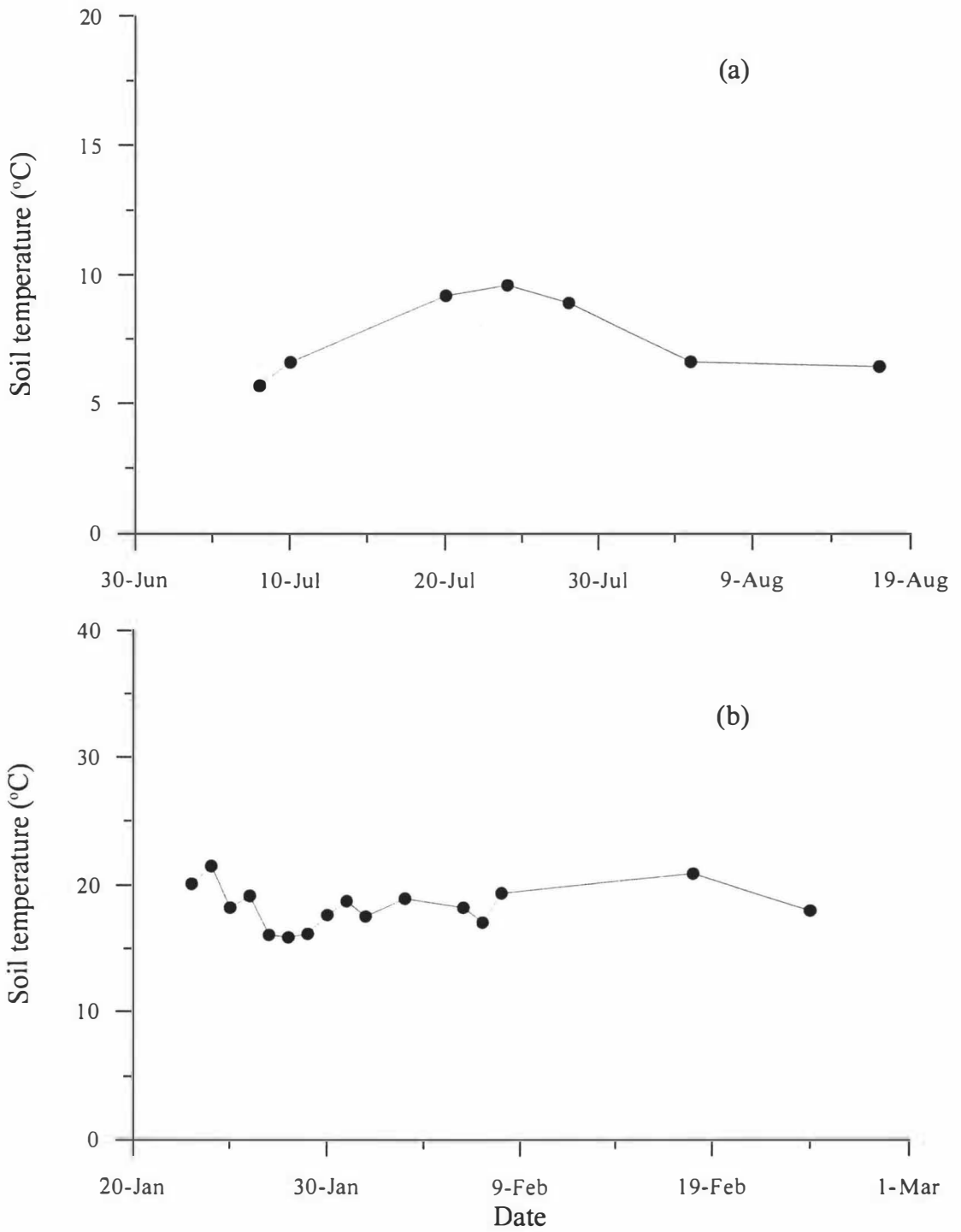
Figure 7.1 Soil temperature (10 cm depth) during the experiment
in winter, 1993 (a) and in summer, 1994 (b)

7.3 RESULTS AND DISCUSSION

7.3.1 Denitrification rate in relation to grazing events

There was generally no difference in denitrification rate among the grazing breaks and the control areas before animals were introduced to the paddock on 21 July 1993 in the moist, cool winter (Table 7.1). The rate of denitrification increased steadily 3 days after grazing in the breaks, reached a maximum at around 10 days, and then declined to a value similar to the control site by approximately 14 days (Table 7.1 and Figure 7.2). Although the patterns of denitrification rate following the grazing events were alike among the breaks, the magnitude of the peak varied depending upon the patterns of rainfall and dates of grazing. The highest rate observed in this study was 0.087 mg N_2O-N kg^{-1} soil day^{-1} on the tenth day after grazing in break No. 6 (Table 7.1).

In 1994 the summer was very dry with little rain for several weeks before the study period. Rates of denitrification were very low in both the grazed and the control sites in this dry, warm period (Figure 7.3). After a brief rain during the night of 24 January, peaks of denitrification rate were observed on 25 and 26 January. The grazing event by itself did little to increase denitrification rate in this season, although the rate was slightly higher from the grazed site than that from the control site immediately after this rainfall event (Figure 7.3). This marginal difference persisted for only 2 days (Figure 7.3).

Table 7.1 Denitrification rates (mg N_2O-N kg^{-1} d^{-1}) (mean values ± SD) during the experiment in winter, 1993

Area Sampling date	Control 1	Control 2*	Break 1	Break 2	Break 3	Break 4	Break 5	Break 6	Break 7	Break 8
			Grazing date							
			21 Jul	22 Jul	23 Jul	24 Jul	25 Jul	26 Jul	27 Jul	28 Jul
08 Jul	0.018 (0.0058)	0.017 (0.0077)	0.021 (0.0064)	0.022 (0.0035)	0.017 (0.0043)	0.018 (0.0039)	0.016 (0.0051)	0.017 (0.0073)	0.016 (0.0050)	0.016 (0.0033)
10 Jul	0.015 (0.0036)	0.013 (0.0041)	0.020 (0.0050)	0.016 (0.0052)	0.016 (0.054)	0.015 (0.0070)	0.020 (0.0079)	0.014 (0.0060)	0.020 (0.0022)	0.016 (0.0023)
20 Jul	0.018 (0.0041)	0.015 (0.0088)	0.017 (0.0076)	0.019 (0.0062)	0.014 (0.0060)	0.017 (0.0068)	0.018 (0.0068)	0.018 (0.0026)	0.015 (0.0051)	0.015 (0.0026)
24 Jul	0.022 (0.0079)	0.020 (0.0055)	0.067 (0.014)	0.025 (0.0055)	0.029 (0.0070)	0.029 (0.0060)	0.023 (0.0050)	0.021 (0.0040)	0.021 (0.0053)	0.022 (0.0052)
28 Jul	0.026 (0.0068)		0.081 (0.020)	0.068 (0.014)	0.065 (0.023)	0.063 (0.017)	0.031 (0.0060)	0.033 (0.0086)	0.032 (0.0035)	0.031 (0.0051)
05 Aug	0.021 (0.013)		0.022 (0.0078)	0.018 (0.0044)	0.036 (0.0090)	0.038 (0.012)	0.042 (0.012)	0.087 (0.024)	0.060 (0.0083)	0.058 (0.0029)
17 Aug	0.024 (0.0078)		0.023 (0.0041)	0.026 (0.013)	0.019 (0.0040)	0.023 (0.0086)	0.026 (0.0048)	0.027 (0.0021)	0.021 (0.0067)	0.024 (0.0024)

*, Control area 2 was destroyed by grazing animals on 28 July.

Figure 7.2 Effect of grazing on denitrification rate during the experiment in the cool, moist winter in 1993 (Data in all the grazed breaks on 28 July and 5 August, selected from Table 7.1) (bars represent SD)
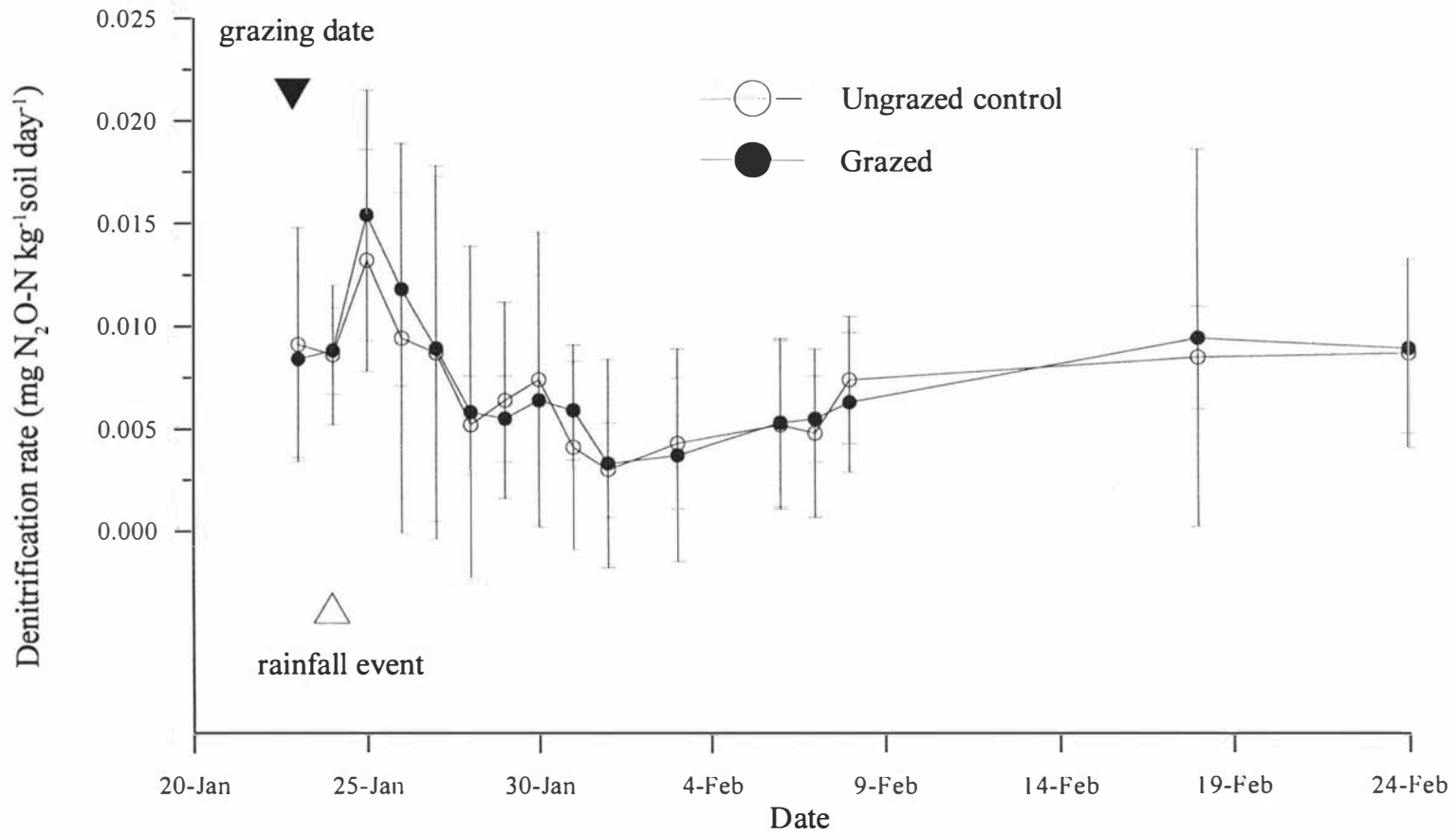
Figure 7.3 Denitrification rates during the experiment in summer, 1994 (bars represent SD)

7.3.2 Soil mineral nitrogen and denitrification

Changes in soil NH_4^+-N and NO_3^--N covering the study periods are presented in Tables 7.2a, b, Figures 7.4 and 7.5 a, b for the studies in winter and summer, respectively. The concentrations of soil NH_4^+-N increased rapidly during the first few days after grazing in both moist and dry seasons. Afterwards, they decreased during the following 10 and 5 days, in the moist and dry soils, respectively. Thereafter, NH_4^+-N remained relatively constant (Figures 7.4 and 7.5a). Significant increases in the concentration of soil NO_3^--N in the grazed breaks occurred 3-4 days after a grazing event in winter. This higher NO_3^--N concentration, compared with the control area, remained in the grazed pasture soil for about 10 days (Table 7.2b and Figure 7.4). Under dry conditions in summer, higher NO_3^--N concentrations were detected in the soil one day after grazing. These higher concentrations of NO_3^--N persisted in the grazed site until a month later, when the observations were discontinued (Figure 7.5b).

The stimulating effect of grazing on denitrification rate in winter may have been due to the N returned in animal excreta. The repetitive patterns of enhanced soil NO_3^--N accumulation and denitrification rate observed in the moist, cool winter period (Figures 7.2 and 7.4), as a consequence of grazing, suggest that the accumulation of NO_3^--N induced by grazing may provide a substantial supply of substrate for denitrification, especially during those times when soil moisture conditions are conducive to denitrification. However, a significant difference in NO_3^--N concentration in the dry soil, between the grazed and control areas during summer (Figure 7.5b), failed to induce any marked increase in denitrification rate in pasture (Figure 7.3). This can be explained by the low soil moisture contents and high O_2 concentrations present in soil under such conditions (Chapter 6) which inhibit denitrification.

Table 7.2a Soil mineral nitrogen concentration (mg NH_4^+-N kg^{-1}) (mean values ± SD) during the experiment in winter, 1993

Area Sampling date	Control 1	Control 2*	Break 1	Break 2	Break 3	Break 4	Break 5	Break 6	Break 7	Break 8
			Grazing date							
			21 Jul	22 Jul	23 Jul	24 Jul	25 Jul	26 Jul	27 Jul	28 Jul
08 Jul	6.25 (1.17)	8.62 (1.54)	6.89 (3.71)	9.69 (3.93)	5.54 (1.35)	6.65 (1.39)	6.83 (2.22)	8.58 (0.55)	5.68 (1.68)	5.58 (4.21)
10 Jul	7.48 (1.59)	8.91 (3.30)	8.90 (3.12)	6.88 (1.82)	10.0 (2.93)	10.1 (3.49)	9.87 (2.62)	9.70 (3.25)	10.8 (1.76)	7.71 (4.73)
20 Jul	11.36 (1.69)	15.5 (3.39)	10.8 (2.85)	12.3 (1.40)	8.90 (3.18)	9.40 (1.98)	8.60 (1.37)	9.11 (4.31)	11.7 (5.40)	8.79 (2.59)
24 Jul	8.90 (0.96)	9.70 (1.02)	14.6 (2.81)	31.1 (8.04)	18.7 (4.97)	11.2 (1.69)	10.5 (2.95)	9.32 (1.81)	12.4 (1.14)	10.6 (2.69)
28 Jul	10.37 (1.84)		13.2 (4.15)	14.2 (3.84)	18.6 (1.83)	19.4 (6.44)	21.0 (6.72)	24.3 (8.10)	29.6 (3.35)	12.7 (2.74)
05 Aug	9.70 (3.63)		10.4 (2.80)	10.7 (3.80)	9.66 (3.93)	15.3 (3.00)	14.6 (4.53)	12.0 (5.85)	16.6 (3.68)	14.6 (1.77)
17 Aug	8.51 (2.00)		8.06 (1.66)	10.8 (3.60)	11.0 (3.79)	10.9 (5.17)	9.05 (1.68)	11.1 (5.28)	9.31 (3.21)	10.2 (1.52)

*, Control area 2 was destroyed by grazing animals on 28 July.

Table 7.2b Soil mineral nitrogen concentration (mg NO_3^--N kg^{-1}) (mean values ± SD) during the experiment in winter, 1993

Area / Sampling date	Control 1	Control 2*	Break 1	Break 2	Break 3	Break 4	Break 5	Break 6	Break 7	Break 8
Grazing date			21 Jul	22 Jul	23 Jul	24 Jul	25 Jul	26 Jul	27 Jul	28 Jul
08 Jul	1.02 (0.24)	1.16 (0.39)	1.42 (0.41)	1.31 (0.61)	0.95 (0.57)	1.12 (0.40)	0.89 (0.47)	0.96 (0.42)	1.24 (0.84)	1.32 (0.39)
10 Jul	0.94 (0.08)	0.99 (0.42)	1.01 (0.50)	1.04 (0.47)	1.25 (0.59)	0.94 (0.26)	0.86 (0.40)	1.07 (0.63)	1.25 (0.69)	1.48 (1.01)
20 Jul	0.61 (0.13)	0.82 (0.28)	1.10 (0.67)	1.00 (0.50)	1.32 (0.46)	1.19 (0.48)	0.92 (0.30)	1.00 (0.74)	1.25 (0.67)	1.26 (0.38)
24 Jul	1.14 (0.24)	1.04 (0.56)	2.70 (0.55)	1.30 (0.91)	1.40 (0.48)	1.80 (1.52)	1.04 (0.42)	0.96 (0.32)	1.47 (1.19)	1.01 (0.66)
28 Jul	0.86 (0.37)		2.30 (1.00)	2.34 (0.90)	2.39 (0.75)	2.58 (0.50)	1.93 (0.75)	1.10 (0.80)	1.11 (0.58)	1.04 (0.65)
05 Aug	0.79 (0.44)		1.67 (0.89)	1.54 (1.05)	2.47 (1.03)	2.33 (0.81)	3.12 (1.10)	3.82 (1.17)	3.38 (2.00)	3.70 (1.61)
17 Aug	0.91 (0.63)		0.58 (0.24)	0.83 (0.50)	0.85 (0.41)	1.15 (0.61)	0.85 (0.67)	0.99 (0.30)	1.27 (0.70)	1.14 (0.48)

*, Control area 2 was destroyed by grazing animals on 28 July.
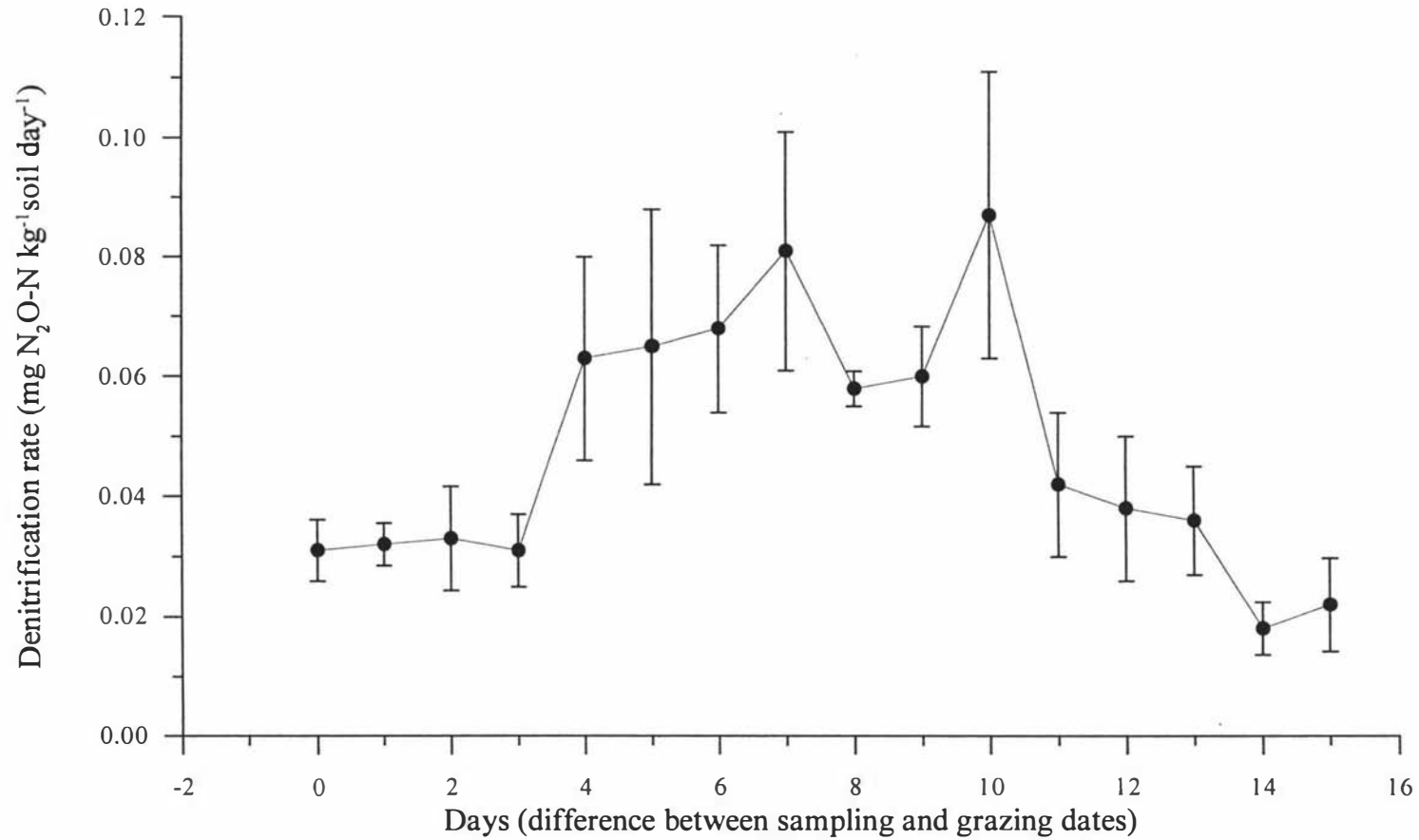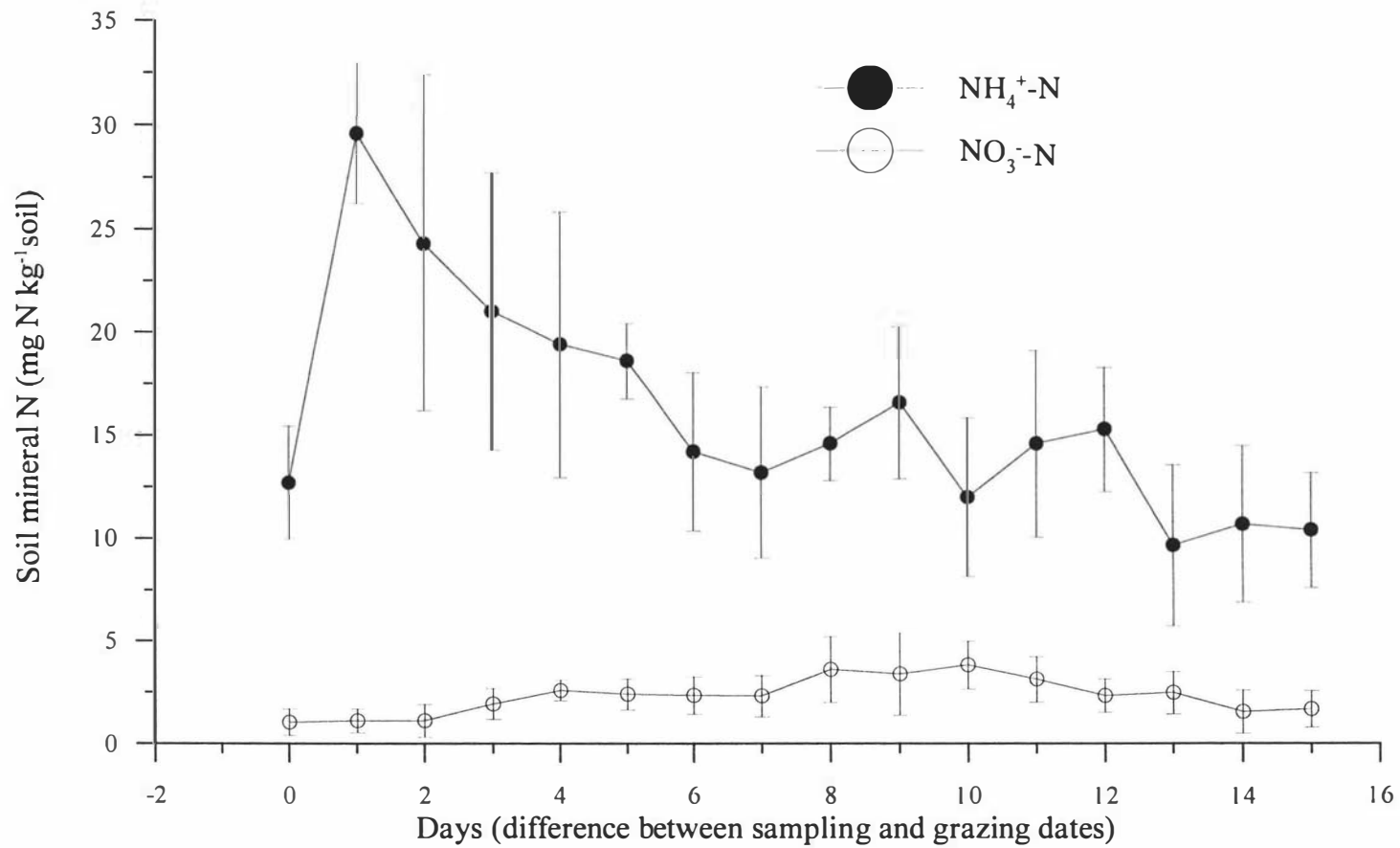
Figure 7.4 Soil mineral nitrogen concentrations during the experiment in the cool, moist winter in 1993
(Data in all the grazed breaks on 28 July and 5 August, selected from Tables 7.2a and 7.2b. bars represent SD)
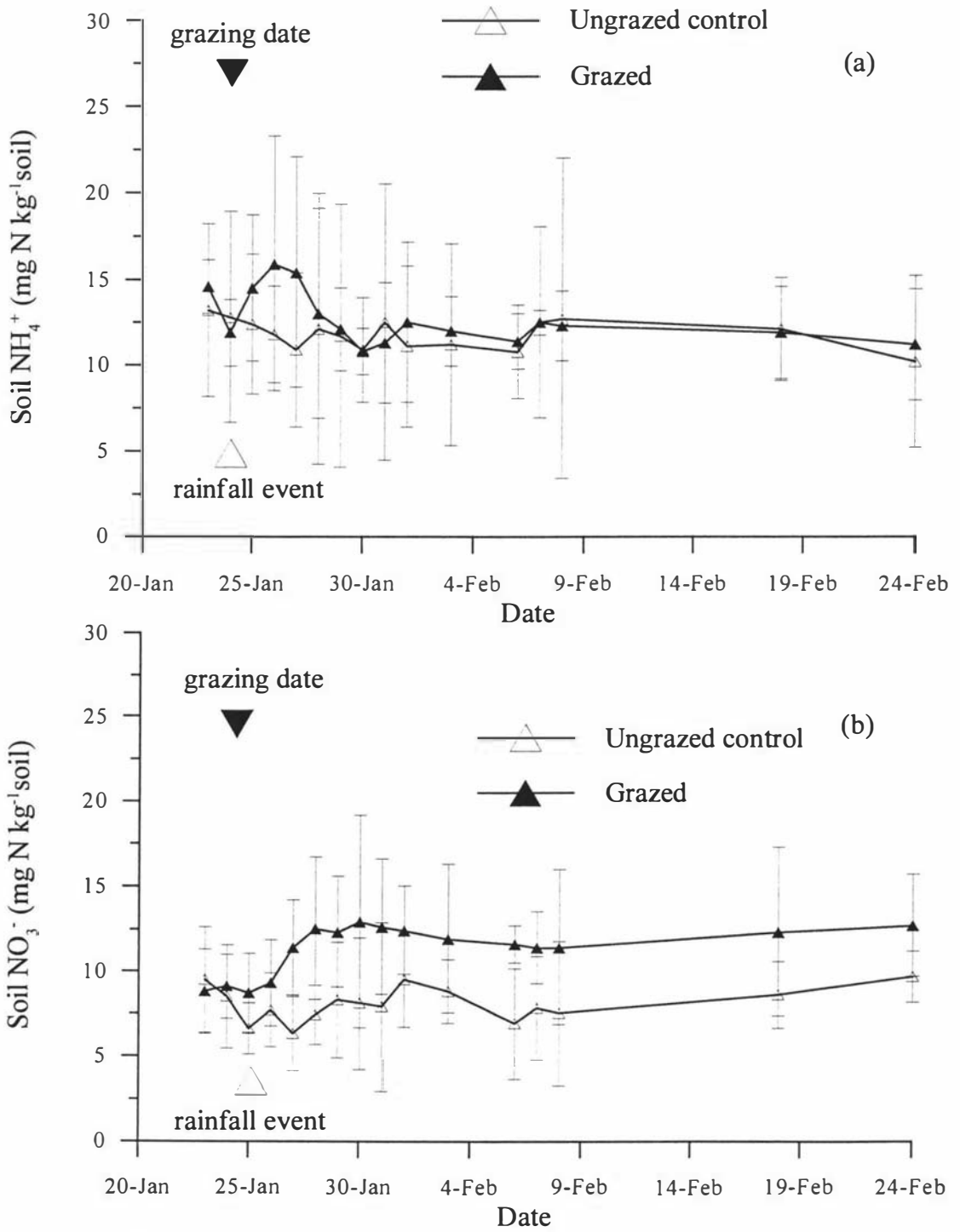
Figure 7.5 Soil mineral nitrogen concentrations during the experiment in summer, 1994
(a, NH_4^+-N; b, NO_3^--N) (bars represent SD)

7.3.3 Soil moisture and denitrification

The soil water contents clearly show the difference in moisture status during the two experiments (Figures 7.6a and 7.6b). The patterns of denitrification rates following the grazing events in the two seasons reflect the differences in soil moisture conditions during the two experiments. The lack of a stimulating effect of grazing in summer was likely the result of low soil water, the soil moisture contents being less than 20% (ww^{-1}) on most of the sampling dates (Figure 7.6b). On 25 and 26 January denitrification rates marginally increased in the grazed area, which is likely to be due to increased soil water contents as a result of rainfall on the previous night, in combination with increased NO_3^--N concentrations following nitrification of urine or dung-N after the grazing event. However, the fluctuations in soil moisture itself in the winter period (Figure 7.6a) had relatively little influence on changes in denitrification rate, compared to soil NO_3^--N concentrations after grazing.

7.3.4 Soil respiration and denitrification

Slightly greater rates of soil CO_2 emission were observed during the first 10 days or so after grazing in both seasons (Table 7.3, Figures 7.7 and 7.8). The peak rates of CO_2 emission seemed to occur within a few days of grazing both in the moist, cool season and in the dry, warm season. Denitrification and respiration rates after grazing appeared to be closely associated during the experiment conducted under the moist, cool winter condition (Figures 7.2 and 7.7). The grazing event may have provided conditions conducive to denitrification, by supplying more C to denitrifying organisms. This greater availability of C may be due to the addition of plant residues and deposition of animal excreta during grazing and,
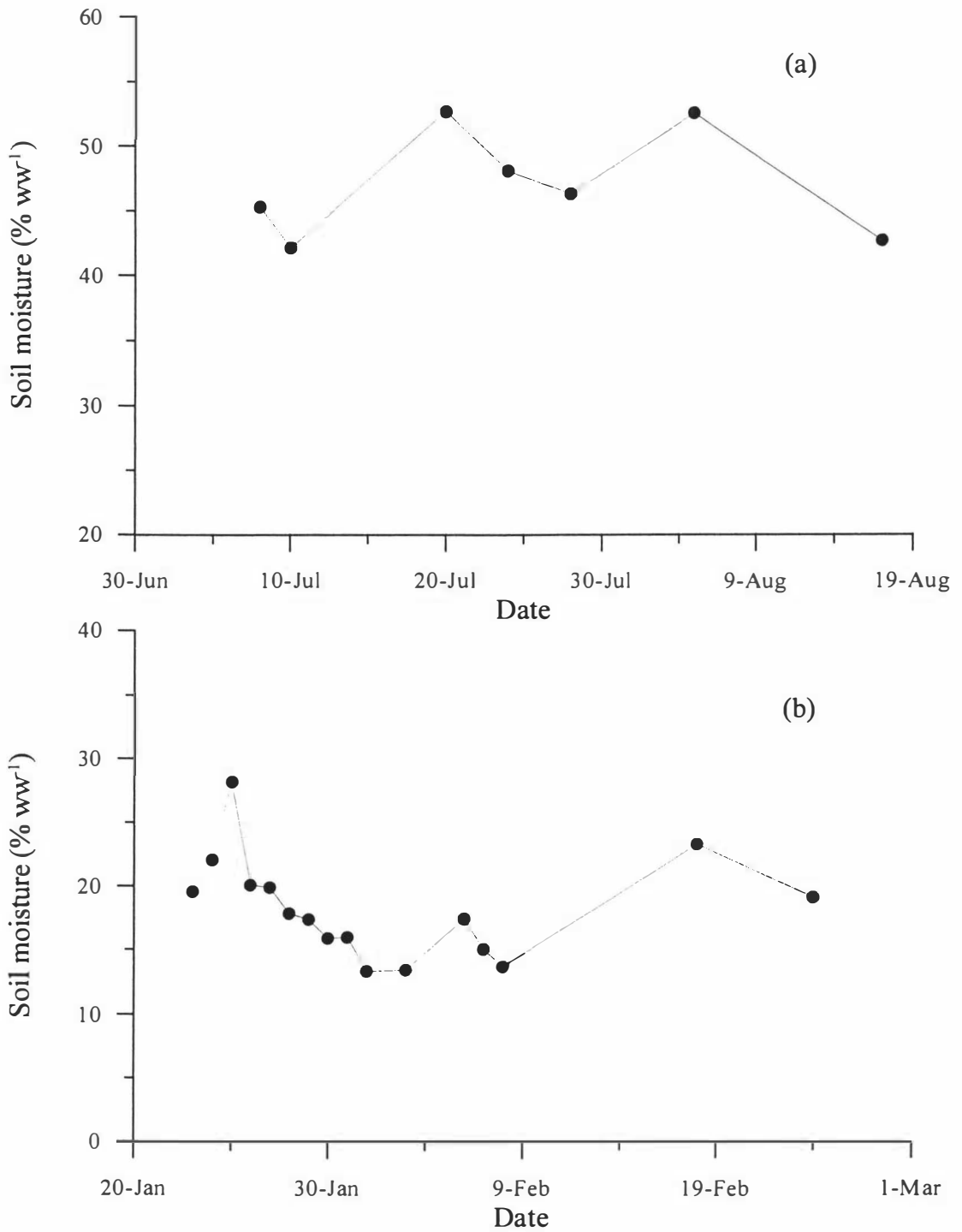
Figure 7.6 Soil moisture contents during the experiment
in winter, 1993 (a) and in summer, 1994 (b)

Table 7.3 Soil respiration rates ($mg\ CO_2\text{-}C\ kg^{-1}\ d^{-1}$) (mean values ± SD) during the experiment in winter, 1993

| Area	Control 1	Control 2*	Break 1	Break 2	Break 3	Break 4	Break 5	Break 6	Break 7	Break 8
Sampling date						Grazing date				
			21 Jul	22 Jul	23 Jul	24 Jul	25 Jul	26 Jul	27 Jul	28 Jul
08 Jul	16.7 (1.40)	15.9 (5.41)	16.3 (6.55)	16.8 (4.82)	16.9 (2.44)	15.4 (2.76)	16.3 (0.68)	15.9 (1.82)	17.5 (2.58)	14.4 (3.06)
10 Jul	16.2 (3.02)	16.0 (7.68)	15.9 (1.62)	15.7 (5.56)	15.7 (4.99)	16.6 (2.64)	15.7 (3.00)	16.5 (4.67)	16.5 (2.79)	16.4 (3.24)
20 Jul	15.5 (1.32)	16.3 (2.29)	15.6 (1.61)	17.8 (7.06)	13.9 (3.74)	15.5 (3.77)	17.1 (2.26)	15.9 (3.52)	14.7 (1.07)	17.4 (3.01)
24 Jul	16.5 (1.95)	15.3 (2.91)	22.6 (6.72)	24.8 (2.78)	19.4 (5.44)	14.7 (2.46)	16.3 (5.77)	15.3 (6.27)	16.9 (5.39)	17.8 (2.73)
28 Jul	16.9 (1.29)		19.0 (4.22)	18.7 (4.68)	17.1 (3.11)	24.0 (4.02)	22.3 (3.67)	20.0 (3.96)	19.1 (6.83)	16.6 (4.10)
05 Aug	19.3 (3.76)		17.9 (4.27)	16.1 (3.30)	19.6 (4.64)	20.9 (7.15)	19.5 (5.52)	19.0 (4.11)	19.4 (2.57)	18.9 (4.28)
17 Aug	18.3 (6.00)		18.4 (1.99)	17.3 (2.60)	17.1 (4.80)	17.7 (6.92)	16.6 (0.21)	16.0 (4.38)	16.4 (3.11)	15.9 (2.35)

*, Control area 2 was destroyed by grazing animals on 28 July.
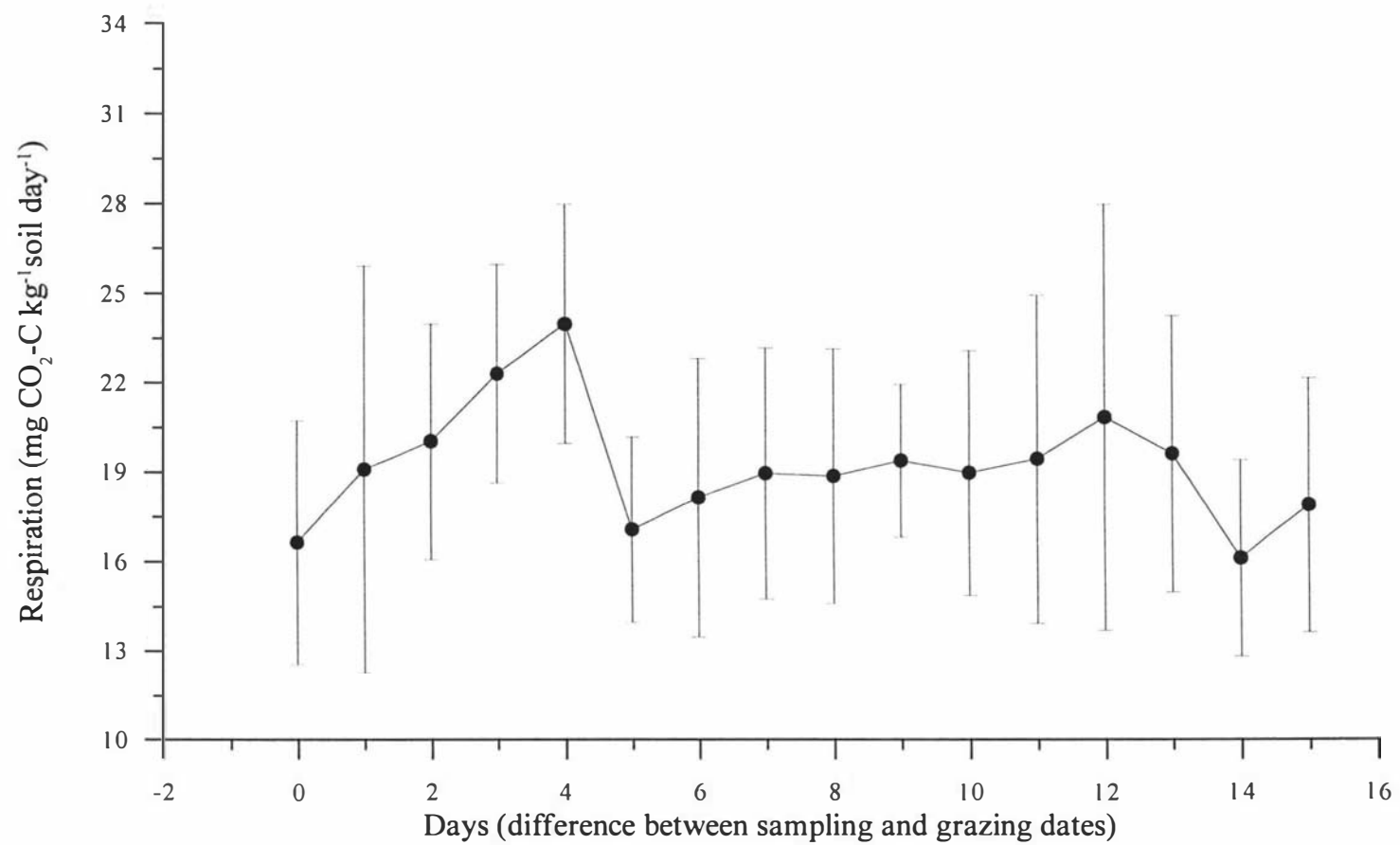
Figure 7.7 Soil respiration rates during the experiment in the cool, moist winter in 1993
(Data in all the grazed breaks on 28 July and 5 August, selected from Table 7.3) (bars represent SD)

Figure 7.8 Soil respiration rates during the experiment in summer, 1994 (bars represent SD)

possibly, some surface "cultivation", especially in wet soils. In addition to providing an energy source to denitrifying organisms, the grazing event may also result in more anaerobic environments for denitrification through O_2 consumption during decomposition of plant residues and animal faeces. Previous researchers have recognized the importance of a rapid onset of anaerobiosis, from generation of CO_2, to initiate denitrification (Smith and Tiedje, 1979a; Sherlock and Goh, 1983). Greater compaction of the soil after grazing would also lead to more anaerobic sites when the soil was wet in the winter.

7.3.5 Denitrification enzyme activity in relation to grazing events

Denitrification enzyme activity of the soil was substantially higher a few days after grazing in the winter (Figure 7.9). In contrast, the difference was not statistically significant between the grazed and control sites in the summer (Figure 7.10). The increase in denitrification enzyme activity in the winter reflects an enhanced microbial population in the soil for denitrification after the grazing. The increase in microbial population was probably caused by both C and N additions to the topsoil during the grazing events. Low NO_3^--N concentrations prior to grazing may have limited denitrification enzyme induction (Firestone, 1982). Promotion of anaerobic conditions by grazing could be also another reason for higher denitrification microbial population.
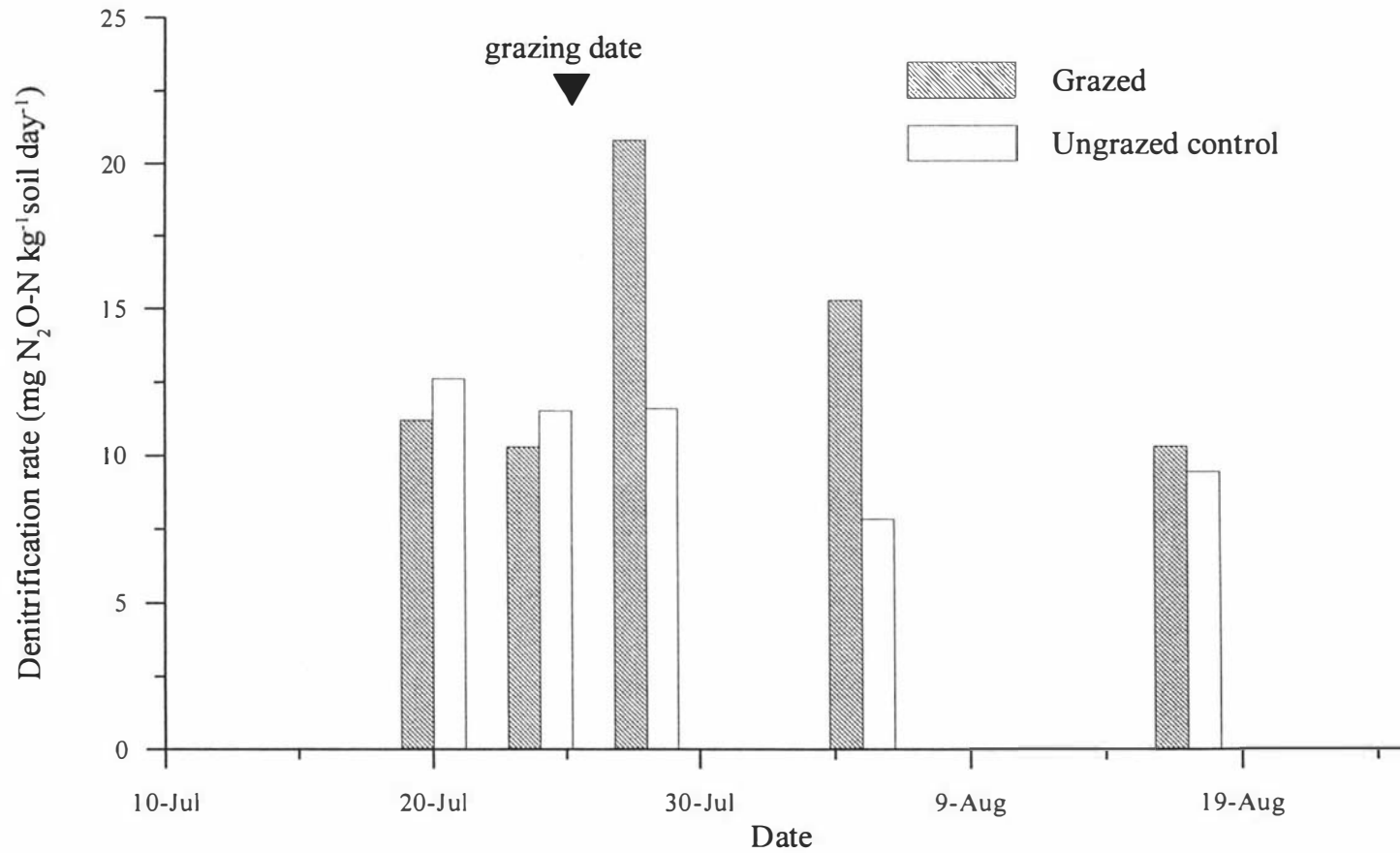
Figure 7.9 Denitrification enzyme activities before and after grazing in "break 5" and the control area during the experiment in winter, 1993
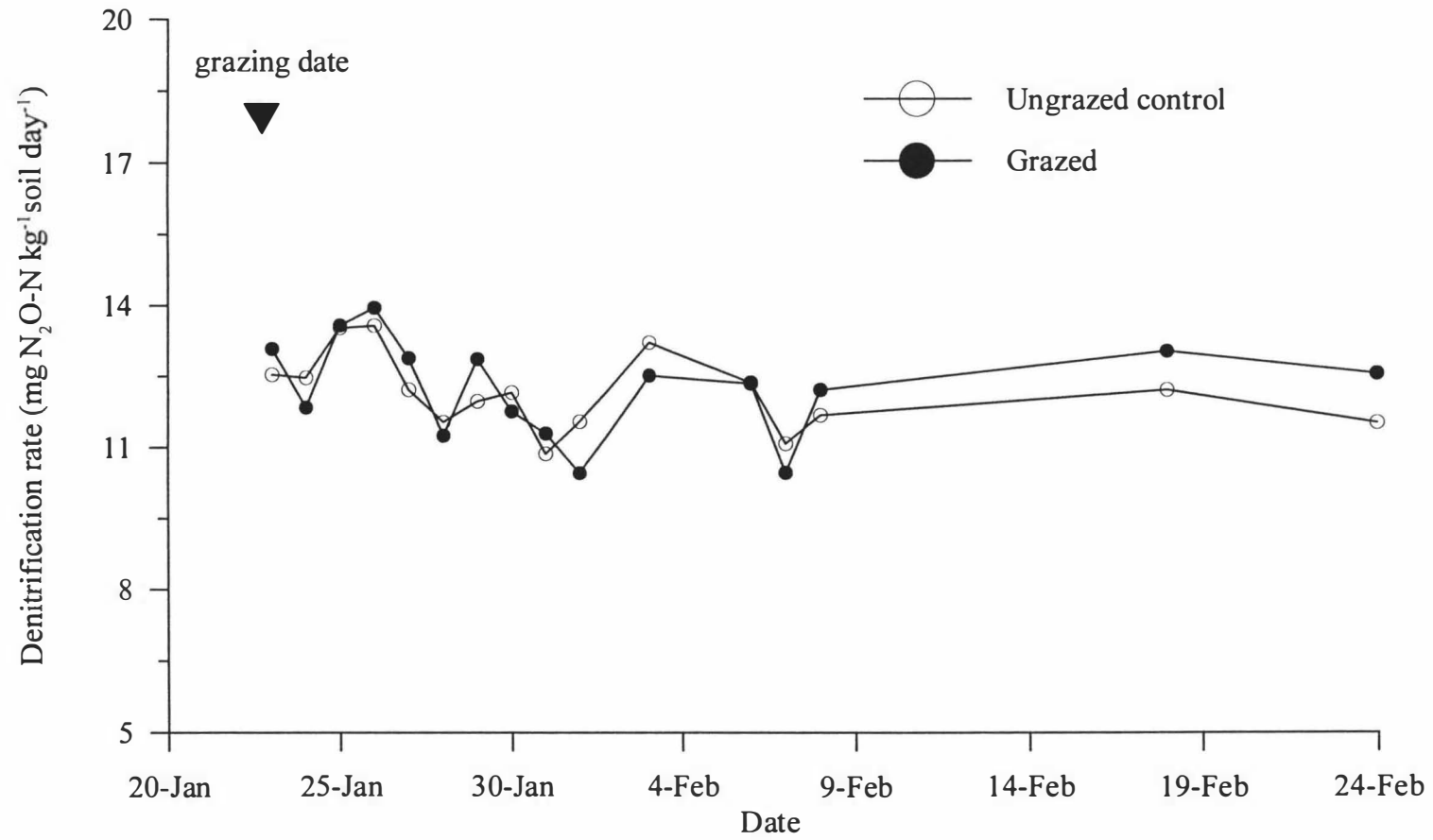
Figure 7.10 Denitrification enzyme activities during the experiment in summer, 1994

7.3.6 Soil pH and denitrification

The soil pH values measured in different breaks and the control area are presented in Table 7.4, Figures 7.11 and 7.12 for the winter experiment and the summer experiment, respectively. A slight increase in soil pH was found for a few days immediately after grazing in both experiments. The rate of denitrification can be affected by pH, generally being low in acid conditions and more rapid at slightly alkaline pHs (Nommik, 1965; Bremner and Shaw, 1958). Hydrolysis of urine-N can create high pH conditions in patches temporarily (Doak, 1952) and thereby the higher rate of denitrification we observed may be partly due to the higher pH in the urine patches after the grazing event in winter.

7.3.7 Nitrogen losses through denitrification directly induced by the grazing in winter

Integration of the daily rates of denitrification over time resulted in total denitrification N losses about 0.17 and 0.45 kg N ha^{-1} for about two weeks after grazing events in the control and grazed areas in winter. Therefore, the direct N loss through denitrification induced by the grazing event was 0.28 kg N ha^{-1} over two weeks following the grazing. A dairy cow produces 20 litres of urine per day with a mean N concentration of 0.9% (Hutton *et al.*, 1967). Therefore, about 0.18 kg of N can be deposited in urine per cow per day. In the winter grazing about 54 kg N per hectare can be returned by 300 cows to the paddock under the block-grazing system in use. Thus less than 1% of the N added in urine was lost by denitrification in the two weeks following grazing. The low losses of N found in our study might be the result of low soil temperatures in winter, as other factors, such as soil water content and NO_3^--N concentration, were conducive to denitrification.

Table 7.4 Soil pH values during the experiment in winter, 1993

Area	Control 1	Control 2*	Break 1	Break 2	Break 3	Break 4	Break 5	Break 6	Break 7	Break 8
Sampling date			Grazing date							
			21 Jul	22 Jul	23 Jul	24 Jul	25 Jul	26 Jul	27 Jul	28 Jul
08 Jul	6.16	6.09	6.17	6.15	6.02	5.91	5.94	5.85	6.04	6.15
10 Jul	6.05	5.98	6.04	6.14	6.08	6.02	6.08	5.92	5.96	6.11
20 Jul	5.95	6.11	6.04	5.89	5.97	6.00	5.94	6.00	5.98	5.89
24 Jul	6.03	6.00	6.21	6.39	6.24	6.05	5.88	5.95	6.04	5.87
28 Jul	5.98		6.08	6.04	5.97	6.10	6.16	6.41	6.35	6.11
05 Aug	5.90		5.91	6.12	5.95	6.14	6.08	6.01	6.00	5.99
17 Aug	5.92		6.09	5.98	6.02	5.94	5.92	5.93	6.05	5.96

*, Control area 2 was destroyed by grazing animals on 28 July.
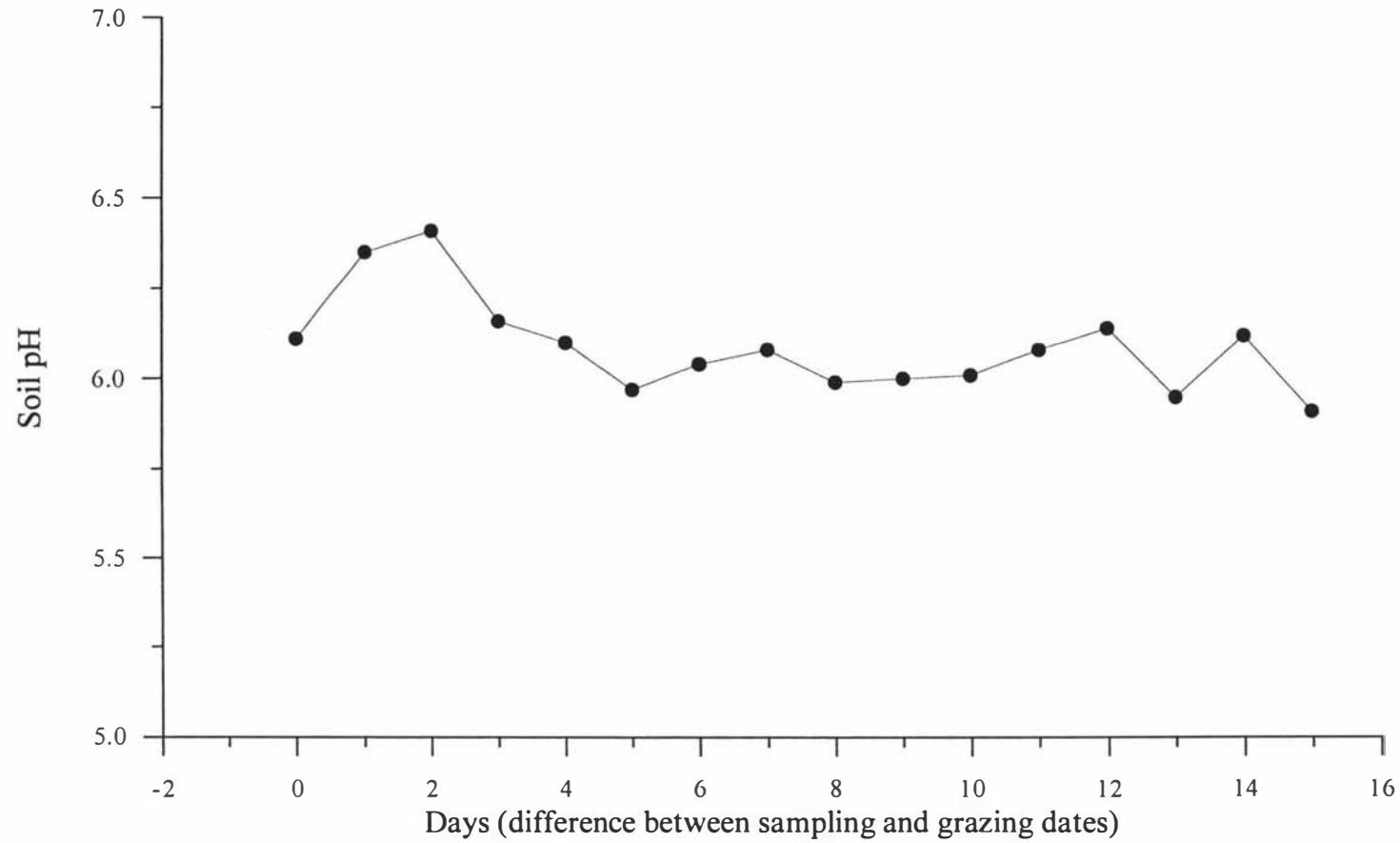
Figure 7.11 Soil pH values during the experiment in the cool, moist winter in 1993
(Data in all the grazed breaks on 28 July and 5 August, selected from Table 7.4)

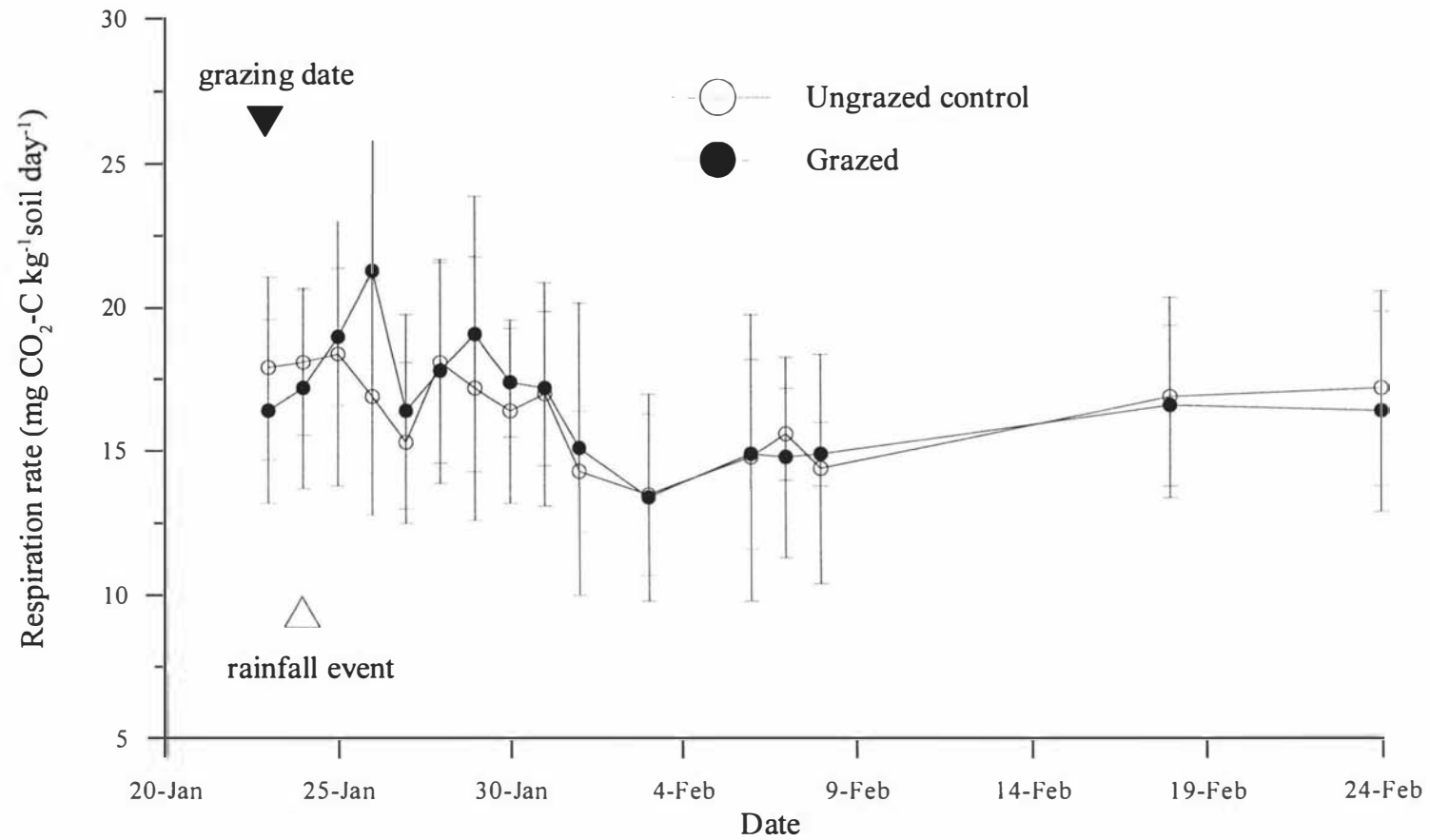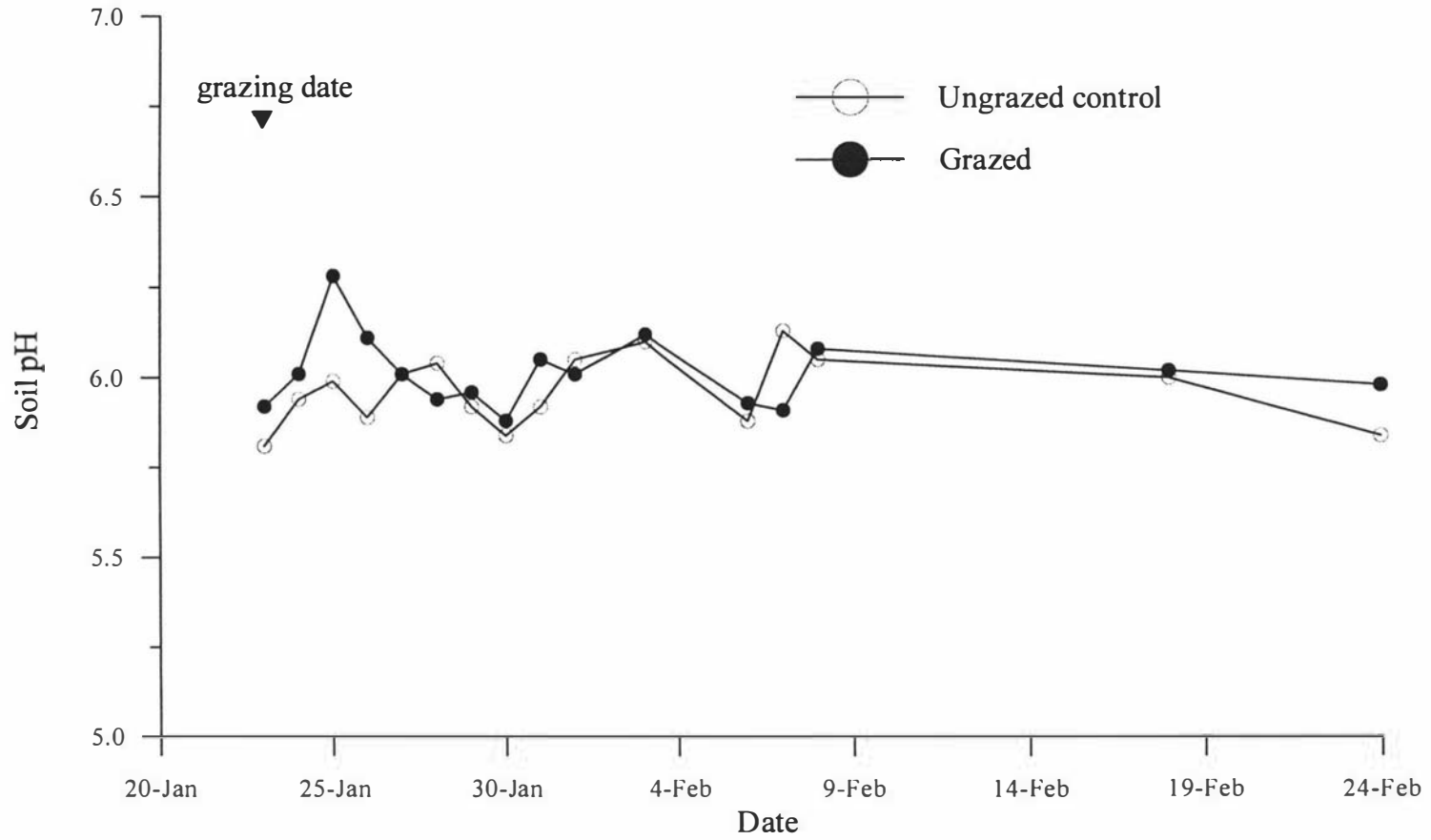Figure 7.12 Soil pH values during the experiment in summer, 1994

The results of our study also suggest that a substantial amount of NO_3^--N may have been leached below the sampling depth. As expected, the NH_4^+-N concentration was high for a few days after grazing. Thereafter, the NH_4^+-N concentration rapidly declined to control concentrations (Figure 7.4). However, this decrease in NH_4^+-N did not result in a proportional increase in the soil NO_3^--N concentration (Figure 7.4). Indeed, soil NO_3^--N concentration also decreased by about 3.5 kg N ha^{-1} until it reached the control level at about 14 days after grazing. Considering 54 kg N ha^{-1} was returned in urine and only 0.28 kg N ha^{-1} was lost through denitrification in the 14 days after grazing, leaching losses of NO_3^--N may have been significant during that period, since N uptake and immobilization rates by plants and microorganisms in soils in winter are generally very low (Ruz-Jerez, 1991). Other studies have also suggested that the losses of NO_3^--N through leaching during wet seasons (Holland and During, 1977; Ball *et al.*, 1979; Steele, 1982) may affect the accumulation of NO_3^--N in soil and the amount of N lost by denitrification.

7.4 CONCLUSIONS

With regard to temporal variability of the denitrification rates in the study pasture, we have recognized that one of the important factors affecting this variability is soil moisture content (Chapter 6). The results reported in this chapter suggest that the grazing animals also make a relatively important contribution to temporal variability of denitrification rate in pasture.

The effect of animal grazing on denitrification rates may be stimulated by higher NO_3^--N concentrations in the surface soil, as well as more available-C and higher denitrification enzyme activities after grazing in winter. However, the influence of grazing on

denitrification rates was only short lived (<2 weeks) during the winter, and the absolute amounts of N lost were very low compared with the amounts of N returned by the grazing animals. It may be that denitrification N losses after grazing during the winter period were limited by the soil temperature, and were therefore low compared with other possible N losses from the system.

During the dry, warm summer, denitrification rates were very low, although NO_3^--N concentrations in the grazed site were high. Soil water status probably had a large limiting effect on denitrification rates in summer. The present results also suggest that increase in soil water content could enhance denitrification rate in summer and return of animal excreta in a pasture soil could be a source of increased N loss. Therefore, the impact of grazing events should be considered during any attempt to quantify denitrification N loss from pastures.

CHAPTER 8

STUDY ON LIMITING FACTORS AFFECTING

DENITRIFICATION

8.1 INTRODUCTION

In previous chapters, spatial and temporal variations in soil denitrification in a dairy-farm pasture have been investigated. The large spatial and seasonal variations observed in denitrification rates are often attributed to changes in the availability of NO_3^--N, soluble-C, and soil water. However, few studies have investigated the actual factors limiting the denitrification process in pastures.

In this study individual soil cores were assessed for denitrification rate and then amended with water, NO_3^--N or soluble-C and the denitrification rate was remeasured. It was hoped by this process to identify the factors most limiting denitrification rate in the pasture soils.

8.2 MATERIALS AND METHODS

Samples were collected on eight occasions in three contrasting seasons from a flat land site in a dairy-farm pasture. On two occasions samples were taken from grazed and ungrazed areas in order to investigate the direct effect of grazing. The site description, soil details, and techniques for measuring denitrification in individual soil cores from the field were

presented in Chapter 5. The grazing management on the two occasions in the winter of 1993 and the summer of 1994 was as described in Chapter 7.

Some soil properties on each of the sampling dates are given in Table 8.1. One hundred and twelve soil cores were collected randomly from the flat land site at each sampling time. After the last gas sampling for field denitrification measurement in individual cores (Chapters 5 and 6), soil cores were removed to the laboratory and treated as follows. Of these 112 cores, 20 received no additional treatment, 12 were saturated with distilled water, 20 were amended with approximately 50 μg NO_3^--N g^{-1} soil and were saturated, 20 were amended with approximately 300 μg glucose-C g^{-1} soil and were saturated, 20 were amended with both approximately 50 μg NO_3^--N g^{-1} soil, and approximately 300 μg glucose-C g^{-1} soil and were saturated, and 20 received no N or C amendments but were incubated under unsaturated and anaerobic conditions. To achieve the desired N and C concentrations in soil cores, tests were made to decide how much water was needed to saturate a single soil core. The appropriate concentrations of KNO_3 or glucose solutions were then calculated to provide the required quantities of N and/or C. Soil cores were carefully dipped into the solutions to absorb the required water and to obtain the enhanced NO_3^--N and/or C concentrations. Table 8.2 presents information on the variation in soil properties between amended cores sampled on 25 January 1993. To obtain the anaerobic incubation conditions in the final treatment, PVC tubes were flushed with pure N_2 3 times before the tubes were sealed. In the other treatments, incubation was under an aerobic condition. All cores were then incubated in the PVC tubes with 6 ml of C_2H_2 at 25°C, and gas samples for N_2O and CO_2 analyses were collected and analysed as described in Chapters 3 and 5.

Table 8.1 Soil properties on sampling dates

Climatic conditions	Date	Moisture (% ww^{-1})	Nitrate (mg NO$_3^-$- N kg^{-1})	Respiration (mg CO$_2$-C kg^{-1} d^{-1})	DEA (mg N$_2$O-N kg^{-1} d^{-1})[a]	Temperature (°C)
Warm-moist	17 November 1992	35.00	0.58	18.95	3.50	18
	08 December 1992	38.13	0.56	18.64	3.84	20
	07 October 1993	39.96	0.84	N.D.	3.05	14
Warm-dry	25 January 1993	26.64	10.07	14.98	4.00	19
	30 January 1994[b]	15.85	8.10	16.40	4.07	18
	30 January 1994[c]	15.96	12.9	17.40	4.27	18
Cool-wet	09 June 1993	51.25	1.91	16.22	3.40	13
	20 July 1993	52.60	0.61	17.06	N.D.	10
	05 August 1993[b]	47.03	0.79	19.29	N.D.	7
	05 August 1993[c]	48.54	3.12	20.85	N.D.	7

[a], Denitrification Enzyme Activity (as measured under anaerobic and saturated conditions after amendments with NO$_3^-$-N and glucose-C),
[b], Ungrazed control site,
[c], Grazed site.

Table 8.2 Variation in soil properties in individual soil cores after application of treatments to samples collected on 25 January 1993

	Treatment	Control	Saturation	NO_3^--N amendment plus saturation	Glucose-C amendment plus saturation	NO_3^--N and glucose-C amendment plus saturation	Unsaturated but anaerobic
Moisture	Mean (% ww^{-1})	26.33	54.15	55.14	53.98	54.57	25.14
	Standard deviation	2.43	3.25	2.68	3.89	4.04	3.82
Nitrate concentration	Mean (mg NO_3^--N kg^{-1})	11.04	12.84	48.22	15.79	47.93	7.08
	Standard deviation	7.78	4.75	5.46	10.90	5.54	5.03
Respiration rate	Mean (mg CO_2-C kg^{-1} d^{-1})	30.29	30.13	35.79	59.99	59.49	5.91
	Standard deviation	3.95	3.51	2.12	2.24	2.57	1.17
Denitrification rate	Mean (mg N_2O-N kg^{-1} d^{-1})	0.0207	0.6822	0.4024	0.3742	3.219	0.02123
	Standard deviation	0.0103	0.1947	0.02393	0.1926	0.1924	0.02169

Soil NO_3^--N concentrations in each individual core, immediately following the incubation procedure, were analysed using the method described in Chapter 3. Original NO_3^--N in each soil core was estimated by adding the amounts of the measured NO_3^--N and denitrified N during the incubation.

Due to the large spatial variation in denitrification rates among replicates in the field, the mean soil denitrification rates were calculated using Uniform Variance Unbiased Estimators (Chapter 5). The arithmetic means of replicate denitrification rates were calculated for the laboratory data. Statistical analyses were performed using the Statistical Analysis System (SAS) (SAS Institute, 1985).

8.3 RESULTS

8.3.1 Responses of denitrification to treatments

The effects of soil amendment and subsequent incubation in the laboratory on denitrification rate are presented in Figure 8.1 for samples collected when soils were warm and moist (October-December); in Figure 8.2 for samples collected when soils were warm and dry (January); and in Figure 8.3 for samples collected when soils were cool and moist (July-August). In each figure the filled circles and squares represent the mean rates of denitrification in the 12-20 (depending on treatment) individual cores at field temperature prior to amendment and incubation in the laboratory at 25°C, respectively. The initial mean rates were generally similar across treatments although there was some variation reflecting the large variability between individual cores that has been identified in Chapter 5.
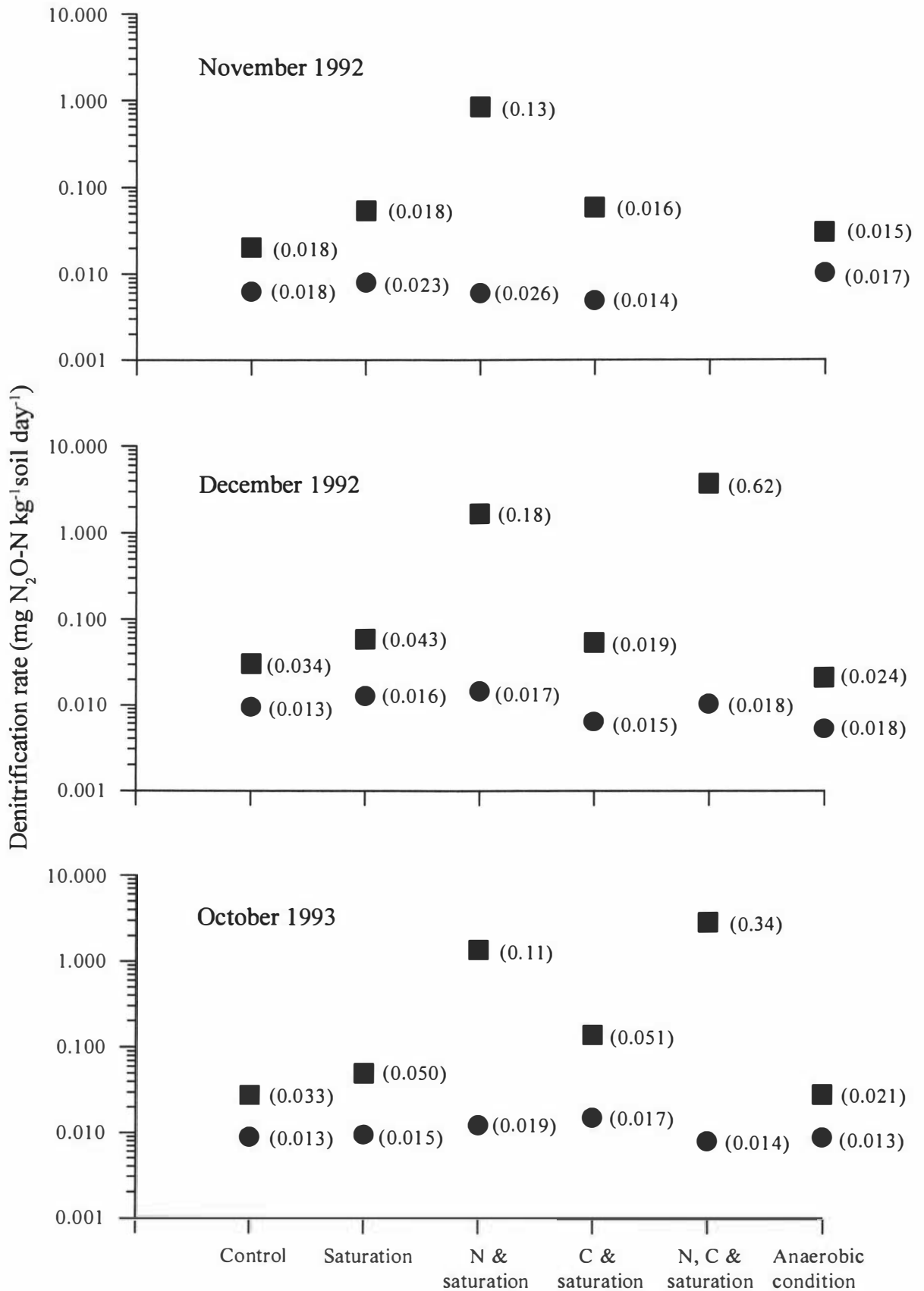
Figure 8.1   Denitrification rates in untreated soil cores collected in warm moist seasons
and incubated at field temperature (circle) and in the same cores
after application of treatments and incubated at 25ºC (square).
(Number adjacent to each data point indicates SD)
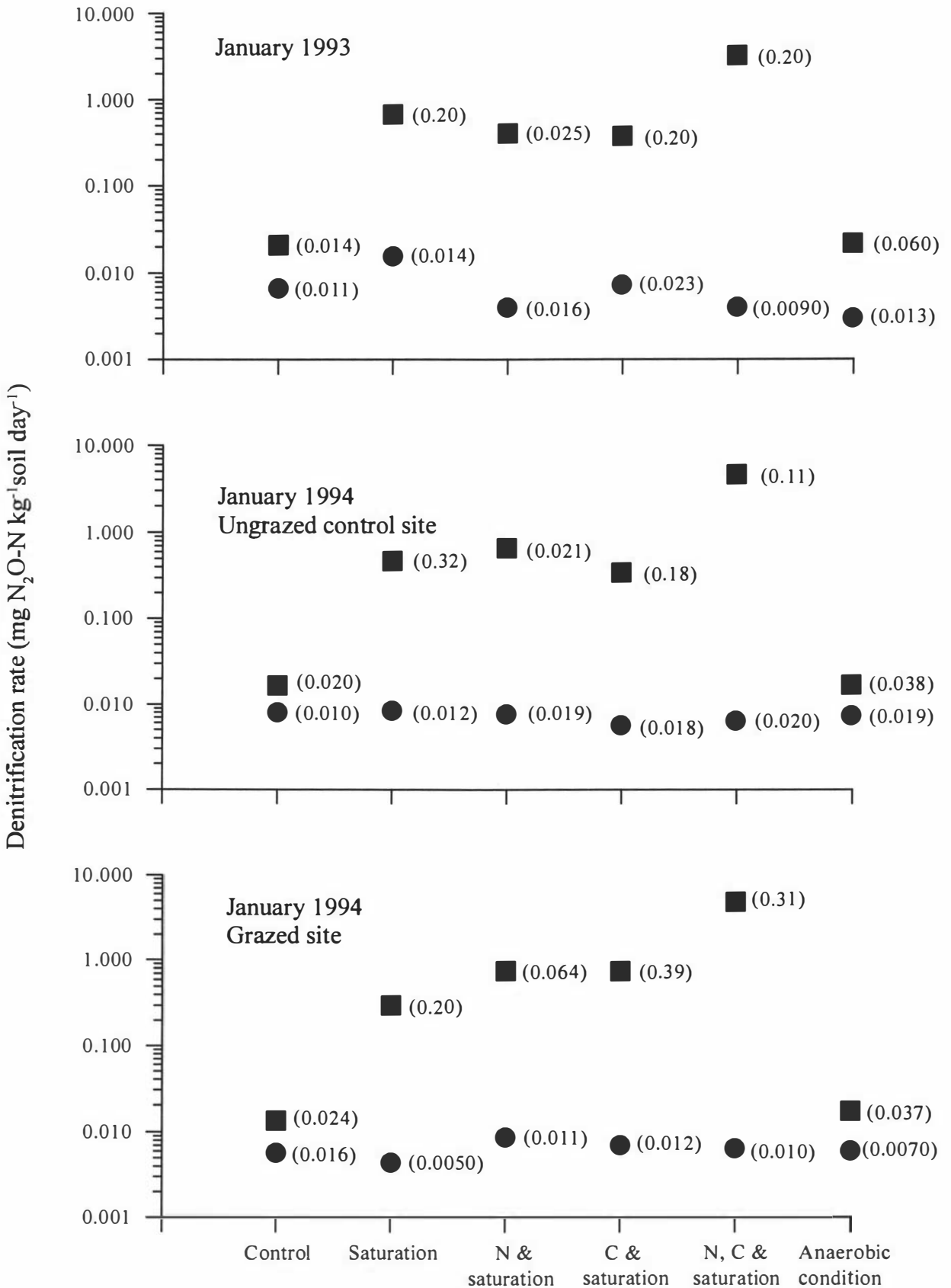
Figure 8.2 Denitrification rates in untreated soil cores collected in warm dry seasons and incubated at field temperature (circle) and in the same cores after application of treatments and incubated at 25°C (square). (Number adjacent to each data point indicates SD)
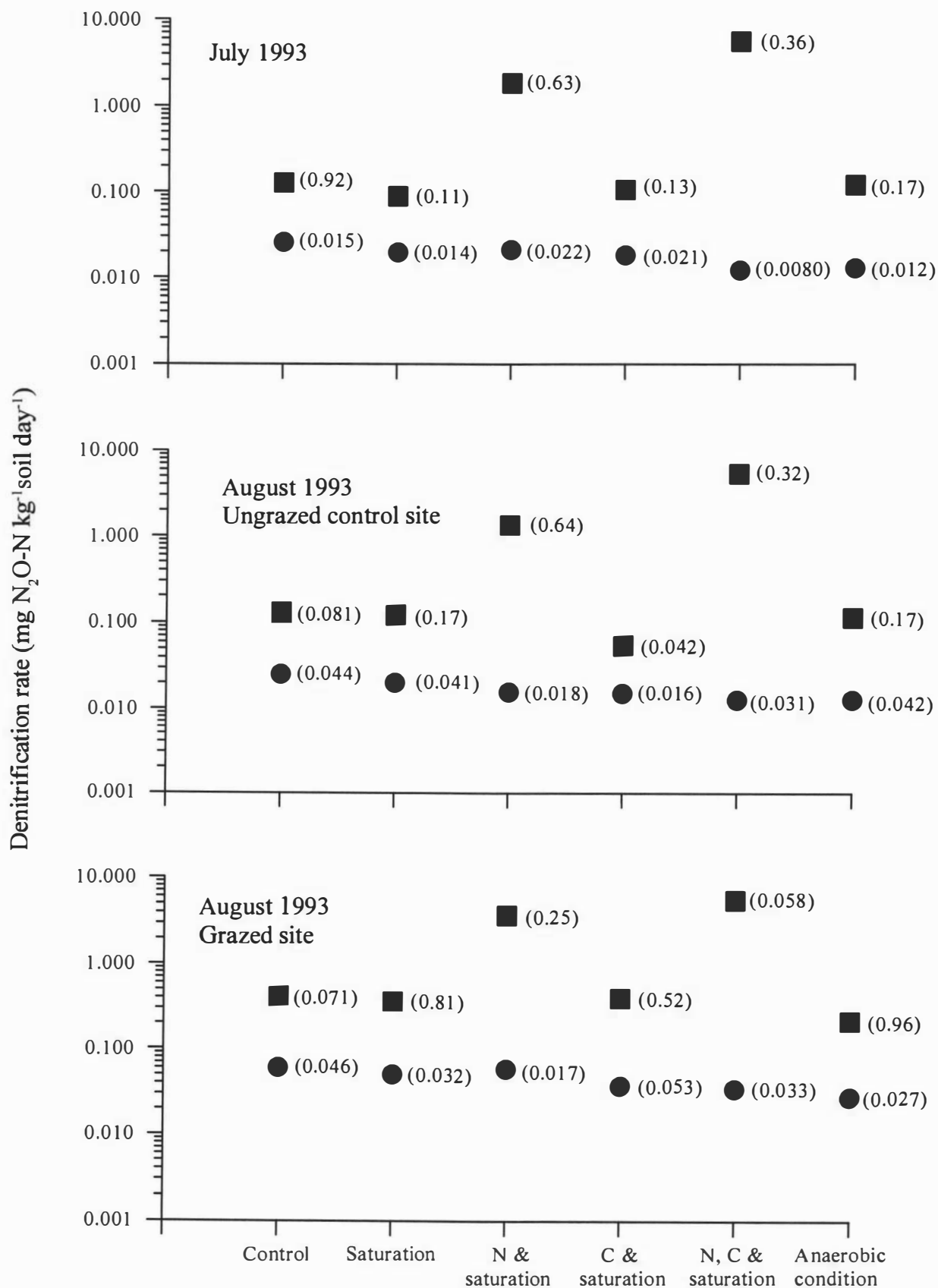
Figure 8.3 Denitrification rates in untreated soil cores collected in cool wet seasons
and incubated at field temperature (circle) and in the same cores
after application of treatments and incubated at 25°C (square).
(Number adjacent to each data point indicates SD)

On each sampling occasion incubation of the unamended control cores at 25°C in the laboratory increased the rate of denitrification above that measured at the cooler field temperature (Figures 8.1-8.3). As would be expected this difference was greatest in the cool, wet season.

In the amended treatments denitrification rates were also higher than in the original samples incubated at field temperature (Figures 8.1-8.3). In some treatments and at some sampling times these increases in denitrification rate were very large - sometimes greater than three orders of magnitude.

The response of denitrification rates to saturation depended on sampling times (Figures 8.1-8.3). During the warm, moist season, the increases in denitrification rate obtained by saturating the soil cores and incubating at 25°C were higher than in the control cores but were consistently lower than the increases in denitrification rate in NO_3^--amended soils (Figure 8.1). In contrast, during the warm, dry period saturation alone was sufficient to induce a very large increase in denitrification rate (Figure 8.2). However, there was no significant difference in the increase in denitrification rates between saturated and control cores collected during the cool, wet season (Figure 8.3).

Regardless of the season, denitrification rates were always strongly enhanced by NO_3^- additions, although the responses to added N in the warm, dry season were not as large as those in the other seasons (Figures 8.1-8.3). The very large increases in denitrification rate after amendment with NO_3^--N in all seasons suggest that availability of NO_3^--N is likely to be one factor limiting denitrification in this pasture soil. The response of denitrification rate to glucose-C solution addition also differed with season, with the largest increase being

found in the warm, dry season (Figures 8.1-8.3). In most cases, the effects of glucose-C on denitrification were much less than those of NO_3^--N (Figures 8.1-8.3). The maximum rates of denitrification were observed when both NO_3^--N and glucose-C solutions were added (Figures 8.1-8.3). These maximum denitrification rates were similar irrespective of when the soils were sampled. Anaerobic incubation of soil cores had little effect on the increase in denitrification rates compared with the control in all seasons (Figures 8.1-8.3).

8.3.2 Relationships between NO_3^- concentration, C availability and denitrification rate

Examples of the relationship between soil NO_3^--N concentration and denitrification rate for each set of treated cores on three sampling dates (representing the three contrasting seasons) are presented in Figures 8.4-8.6. Good linear relationships between denitrification rate and soil NO_3^--N concentration were observed after saturation of soil cores collected during both the warm, moist and warm, dry seasons, or after saturation and addition of glucose to soil cores collected in all three seasons (Figures 8.4-8.6). Under anaerobic incubation conditions, denitrification rates were proportional to soil NO_3^--N concentrations in cores collected during the warm, dry season (Figure 8.5). A positive relationship between denitrification rate and NO_3^--N concentration was also observed in the control soils sampled in the cool, wet season (Figure 8.6).

Similar plots of denitrification rates and soil respiration rates gave good linear relationships when soils were incubated after NO_3^--N amendment and saturation in all three seasons (Figures 8.7-8.9). A good relationship between denitrification rate and soil respiration rate was also found when field moist soils were incubated in the laboratory with no amendment in the warm, dry season (Figure 8.8).
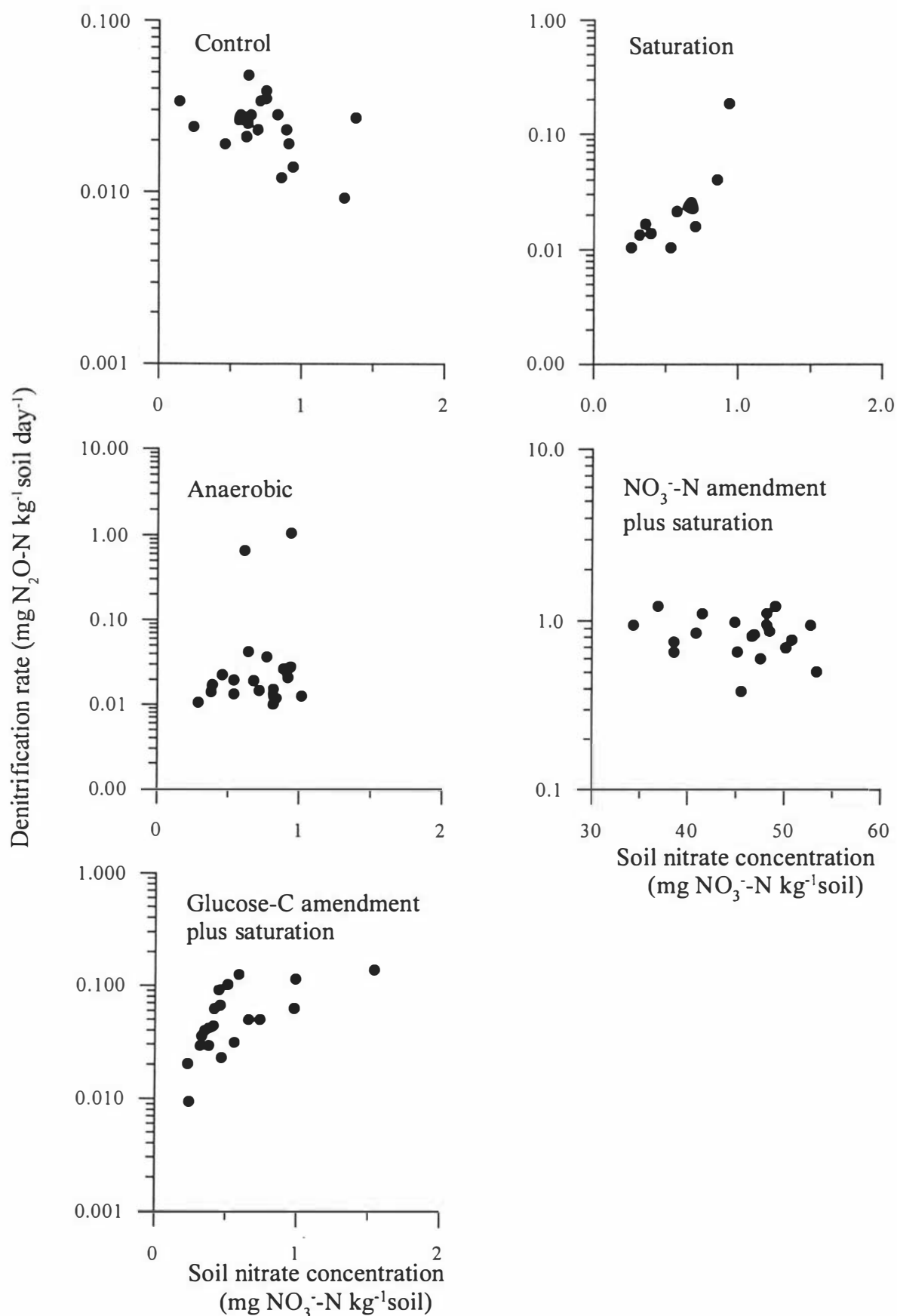
Figure 8.4 Relationship between denitrification rates and soil nitrate concentrations
in cores receiving the indicated treatments (17 November 1992)
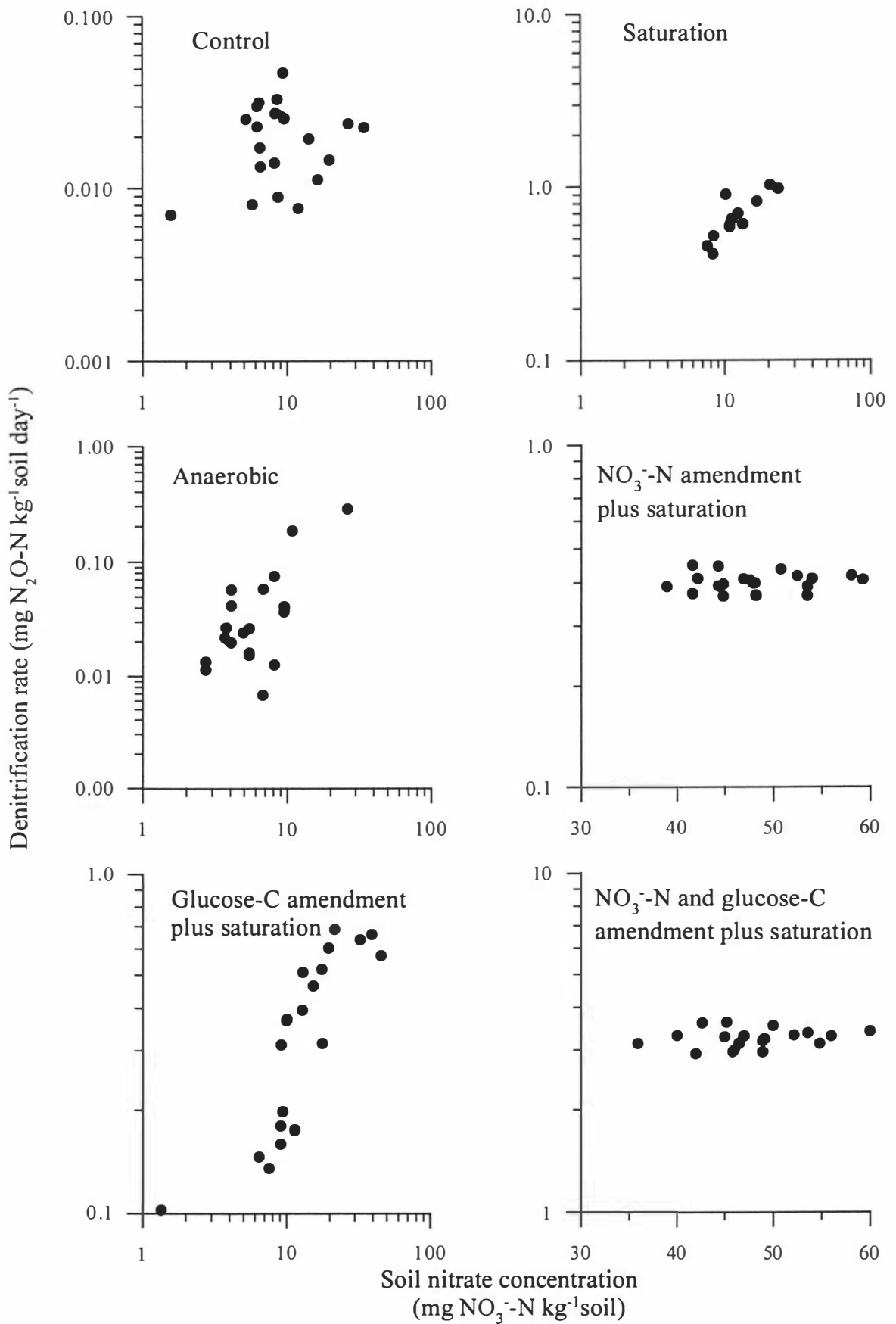
Figure 8.5 Relationship between denitrification rates and soil nitrate concentrations in cores receiving the indicated treatments (25 January 1993)
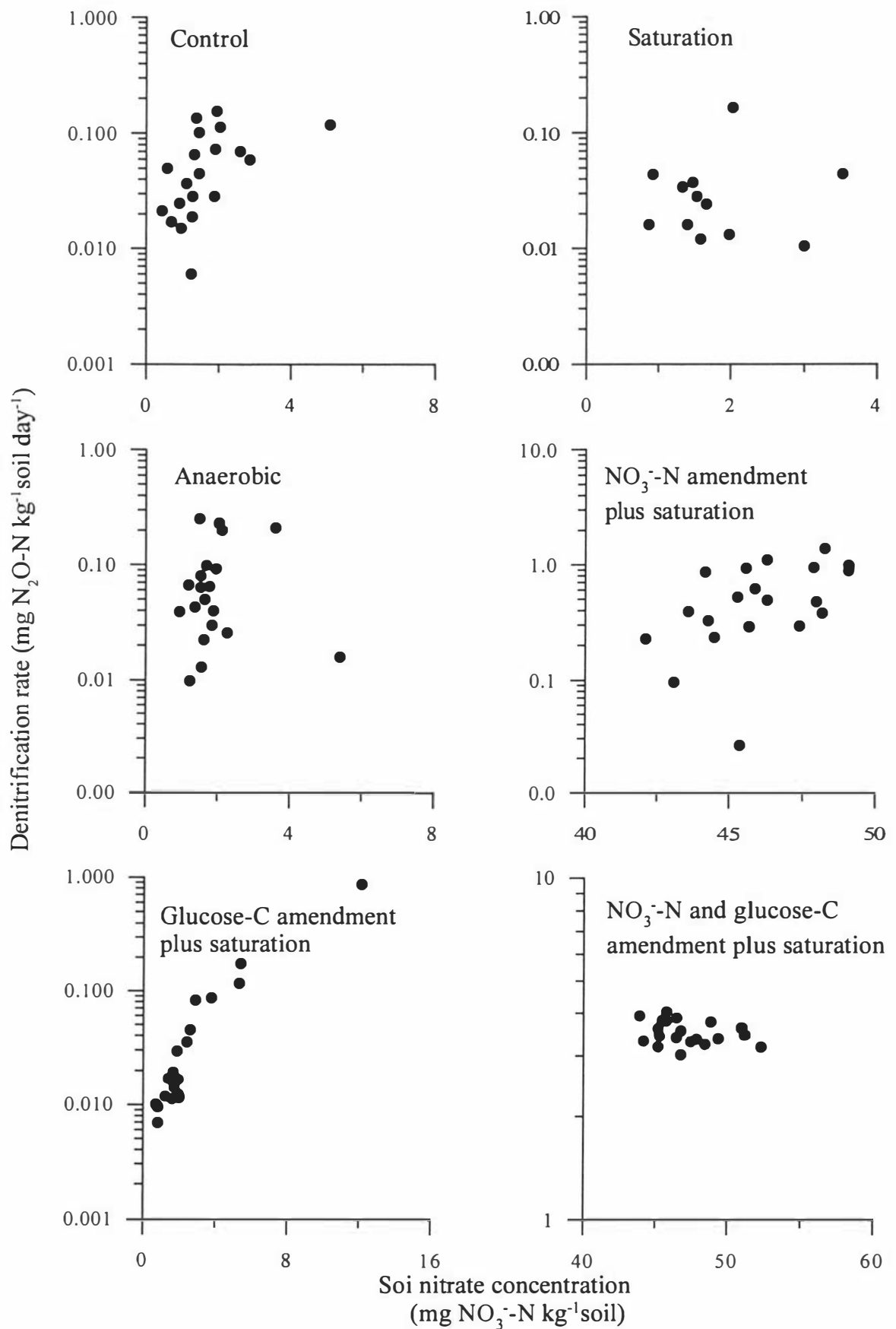
Figure 8.6 Relationship between denitrification rates and soil nitrate concentrations
in cores receiving the indicated treatments (9 June 1993)
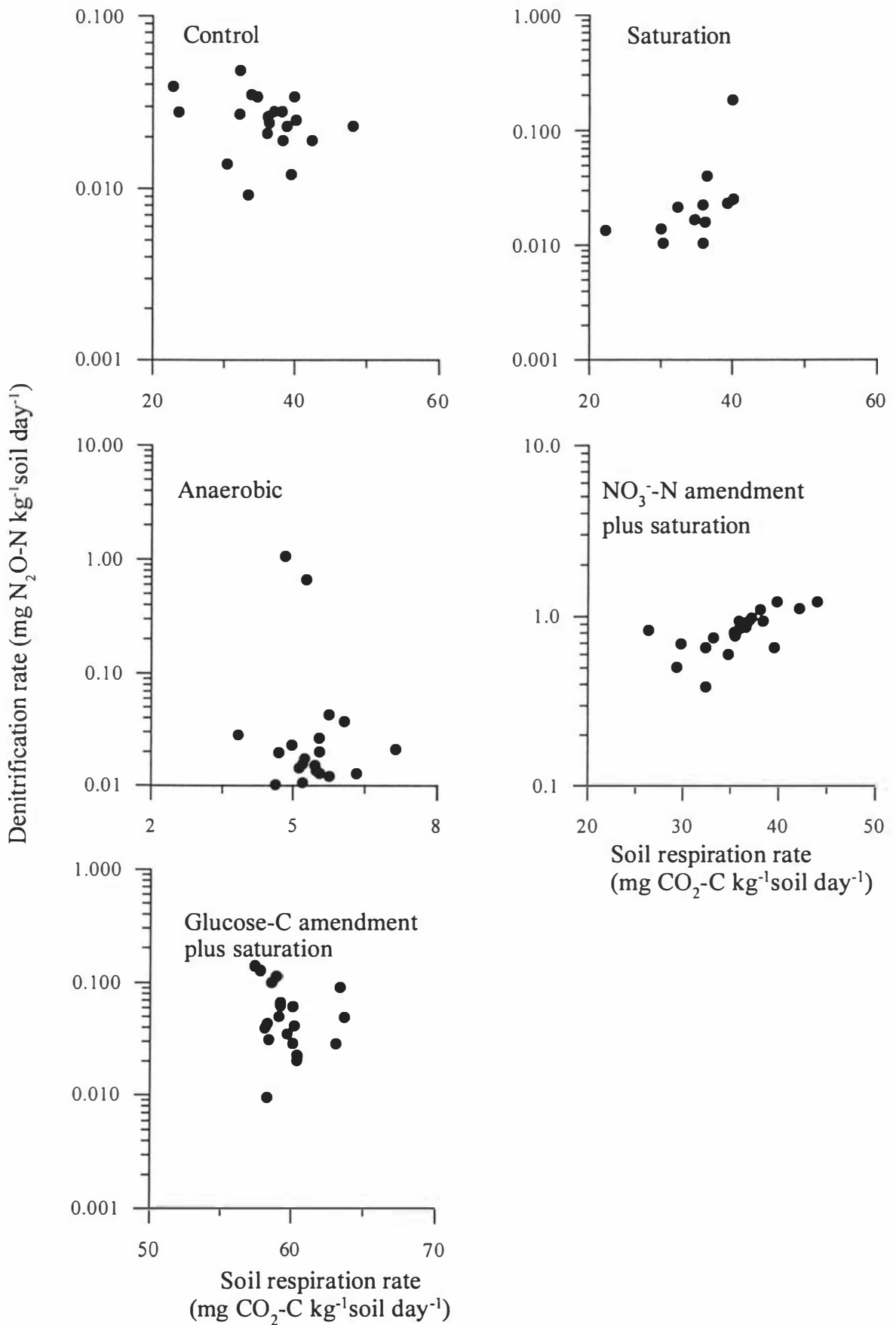
Figure 8.7   Relationship between denitrification rates and soil respiration rates
in cores receiving the indicated treatments (17 November 1992)
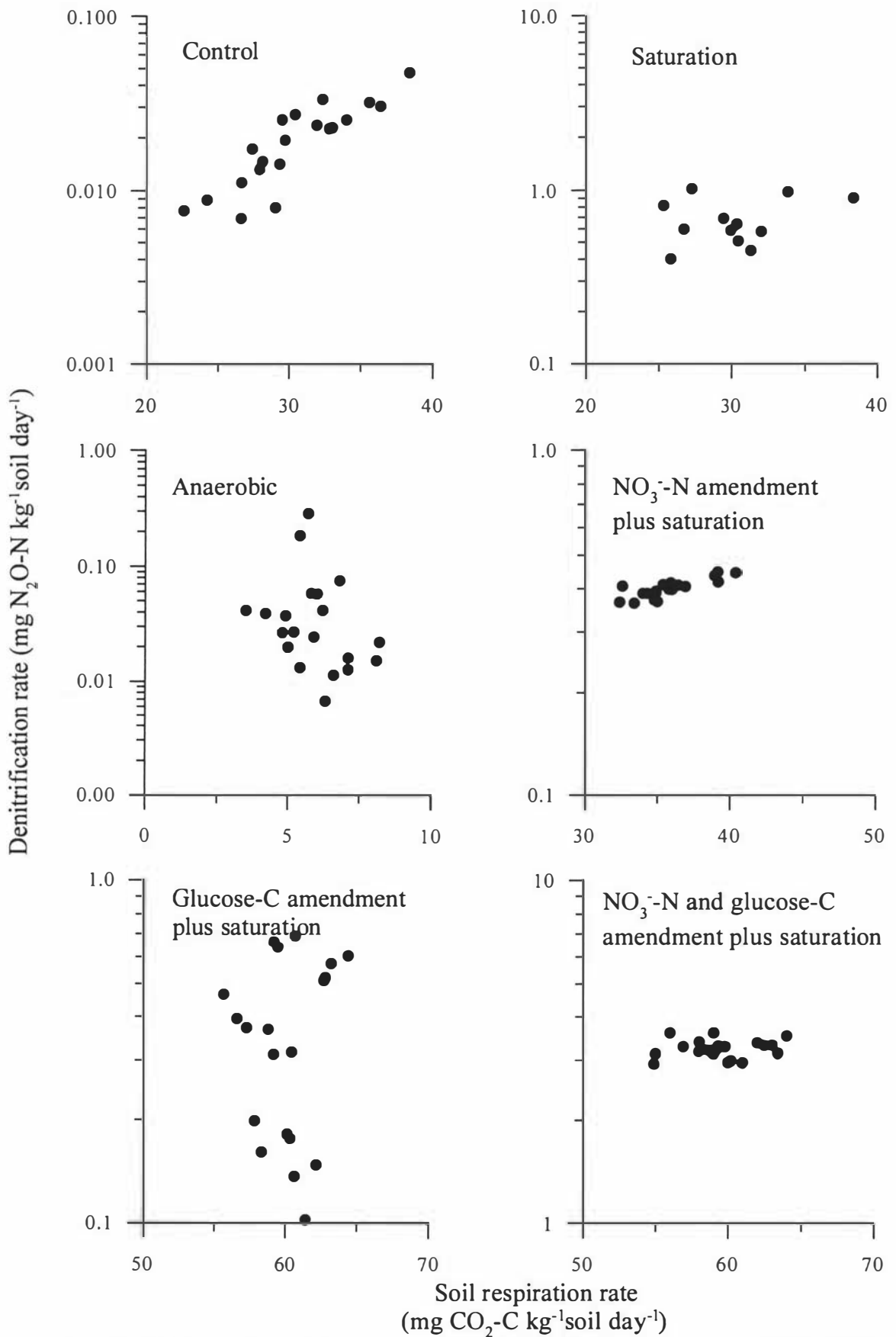
Figure 8.8 Relationship between denitrification rates and soil respiration rates in cores receiving the indicated treatments (25 January 1993)
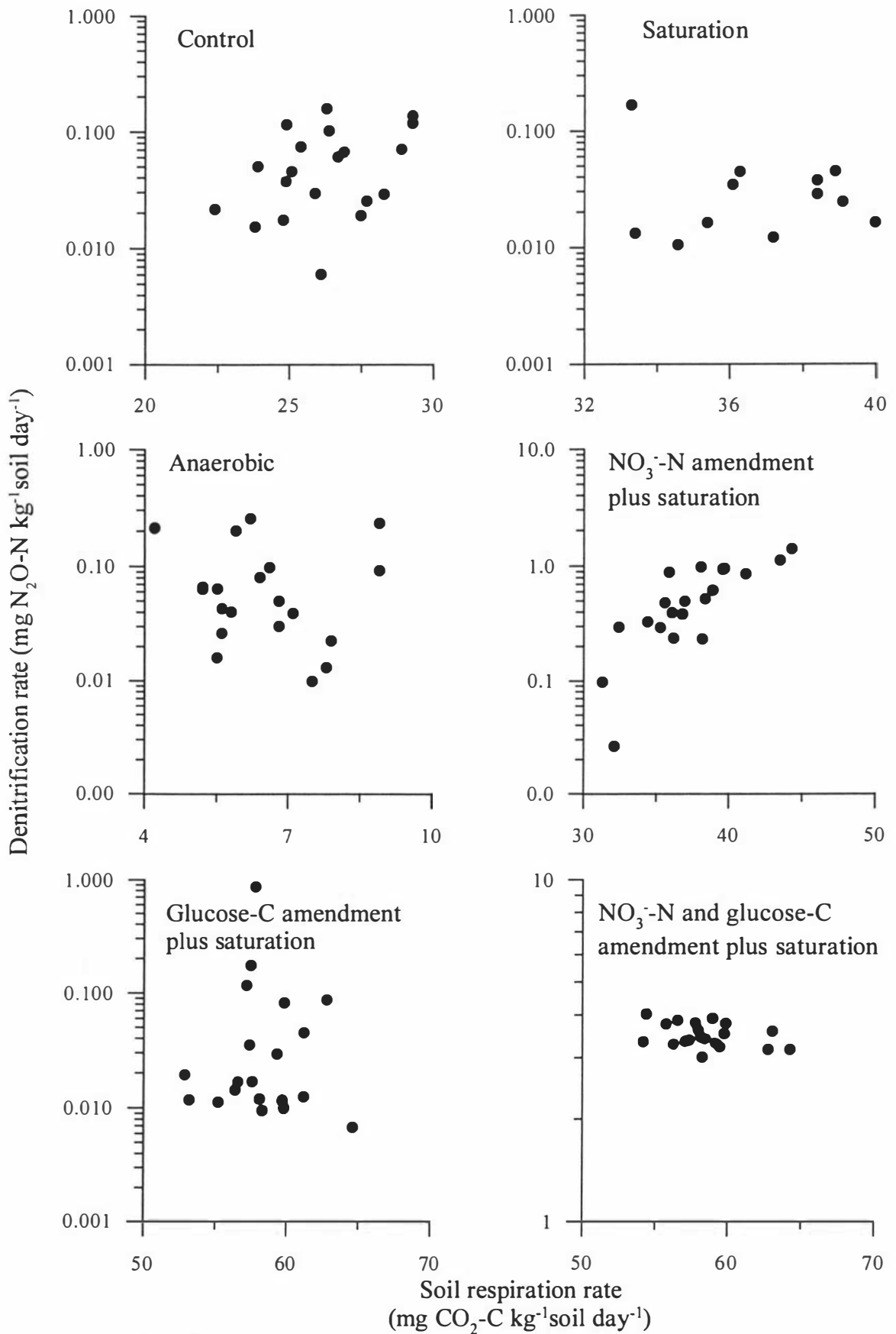
Figure 8.9 Relationship between denitrification rates and soil respiration rates in cores receiving the indicated treatments (9 June 1993)

8.4 DISCUSSION

8.4.1 Influence of soil temperature and soil water content on denitrification

Biological denitrification is an anaerobic microbial process that depends on temperature (Knowles, 1982). The data from this study confirm that denitrification in this pasture soil was limited by temperature in all seasons relative to the rates observed after incubation at 25°C in the laboratory (Figures 8.1-8.3).

In the majority of cases during our study, the soil cores that were saturated had higher rates of denitrification than those in the control (Figures 8.1-8.3). This agrees with the results of many other authors (Bremner and Shaw, 1958; Grundmann and Rolston, 1987; Myrold, 1988) who have demonstrated that soil water content is a major factor determining the rate of denitrification. Water in soil pores controls denitrification through affecting both soil aeration and substrate movement.

Denitrification requires anaerobic conditions, hence, many studies have demonstrated that the rate of denitrification can increase when O_2 concentration in soil decreases (e.g. Parkin and Tiedje, 1984; Tiedje, 1988; Arah *et al.*, 1991). Surprisingly, the results of this study revealed a general lack of denitrification rate response to removal of O_2 (Figures 8.1-8.3). This may suggest that factors other than O_2 status in this pasture were more important in controlling denitrification.

With little apparent effect of O_2 concentration on denitrification, we suggest that the observed effect of soil water content on denitrification may have been due to the easier

diffusive movement of NO_3^- or soluble-C to the microsites where denitrification was occurring in this pasture. This effect was most noticeable in samples collected in the warm, dry summer season (Figure 8.2) when initial soil moisture was at its lowest. Other studies have also suggested that diffusion can limit NO_3^-, or even C, availability to denitrification even when these materials are present at relatively high concentrations in well-aggregated soils (Ryden, 1983; Myrold and Tiedje, 1985a).

8.4.2 Availability of nitrate in soil associated with denitrification

Dramatic increases in denitrification rate after addition of NO_3^- solutions were observed in our study in all seasons. This suggests that low availability of NO_3^- may be a principal factor keeping the denitrification rate low in this pasture (Chapter 6). This result was consistent with other studies in different soils (Keller *et al.*, 1988; Elliott *et al.*, 1991).

It is interesting to note therefore that in the studies on the spatial and temporal variation of denitrification in the study area (Chapters 5 and 6) denitrification rate was not strongly related to soil NO_3^--N concentrations. The smallest effect of NO_3^- addition was found in summer (Figure 8.5) probably because there were relatively high concentrations of soil NO_3^--N at that time. But even then denitrification was still substrate limited after water addition (Figure 8.2). This may be because NO_3^- was rapidly depleted in the denitrification sites, and diffusion of NO_3^- could then limit denitrification rate (Murry *et al.*, 1989; Ambus and Christensen, 1993). Although it has been suggested that NO_3^- does not limit denitrification in most agricultural soils (Parkin and Robinson, 1989), in those cases soil NO_3^--N concentrations were generally higher than in the pasture soil studied here.

8.4.3 Availability of carbon in soil associated with denitrification

Carbon availability has been recognized as one of the most important factors controlling the denitrification rate and the spatial variability in denitrification rate (Burford and Bremner, 1975; Parkin, 1987; Christensen *et al.*, 1990a and b; Weier *et al.*, 1993). In this study we found that the response of denitrification to C addition was not much greater than that obtained by the addition of water alone (Figures 8.1-8.3). Data in Chapter 5 also indicated that the distribution of available-C in this pasture was not the main reason for high spatial variation in denitrification rate. It seems likely that soil C was not an important regulatory factor for denitrification in this pasture. This is consistent with some other field studies in pastures (e.g. Elliott *et al.*, 1991) and contrasts with the results in some cropped soils (e.g. Christensen *et al.*, 1990a and b). These results could be explained by the relatively high organic-C concentrations in our study pasture compared with many cropped soils.

The small effect of added glucose-C may also be due to immobilisation of available N in this N-limited soil in the presence of relatively high levels of available-C. This is supported by the observation that denitrification rate appeared to be correlated with soil respiration when N was made non-limiting by amendment with NO_3^- and saturation (Figures 8.7-8.9). Therefore, the effect of C on denitrification may be influenced by other soil factors in this pasture. The smaller response of denitrification rate to water plus glucose additions in winter and spring compared to summer reflects the lower available NO_3^- concentrations in those seasons (Figures 8.1-8.3). The greater quantities of denitrification in soil cores receiving both NO_3^- and glucose further suggest that at high NO_3^- concentration available-C may limit denitrification. This stimulation of denitrification may be due to either provision

of C directly to denitrifying organisms or stimulation of the other heterotrophic organisms activity leading to reduced O_2 levels in the soil (Beauchamp *et al.*, 1989). This in turn may stimulate the denitrification rate if the denitrifiers have access to NO_3^- (Sexstone *et al.*, 1985).

8.4.4 Influence of grazing on amendment effect

The results in this study again indicate that grazing events can influence denitrification from this pasture. Since NO_3^- concentration, available-C content, and denitrification enzyme activity were increased by grazing events (Chapter 7), the effects of added substrates or water were slightly greater than that in the control sites (Figures 8.2 and 8.3). However, the effect of grazing on observed denitrification response to the various amendments was less than the seasonal effects observed.

8.5 CONCLUSIONS

Data from these experiments suggested that denitrification rates in all seasons were limited by availability of NO_3^-, in particular, the accessibility of NO_3^- to the microsites of denitrification in the soil. Low availability and accessibility of NO_3^-, influenced by low water contents, were considered to be most important factors limiting denitrification in the warm, dry summer, whereas low absolute concentrations of NO_3^- were important in the other seasons. The interactive effects of NO_3^-, C and water on denitrification were clearly demonstrated in this study. The use of NO_3^- concentration or CO_2 production as predictor variables for denitrification rate is reasonable in light of observations of good associations between denitrification rates and NO_3^--N concentrations or CO_2 emission rates under no C

and N limiting, respectively. However, it is difficult to establish good correlations between denitrification rate and NO_3^--N concentrations or CO_2 emission rates on a single soil core in the study paddock for the whole year (Chapter 6). This may be due to the various limitations of C or N in different seasons, as found in this chapter.

CHAPTER 9

A PRIMARY STUDY ON THE EFFECT OF SOIL NITRATE CONCENTRATION ON DENITRIFICATION AS AFFECTED BY DIFFUSION AND NITRIFICATION

9.1 INTRODUCTION

In the field studies reported in Chapters 6 and 7, denitrification rates appeared to be related better to soil moisture content than to the concentration of soil NO_3^--N. In contrast, in Chapter 8 it was demonstrated that although dipping soil cores in water led to an increase in denitrification rate, a very much larger increase in denitrification rate was observed after soil cores were dipped into a dilute NO_3^--N solution in most seasons.

These two observations can be reconciled if it is the rate of transfer of NO_3^--N into the microsite that limits denitrification, rather than the absolute amount of NO_3^--N in the soil. In this case increasing soil moisture may enhance denitrification by reducing aeration (Sexstone *et al.*, 1985) and also by facilitating diffusion of NO_3^--N to the microsite of denitrification (Phillips *et al.*, 1978).

As the major source of N in the soil is in organic forms (Haynes, 1986), NO_3^--N, the substrate for denitrification, has to be produced from organic N by a series of microbial processes including ammonification and nitrification (Jarrell, 1990). Accordingly, in

situations when denitrification is regulated by the rate of arrival of NO_3^--N at reducing microsites, the rate of denitrification could partly reflect both the diffusion rate of NO_3^--N and/or the rate of nitrification in contiguous zones.

Previous studies have indicated that in agricultural soils, denitrification and nitrification can occur simultaneously in separate microsites at an aerobic-anaerobic interface (Starr *et al.*, 1974; Knowles, 1978). The rate of denitrification might then be controlled by the concurrent rate of nitrification. If this is so then the method used to study denitrification should not affect the nitrification process.

This is not the case during conventional denitrification studies, because the acetylene block also inhibits nitrification (Walter *et al.*, 1979; Oremland and Capone, 1988). The presence of as little as 0.1% (vv^{-1}) acetylene has been found to completely inhibit nitrification (Walter *et al.*, 1979). This concentration is far below that required to affect N_2O reductase in denitrification (Walter *et al.*, 1979; Mosier, 1980) and in the past scientists have made use of this to distinguish denitrification and nitrification as sources of N_2O produced from soils (Davidson and Swank, 1986; Klemedtsson *et al.*, 1988). More information is however required on whether inhibition of nitrification with acetylene can affect denitrification rates in short-term assays.

In the study presented in this chapter, we attempted to evaluate the use of acetylene in measurement of denitrification and also the movement of NO_3^--N on the measurement of the rate of denitrification in the soil. We used different acetylene concentrations to selectively inhibit nitrification and denitrification, in order to determine the importance of NO_3^--N produced by nitrification on denitrification in contiguous zones in soil.

9.2 MATERIALS AND METHODS

The soil used was a Tokomaru silt loam with a pH of 5.8 and total C of 4.8%. During soil sampling obvious urine and dung patches in the paddock were avoided to reduce the initial N concentration of the soil. Before use, the field soil was sieved to <6 mm. In the investigation of NO_3^--N diffusion on denitrification, a factorial experiment was conducted at three moisture contents (25%, 39% and 67% ww^{-1}) and three concentrations of soil NO_3^--N (1.3, 6.3 and 26.3 $\mu g\ NO_3^-$-N g^{-1} soil). The designed moisture and NO_3^--N concentrations were achieved by adding various amounts of H_2O and KNO_3 solution to the soil. The treated soil samples, equivalent to 80 g of oven-dry soil were placed in 1.1 litre incubation vessels, each sealed with a lid fitted with a rubber septum. Aerobic incubation conditions were maintained in these treatments. There were three replicates of each treatment. Approximately 10% of the volume of the air headspace was replaced with 60 ml of purified acetylene. The samples were incubated in a constant temperature chamber at 25°C. Gas samples were taken at 1, 6, 24, 36, 60, 100, 126 and 150 hours after the incubation started. The technique of gas sampling and analysis for N_2O have been presented in Chapter 3. Exchangeable NO_3^--N and soil moisture content were determined in the bulk sample before the incubation, and in samples from individual treatments at the conclusion of the experiment.

Treatments in the second experiment to investigate inhibition of nitrification by acetylene in denitrification measurements consisted of a control and acetylene at both 0.1 and 10% vv^{-1}. This study was conducted at two moisture contents (25% and 39% ww^{-1}), with soil samples equivalent to 300 g of oven-dry soil placed in 1.1 litre incubation vessels. There were also three replicates of each treatment. The samples were placed in a constant

temperature chamber at 25°C. The soils were aerated with laboratory air and thoroughly mixed at 12, 24, 48 and 96 hours after the incubation started. Acetylene at the appropriate concentration was added to the incubation vessels each time after aeration. Gas samples for N_2O analysis were taken before each aeration, and small samples of soil were taken for NO_3^--N analysis at each aeration.

9.3 RESULTS

9.3.1 Diffusion experiments

Denitrification rates increased greatly with increasing soil moisture content (Figures 9.1-9.3). The initial high rates of denitrification in the soils incubated at 67% (ww^{-1}) remained constant until more than 90% of the NO_3^--N initially in the soil was denitrified (Figure 9.1). In contrast, the initial rates of denitrification decreased with incubation time at a moisture content of 39% ww^{-1} (Figure 9.2), and denitrification virtually ceased after one-and-a-half days incubation in the driest soils with a moisture content of only 25% ww^{-1} (Figure 9.3). This was despite analyses of soil NO_3^--N from individual treatments after incubation showing that more than 75% and 95% of initial soil NO_3^--N still remained in the soil samples at soil moisture of 39% and 25% (ww^{-1}), respectively (Table 9.1).
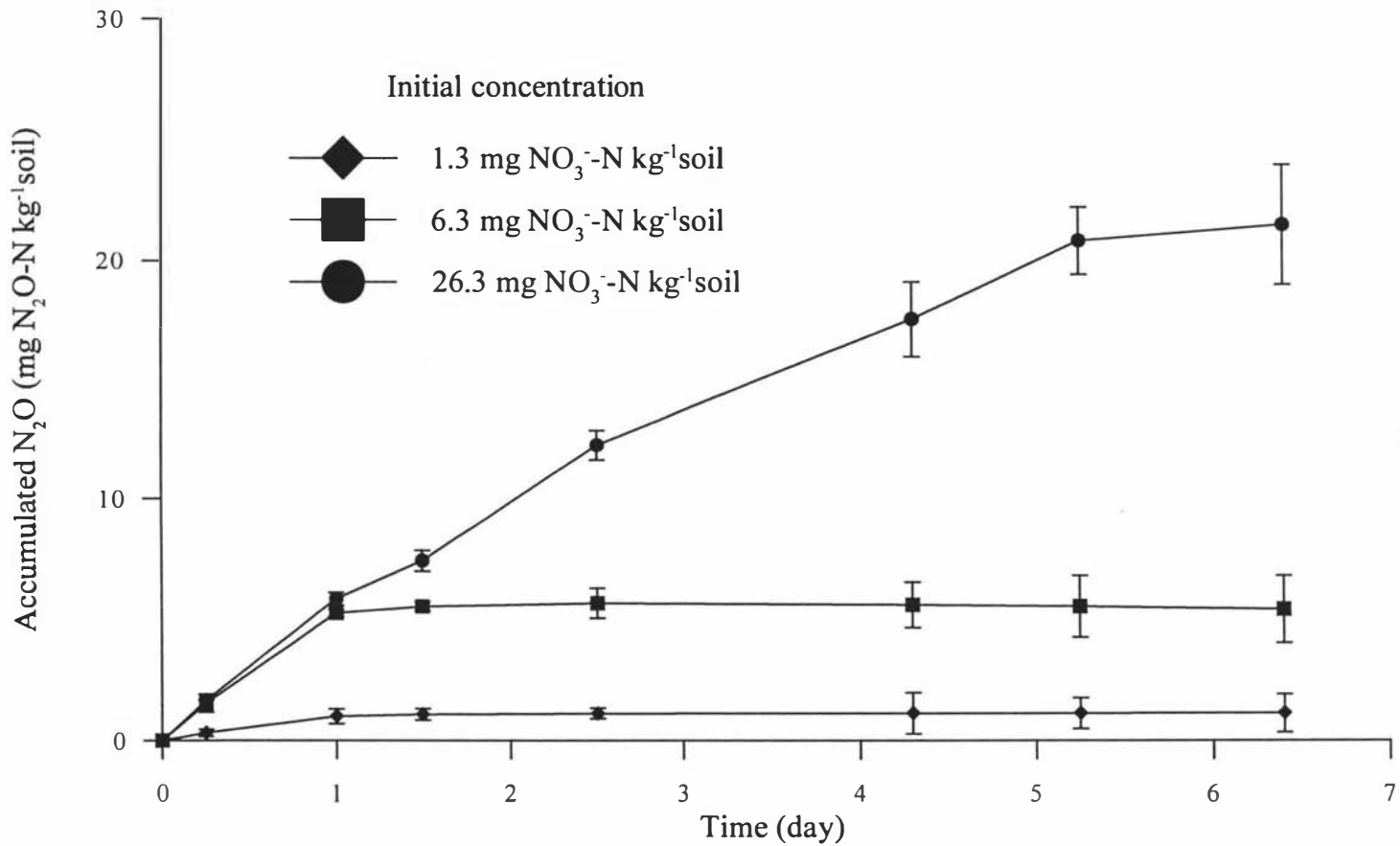
Figure 9.1 Accumulated N_2O from denitrification in soils incubated at 67% (ww⁻¹) moisture content and containing different initial concentrations of NO_3^--N (bars represent SD)
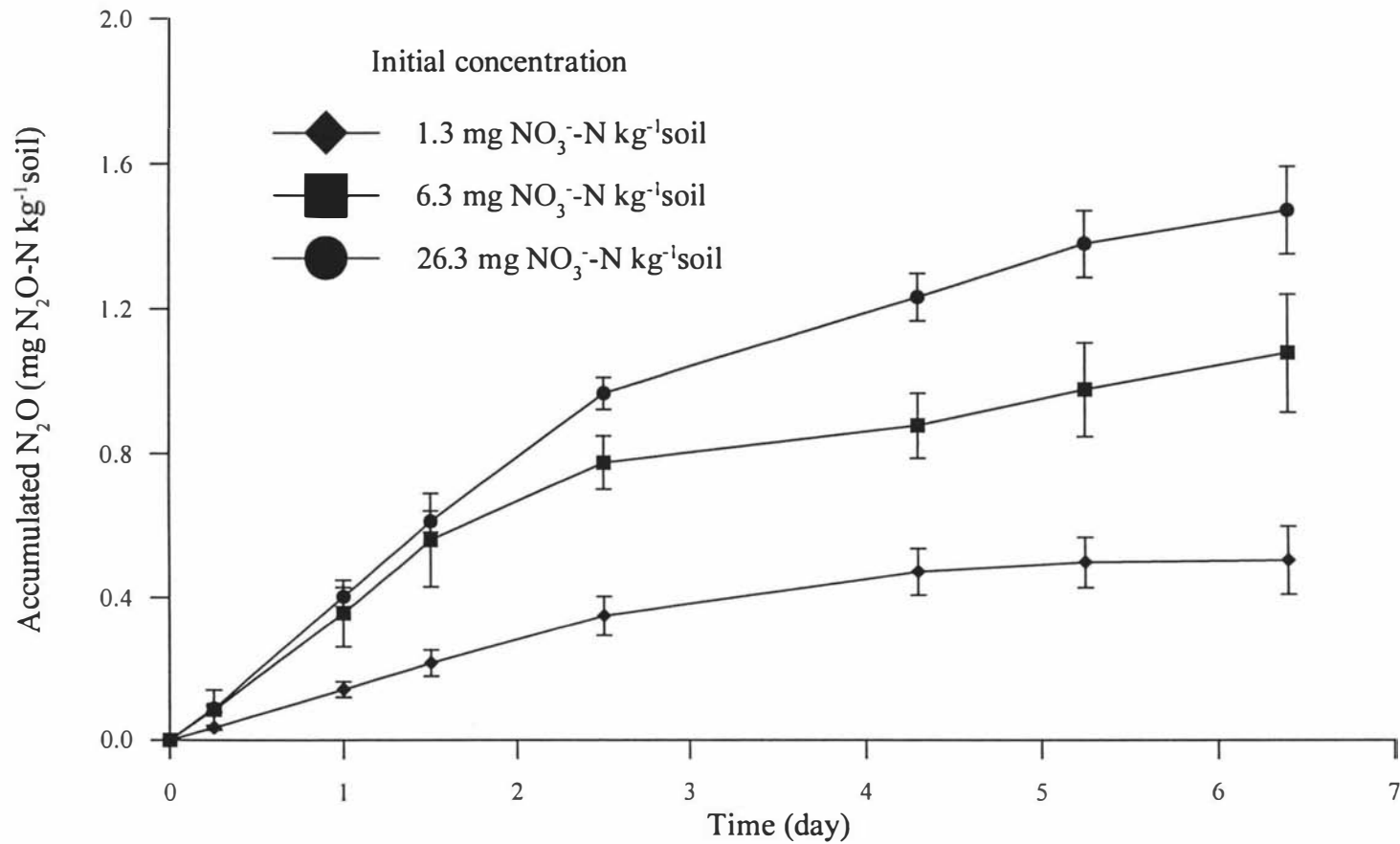
Figure 9.2 Accumulated N_2O from denitrification in soils incubated at 39% (ww^{-1}) moisture content and containing different initial concentrations of NO_3^--N (bars represent SD)
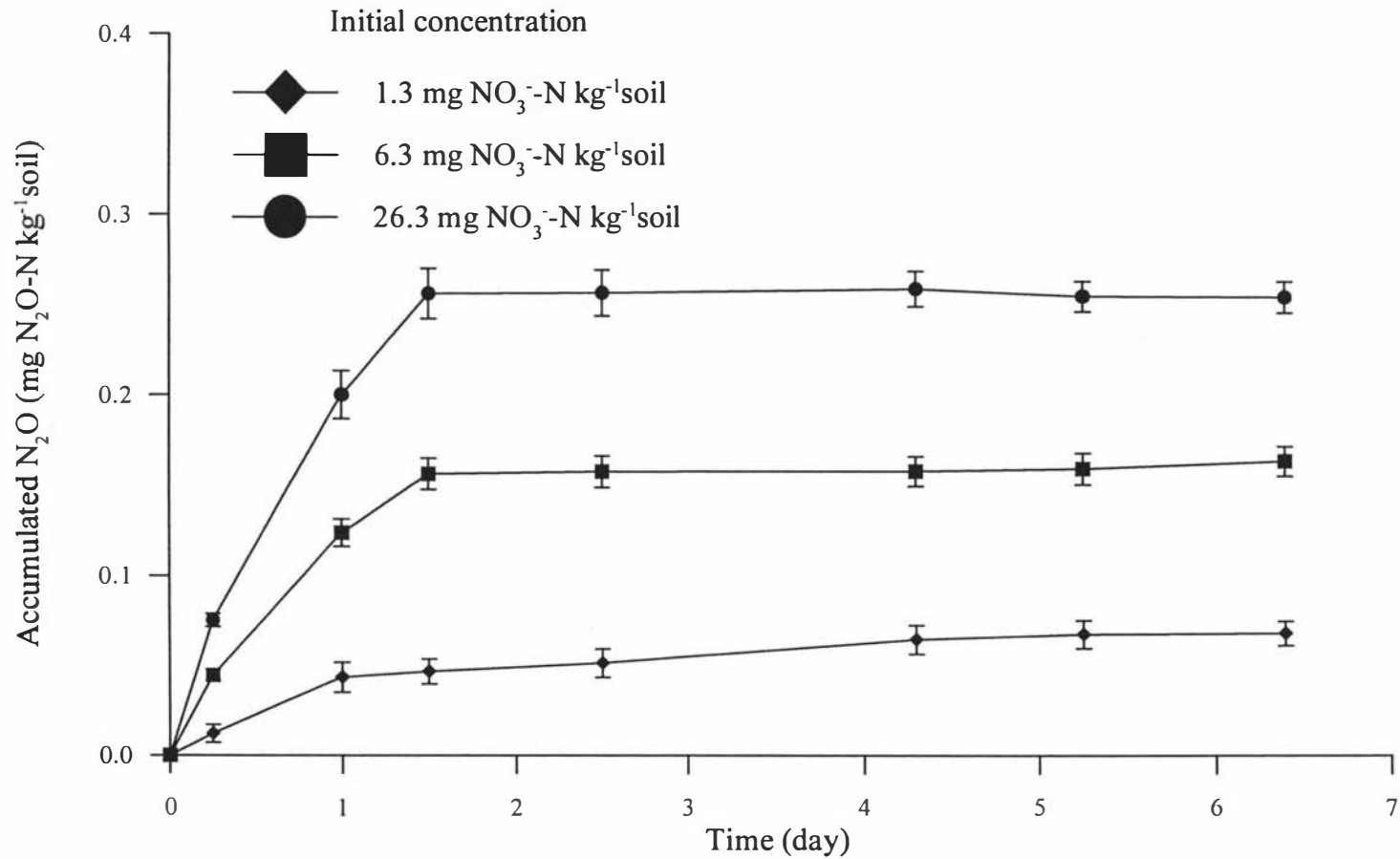
Figure 9.3 Accumulated N_2O from denitrification in soils incubated at 25% (ww^{-1}) moisture content and containing different initial concentrations of NO_3^--N (bars represent SD)

Table 9.1 Change in NO_3^--N concentration in bulk soil during incubation

Soil moisture (% ww^{-1})	NO_3^--N (mg NO_3^--N kg^{-1}) (before incubation)	NO_3^--N (mg NO_3^--N kg^{-1}) (after incubation)
	1.3	1.0
25	6.3	5.7
	26.3	25.9
	1.3	0.8
39	6.3	5.3
	26.3	25.2
	1.3	0
67	6.3	0
	26.3	4.1

9.3.2 Inhibition of nitrification by acetylene in relation to denitrification

Data for N_2O emission rates in soils of 25% (ww^{-1}) moisture content are plotted in the histograms (Figure 9.4). When the full acetylene block was in place (10% vv^{-1}) for measuring full denitrification, N_2O emissions in the short-term (within 24 hours) increased some two-and-a-half-fold compared to that from the samples in which acetylene had not been applied (Control). When the low level acetylene (0.1% vv^{-1}) was in place, N_2O emission was virtually unchanged in the short-term (within 24 hours) compared to the control, indicating that this low concentration of acetylene did not interfere with product gas ratios (N_2O/N_2) by denitrification from the soil. Over time, N_2O emissions from both the low and high levels of acetylene treatments declined, whereas N_2O emission continued at a more or less constant rate in the control. Inorganic N measurement showed that NO_3^--N fell a little in both treatments receiving acetylene (Figure 9.5). But in the control, soil NO_3^--N increased progressively across the whole incubation experiment (Figure 9.5).

Figure 9.4 N_2O emission rates in soil incubated at 25% (ww^{-1}) moisture content with different concentrations of acetylene

Figure 9.5 Concentrations of NO_3^--N in soil incubated at 25% (ww^{-1}) moisture content with different concentrations of acetylene

Figure 9.6 gives the rate of N_2O produced in the different treatments at a higher moisture content (39% ww^{-1}). The patterns of change in N_2O production rate over time at this higher moisture content were similar to those at the lower moisture content under both acetylene treatments (Figures 9.4 and 9.6), but the rate of N_2O emission from the control increased over time (Figure 9.6), whereas the rates were constant over time in the control at the lower moisture content (Figure 9.4). Data for soil NO_3^--N concentrations remaining after incubation were consistent with the differences between original NO_3^--N in soils before incubation and N_2O-N emission loss in both treatments receiving acetylene (Figure 9.7). But soil NO_3^--N increased with incubation time in the control (Figure 9.7), and the NO_3^--N concentration was slightly lower than when soil was drier (Figures 9.5).

9.4 DISCUSSION

9.4.1 Nitrate-N concentration at denitrification sites

High denitrification activity in soil has been found to occur in "hot-spots" of particulate organic matter on a microscale (Parkin, 1987) and in macroscopic aggregates where microbial activity produces anaerobic conditions (Smith, 1980). A continuous supply of NO_3^--N is required for denitrification to continue in these spots and thus NO_3^--N in the bulk soil needs to move to the anaerobic sites where denitrification can occur. Soil conditions, such as soil moisture content and NO_3^--N concentration, can control the movement of NO_3^--N, and consequently perhaps affect denitrification rate.

In this study, when the soils were saturated, the initial high rate of denitrification continued until most of the NO_3^--N in the soil was consumed at all initial NO_3^--N concentrations
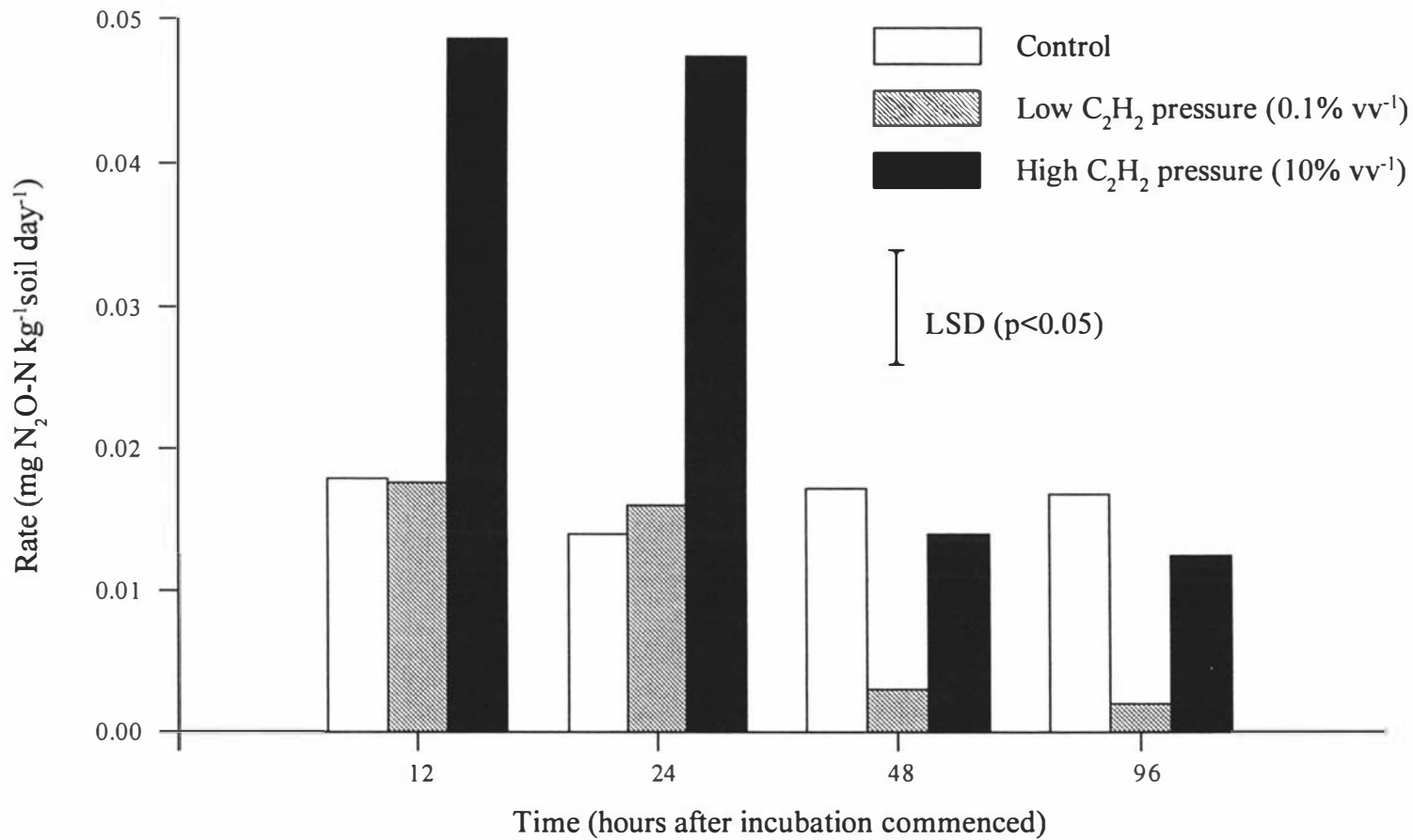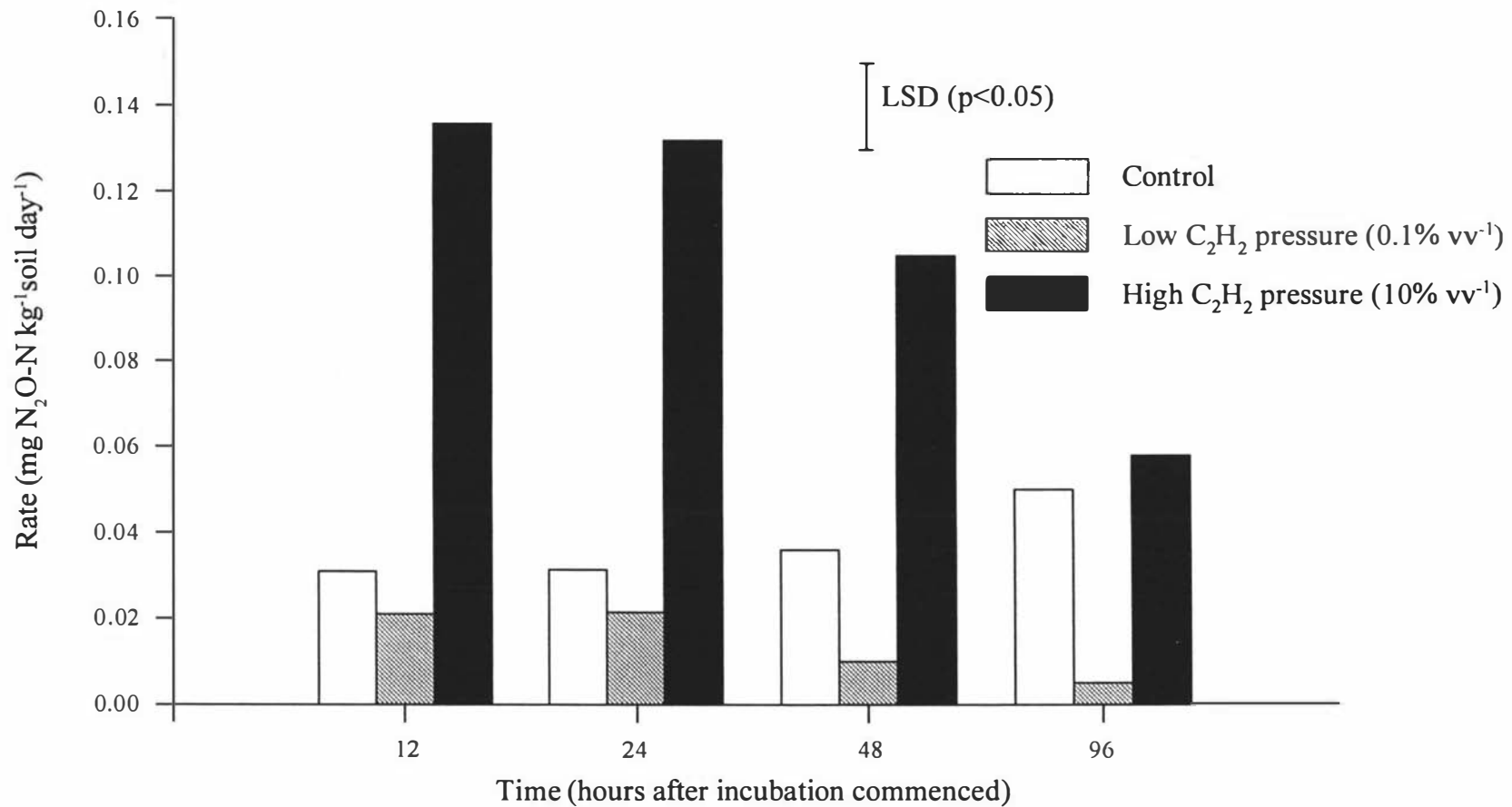
Figure 9.6 N_2O emission rates in soil incubated at 39% (ww^{-1}) moisture content with different concentrations of acetylene

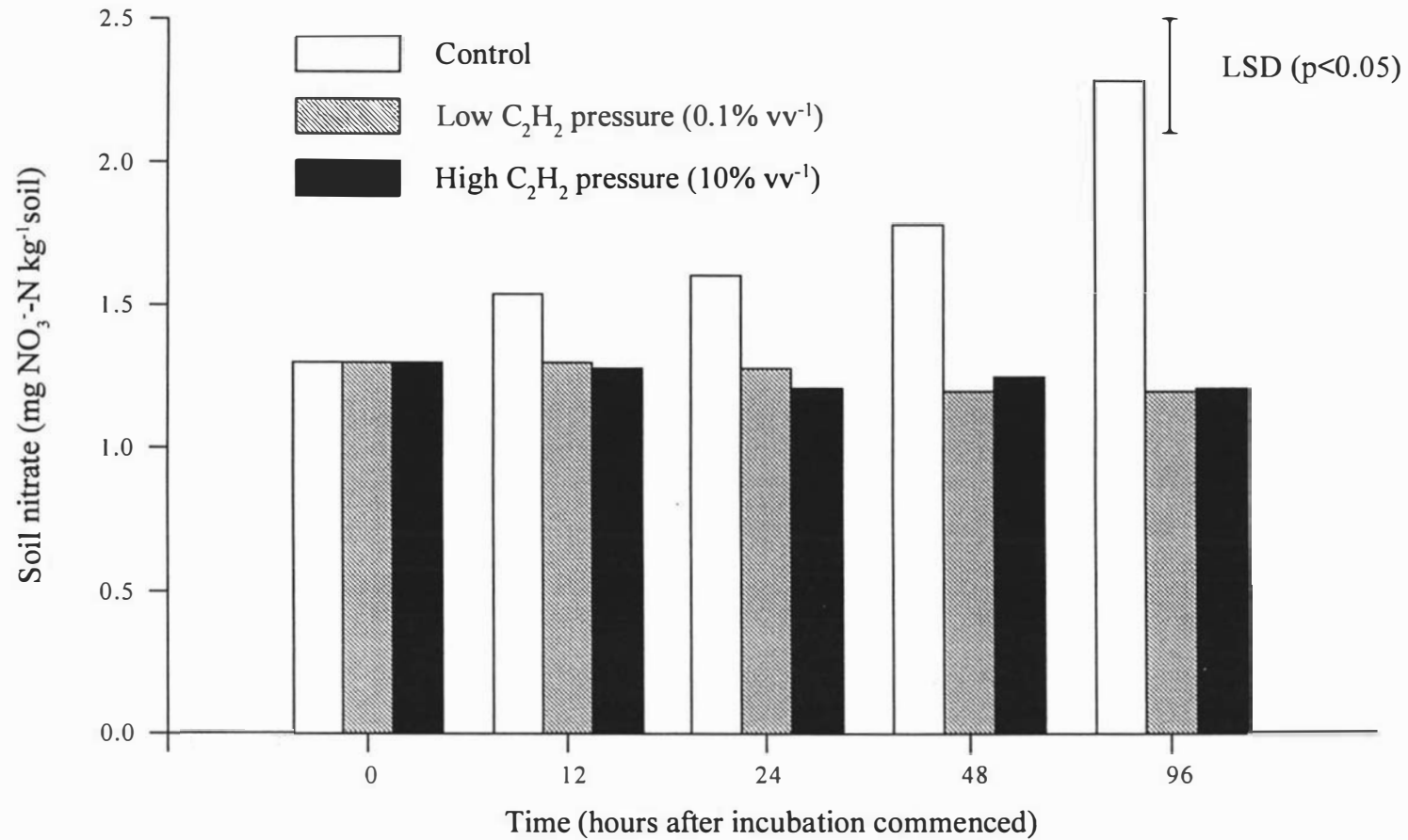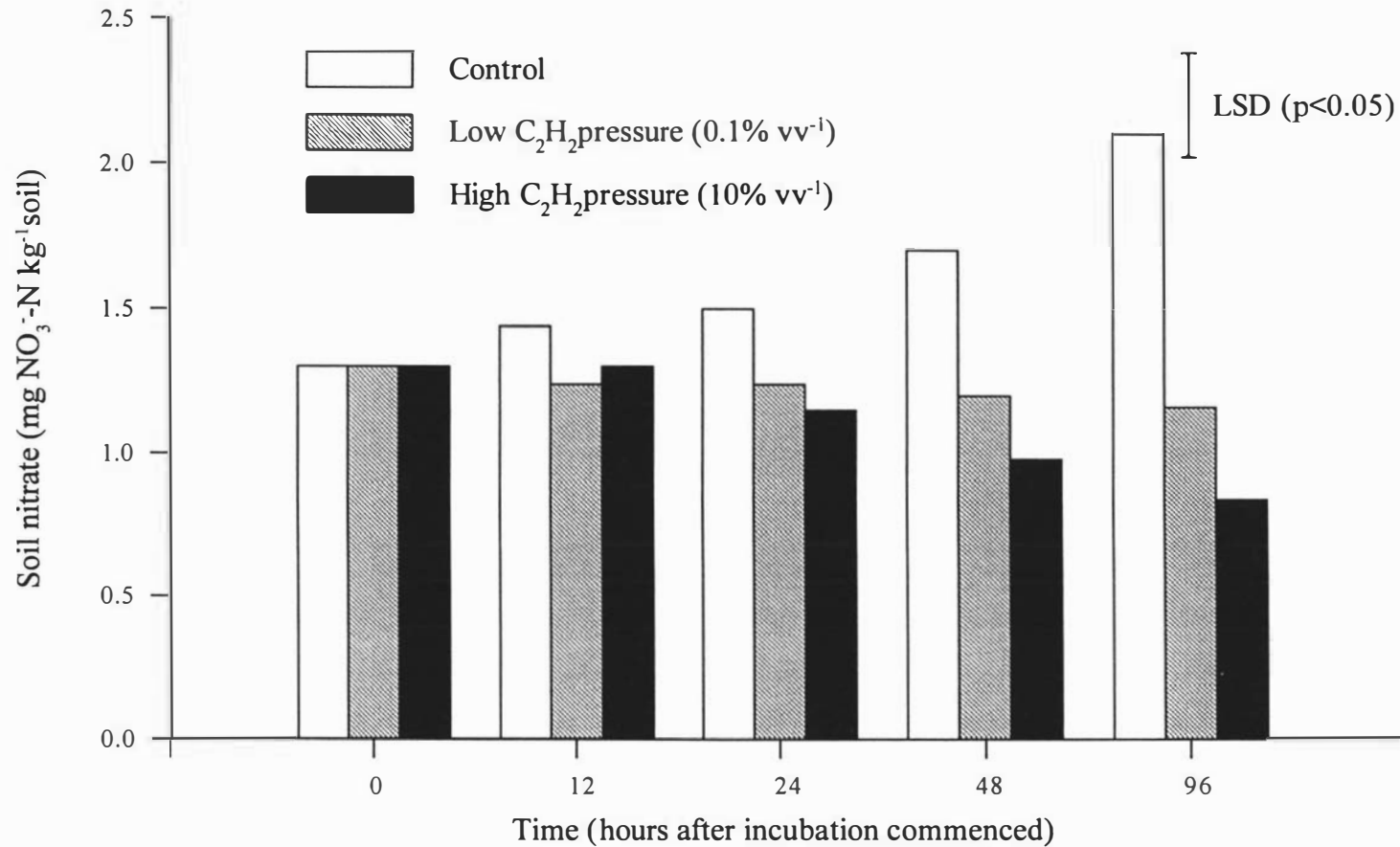Figure 9.7 Concentrations of NO_3^--N in soil incubated at 39% (ww^{-1}) moisture content with different concentrations of acetylene

(Figure 9.1). In this situation, the high moisture content favoured both NO_3^--N movement and the creation of anaerobic sites. This allowed the denitrification rate to follow first order kinetics at the concentrations of NO_3^--N in our study (Chapter 3; Firestone, 1982; Tiedje, 1988). Data in Figures 9.2 and 9.3 demonstrate that in the drier soils denitrification activity slowed markedly after initial periods of relatively high denitrification activity. This may suggest that all the NO_3^--N at denitrification sites had been rapidly consumed, and that the remaining NO_3^--N in the rest of the soil could not reach the sites to replace the denitrified NO_3^--N, because its movement was restricted in these soils containing lower moisture.

Data for bulk soil NO_3^--N (Table 9.1) provide some supports to this suggested explanation. The small drop in NO_3^--N in the bulk soil may reflect a marked drop at or near the denitrification sites and little change in NO_3^--N concentration throughout the rest of the soil. A model developed by Myrold and Tiedje (1985a) also suggested that NO_3^--N diffusion may become limiting for denitrification in soil aggregates with a radius larger than 2 mm.

9.4.2 Influence on denitrification of nitrification inhibition by acetylene

N_2O gas production may arise from both denitrification and nitrification in agricultural soils (Bremner and Blackmer, 1978; Breitenbeck et al., 1980; Robertson and Tiedje, 1987; Klemedtsson et al., 1988), and the predominant N_2O production processes have been found to vary under different conditions (Robertson and Tiedje, 1987). Since a low level of acetylene can fully inhibit nitrification and this level of acetylene will not affect the conversion of N_2O to N_2 in denitrification (Walter et al., 1979), the insignificant differences in the amounts of N_2O produced from the soil with the low level of acetylene treatment (0.1% vv^{-1}) or without acetylene treatment at 12 and 24 hours after the incubation

commenced (Figures 9.4 and 9.6) may indicate that the measured N_2O was mostly produced by denitrification in the control soil and the soil treated with the low level of acetylene. The differences in N_2O emission rate at 12 and 24 hours after the incubation commenced between the control or the 0.1% acetylene treatment and the 10% acetylene treatment (Figures 9.4 and 9.6) reflected N_2 production from denitrification in the control and the soil treated with the low level of acetylene, assuming the total denitrification ($N_2 + N_2O$) rates were the same initially in all three treatments.

After 24 hours there was a significant decrease in the rate of denitrification in both treatments receiving acetylene, but not in the control which, if anything, increased with time at the high moisture content (Figures 9.4 and 9.6). Although only N_2O production (not N_2 + N_2O as measured in the high acetylene treatment) was measured in the control, the N_2O production rate in the control was slightly larger than the total denitrification rate (N_2 + N_2O) measured with the high level of acetylene (Figure 9.4). This indicates that the total denitrification rate in the control was likely to be larger than in the acetylene treatments after 24 hours. Data in Figures 9.5 and 9.7 demonstrate an effect of both low and high levels of acetylene on nitrification. Therefore, the decrease in denitrification rate in the presence of both acetylene treatments (Figures 9.4 and 9.6) may be due to a limited NO_3^--N supply in the soil . However, the NO_3^--N concentration in the bulk soils receiving acetylene did not change much with time (Figures 9.5 and 9.7), suggesting that the decrease in NO_3^--N concentration would have only occurred in the small denitrification sites.

More NO_3^--N produced by nitrification in the control than in both soils receiving acetylene during the 96 hours incubation (Figures 9.5 and 9.7) may suggest that the larger N_2O emission rates in the control at longer times (Figures 9.4 and 9.6) were due to the NO_3^--N

produced in the soil. This suggestion raises a concern for long-term denitrification measurements made with acetylene inhibition, since the concurrent inhibition of nitrification by acetylene may affect the rate of denitrification when NO_3^--N supply is limited (Tiedje *et al.*, 1989).

At times less than 24 hours however there was no evidence of acetylene reducing denitrification activity (Figures 9.4 and 9.6). Therefore, use of acetylene in denitrification measurements should perhaps be limited to short-term (<1 day) studies (Walter *et al.*, 1979). Other studies have also claimed that prolonged exposure to acetylene can stimulate denitrification where NO_3^--N is not limiting (Yeomans and Beauchamp, 1982; Cooke and White, 1988), as soil microorganisms may use acetylene as a C source.

9.5 CONCLUSIONS

With an increase in soil moisture content, initial denitrification rates were higher and continued for a longer time, resulting in an apparently greater proportion of the soil NO_3^--N being denitrified. This reflects the effect of diffusion rate of NO_3^--N on denitrification in the soil.

The acetylene blockage technique could under-estimate denitrification rate when soil NO_3^--N concentrations were low, by simultaneously blocking nitrification and thereby limiting provision of NO_3^--N substrate. This problem could be more serious in under-estimation of denitrification when the soil is dry. However, our results indicate that the major impact would not be observed during a 24 hours incubation of the tested soil.

CHAPTER 10

SYNTHESIS AND SUMMARY

10.1 INTRODUCTION

Studies of denitrification can be traced back a long time. However, the number of these studies has increased greatly since the acetylene inhibition technique for measuring denitrification was developed, and many contributions have been made towards a better understanding of the mechanisms of denitrification within the last decade. With both agricultural and environmental concerns about denitrification, research has now focused on quantitative measurement of the magnitude of nitrogen (N) loss through denitrification in the field.

Studies of the N cycle in New Zealand pastures have indicated large N losses from the cycle. It is possible that denitrification is one of the important processes causing these losses. At present however there are few quantitative estimates of the contribution of denitrification in pasture soils to regional and global N cycling. The present study was undertaken to quantify the extent of N loss through denitrification in a paddock within an intensive dairy-farm, and to examine the relationships between denitrification rate and various environmental and soil factors.

10.2 NITROGEN LOSS THROUGH DENITRIFICATION

A short-term assay for measuring soil denitrification enzyme activity (DEA) in the field was developed. It involved anaerobic incubation of the saturated soil samples with non-limiting NO_3^--N and available-C and the measurement of N_2O emission rate in the presence of acetylene at a constant laboratory temperature (Chapter 3). Field-moist samples of soil retained their DEA during 5 days' storage at either 2 or 20°C, but thereafter the DEA declined with time. Drying and storage of air-dried soil also affected DEA. To avoid any effect of growth of denitrifying organisms on DEA in the assay, the incubation period should not exceed 5 h at 20°C, and the optimum concentrations for NO_3^--N and glucose-C in the assay were 50 and 300 μg g^{-1} soil, respectively.

Variations in denitrification activity with depth in soils under pasture at different times of the year were studied (Chapter 4). Denitrification enzyme activities (DEAs), measured in the soil under saturated and anaerobic conditions in the presence of non-limiting C and NO_3^--N, had their maximal values in the surface soil and generally decreased exponentially with depth, regardless of sampling times in different seasons. But the DEA was still considerable even at a depth of 20-40 cm, which indicates that microorganisms capable of denitrification occurred at depth in these soils under pasture. In addition, DEAs did not show marked temporal change between the early summer and early winter samples, nor before and after a rainfall event in the autumn

Field denitrification rates were measured in a dairy-farm paddock over about an 18 months period using *in situ* cores with the acetylene block technique. High coefficients of variation and skewed distributions of denitrification rate were observed. A log-normal distribution

provided a better fit than a normal distribution in 76 out of the 82 data sets from all topographical sampling sites and measurement dates (Chapter 5). The study indicated that the spatial variation in denitrification rate was lower in relatively dry soil, increased with rainfall, but eventually decreased again in very wet soil after prolonged rainfall. Large variation among denitrification rates also occurred after grazing.

An analysis of the spatial dependence of the variability at the flat land site indicated that either there was no spatial dependence of the denitrification rate or that the spatial dependence was very short range and less than the minimum sampling lag of 0.3 m, irrespective of sampling times.

There were, however, distinct differences in the mean denitrification rate at the different topographical sites in the dairy-farm paddock being studied. Denitrification rates were generally highest in the compacted gateway and moist gully areas.

Denitrification followed a marked seasonal pattern in the study paddock, with the rate being highest in cool, wet winter conditions and lowest during the warm, dry summer (Chapter 6). However, denitrification rates increased after rainfall events for a short period in the summer (Chapters 4, 6 and 7). The annual loss of N through denitrification in this paddock appeared to be only around 4.5 kg N ha^{-1}. This is very low compared to other possible pathways of N loss.

In the moist, cool winter, animal grazing at a high stocking rate increased denitrification rate significantly for about 2 weeks after grazing (Chapter 7). However, the total N loss through denitrification during that period was small, with less than 1% of the N returned

in urine by the grazing animals being lost through denitrification in the 2 weeks following grazing. In the dry, warm summer, no systematic effect of grazing at a low stocking rate on denitrification was observed, even though higher concentrations of soil NO_3^--N persisted for a long period after the grazing event.

10.3 FACTORS REGULATING DENITRIFICATION

In developing optimum conditions for the short-term denitrification enzyme activity assay, both available-C and NO_3^--N were found to limit denitrification rate under the anaerobic and saturated incubation conditions of the laboratory test (Chapter 3). A study of the factors most limiting denitrification in soils from different depths on several sampling occasions throughout a year again revealed that at all sampling times available-C limited denitrification activity, particularly in the subsurface soils (Chapter 4). Nitrate-N also limited the denitrification activity at some depths, especially in the surface soils when native soil NO_3^--N concentrations were low (Chapter 4). This study also suggested that heavy rainfall may wash some soluble-C and NO_3^--N from the surface down to the subsurface soils, and this may cause an increase in the field denitrification rate at depth after rainfall.

Higher field denitrification rates were generally observed when soil moisture contents were high for extended periods in winter, and during brief periods after rainfall events in the other seasons (Chapters 4, 6 and 7). However, the observed temporal pattern of denitrification rate in the field was not associated with the temporal changes in soil NO_3^--N concentration. The effect of animal grazing on denitrification rate was also influenced by soil conditions, particularly soil moisture content, in different seasons. Denitrification rates

significantly increased for about 10 days after grazing in the winter when soil moisture contents were conducive to denitrification in the soil.

A statistical approach was used to examine the effects of environmental and soil factors on denitrification rate in the field, both temporally and spatially in the study (Chapter 6). Correlation and multiple regression analyses indicated that the relationships between edaphic variables and denitrification rate in single soil cores were not consistent across the sampling sites in the paddock, and differed among the sampling dates (Chapter 6). Relationships between point measurements of denitrification and other edaphic factors were also poor for the combined data set, comprising all the sampling sites over the year. However, the study indicated that relationships between denitrification rate and NO_3^--N concentration, as well as with soil respiration rate, varied between the data sub-sets depending on the soil moisture content. It was observed that weak relationships between denitrification rate and NO_3^--N concentration, and relatively good relationships between denitrification rate and respiration rate, existed at low soil moisture contents. Opposite results were obtained at high soil moisture contents (over about field capacity). At low soil moisture contents, diffusion of NO_3^--N to denitrification sites may be more limited by soil moisture than by the concentration of NO_3^--N. Whereas denitrification rates were more closely related to soil NO_3^--N concentration when soil moisture contents were high. The better relationship between denitrification rate and respiration rate at low soil moisture contents may suggest that O_2 consumption by respiration is necessary to produce anaerobic sites which can then stimulate denitrification in the dry soils.

Mean denitrification rates in the field from individual dates were positively correlated to soil moisture content, and negatively correlated to all other measured variables in almost all the sampling sites and also for the aggregated data for the whole paddock (Chapter 6). Regression

equations derived from the mean-value data for each sampling date improved the prediction of the observed denitrification rate, compared to those from the individual core data sets. Soil moisture content and NO_3^--N concentration together accounted for 51% of the observed variability in mean denitrification rate in the field.

The statistical analysis of factors correlated to denitrification rate discussed above suggests that some of the large spatial and temporal variation observed in soil denitrification rate in the study paddock could be attributed to spatial and temporal changes in soil water content, availability of NO_3^--N, available-C level, soil temperature and to complex interactions between these variables. Further laboratory experiments were then conducted to examine more closely the factors most limiting denitrification rate in different seasons and to understand the causes of the variation in denitrification in the study pasture (Chapter 8).

Soil temperature in the field was found to limit denitrification rate in all seasons relative to the denitrification rate measured at 25°C in the laboratory. This temperature effect was greatest in the cool, wet season. In the study area, the high soil water contents in the winter necessary for the denitrification process were associated with low temperatures. During this period, soil NO_3^--N supply for denitrification was also restricted, and the rates of denitrification increased when a solution of NO_3^--N was added to the soil cores. Although the limiting effect of soil NO_3^--N on denitrification rate in the summer was not as apparent as in the winter, the rate of denitrification was still enhanced by amendment with NO_3^--N solution. The increase in denitrification rate after addition of NO_3^--N in all seasons suggests that the availability of NO_3^--N was likely to be a principal factor limiting denitrification in this pasture soil.

A large increase in denitrification rate was obtained by saturating the soil cores collected in most

seasons, particularly during the warm, dry period. However, little enhanced effect on denitrification rate by anaerobic incubation of soil cores was observed. These results suggest that the observed effect of water addition on denitrification rate may have been due to the easier diffusive movement of NO_3^--N, or possibly soluble-C, to the microsites where denitrification was occurring in this pasture, and creation of anaerobic sites in the soil may not have been as important to the increase of denitrification rate. In the case of NO_3^--N, this suggestion was supported by the results from an investigation into the effect of the movement of NO_3^--N on the denitrification rate in the soil (Chapter 9).

The suggestion that the rate of diffusion of NO_3^--N into the microsite can limit denitrification rate may account for the two apparently contradictory observations reported in Chapters 6 and 8, in which denitrification rates appeared to be related better to soil moisture contents than to the concentrations of soil NO_3^--N in the field (Chapter 6) while, in contrast, large increases in denitrification rate were obtained after soil cores were dipped into diluted NO_3^--N solutions. Thus in winter when soil moisture contents are high the rate of diffusive movement of NO_3^--N into microsites is influenced strongly by the concentration of NO_3^--N in the bulk soil. In summer however the soil moisture content may be the more important determinant of the rate of NO_3^--N diffusion.

A large effect of available-C on denitrification rate was observed in soil incubated under anaerobic, saturated conditions (Chapters 3 and 4), but the response of denitrification rate in individual soil cores to C addition alone was not significant (Chapter 8). These results suggest that C levels may limit denitrification rate when other factors are optimal for denitrifiers in the soil, but that C will be a less important factor in controlling denitrification rate in this pasture, when other factors are also affecting the rate of denitrification and its spatial and temporal

variation. Carbon may have a role in stimulating O_2 consumption by respiration in the soil to promote denitrification rate, as a slight correlation was observed between denitrification rate and respiration rate in the individual soil cores (Chapter 6) and a good linear relationship was also observed between denitrification rate and respiration rate when soil was incubated after NO_3^--N amendment (Chapter 8).

As discussed above, the availability of NO_3^--N for denitrification rate can be influenced by the soil moisture content and the absolute amount of NO_3^--N in the soil. In addition to the soil moisture content and NO_3^--N concentration, concurrent nitrification processes in soil can also influence the supply of NO_3^--N for denitrification (Chapter 9). The inhibition of nitrification by acetylene in the soil during measurement of denitrification using the acetylene inhibition technique may therefore affect the availability of NO_3^--N for denitrification. However, the results of this study suggested that inhibition of nitrification by acetylene did not affect the short-term measurement of denitrification rate.

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