THE INFLUENCE OF NITRIFICATION IN DETERMINING THE SUPPLY, DISTRIBUTION AND FATE OF NITROGEN IN GRASSLAND SOILS

by

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ABSTRACT

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David John Hatch

The microbiology of nitrification has been extensively studied, but the ecology and environmental impact of the process has received less attention. The reason for this has more to do with the difficulties of conducting field experiments to examine the links with other processes which cause losses of nitrogen, than a failure to appreciate its importance in agricultural systems. This project was designed to overcome some of the limitations of existing field techniques to enable simultaneous measurements of nitrification and the major processes of N supply (mineralization) and N loss (denitrification and leaching) to be examined.

The study proceeded in three distinct phases: firstly, soils with contrasting N management histories were examined, using laboratory assays for *potential* activities. Clear differences which resulted from higher N inputs were established, with correspondingly higher nitrifying activities. For example, in a fertilized soil, ammonia-oxidizers produced 48.4 compared with 1.3 nM NO_2^- g^{-1} soil h^{-1} in an unfertilized soil. Potential nitrite-oxidizing rates were 93.4 and 62.5 nM g^{-1} h^{-1}, respectively. Assays of enzyme kinetics, therefore confirmed the higher nitrifying activity in the fertilized soil, but demonstrated a lower affinity of the enzyme for NO_2^- substrate, with K_m values of 436 and 310 μM NO_2^--N, respectively. Nitrifying rates in soils from grass-clover swards were intermediate between the fertilized and unfertilized soils.

Secondly, a new field incubation technique was developed and used to obtain *actual* rates by concurrent measurements of the major N cycling processes. A strong correlation was established between nitrification and denitrification ($r^2 = 0.98$). The measurements were verified by comparison with other independent methods. Net rates of nitrification in the same soil type ranged from 0.55 - 1.17 kg N ha^{-1} d^{-1}, with the highest rates in the fertilized soil. Over 70% of the mineralized N was nitrified, of which 80% was subsequently lost (i.e. either denitrified or leached).

Thirdly, the practical implications of these findings were examined in greater detail using ^{15}N labelling techniques which enabled process rates (net and gross) to be established in a model of the N cycle. When nitrification was inhibited, there were no significant differences between gross or net mineralization rates in the soils from the three swards, which indicated that N-immobilization could be directly influenced by the level of nitrifying activity in these soils.

The influence of nitrification in determining the pathways of N loss from grassland soils was quantified in this study. From a detailed investigation of the processes involved in N cycling, it was deduced that nitrification was also one of the major factors in determining the outcome of competition for inorganic N between plant and microbial biomasses.

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Special thanks should go to my wife Rosemary and son Robert who have supported and encouraged me throughout and to whom I now promise some quality time.

I should like this work to be dedicated to the memory of my father:

Frederick John Hatch (1920-1986)

AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

This study was carried out at the Institute of Grassland and Environmental Research, North Wyke, Okehampton, Devon: the work formed part of a MAFF commission with IGER.

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Publications: (%Iniated; %Executed; %Wrote)
Hatch, Jarvis and Parkinson 1996 (90; 100; 90)
Lovell and Hatch 1997 (90; 60; 60)
Hatch, Jarvis and Parkinson, 1998 (80; 100; 70)
Ledgard, Jarvis and Hatch 1998 (0; 20; 5)*
Clough, Jarvis and Hatch 1998 (0; 30; 10)*
Bhogal, Hatch, Shepherd and Jarvis (in press)*
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Posters:
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British Grassland Society, Field Meeting at IGER, September 1997
Seale Hayne Faculty of Agriculture & Land Use, June 1998
University of Oslo, Norway, September 1998

Signed .....

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CHAPTER 1 Introduction and literature review

1.1 The Nitrogen cycle in grassland soils

Nitrogen (N) is a ubiquitous element which makes up 78.1% (by volume) of the atmosphere and is a component of all soils, comprising less than 0.1% of desert soils to over 2% of highly organic soils (Haynes, 1986). These vast sources of N, however, are unavailable to most forms of terrestrial life, which must rely on the activities of specialized microorganisms in order to obtain the N needed for the vital building blocks of life (e.g. amino acids, proteins). Only a few soil organisms (e.g. *Rhizobium*, *Azotobacter*, *Azospirillum*) are able to fix N from the atmosphere by conversion of dinitrogen (N_2) to ammonium (NH_4^+) using the nitrogenase-enzyme system (Sprent and Sprent, 1990).

In contrast, a host of heterotrophic organisms are able to assimilate organically-bound soil N, through the decomposition of soil organic matter (Dickinson and Pugh, 1974). The process of hydrolysis and catabolic breakdown of complex compounds into inorganic forms is termed mineralization and culminates in the release of NH_4^+ (ammonification). The energy made available to the decomposer organisms by this process enables the uptake of NH_4^+ for incorporation into cell components (Jansson and Persson, 1982). This anabolic process is termed immobilization and is concomitant with mineralization, although the net result of the opposing processes is, to some extent, dependent on the C:N ratio of the substrate; the critical C:N ratio for net mineralization is considered to be <25 - 30 (Harmsen and van Schreven, 1955), but is likely to vary between different ecosystems. Thus, net mineralization results in release of NH_4^+, whereas net immobilization results in a decrease in available soil inorganic N.

The N cycle is kept in approximate balance by the activity of another group of organisms (the denitrifiers), which are capable of returning fixed N to the atmosphere by the reduction of nitrate to nitrous oxide (N_2O) and N_2 (Knowles,1982; Firestone, 1982). Equilibrium is, therefore, maintained by the transport of N between the lithosphere and the troposphere, and is facilitated by a further group of microorganisms (the nitrifiers) which converts NH_4^+ released from net ammonification into nitrate (NO_3^-), the substrate for denitrification (see Fig. 1.1).

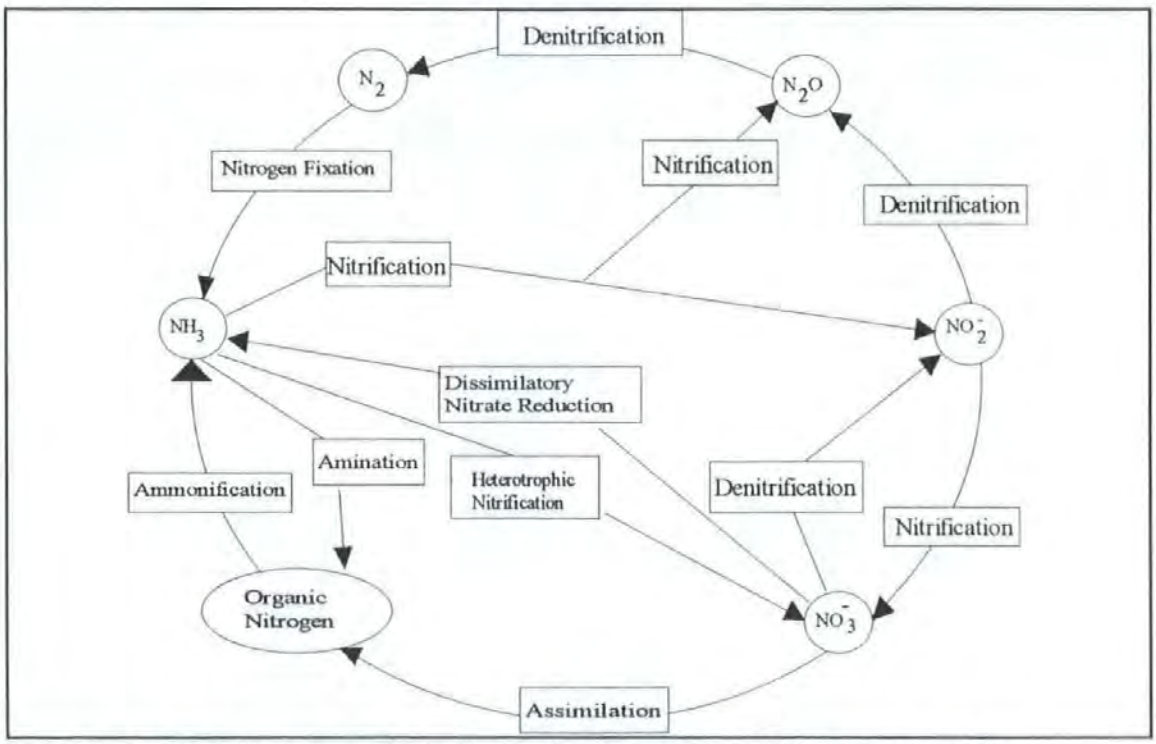


Figure 1.1 The Nitrogen cycle (redrawn from Kuenen and Robertson, 1988)

The importance of this stage in the N cycle is central to this investigation which seeks to test the hypothesis that:

"Nitrification occupies a pivotal role in the N cycle of productive grassland soils by determining the supply of substrate (nitrate) to the two major processes involved in losses of N, i.e. denitrification and leaching".

This chapter describes the nitrification processes and the factors controlling the activity of the nitrifying organisms, which relate to the soil ecosystem. Our current understanding and areas where information is inadequate, or inconclusive are highlighted.

1.2 Nitrification by autotrophic organisms

Nitrification is generally defined as the biological oxidation of ammonia (NH_3) to NO_3^-, with nitrite (NO_2^-) as an intermediary product. Much of our present understanding of the nature of the process of nitrification stems from the early pioneering work of the Russian microbiologist, Sergei Nikolaevitch Winogradsky (1856-1946). In 1922, after an absence of nearly 35 years from scientific research, he returned to continue his investigations and reviewed the literature of the intervening years; he concluded that little new knowledge had been acquired to add to that of his earlier work. For an account of the life of Winogradsky, see Waksman (1946).

Almost half a century after Winogradsky's death, a wealth of literature has now been amassed, with contributions covering a wide range of factors concerned in the process of nitrification. These have been extensively reviewed under different environmental headings, including marine and estuarine waters (Kaplan, 1983; Ward, 1978), water and sewage treatment plants (Focht and Chang, 1975; Wanielista and Eckenfelder, 1978; Winkler, 1981; Painter, 1970) and soils (Alexander, 1965; Focht and Verstraete, 1977; Belser, 1979; Schmidt, 1982; Haynes, 1986). However, the ecology of nitrifying bacteria and the factors controlling the growth and development of the organisms in their natural environments are still poorly defined, not least in agricultural soils, where edaphic factors (i.e. the soil chemical and physical properties which influence growing organisms) are usually disturbed

in some way when the measurements are made.

Winogradsky's major contribution (Winogradsky, 1890) was to identify two distinct genera of chemoautotrophic bacteria which are responsible for oxidizing nitrogen (N) in its most reduced form (NH_3), through to the most oxidized form (NO_3^-). He called the ammonia-oxidizing and nitrite-producing bacteria *Nitrosomonas*, and the nitrite-oxidizing and nitrate-producing bacteria were named *Nitrobacter*. The family *Nitrobacteraceae* (in the order *Pseudomonadales*) are slow growing, gram-negative aerobes with optimum activity at temperatures of between 30 and 37°C and a neutral to alkaline pH, with no growth at pH < 4.0 (Focht and Verstraete, 1977). They were shown to be true autotrophs, i.e. having no need of organic substrates because all their requirements for carbon are obtained from carbon dioxide (CO_2), and their energy needs are met by the oxidation of ammonia and nitrite, as shown by the following equations:

$$NH_4^+ + \frac{3}{2}O_2 \rightarrow NO_2^- + H_2O + 2H^+ \qquad \Delta F = -84.0\ kcal$$

$$NO_2^- + \frac{1}{2}O_2 \rightarrow NO_3^- \qquad \Delta F = -17.8\ kcal$$

Where ΔF = Change in free energy

[from Russell, 1973]

The first oxidation stage involves the transfer of six electrons from the N in ammonia, with

a valency of 3-, through to the N in nitrite at a valency of 3+. The final step involves a further pair of electrons to produce nitrate, which has an oxidation state of 5+. The energy yield from these oxidizing reactions is very low, which makes the nitrifiers poor competitors against heterotrophic organisms. Fenchel and Blackburn (1979) calculated that *Nitrosomonas* generates only 1 to 4% and *Nitrobacter* from 3 to 10% of the cell material that might be expected from a heterotroph, from equivalent sources of available energy.

There are five genera of ammonia-oxidizers known to exist in soil: *Nitrosomonas*, *Nitrosolubus*, *Nitrosovibrio*, *Nitrosospira* and *Nitrosococcus* and one nitrite-oxidizer, *Nitrobacter* (Belser, 1979). Since *Nitrosomonas* and *Nitrobacter* are the two most commonly isolated genera in terrestrial habitats, they have been assumed to be the most abundant. This is based on the principle of 'competitive exclusion', which proposes that a single species excludes all others from a generalized NH_4^+ oxidizing niche (McLaren and Ardakani, 1972) . This view has been challenged (Belser and Schmidt, 1978a), at least in the case of the ammonia-oxidizers, and may in reality only reflect their ability to out-compete other organisms in liquid culture. These authors found *Nitrosomonas* and *Nitrosospira* to be more common than *Nitrosolobus*, but in another study on a Rothamsted soil, *Nitrosolobus* was found to predominate (MacDonald, 1979). Assessments of nitrifier populations by standard techniques of serial dilution and isolation, e.g. the Most Probable Number (MPN) method (Alexander, 1982), can give misleading results. This was demonstrated by Rennie and Schmidt (1974), who found counts of soil nitrifiers to be four orders of magnitude less by the MPN method, than determinations based on direct microscopic counting, using the fluorescent antibody (FA) stain (Bohlool and Schmidt, 1980). In another study, Johnson and Sieburth (1976) established the presence of

Nitrosomonas and *Nitrobacter* populations in a marine surface slime, by *in situ* electron microscopy, but were unable to culture the organisms by conventional methods. Belser and Schmidt (1978b) used FA staining and a modified isolation technique to show the existence of considerable serological diversity, particularly within the *Nitrosospira* genus.

All the nitrifiers, with the exception of some strains of *Nitrobacter winogradsky*, are obligate chemolithotrophs (obtaining their energy from simple inorganic reactions). The currently recognized genera of chemolithotrophic nitrifiers is shown in Table 1.1.

Table 1.1 The Family Nitrobacteraceae (From Belser, 1979)

Oxidation	Genus	Species	Habitat
Ammonium to	*Nitrosomonas*	*europaea*	Soil, water, sewage
nitrite	*Nitrosolobus*	*multiformis*	Soil
	Nitrosovibrio	*tenuis*	Soil
	Nitrosospira	*briensis*	Soil
	Nitrosococcus	*nitrosus*	Soil
		oceanus	Marine
		mobilis	Marine
Nitrite to	*Nitrobacter*	*winogradsky*	Soil, water
nitrate	*Nitrospina*	*gracilis*	Marine
	Nitrococcus	*mobilis*	Marine

1.3 Heterotrophic nitrification

For many years after Winogradsky's discovery, nitrification was thought to be the sole preserve of autotrophic bacteria, and it was another 50 years or so, before the first evidence of N oxidation by heterotrophic organisms was presented (Nelson, 1929). It has now been shown that heterotrophic bacteria (e.g. *Arthrobacter*), as well as fungi (e.g. *Aspergillus*) and also some *Actinomycetes*, are able to nitrify in pure culture under laboratory conditions (Focht and Verstraete, 1977) and are potential nitrifiers in soil (Kilham, 1986). The occurrence of heterotrophic nitrification, however, may be masked in natural systems since the process is not obligatory for the growth of the organisms. Higher yields of energy can be gained by heterotrophs from the oxidation of carbon (C) in organic substrates, than might be obtained from the small amounts of reduced N which appear to be oxidized during nitrification (Kilham, 1986). In view of this, the standard definition of nitrification as a strictly inorganic reaction is generally considered too restrictive, and an alternative definition of nitrification as:

"The biological conversion of N in organic or inorganic compounds from a reduced to a more oxidized state",

which was previously proposed by Alexander *et. al.* (1960), has now become more widely adopted. This definition is to be preferred as the precise biochemical pathway for heterotrophic nitrification is still uncertain and it may involve either an organic or inorganic pathway or a combination of both options, as shown in Fig. 1.2.

There are still doubts surrounding the role of heterotrophic nitrification in natural ecosystems (Schmidt, 1982) which may only become important in very acidic soils. For example, when NH_4^+ was added to an acid soil, the basal rate of nitrification was not

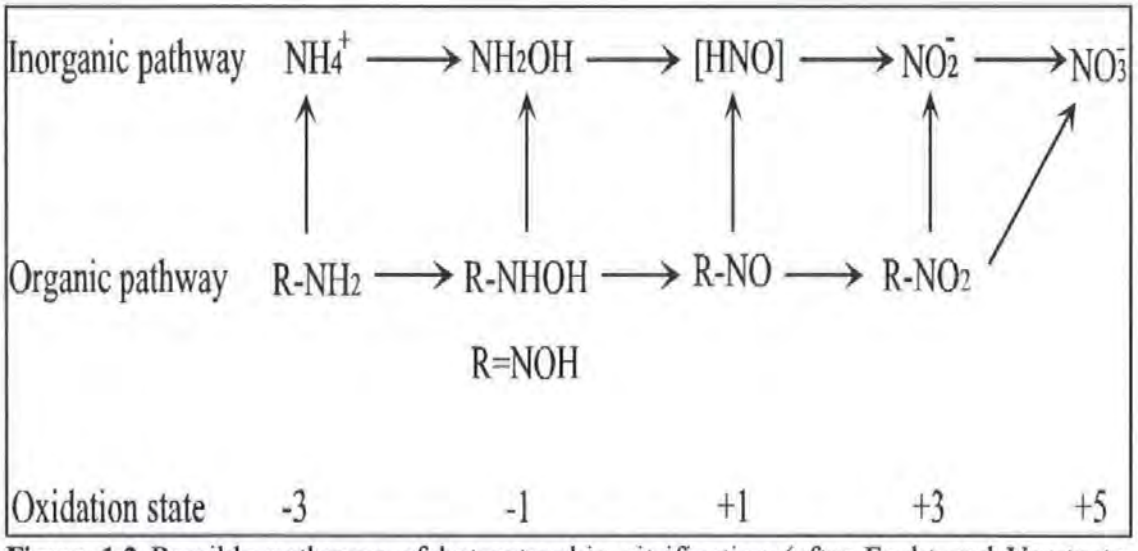


Figure 1.2 Possible pathways of heterotrophic nitrification (after Focht and Verstaete, 1977).

stimulated by the additional substrate (Weber and Gainey, 1962), suggesting the absence of any autotrophic component. In another study, evidence of nitrifying activity was detected even though nitrifiers could not be isolated; the addition of lime also failed to increase the measured rate of nitrification (Ishaque and Cornfield, 1972). The fact that nitrification occurs in situations where autotrophic organisms would not be expected to be active, or indeed in environments from where autotrophs cannot be isolated, provides only circumstantial evidence that the source of nitrification is heterotrophic (Focht and Verstraete, 1977). It is possible that unidentified species of autotrophs, which are adapted to such conditions, may yet exist. In any case, nitrification rates obtained from cultures grown axenically (i.e. in pure isolated cultures) are 10^3 to 10^4 times lower for heterotrophs than for autotrophs (Focht and Verstraete, 1977) and their contribution in natural systems may, therefore, be of little importance and only significant where autotrophs do not thrive.

The use of selective inhibitors may present the best opportunity for unravelling the relative contributions of different nitrifying processes in mixed populations. Acetylene (C_2H_2) has

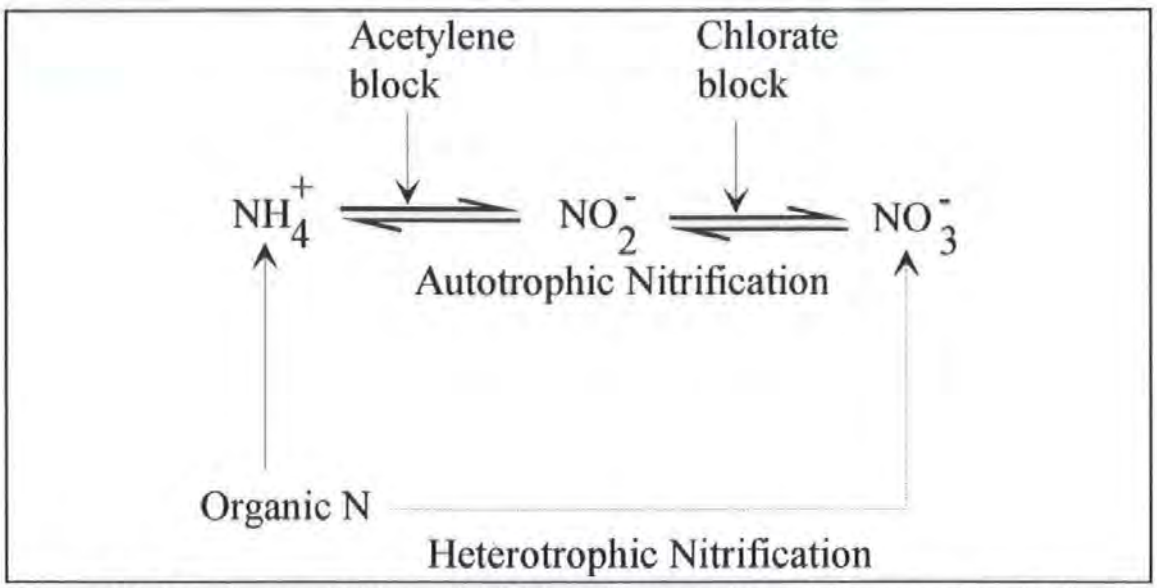


Figure 1.3 Acetylene and Chlorate inhibition of autotrophic and heterotrophic nitrification (from Schimel *et al.*, 1984).

been shown to be an effective physiological block of autotrophic NH_4^+ oxidation to NO_2^- (Berg *et. al.*, 1982), as a non-competitive inhibitor of NH_3 oxygenase which does not inhibit heterotrophic nitrification (Hynes and Knowles, 1982). Chlorate will inhibit the autotrophic oxidation of NO_2^- to NO_3^- (Hynes and Knowles, 1983) and both chlorate and C_2H_2 have been used in a soil perfusion column to demonstrate the dominance of autotrophic nitrification in an agricultural soil maintained at pH values ranging from 4.5 to 7.5 (Kilham, 1987). In the same study, nitrification in an acid soil under coniferous plantation was shown to be predominantly heterotrophic. The blocking effect of these two inhibitors is illustrated in Fig. 1.3. More recently, the stable isotope ^{15}N has been used in a modified application of the mean pool dilution technique to enable the separate pathways of autotrophic and heterotrophic nitrification to be evaluated (Barraclough and Puri, 1995). In this study only 8% of the observed nitrification in an acid woodland soil was attributed to heterotrophic activity, so that whilst biochemical blocks are effective in separating heterotrophic from autotrophic nitrification, their specificity is difficult to prove.

1.4 Other nitrification processes

Two other nitrifying processes have been described in soils, but their significance is unclear. Methylotrophic bacteria, capable of oxidizing methane and other C-containing compounds have been shown to also oxidize NH_4^+ (Dalton, 1977) and a strictly non-microbial chemical oxidation can occur in soils exhibiting high manganese oxide content (Bartlett, 1981).

1.5 Controls on nitrification

The term 'climax vegetation' is used to describe the end-point in the successional development of plant communities, which approach a steady-state when the vegetation has come into approximate equilibrium with the local conditions. For various successional stages leading up to climax vegetation, characteristically low NO_3^- levels were demonstrated for different soils, the lowest level being generally associated with the climax stage itself (Rice and Pancholy, 1972, 1973). These workers suggested that nitrification was inhibited under these conditions by increasing levels of tannins. However, Bohlool *et al.* (1977) could find no evidence of inhibition using pure cultures of nitrifiers with high concentrations of added tannins.

Similarly, unfertilized grassland soils generally have very little NO_3^- (Richardson, 1938) and small, but variable, NH_4^+ contents. This observation led to speculation of allelopathy (the adverse influence that one living organism has on another by the secretion of a chemical inhibitor) associated with grass roots which were presumed to release substances into the rhizosphere that could inhibit nitrification (Theron, 1951). This hypothesis provided a plausible explanation of the controls which might govern low-input systems;

the conservation of the limited supply of nutrients under these circumstances, would be an essential stratagem to maintain the equilibrium. However, the difficulty in separating apparent allelopathic inhibition from other naturally occurring controls remains, and the latter constraints should first be eliminated before allelopathy can be invoked. Hence, Robinson (1963) found that low rates of nitrification in a grassland soil could be increased when soil pH and substrate in the form of urea were amended. Clark and Paul (1970) reviewed the literature on allelopathy in grassland, but the outcome remained inconclusive. More recently, Bremner and McCarty (1993) have looked again at the evidence, both for and against allelopathic interactions, and suggested that even where allelopathy is suspected, such effects are negligible.

An alternative explanation for low nitrification rates is that of nutrient-deprivation. For example, an inadequate phosphate supply was shown to limit NO_2^- oxidizing bacteria in a savanna grassland soil (Purchase, 1974a,b). The role of mycorrhizae may be of particular interest in this respect as they are known to be effective scavengers of phosphate and also preferentially take up N in the NH_4^+ form. In this way mycorrhizae could act as direct competitors with nitrifiers for substrate and could, therefore exert a controlling influence in climax systems (Verstraete, 1981).

1.5.1 Temperature

The two physical factors which most affect nitrifier populations are temperature and moisture (Belser, 1979). As might be expected from the effects on microbes in general, a rise in temperature results in increased activity up to an optimum point. If the optimum temperature is exceeded, further increases cause a decline in activity until a stage is reached

from which the ultimate recovery to previous rates is permanently impaired. Evidence from the literature would suggest that autotrophic nitrification is more sensitive to high temperatures than the process of ammonification (Etinger-Tulczynska, 1969; Ishaque and Cornfield, 1974; Myers, 1975). Consequently, the supply of substrate (NH_4^+) at elevated temperatures may exceed the oxidizing capacity of the nitrifiers and NO_2^- can accumulate (Mahandrappa, *et al.*, 1966). Nitrification activity is usually optimal between 25 and 35 °C (Justice and Smith, 1962) and was found to be almost completely inhibited at 40 °C by Keeney and Bremner (1967). However, these authors found that ammonification continued to be stimulated up to 40 °C, so that nearly all the NH_4^+ produced remained in this form, whereas at lower temperatures, virtually all of the mineralized N was converted to NO_3^-. *Nitrosomonas* has a high activation energy equivalent to a Q_{10} of 3.0 (Q_{10}= the ratio of the rate of progress of a reaction, at a given temperature, to the rate at a temperature 10 °C lower), but *Nitrobacter* has a Q_{10} of only 1.7 (Belser, 1979). Increased temperature, therefore, stimulates NH_4^+ oxidation to a greater extent than NO_2^- oxidation. Model-based predictions by Laudelout and co-workers suggested that when populations of these two nitrifiers were equal, high temperatures would lead to an accumulation of NO_2^-, and they were able to demonstrate that this could occur in practice (Laudelout *et al.*, 1974,75,76).

Low temperatures affect both ammonification and nitrification rates, but have a greater influence on the latter process (Tyler *et al.*, 1959). Fluctuating, low temperatures are more inhibitory than constant low temperatures, and it has been suggested that the effect on the microbes may be bacteriocidal (Campbell and Bierderbeck, 1972). Indiginous nitrifiers can alter their temperature optima in response to climatic variations (Mahendrappa *et al.*, 1966).

For example, in a tropical soil the optimum was found to be 35 ^{0}C with nitrification continuing up to 50 ^{0}C (Myers, 1975), whereas in a soil from Alberta (Canada) the optimum was only 20 ^{0}C and almost completely ceased at 30 ^{0}C (Mahli and McGill, 1982). Nitrification activity was still detected in Alberta soils even when the ground was frozen (Mahli and Nyborg, 1979), but nitrification is more generally inhibited until the soil has warmed to at least 4 or 5 °C (Anderson and Boswell, 1964).

1.5.2 Moisture and Aeration

The rate of diffusion of oxygen (O_2) through air is ten thousand times faster than through water (Smith and Arah, 1990) so that changes in soil moisture content will directly affect the aerobicity of soils. The oxidation of NO_2^- to NO_3^- by *Nitrobacter* was shown by Aleem *et al.*, (1965) using ^{18}O, to involve molecular O_2 generated from water and not obtained directly from atmospheric O_2. Although the soil liquid phase is the source of both the O_2 and HCO_3^-, which are utilized in nitrification, Schmidt (1982) suggests that utilizable C is unlikely ever to be limiting to the autotrophic soil nitrifiers, but O_2 frequently is. For example, the solubility of O_2 would allow a concentration of 0.315 m*M* to develop in soil solution (at 15 °C) in equilibrium with air at atmospheric pressure, but Schmidt (1982) describes certain conditions that can promote O_2 depletion, *viz.* (i) high soil moisture content, which fills soil pores and restricts recharge of O_2 from the gaseous phase; (ii) high soil temperatures, which reduce the solubilty of O_2 and increases O_2 demand by heterotrophic microorganisms; and (iii) readily-oxidizable organic matter, which also increases heterotrophic O_2 demand. A differential effect of limited O_2 supply on the two main nitrifier populations may be deduced from the work of Laudelout *et al.*, (1976) who found the K_m (the concentration of substrate at which the rate of reaction proceeds at half

the maximum rate) for O_2 to be smaller for *Nitrosomonas* (0.016 m*M*) than for *Nitrobacter* (0.062 m*M*). Under conditions of low aeration and high temperature, their model predicted complete temporal separation of NH_4^+ oxidation and NO_2^- oxidation; the NH_4^+ oxidizers could out-compete the NO_2^- oxidizers for O_2 and growth of the latter would be inhibited. This differs from the purely temperature-driven effect described above, where high temperatures alone did not inhibit the activity of *Nitrobacter*. In natural habitats, both groups of nitrifiers would compete poorly with heterotrophs, which have much lower K_m values (0.001 m*M*) for O_2 (Focht and Verstraete, 1977).

When soil moisture tension approaches saturation point (0 kPa) nitrification ceases completely (Miller and Johnson, 1964; Malhi and McGill, 1982) or occurs only very slowly (Sabey, 1969). At moderately high moisture levels (1-10 kPa) nitrification is enhanced so long as aeration is adequate (Dommergues, 1966). At the "permanent wilting point" (-1500 kPa) nitrification can still occur (Miller and Johnson, 1964; Dubey, 1968; Sabey, 1969) and below this point nitrifiers are more inhibited than ammonifiers (Dommergues, 1966). Even severe desiccation probably only decreases the size of the population (Belser, 1979) and a proportion of it can remain viable in air-dried soil for as much as 14 years (Alexander, 1965). When soils were re-wetted after prolonged adverse conditions of high temperatures, high solute concentrations and desiccation during a four month dry period in a Moroccan coastal soil, activity quickly recovered following the onset of the first rainfall (Chiang *et al.*, 1972). Thus, where increasing soil moisture content tends to enhance nitrification, provided O_2 is available, the nitrifying activity in dry soils will vary with soil texture along with those properties which govern osmotic potential (Russell, 1973).

1.5.3 pH effects

The sensitivity of nitrifiers to pH has been recognized for some time, but was probably the main reason for the general misconception of earlier workers that successful isolation and culture could only be achieved by the inclusion of solid particles (usually $CaCO_3$) in the culture medium. The likely benefit of adding $CaCO_3$ was to neutralize acid produced by the organisms, but when the pH was controlled the requirement for particulate matter was shown to be unnecessary, either for *Nitrobacter* (Goldberg and Gainey, 1955) or for *Nitrosomonas* (Engel and Alexander, 1958).

The pH optimum for the growth of nitrifiers is the alkaline side of neutral (Painter, 1970) which, in soils, encourages autotrophs to become the dominant species. Below pH 4.5 (e.g. in acid forest soils) autotrophs are inhibited (Sarawat, 1982) and nitrification is more likely to be heterotrophic. However, it is possible that some strains of autotrophic nitrifiers also exist which have adapted to acid conditions and in soils of pH 4 to 5, nitrification is often still detected (Weber and Gainey, 1962; Walker and Wickramsinghe, 1979; Federer, 1983). Conclusive proof of adaptation is hard to find since microsites may develop in acid soils which are at a higher pH than is indicated by bulk soil measurements. Liming can increase the numbers of autotrophs by several orders of magnitude (Soriano and Walker, 1973), but in soils with pH >7.5 the equilibrium between NH_4^+ and free ammonia (NH_3) moves in favour of the latter, which is particularly toxic to *Nitrobacter* (Morrill and Dawson, 1967).

At low soil pH the inhibition curve is a function of the equilibrium between NO_2^- and nitrous acid (HNO_2); whilst acidity may be directly inhibitory, mobilization of aluminium is also implicated as a limiting factor (Brar and Giddens, 1968).

1.5.4 Substrate

The major limitation to autotrophic nitrification is likely to be the availability of substrate itself (Alexander, 1965) and, therefore, the whole process is under the control of ammonification (mineralization). The inadequacy of this supply can be demonstrated in the soil by the addition of NH_4^+, which can greatly stimulate the growth of the nitrifier population (McLaren, 1971; Ardakani *et al.*, 1974). Thus, additional inputs of NH_4^+ from other sources such as fertilizers, atmospheric deposition and hydrolysis of organic compounds in urine, will also exert an influence on nitrifying activity. The processes generally proceed in the order of the fastest, which is NO_2^- oxidation (a fact borne out by the negligible amounts of NO_2^- generally found in soil), followed by NH_4^+ oxidation, with the slowest process being ammonification. Measurements of nitrification rates based entirely on NO_3^- production could be misleading; nitrifying activity can only proceed at the rate dictated by NH_4^+ production.

The nitrifying organisms involved in the two stages of nitrification are sensitive to their own substrate, but are more sensitive to the substrate of the other organism (Painter, 1970). 'Substrate repression' by NH_4^+ is in the range 400-800 μg N g^{-1} dry soil (Haynes, 1986), but the inhibitory effect of high NH_4^+ concentrations has also been attributed to the toxicity of NH_3 (Broadbent *et al.*, 1957; Stojanovic and Alexander, 1958) or to the lowered pH caused by adding $(NH_4)_2SO_4$ (Justice and Smith, 1962; Mahli and McGill, 1982). 'End product repression' can also occur, with NO_2^- concentrations ranging from 1400 to 4200 mg N l^{-1} adversely affecting the growth of *Nitrosomonas* (Painter, 1970). Similarly, high concentrations of NO_3^- can inhibit NO_2^- oxidation by *Nitrobacter* (Boon and Laudelout, 1962).

Nitrosomonas and *Nitrobacter* oxidize about 35 and 100 atoms of N, respectively, for the fixation of one molecule of CO_2 (Alexander, 1965). About three times as much nitrogenous substrate, therefore, is required for the growth of NO_2^- oxidizing bacteria than for NH_4^+ oxidizing bacteria. Cell yields calculated from this relationship and from cell counts suggest that from 1 to 4 x 10^4 cells of *Nitrobacter* $\mu g^{-1}N$ are produced from the oxidation of NO_2^-, and about three times this number of cells of *Nitrosomonas* are produced from the oxidation of an equivalent amount of NH_4^+-N (Focht and Verstraete, 1977). The 'generation time' (time taken for the population to double itself) for *Nitrobacter* has been reported to be as short as 7 hours and for *Nitrosomonas* to be about 11 hours by Alexander (1965) using culture solutions. In natural systems, Morrill and Dawson (1967) showed the number of *Nitrosomonas* bacteria did slightly exceed those of *Nitrobacter*, but were about the same when the soil was slightly acid (pH 6.4).

The affinity of the autotrophs for their substrates is indicated by K_m values (assessed in the temperature range of 20 to 30 ^{0}C) of between 1 and 10 mg N l^{-1} and 5 and 9 mg N l^{-1}, for NH_4^+ and NO_2^- oxidation, respectively (Focht and Verstraete, 1977). Substrate concentrations in soil are generally likely to be equal to, or less than the K_m values and, therefore, oxidation rates will be dependent on the substrate concentration.

For this reason and those outlined above, it is clear that over-reliance on NO_3^- levels as indicators of nitrifying activity is inadvisable; the determination of soil NO_3^- in isolation has contributed little to an understanding of the dynamics of the process (Schmidt, 1982). Indeed, Jansson (1958) relegates the nitrate ion to a minor role in the N cycle in soils in favour of NH_4^+, which is represented as the key form, undergoing continual consumption

and renewal by the microbial biomass. Competition for NH_4^+ is placed in the order heterotrophs>nitrifiers>plants; NO_3^- then represents the inorganic N which is surplus to the requirements of the general soil biomass. Jackson *et al.* (1989) also found that plants were less successful competitors for NH_4^+ than microbes. Besides microbial immobilization, other competing processes may be responsible for lowering the NO_3^- level in soil, e.g. plant uptake, denitrification and losses through leaching. Furthermore, even if the complication of NO_3^- uptake by plants is avoided, Russell (1973) lists four conditions which must be met before NO_3^- will accumulate in a fallow soil: (1) there must be an adequate supply of readily decomposable organic matter; (2) the soil must be kept free of weeds; (3) the soil must be kept moist, but not necessarily continuously; (4) there must not be too much rain, or NO_3^- will be removed by leaching. In attempting to accurately measure nitrification in the field, therefore, all of these naturally occurring factors need to be either eliminated, accounted for, or controlled.

Changes under carefully defined conditions could give important indicators which could help to characterize soil inorganic N status and behaviour. For example, Jarvis and Barraclough (1991) found that the ratio of NO_3^- : NH_4^+ widened in a grassland soil with increasing N fertilizer rates. In the same study, when a common application rate of N was subsequently applied, differences in this ratio relating to the previous treatment remained, which suggested a change in nitrification had taken place. Increases in nitrifying potential, however, can be an undesirable development in stable ecosystems. The end-product of the process (NO_3^-) is a more mobile ion than the NH_4^+ ion (which is retained on cation exchange sites of soil particles,) and is, therefore, more vulnerable to loss.

1.6 Coupling of processes

Knowles (1978) has proposed that a close coupling may exist between the processes of nitrification, denitrification and possibly also N fixation, through intermediates which are common to different parts of the N cycle (Fig. 1.1). Thus, N_2, NO_3^-, NO_2^- and N_2O may all be represented as both substrates and products providing coupling links between the various processes, as illustrated in Fig.1.4. Knowles (1978), suggested that NO_3^- could provide a coupling between nitrification and denitrification in two ways. First, by 'successive coupling', whereby conditions conducive to nitrification (*viz.* dry/aerobic soils) produce an accumulation of NO_3^-. Conditions then develop which are more appropriate to denitrification (*viz.* wet/anaerobic soils), during which NO_3^- is reduced to N_2O and N_2. The second way is by 'simultaneous coupling', which proceeds from adjacent microsites. Nitrifying activity in an aerobic site near the surface of a saturated soil generates NO_3^-, which is transported downwards to an anaerobic microsite in the mobile water-phase, where the process of denitrification predominates. Wetting and drying cycles which can stimulate mineralization in soils (Birch, 1964) may, therefore, also promote further losses of N from the soil (Patrick and Wyatt, 1964).

The identification of N_2O as an intermediate product of NH_4^+ oxidation (Yoshida and Alexander, 1970) can also be envisaged as providing a possible coupling between nitrification and denitrification (see Fig. 1.4) and in addition, has implications for the environment in respect of ozone destruction (Crutzen, 1970). Under strictly aerobic conditions, N_2O emission was demonstrated during nitrification of fertilizer N applied to several different soils (Bremner and Blackmer, 1978). However, the existence of isolated anaerobic microsites in otherwise aerobic soil conditions, can be brought about by the rapid

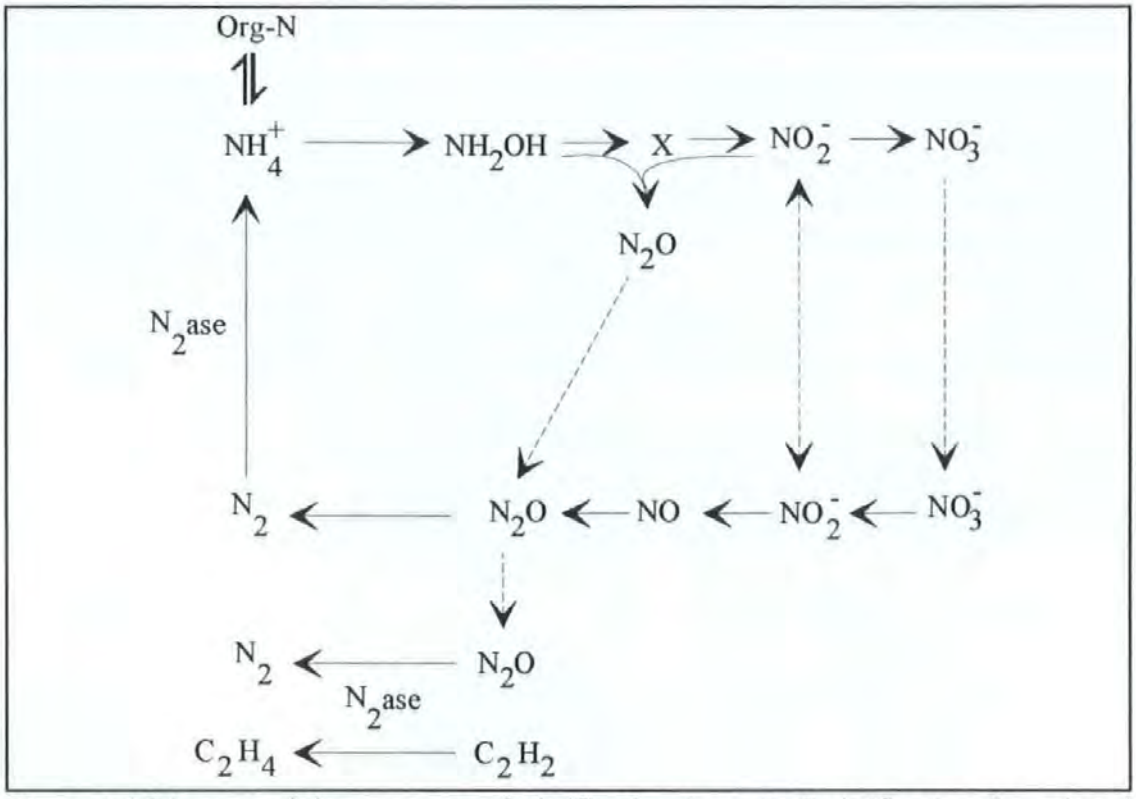


Figure 1.4 Diagram of the processes of nitrification (upper), denitrification (lower) and N_2 fixation (bottom), with possible coupling links shown by dashed lines (from Knowles, 1978).

decomposition of plant litter (with the associated consumption of O_2), producing 'hotspots' of denitrification (Tiedje, 1987). This would tend to obscure the true source of N_2O. However, there is now increasing evidence to show that the processes of nitrification and denitrification can be combined in conditions which do not necessarily favour either process, and that the organisms responsible are less sensitive to O_2 supply than was previously thought (Kuenen and Robertson, 1994). More recently, the relative contributions of the two processes to the flux of N_2O have been resolved using differential labelling of the respective N pools and examination of the ^{15}N-distribution in the accumulated N_2O (Stevens *et al.*, 1997). The possibility of a further coupling between N fixation and processes which involve N_2 and N_2O (Fig. 1.4) has been suggested, since nitrogenase can reduce both of the end-products of denitrification (Knowles, 1978).

A need for "simultaneous investigations of the interactions between nitrifying and reducing (particularly denitrifying) microorganisms", was identified as the dominant theme of the conference on: "Nitrification and Reduction of Nitrogen Oxides" (1976), by the chairman (Payne, 1978). The fact that his plea for more integrated ecological studies failed to produce an adequate response in succeeding years, is due more to the high degree of difficulty encountered in combining these studies and the complex physiology of the soil nitrifiers and their microhabitat, than to any diminution of interest in the topic. A similar request for studies to be undertaken in which natural conditions remained largely unaltered, was also made by Schmidt (1982). He stated that "microbiological data on the nitrifiers in relation to the processes as carried out in nature, would contribute considerably to an understanding of the basis for a particular nitrification rate, and to the reliability of models to predict rates at which nitrification takes place under various environmental conditions," but also cautioned that "a major difficulty in studying nitrification, especially in soil, is that the process interfaces with other components of the N cycle."

It is the general aim of the present work to attempt to measure nitrification rates in soils under different grassland management regimes in the natural environment, with the contemporaneous assessment of N losses (through the processes of denitrification and leaching), to try to relate changes to possible coupling and/or stimulation of the processes involved.

The specific aims of the project are stated at the start of the next chapter and the means by which they are to be achieved are also described and elaborated further in succeeding chapters.

CHAPTER 2 Development of a soil incubation system for the field measurement of nitrification

2.1 Project aims

a) To **develop** a new technique for the field measurement of nitrification, with simultaneous estimates of losses of nitrate by denitrification and leaching. *The approach is firstly, to identify the limitations associated with existing methods and secondly, to attempt to overcome those aspects which most directly interfere with natural fluctuations in environmental conditions (eg. temperature, rainfall, evaporation).*

b) To **link** the *in situ* measurements of nitrification with other processes, to gain an understanding of the controlling factors determining the fate of nitrate. *Nitrification activity will be assessed in actual and potential terms and related to measured losses of nitrate by denitrification and leaching.*

c) To **examine** the practical implications of these findings under different grassland management regimes. *Many aspects of N cycling in grassland soils are investigated in isolation from related processes; the aim of this work is to combine different measurements in a microcosm of the field environment.*

2.2 Background to existing methodology

Since any limitation in the process of mineralization, which supplies substrate (NH_4^+) to the process of nitrification, will necessarily determine the maximum rate at which the latter

proceeds (see Section 1.5.4), evaluation of nitrifying rates will be more meaningful if concomitant assessments of both processes can be made.

Recent advances in the use of the stable isotope ^{15}N (Barraclough, 1991; Barraclough and Puri, 1995) have enabled measurements to be made of **gross** rates of several of the processes involved in N cycling in soil (including nitrification), but because of the expense and complexity of the analytical equipment and materials required, these methods are best suited to short-term evaluations that make direct comparisons between treatments. The ^{15}N technique provides a powerful tool for the researcher, to add to the other methods available for field measurements, but is less well-suited to long-term seasonal measurements, which are required in order to construct balances of the flows of N in agricultural systems. The ^{15}N technique will be discussed in detail in later sections and will be employed to assess **specific** rates of processes, under well-defined conditions, to supplement measurements of **potential** rates obtained from laboratory incubations.

In order, therefore, to obtain **net** measurements of the processes connected with nitrification, that may be related in seasonal terms to losses of N from the soil, an improved field incubation technique was developed to meet these requirements. The existing methods rely generally on an assessment of the incremental (net mineralization) or decremental (net immobilization) change in inorganic N ($NH_4^+ + NO_3^-$). Soil samples taken at the start of an incubation are compared with a similar sample incubated in conditions that are intended to resemble closely the environmental fluctuations in the field. The methods adopted by workers to measure mineralization can roughly be divided into two soil sampling approaches: 1) disturbed soil and 2) undisturbed soil cores. Disturbed soil samples, either

crumbled by hand or sieved, may be incubated in the field by burying the sample in a polyethylene bag (Eno, 1960; Westerman and Crothers, 1980; Raison *et al.*, 1987). The advantage of this approach is that losses of NO_3^- by leaching are prevented, which might otherwise be overlooked in the evaluation. Losses of N by denitrification may, however, still result in an under estimate of mineralization and further inaccuracies can be caused by alterations to the soil structure. The use of relatively undisturbed soil samples in the form of intact soil cores will help to overcome some of these disadvantages (Cabrera and Kissel, 1988; Sierra, 1992), but losses can still occur as a result of denitrification. For this reason, Hatch *et al.* (1990, 1991) used acetylene (C_2H_2) in the headspace of the incubating vessel to prevent nitrification and, therefore, avoid the production of NO_3^-, which might otherwise be denitrified to gaseous forms (N_2O and N_2) which are more difficult to quantify. The assessment of net mineralization in this case, relied upon the measured accumulation of NH_4^+ during an incubation of 14 days.

All these methods suffer from the shared problem of using samples that are effectively isolated, to varying extents, from the normal fluctuations in soil water due to evaporation and precipitation during the period of incubation. Gill *et al.* (1995) attempted to minimise these effects by using a shortened incubation period of 7 days, but they were unable to find any significant effect of soil water content (SWC) on mineralization rates, although the literature would suggest that wetting and drying of soil has a major influence on the process (Birch, 1964; Stanford and Epstein, 1974). In addition, the sides of the soil cores are exposed during incubation so that the increase in aeration may falsely elevate rates of mineralization.

2.3 Description of equipment used in the novel incubation system

The novel incubation system (Fig. 2.1) incorporates a PVC incubation tube (length 200 mm, i.d. 38 mm, o.d. 49 mm) which acts both as the soil coring tool (when fitted with a detachable cutting ring) and as a permanent sheath in which the soil core is held during incubation (Fig. 2.1a). A soil core is obtained by driving the PVC tube into the ground, using a club hammer and metal striking cap. The correct depth of core (150 mm length) is determined when the soil surface reaches a set of four holes (3 mm diameter) drilled around the tube (50 mm from the top and 150 mm from the base of the tube) which are equally spaced to provide outlets for surplus water to drain by lateral flow from the soil core surface. An O-ring (neoprene, 5 mm diameter) is positioned immediately below the drainage holes, in a groove cut on the outside of the tube.

The PVC incubation tube containing the soil core is extracted from the ground by means of a larger metal soil corer (80 mm diameter i.d., 300 mm length). The metal corer is placed over the top of the protruding incubation tube and driven into the ground to a marked position equivalent to the depth of the soil core (Fig. 2.1b). In this way, the metal corer is used to extract the tube, together with an additional outer soil core, consisting of a cylindrical section which forms an annular ring (30 mm width) around the incubation tube (Fig. 2.1c). The soil from the outer core is carefully peeled away from the PVC tube and retained as a closely representative sample of the inner soil core sample. The inorganic N content of the outer core provides a baseline 'starting' value for subsequent comparisons with the field incubated soil core.

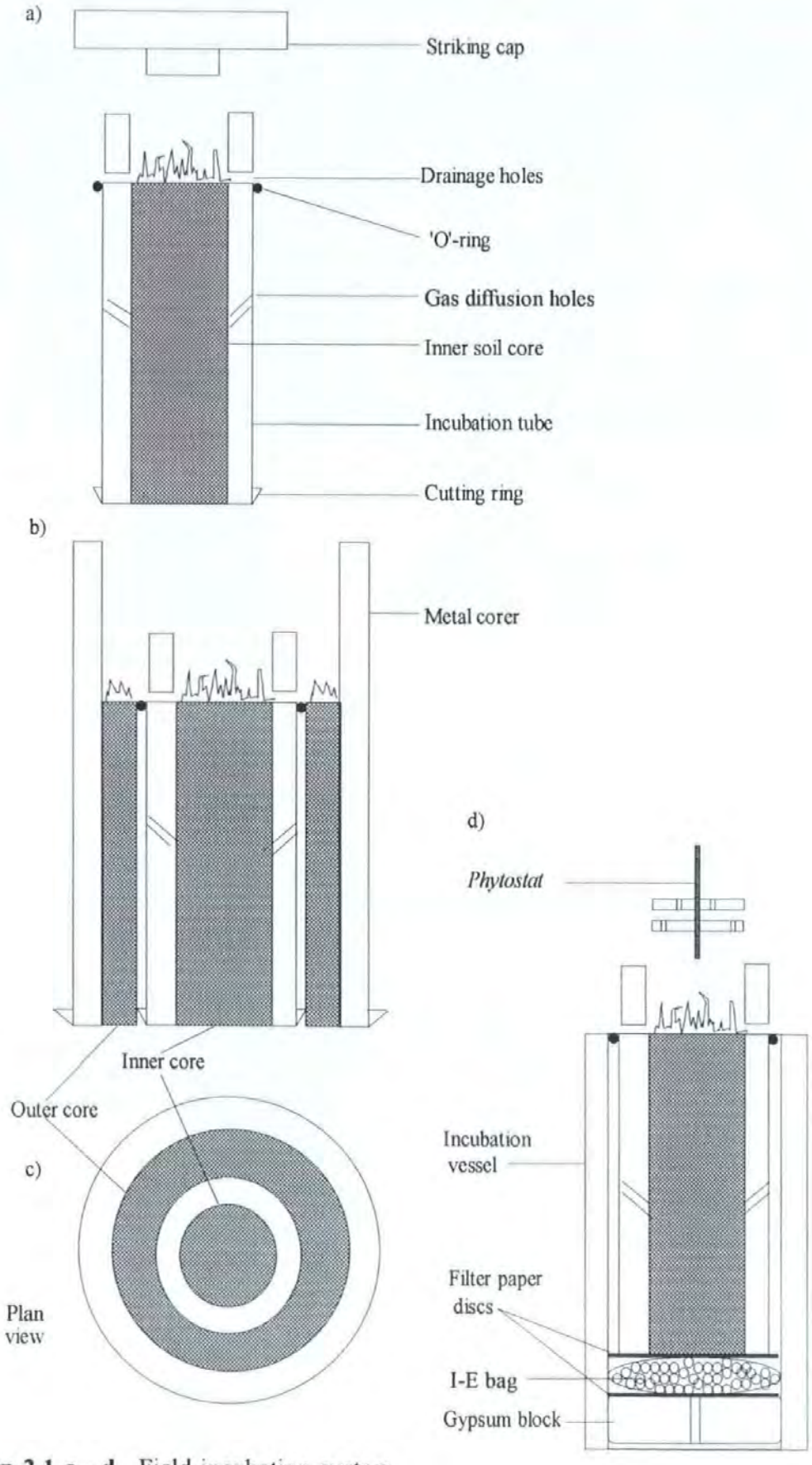


Figure 2.1 a - d. Field incubation system

An incubation vessel, consisting of a PVC tube (length 175 mm, 50 mm i.d., 60 mm o.d.), is driven into a suitably sized hole in the ground, so that the top of the tube is level with the soil surface, to provide a receptacle for the incubation tube (Fig. 2.1d) The inner edge of this vessel is machined to provide a groove into which the O-ring on the incubation tube locates to make a gas-tight connection between the two tubes. When the incubation tube is held in this position, it is suspended 25 mm above the soil at the bottom of the incubation vessel. Capillarity between the base of the soil core and the undisturbed soil layer is re-established and maintained through two circular layers of fine nylon (48 mm diam., 18.1 cm^2, 355 μm mesh, Lockertex, Warrington, Cheshire) sewn together with nylon thread in the shape of a 'tea-bag', leaving a 6 mm gap in the perimeter. Using a small funnel, 4 g of wet ion-exchange (I-E) resin beads (20-50 mesh) are placed into the bag through this opening (see Section 2.4.1), which is then sealed using cyanoacrylate adhesive. The I-E bag is sandwiched between two filter paper discs (47 mm diameter, grade 40, Whatman Ltd.) to protect the resin and improve contact between surfaces. When the I-E bag is fully wetted it occupies a space 10 mm deep and the remaining space is filled with a gypsum block (47 mm diameter, 15 mm deep: see Section 2.6). The gypsum block makes good contact with the undisturbed soil layer at a depth of 175 mm from the soil surface and acts as a passive and inert medium for the movement of soil water between the incubated core and the surrounding soil.

Plant growth in the soil core is suppressed by means of a specially constructed device, referred to here as a *phytostat*, which prevents light from entering the incubation tube. It consists of a pair of PVC discs (37 mm diameter), centrally mounted on a metal shaft (6 mm diameter, 50 mm length) each of which has four holes (3 mm diameter) drilled through

the discs and spaced equally around the perimeter, 5 mm from the outer edge. The discs on the shaft are rotated so as to place them eccentrically to each other, with no vertical alignment between the holes. In this way, the *phytostat* permits water movement both in and out of the incubation tube, but light is excluded and plant growth prevented.

2.3.1 Summary

The novel incubation system has the following potential advantages over existing methods of field incubation:

1) Assessment of 'starting' values is based on soil which is closely associated with the soil core sample taken for incubation.
2) Natural wetting and drying cycles continue during incubation.
3) Aerobicity of incubated soil cores remains largely unaltered.
4) Losses of NO_3^- by leaching and denitrification can be assessed.

2.4 Laboratory tests on component parts of the incubation system

The incubation system comprises a means of assessing losses of NO_3^--N by leaching using ion-exchange (I-E) resin bags and denitrification by a nitrification block method using acetylene (C_2H_2). Each of these aspects will be examined in this section.

2.4.1 I-E resins

Ion-exchange materials are insoluble matrices containing labile ions, which are capable of exchanging with other ions in the surrounding medium, but remain physically unchanged throughout. Ion-exchange is a stoichiometric process (the ion equivalents entering the resin are equal in charge to those leaving it), but resins exhibit a preference for certain ions (the

'selectivity coefficient'), so that the affinity of the resin for a particular ion needs to be considered carefully. Efficient ion-exchange is achieved when the ion which is to be absorbed has a considerably greater affinity for the resin than the ions already occupying the exchange sites.

Anion exchange resins derive their properties from the amino groups in the resin structure. The resin chosen to intercept NO_3^- in soil solutions was Dowex 1-x 8 (a strongly basic resin supplied in the Cl^- form by Fluka Chemicals, Gillingham, Dorset). The exchange capacity of the resin is 1.33 mol dm^{-3} (or 1.33 meq. ml^{-1} wet resin), operating over the full pH range and up to a maximum temperature of 150 °C. The mesh size of the material used in the construction of the bags is constrained by the need to prevent leakage of resin whilst at the same time allowing for efficient desorption through the free movement of the extractant between the resin beads. Earlier tests on nylon materials showed that mesh sizes of <200 μm caused air bubbles to become entrapped, which prevented the bags from becoming fully immersed in the extractant, leading to incomplete desorption.

2.4.2 Tests on I-E bags

The method of estimating NO_3^- leaching by I-E resin bags described by Lajtha (1988) was examined in greater detail to test its suitabilty to meet the requirements of the novel incubation system. Briefly, anion-exchange resin (Dowex 1-x 8) was converted to the bicarbonate form by three successive rinses in 0.5 *M* $NaHCO_3$. The I-E bags were rinsed

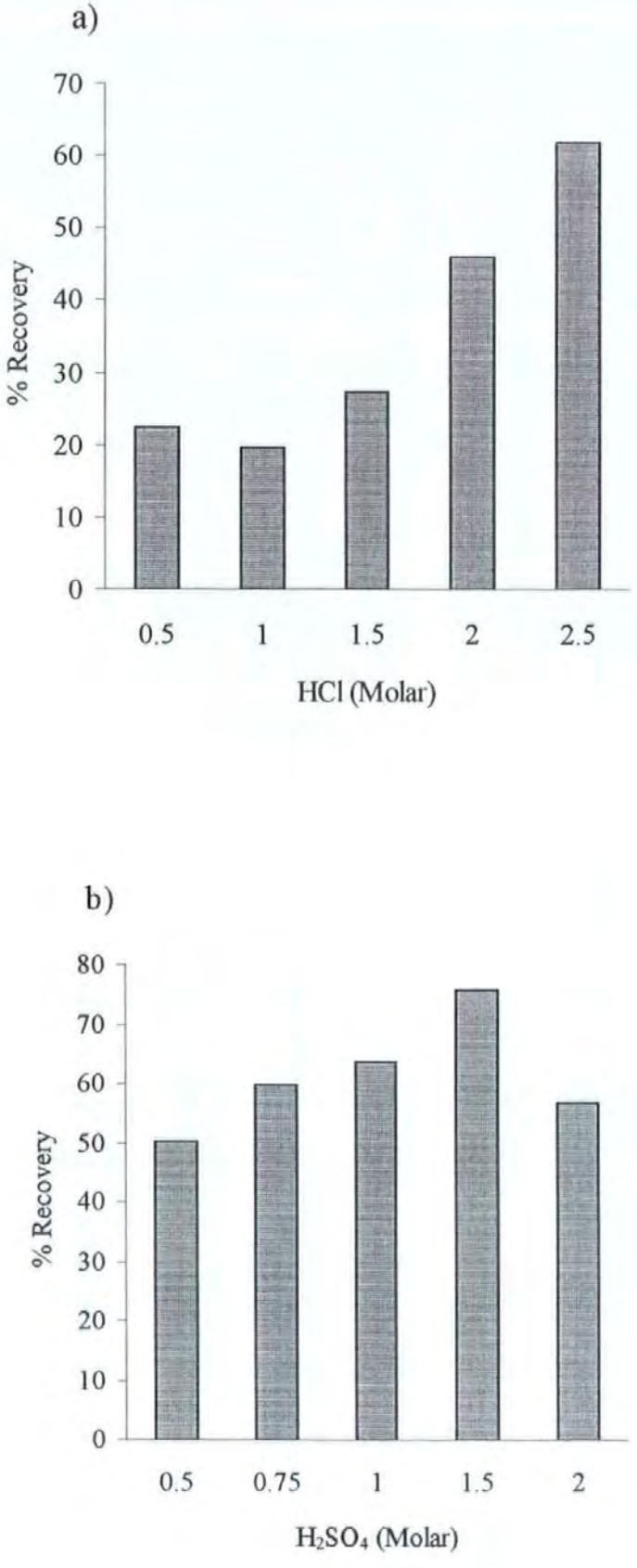


Figure 2.2 Mean recoveries of NO_3^--N from I-E resin bags using a) HCl (n = 3) and b) H_2SO_4 (n = 2) extractants.

in deionised water and spun dry in a hand-operated centrifuge. For the purpose of the present study a domestic salad shaker (*Moulinex*) was found to be satisfactory. The I-E bags were each immersed in 200 ml of 3.6 m*M* $NaNO_3$ containing 50 mg l^{-1} NO_3^--N, for 2 h with occasional stirring, then rinsed in deionised water and spun dry, as before. Samples of the original NO_3^- solution, along with samples of the solution that had been exposed to the I-E bags, were kept for analysis. The I-E bags were extracted using three 50 ml rinses of 0.5 *M* HCl (Lajtha, 1988) and made to standard volume (200 ml) with deionised water. The recommended concentration of 0.5 *M* was extended to include 1.0, 1.5, 2.0 and 2.5 *M* HCl (3 reps. to each concentration) in the range of extractants. The efficiency of these extractants was expressed in terms of the mean recoveries of the known NO_3^- loadings and calculated for each I-E bag from the difference between the extractant concentrations, analysed before and after exposure to the resin (Fig. 2.2a). Only poor recoveries of NO_3^- were found at the lowest concentration of extractant 0.5 *M* (22.4%) and recoveries increased exponentially to the highest concentration of 2.5 *M* (61.9%), suggesting impractically high concentrations of HCl would be required for near complete recovery.

The choice of HCl seemed to be of doubtful value in meeting the requirements of the present study and was probably adopted by the previous author as a compromise to enable both NO_3^- and PO_4^{---} to be extracted from soil solutions using the same resin. Also, replacement of the Cl^- functional group with HCO_3^- introduced an additional complicating step, with no apparent benefit in terms of affinity for NO_3^- and the further disadvantage that the volume of the resin increased by some 20%. The order of ion affinities for anion exchange resins established by Kunin and Myers (1947) is:

$$Cl^- < PO_4^{--} < NO_3^- < SO_4^{--}$$

It would appear, therefore, that SO_4^{--} is the more effective ion to displace NO_3^-, which in turn, should readily displace Cl^- from the resin. Indeed, Cl^- was adopted as the functional group to be exchanged with NO_3^- by Wyland and Jackson (1993), who also found that recovery was enhanced when SO_4^{--} was present in the solution and 2 *M* KCl was used as the extractant.

Recovery tests were repeated with Cl^--charged resins, combining a longer exposure to NO_3^- solutions (24 h), so that additional information on the capacity of the I-E bags could be obtained. Extractant solutions containing SO_4^{--} were considered in either the potassium or sodium form of the salt, but neither had the required solubility to achieve sufficiently high concentrations of the anion. Using duplicate I-E bags for each concentration to establish the trend, a range of H_2SO_4 extractants was tried and included 0.5, 0.75, 1.0, 1.5 and 2.0 *M* solutions. Recovery of NO_3^- from the I-E bags was increased compared with HCl extractant and the maximum recovery improved to 75.7% (Fig. 2.2b).

The possibility that the increasing acidity of the extractant solutions was interfering with the NO_3^- analysis was checked by re-analysing the whole set of extractions. Each sample was divided into two sub-samples: one from each pair remained unchanged and the other was neutralized by titration with 0.375 *M* Na_2CO_3. No significant difference was found between the two sets of samples, which were closely correlated ($r^2 = 0.864$), so that the increased recovery values can properly be attributed to the greater affinity of the resin for SO_4^{--} over NO_3^- (Fig. 2.3).

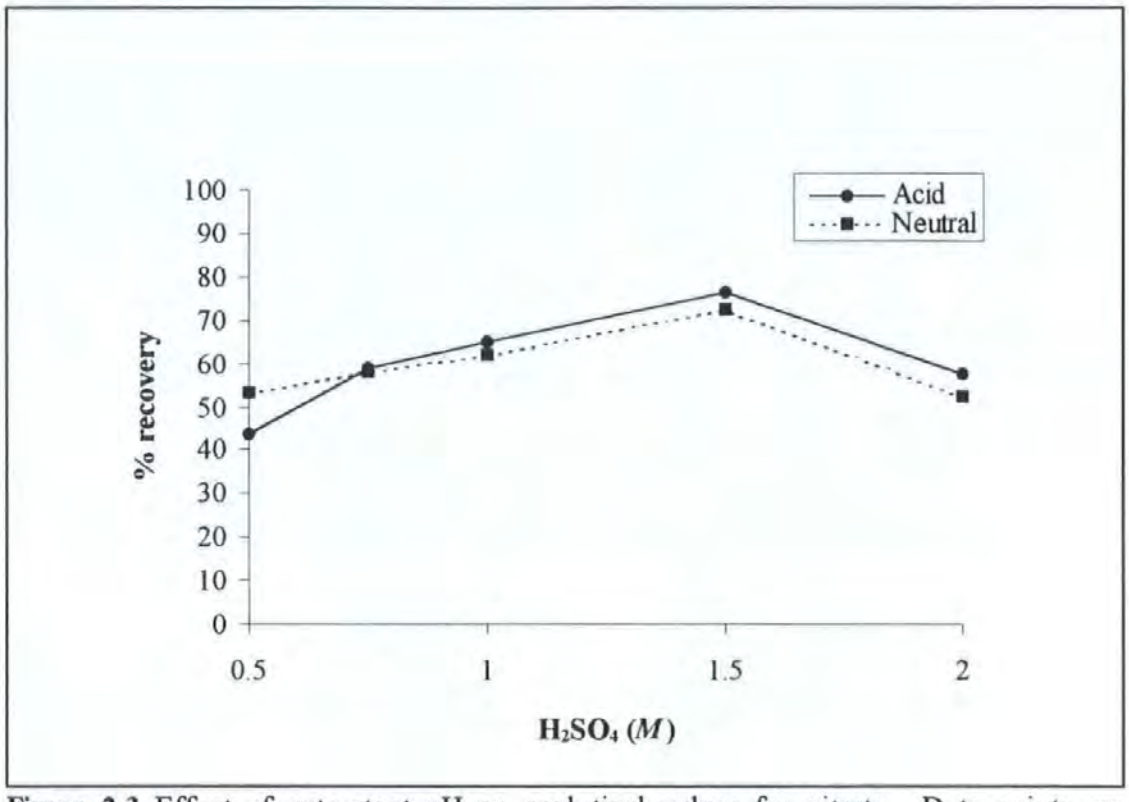


Figure 2.3 Effect of extractant pH on analytical values for nitrate. Data points are means (n = 2).

A comparison was also made of the effect of increasing the extraction time to see if any improvement could be obtained in recovery. Four replicates each of the NO_3^- pre-loaded I-E bags were extracted in 1.5 *M* H_2SO_4, by either leaving to soak for 24 h with occasional stiring, or shaken vigorously for 1 h. Mean recoveries were 77.7% (± 5.16) and 77.2% (± 1.40) for 24 h and 1 h, respectively, and were not significantly different (P = 0.05). The exchange capacity of the resin was found to be >2 mg NO_3^--N g^{-1} (wet resin), which meant that < 10% of the theoretically available exchange sites were occupied. Average weekly winter rainfall (January, South West England) is about 30 mm and the average NO_3^--N concentration in leached soil water (for grazed fields receiving 200 kg N ha^{-1} y^{-1}) is about 10 mg l^{-1} (Scholefield *et al.*, 1993). Each soil core (with a surface area of 11.4 cm^2) could, therefore, potentially leach 342 µg N during an incubation lasting one week, i.e. well within the exchange capacity of the I-E resin.

2.4.3 Summary

The I-E bags were used in the Cl^- form and the recommended regenerant was taken from a technical handbook (Ion-exchange resins, BDH Chemicals Ltd. Poole, England) specifying HCl (5% w/v): 350 ml for 100 ml moist resin (5.7 ml wet resin weighs 4 g). The method adopted to recover absorbed NO_3^- was to immerse the I-E bag in 50 ml of 1.5 *M* H_2SO_4 extractant for 1 h with shaking, and then to rinse the bag with two further 50 ml batches of the extractant, finally making to standard volume (200 ml) with rinses of deionised water.

2.5 Loading / flowrate effects on absorption by I-E bags

The efficiency of absorption of NO_3^- by I-E bags was evaluated further using a perfusion system (see Fig. 2.4; Plate 2.1).

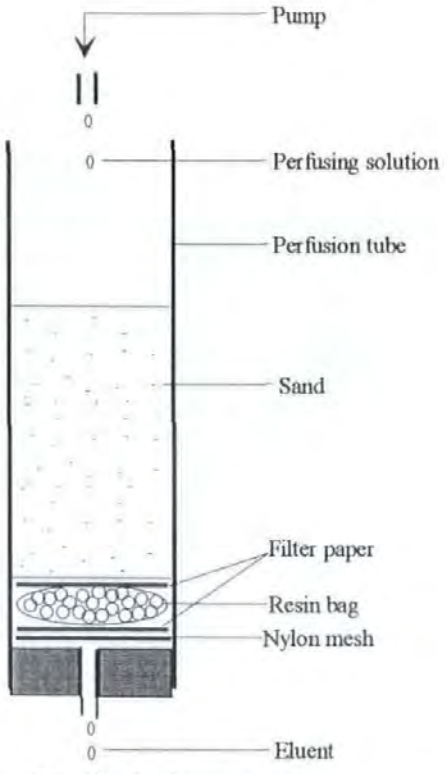


Figure 2.4 Perfusion system

Plate 2.1 Perfusion tubes and pump

Twelve perspex leaching tubes (325 mm long, 50 mm i.d.) were supported in upright positions and each was packed to a depth of about 150 mm with 200 g acid washed, dry horticultural sand (screened to <1 mm). The lower ends of the tubes were stoppered with rubber bungs (size 49), centrally drilled to hold a length of glass tubing (70 mm long, 5 mm o.d.). The glass tubing was positioned so that the upper end was flush with the surface of the bung to provide a free-draining outlet from the perspex tube. The outlet was covered by a circular piece of fine nylon gauze (48 mm diameter, 140 μm mesh) to retain the sand. Perfusate (100 ml) containing either 100, 50, 25 or 10 mg l^{-1} NO_3^--N was used to wet the sand, with each concentration replicated three times. The perfusion tubes were allowed to drain freely overnight and then anion-exchange resin bags (in the Cl^- form, as previously described), sandwiched between two filter paper discs (47 mm diameter, grade 40, Whatman Ltd.), were placed beneath the sand and above the nylon mesh. Positioning of the I-E bags was achieved by briefly inverting the perfusion tube. A plunger was employed to retain the sand and care was taken to avoid displacing perfusate, so that the sand remained primed at the point where drainage would resume when more perfusate was added. In this way, precise timing of flowrates through the I-E bags was achieved and commenced from when eluent flowed from the outlets. Perfusate was supplied to the top of the tube from a peristaltic pump at either 50 or 100 ml 6 h^{-1}. Previous tests on alternative means of controlling the rate of perfusion, consisted of connecting the bases of the perfusion tubes to a peristaltic pump with a pull-through flow system (Killham, 1987). However, this arrangement was less satisfactory for the purposes of the present tests, because 'fingering' (by-pass flow) occurred between the sand and the wall of the tubes and high concentrations of NO_3^- were detected in the eluents.

Good recoveries were sustained over the range of NO_3^- concentrations applied in perfusates at 50 ml 6 h^{-1}, which simulated a rainfall event of *c.* 7 mm h^{-1} (Fig. 2.5). At the lowest NO_3^- loading (10 mg N l^{-1} x 50 ml 6 h^{-1}), which represented an increase of nearly 50% in the (anticipated) N-loading in eluents from field incubated soil cores, over 90% was recovered in the I-E bags. At twice this flowrate, simulating extreme rainfall events of *c.* 15 mm h^{-1} (sustained for 6 h), the recoveries were less uniform and ranged from 77 - 94%. The low recovery of 77% from the highest loading (100 mg N l^{-1} x 100 ml) was to be expected, since it exceeded the known capacity of the resins by >20%. Performance of the I-E bags was, therefore, well within the necessary limits required to extract NO_3^- from soil solutions, but measurements of potential leaching losses from incubated soil cores will be adjusted using the established recovery factor of 90%.

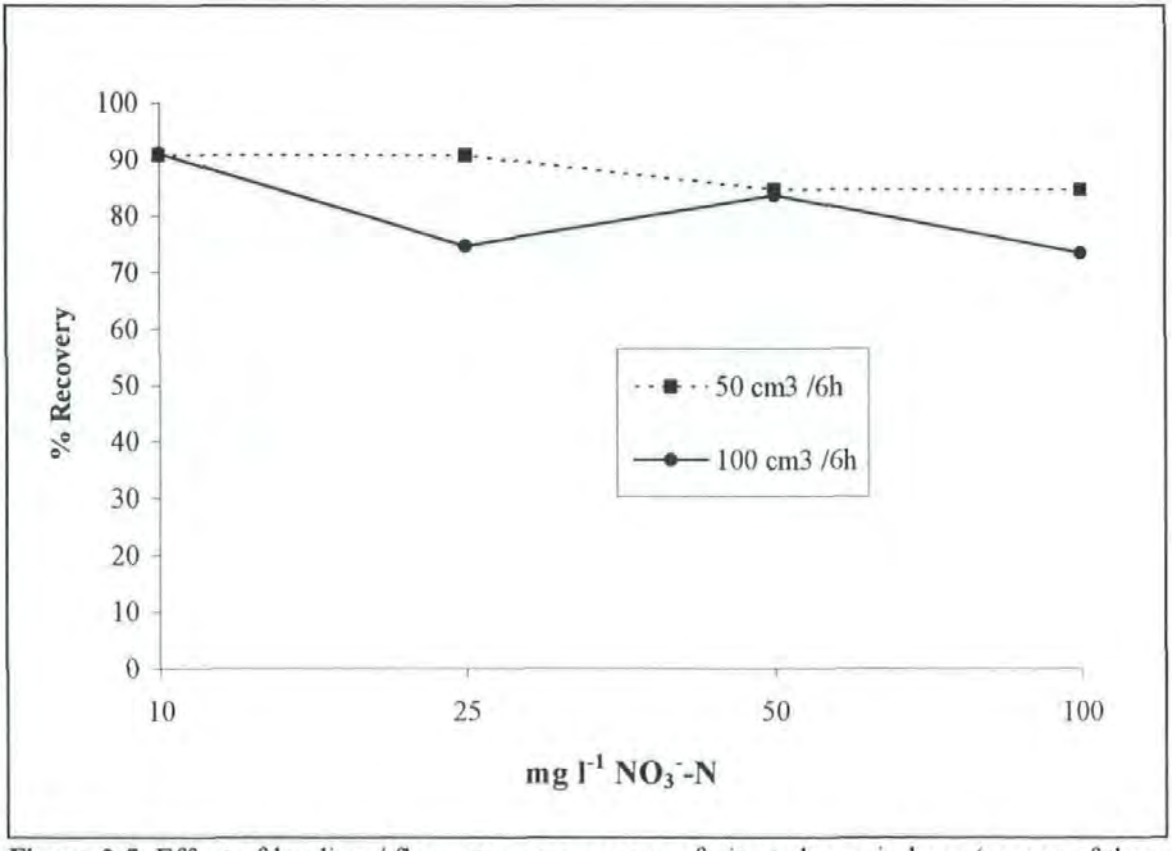


Figure 2.5 Effect of loading / flowrates on recovery of nitrate by resin bags (means of three replicates).

2.6 Inert porous blocks

The incubation system described in Section 2.3 specifies the requirement for a supporting structure which is both inert and porous to separate the I-E bags from contact with the soil layer beneath the incubated soil cores. The 'porous block' must ensure that the I-E bags are only exposed to NO_3^- in the mobile water phase emanating from the incubated soil core and not affected to any large extent by the undisturbed soil layer beneath the incubation vessel (Fig. 2.1d). In addition, water tension in the soil core had to be maintained and various materials were considered and tested for the necessary properties. The results from field tests on the two most promising materials are described below.

2.6.1 *Oasis* blocks

Cylindrical blocks (47 mm diameter, 15 mm deep) made from *Oasis* (an open cell expanded foam used by florists to provide water to plants under capillary tension), were tested for their suitability. Soil cores (4 replicates) were field incubated for 7 d periods using the new system lasting for a total of 35 d (total precipitation of 32.1 mm). Soil water contents (SWC) of the soil cores were compared with the surrounding soil (Fig. 2.6) using a Student t-test. Mean SWC of soil cores was significantly increased (by an average of 22%) compared with soil which was not in contact with the *Oasis* ($P < 0.05$). The water-holding capacity of *Oasis* is almost 1 ml cm^{-3}, which amounts to a total of 28 ml water per block. The water content of the soil core at field capacity (Hallsworth series) was about 20 ml, representing a potential increase of 140% if all of the available water were taken up by the soil core. The likelihood of water-logging, particularly at the base of the soil core was high and the choice of *Oasis* was rejected.

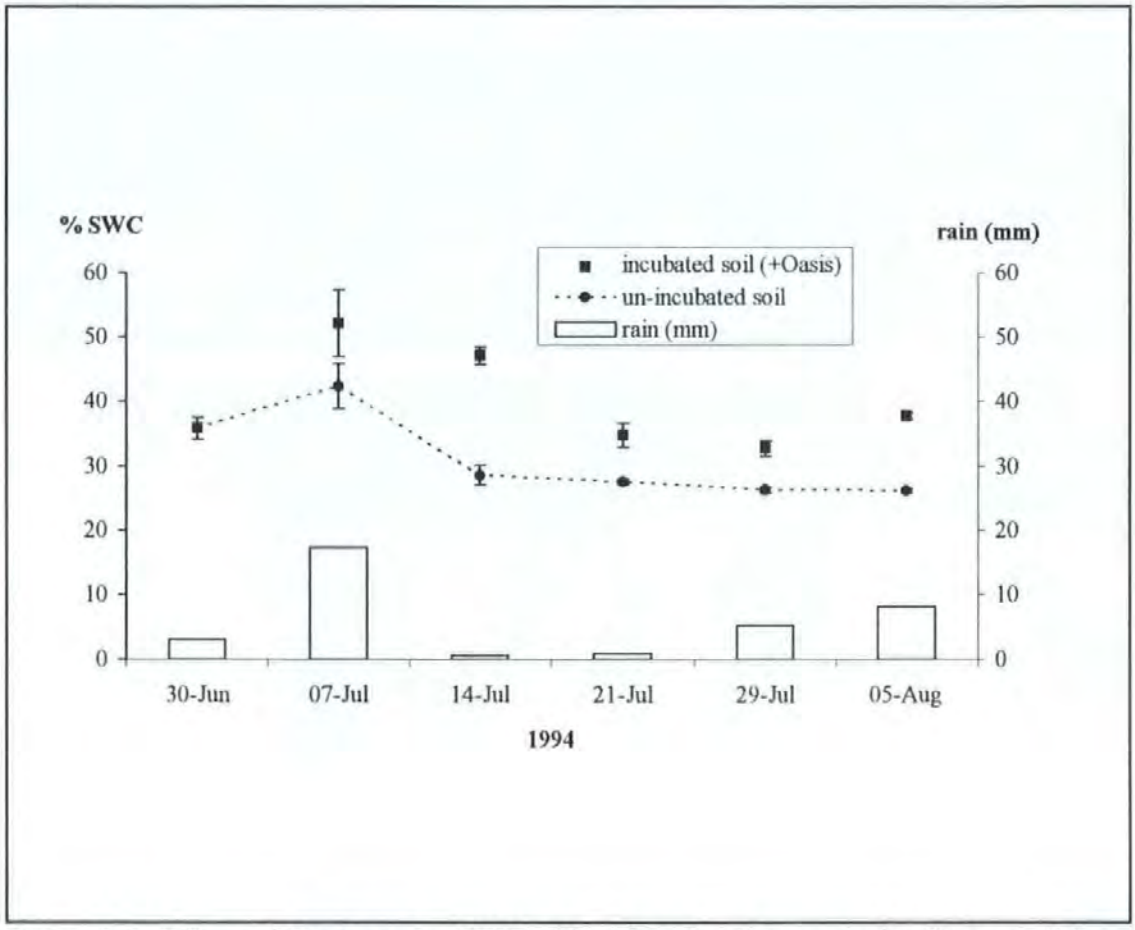


Figure 2.6 Effect of *Oasis* on the SWC of incubated soil cores under field conditions. Vertical bars = SEMs (n = 4)

2.6.2 Gypsum blocks

Calcium sulphate ($CaSO_4$) occurs naturally and as the dihydrite, $CaSO_4.2H_2O$, is known as gypsum. When gypsum is heated to 97°C, loss of water occurs to form the hemihydrate:

$$2(CaSO_4 \bullet 2H_2O) \rightleftharpoons (CaSO_4)_2 \bullet H_2O + 3H_2O$$

The product of the forward reaction is 'Plaster of Paris'. When water is added, the reaction is reversed and a solid mass of interlocking crystals of gypsum are formed, accompanied

by a slight expansion and generation of heat. Gypsum blocks (47 mm diameter, 15 mm deep) were individually moulded using 25 g of 'Plaster of Paris' in a 1:5 ratio with water (w/v) to achieve a thick, but still fluid mixture. The blocks were allowed to cure for 12 h and then a 6 mm hole was drilled through the centre to prevent any pools of water from forming on the top of the block. The water holding capacity (± SEM, 11 d.f.) of the gypsum block was 9.0 ml (± 0.34) which was decreased slightly to 8.7 ml (± 0.33) when a 30 cm tension was applied. Incubated soil cores (4 replicates) were compared over 7 d periods for a total of 28 d (total precipitation of 152.3 mm) and were not found to be significantly different from non-incubated soil (Fig. 2.7).

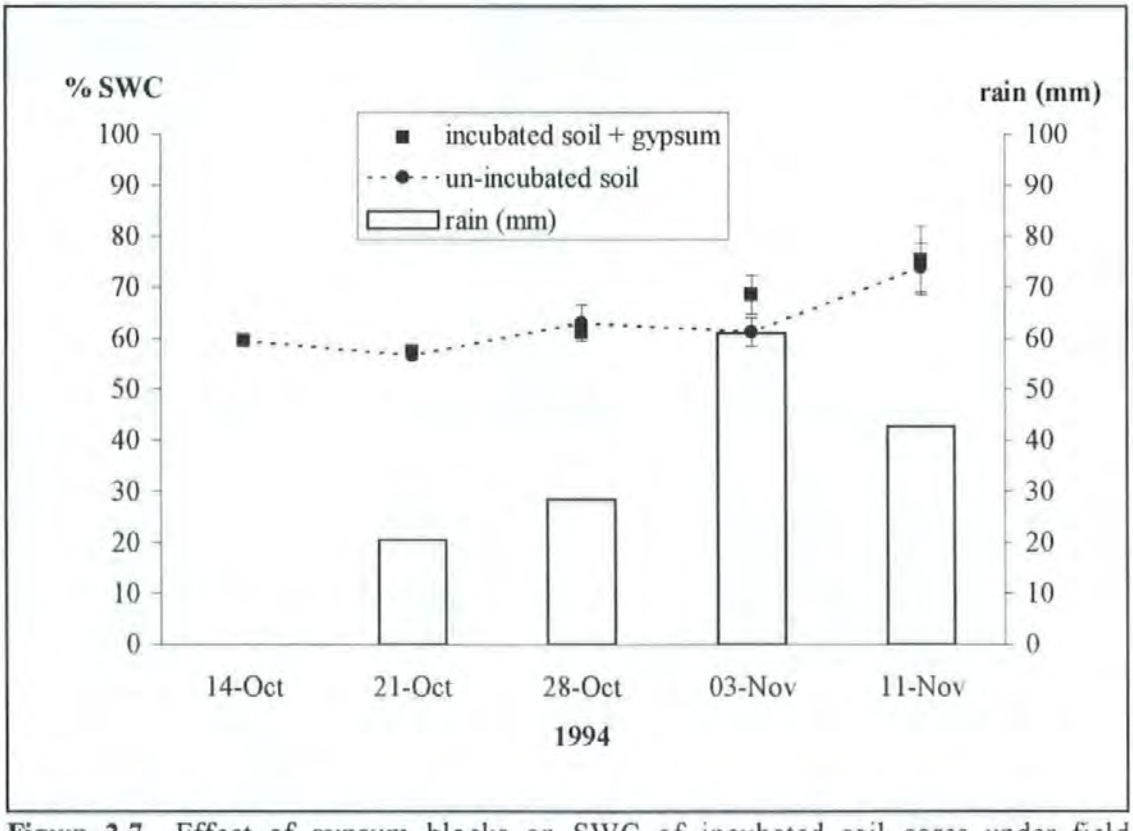


Figure 2.7 Effect of gypsum blocks on SWC of incubated soil cores under field conditions. Vertical bars are SEMs (n = 4)

Data points have not been joined for the soil cores on either graph, because they represent

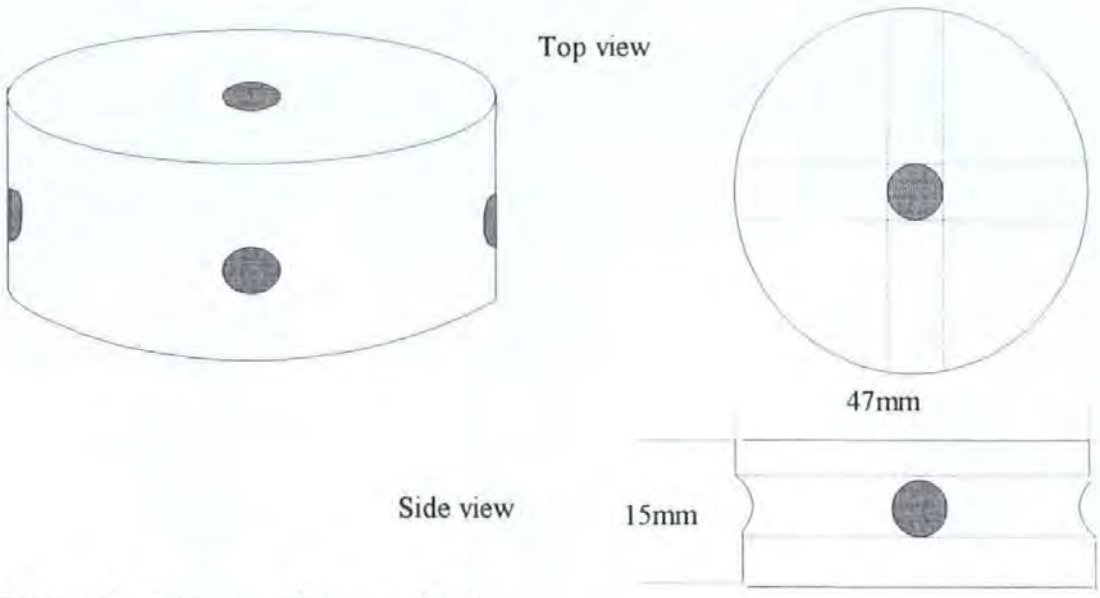


Figure 2.8 Diagram of gypsum blocks

non-contiguous measurements from successive individual samples, whereas the data from the surrounding soil are repeated measurements from the same sample set. Gypsum blocks were incorporated into the field incubation system and were modified to accommodate controlled-release capsules (see following sections describing C_2H_2 infusion of soil cores) by drilling four further holes around the periphery of the block (Fig. 2.8).

2.7 Acetylene inhibition of nitrification

The recognition of the inhibitory effect of C_2H_2 on autotrophic nitrification (Walter *et al.*, 1979), also presented a means by which denitrifying activity could be suppressed through restricted substrate (NO_3^-) supply. Warren (1988) employed C_2H_2 in laboratory incubations to inhibit nitrification, thereby denying denitrification; the amount of accumulated NH_4^+ was taken as an estimate of net mineralization. Ryden (1982) showed that only low concentrations of C_2H_2 were needed to inhibit nitrification and mineralization was not affected. Hatch *et al.* (1990) further developed the method for field application by including 2% C_2H_2 in the headspace of incubation vessels. Subsequent refinements (Gill

et al., 1995) ensured that acetone (which is a contaminant of commercially supplied C_2H_2) was removed by two sequential water-traps to avoid introducing additional sources of energy. Another consideration is that degradation may occur in the soil, after a variable lag phase of up to 8 d, through the development of a C_2H_2-metabolizing population (Terry and Duxbury, 1985), which can then increase rapidly (Topp and Germon, 1986). Further difficulties associated with C_2H_2-inhibition have been identified by Smith and Arah (1990), who pointed out that soil types that would be expected to have the highest denitrifying capacity (e.g. heavy clay/anaerobic soils), often gave lower than expected rates, due to poor infusion of the gas through water-filled pores. Jackson *et al.* (1989) attempted to overcome this problem by infusing soil cores with C_2H_2 dissolved in water, but this caused increases in soil water content.

Calcium carbide (CaC_2) was used to generate acetylene gas for lighting purposes in the early days of the motor industry (1 kg CaC_2 yields 300 l of gas); a controlled release of gas was achieved by altering the rate of addition of water onto the carbide granules.

The reaction of carbide with water is:

$$CaC_2 + 2H_20 = C_2H_2 + Ca(OH)_2$$

Controlled release technology has been pioneered in the pharmaceutical industry using coatings of sugars or water soluble celluloses, but the difficulties of applying water soluble coatings to a water-activated ingredient (*viz.* CaC_2), made them unsuitable for the present application. Previously, the majority of (medicinal) enteric coatings were applied as

solutions in organic solvents. However, because of the hazards associated with such solvents, e.g. cost, safeguards and disposal problems, these have been largely discarded in favour of aqueous solutions. Proprietary mixtures of water soluble celluloses (e.g. hydroxypropylmethylcellulose, HPMC) with water-insoluble celluloses (e.g. ethyl cellulose, EC) were tested in the present study. Combinations of mixtures (HPMC : EC) from 9:1 (soluble) to 5:5 (nearly insoluble) as 10% mixtures (w/v) in an organic solvent (50:50 methylene chloride/ethanol) were examined. Unfortunately, the difficulty of obtaining an even coating over irregular lumps of carbide, ultimately meant that this technique had to be rejected. An alternative approach of coating carbide with waxes was used by Banerjee and Mosier (1989) to achieve long-term persistence in soil. However, the method described by these authors was better suited to coating large particles of carbide and the encapsulating method gave variable and prolonged delays in the release of C_2H_2 which did not meet the requirements of the present short-term incubations.

Gelatine capsules are employed to package medicinal formulations and in the past have also been coated to achieve delayed dissolution. Coatings such as beeswax, carnuba wax and shellac were used and, for some applications, the gelatine itself was modified chemically. Reactive side-chain groups in gelatine consist of amino, carboxyl and hydroxyl groups, of which the amino groups are the most amenable to chemical modification and the hydroxyl groups the least so. Reagents which are capable of reacting with two amino groups can cross-link within the gelatine matrix to give increased solution viscosity and a higher gel melting point, even to the extent that the gelatine becomes insoluble. Exposure to formaldehyde renders the gelatine capsule increasingly insoluble, but still porous to water. Pharmacological use of formaldehyde has now been discontinued, largely because of 'after-

hardening' of the capsules which can happen naturally through impurities in the gelatine (chelation of heavy metals), or at elevated temperatures (> 40° C).

To overcome some of the problems associated with contamination and persistence of C_2H_2 in soil, the novel incubation method (a) restricts exposure of soil cores to < 8 d and (b) replenishes metabolised C_2H_2 by a controlled infusion of the gas using gelatine capsules containing CaC_2, as described below.

2.7.1 Formulations for controlled-release capsules

Hard-shell gelatine capsules (size 3; Farillon, Romford, UK) were pre-treated by exposure to formaldehyde vapour in a desiccator for 72 h. This process softens the capsule, but rigidity was re-established by heating at 100 °C for 30 mins. During the heating stage, the original shape of the softened capsule was preserved by placing the open 'halves' on dress-making pins, mounted vertically in a balsa wood stand. Four types of controlled release capsules were prepared, each containing 100 mg CaC_2 granules (< 2 mm) which were coated to delay the release of C_2H_2 gas, as follows:

Type 1: Uncoated, with rapid gas release from d0 - d1

Type 2: Coated in paraffin wax, with a delayed gas release from d2 - d3

Type 3: Coated in a mixture of paraffin wax / beeswax (50/50), with a delayed gas release from d4 - d5

Type 4: Coated in beeswax, with a delayed gas release from d6 - d7

The gas release times are nominal and the success of the capsules is dependent upon the persistence of C_2H_2 as a nitrification inhibitor and on a degree of overlap between release

times for the different types of capsules. The pre-drilled holes in the gypsum blocks (see Fig. 2.8) each holds one of the four types of capsules, which together generate a total of 30 ml of C_2H_2.

Waxes were refrigerated before use and fine shavings were obtained using a 'cheese grater'. The capsule base was filled to about 1/4 full with wax shavings and carbide granules (approx. 100 mg) were packed above the wax to the brim of the capsule. The capsule top was filled with more wax shavings and the two halves were pressed firmly together. Coating of the carbide granules was achieved by immersing the filled capsules in molten wax, which imparted the appropriate amount of heat to melt the wax inside the capsule, without deforming the gelatine. The granules were coated evenly by rolling the capsules around in the molten wax until the wax inside was thoroughly dispersed. The capsules were then removed and any wax adhering to the outside was wiped off with absorbent paper. When the capsules had cooled, the carbide granules were observed to have become encased in a fine matrix of wax and could be stored in a desiccator until needed. On exposure to water, the capsule softens, which distorts the wax matrix, so that the carbide granules become wetted progressively; the reaction releases C_2H_2 which further disrupts the wax matrix and enables the ingress of more water.

2.7.2 Laboratory tests on controlled release capsules

Soil cores (150 mm deep) were obtained using incubation tubes which were then placed into incubation vessels (see Fig. 2.1) embedded in moist sand to simulate the supply of soil water under tension and kept in a fume hood at 20 °C. The incubation tubes were modified to improve infusion of C_2H_2 into the soil core: four upward sloping holes were drilled (3.5

mm diameter, 75 mm from soil surface) at angles of 30° and equally spaced around the tube to avoid by-pass flow of leachate from the sides of the tube. A gas sampling probe was constructed from stainless steel (s.s.) tube (1 mm diameter, 125 mm long) with a rubber suba seal (No. 13) fitted to one end and a microporous (s.s.) mesh (hydrophobic, but permeable to gas) attached to the other end. Gas sampling probes were inserted into each of four soil cores (50 mm from the surface) and a gypsum block was placed beneath each soil core at the bottom of the incubation vessel and in contact with the wet sand. Each gypsum block contained a different capsule (100 mg CaC_2), representing one of the four types described above. After 1, 2, 4 and 5 d, samples of the soil atmosphere from around the probes were taken by withdrawing gas through the suba seal with a syringe. The first 0.5 ml was discarded and then a further 2 ml were withdrawn slowly and injected into the sample loop (0.5 ml) of an *Autofim** portable gas chromatograph, fitted with a flame ionisation detector.

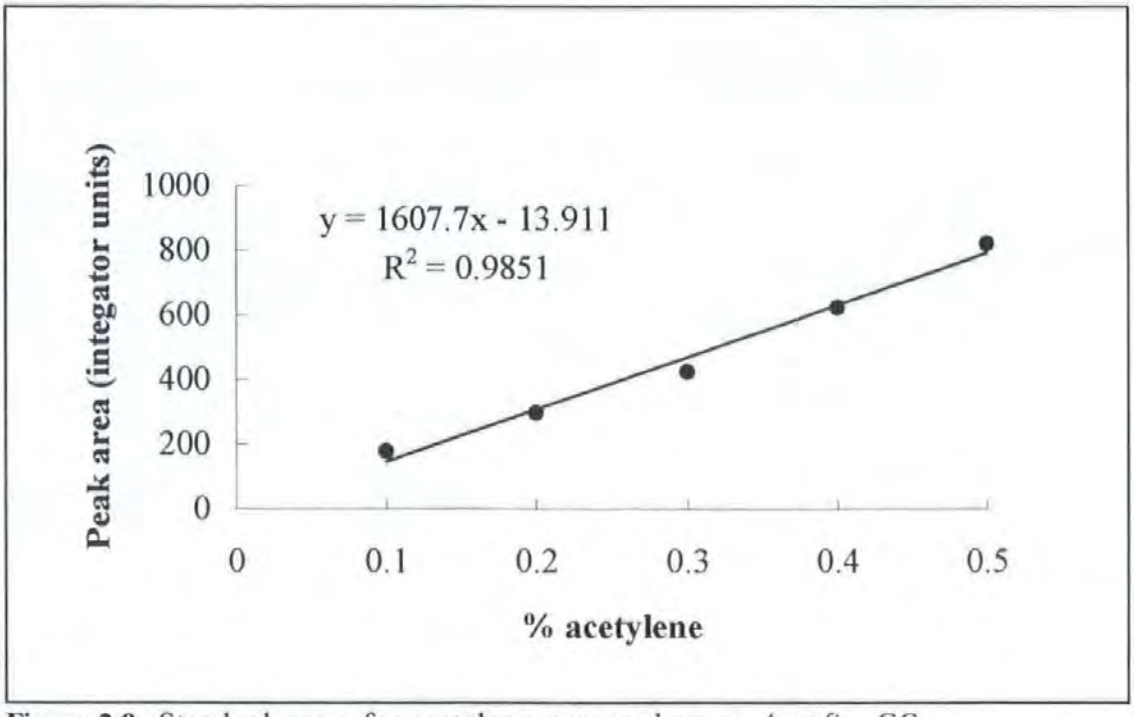


Figure 2.9 Standard curve for acetylene measured on an *Autofim* GC.

The samples were compared against a set of C_2H_2 standards prepared from a cylinder of commercial-grade C_2H_2. The response of the instrument was linear (see Fig. 2.9) and the results are shown in Table 2.1.

Table 2.1 Acetylene (%) in soil atmospheres at 50 mm from surface of soil cores, incubated at 20 °C with controlled release capsules.

Capsule type		Incubation period			
		1 day	2 days	4 days	5 days
Type 1	Uncoated	0.3	0.04	0.04	-
Type 2	Paraffin wax	0.16	0.04	0.04	0.03
Type 3	Paraffin/Beeswax	0.07	0.04	0.04	-
Type 4	Beeswax	-	-	-	0.04

*The author gratefully acknowledges the loan of the *Autofim* instrument from Research Engineers Ltd., Orsman Road, London, N1 5RD for the duration of the test.

Concentrations of C_2H_2 in the soil atmosphere at the end of a 5 d incubation (Table 2.1) were in the range found to be completely effective in preventing nitrification by other workers, *viz.* 0.01% (Berg *et al.*, 1982) to 0.1% (Walter *et al.*, 1979). The concentrations determined in the soil cores are likely to be elevated to some extent when the full complement of capsules is utilized, since the effect will be to reinforce the impact of individual capsules.

2.7.3 Conclusions

In this chapter, the successful development of a new field incubation technique was described. The individual components of the system were thoroughly tested under laboratory and field conditions and were shown to meet the required specifications. Ion-exchange resin had sufficient capacity and a high affinity for NO_3^- to extract the N leached from soil cores. Encapsulated CaC_2 (coated in wax) produced a controlled-release of C_2H_2 on exposure to moist soil. The gypsum blocks provided a porous medium to support the capsules, without significantly affecting soil water conditions during incubation. Conclusive evidence of the efficiency of the controlled release capsules in preventing nitrification in soil cores could only be obtained by measuring changes in NO_3^--N under conditions of field incubation (see Section 5.2.3). A more comprehensive examination of the incubation system was undertaken in spring and autumn (1996), during which times the prevailing conditions are often conducive to high losses of NO_3^- through denitrification and leaching, respectively (see Chapter 5).

In the next chapter, the new incubation system was further tested under field conditions. Based on these initial data, the extent of sample variability was shown; improved methods were devised and protocols for analysis of soil samples were established. The development phase of the project culminated in a model to enable several N cycling processes to be measured simultaneously using sets of paired incubations ($\pm C_2H_2$).

CHAPTER 3 Initial field testing of the soil incubation system and protocols for analysis of soil samples

3.1 Initial field tests of incubation system

Temperature and water content are major factors influencing both mineralization and nitrification rates (Miller and Johnson, 1964). Both variables were discussed in Section 1.5 in relation to nitrification, and operating limits for the incubation system were examined for soil water content (see Section 2.6). However, in previous studies using field measurements of net mineralization, temperature was found to exert the greater influence on rates (Hatch *et al.*, 1990; Gill *et al.*, 1995). This chapter describes the performance of the incubation system under field test conditions and the extent to which the temperature of the soil core was affected. The information obtained from monitoring changes in inorganic N levels during incubation and the protocols adopted to ensure consistent extractions of soil N are also detailed. These earlier tests did not, however, include C_2H_2 infusion of soil cores for measurements of denitrification; elaboration of relevant calculations are presented at the end of this chapter in the form of a simple model and its application is to be discussed in subsequent chapters. The incubation system, incorporating the full complement of field measurements (net mineralization, nitrification, denitrification and leaching), enabled the major N cycling processes to be investigated.

3.1.1 Temperature within the incubated soil core

The incubation system was tested on two separate occasions over 7 d periods (21/6/94-27/6/94 and 7/7/94-13/7/94), by comparing the temperature within two incubated soil cores with that of the surrounding soil, using platinum resistance (2 kΩ) thermometers

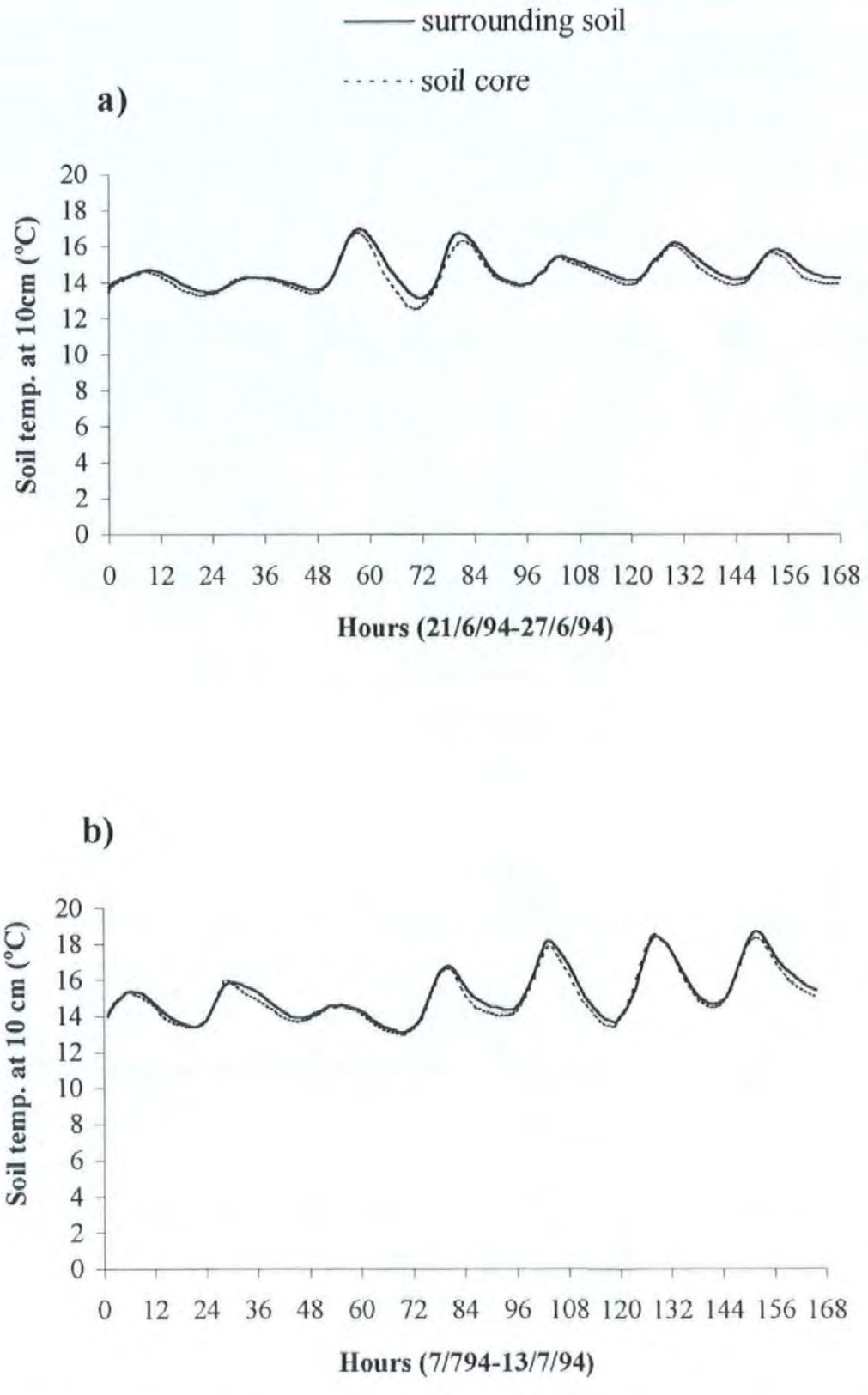


Figure 3.1 Temperature of incubated soil cores compared with surrounding soil: measurements began at 0900h at the start of each 7d period in a) June 1994, b) July 1994.

(thermistors). A thermistor was inserted at 100 mm depth into each soil core and two others were placed in the surrounding soil (at the same depth): temperature was then recorded hourly. Data were stored on a Delta-T data logger (Delta-T Devices, Burwell, Cambs.) and the mean temperatures of soil cores and surrounding soil were compared using a paired sample t-test, for each 7 d period. The daily temperature patterns (Fig. 3.1a and b) between the incubated soil cores and the surrounding soil were highly correlated during both periods (r^2 = 0.976 and 0.993, respectively). A small, but significantly (P <0.001) lower mean temperature was recorded within the soil cores compared with the surrounding soil during both measurement periods (differences between means of 0.28 °C and 0.23 °C, respectively). It was evident that these small differences were associated chiefly with the periods following peaks in diurnal temperature fluctuations (around noon) and persisted until the lowest temperatures were reached (around 0800h). Thus, the small difference between an incubated soil core and the surrounding soil was most pronounced when the air temperature was declining. The soil core, insulated within the PVC tube, lost slightly more heat than the surrounding soil, which was buffered by having the greater mass. During the warming-up phase of the diurnal cycle, the temperatures measured within and outside the incubation tube were in close agreement.

It was concluded that enclosure of soil cores in the incubation system for periods lasting 7 d had no cumulative effect on the temperature within the soil core and only a small overall difference was recorded on a day-to-day basis. If the Q_{10} of the rate of nitrification is taken to be from 1.7-3.0 (see Section 1.5.1), then the process could be slowed by up to 8% during that part of the day when the temperature of the incubated soil core falls by *c.* 0.25 °C.

3.2 Experimental sites

It has been suggested (Jarvis and Barraclough, 1991) that nitrifying activity may be increased by applications of N fertilizer. Based on this observation, the present investigation seeks to extend these findings and to relate them to N inputs and losses by direct measurement of the processes involved. In order to interpret the effects of systems of field management correctly, a long-term experiment with well established treatments was needed for the investigation. These requirements were provided by a field drainage experiment (Armstrong and Garwood, 1991; Scholefield *et al.*, 1993) at the Institute of Grassland and Environmental Research (IGER) site on Rowden Moor (OS Grid ref. SX 651995) at the North Wyke Research Station in Devon. At this site, hydrologically isolated field lysimeters, each of 1 ha long-term managed grassland, allow the comparison of the effects of field drainage with undrained soil. The site has been under permanent grassland management for over 40 years on a poorly drained clay loam soil, classified as a clayey, non calcareous pelosol of the Hallsworth series derived from the Culm Measures (a typical *pelo-stagnogley* soil (Findlay *et al.*, 1984; USDA = *typic haplaquept*; FAO = *dystric gleysol*). The combination of a high annual rainfall (on average, 1050 mm) and a soil with a high clay content (*c.* 36%), means that for much of the winter period the soils are water-logged and without artificial drainage, a large proportion of the rainfall is removed in run off through surface or sub-surface lateral flow. Surface run off and drainage waters from each plot are separately channelled to outflow weirs to enable the measurement of flow rates and the collection of water samples for analysis.

Three experimental treatments within the experimental system were identified which are likely to provide contrasting features and may, over several years, have affected the

processes concerned in this investigation. The treatments selected for the study have all been installed with a system of field drainage (field drains 40 m apart and 85 cm deep with a porous backfill with temporary mole drains 2 m apart and 55 cm deep), that has been shown to increase mineralization rates compared with undrained soil, through improved aeration (Blantern, 1991). The selected treatments were continuously grazed by cattle during the growing season and since 1983 have either received 200 kg N ha^{-1} y^{-1} in 9 equal applications at 3 weekly intervals (*NF* treatment), or have remained unfertilized since 1983 (*ZN* treatment), or relied on inputs of fixed N from a mixed sward, which since 1988 contained clover (*GC* treatment). The treatments are represented by two replicate plots, except in the case of *ZN*, with only a single replicate. These treatments form part of a larger experiment (Tyson *et al.*, 1993) that has been used in previous studies on nutrient cycling (Scholefield *et al.*, 1993), but due to subsequent changes, now represent the only unchanged elements of the original experimental design of 13 years ago (Fig. 3.2).

3.2.1 Field measurements of mineralization / nitrification

A field incubation system based on 150 mm deep soil cores (as described in Section 2.3) was used to examine mineralization / nitrification in two of the long-term treatments, described above. A comparison was made between the *ZN* plot and one of the replicate plots from the fertilised treatment (*NF*, block B). Soil cores (4) were taken at random from within the grazed areas of these plots each week, starting on 7 October 1994 and lasting for a period of four weeks. Four replicate soil cores, enclosed in incubation tubes, were placed in the ground in incubation vessels and grouped together under cages (1.0 x 1.6 m) to protect them from grazing animals. The soil from each incubation tube, together with soil from the outer cores (representing 'start' values), were crumbled, thoroughly mixed and

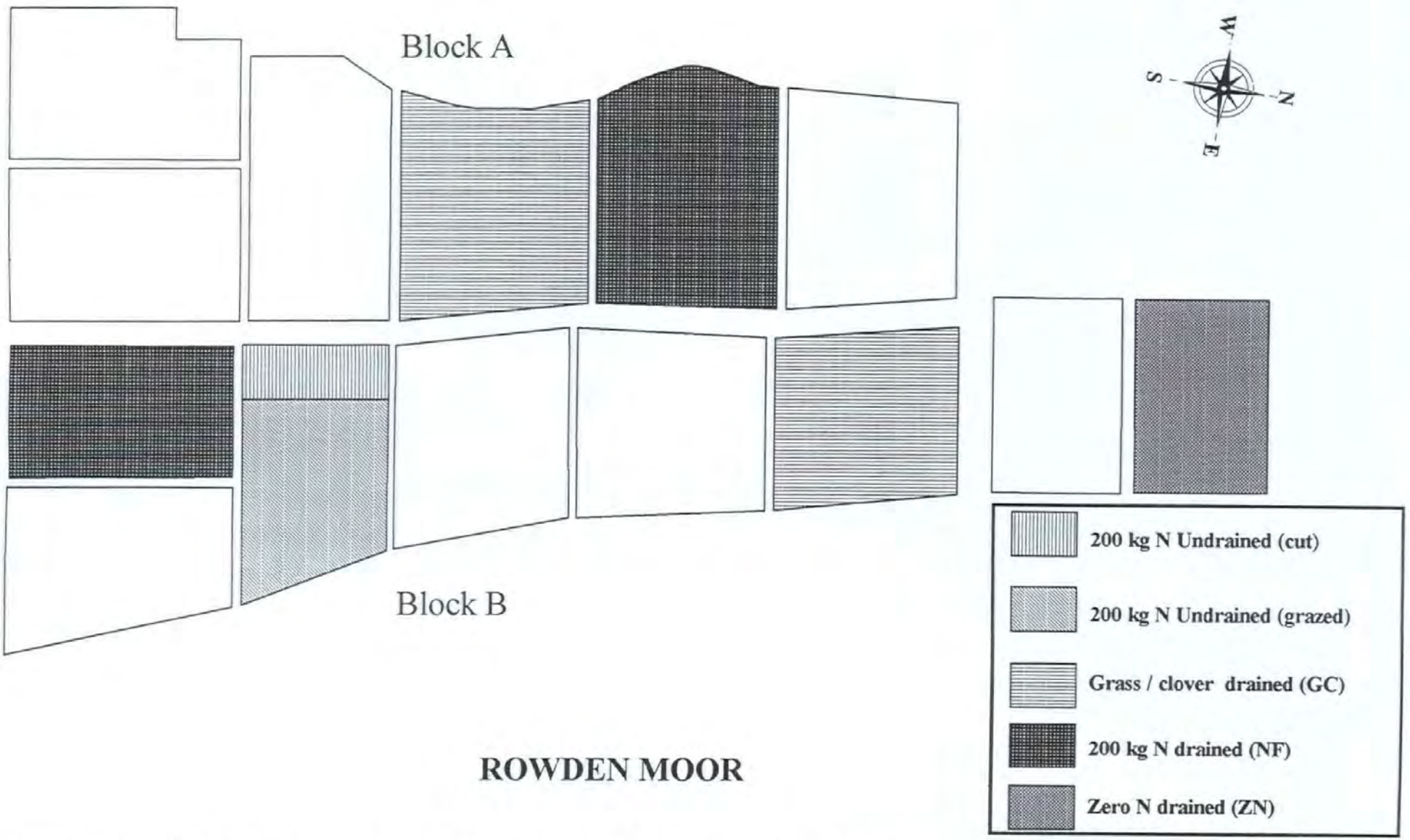


Figure 3.2 Map showing experimental layout of treatments used in the present study (1994)

stones and pieces of undecomposed, identifiable plant material removed. Fifty grammes moist soil were shaken with 100 ml 1 *M* KCl for 1 h and the suspension was filtered using Whatman No. 1 filter paper, previously washed with 1 *M* KCl solution. Soil extracts were analysed colorimetrically for NH_4^+ by the indophenol reaction (Krom, 1980) and for NO_3^- by hydrazine reduction (Kamphake *et al.*, 1967; Kempers and Luft, 1988) using a Skalar segmented flow analyser (Skalar, U.K. Ltd., Osbaldwick, York). Net mineralization rates were calculated as the difference in the inorganic N at the start and end of each incubation period. Net nitrification was calculated from the change in NO_3^- content from the 'start' values, and included NO_3^- absorbed from the soil core by the I-E bags. Soil inorganic N and soil water content (SWC) were determined from the non-incubated soil obtained at the start of each incubation period.

3.2.2 Results from initial field measurements

Mean rates of net mineralization (+) or immobilization (-) for the period (Fig.3.3a) were -0.24 and 0.05 kg N ha^{-1} d^{-1} ± 0.166 (*ZN* and *NF*, respectively), but were not significantly different. Only on one occasion (*viz.* 28 Oct. 1994) was there a significant difference in rates (P <0.05). The whole data set was statistically analyzed using *REML* (restricted maximum likelihood) by treating 'Weeks' (the sampling frequency) as a random effect and 'Treatments' (Units: *ZN* and *NF* from individual soil cores) as a fixed effect. The variance components for net mineralization were 0.0739 (Weeks) and 0.1445 (Units). It was evident that the variability in the data set due to spatial effects (soil coring) was almost twice that associated with temporal effects (sampling frequency). Thus, it was not possible to distinguish between environmental influences that might have been expected to affect net mineralization over the sampling period and the inherent variation within individual soil

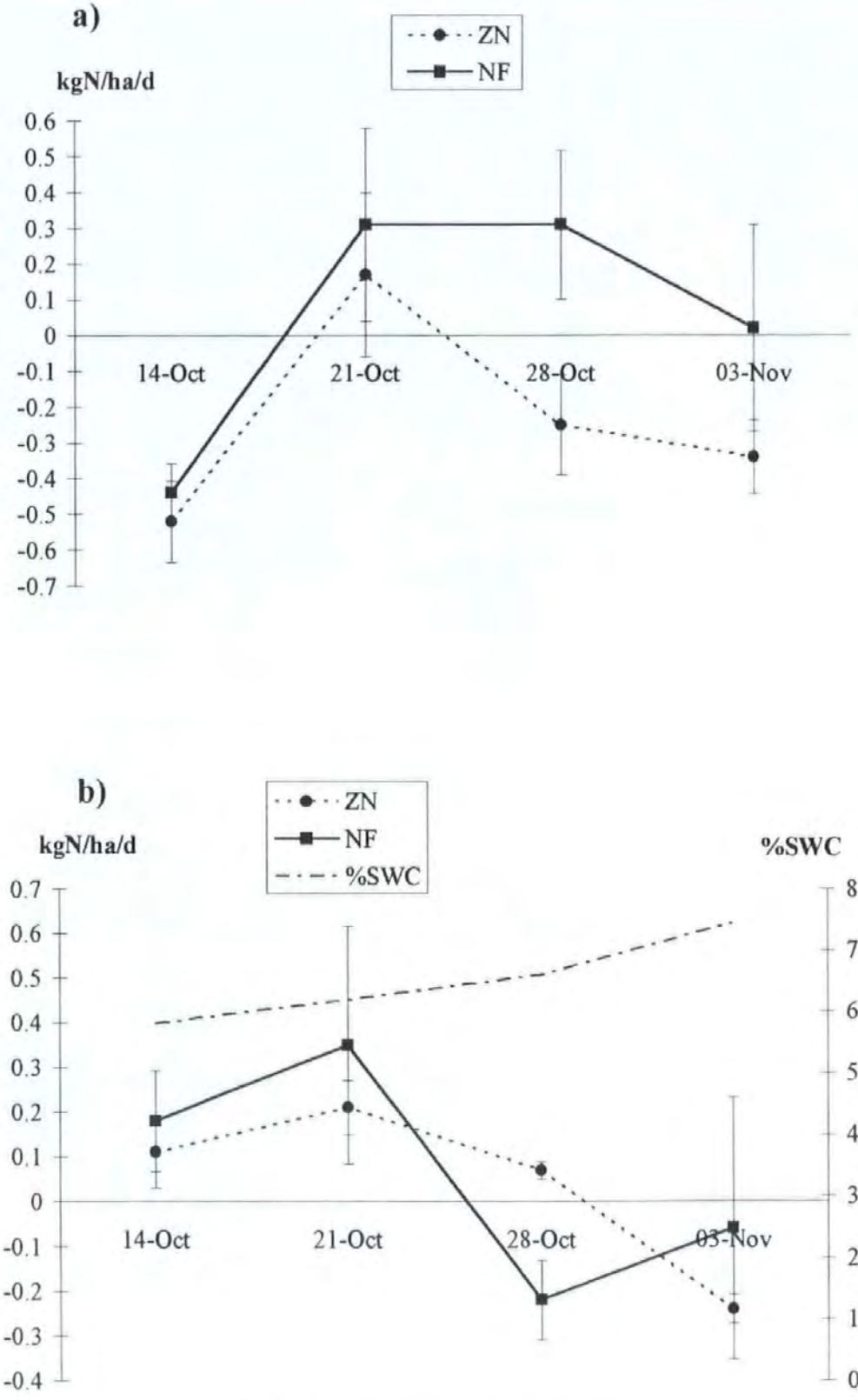


Figure 3.3 a) Net mineralization and b) nitrification in an unfertilized (*ZN*) and fertilized (*NF*) grazed sward.

cores. Such variation might well be expected in highly heterogeneous soils, which were also grazed and demonstrates the necessity for increased replication, particularly as the system depends upon destructive sampling.

Nitrification rates (Fig.3.3b) ranged from 0.01 to 0.366 and from 0.063 to 1.132 kg NO_3^--N ha^{-1} d^{-1} (*ZN* and *NF*, respectively), but on some occasions negative rates were recorded in both treatments, indicating that recovery of NO_3^- was incomplete. Whilst care was taken to limit or remove competition for NO_3^- (by suppressing plant uptake and monitoring for leached N), denitrification and microbial immobilization could still deplete the inorganic N pool in the soil core and lead to misinterpretations of rates of net nitrification. It was noticeable that the negative values for nitrification were coincident with an increase in SWC, following the return of the soil to field capacity and a tendency to waterlogging in the upper horizon (SWC >60%). Anaerobic conditions, conducive to denitrification, could have developed and may have led to unquantified losses of NO_3^--N. Mean rates of nitrification were 0.037 and 0.060 ± 0.113 kg N ha^{-1} d^{-1} (*ZN* and *NF*, respectively) with spatial variances being more than three times those which were temporally attributable (0.094 and 0.028 kg N ha^{-1} d^{-1}, respectively).

3.2.3 Assessment of inorganic N in soils

Amounts of inorganic N in the soil from the two treatments were assessed from non-incubated soil obtained at the start of each incubation period (Fig. 3.4). Ammonium : nitrate ratios were generally higher in *ZN* than *NF* (means of 2.3 : 1 and 1.8 : 1, respectively), except on 3 November, when *NF* had a higher ratio (P <0.05). The mean levels of NO_3^--N in *ZN* were lower than *NF* on all sampling dates (2.62 and 3.49 ± 0.438

kg N ha^{-1}, respectively), but were only significantly different on 28 October (P <0.05), which suggests greater nitrifying activity in the latter. Levels of NH_4^+-N were similar in both treatments with means of 6.15 (*ZN*) and 6.12 (*NF*) ± 0.773 kg N ha^{-1}.

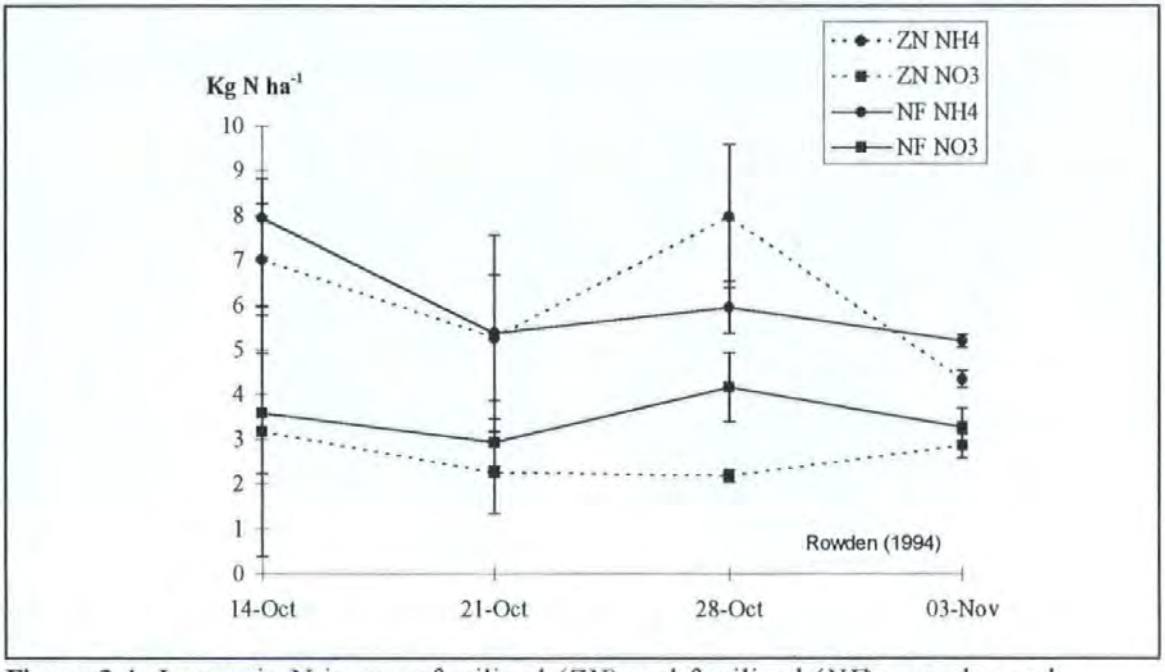


Figure 3.4 Inorganic N in an unfertilized (*ZN*) and fertilized (*NF*) grazed sward.

3.2.4 Summary

The initial field tests provided indications of increased nitrifying activity in *NF* compared with *ZN*, but the absence of firmer evidence was due to the high degree of variation in the data. Nevertheless, three key areas were identified where improvements to the assessment of the processes of mineralization and nitrification could be made:

i) Improve the recovery and consistency of soil inorganic N extractions

ii) Increase the replication using more incubated soil cores / treatment

iii) Assess losses of NO_3^--N attributable to denitrification

The first of these requirements are discussed in the following sections and the other two aspects are the subject of future chapters.

3.3 Protocols for analysing soil samples

The extraction of inorganic N from soil (*viz.* NH_4^+, NO_2^- and NO_3^-) is most commonly achieved by displacement of the ions with a high salt concentration (e.g. KCl or K_2SO_4) in the extractant. Various concentrations of salts have been favoured, but the objective is not necessarily to achieve a complete recovery of the inorganic N from the soil, but rather to provide an index of the availability of soil N for plant uptake. Displacement of the negatively charged ions (NO_2^- and NO_3^-), which are not strongly attracted to soil components, could be achieved simply by dispersion of the soil particles in water (using a blender or shaker). However, ammonium (NH_4^+), which has a positively charged ion, is attracted to the negatively charged surfaces of clay particles and organic matter and displacement depends to some degree on the protection afforded by the soil structure. Displacement of NH_4^+ is achieved by overwhelming the sites with an alternative cation. To achieve effective displacement, a high ratio of extractant ions : NH_4^+ is required and substitution of cations on the soil-exchange sites is facilitated by using a high extractant volume : soil ratio. Ryden *et al.* (1987) employed 1 *M* KCl in a 2 : 1 (extractant : soil) ratio for inorganic soil analysis, but 2 *M* KCl has been more generally favoured (Bremner, 1965). Ratios of 2 : 1 (Jackson *et al.*, 1988), 3 : 1 (Cabrera and Kissel, 1988), 5 : 1 (Davidson *et al.*, 1991) and 10 : 1 (Donaldson and Henderson, 1990) have also been utilized, especially where the major portion of N is likely to be in the NH_4^+ form: *viz.* during anaerobic incubation or with nitrification inhibitors (e.g. N-serve, Didin and C_2H_2). To ensure uniformity, all further work in the present study employed 2 *M* KCl as the standard extractant and tests were carried out to establish suitable protocols for assessing ammonification and nitrification in field incubated soil cores.

3.3.1 Effect of extraction shaking time

Soil samples were collected from the long-term experimental site from an undrained treatment (receiving 200 kg N ha^{-1} y^{-1}) which was divided into areas that were permanently grazed by cattle throughout the grazing season and also from additional areas that were reserved for conservation of herbage for winter feed (Fig. 3.2). The latter received applications of slurry that were equivalent to the over-winter output of housed animals from the grazed areas. Soil was collected with a 37 mm diameter corer to a depth of 150 mm on 27 June 1995 from the cut and grazed areas to provide four representative samples from each (4 cores to each bulked field sample). Samples for extraction were prepared within 1 h of collection by crumbling the soil cores to < 6mm (stones and large roots discarded) and a 100 g sub-sample was shaken vigorously on a rotary shaker with 2 *M* KCl (200 ml). The standard shaking time of 1 h was extended to include 2 and 3 h. The samples were then filtered using Whatman No. 1 filter paper and stored in a freezer, until they were analysed for NH_4^+ and NO_3^- on an autoanalyser (as described earlier). Results were expressed on a dry soil basis, taking into account the volume of water present in the soil sample.

Table 3.1 The effect of shaking time on the extraction of inorganic N with 2 *M* KCl (μg N g^{-1} dry soil). Mean values are shown with SEMs in parentheses (n = 4).

Treatment (undrained)	1 h	2 h	3 h
200 N Cut	34.4 (± 7.38)	33.8 (± 6.62)	37.6 (± 7.38)
200 N Grazed	30.2 (± 5.53)	31.2 (± 5.17)	33.6 (± 5.42)

Data were analysed by *ANOVA* and are shown in Table 3.1. There were no significant differences between mean values obtained from the four replicates representing cut or grazed treatments, or with extended shaking times. A standard extraction involving vigorous shaking for 1 h was, therefore, adopted both for convenience and efficiency of operation.

3.3.2 Effectiveness of KCl extraction on recovery of added N to soil.

Soil was collected from the Rowden experimental site using a 25 mm diameter corer to a depth of 100 mm. A composited sample, representative of the Hallsworth soil series, was obtained by thoroughly mixing sieved (4 mm) soil from 120 cores from which the stones and large pieces of root material were discarded. Sufficient moist soil was weighed into 24 conical flasks so that each contained the equivalent of 25 g dry soil. Half of the flasks received 2 ml of 22.3 m*M* NH_4NO_3 solution to provide 25 μg N g^{-1} dry soil (both as NH_4^+ and NO_3^--N). The other 12 flasks received 2 ml demineralised water which raised the soil water content to approximately that of field capacity (*c.* 60%). The additions of liquid were made using a dropping pipette and were evenly distributed over the soil samples: flasks were then loosely covered and left to stand overnight at 20 °C. Inorganic N was extracted by adding 2 *M* KCl in ratios of either 2 : 1, 5 : 1 or 10 : 1 (KCl (vol) : dry soil wt.) to four replicate flasks for each ratio set. The flasks were shaken vigorously on a rotary shaker for 1 h and the suspensions were filtered through Whatman No. 1 paper (previously rinsed with 2 *M* KCl). Soil extracts were frozen for storage and then thawed out in a refrigerator, before being analysed for NH_4^+ and NO_3^- (as described in Section 3.2.1).

Recovery of added N was over 80% with all three extractant : soil ratios tested, but was

more variable in the 2 : 1 set (Table 3.2). A possible explanation is that the clay particles in the Hallsworth soil (*c.* 36% clay content) were less well dispersed in the more viscous soil suspensions. The unrecovered N was probably immobilized during the equilibration period into organic forms which were not assessed. Incomplete recoveries were anticipated and consistent with the findings of other workers (Bristow *et al.*, 1987): the 'disappearance' of N would have been facilitated by the warm, moist and well-aerated conditions in the flasks stimulating N-immobilization by the soil microbial biomass. However, the higher recovery found with the 10 : 1 set was confounded by greater variabilty in NH_4^+-N contents, which was attributed to the increased dilution of the extracts and a consequent effect on analytical precision. On balance, therefore, the most consistent (i.e. least variable) recoveries were achieved with ratios of 5 : 1, particularly for NH_4^+ which is the prime concern of investigations using incubated soil cores with nitrification inhibitors.

Table 3.2 Mean % recovery (SEM) of NH_4NO_3 added to soil and extracted with 2 *M* KCl (n = 4).

KCl : soil ratio	NH_4^+-N	NO_3^--N	Combined recovery
2 : 1	80 (20)	88 (20)	85 (20)
5 : 1	81 (2)	81 (5)	81 (3)
10 : 1	95 (8)	84 (2)	87 (4)

3.4 Calculations of N cycling processes using field incubation data

The difficulties of assessing N cycling processes in a field situation were illustrated in Section 3.2 and centred mainly on the incomplete recovery of NO_3^--N from the incubated

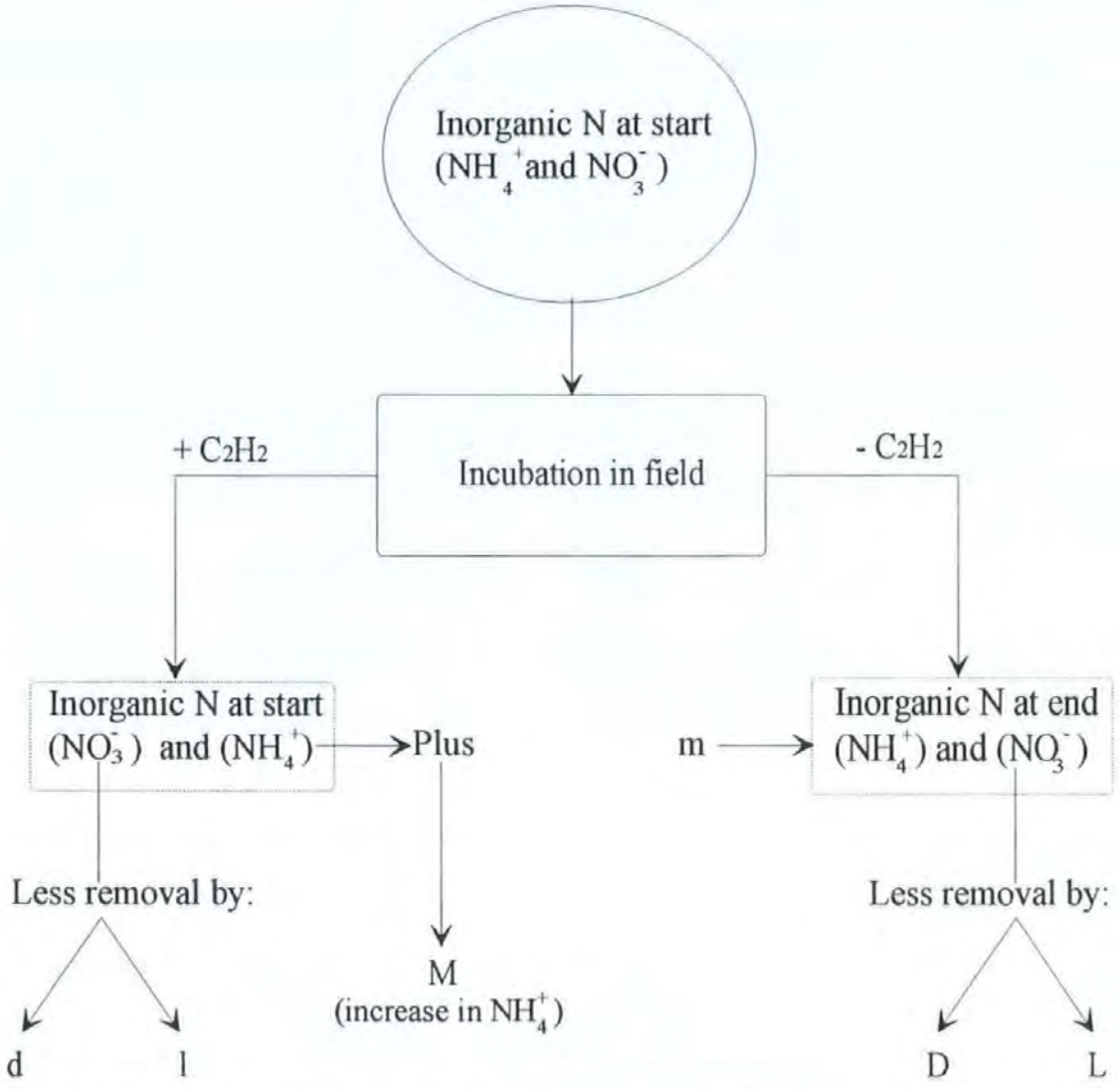


Figure 3.5 Diagram showing paired incubations with and without C_2H_2

soil cores. Before an effective field evaluation of these processes could be made, it was also essential to quantify losses of N through denitrification. The problem was approached by arranging two soil incubations, either with or without C_2H_2, so that denitrification was denied in the first ($+C_2H_2$) and allowed to proceed in the second ($-C_2H_2$) as shown in Figure 3.5. Incubations are arranged to run in parallel and the processes of net mineralization, nitrification, leaching and denitrification can then be derived from the following model:

$M = m + D + L$, therefore, $D = M - (m + L)$

and

N = [Increase in NH_4-N ($+C_2H_2$)] - [Increase in NH_4-N ($-C_2H_2$)]

(Assumes immobilization is same for incubations ± C_2H_2)

where:

M = net mineralization

m = residual net mineralization (after losses through D and L)

D = denitrification (of original plus new sources of NO_3^-)#

L = leaching potential (obtained from ion-exchange resin bags)*

N = nitrification

d = denitrification confined to original nitrate at start of incubation

l = leaching measured with resin bags (check on effectiveness of C_2H_2)

Notes: Change in original NO_3^--N content ($+C_2H_2$) is net of any removal in denitrification (d) and/or leaching (l), where *de novo* NO_3^--N production is inhibited. For controls: #D is not adjusted by d (to give **actual** N denitrified in measurement period); *L is adjusted by l (to give **potential** leaching of new NO_3^- only), defined as (see Fig. 3.5): d = denitrification (confined to original NO_3^- at start of incubation); l = leaching of original NO_3^- (from I-E bags to check effectiveness of C_2H_2).

The development of the completed incubation system, with the facility for evaluating *in situ* denitrification losses by continual C_2H_2 infusion (described in Chapter 2), required comprehensive field tests to assess the potential role of nitrification as a controlling influence on the processes of N cycling. The next chapter examines soils from defined management systems for evidence of any differences in nitrifying activity that might be related to differences in the observed losses of N.

CHAPTER 4 The effect of field management systems on the processes of decomposition and nitrification

4.1 Experimental layout

During the 1995 grazing season, sub-plots were established within the main treatments previously described in Section 3.2. There were two major reasons for these modifications:

i) To establish the extent to which long-term management systems may have altered rates of mineralization and immobilization turnover (*MIT*) and nitrification; it was important that the findings were divorced from the possible masking effect of inputs of fertilizer and excretal returns during the current season.

ii) The original experimental design, based on large scale plots of 1 ha, provided only two field replicates each of treatments *NF* (200 kg N ha^{-1} y^{-1}) and *GC* (grass/clover) and only one replicate of *ZN* (unfertilized) treatment (Fig. 3.2), which placed a considerable constraint on statistical analyses. The field replicates were, therefore, divided into sub-plots (7m x 7m) within the main treatment which were individually fenced to exclude animals and were cut regularly to maintain the sward at a height comparable with the grazed areas. Fertilizer N was not applied in 1995.

There were four replicate sub-plots in the *ZN* treatment (no. 1-4) and two replicate sub-plots in each of the main field treatments: *NF* Block A (no. 5-6), *NF* Block B (no. 7-8); *GC* Block A (no.9-10) and *GC* Block B (no. 11-12) as shown in Figure 4.1. Some properties of the soil from these treatments which are considered to be of particular importance in the context of *MIT* studies are shown in Table 4.1.

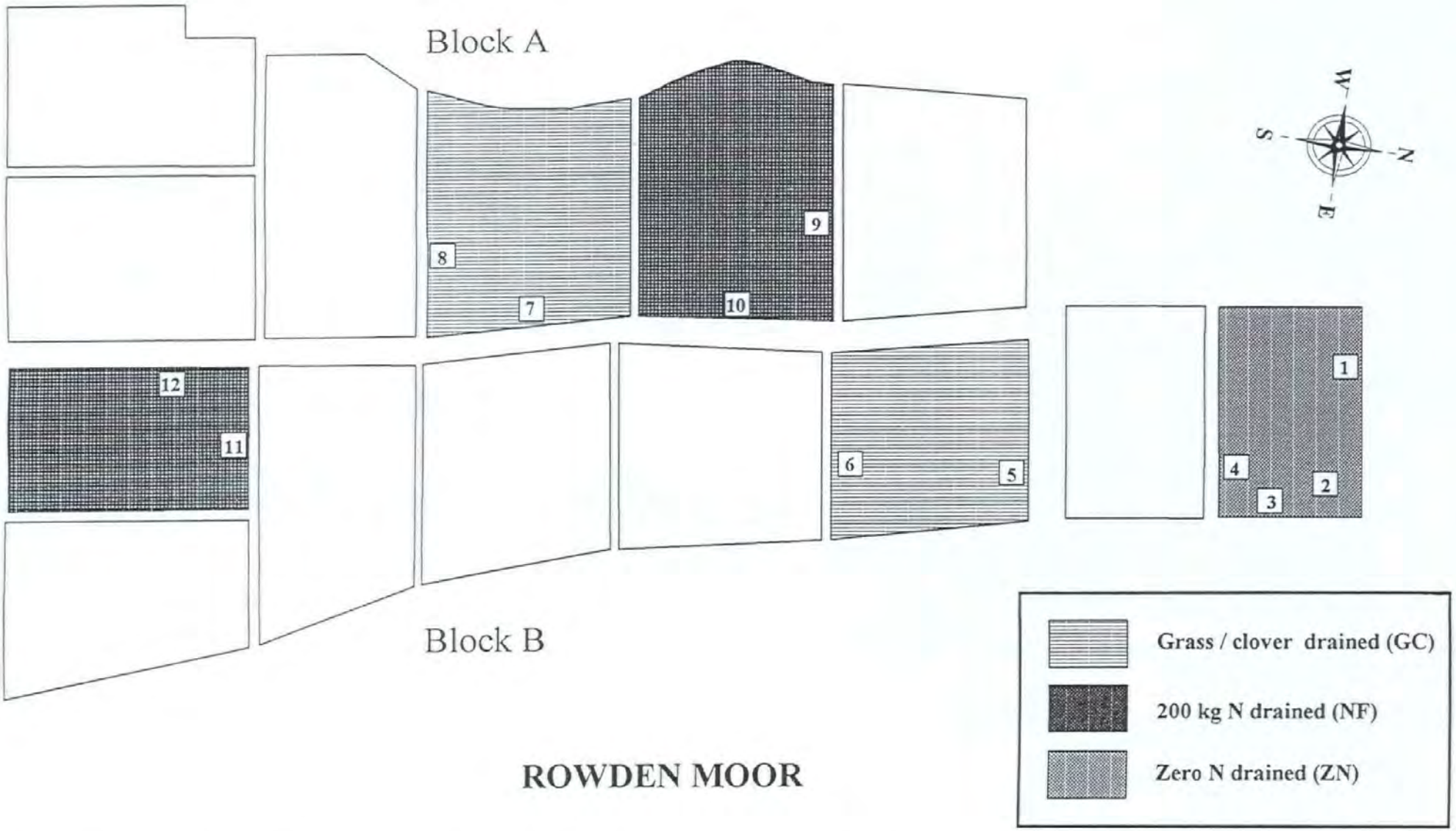


Figure 4.1 Map showing experimental layout and subplots of treatments used in the present study (1995-8) Scale: 1cm=25m

4.2 Mineralization measurement

Three approaches to measuring mineralization were adopted: (i) net mineralization by a field incubation technique; (ii) potential mineralization by anaerobic incubation; (iii) an index of potential mineralization based on chemical extraction. The analyses were all performed on soil samples obtained in June 1995 from the sub-plots described earlier, which had not been grazed or fertilized since the grazing season of 1994.

Table 4.1 Some important properties of the soils from the main field treatments.

Treatment (drained)	Total N ($mgNg^{-1}$)	Organic C ($mgCg^{-1}$)	C : N	pH		Bulk density	Water content at field capacity (%) ($g\ g^{-1}$ dry soil)
				water	$CaCl_2$		
ZN	5.9	57.5	9.7	5.3	4.7	0.88	67
GC	5.1	53.6	10.5	5.5	4.7	0.99	63
NF	6.8	70.5	10.4	5.6	5.0	0.95	64

4.2.1 Field incubation

Using a 25 mm diameter soil corer, eight soil cores to a depth of 150 mm were extracted from each of the four replicate sub-plots from the main treatments. The cores were placed in 1l glass Kilner jars (8 per jar) and 2% C_2H_2 was added to the headspace to prevent nitrification. The jars (12) were incubated in the field in suitably-sized holes for 6 d, using the technique reported by Hatch *et al.* (1990, 1991). At the same time, further soil cores were taken which were analysed for inorganic N, as described previously, but the ratio of KCl to soil (see Section 3.3.2) was increased to 5 : 1 (vol. : wt.). Net

mineralization, in the absence of nitrification, was determined from the change in NH_4^+-N between the incubated soil samples and those taken at the start.

4.2.2 Anaerobic incubation

An anaerobic incubation method (Lober and Reeder, 1993), based on a modification of an earlier technique (Waring and Bremner, 1964), was used to obtain an estimate of potentially mineralizable N in a subset of soil samples obtained at the same time as field evaluations were carried out. Fresh soil was screened to pass a 2 mm sieve and the equivalent of 5 g dry soil was placed into 60 cm^3 polypropylene syringes (12) fitted with Luer-Lok taps (3 treats. x 4 reps.). Demineralized water (13 cm^3) was added and entrapped air bubbles were dislodged using a hand-held vibrator. The plungers were then inserted into the syringes and all the remaining air was expelled through the tap, which was then closed. The syringes were clamped to a stand using saddle clips and placed in an incubator for 7 d at 35 °C. Following incubation, 37 cm^3 of 2.7 *M* KCl were added to each syringe, resulting in a final extractant concentration of 2 *M* KCl and a 10 : 1 ratio of KCl to soil (vol. : wt.). The plungers were then withdrawn sufficiently to introduce headspaces of approximately 10 cm^3 and the stand was firmly attached to a rotary shaker with the syringes held in a horizontal plane. The syringes were shaken vigorously for 1 h and the contents filtered through Whatman No. 1 paper (pre-rinsed in 2 *M* KCl). Filtrates were analysed for NH_4^+-N on an autoanalyser, as previously described. Mineralizable N was calculated as the difference between post and pre-incubation NH_4^+-N concentrations.

4.2.3 Chemical extraction

Samples from the subset of soil cores, taken from the three field treatments (4 replicates),

were air-dried and ground to pass a 2 mm sieve. Mineralizable N was estimated using the method described by Whitehead (1981), *i.e.* 10 g dry soil were boiled for 1 h with 50 ml 1 *M* KCl and the extract was then filtered, made to standard volume and analysed for NH_4^+-N and NO_3^--N as before.

Table 4.2 Mineralization estimates by three methods. Mean values are shown (μg N g^{-1} dry soil) with SEMs in parentheses (n = 4).

Treatment (drained)	Anaerobic incubation	Field incubation	Boiling KCl extraction
ZN	290 (12.8)	6.70 (0.540)	31.7 (2.68)
GC	242 (9.2)	6.16 (1.262)	30.4 (1.60)
NF	264 (40.0)	13.73 (0.639)*	33.2 (1.66)
Significance	n.s.	*sig. diff.	n.s.

*$P < 0.05$

4.2.4 Results of mineralization measurements

The three methods for assessing mineralization represent contrasting approaches to the problem of predicting the amounts of N which may be released from the soil O.M. fraction during a defined period of incubation. The lowest values were obtained from the field incubation measurements (Table 4.2) which provided an estimate of the **actual** rate relating to the period of incubation (6d) and were derived from readily degradeable sources of organic N. Net mineralization in the fertilized treatment (*NF*) was > two-fold ($P < 0.05$) that of the unfertilized treatments (*GC* and *ZN*). The chemical extraction (KCl) indices

were up to 5-fold higher than with field incubation values, but there were no significant differences between treatments. Previous studies (Hatch *et al.*, 1990, 1991; Gill *et al.*, 1995) have found this method to be relatively insensitive to the effects of field management systems, but probably more suited to identifying differences between soil types (Whitehead, 1981). Anaerobic incubation provided an estimate of **potential** seasonal mineralization with values which were nearly an order of magnitude larger than other methods for all three treatments; the highest value that was found with the *ZN* treatment was not significantly different from the other treatments (*NF* and *GC*).

4.3 Microbial biomass

The Rowden experimental site was sampled again in autumn 1995, shortly after the soil had returned to field capacity (approximately 60% SWC, by weight) as indicated by the first appearance of drainage water flowing from the field drains. Ten soil cores (25 mm diameter) to a depth of 150 mm were taken from each treatment sub-plot (*ZN*: plots 1-4; *NF*: plots 5-8 and *GC*: plots 9-12, see Figure 4.1) The soil was crumbled to pass a 4 mm sieve and stones and identifiable pieces of root were rejected. Prepared soil samples were stored at 4° C in sealed polyethylene bags until required. Soil microbial biomass (*SMB*) activities were evaluated in terms of both basal respiration (as an indication of **intrinsic** activity) by the method described by West and Sparling (1986) and substrate-induced respiration (as an indication of **potential** activity) by the method described by West (1986).

4.3.1 Basal respiration

Moist soil (4 field reps. per treatment), equivalent to 10 g dry weight, was placed into 72 ml serum bottles (24) and left for 30 minutes to equilibrate at 25 °C. The bottles were then

sealed with subaseals (No. 21, Fisons, Loughborough, Leics.) and incubated for a further 18 h. Headspace gas samples were withdrawn (2 ml) and analysed for CO_2 using a gas chromatograph fitted with a thermal conductivity detector and a packed column (*Porapak Q*). Basal respiration was expressed as µl CO_2 g^{-1} (dry soil) produced h^{-1}.

4.3.2 Substrate-induced respiration

Following an 18 h incubation, the bottles (24) were vented and all received sufficient glucose solution (*c.* 20 ml) to provide 20 mg g^{-1} (dry soil), resulting in a 2 : 1 (vol./soil wt.) ratio. Half the bottles (12) also received cyclohexamide at 60 mg g^{-1} (dry soil) incorporated with the glucose solution to inhibit eukaryotic (fungal) respiration. The mixtures were swirled to disperse the soil and left to equilibrate for 30 minutes at 25 °C. The bottles were then stoppered (t_0) and incubation continued with samples withdrawn from the headspace at 30 minutes (t_1) and again at 150 minutes (t_2) for CO_2 analysis, as above. The hourly rate of bacterial respiration was determined from the increment in the amounts of CO_2 produced between $t_1$and t_2, divided by 2, and expressed as µl CO_2 g^{-1} dry soil h^{-1}.

4.3.3 *SMB* activity

There were no significant differences between treatments in basal respiration rates, nor were there differences when substrate (glucose) was added, although all rates were increased by more than two-fold (Table 4.3). Estimates of biomass-C were obtained from the latter using the following formula of Ocio and Brookes (1990):

$$\mu g\ biomass\text{-}C\ g^{-1}\ (dry\ soil) = 400.4 * (cm^3\ CO_2\ 100\ g\ soil^{-1}\ h^{-1}) + 3.7$$

The amounts of biomass-C were similar in all treatments (sampled in autumn 1995), but

low when compared with the results of Lovell *et al.* (1995), who found biomass-C was highest in the spring. The present estimates may reflect the timing of sampling, or some other effect related to storage (Ross *et al.*, 1980). Although not statistically different, substrate-induced respiration indicated that just over half the total respiration in the unfertilized treatments (*ZN* and *GC*) and slightly under half in the fertilized treatment (*NF*) could be attributed to bacteria when fungal respiration was inhibited.

Table 4.3 Soil microbial biomass respiration rates. Mean values (μl CO_2 g^{-1} h^{-1}) are shown with SEMs in parentheses (n = 4). All values expressed on a dry soil wt. basis.

Treatment (drained)	Basal activity	Substrate-induced	Bacterial respiration	%Bacterial respiration	Biomass (μg C g^{-1})
ZN	3.76 (0.224)	7.98 (0.705)	4.61 (0.239)	57.8	323
GC	3.80 (0.221)	7.86 (0.589)	4.37 (0.125)	55.5	318
NF	3.47 (0.046)	8.85 (0.623)	4.37 (0.589)	49.3	358

4.4 Laboratory tests of nitrifying activity

Soils from the stored subset of samples, examined previously for *SMB* activity, were also used to estimate nitrification potentials by two techniques: (i) perfusion and (ii) soil slurries.

4.4.1 Nitrification potential using a perfusion system

The perfusion tubes employed in testing the performance of resin bags (Section 2.5) were modified to enable eluent to be collected from soil columns. Preliminary tests using free-

draining columns filled with a soil and sand mixture to assess nitrifying activity (after Kilham, 1987) were inconclusive, as little or no NO_2^- (the primary product) could be detected in the eluents. The reason for this was unclear, but the high ratio of sand : soil that was necessary to ensure free drainage through the clay soils, resulted in flow characteristics which may not have allowed sufficient contact between the soil particles and the substrate in the perfusate. Ultimately, the method which was adopted had a modified outlet in the form of a manometer-tube overflow (Fig. 4.2) and used the equivalent of 50 g dry weight of fresh soil (4 mm sieved) mixed with 150 g of acid-washed, horticultural-grade silver sand (screened to < 1 mm) and autoclaved before use. The outlet from the perfusion tubes, via a rubber bung, was covered by a disc of fine nylon mesh (140 μm) and then by a filter paper (Whatman No. 40, 47 mm diam.) and the soil and sand mixture was packed loosely into the tubes above the filter paper to a depth of about 150 mm. When fully wetted and freely drained, the mixture retained about 50 ml liquid.

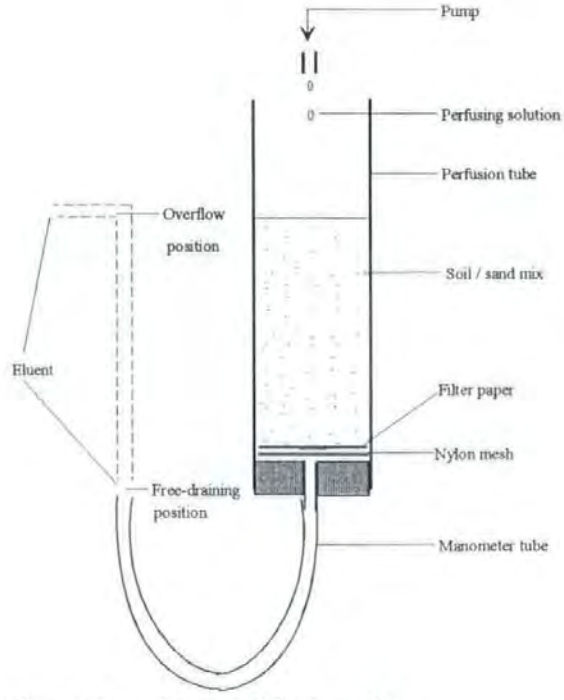


Figure 4.2 Soil perfusion tube with modified outlet

Soils from each of the main treatments (*NF*, *GC* and *ZN*: four field replicates of each) were allowed to equilibrate in 12 perfusion tubes at 20 °C for three days to minimise the effects of storage. With the manometer tube outlet placed level with the base of the column (the free-draining position), pumps were set to deliver phosphate buffer (see Section 4.4.2) at a rate of 50 ml 3 h^{-1} (once in every 24 h) to adjust the soil gradually to pH 7.5. After this period, the soil / sand mixture had consolidated to a depth of about 100 mm. On the third day, the perfusate was changed to include 2 m*M* $(NH_4)_2SO_4$ and 15 m*M* $NaHClO_3$ in buffer solution to evaluate ammonia-oxidizing activity and was supplied at 17 ml h^{-1}. Chlorate is an effective inhibitor of NO_2^- oxidation (see Berg and Rosswall, 1985) because the nitrifiers convert ClO_3^- to chlorite, which is particularly toxic to *Nitrobacter*. At the same time, the manometer tube was raised to be level with the surface of the soil / sand mixture (the overflow position), so that the column was flooded before drainage occurred. After 3 h perfusion, drainage began and eluent was collected hourly over the following 6 h. Each set of eluents was immediately analysed for NO_2^-, using a flow-injection analyser (FIA model 5020, Perstorp Analytical, Maidenhead, Berks.) to measure the absorbance (λ = 540 nm) of the purple azo-dye, produced by the reaction of NO_2^- with sulphanilamide. The response of the instrument (Beer's Law) was non-linear (over concentration range 0 - 250 µM, cell path length 10 mm) and the standard curve was best described (r^2 = 0.9999) by a polynomial expression of the form :

$$y = ax^2 + bx + c$$

Where y = the absorbance reading for the samples.

The formula was re-arranged to solve x (the concentration of NO_2^- in the samples) as follows:

$$x = \frac{-b \pm \sqrt{b^2 - 4a(c-y)}}{2a}$$

The process was repeated with a new set of soil samples in the perfusion tubes, equilibrated to pH 7.5 for three days as before. The perfusate was then replaced with a fresh buffer solution containing 2 m*M* Na_2NO_2 (without chlorate) to evaluate nitrite-oxidizing activity. An inhibitor of autotrophic ammonia-oxidation was also included in the perfusate so that no <u>new</u> NO_2^- was produced during the incubation. Other workers have previously used the inhibitor nitrapyrin (N-serve), but this has now been withdrawn from commercial use. An alternative product was tested, which is marketed under the trade name of *Didin-liquid* (Omex Agriculture Ltd., Kings Lynn, Norfolk, U.K.) and contains 40% w/w dicyandiamide (DCD) as the active ingredient. The inhibitor was added to the perfusate at a rate of 100 µg *Didin* g^{-1} dry soil, or 0.004% (w/v) of the active ingredient (DCD) in the perfusate: comparable to the concentration of nitrapyrin adopted by Dusek (1995). Tests on DCD in conjunction with NH_4^+ substrate and chlorate in the perfusate, showed this rate to be completely effective in blocking nitrite production in soils from *ZN* and *GC* and only < 0.01% of the substrate was oxidized in *NF* soils. The small amount of NO_2^- produced in this case could have occurred because of incomplete blocking due to some property of the soil structure, or it may suggest the presence of an heterotrophic nitrifying component in the soil from the fertilized treatment. Nitrifying activity was calculated as nMoles NO_2^--N g^{-1} (dry soil) h^{-1} by taking a linear regression through data points from four replicate columns of hourly rates of NO_2^-, either produced (ammonia-oxidation), or consumed (nitrite-oxidation).

Table 4.4 Nitrifying activity measured with perfusion tubes. Mean values are shown with SEMs in parentheses (n = 4).

Treatment (drained)	Ammonium oxidation (nM NO_2^- g^{-1} h^{-1})	Nitrite oxidation (nM NO_2^- g^{-1} h^{-1})
ZN	0.96 (0.069)[a]	0.42 (0.015)[a]
GC	1.46 (0.089)[b]*	0.48 (0.024)[b]***
NF	1.77 (0.160)[b]***	0.59 (0.023)[c]***

Numbers in columns followed by different letters are sig. diff.(* $P < 0.05$; *** $P < 0.001$).

4.4.2 Results of measurements of nitrifying activity using perfusion tubes

The whole data set was analysed using a **grouped regression analysis** to establish whether the slopes of the time courses (i.e. the rates of nitrification per unit wt. dry soil, see Fig. 4.3a and b) were statistically different. There were significant differences in nitrifying activities between all three treatments for both NH_4^+ and NO_2^- oxidizing activity, as shown in Table 4.4, except for the difference between rates for the *NF* and *GC* treatments (NH_4^+-oxidizers only), which was at a lower level of significance (P = 0.063). The NH_4^+-oxidation rates (nMoles g^{-1} h^{-1}) were at the bottom end of the range found with New Zealand grassland soils (1.4 - 407) by Steele *et al.* (1980) and much lower than those from arable soils (100 - 800) surveyed by Kilham (1987) using perfusion techniques. After 4 h perfusion, the linearity in NH_4^+ oxidation rates ceased in *NF* and *GC* (Fig. 4.3a), possibly due to poisoning of the enzyme complex. It is possible that the chlorate (on conversion to chlorite) could have adversely affected NH_4^+ oxidation (Groffman, 1987), although it has been found to be much more selective against NO_2^- -oxidizers and not to affect NH_4^+

oxidizers (Belser and Mays, 1980). In this respect, it is possible that perfusion may re-inforce any adverse inhibitory effects by continual supply/replacement of the inhibitor, which could have a more pronounced effect than the single dosage employed with incubated soil slurries (see Section 4.5).

4.4.3 Phosphate buffers

Although many workers recommend that measurements of potential nitrification are performed on soils buffered to near neutral, or slightly alkaline pH (Belser and Mays, 1980; Kilham, 1987; Dusek, 1995), none gives detailed instructions on the formulation of suitable buffer solutions. An empirical approach was adopted to establish the combination of 2.5 m*M* phosphate solutions required to produce a pH 7.5 phosphate buffer. A series of titrations was made with 100 cm^3 of 2.5 m*M* Na_2HPO_4 against a titre of 2.5 m*M* KH_2PO_4 and then the reverse titration was repeated with Na_2HPO_4 as the titre. A plot of pH against molar ratio of Na_2HPO_4 : KH_2PO_4 gave a linear relationship ($r^2 = 0.9997$) described by the following equation:

$$y = 0.4272\ Ln\ (x) + 6.7691$$

where y = pH

and x = the molar ratio of Na_2HPO_4 : KH_2PO_4

Using this equation, a buffer solution of pH 7.5 was prepared by mixing 2.5 m*M* phosphate solutions in a 5.54 : 1 (vol / vol) ratio of Na_2HPO_4 : KH_2PO_4. The buffer solution was checked using a pH meter and found to be pH 7.5 ± 0.1.

4.5 Potential activity of nitrifiers in buffered soil slurries

The low overall potential nitrifying activities recorded with perfusion tubes, despite

buffering to pH 7.5, may either reflect the intrinsically low pH of the soils (see Table 4.1), allelopathic mechanisms of long-term systems (see Section 1.5) or poor substrate / enzyme contact. However, set against the last possibility is the fact that perfusion avoids 'end-point repression' by removing the product as it is formed (see Section 1.5.4). Thus, the natural competition for N by immobilization into plants and microbial biomass is simulated. The results from the perfusion tubes were compared with other methods using incubated soil slurries to validate the findings.

4.5.1 Potential activity of ammonia-oxidisers in buffered soil slurries

Samples (10 g dry soil equivalent) of stored soil from the four field replicates of the main treatments (*NF*, *GC* and *ZN*) were weighed into twelve polyethylene bottles with screw caps and pre-incubated at 20 °C for three days at a SWC of 85% field capacity to avoid waterlogging. Evaporative losses were minimized by capping the bottles, which were vented each day to maintain aerobic conditions. In the method described by Dusek (1995), pre-incubation was found to overcome the effects of sieving and storage, resulting in reduced variances of measured factors. At the start of incubation, 50 ml phosphate buffer (pH 7.5) with chlorate (15 m*M*) and 2 m*M* $(NH_4)_2SO_4$ were added to the bottles which were transferred to a water bath at 25 °C. The bottles were shaken for 8 h and samples of the suspensions (2 ml) were withdrawn hourly, immediately centrifuged at 4000 g (4800 rpm) at 4 °C for 30 minutes and the supernatant was then directly analysed for NO_2^-, as previously described. Potential activity was calculated as the rate of NO_2^- accumulation using a simple linear regression and allowance was made for the progressive reduction in the volume of the slurry due to sequential sampling.

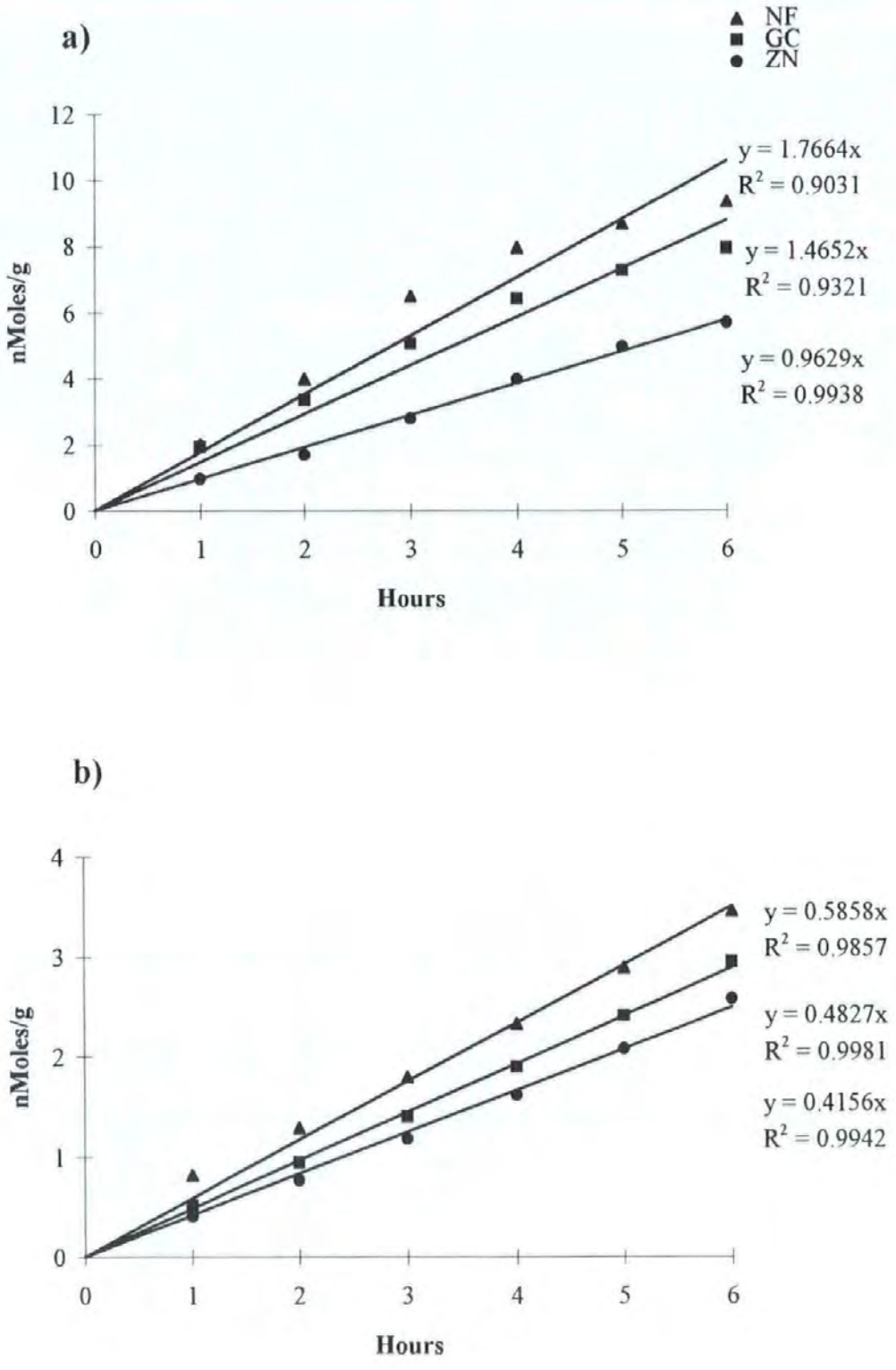


Figure 4.3 Nitrification potential from soil perfusion columns: a) Ammonium and b) Nitrite - oxidation.

4.5.2 Potential activity of nitrite-oxidizers in buffered soil slurries

The two contrasting treatments of *NF* and *ZN* were selected for assays of the kinetics of the NO_2^--oxidizer enzyme using the method of Belser and Mays (1980). A composite sample of the stored (4 °C) field moist soil (200 g dry soil equivalent), bulked from the four field replicate sub-plots of each treatment, was pre-incubated for three days at 20 °C at a SWC of 85% field capacity. The soil sample was then blended with 1l phosphate buffer (pH 7.5) containing 100 $\mu g\ g^{-1}$ (dry soil *) Didin liquid.* Aliquots (50 ml) of the slurry (containing the equivalent of 10 g dry soil) were withdrawn with an automatic pipette, whilst vigorous stirring of the mixture was maintained and were transferred into a set of 125 ml polyethylene bottles (12 for each treatment). Duplicate bottles each received 2 ml additions from stock solutions of Na_2NO_3 to produce final concentrations of 50, 100, 150, 200, 300, or 400 μM in the slurries. The bottles were placed in a water bath at 25 °C and shaken continuously. After 1, 2, 3, 4, 6, 8, 11 and 22 h, samples were taken (2 ml), centrifuged at 4,800 r.p.m. (4 °C) for 30 minutes and analysed immediately for residual NO_2^-, as before. Potential activity was calculated as the rate of NO_2^- disappearance for each substrate concentration using a simple linear regression. In addition, the affinity of nitrite-oxidizing enzymes for substrate was evaluated using Michaelis-Menten kinetics.

4.5.3 Results of ammonium-oxidation measurements using incubated soil slurries

The data were analysed using the grouped regression method as before. Overall activities of the nitrifiers were higher with slurry incubation than with perfusion tubes. Rates (nM $NO_2^-\ g^{-1}\ h^{-1}$) were obtained from the slopes of fitted linear regression lines (Fig. 4.4a) and increased significantly ($P < 0.001$) from 0.82 (± 0.231) in *ZN*, to 16.9 (± 1.75) in *GC*, to 26.2 (± 2.05) in *NF*. An apparent lag in initial oxidizing activity of about 4 h was evident

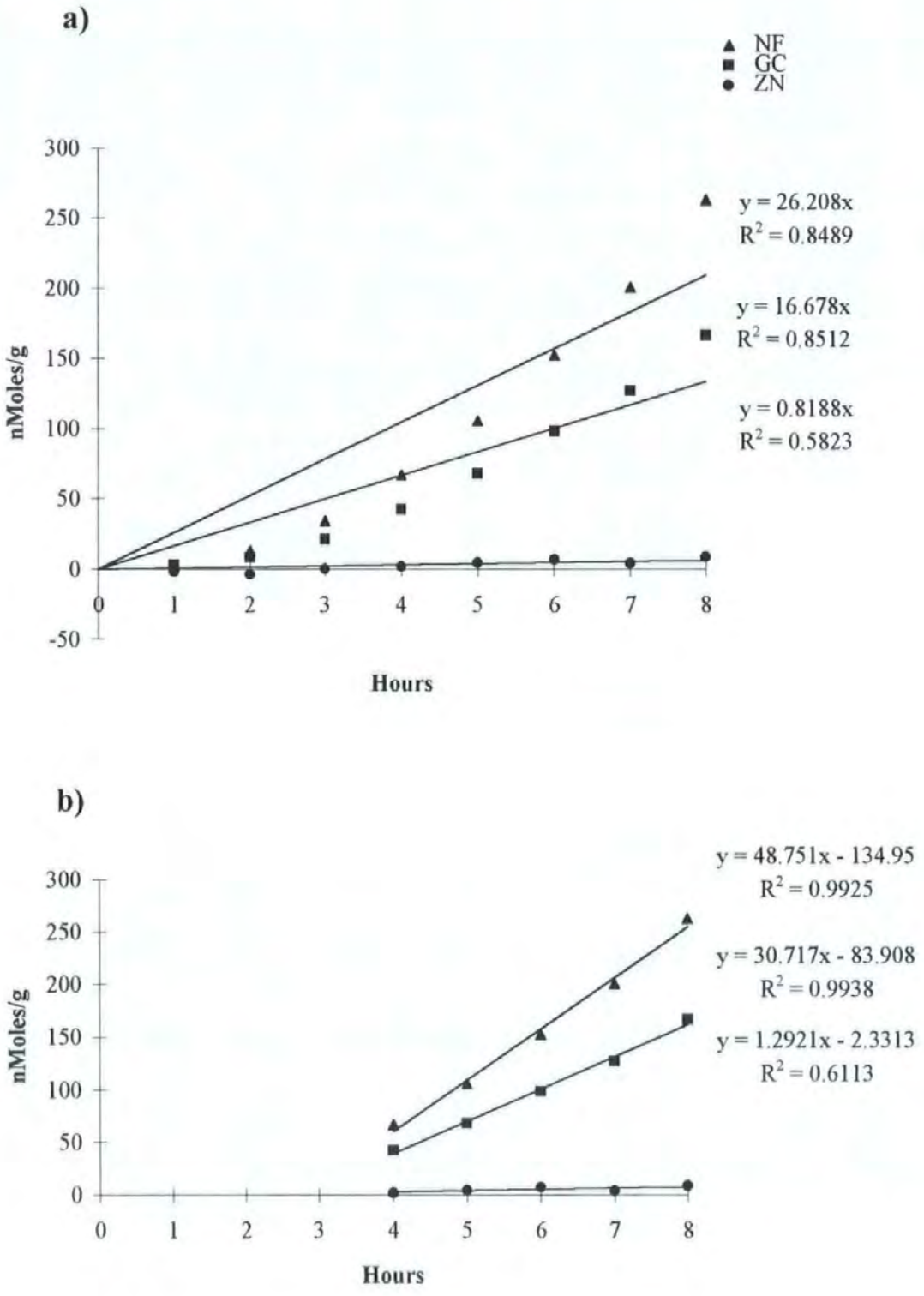


Figure 4.4 Nitrification potential (NH_4^+-oxidation) from incubated soil slurries: regressions of mean hourly data from a) start and b) after 4 h lag.

from the plotted data so that when re-plotted using data for 4-8 h only (Fig. 4.4b), the steady state rates were nearly two-fold higher than the slopes of the 0-8 h data set: *viz.* 1.29, 30.72 and 48.75 n*M* NO_2^- g^{-1} h^{-1} for *ZN*, *GC* and *NF*, respectively. These rates are well within the ranges found by other workers who have employed incubated slurries of grassland soils, *viz.* 0-236 (Sarathchandra, 1978); 21-100 (Belser and Mays, 1982); 13-39 (Berg and Rosswall, 1987); 60-100 (Dusek, 1995) and (using arable soil) 6-36 (Hojberg *et al.*, 1996).

4.5.4 Kinetics of nitrite-oxidation from incubated soil slurries

The following extract is taken from Lehninger (1981): "At low substrate concentrations [S] the initial reaction velocity (v_0) is nearly proportional to the substrate concentration, so that the reaction is approximately first order. With increasing substrate concentrations, the increase in v_0 is less and no longer proportional to [S], so that the reaction is mixed order. With further increases in substrate concentration, the reaction rate becomes independent of [S] and asymptotically approaches a constant rate (see Figs. 4.5a and 4.6a). In this range of [S], the reaction is essentially zero order and the enzyme is deemed to be saturated with its substrate. All enzyme-catalysed reactions show the saturation effect, but there is a wide variation in the concentration of the substrates required to produce it."

The Michaelis-Menten equation defines the rate equation for an enzyme-catalysed reaction (single substrate), in terms of the mathematical relationship between v_0, V_{max}, [S] and K_m:

$$v_0 = \frac{(V_{max} \cdot [S])}{K_m + [S]}$$

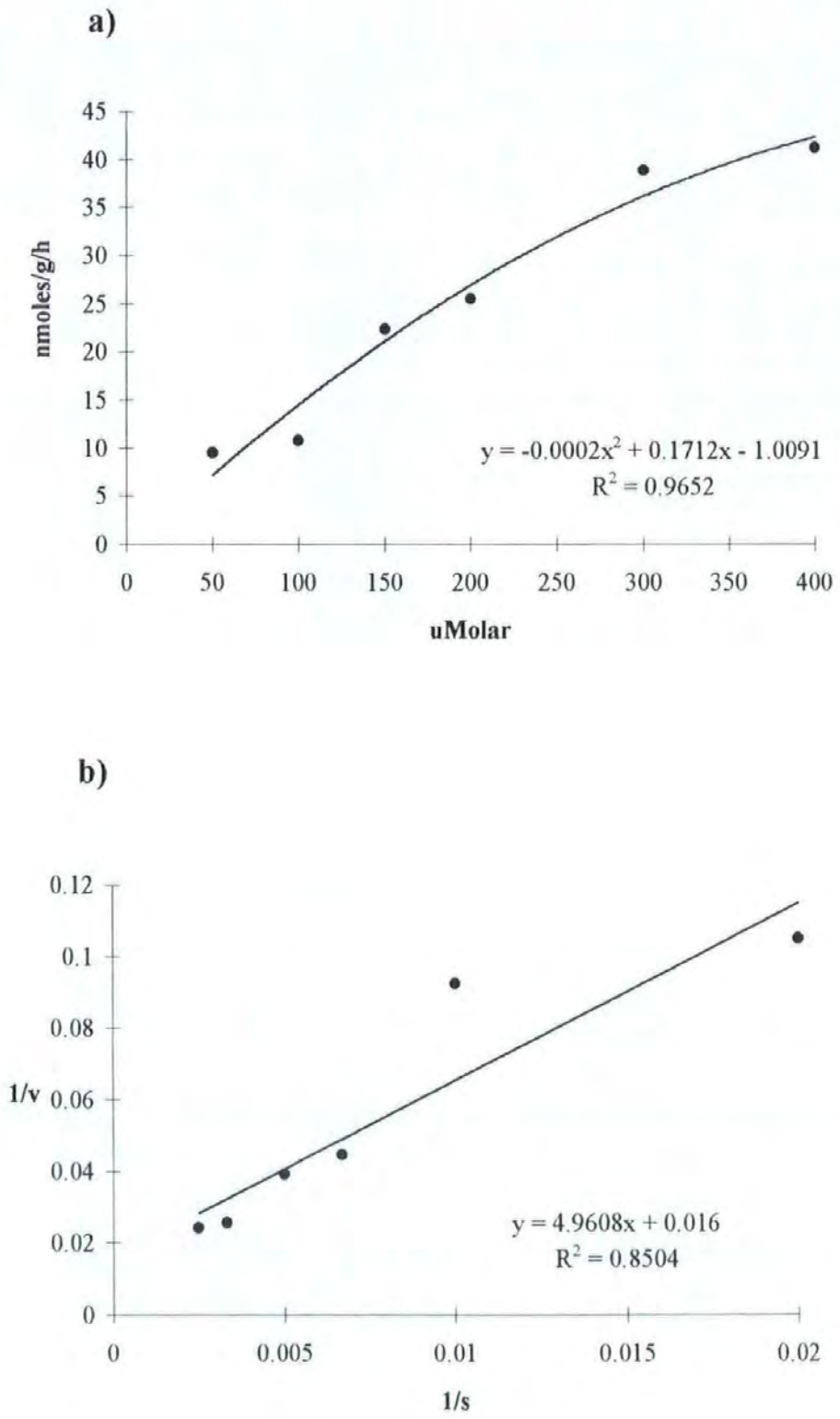


Figure 4.5 Enzyme kinetics of nitrite-oxidizers in unfertilized soil (*ZN*): a) Michaelis-Menten plot b) Lineweaver-Burke plot.

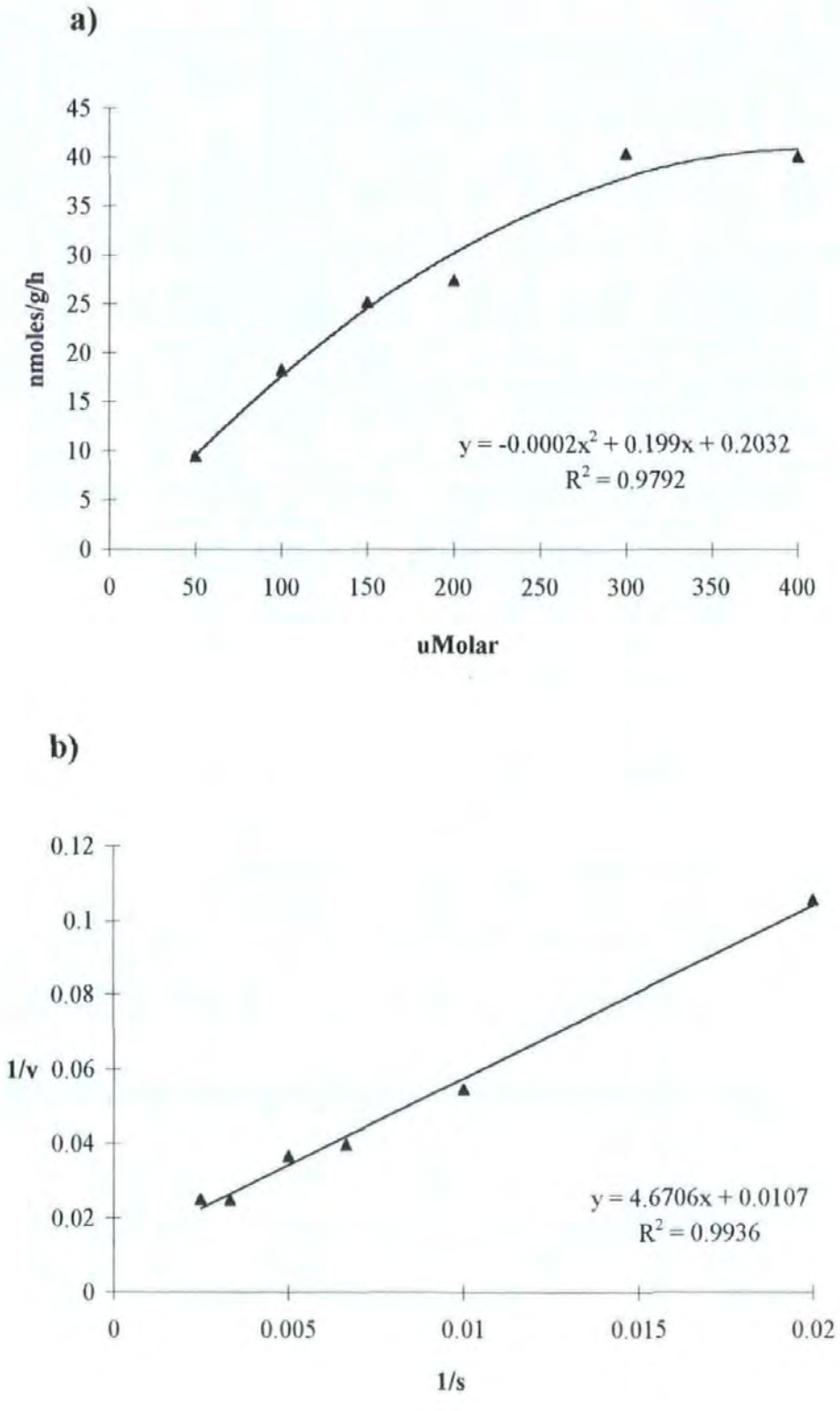


Figure 4.6 Enzyme kinetics of nitrite-oxidizers in fertilized soils (*NF*): a) Michaelis-Menten plot b) Lineweaver-Burke plot.

When the initial reaction rate (v_0) is exactly one-half the velocity (V_{max}), then an important numerical relationship exists where:

$$K_m = [S]$$

Thus, K_m (the Michaelis-Menten constant) can be derived and is equal to the substrate concentration at which the initial velocity is half maximal (1/2 V_{max}). However, because the reaction rate approaches V_{max} asymptotically, accurate evaluation of the rate is not possible, since the point of convergence is with an infinite concentration of substrate. A useful transformation of the Michaelis-Menten equation involves plotting 1/v against 1/[S], the so-called 'double reciprocal' or Lineweaver-Burke plot (see Figs. 4.5b and 4.6b). The line then has a slope of K_m/V_{max} and an intercept of $1/V_{max}$ and hence both K_m and V_{max} can be accurately determined. Additionally, the **specific affinity** of the enzyme for the substrate can be derived from V_{max}/K_m, which describes the nutrient-sequestering ability of the nitrifying population, since it combines the qualitative (K_m) and quantitative (V_{max}) components of the metabolic process.

Potential nitrite-oxidizing rates (V_{max}) were 62.5 (*ZN*) and 93.4 (*NF*) nMoles g^{-1} h^{-1}, with K_m values of 310 and 436 μM NO_2^--N, respectively, and were in close agreement with data for a grassland soil from Both *et al.* (1992). Thus, confirmation of the higher nitrifying activity of the fertilized soil was obtained, but a lower affinity of the enzyme for NO_2^- substrate was indicated by the larger K_m in *NF*. However, when the specific affinities (V_{max}/K_m) of the enzymes are calculated for the soils from the two treatments, there appears to be only a small difference in the ability of the two nitrifying populations to oxidize NO_2^- (202 and 214 10^{-6} l g^{-1} h^{-1}, respectively). Compensatory processes apparently exist, which enable the relatively inactive nitrifying population (*ZN*) to develop a higher affinity for

the NO_2^- substrate, in contrast to the higher oxidizing potential of the fertilized soil (*NF*). Hence, a decline in V_{max} appears to have been compensated for by a decrease in K_m (lower K_m = higher affinity) and vice versa, as Both *et al.* (1992) also observed. The ecological advantage of this stratagem would seem to be that highly toxic NO_2^- (to both nitrifiers and plant-life) can still be effectively converted to the harmless and mobile nitrate-ion, even in systems which are not adapted for rapid nitrification. Hence, NO_2^- is seldomly found in agricultural soils in any appreciable amounts and usually < 0.1 $\mu g\ g^{-1}$ soil (Van Cleemut and Samater, 1996) unless conditions which are inhibitory to the oxidation of NO_2^- develop, e.g. high NO_3^- concentration (Boon and Laudelot, 1962); high temperature (Mahandrappa, 1966); low aeration coupled with high temperature (Laudelot *et al.*, 1976).

4.6 Conclusions

The marked differences in nitrifying activities in the soils under the three management regimes (*ZN* < *GC* < *N*F) represent subtle responses to the contrasting N-input systems. The measurements reported here were made during a growing season in which fertilizers were withheld and grazing animals excluded, so that the changes were clearly associated with previous conditions and did not merely reflect current inputs of N. There are implications in these findings for N cycling, in general and for the fate of mineralized N linked to nitrifying activity, in particular. Laboratory evaluations of *SMB* and potential mineralization would suggest that such tests are unlikely to predict differences in actual mineralization and the subsequent oxidation of released NH_4^+ needs be assessed in situations where competing processes are allowed to prevail naturally. Further work is required to establish the extent to which nitrification may influence other processes of N cycling and these aspects are discussed in more detail in Chapter 5.

CHAPTER 5 Concurrent measurements of net mineralization, nitrification, denitrification and leaching from field incubated soil cores

5.1 Experimental strategy

The **development** and **testing** of the improved field incubation (tube) method for measuring net nitrification concluded the first of the three major aims of this project (see Section 2.1). The second aim was to **link** the *in situ* measurements of nitrification with other N cycling processes. Experiments to date have been based on established field sites, where the combination of a high annual rainfall (on average, 1050 mm) and a soil with a high clay content (*c*.36%) means that for much of the winter period the soils are waterlogged. Conditions conducive to high losses of N through denitrification and leaching, therefore, often prevail in spring and autumn, when soil temperatures do not fall generally to levels which would inhibit microbial activity.

Two field experiments were therefore designed in order to exploit the particular soil and environmental conditions associated with these seasonal weather patterns. Investigations into possible linkages between the supply of NO_3^- (via nitrification and/or fertilizer) and the fate of soil N through various loss processes could then be conducted. In previous experiments, fertilizer applications were discontinued and grazing animals excluded to assess the validity of the methodology by decreasing the variability between measurements within a carefully defined set of conditions. In the following series of investigations, normal fertilizer regimes were incorporated into the schedule and cattle were only excluded from sampling areas for the duration of the measurement periods. In the autumn experiment, a field site with freely drained soil was also included to enable the important

component of N leaching to be more closely examined.

5.2 Investigation of microbial activity following spring applications of N

In spring 1996, microbial activity was examined over a ten week period following the first and second seasonal applications of N, by taking 'spot' measurements of *SMB*, total C and total N contents, and respiratory activity. At the same time, using the improved field incubation system, net rates of ammonification, nitrification and denitrification were obtained from an integration of measurements taken over this period, to investigate associated responses in microbial activity. Before turnout, cattle-proof enclosures were erected to provide four small sub-plots (5 x 5 m) in the 200 kg N ha^{-1} drained treatment (NF, Block A: see Fig 4.1) of the Rowden Drainage Experiment. The field drains ceased flowing after April 1996, so that further leaching of soil N was unlikely. I-E bags were, therefore, omitted from the incubation vessels and larger gypsum blocks (depth 25 mm) were constructed to occupy the space between the incubation tube and the undisturbed soil layer. On 27 March 1996, the first fertilizer was applied at a rate of 40, 26 and 50 kg ha^{-1} of N (as NH_4NO_3), P and K, respectively. Fertilizer was spread by hand in two directions (at right angles across the plots) to ensure uniformity of application. Measurements began after the first application of fertilizer, with a further application of N (60 kg ha^{-1}) only on 26 April and continued for 10 weeks until 6 June 1996. During the experimental period, the weather was generally wet and dull, e.g. May was colder (mean temperature of 8.9 °C) and wetter (with 80.7 mm rain) than average (30 year monthly means of 10.7 °C and 65.2 mm, respectively).

5.2.1 Net ammonification, nitrification and denitrification

The novel ('tube') field incubation technique was employed to measure *in situ* net ammonification, nitrification and denitrification during the experimental period. Each week, 12 cores enclosed in PVC tubes (37 mm diameter, 150 mm length) were extracted (three cores per sub-plot) along with 12 paired soil samples (extracted as outer concentric layers from around the PVC tube) using a larger corer (80 mm diameter, 300 mm length). The outer soil cores were extracted within 1 h of collection in 2 *M* KCl (soil:extractant volume ratio of 1:5) and analysed for NH_4^+ and NO_3^--N. The sheathed cores were incubated in the field for 7 days: six cores received a continual, slow infusion of C_2H_2 (from controlled release capsules, see Section 2.7.1) and the other six cores were incubated without C_2H_2. At the end of each 7 day incubation period, the sheathed cores were removed and extracted, as before and a new set of cores were obtained for incubation. Net rates for the N transformation processes were calculated from previously established relationships (see Section 3.4). Thus, an integrated measure of net mineralization (ammonification) was obtained from the increase in ammonium (NH_4^+)-N in the six cores incubated in the presence of C_2H_2, over the ten week period. An estimate of net nitrification was obtained from the difference in NH_4^+-N produced in the set of cores incubated with C_2H_2 minus that produced in cores incubated without C_2H_2 (i.e. the amount of NH_4^+-N that would have been nitrified, assuming immobilization was the same in both sets). Denitrification was estimated by the difference in NO_3^--N accounted for with (denitrification denied) and without (denitrification not denied) C_2H_2 .

5.2.2 Soil microbial biomass (*SMB*)

During the period of the experiment, *SMB* was investigated on five separate occasions at

intervals of 14 days. Soil was sampled using a corer (2.5 cm diameter, 15 cm deep) and the soil cores taken from each sub-plot were separately crumbled and sieved (4 mm) and stones, above-ground plant material and visible roots were discarded. A sub-sample was dried overnight at 105 °C for determination of water content. Established techniques (Brookes *et al.*, 1985; Vance *et al.*, 1987) were employed to identify and characterize *SMB* using the fumigation/extraction method (FE). On the days when soil was collected, duplicate samples of moist soil (12.5 g) were weighed into 60 ml glass bottles and the first of each pair was extracted with 50 ml 0.5 *M* K_2SO_4 by shaking the mixture for 1 h on an end-over-end shaker. The suspension was then filtered (Whatman No. 1) and the filtrate frozen for storage. The second sample was fumigated in a desiccator, lined with wet tissue paper and containing 35 ml ethanol-free chloroform ($CHCl_3$) in a beaker with glass boiling granules. Ethanol (included as a commercial stabilizer) was first removed from 50 ml $CHCl_3$ (to avoid erroneously high measurements) by shaking with 15 ml water in a separating funnel and then drawing off the $CHCl_3$ from below. The process was repeated twice more, each time discarding the water phase (containing the ethanol contaminant); the last traces of water were finally removed from the $CHCl_3$ by adding fused granules of $CaCl_2$. The desiccator was evacuated and soil samples fumigated for 24 h, after which the beaker of $CHCl_3$ was removed. Residual $CHCl_3$ vapour was dispersed by alternating between evacuation and a controlled release of the vacuum back to atmospheric pressure. The soil was then extracted, as for the unfumigated sample, and frozen for storage. The extracts were used to determine biomass C and N, and results were expressed on an oven dry soil basis.

Biomass N The fumigated (F) and unfumigated (NF) extracts were thawed in a refrigerator

prior to Kjeldahl digestion which involved boiling 25 ml extract (including glass boiling granules) with 0.6 ml 0.19 *M* $CuSO_4$ and 10 ml concentrated H_2SO_4. The digestion process was found to proceed more gently when the acid was thoroughly incorporated in the mixture and allowed to stabilize overnight before any heat was applied. The temperature of a heating block was first raised gradually to 150 °C and when this was reached, the digest boiled vigorously. Further heat was then applied up to 200 °C and maintained until the volume had been reduced to about 10 ml (with no water remaining). Final digestion was at 400 °C, refluxed for 3 h. After cooling, the digest was made to standard volume (75 ml) with water, mixed and stored at 4 °C. The total N content (as NH_4^+-N) was determined colorimetrically (flow injection analyser). Biomass N was calculated using the formula of Jenkinson (1988):

Biomass N = 2.22 (Kjeldahl N in F extracts - Kjeldahl N in NF extracts)

Biomass C Sub-samples of the F and NF extracts (8 ml) were digested with 2 ml 0.067 *M* $K_2Cr_2O_7$, 70 mg HgO and 15 ml of a 2:1 v/v mixture of H_2SO_4 and H_3PO_4 (both as concentrated acids). After boiling under a water reflux for 30 min, the excess dichromate was determined from a back titration with standardized 0.33 *M* $(NH_4)_2Fe(SO_4)_2.6H_2O$ in 0.4 *M* H_2SO_4. Biomass C was calculated using the formula of Vance *et al.* (1987):

Biomass C = 2.64 (organic C in F extracts - organic C in NF extracts)

Biomass activity Sub-samples of the sieved soil from each sampling occasion were also placed in sealed serum bottles at 22 °C and CO_2 concentrations in the headspace were measured initially, and again after 4 hours of incubation, using an I.R. gas analyser. Specific respiration was calculated as μg CO_2-C produced by 1 mg biomass-C per g dry

soil per hour. An alternative approach to measuring substrate-induced respiration (SIR) to the previous method (see Section 4.3.2) was used, which employed 'dry' (as opposed to liquid) glucose additions (5 mg glucose per g equivalent dry weight of soil) which can give more sensitive responses (Anderson and Domsch, 1978). Selective respiratory inhibition (Anderson and Domsch, 1975) was again used to discriminate between fungal and bacterial components (see Section 4.3.2).

5.2.3 Results from the spring studies

A net release of NH_4^+-N corresponding to the pattern of *SMB* activity was recorded (Fig. 5.1a) with steadily declining rates of net ammonification following the first spring application of N, *viz.* 1.80 to 0.88 kg N ha^{-1} d^{-1}. After the second N application, rates increased significantly to 2.20 kg N ha^{-1} d^{-1} ($p<0.05$) when compared with samples taken immediately prior to fertilizer application (*viz.* 0.82) and a steady decline in rates was again repeated, reaching a minimum of 0.57 followed by a slight increase to 0.89. Therefore, N applications appeared to have directly stimulated rates of ammonification, which has been referred to as a 'priming effect' by other workers (see Haynes, 1986). Nitrification of the released NH_4^+-N was rapid and suggested a similar pattern in activity to ammonification (Fig. 5.1b), with maximum rates initiated by the two fertilizer events, 1.6 and 1.38 kg N ha^{-1} d^{-1}, respectively. However, the rates of the two processes were only weakly correlated ($r^2=0.41$) with 33% of the variation accounted for; 63% of the net NH_4^+-N released was nitrified to NO_3^--N. Denitrification of NO_3^--N also appeared to have been stimulated by the N fertilizer applications (Fig. 5.2) with maxima of 0.75 and 0.54 kg N ha^{-1} d^{-1} recorded and an overall loss of N for the period of 20.8 kg (representing 36% of the nitrified N).

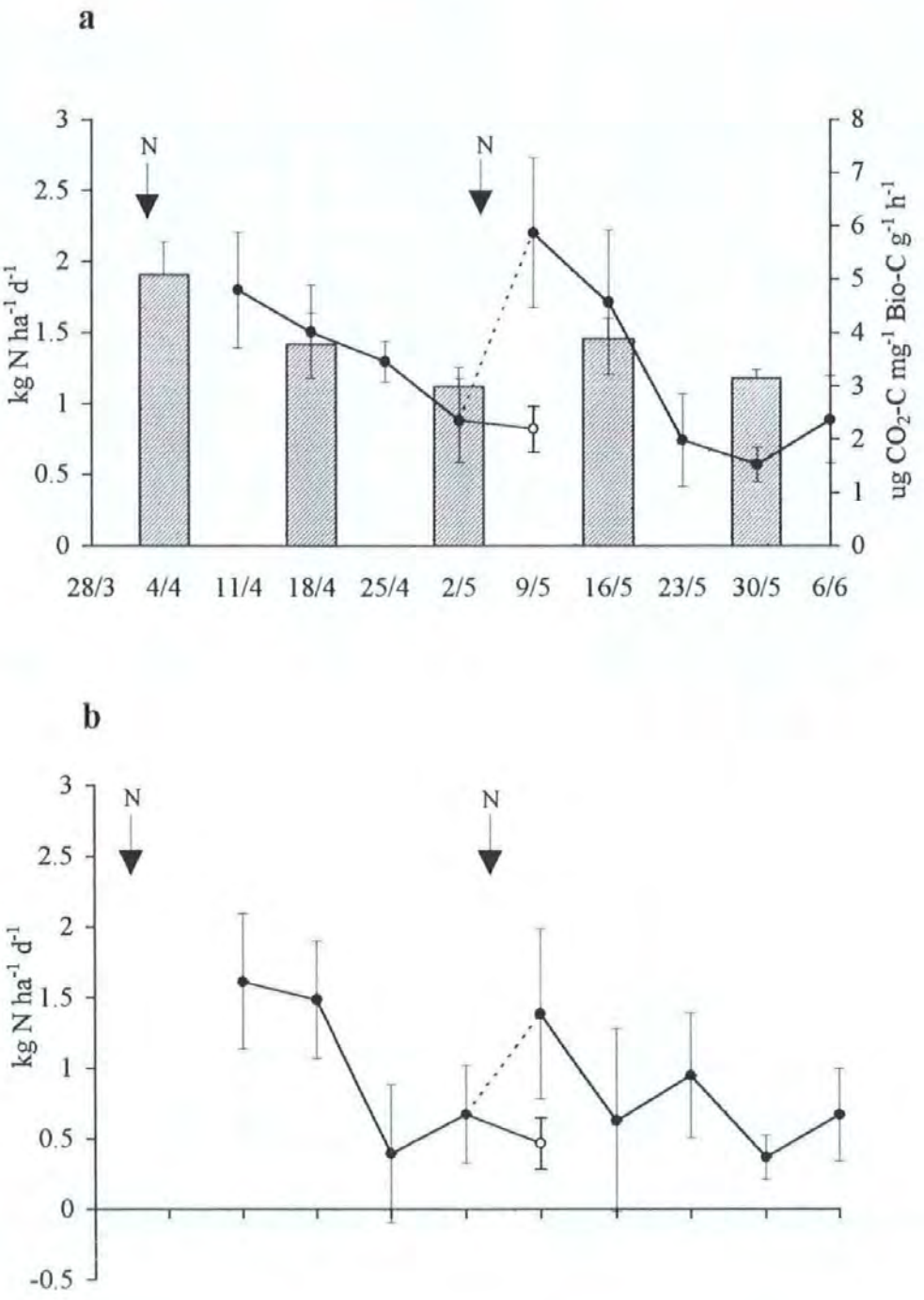


Figure. 5.1 a Net ammonification (kgN ha^{-1} d^{-1}) following N applications (closed circles ±SEM) and after witholding N (open circle ±SEM), and specific respiration (μgCO_2-C mg^{-1}Bio-C g^{-1} h^{-1}) of biomass (bars +SEM), in soil from a fertilized grass sward during spring (n=4). **b** Nitrification (kgN ha^{-1} d^{-1}) following N applications (closed circles ±SEM) and after witholding N (open circle ±SEM), in soil from a fertilized grass sward during spring 1996 (n=4).

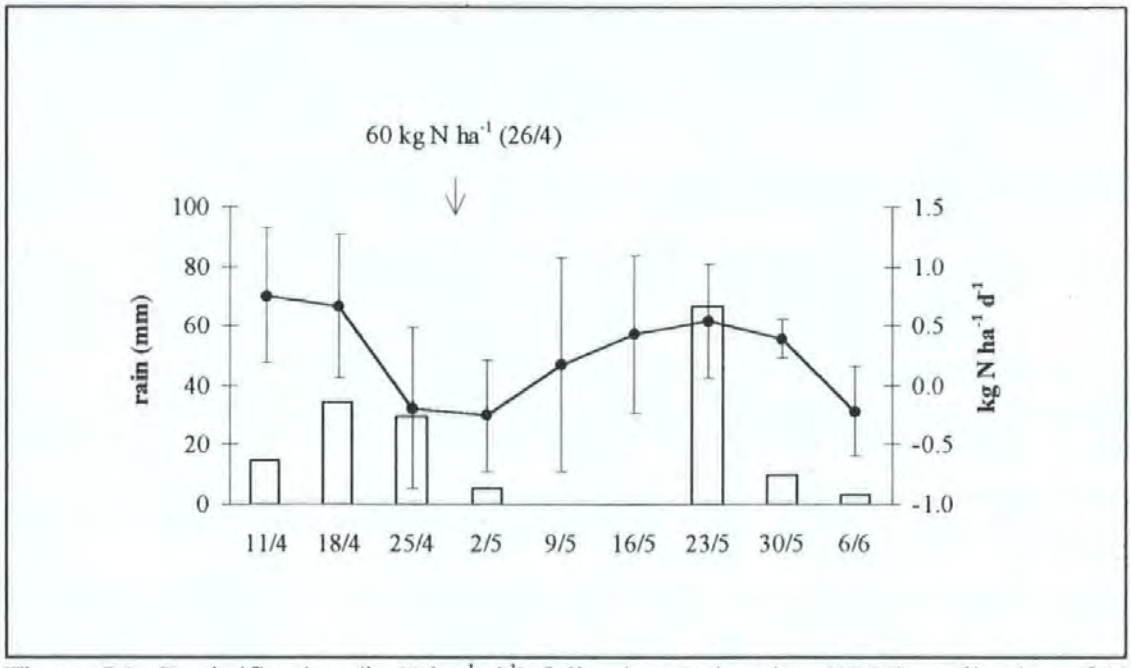


Figure 5.2 Denitrification (kgN ha^{-1} d^{-1}) following 2nd spring (1996) application of N (closed circles ±SEM) and weekly precipitation (mm) shown as bars (n=4)

When rates of denitrification and nitrification were compared (omitting negative or zero rates) the correlation between the two processes was poor (r^2=0.16). However, one of the data points was shown to be associated with a high residual error and low leverage (Cooke's distance residuals/leverage test), but with no obvious/recorded analytical error. A considerably stronger correlation was obtained (r^2=0.98) when this outlying point was omitted from the regression (Fig. 5.3). Thus, circumstantial evidence for a direct linkage between the processes was obtained, suggesting that the supply of NO_3^--N from fertilizer and/or nitrification can strongly influence the rate of denitrification. No significant diffference was found between the mean NO_3^--N contents (μg N g^{-1}) of soil sampled before (2.6 ±0.25) and after incubation with C_2H_2 (2.2 ±0.45), which confirmed the effectiveness of the controlled-release capsules in preventing nitrification.

Despite these independent measurements showing enhanced microbial activity, the overall

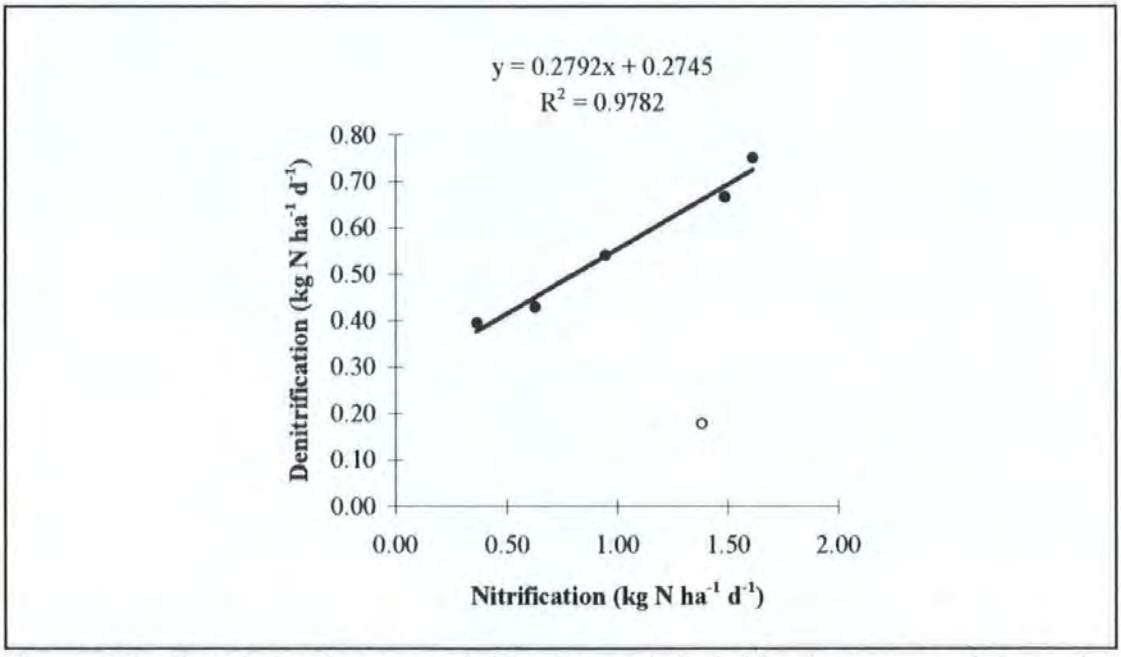


Figure 5.3 Correlation between nitrification and denitrification; one outlying value (open circle) omitted from regression analysis

size, in terms of biomass-C, was unchanged. Thus, *SMB* (measured by FE) contained nearly 1000 mg C per kg (dry) soil, but this did not alter significantly during the ten week period of the experiment (Table 5.1).

Table 5.1 Total C and total N in *SMB* (dry soil wt. basis) from a fertilized grass sward during spring 1996 (4 reps.)

Sampling dates	Biomass C		Biomass N		Biomass
	mgC kg^{-1} soil	SEM	mgN kg^{-1} soil	SEM	C:N
4 April	922.2	103.53	148.2	11.63	6.22
18 April	947.7	69.61	149.6	22.29	5.73
2 May	996.4	49.85	158.8	14.46	6.35
16 May	993.4	115.78	158.6	13.34	6.21
30 May	1184.0	56.95	183.3	5.81	6.45

Biomass N content was also unchanged during the experiment with an overall mean value

of 160 mg N kg^{-1} (dry) soil; consequently the C:N ratio of the biomass averaged 6.2 throughout (Table 5.1). Other studies have shown the *SMB* to be remarkably stable under field conditions (Bristow and Jarvis, 1991; Witter *et al.*, 1993) and additions of fertilizer N had no effect on biomass size over the course of one year (Lovell *et al.*, 1995). However, total *SMB* (as measured by FE) does not necessarily reflect its activity, since the proportions of active and inactive components may change due to environmental factors (Wardle and Parkinson, 1990), or with additions of substrate (Ocio and Brookes, 1990). **Substrate-induced respiration** (SIR) had rates between 20.3 and 27.5 μl CO_2 g^{-1} h^{-1}, but also showed no discernable trend with time. There were also no marked changes in the ratio of fungi : bacteria (assessed by selective repiratory inhibition) over the period of the experiment; overall the mean ratio was 3.0 : 1, indicating that the biomass contained a consistently higher proportion of fungi. **Specific respiration** (Fig. 5.1a), which more closely reflects the metabolic activity of the biomass, showed a decrease from a maximum of 5.1 (μg CO_2-C mg^{-1} biomass-C g^{-1} h^{-1}) immediately after the first application of N, to a significantly lower level of 3.0 ($p<0.05$) by week 5. Following the second application of N, this downward trend was halted and by week 7, specific respiration was no longer significantly different from the initial value. After a further two weeks (week 9), however, specific respiration had again fallen significantly ($p<0.05$) below the initial value. This response to N suggests that the activity of the microbial population may have been limited to some extent by an inadequate supply of N. In this experiment, therefore, additions of fertilizer N coincided with increased *SMB* activity, without the size of the biomass (as assessed by FE extraction) being measurably affected. Lovell and Jarvis (1996) found that in soil treated with urine, biomass-mediated processes also increased substantially, whilst biomass size remained the same. Joergensen (1996) has proposed that there is an active

component of the *SMB*, rather than its overall size, which varies during the year in response to altered conditions.

The existence of a large, but mainly dormant bacterial population, suggested by Paul and Voroney (1980), may partly explain the relatively stable component identified by FE extraction. The other changes observed in various aspects of *SMB* activity are most likely to be as an indirect effect of the increased inorganic N which increases the supply of C substrates through enhanced root growth and exudation into the rhizosphere (Fauci and Dick, 1994). Respiratory activity is relatively easy to measure and could provide an indicator of short-term responses in microbial activity to external influences. In the case of nitrifying processes the increase in substrate (NH_4^+-N), supplied either directly from fertilizer, or indirectly as a result of stimulated mineralization (i.e. from organic N), appears to have increased the rate of nitrification, as was recently shown by Watson and Mills (1998). Thus, supporting evidence for the role of nitrification in responding to N and promoting losses through denitrification has been obtained and was examined further in the next experiment.

5.3 Investigation of N transformations in autumn/winter

In the autumn experiment, measurements followed a growing season with grass under conventional silage management, so that nitrifying activity *per se* could then be assessed without the complication of further inputs of fertilizer N. Two soil types, with contrasting drainage characteristics, were selected at the farm of the Institute of Grassland and Environmental Research, Okehampton, Devon, UK to test further the performance of the 'tube' incubation method. The first was a freely drained, reddish gravelly loamy soil over

Permian breccia from the Crediton Series: a typical brown earth (Findlay *et al.*, 1984). The second was a poorly drained clay loam soil of the Hallsworth series (see Section 3.2 for full classification). Both soil types had been under a 3-cut grass silage management system with similar inputs of N for the last 5 and 4 years, but had previously contrasting histories of continuous arable cropping and long-term grassland, respectively. Some important chemical and physical properties of the soils are presented in Tables 5.2, 5.3 and 5.4.

Table 5.2 Soil particle size distribution (%) in the Crediton and Hallsworth soil series (0-150 mm depth). Particle sizes in mm.

Soil series (drainage)	Clay (<0.002)	Silt (0.002- 0.02)	Fine sand (0.02-0.2)	Coarse sand (0.2-2.0)	OM* (%)
Crediton (free)	24.2	10.3	14.9	51.7	5.6
Hallsworth (poor)	48.5	12.7	16.3	7.7	14.1

*OM denotes organic matter (not including living roots) assessed by loss of mass on ignition

Table 5.3 Percentage by volume of soil, stone, water- and air-filled pore spaces at field capacity in freely and poorly drained soils at IGER, North Wyke, UK (January 1997)

Soil series (drainage)	Fine soil (%)	Stone >2 mm	Pore space (% volume)			Dry bulk density (g soil cm^{-3})
			Water	Air	Total	
Crediton (free)	44.9	5.0	35.7	14.4	50.1	1.19
Hallsworth (poor)	27.2	0.5	46.8	25.5	72.3	0.72

Table 5.4 Some properties of the freely and poorly drained soils (0-150 mm depth) measured at the end of the experimental period (oven dry weight basis)

Soil series (drainage)	Inorganic N ($\mu g\ N\ g^{-1}$)	$NH_4^+ : NO_3^-$	Total N ($mg\ N\ g^{-1}$)	Organic C ($mg\ C\ g^{-1}$)	C : N	pH
Crediton (free)	3.4	3.8	3.3	24.0	7.3	5.3
Hallsworth (poor)	5.9	3.9	6.3	57.8	9.2	5.1

Each soil type was represented by four replicate plots (500 m^2). In 1996, N applications to freely and poorly drained soils amounted to 285 and 297 ($kg\ ha^{-1}$) fertilizer and 128 and 98 ($kg\ ha^{-1}$) slurry total N, respectively. Three silage cuts were made in 1996, the last being on 1 October for both soil types. From 31 October 1996 to 3 January 1997, measurements were made using the 'tube' incubation system; two paired soil cores (each including associated outer cores) were taken at random from within each of the field plots every 4-5 days (11 days on one occasion) in incubation tubes, one soil core to be incubated with C_2H_2 and the other without C_2H_2. Each of the 16 cores was then incubated in the field in incubation vessels with an I-E bag and gypsum block (15 mm depth) placed beneath each core, half of the blocks (8) contained controlled release capsules (incubations with C_2H_2). At the end of each incubation period, the soil core was removed and NH_4^+ and NO_3^--N were extracted using 2 *M* KCl extractant (5 : 1, KCl : fresh soil) together with a new set of outer cores, representing the starting 'baseline value' for the succeeding incubation. The NO_3^- absorbed in the I-E bags was extracted with 1.5 *M* H_2SO_4, as before

(see Section 2.4.3) and the recovery factor of 90% was used to adjust estimates of potential leaching, based on the efficiency of NO_3^- recovered in previous tests.

Denitrification was also measured independently over the experimental period by the 'jar incubation' method (Ryden *et al.*, 1987), using 8 cores (100 mm deep x 25 mm diameter) per jar (four replicate jars per soil type). At the end of the experiment, over-winter herbage production from the swards on the two soil types was assessed by taking four replicate quadrats ($1 m^2$) to a cutting height of 40 mm. The herbage was dried at 85 °C for 12 h in a forced-draught oven, ground and analysed for total N content using a Carlo Erba CN Analyser. Potential nitrifying activity was estimated from soil cores obtained at the end of the experiment (four replicates of 10 bulked cores: 150 mm deep x 37 mm diameter). The soil was sieved (6 mm) and fresh soil (equivalent to 10 g dry weight) was incubated in a water bath at 25 °C as a 'slurry' (50 ml) of 2 m*M* $(NH_4)_2SO_4$, 2 m*M* phosphate buffer (pH 7.5) and 15 m*M* $NaHClO_3$, as before and the rate of NO_2^- production over 8 h was measured (see Section 4.5.1).

Leaching of NO_3^--N was independently assessed using ceramic suction cups (25 mm diameter, 55 mm length) inserted at a depth of 600 mm in the freely drained soil and at 300 mm in the poorly drained soil (12 replicates to each soil type). Estimates of net mineralization were also independently obtained by the method of Hatch *et al.* (1990) using a 7 d field incubation of 4 cores (125 mm deep x 37 mm diameter) per incubation vessel, with C_2H_2 to inhibit nitrification. There were four replicate incubations to each soil type. The original method was altered slightly so that the assessment of net mineralization was based only on the change in soil NH_4^+-N during incubation, rather than the overall change

in inorganic N (i.e. $NH_4^+ + NO_3^-$). This was based on the assumption that, as shown in previous studies (Hatch *et al.*, 1991; Gill *et al.*, 1995), C_2H_2 is an effective inhibitor of nitrification and there would be no increase in NO_3^- levels.

5.3.1 Methods of soil analyses

Total C, total N and pH were also measured on oven-dried sub-samples of the bulked soils. Soil particle size analysis (Piper, 1950) was obtained from air-dried (30 °C) samples, crushed to pass a 2 mm sieve. Sub-samples, 100 g soil (Crediton series) or 50 g (Hallsworth series) were mixed with 5 ml saturated solution of sodium oxalate, 5 ml 1 *M* NaOH and 10 ml hydrogen peroxide (6 % H_2O_2 v/v). Sufficient deionized water was added to saturate the samples, which were broken up using a pestle into a smooth lump-free paste. The Hallsworth soil also had a high O.M. content and required further additions of H_2O_2 and gentle heating on a water bath to disperse the aggregates and destroy the O.M. The reaction was completed when the mixture no longer frothed (> 24 h, in the case of Hallsworth soil). The remaining H_2O_2 was then destroyed by boiling for a few minutes. The mixtures were then vigorously stirred for about 15 min (30 min for the clay soil) and washed into a Bouyoucos cylinder, using a wash bottle. Further water was added up to the 1205 ml mark (100 g sample) or the 1130 ml mark (50 g sample) with the hydrometer in place. The cylinder was then inverted (without the hydrometer), ten times and then allowed to settle. Both the time and temperature of the mixture were noted and after 3 min, the hydrometer was gently inserted and read at 4 min. Prior to taking the reading, the hydrometer was depressed slightly and allowed to settle, to avoid an erroneous reading from froth or surface tension effects. A few drops of amyl alcohol (=Pentan-1-ol) helped to disperse the froth to make the recording clearer. The hydrometer was then removed and

the mixture left to settle further for 2 h, after which another hydrometer reading was obtained, along with the temperature of the mixture, at that time. Readings were corrected from the calibrated value (19.5 °C), by adding 0.3 for every 1 °C above this value and deducting 0.3 for every 1 °C below it. The second reading (representing only the clay component) was subtracted from the first reading (representing silt + clay components) to give the silt only component. Percentages were obtained by dividing these component parts by the amount of soil from which they were derived (on a dry soil weight basis), multiplied by 100. The liquid was decanted from the cylinder through a 70 mesh sieve over a bowl. After sieving in water for 1 min, the coarse sand (on the sieve) was transferred to an evaporating dish and dried in an oven (105 °C), whilst the remaining contents of the bowl were washed into a 1 l beaker to a depth of 10 cm. The fine sand was separated from the silt by vigorously stirring the sediment, followed by a period of settling-out which was determined from the temperature of the mixture, according to the following table:

Table 5.5 Settling time required before decantation of silt to retain fine sand, as determined from the temperature of the washings, after removal of coarse sand

°C	16	17	18	19	20	21	22	23	24	25
secs	320	310	300	300	288	280	270	270	260	255

The process was continued until the liquid ran clear and the fine sand was then dried in an evaporating dish, as before. The coarse and fine sands were expressed as percentages of the original (dry) soil sample. Organic matter content (not including living root matter) was obtained from the loss of weight on ignition of soil placed in a muffle furnace and

heated overnight at 600 °C and expressed as a percentage component of the dry soil.

5.3.2 Results from the autumn studies

Net rates (kg N ha^{-1} d^{-1}) of mineralization in the soils measured by tube incubation (Fig. 5.4a and b) ranged from 0.28 - 1.64 (freely drained) and 0.55 - 1.17 (poorly drained). The mean daily rates (kg N ha^{-1}) for the two soils over the measurement period were significantly different ($p < 0.001$), 0.59 (± 0.064) in freely drained soil compared with 0.88 (± 0.056) in poorly drained soil. Cumulative totals for the net release of mineralized N from soil organic matter (Table 5.6) compared favourably with independent measurements using the jar incubation technique over the same measurement period.

Table 5.6 Comparison between the tube incubation method and alternative methods of measuring several N transformation processes over 64 days (31/10/96-3/1/97)

Process	Freely drained soil (kg N ha^{-1})		Poorly drained soil (kg N ha^{-1})	
	Tube incubation /resin bags*	Jar incubation /ceramic cups#	Tube incubation /resin bags*	Jar incubation /ceramic cups#
Net mineralization	34.8 (1.29)	56.1 (0.58)	53.9 (0.39)	43.2 (1.17)
Nitrification	20.2 (2.42)	-	38.7 (0.97)	-
Denitrification	7.2 (3.39)	5.3 (0.15)	24.2 (3.51)	7.1 (1.76)
Leaching	3.7 (0.71)*	3.7 (1.73)#	6.9 (0.88)*	3.8 (2.05)#

Data are means with ± SEMs shown in parentheses

Coefficients of variation were higher than would be anticipated under strictly controlled analytical conditions, but of the order commonly encountered with field measurements in grassland soils (mean values of 52 % and 38 %, for freely and poorly-drained soil,

respectively). In the freely drained soil, 58 % of mineralized N was nitrified, compared with 72 % in the poorly drained soil. Daily rates (kg N ha^{-1} d^{-1}) of nitrifying activity ranged from 0.03 - 0.82 (Fig. 5.4a) and 0.37 - 0.95 (Fig. 5.4b), with mean daily rates for the whole period of 0.24 (± 0.072) and 0.60 (± 0.074), respectively. No independent method for assessing nitrifying activity in the field was available, but laboratory measurements of potential nitrification (where NH_4^+-N substrate was non-limiting) gave rates of 14.7 and 8.3 nMoles NH_4^+ oxidized g^{-1} (dry soil) h^{-1}, obtained from the slopes of linear plots of NO_2^- production over 8 h (r^2 = 0.99 and 0.97) in the freely and poorly drained soil, respectively. Thus, higher ($p < 0.001$) nitrifying rates were found in the field in the poorly drained soil, but the greater potential activity was in the freely drained soil.

Other factors which might have been expected to correspond with different rates of nitrification were similar (Table 5.4), e.g. soil pH and the ratios of NH_4^+ : NO_3^-. Nitrification was related to rates of mineralization ($p < 0.001$) in both the freely drained ($r^2 = 0.41$) and in the poorly drained ($r^2 = 0.52$) soil, as might be expected, since the supply of substrate (*viz.* NH_4^+) is determined by the rate of mineralization. Also, NH_4^+ contents exceeded those of NO_3^- in both soil types, which confirmed this dependence and showed that there was a lag in the oxidation of NH_4^+ by the nitrifying population which allowed NH_4^+ to accumulate.

Rates (kg N ha^{-1} d^{-1}) of loss of N by denitrification (estimated by differences in inorganic N from weekly tube incubations ± C_2H_2) ranged from 0.04 - 0.52 (Fig. 5.5a: freely drained) and from 0.17 - 1.08 (Fig. 5.5b: poorly drained). The mean rates (kg N ha^{-1} d^{-1}) differed between the two soils ($p < 0.01$), i.e. 0.05 (± 0.086) compared with 0.40 (± 0.079),

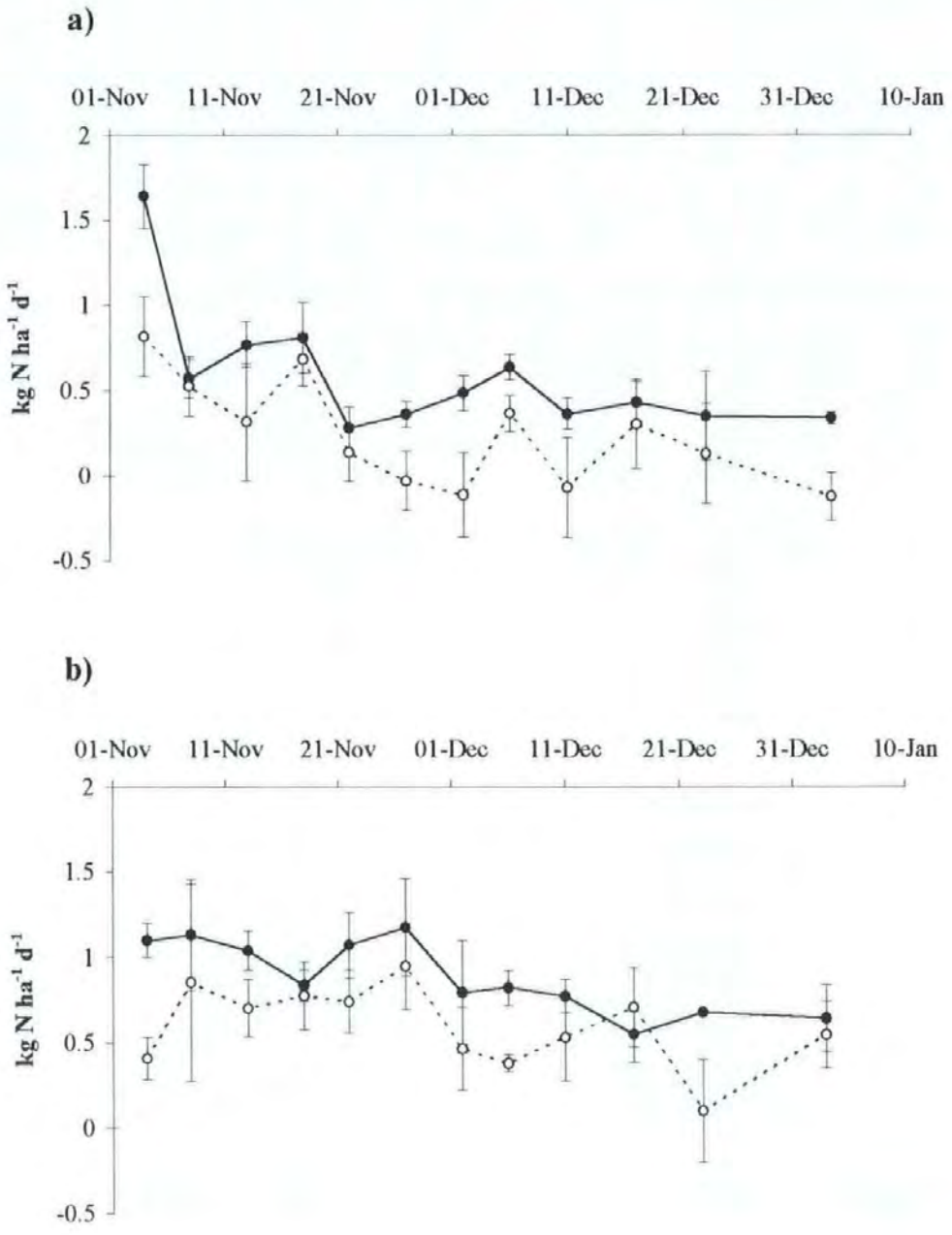


Figure 5.4 Weekly measurements shown as rates (kg N ha^{-1} d^{-1}) of net mineralization (closed circles ± SEM) and nitrification (open circles ± SEM) in a) freely and b) poorly drained soil over an experimental period (64 d) in autumn/winter 1996/7 (n = 4)

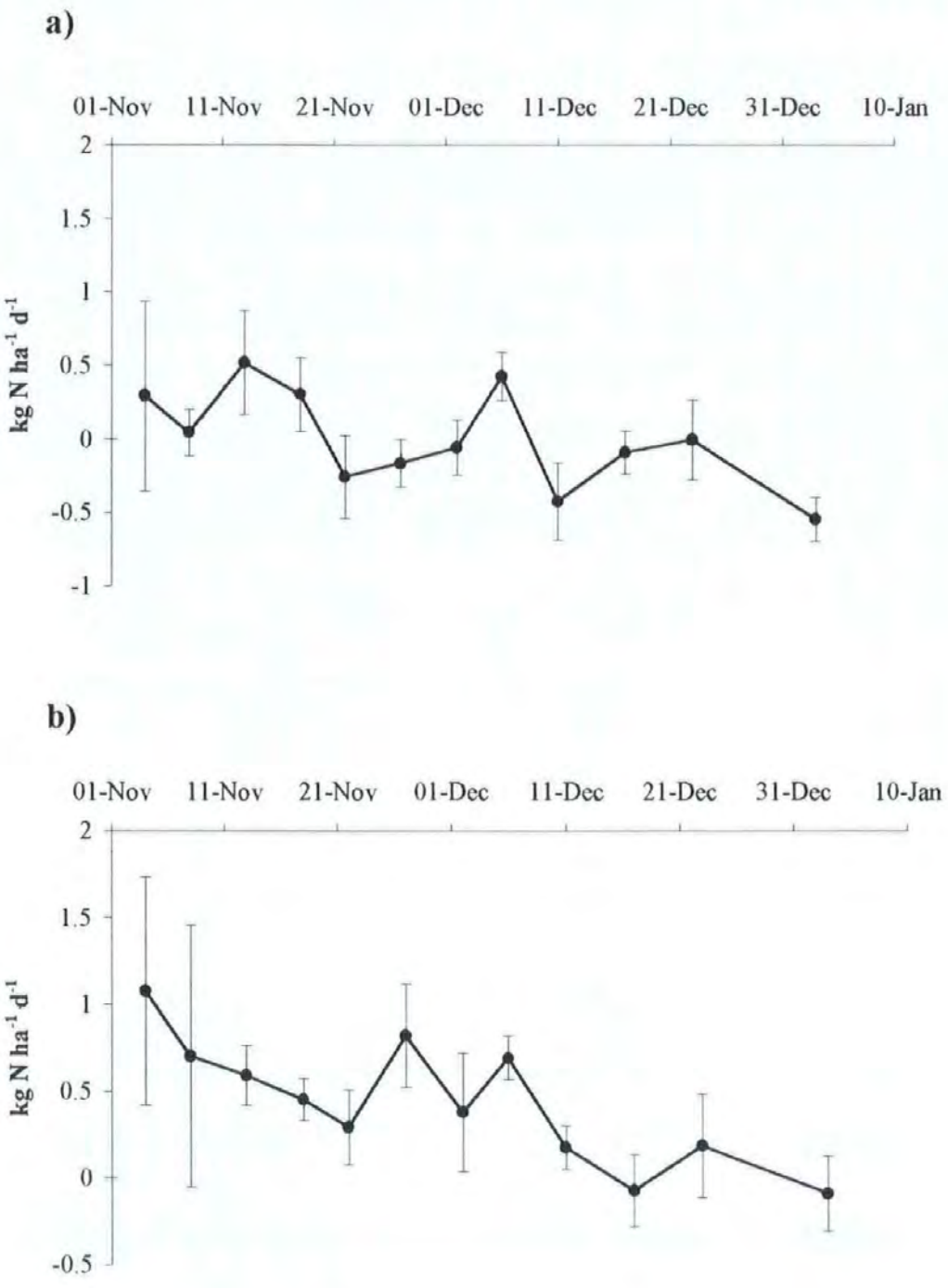


Figure 5.5 Weekly measurements shown as rates (kg N ha^{-1} d^{-1}) of denitrification in a) freely and b) poorly drained soil over an experimental period (64 d) in autumn/winter 1996/7. Vertical bars are ± SEM (n = 4)

respectively. Daily rates of denitrification were closely correlated with rates of nitrification in the freely drained soil ($p < 0.001$), with $r^2 = 0.67$ (one outlying value was removed with high residual error, but with low leverage, Fig. 5.6a). However, rates of denitrification also included some negative values which were assumed to be zero (i.e. at or near a rate below the level of detection) in the calculation of the cumulative total (Table 5.6). In the poorly drained soil, there were fewer negative values (Fig. 5.6b) and the two processes were again significantly correlated ($p < 0.001$), with $r^2 = 0.68$ (two outlying values removed with high residual errors, but with low leverage). The cumulative total was > 3-fold larger than that of the freely drained soil (Table 5.6).

The cores remained tightly fitted in the incubation tubes during incubation, so that preferential leaching down the sides was unlikely and shrinkage of the soil within the tubes did not occur. Both of the techniques used for measuring leaching (by I-E bags or ceramic suction cups) and those used for denitrification (by difference in the tube incubation, or by jar incubation) were in close agreement in the freely drained soil (Table 5.6). In the poorly drained soil, however, the estimates were greater with the tube incubation method for both processes (Table 5.6). The N unaccounted for in measurements taken by tube incubation over 64 d (i.e. net mineralization minus losses from denitrification and leaching) amounted to 24.0 and 22.8 kg N in the freely and poorly drained soil, respectively. Some of this N loss could be attributed to uptake in the autumn/winter herbage growth (assessed from herbage sampled from these plots at the end of the experimental period) which yielded 26.2 (± 1.60) and 55.8 (± 6.81) kg N, respectively. No assessment was made of the N in herbage at the start of the experimental period (October 1996), so it is possible that more N was present in re-growth (i.e. before soil measurements began) following the last silage

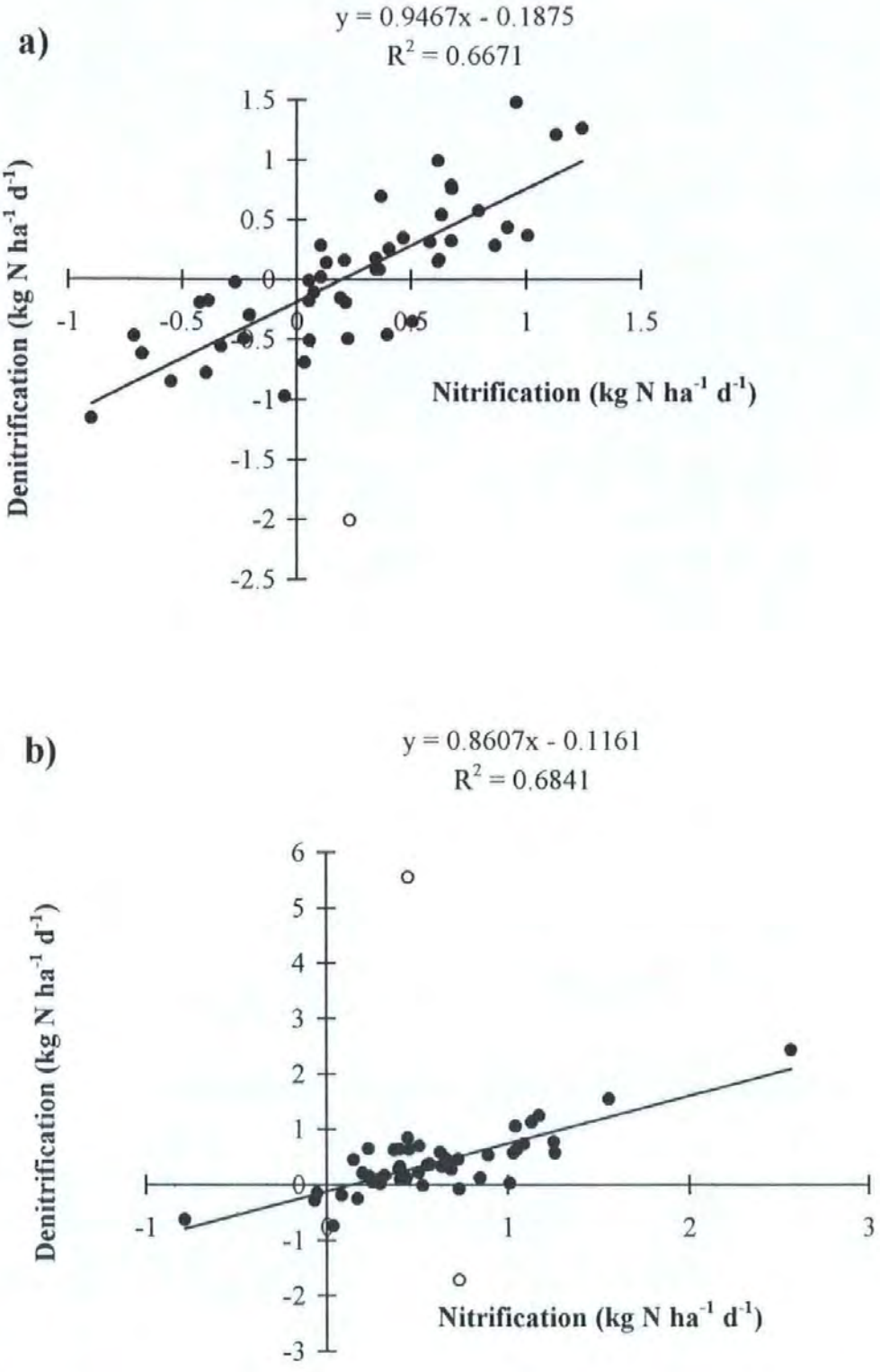


Figure 5.6 Correlations (closed circles) between nitrification and denitrification in **a)** freely drained soil and **b)** poorly drained soil from weekly measurements: outlying values (open circles) omitted

cut on the poorly drained soil (due to the wetter soil conditions) and was therefore over-estimated, whereas growth on the drier soil had ceased after cutting and regrowth only occurred after the soil had returned to field capacity. This discrepancy would have been carried forward into the over-winter assessment of uptake from N mineralized during the measurement period, but nevertheless provides an estimate of removal into herbage. Losses of N through senescence and transport into roots, which were not accounted for, would also contribute to errors in the estimates on both soil types.

5.3.3 Conclusions from the autumn/winter studies

The tube incubation method allows concurrent measurements of the major N transformation processes (mineralization, nitrification, denitrification and leaching) to be made which would not otherwise be possible using existing techniques. Foremost amongst the advantages is the opportunity to extend the period of measurement indefinitely, which would not be practical with other methods, particularly for gross measurements using ^{15}N methodology (due to the expense and other technical limitations, see Section 6.1). An additional benefit of the improved incubation method is that integrated estimates of the various processes are obtained for defined periods (which avoids problems of long-term exposure/supply of soil to C_2H_2, see Section 2.7), rather than the alternative method of extrapolating from 'spot' measurements. The present experiment, which was designed to illustrate the use of the technique under field conditions, showed that the processes of net mineralization, nitrification and denitrification were closely associated. Such associations might be expected, given the substrate/product relationships which exist, although supporting evidence is limited. The effect that antecedent fertilizer inputs can have on increasing the rate of nitrification has been shown recently (Jarvis and Barraclough, 1991;

Hatch *et al.*, 1996; Watson and Mills, 1998) and this will have implications in terms of the supply, distribution and fate of N in soils. Further possible correlations might also have been expected between nitrifying activity and the removal of nitrate through leaching. However, the lack of drainage in December 1996 (driest December since 1959) precluded meaningful comparisons.

Errors associated with the measurements were within the range typically encountered in field experimentation. However, some negative values were found for nitrification, denitrification and also leaching which were obviously incorrect, whereas negative rates for mineralization are probably more correctly interpreted as net immobilization. The reason for these discrepancies was probably the combination of inherently low rates and high spatial variation between replicates. Greater replication (e.g. 6-8 incubation tubes to each treatment) would be required to better resolve temporal changes in these processes. In overall terms, the values obtained with tube incubation and other methods compared well, with the exception of denitrification in the poorly drained soil. Lower than expected estimates of denitrification in 'heavier' soils are recognized as particular problems of the jar incubation method (Smith and Arah, 1990). This may be due to enhanced aeration and an inability to ensure satisfactory infusion of C_2H_2 to fully inhibit nitrous oxide reductase activity (i.e. N_2O to N_2). A marked advantage of the tube incubation method is, therefore, the enclosure of the soil core in a sheath to prevent exposure of the sides of the soil cores to unnatural aeration and the improved method of providing a sustained infusion of C_2H_2 via the base of the incubation tube and by gas diffusion holes placed around the sides (see Fig. 2.1). Degradation of C_2H_2 by C_2H_2-metabolizing populations is also recognized as a potential problem (with the possibility of wider effects on C cycling processes), but is one

which usually only develops after a lag phase of up to 8 d (Terry and Duxbury, 1985). The greater variability in the data relating to denitrification in the freely drained soil, however, was probably caused by an inadequate supply of C_2H_2 from the controlled-release capsules, because of very dry soil conditions. It may be necessary in such circumstances, periodically (e.g. halfway through the incubation period) to re-wet the gypsum blocks to ensure that the reaction with CaC_2 granules proceeds satisfactorily.

Differences in assessments of nitrifying activities (actual and potential) are less easy to reconcile. The greater potential nitrifying activity in the freely drained than the poorly drained soil suggests that a more active, or abundant population of nitrifiers may have developed in the freely drained soil in response to aerobic conditions more favourable for growth of the bacteria. The higher (actual) field rate of nitrification found with the poorly drained soil is, however, compatible with having a higher rate of net mineralization (since the supply of NH_4^+ substrate will be the rate-limiting step). It is also possible that this soil type, which is more highly structured, may have microsites with a higher pH which would favour nitrification, despite the apparent similarity in pH of the two soils when measured on disturbed soil samples. Such differences would not be a factor in the laboratory incubations which were based on soil 'slurries' buffered at pH 7.5.

Estimates of N leached using both I-E bags and cup-samplers were low and reflect the unusually dry winter period of 1996/7 and generally lower losses associated with cut (as opposed to grazed) swards (Ryden *et al.*, 1982). It would, therefore, be of interest to assess leaching under grazed swards in a more typical drainage period, but a considerable increase in replication would be required to overcome the greater variability in soil inorganic N

content induced by excretal returns. The 'paired' sampling approach, adopted in the present method, has an advantage over random sampling since spatial variation is largely accounted for by closely linking the 'start' cores with the incubated cores (*viz.* concentrically positioned cores). I-E bags also have the advantage over cup-samplers of operating in a 'passive' mode to the passage of soil water, whereas cup-samplers may modify the flow characteristics of soil water and are less satisfactory in 'heavier' soils (Hatch *et al.*, 1997). The tube incubation method also overcomes many of the other problems associated with enclosed incubation vessels, causing only minimal disturbance to the soil which allows natural wetting/drying processes to proceed and avoids large changes in the soil atmosphere/temperature status. Finally, the low cost of the equipment involved in the tube incubation method enables large scale monitoring for prolonged periods of experimentation to be undertaken, as well as providing the opportunity for examining the linkage between N cycling processes.

The extent, however to which nitrification may exert a controlling influence over components of the N cycle can only be determined further from direct measurements of the gross rates of the processes involved. Chapter 6 describes the final part of the project which was devoted to **examining** the practical implications of these findings under different management regimes and thereby addresses the third aim of the project as stated in Section 2.1. The natural stable isotope of nitrogen (^{15}N) was employed, using techniques of 'mean pool dilution', to determine rates of the key processes. The tube incubation method was adapted to provide microplots for the ^{15}N enrichment experiment and simultaneous estimates of net process rates. The development of the equipment, experimental techniques and the calculations involved in resolving these processes are described.

CHAPTER 6 Development of a multi-point soil injector to measure gross and net rates of the major N cycling processes

6.1 Introduction: concepts and constraints

A further development of the tube incubation technique (previously described in Chapter 2) was undertaken in order to combine net measurements of mineralization, immobilization and nitrification with gross rates of the same processes, obtained using standard ^{15}N pool dilution methodology (Kirkham and Bartholomew, 1954; Barraclough, 1991). The basic concept was to provide an alternative calibration of the tube incubation technique, together with simultaneous assessments of the gross rates of the major processes (using a specially designed ^{15}N soil injector) to underpin the findings of the previous chapter.

A popular approach employed in field evaluations using the ^{15}N mean pool dilution technique involves sets of paired microplots (open-ended tubes inserted into the ground and enclosing undisturbed soil profiles). The microplots are enriched with a source of the stable isotope of nitrogen (^{15}N): $^{15}NH_4^+$-N for gross rates of mineralization/immobilization and $^{15}NO_3^-$-N for gross rates of nitrification (Barraclough, 1991; Monaghan, 1995; Davidson *et al.*, 1991). An advantage of this approach is that only the products, rather than the substrates of these processes are supplemented, which avoids stimulation and possible disturbance of the steady state. Following injection (at t0), assessment of the starting point enrichment is made (after 24 h equilibration period) by removing one of the paired microplots (t1) and then comparing this with the resultant enrichment in the remaining microplot, after a defined period of field incubation has elapsed (t1+).

The underlying assumption is that both native ^{14}N sources and ^{15}N enrichments are jointly removed and therefore make no impact on the overall isotopic ratio, provided that three conditions are satisfied (Kirkham & Bartholomew, 1954):

1. Process rates must remain constant over the period of measurement
2. The incubation period must be short enough to avoid remineralization of labelled sources of N which have become immobilized
3. There must be no significant discrimination between isotopic sources of ^{15}N and ^{14}N sources by the processes under study

A fourth consideration, which is of particular importance when using intact cores (Davidson *et al.*, 1991) is that the inorganic N pools (NH_4^+ and NO_3^-) within the soil microplot should be uniformly enriched with the isotope source to avoid erroneous results.

A technical protocol was adopted which to a large extent, satisfied the requirements of the first three conditions and special equipment was designed to fulfill the fourth. Compliance with the first of these conditions was achieved by adopting a relatively short incubation period of 4 d, at a time when weather conditions were stable. The shortened incubation period also enabled problems associated with the second condition to be avoided; Barraclough (1991) has suggested that a period of no more than 3-5 d is suitable for soils at temperatures up to 23 °C. A 4 d incubation was adopted as a compromise to also produce a measurable change in inorganic N to allow accurate and simultaneous 'tube' incubation measurements (for net rates). It was not possible, however, to ensure compliance with the third condition, but this is thought unlikely to present problems when soil enrichments are employed that greatly exceed the natural abundance of ^{15}N (Davidson *et al.*, 1991). Specially designed apparatus was constructed in order to achieve uniform

enrichment of undisturbed soil and is described below. Process rates were derived from the decline in the initial enrichment of the soil N pool due to the addition of unlabelled 'native' ^{14}N (ammonification and nitrification). Consumptive processes (immobilization + gaseous losses) were assessed by the rate of removal of the ^{15}N label, but could also have been affected by addition of the isotope (substrate). Alternative estimates of immobilization were examined, based on the appearance of ^{15}N in chloroform-labile microbial biomass.

Initial analysis of soils from the experimental sites suggested that inorganic N levels (NH_4^+ plus NO_3^-) could range up to 20 μg N g^{-1} dry soil, and the N-enriched source would add no more than an equivalent amount of N. Aspects to be considered when deciding on soil enrichment include the need to add sufficient isotope at the start of field incubation to be present in measurable amounts at the end. In practice, this means that an enrichment of >0.6 At.% (i.e. at least twice natural abundance) is desirable at the end of the incubation. Another constraint is the need to keep the enrichment levels at the start of the incubation within the upper limits of the capabilities of the mass spectrometer (MS) (approximately 12 At.%) to avoid 'spiking' samples with an unlabelled N source in order to prevent the collector of the MS from saturating. The disturbance of the 'steady state' must also be kept to a minimum to avoid excessive perturbations in the rates of processes to be measured. It is also necessary to make measurements of process rates close to the linear phase of the isotope dilution curve where processes obey zero-order kinetics to maximise accuracy (Barraclough, 1991). Because rates of mineralization in grassland soils are likely to be high, the initial enrichments were increased to 25 At.% $^{15}NH_4^+$ and to 20 At.% NO_3^- (since rates of ammonification are most likely > nitrification; see Section 5.2.3).

6.2 Equipment for ^{15}N enrichment of undisturbed soil

Enclosures for the microplots were fashioned from PVC tubes (175 x 100 mm) with chamfered cutting edges (30°) at one end. Instead of paired microplots, the current technique employed individual microplots which were inserted in the ground (150 mm depth) using a drop-hammer device. A multi-point soil injector (Plate 6.1) was designed which fitted onto the rim of the microplots, based on a modified design of a gas injector described by Murphy *et al.* (1998), but adapted for liquid additions. Injection of the liquid was via a cluster of 13 spinal needles (14 gauge stainless steel, 150 mm length), mounted in brass couplings in a perspex manifold (Fig. 6.1a) and held in place with knurled screw caps. The pointed ends of the needles were sealed with epoxy resin and drilled with two side-port openings. Flow restrictors were placed over the top end of the needles to increase the back pressure within the manifold to help maintain an even flow of liquid through the needles. The flow restrictors consisted of discs (7 mm diameter) cut from an open cellular (permeable) foam material (ear-defender plugs). The manifold and needles were supported on three polished stainless (*ram*) steel rods (16 mm o.d.) which allowed the assembly to move a distance of 150 mm: equivalent to the length of the needles (Plate 6.1). In the primed position, the needles were fully extended and a 60 ml syringe (containing 40 ml liquid) was placed in a cradle and connected to the inlet of the manifold by silicon tubing using Luer-lock fittings. A handle winder, mounted on screw studding (25 mm o.d., *Acme* 5 t.p.i) was synchronised with the movement of the plunger and the travel of the needles through the soil profile. In this way, injection of liquid was always uniformly distributed, regardless of the rate at which the needles were withdrawn through the soil (see Section 6.3.1).

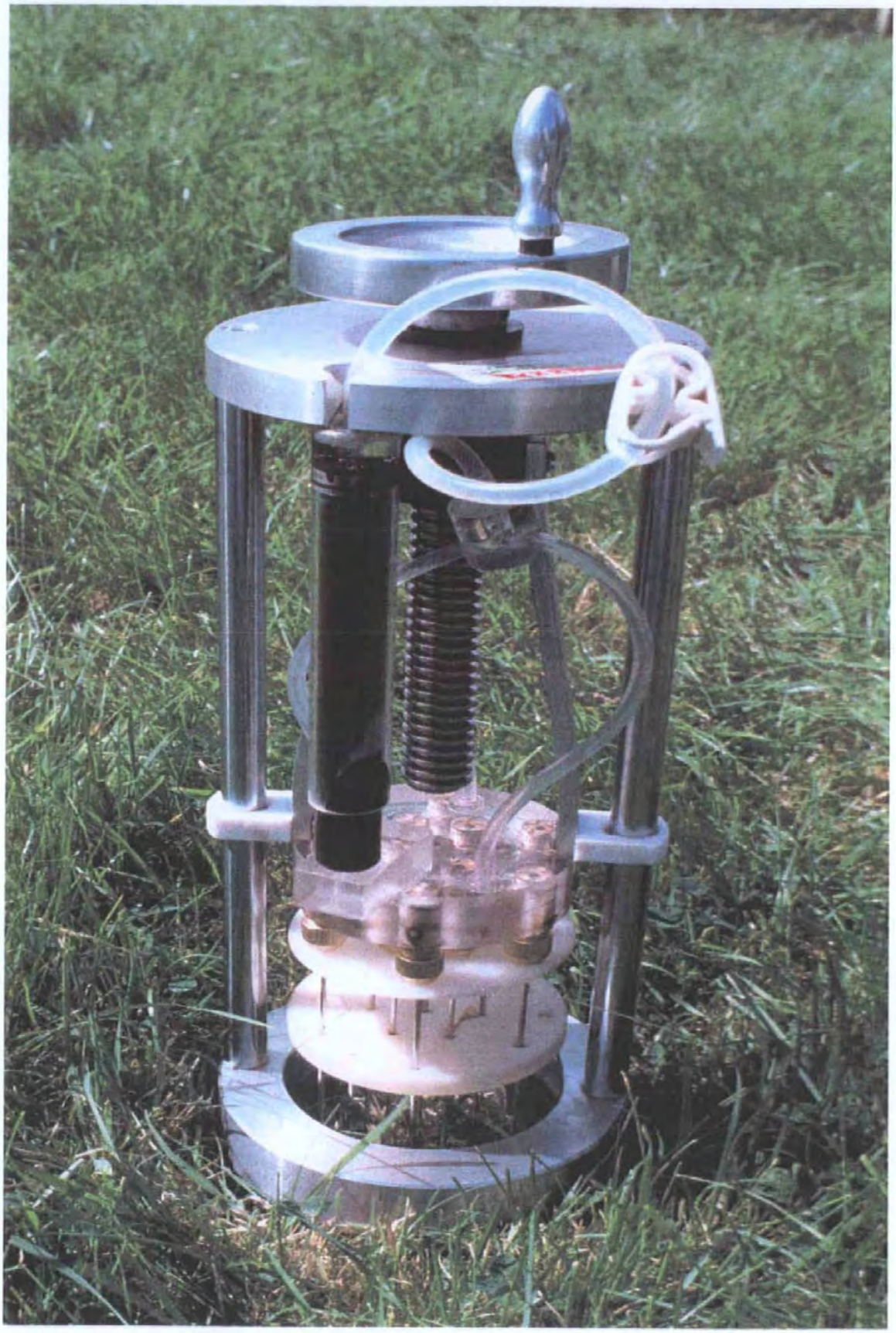
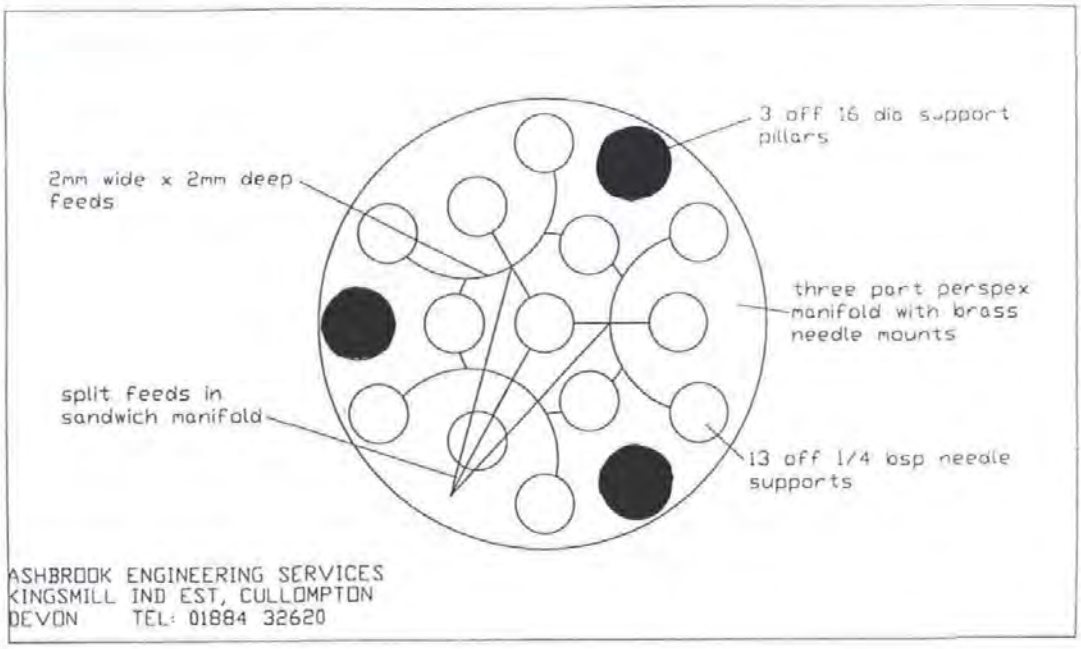


Plate 6.1 ^{15}N soil injector

a)



b)

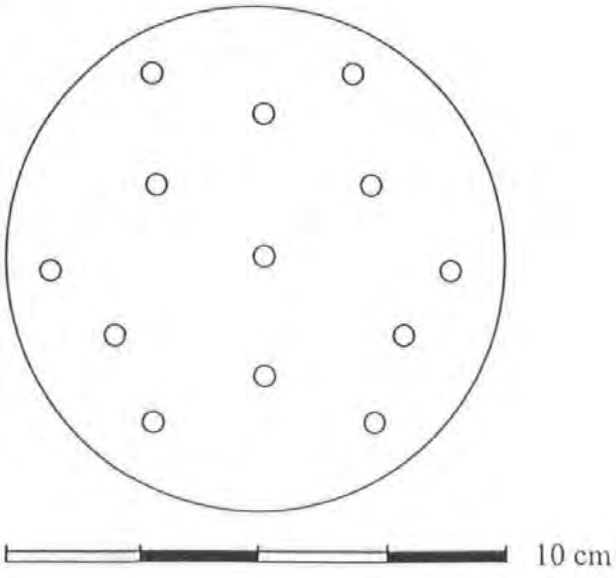


Figure 6.1 a) Computer-aided design/manufacture (CAD/CAM) drawing of the perspex manifold from the ^{15}N soil injector

b) Template showing location of hypodermic needles in the manifold and identical pattern used for polypropylene templates: (i) soil surface template with spiked feet (TA), (ii) thicker template used for making perpendicular holes (TB), (iii) needle-guide template (TC)

6.2.1 Injection of ^{15}N

A polyethylene (100 mm diameter, 10 mm deep) template (TA), which fitted closely inside the microplot, was pressed on to the surface of the soil and kept in place by three spiked feet. It contained 13 countersunk holes in a pattern which was designed to distribute isotope evenly throughout the microplot (Fig. 6.1b). A second polyethylene (100 mm diameter, 40 mm deep) template (TB) was placed on top of TA and contained the same pattern of holes, which were aligned with TA using two metal spikes (one through the centre hole and the other through a peripheral hole) inserted into the soil. A wooden handled spike (190 mm) was used to make guide holes in the soil to a depth of 150 mm via both templates. The thicker template (TB) ensured that the guide holes were perpendicular to the soil surface in order to avoid bending the needles (n.b. this technique is most suited to investigations confined to the upper organic layer of soils where the major proportion of microbial activity occurs and in soil types which contain few stones). The guide spikes and TB were then removed and the injector positioned over the microplot. The needle cluster was aligned with TA by means of a needle-guide template (TC, Fig. 6.2) which could be positioned near to the needle points to enable them to be aligned with the countersunk holes on TA. The needles were then gently introduced into the soil, taking care to present the whole needle cluster evenly, until the base plate of the injector was seated on top of the microplot rim. By rotating the handle winder, the needles were withdrawn steadily through the soil profile whilst the plunger was advanced into the syringe barrel to force liquid (40 mls) from the manifold.

6.3 Experimental details

The experimental layout adopted in Section 4.1 was repeated, but for this experiment nine

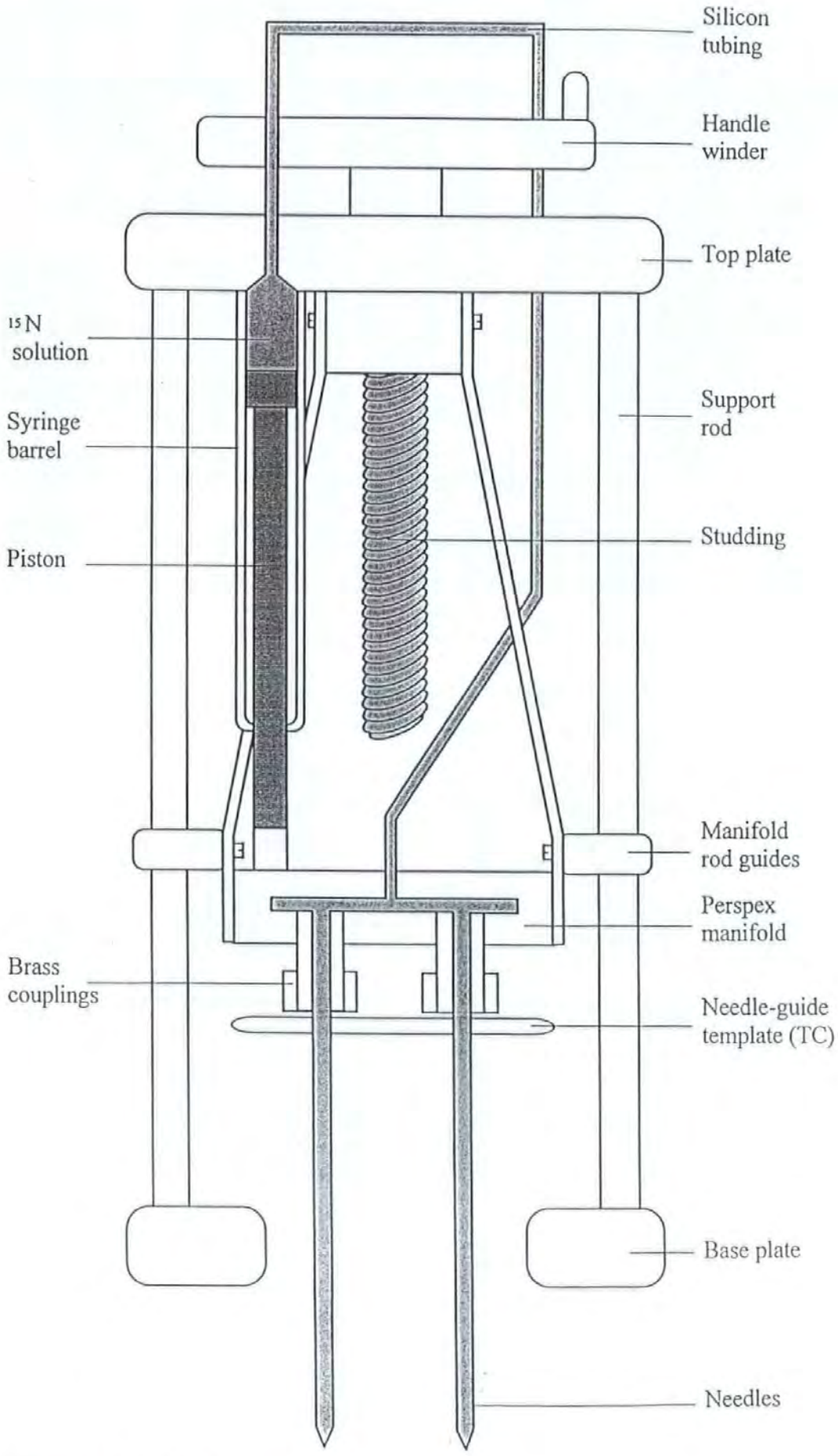


Figure 6.2 ^{15}N Soil injector

individual plots (30 m^2) were re-sited to provide new sets of three replicates for each of the three base treatments. As before, the treatments consisted of long-term (>15 years) grazed pastures which were drained by a system of field and mole drains (see Section 3.2). During the grazing season of 1997, animals were excluded from these plots and the swards were maintained in a 3-cut silage system. The three base treatments were: fertilized grass swards receiving 200 kg N ha^{-1} y^{-1} (*F*); unfertilized swards containing a mixture of clover (approximately 10% by weight) and grass (*C*); unfertilized grass only swards (*Z*). In April 1997, all the plots received P and K (25 and 50 kg ha^{-1}, respectively) and the *F* plots also received 40 kg N ha^{-1}. The nine plots were cut in June and again in July followed by fertilizer applications of 60 kg N ha^{-1} to the *F* plots on both occasions. Thus, by the start of the measurement period, the *F* plots had received a total of 160 kg N ha^{-1}, i.e. 80% of the intended annual application.

One week before commencement of measurements (6 August), 54 microplots were inserted in these swards (6 microplots to each of 3 replicate plots x 3 treatments). The microplots were left for one week for the swards and soils to recover from any disturbance and then on 13 August (t0), herbage from within the microplots was cut level with the rim (25 mm above the soil surface). Each microplot was injected uniformly with a ^{15}N solution throughout the soil core profile, using the new soil injector. In each of the base treatments, 12 replicate microplots received a solution containing 25 At. % $^{15}NH_4^+$ and the other 6 microplots received 20 At. % $^{15}NO_3^-$ to follow the rates of gross mineralization and nitrification, respectively. After 24 h equilibration (14 August), a soil core was removed (t1) from the centre of each microplot, using an incubation tube and cutting ring, as described in Section 2.3. The soil remaining in the microplot, which consisted of a

concentric ring of soil with a hollow centre, was removed from the microplot and the inorganic N extracted within 1 h using 2 *M* KCl. The incubation tube, containing a soil core with the same enrichment as the t1 soil, was placed in an incubation vessel in the field for a further 96 h incubation (without *phytostats* to allow normal plant uptake processes to proceed during the measurement period). Half of the microplots which had received $^{15}NH_4^+$ were incubated over gypsum blocks without controlled-release capsules (no inhibitor, A-data set) and the other half (plus inhibitor, I-data set) were incubated with capsules to provide an infusion of C_2H_2 into the soil core (see Section 2.3). The microplots receiving $^{15}NO_3^-$ were also incubated without C_2H_2 (N-data set). As there was little likelihood of leaching from the soil at that time of year (SWC had fallen below field capacity), I-E resin bags were omitted from the incubation vessels and specially constructed gypsum blocks (25 mm height) occupied fully the space beneath the soil cores. On 16 August, the incubation tubes with controlled release capsules (plus inhibitor, I-data set) were removed briefly to enable the gypsum blocks to be re-wetted (10 mls water), as soil conditions were particularly dry. This ensured that the reaction with CaC_2 proceeded satisfactorily so that the infusion of C_2H_2 was maintained.

At the end of the incubation period (18 August), the soil cores were removed (t5) and analysed within 1 h (as above). Over the experimental period (13-18 August), the mean maximum and minimum temperatures were 23.4 and 12.7 °C, respectively. Soil temperature at 100 mm depth averaged 17.3 °C, with a total of 49.2 hours of sunshine. Apart from precipitation on 12 August, when 3.9 mm rain was recorded, the experimental period remained dry and warm during a prolonged spell of settled weather conditions.

6.3.1 Testing uniformity of soil ^{15}N injection

An additional 8 microplots were used to test the uniformity of ^{15}N injection: four received 25 At. % $^{15}NH_4^+$ and four received 20 At. % $^{15}NO_3^-$, as described previously. After 24 h equilibration period (t1, 14 August), cores were removed from the centres of the microplots, using incubation tubes. The soil from the tubes, together with the soil from the associated outer cores, were extracted immediately with KCl to compare the uniformity of enrichments achieved by ^{15}N injection at the starting point of an incubation period, i.e. 'start values' from the outer core and the corresponding 'start values' for soil cores undergoing a period of incubation.

6.4 Soil/plant processing

On each day of sampling (t1 and t5), soil was removed from microplots and incubation tubes and passed through a 6 mm mesh sieve. Stones were discarded, but all plant material (shoots plus roots) which was retained on the sieve was carefully removed, washed clean of soil over a 200 µm mesh sieve and oven dried at 105 °C. The dried plant material was then weighed and ground to a fine consistency using a succession of grinders: hammer mill (JKA, Model A20) and ball mill (Glen Creston). Soil sub-samples (50 g fresh) were extracted with 250 ml of 2 *M* KCl for analysis of NH_4^+ and NO_3^- (Scalar autoanalyser) and the extracts were frozen on the day of collection (t1 and t5). Other soil samples (100 g fresh) were used to assess the gravimetric water content, or extracted (12 g fresh) in 0.5 *M* K_2SO_4 for analysis of the *SMB* (as described in Section 5.2.2).

6.5 Diffusion of ^{15}N labelled solutions

The inorganic N enriched with ^{15}N was recovered from soil extracts using a diffusion

technique described by Stark and Hart (1996). A VG 602E isotope ratio mass spectrometer (see Fig. 6.3), linked to a Carlo Erba C N analyser and controlled by Europa ANCA software/interface, was used to measure the ^{15}N enrichment of the soil extracts and required at least 100 μg N to ensure a robust signal. Sufficient KCl extract to contain either 140 or 200 μg N (initial tests suggested recoveries of 70% and 50% for NH_4^+ and NO_3^- contents, respectively) were placed into 250 ml wide-neck polyethlene bottles. A pair of acidified traps was added to each bottle, consisting of two discs of filter paper (Whatman No. 1, 7 mm diameter), each treated with 5 μl 2.5 *M* $KHSO_4$. The pair of discs were sealed in a 70 mm strip (4 mm apart) of 12.5 mm wide PTFE pipe sealing tape (Teflon) using an 11 mm diameter plastic culture tube to press a concentric circle around each disc. In the case of $^{15}NH_4^+$ labelled N, the extracts were diffused for 6 d at 25 °C with 0.3 g of MgO, whereas $^{15}NO_3^-$ labelled N was first diffused for 7 d in open bottles (without lids) which were swirled daily to drive off NH_3. After this time, water was added to replace evaporative losses and an extra scoop (0.3g) of MgO plus 0.5 g of Devarda's alloy was added to the extract; the bottles were sealed tightly with a screw lid and left for a further 6 d to diffuse. Devarda's alloy releases H_2, in addition to the NH_3 liberated by MgO and leakage from the screw cap was avoided by inverting the bottles during the diffusion process so that the gases were entrapped in the closed end of the bottles. Bottles which do leak can be identified readily by seepage of liquid and therefore discounted. Soil microbial biomass (*SMB*) was extracted from the soils by the fumigation-extraction (F-E) method (Brookes *et al.*, 1985; Vance *et al.*, 1987). The extracts were used to determine biomass C, by dichromate oxidation with back-titration against ammonium ferrous sulphate, and biomass N by Kjeldahl digestion, followed by flow injection analysis.

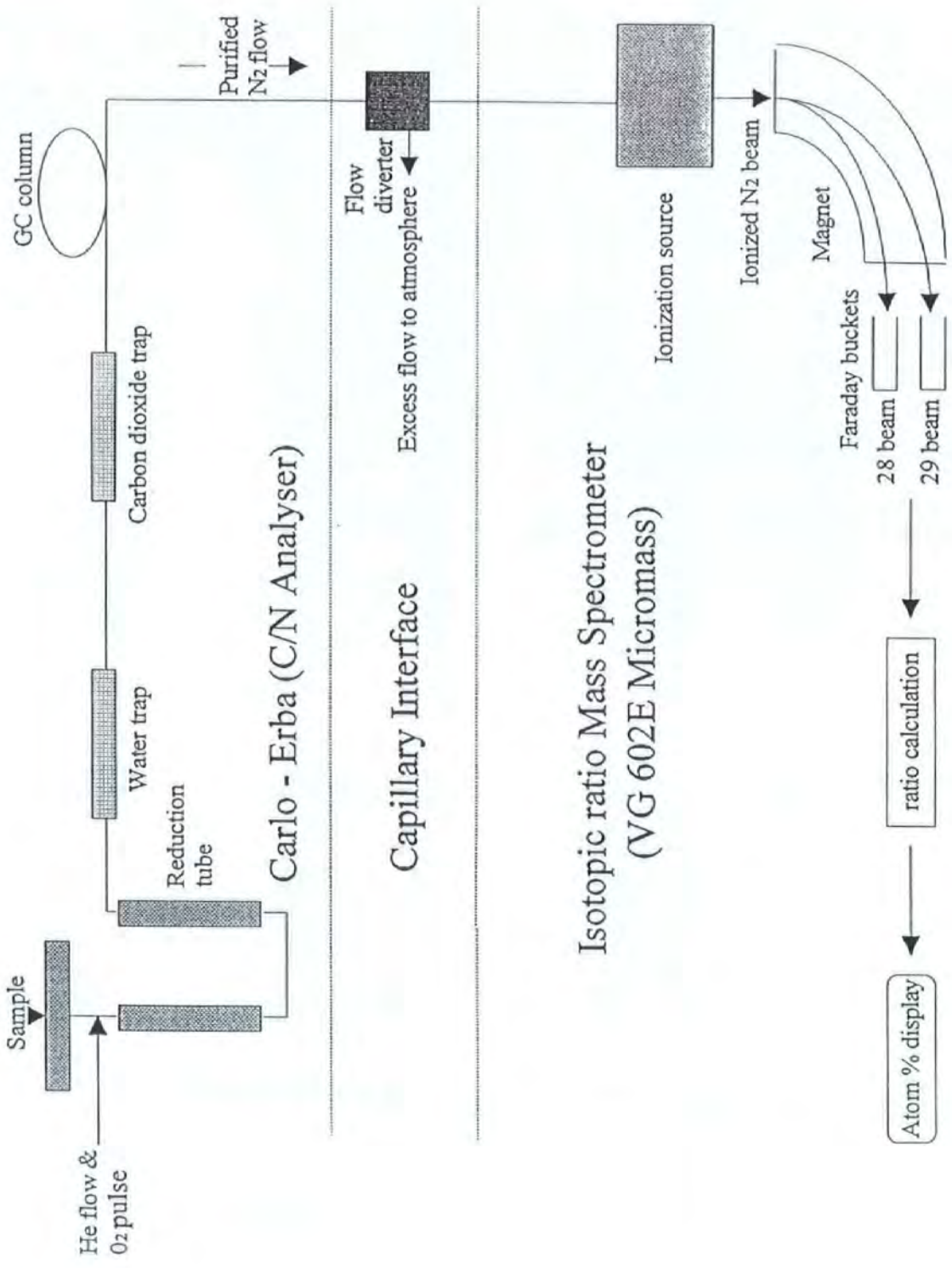


Figure 6.3 Schematic diagram showing a C N analyser linked to an isotope ratio mass spectrometer.

Biomass C and N were calculated using extraction correction factors of 2.64 and 2.22, respectively (see Section 5.2.2). Analysis of the Kjeldahl digests (containing 140 μg N) for ^{15}N enrichment used the same procedure as for KCl extracts, but in this case the solutions in the diffusion bottles were made sufficiently alkaline (pH >11.0) to liberate $^{15}NH_3$, by the addition of 10 *M* NaOH, instead of MgO.

After diffusion, the PTFE strips were recovered, rinsed in deionised water and the top layer of PTFE was peeled back with forceps to expose the filter paper discs. Each pair of discs was impaled on a single stainless steel pin and dried in a desiccator over concentrated H_2SO_4 for at least 4 h. The pairs of dried discs were placed together in one tin capsule (8 x 5 mm) and analysed for ^{15}N enrichment by mass spectrometry. The two discs provided a safeguard against failure or incomplete diffusion by one of the discs, since the other would still have had sufficient capacity to absorb all of the labelled N source.

6.5.1 Standards and controls

Additional extracts (75 ml) of 2 *M* KCl containing 100 μg N (as $^{15}NH_4^+$) were placed in bottles with acid traps (diffused standards) covering a range of standards: 0, 2.5, 5.0, 7.5 and 10.0 At. % ^{15}N (see Appendix 1 for calculations). Other acid traps were first 'spiked' with ^{15}N in the same range and analysed in tin capsules (non-diffused standards). The bottles were incubated, as above, with 0.3 g MgO. Blank-correction of the samples was made using the standard controls to determine the extent of contamination, from the decline in enrichments between non-diffused and diffused standards. The effectiveness of the first stage of the diffusion process was also examined (i.e. liberation of all $^{15}NH_4^+$), by adding 100 μg N (as 5 At. % $^{15}NH_4^+$) to four replicate bottles, plus 100 μg N (as 0.3663

At. % $^{15}NO_3^-$ *viz.* natural abundance). Four further replicates received only 100 μg N as 0.3663 At. % $^{15}NO_3^-$. The first set was diffused with MgO (in bottles vented to remove NH_3) and then both sets (8 bottles) were diffused again, this time with Devarda's alloy (+ extra MgO). Acid traps were only added at the second stage to capture any labelled N souces remaining in the bottles.

6.6 Other measurements

C-mineralization was estimated from the amount of CO_2 respired from field incubated soil cores according to a modified technique described by Gilmour *et al.* (1985). At t1, pairs of fresh soil cores (25 x 150 mm) were removed from each of the three replicate plots and each core was placed in an individual 1l Kilner jar (6 replicate jars per treatment) containing a 70 ml serum bottle with 25 ml 0.5 *M* NaOH. Six other jars without soil, but containing alkali were included as controls. The jars were placed in holes in the ground for 96 h, after which the bottle of alkali was removed and stoppered (t5). The amount of CO_2 respired was calculated after addition of 10% $BaCl_2$ and back titration of the excess alkali against 0.1 *M* HCl (see Appendix 2).

Net N-mineralization was estimated using the technique described by Hatch *et al.*,1990. Four soil cores (37 mm diameter) were analysed from each replicate plot for NH_4^+-N content at the start (t0) of the measurement period (see Section 4.2.1). Four additional cores were taken from the same plots and placed in 1l Kilner jars (3 replicate jars to each treatment) with 2% C_2H_2 in the headspace for an incubation lasting 7 d. At t7, the cores were removed and analysed, as for the t0 cores. Net mineralization was calculated from the difference in NH_4^+-N contents between the 'start' and incubated cores (t7 - t0) and

expressed as a net rate per day.

6.7 Results and discussion

Each needle in the ^{15}N soil injector delivered on average 2.99 ml with a precision of ± 0.275 ml, which was estimated to have increased the soil water content of the microplots by not more than 5% (dry weight soil basis). In the test microplots, no significant differences were found between the inorganic N pool sizes of either NH_4^+ or NO_3^-, nor in their ^{15}N enrichments (24 h after injection, t1), when the inner soil cores (incubation tubes) were compared with the associated outer cores (extracted on the same day, t1). Thus, the comparison between cores extracted at t1 and those incubated until t5 was valid in terms of the uniformity of initial ^{15}N enrichments. Diffusion effficiency was subjected to the severest test under laboratory conditions (see Section 6.5.1), since any carry-over of labelled $^{15}NH_4^+$ would be readily identified as an elevated enrichment in the NO_3^- component (added at natural abundance) which was also reduced to NH_4^+ before diffusion. The mean enrichment (At. % ^{15}N) of controls without ^{15}N addition was 0.389 ± 0.0016, compared with 0.378 ± 0.0009 in the controls to which $^{15}NH_4^+$ was added. The difference was significant (P <0.001) and probably arose through contamination (^{14}N) in the extra MgO used in the venting stage, which would have the effect of diluting the ^{15}N natural abundance ratio. Since there was no evidence, therefore of an elevated enrichment attributable to carry-over of $^{15}NH_4^+$, it was concluded that the diffusion stage was effective in distinguishing between the two forms of inorganic N. Similarly, where other amendments were equal (*viz.* MgO and Devarda's alloy) the apparent contamination was insignificant, so that the correlation between standards (diffused vs non diffused) was extremely close (r^2 = 0.9994) with an intercept for the fitted line of 0.3772 At. % ^{15}N,

which is close to natural abundance. Standards (diffused and non diffused) were included in all subsequent analytical runs to estimate the extent of any contamination. However, no correction for blank contamination was found to be necessary with enrichments which greatly exceeded natural abundance, or in sample runs where close correlations were established between diffused and non diffused standards (i.e. with uniformly low levels of contamination). The calculation required for blank-correction of low enrichment samples (Stark and Hart, 1996) is, nevertheless, reproduced in Appendix 3 for reference.

In the experimental microplots, prior to injection of ^{15}N (t0), the inorganic N contents of the soils were about 5 μg NH_4^+-N g^{-1} soil in both *F* and *C* treatments (less in *Z*) and also of NO_3^- in *F*, but with approximately one-tenth of these concentrations (0.5 μg N g^{-1} soil) of NO_3^- in *C* and *Z* treatments (Table 6.1).

Table 6.1 Inorganic N contents (μg N g^{-1} dry soil ±SEM) of NH_4^+-N pool (A,I) or NO_3^--N pool (N), before injection of 20 μg N g^{-1} dry soil (t0) and at 24 h (t1) and 96 h (t5) after injection (n = 6)

Treatments (N pool)	t0	t1	t5
FA (NH_4^+-N)	5.9 (0.52)	26.2 (1.78)	34.7 (5.06)
FN (NO_3^--N)	5.9 (2.44)	41.2 (9.70)	43.4 (9.14)
FI (NH_4^+-N)	5.9 (0.52)	28.6 (2.66)	35.3 (2.73)
CA (NH_4^+-N)	4.5 (0.31)	24.1 (1.58)	21.8 (2.11)
CN (NO_3^--N)	0.5 (0.15)	28.2 (2.20)	26.5 (2.13)
CI (NH_4^+-N)	4.5 (0.31)	26.7 (2.21)	32.3 (2.53)
ZA (NH_4^+-N)	3.7 (0.44)	24.4 (2.21)	36.7 (8.92)
ZN (NO_3^--N)	0.3 (0.07)	21.2 (0.58)	22.8 (1.26)
ZI (NH_4^+-N)	3.7 (0.44)	20.6 (1.04)	26.7 (5.65)

At t1, following injection of ^{15}N, all treatments increased ($P < 0.001$) by approximately 20 μg N g^{-1} soil, as intended (the exception was *FN*, which probably reflects greater heterogeneity due to previous N inputs) and these concentrations persisted until t5 (Table 6.1). The ^{15}N enrichments of the soils at t1 were broadly similar across all treatments (range 10.8-16.0, Table 6.2), but had declined by about one-third at t5. Consequently, of the total ^{15}N recovered from all treatments at t5 (Table 6.2), the soil retained the major component (22-61%) with more variable amounts recovered in the *SMB* (0-23%); the minor component was in plant material (3-11%). Of the ^{15}N added, therefore, on average 68%, 56% and 72% in total was recovered at t5 in *F*, *C* and *Z* treatments, respectively. These values are in line with total recoveries of added ^{15}N in other studies (Bristow *et al.*, 1987; Schimel *et al.*, 1989), but are at variance in terms of the dominance of the *SMB* competition over plant biomass. Plant biomass was found to be the more important sink for added N by Bristow *et al.* (1987) and Ledgard *et al.* (1998). However, both the present study and other workers (Jackson *et al.*, 1989) found microbial uptake to be the dominant factor in removal of added N, and Hart *et al.* (1993) also found similar recoveries of added ^{15}N in the *SMB*, which ranged from 9-15%. Recovery of ^{15}N by plants (shoots + roots) was similar in all three treatments and increased by about 50% between t1 and t5 (Table 6.2). Recoveries of ^{15}N in the *SMB* were far more variable and in some treatments declined with time, illustrating the transient nature of recently acquired microbial N (Bristow *et al.*, 1987). However, potential sources of error by initial, rapid abiotic reactions (Davidson *et al.*, 1991) were largely avoided by delaying the first sampling for an equilibration period of 24 h, following ^{15}N injection. The competitive success of the *SMB* for available N could represent a major control over the availablity of N for plant uptake and will be discussed in more detail later.

Table 6.2 Mean (n = 6) percentage recovery of ^{15}N from the NH_4^+ pool (A-and I-data sets) and the NO_3^- pool (N-data sets)

Treat.	At.%^{15}N soil (inorganic)		%rec.^{15}N soil (inorganic)		%rec.^{15}N (*SMB*)		%rec.^{15}N (plant)		Total % recovery	
	t1	t5	t1	t5	t1	t5	t1	t5	t1	t5
FA	14.49	9.02	64.0	50.3	5.2	11.7	2.7	4.8	72	67
FN	10.79	9.41	64.6	61.1	0.3	0	1.4	3.0	66	64
FI	15.07	8.81	71.5	51.9	5.2	15.2	2.9	4.8	80	72
CA	14.86	6.49	60.1	22.3	18.3	10.8	5.0	7.8	83	41
CN	13.46	11.24	63.2	49.6	12.6	0	3.9	8.8	80	58
CI	14.49	8.50	64.8	44.9	25.1	18.7	3.4	5.2	93	69
ZA	13.73	8.55	55.7	54.4	15.8	19.5	5.1	7.9	77	82
ZN	16.02	13.44	57.2	51.3	0.1	0	5.1	7.3	62	59
ZI	13.86	8.98	48.1	42.2	45.1	22.8	5.7	10.9	99	76

Process rates (expressed on a per day basis) were calculated from the rates of dilution, or removal of the ^{15}N labelled NH_4^+ and NO_3^- between days 1 and 5 (t5 - t1), following the equations (see Appendix 4) of Kirkham and Bartholomew (1954). Although the lowest values for gross mineralization were found with the *Z* treatments (Table 6.3), no significant differences were found between the treatments. Conversely, the lowest immobilization of NH_4^+ was found with the *F* treatments and this trend was repeated in the immobilization values found in the chloroform-labile pool of the *SMB*, but the data were highly variable and no significant differences were established. It was interesting to note, however, that within each treatment the presence of the nitrification inhibitor (C_2H_2) increased the rate of immobilization of $^{15}NH_4^+$ into the *SMB* by 30%, 48% and 9% for *F*, *C* and *Z* (I-data sets), respectively. This suggests that the treatments with the highest nitrifying activities (see Table 6.4) were most affected by inhibition.

Table 6.3 Gross rates of processes (±SEM) obtained from ^{15}N mean pool dilution data (μg N g^{-1} d^{-1}) n = 6

Treatment	Gross mineralization	Ammonium consumption	Gross nitrification	#Ammonium immobilization	*SMB* immob.
FA	4.1 (1.41)	2.8 (1.26)	1.6 (0.45)	1.2	3.0
FI	4.2 (0.73)	2.5 (0.79)	"	0.9	3.9
CA	5.4 (1.14)	6.0 (1.21)	1.2 (0.33)	4.8	3.3
CI	4.1 (0.63)	2.7 (0.48)	"	1.5	4.9
ZA	3.6 (0.55)	4.0 (0.54)	1.0 (0.06)	3.0	5.5
ZI	2.5 (0.65)	4.3 (1.06)	"	3.3	6.0

#Calculated from Davidson *et al.*, 1991: (NH_4^+ consumption - gross nitrification = NH_4^+ immobilization) **where** NH_4^+ consumption includes the sum of immobilization (i.e. microbial assimilation), autotrophic nitrification, volatilization and other possible fates.

Gross rates of mineralization and NH_4^+ consumption (Table 6.3) were similar between all treatments and close to the soil inorganic pool concentrations prior to injection (Table 6.1). Thus, inherently low inorganic N levels would be expected to be maintained in the 'steady state', unless amended by fertilizer inputs. A useful indication of the relative turnover rates in these treatments can be obtained from calculations of **pseudo-residence times**, defined as "the ratio of the NH_4^+-N pool size : NH_4^+-N consumption rate" (Tietema and Wessel, 1992). Ratios of 2.1, 0.8 and 0.9 (NH_4^+-N pool) and 3.9, 0.3 and 0.4 (NO_3^--N pool) were found with *F*, *C* and *Z* treatments, respectively, representing the number of times the pool size turned over each day. Therefore, turnover of N-pools was faster in the N-limited swards (*C* and *Z*), with the NO_3^--N pools turning over twice as fast as the NH_4^+-N pools. An explanation might be that plants experiencing only a limited mobile N supply, become effective 'scavengers' of sources of available N (Parsons *et al.*, 1991). In the fertilized swards (*F*), the turnover of the NH_4^+-N pool was on a 2-day cycle, whereas the NO_3^--N pool took twice this time to be processed. It is clear then, that high fluxes and a rapid

turnover of the inorganic N pools are features of these grazed grassland soils, as was previously demonstrated by Ledgard *et al.* (1998).

Table 6.4 Nitrification and consumption of nitrate ($\mu g\ N\ g^{-1}\ d^{-1}$ ±SEM) n = 6

Treatment	Nitrification (gross)	Nitrification (Tube)	Nitrate consumption
F	1.6 (0.45)	0.3 (1.47)	1.5 (0.42)
C	1.2 (0.33)	2.6 (1.00)	1.6 (0.47)
Z	1.0 (0.06)	-2.5 (2.32)	0.8 (0.16)

Gross nitrification was also closely matched with NO_3^- consumption (Table 6.4) so that, again, NO_3^- would be unlikely to accumulate unless supplemented by fertilizer. The highest rate of nitrification (gross) was found with *F* compared with *Z* treatments, but this was only at a low level of significance ($P = 0.09$) and with *C* intermediate (Table 6.4). Measurements of net nitrification (by tube incubation) were not consistent with other estimates (Table 6.4) and may have been adversely affected by additions of N (as $^{15}NH_4^+$), since the assessment relied on differences in NH_4^+ produced in the presence and absence of a nitrification inhibitor (C_2H_2). Nitrification rates are dependent upon the supply of substrate (NH_4^+) usually from the mineralization of soil O.M. (i.e. gross mineralization). In the present study, 39, 25 and 33% of the NH_4^+ mineralized was nitrified (based on gross nitrification rates) in *F*, *C* and *Z* treatments, respectively, compared with 72% found with the same soil type in the previous autumn/winter experiment (see Section 5.3.2). Similar rates (12 - 46%) were measured during the growing season of annual grasses in California (Davidson *et al.*, 1990). Thus, the oxidizing capacity of the nitrifiers was exceeded in the summer conditions of the present study, which suggests that the Q_{10} of nitrifiers must be

somewhat lower than that of heterotrophs involved in O.M. decomposition.

Combining the tube incubation technique with the ^{15}N technique enabled simultaneous assessments of gross and net rates. Net rates of mineralization (Table 6.5) obtained with tube, jar, or ^{15}N-calculated rates were in reasonable agreement (particularly with *F* and *C* treatments) and approximately half the gross rates found for each of the treatments (Table 6.3). Estimates of net mineralization in the *C* treatment were intermediate between *F* and *Z*, but there were no significant differences overall between treatments. The lowest microbial activity, as measured by CO_2 respiration (Table 6.5), also appeared to be in *Z*, but again was not significantly different from the other treatments (it should be noted that the simplified method, using intact soil cores, also included respiration by living roots).

Table 6.5 Net mineralization of nitrogen (μg N g^{-1} d^{-1} ±SEM) estimated by three different techniques, and carbon (μg C g^{-1} d^{-1}) measured by CO_2 evolved during jar incubation

Treatment	#Net N min. (calculated)	Net N min. (Tube: n=6)	Net N min. (Jar: n=3)	Net C min. (Jar: n=6)
F	2.9	2.3 (0.66)	1.9 (0.32)	70.5 (6.73)
C	0.6	2.1 (0.84)	1.8 (0.13)	76.0 (5.62)
Z	0.6	0.6 (1.80)	1.3 (0.28)	59.9 (5.28)

#Calculated from the difference between gross min. and NH_4^+ immobilization (using A-data sets)

Additionally, laboratory incubations (Table 6.6) supported the finding that microbial activity, as assessed by specific respiration, was similar in all treatments. In terms of biomass size, both biomass C and N were similar in treatments *F* and *Z* (long-term undisturbed for >50 years), but lower in *C* ($P < 0.05$), which had been ploughed and reseeded in 1982 and had, therefore, not yet reached a new equilibrium.

Plant N uptake data (calculated from ^{15}N recoveries) were very variable (cv = 60.3%), with only low rates recorded which averaged 0.21, 0.44 and 0.51 kg N ha^{-1} d^{-1} for *F*, *C* and *Z*, respectively. This is in the reverse order of what might have been predicted from the known productivity of these swards, but may have been an anomaly of recently cut plants and a rapid response to applied N in those swards containing N-starved plants (viz. *C* and *Z*), compared with the lower uptake ($P < 0.01$) from higher N-status plants (*F*). These results are, therefore to be considered with some reservations and may not be wholly representative of the swards in equilibrium with the management regimes. There was no effect of either the form of N (NH_4^+ or NO_3^-) or inhibitor (C_2H_2) on the relative uptake rates within (A-, N-, or I-data sets) or between treatments (*F*, *C* or *Z*).

Table 6.6 Soil microbial biomass N and C (µg g^{-1} dry soil) and specific respiration (µg CO_2-C g^{-1} dry soil) n = 6

Treatment	Biomass N		Biomass C		Specific
	t1	t5	t1	t5	respiration
F	278.6[a]	294.9[a]	2025[a]	2024[a]	1.39[a]
C	242.3[a]	244.3[b]	1415[b]	1459[b]	1.14[a]
Z	282.9[a]	292.3[a]	1943[a]	1989[a]	1.13[a]

Values which have different letters within columns are significantly different ($P < 0.05$)

Soil microbial biomass (*SMB*) data for immobilization were also very variable (cv = 93.1%), with rates which were lowest in *F* and highest in *Z* and with *C* intermediate, but the differences were not significant (Table 6.3). Some caution must be exercised in the interpretation of immobilization from the rate of appearance of the label in the microbial biomass. Whilst this approach may seem more direct than calculations based on the disappearance of the label from the inorganic N pool (see Appendix 4, equation 2),

questions have been raised concerning uncertainties with the measurement of *SMB* by chloroform fumigation (Voroney and Paul, 1984; Vance *et al.*,1987; Davidson *et al.*, 1989). No adjustment was made to allow for the fraction of microbial biomass N extracted after exposure to chloroform (K_N factor), since an appropriate value for K_N has not yet been widely adopted. An alternative method (see Table 6.3) of calculating ammonium immobilization and net mineralization (by difference from gross mineralization, see Table 6.5) showed that differences in net mineralization are largely determined by rates of immobilization (Fig. 6.4).

The effect of nitrification on rates of N cycling processes was examined in swards with different nitrifying activities and compared with rates measured in the presence of a nitrification inhibitor. Where nitrification was <u>not</u> inhibited (Fig. 6.4a), the lowest rates in net mineralization were associated with soils from the unfertilized swards (*C* and *Z*). Ledgard *et al.* (1998) also found that immobilization was greatest in unfertilized swards, which in turn determined the ultimate differences found in net mineralization rates. Where nitrification <u>was</u> inhibited (Fig. 6.4b), net mineralizaion was not significantly different between the three soils (*F*,*C* and *Z*). The calculated rates for net mineralization ($\mu g\ N\ g^{-1}\ d^{-1}$) shown in Fig. 6.4b were 3.3 (±0.92), 2.6 (±0.97) and -0.8 (±0.97), respectively and were in closer agreement overall (for *F* and *C*) with estimates by 'tube' and 'jar' incubation, than those based on calculations without the inhibitor (Table 6.5). When nitrification is allowed to proceed naturally, other losses may occur (see Section 3.4) which may cause an under-estimate in measured rates. In the present study, the effect of inhibitor (I-data set) was to produce higher values for immobilization of ^{15}N in the *SMB* in all treatments, suggesting that NH_4^+ uptake may have been enhanced when this form predominated.

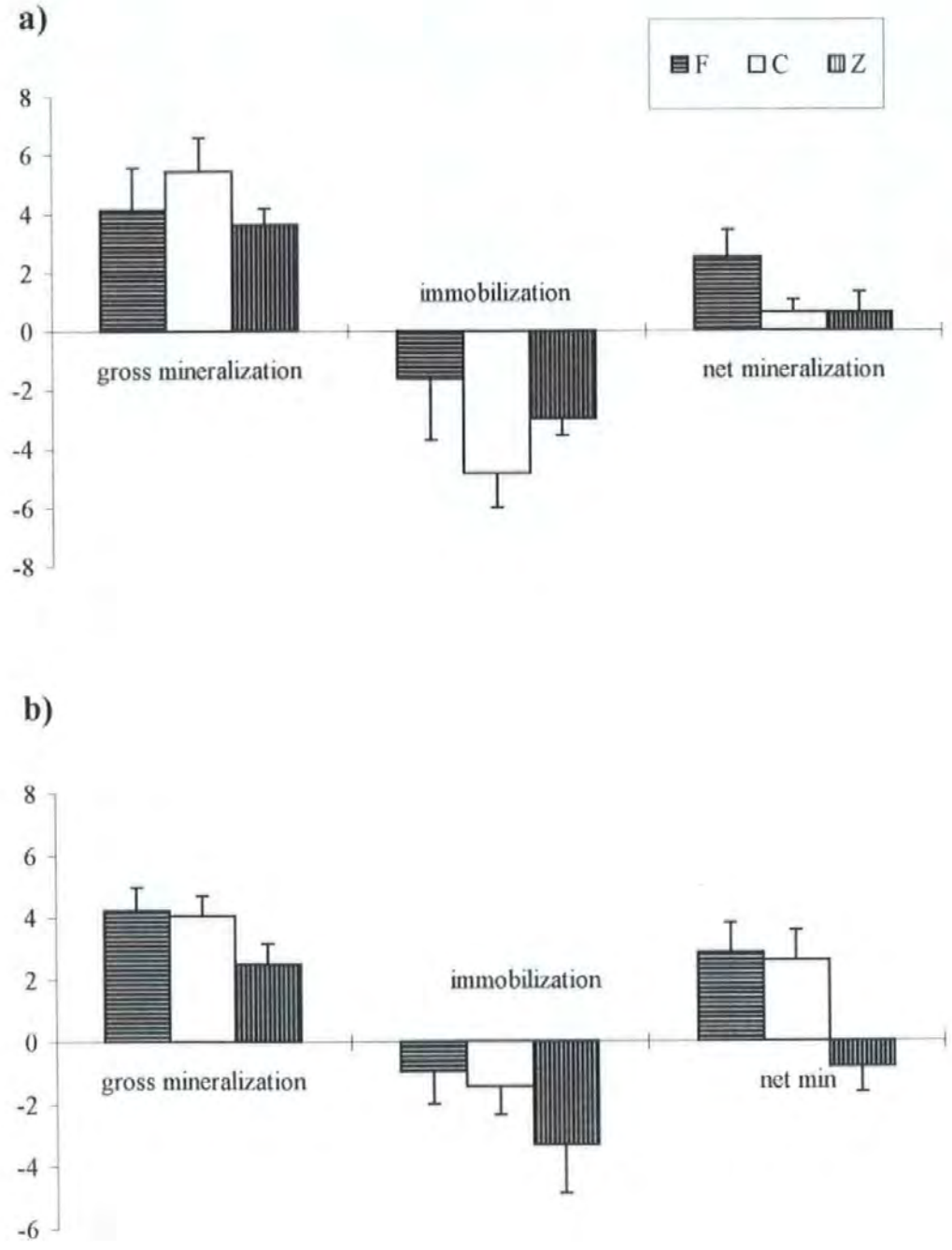


Figure 6.4 Gross mineralization, NH_4^+ immobilization and net mineralization (by difference) from ^{15}N data (μg N g^{-1} d^{-1}) for fertilized (*F*), grass/clover (*C*) and unfertilized (*Z*) swards: **a)** A-data set **b)** I-data set (+ nitrification inhibitor)

6.7.1 Summary

The multi-point soil injector proved to be very effective at delivering ^{15}N evenly throughout the soil profile under investigation, so that accurate measurements of process rates could be obtained. Future use of the apparatus will probably be confined to using separate pairs of microplots (i.e. not concentrically paired), since the combination of tube incubation with ^{15}N techniques was very labour intensive, although justified in terms of verifying techniques. The importance of nitrification to N cycling in grassland soils was established in net terms in the previous chapter and examined in both net and gross terms in this chapter, which provided confirmation of rate measurements. The wider and more subtle role of nitrification in determining the uptake patterns of plant and/or microbial biomass was not resolved completely, in as much as some of the trends were not statistically established and the differences in availability of forms of N were not reflected in plant utilization. These aspects and recommendations for future work, together with an overview of the whole project are discussed in greater detail in Chapter 7.

CHAPTER 7 Overview: project synthesis and conclusions

7.1 Introduction

An overview of the project is discussed in two parts:

1) A synthesis of the data, drawing upon salient points to illustrate the main findings, together with a re-appraisal of the major aims of the project

2) A synopsis of the work, identifying the limitations, implications and priorities for future investigations, culminating in a re-examination of the validity of the hypothesis, which proposed that:

'Nitrification occupies a pivotal role in the N cycle of productive grassland soils by determining the supply of substrate (nitrate) to the two major processes invoved in losses of N, i.e. denitrification and leaching'

7.2 Synthesis of the data

A first step in studying nitrification was to examine soils for differences in their capacity to mineralize soil organic matter and release ammonium, which is the substrate for nitrifying organisms. However, a range of laboratory techniques for evaluating possible differences in mineralization (see Section 4.2.4) and *SMB* size (see Section 5.2.2) and activity (see Section 4.3) in the present soils did not show any differences in potential rates in these processes, even between soils with contrasting management histories. It may well be that such differences can only really be detected in truly dynamic situations under realistic conditions for field evaluation. Thus Gill *et al.* (1995) found that net mineralization was greatest in the fertilized treatments from these soils, but that

withholding N fertilizer did not have any immediate effect on rates of N-turnover. The current study, using the improved field incubation technique, gave similar results (see Section 3.2.2), but also illustrated the difficulties of making measurements in soils with such high spatial variability. In the first phase of the project, improvements in the sampling procedures were devised and introduced (see Section 3.3) to decrease some of the experimental errors; sampling replication was increased to try to overcome some of the variation within treatments. Laboratory assessments were done on bulked samples, derived from mixures of several soil core samples, which helped to decrease the field sampling errors, but this may have stimulated mineralization. Nevertheless, the usefulness of laboratory estimates of potential mineralization as indicators of net rates in the field was limited, since much of the readily degradable organic material is derived from the litter of the current and past seasonal growth; this will not necessarily feature as a source of substrate in laboratory incubation studies. This makes the prediction of N supply particularly difficult. However, a first step towards a predictive tool has come from re-examination of previously published data in an associated study, which has resulted in the identification of some important correlations between soil thermal units (cumulative temperature at 10 cm depth) and net mineralization (Clough *et al.*, 1998).

Aspects of the microbial component of the soils under investigation were examined by standard techniques (see Section 4.3), but appeared to give only incomplete information about the decay process. Lovell *et al.* (1995) found that total biomass was inversely related to net mineralization and Bardgett and Leemans (1995) showed that activity decreased with lower fertilizer inputs, suggesting that much of the biomass may be present in a state of dormancy. Qualitative differences in microbial composition are more difficult

to identify since changes in the activities of the various population components may be only transitory. However, the dominance of fungal over bacterial activity in the fertilized treatment (see Section 4.3.3) is in accordance with the observation that an heterotrophic nitrifying component (resistant to an autotrophic blocking agent) may have been present (see Section 4.4.1). The possibility of an altered balance in the microbial composition of fertilized soils has interesting implications, the investigation of which is probably best undertaken by ^{15}N-labelling techniques in combination with selective metabolic inhibitors. Such investigations, however, need to be at a microbiological scale and were beyond the scope of the present study.

More sensitive assays of nitrifying activities were used to examine the long-term field sites (see Sections 4.4 and 4.5) and clear differences were established between the soils from unfertilized and fertilized grass swards, with soils from the grass/clover swards being intermediate. Given that these differences were shown to have persisted for one year following removal of cattle and fertilizer inputs, elevated potentials for nitrification suggested increases in the potential for losses of N through denitrification and leaching (Hatch *et al.*, 1996). Interestingly, the contrasting fertilizer histories of these soils had not altered the ability of the nitrifiers to oxidize the potentially toxic nitrite ion, as demonstrated by enzyme kinetics assays (see Section 4.5.4). An important insight was provided into the ability of the nitrifying organisms to convert ammonium into the more mobile (and harmless) nitrate ion. 'Nitrification is not necessarily detrimental to ecosystems if it proceeds to the final product (NO_3^-) and may also enhance plant uptake', as will be discussed later.

It may be that the response of mobilization and immobilization turnover (*MIT*) rates to additional inputs from SOM and the effects of environmental fluctuations are extremely transient. In fact, N-supply plays only a supporting role to the processes of *MIT* and it is the availability of readily oxidizable carbon (C) which is often the rate-limiting step, Lovell *et al.* (1996) found that the addition of N to dung (with a C : N ratio of 19.1 : 1) mixed with soil had no effect on either the size, or activity, of the *SMB*. Therefore, even though rates of supply of NH_4^+ from the decomposition of SOM may not apparently differ, other reactions influencing its oxidation to NO_3^- (*viz.* nitrification) are inherently important in determining the fate of N. It was with some justification that Both *et al.* (1992) concluded that "no simple answer can be given to the question as to which of the community (nitrifying) parameters determined in (their) study is most suitable to describe or predict nitrate production in the field." They went on to suggest that "this information can probably best be obtained from intact cores incubated *in situ*."

7.3 Re-appraisal of the main aims

The approach of using field incubated soil cores was adopted in the present study to address the above question posed by Both *et al.* (1992), and to meet the criteria set out in the original aims of the current project which are reproduced here:

a) To **develop** a new technique for the field measurement of nitrification, with simultaneous estimates of losses of nitrate by denitrification and leaching

b) To **link** the *in situ* measurements of nitrification with other processes, to gain an understanding of the controlling factors determining the fate of nitrate

c) To **examine** the practical implications of these findings under different grassland management regimes

Each of the three components above will be reviewed in turn to indicate the extent to which the project has fulfilled the three broad aims. Attention will also be given to the assessment of the implications of the findings and the requirement for future work, where aspects remain unclear or unresolved.

7.3.1 **Development** of a new field incubation technique

The information which was obtained from laboratory-based investigations represented only "snapshots" of particular conditions. The second phase of the project was, therefore, directed towards elucidating the relationships between the process of nitrification *per se* and other transformations of N, using the novel incubation system under field conditions. This new system offers significant improvements over existing methods such as 'jar incubation' (Hatch *et al.*, 1990), 'buried bag' (Eno, 1960) or 'covered tubes' (Raison *et al.*, 1987) and provided simultaneous measurements of the major N cycling processes in a combined approach which had not been attempted previously. The considerable difficulties of measuring changes in mineral N contents from the differences between incubated and non-incubated soil in previously grazed swards (affected by excretal returns) or cut swards (receiving slurry N) had hitherto been avoided assiduously by most other workers. The development of the novel incubation system provided a sampling protocol which enabled closely matching pairs of soil samples to be taken (using concentrically placed soil corers) that overcame many of the problems associated with heterogeneous areas (Hatch *et al.*, 1998). The programme of work included a field incubation study of fertilized/cut grass during spring (see Section 5.2), which was targeted at conditions where potentially high denitrification losses may be coupled to nitrifying activity (Lovell and Hatch, 1997). In autumn 1996 (see Section 5.3), these measurements were repeated on fertilized/cut swards,

following the return of the soil to field capacity, to assess interactions with potential leaching losses. Both experiments showed considerable potential for N loss, particularly through denitrification, and identified possible inaccuracies with some of the existing techniques. In particular, the much higher rates of denitrification found with tube incubation (>3-fold) compared with jar incubation, illustrated how vulnerable well-structured soils can be to disturbance (Hatch *et al.*, 1998) and the likely shortfall in the assessment associated with the latter technique.

The successful development of an effective means of supplying C_2H_2 (a powerful nitrification inhibitor) using controlled-release capsules was another key step in resolving field incubation in open vessels. Whilst C_2H_2 has been used successfully in other studies to assess net mineralization (Hatch *et al.*, 1990; Gill *et al.*, 1995) and denitrification (Ryden *et al.*, 1987), other methods have not utilized the opportunity to resolve several process rates simultaneously by paired incubations ($\pm C_2H_2$). Further, where the scope of investigations can be focused on mineralization (known to be a key process in determining losses, see Blantern, 1991; Jarvis *et al.*, 1996), a simplified version of the tube incubation has been developed (Bhogal *et al.*, in press), which can also be adapted for use in arable soils. The method involves taking account of soil N (before and at the end of the growth period), crop uptake (herbage only), mineralization from soil cores and leaching using ion-exchange resin bags. Possible drawbacks to the use of tube incubation are the need to make contiguous measurements with short intervals between incubations (<14 d) and the difficulties encountered with soil coring in dry or stony soils. Also, the technique does not discriminate between losses of N_2O from denitrification and those attributable to nitrification, although fully recognised as a significant source under certain conditions (see

Section 1.6). The investigation of the origins of N_2O (and NO) was beyond the scope of the present study. For an account of current work on the contributions from these major soil processes, see Jarvis (1997).

7.3.2 Linking *in situ* nitrification measurements with other processes

Close coupling between nitrification and denitrification has been demonstrated in the present study (Hatch *et al.*, 1998) and this has contributed towards our understanding of some of the causes of inefficiency of N use in high input systems. Thus, loss of N from soil O.M. may be initiated by high nitrifying activity which can persist after fertilizer inputs cease (see Section 5.3.2). Also, even though the soil O_2 conditions conducive to these processes are opposite (aerobic and anaerobic, respectively) the opportunity may develop for adjacent soil microsites to provide appropriate conditions due to 'hotspots' of decomposition, which can rapidly consume available O_2 (Tiedje, 1987).

Leaching was measured during an exceptionally dry winter so that meaningful correlations could not be established. However, the soil with the higher nitrifying activity, also lost more N through leaching (see section 5.3.2) so that further investigations of this interaction, under differing conditions of winter drainage, would be warranted. It would seem probable therefore, that the increased supply of N in a more mobile form (NO_3^-) could well lead to increased losses and this has been shown to be the case by other workers (Scholefield *et al.*, 1993).

Whilst validation of the tube incubation technique was provided by comparisons with jar incubation, the known limitations of the latter (isolation of cores from natural drying and

wetting cycles and possible disturbance of aeration and temperature regimes), meant that a more rigorous test was required. In phase three of the project, studies using ^{15}N isotope techniques were introduced to measure gross rates of nitrification and a large part of the study was devoted to the acquisition of the technology and application of the necessary analytical skills (this was facilitated by a British Council funded exchange visit to New Zealand towards the end of 1996). Only by quantifying the total supply of substrate (NH_4^+), was it possible to make definitive statements about the processes concerned from comparisons made between soils exhibiting net differences. Initial information on these relationships was obtained at an early stage of the project in an associated study carried out in the UK with the NZ partner (Ledgard *et al.*, 1998).

The experiment, combining tube incubation with ^{15}N mean pool dilution techniques, showed that the measured process rates were valid. ^{15}N techniques have certain limitations (see Section 6.1), but are generally more precise in the assessment of rates since the ^{15}N label is uniquely identifiable. This avoids the possibility of 'double counting' which may be an artefact of some of the measurements based purely on differences between start and end soil N concentrations. Thus the close agreement between net rates, either measured directly or calculated from ^{15}N labelling, provided an alternative and independent check on techniques.

7.3.3 **Examining** the practical implications

In natural systems with low inputs, nitrification rates are also generally low (see Section 1.5) and the preservation of N (in net terms) is ecologically sound. In such situations, the small, natural inputs from N-fixation (e.g. blue-green algae, *Azotobacter* etc.) and in

precipitation, are usually balanced by minimal losses through leaching and denitrification. Where swards are fertilized and other conditions non-limiting (pH, temperature, etc.), nitrifying activity increases and may persist along with concomitant increases in losses through denitrification and leaching. The maintenance of low, stable levels of inorganic N in grassland soils, despite high rates of *MIT*, suggests a dynamic equilibrium in which the favouring of one pathway must be necessarily at the expense of another. Therefore, with restricted substrate supply and if consumption is dominated by a particular pathway, e.g. by microbial uptake, then plant uptake may be constrained. The wider role implied by microbial discrimination between NH_4^+ and NO_3^- (see Section 6.7) would suggest that the balance between inorganic N forms will be of vital importance in determining uptake.

7.4 Sinks within the soil for mineral N

The major sinks for mineral N within the soil are the microbial and plant biomasses, and denitrification and leaching losses constitute the most important routes to external sinks. The internal N cycle is shown in Figure 7.1, which summarises the data obtained from the ^{15}N experiment (see Chapter 6) in agronomic terms (*viz.* kg N ha^{-1}). The results from the ^{15}N experiment suggest that differences in nitrification are the dominant factors in determining the equilibria between the competing processes of microbial and plant sinks for mineral N. Such differences are also reflected in the relative amounts of NH_4^+ : NO_3^- in the inorganic N pools, with ratios of approximately 1, 10 and 12 : 1 under the three sward types, i.e. fertilized grass (*F*), grass/clover (*C*) and unfertilized grass (*Z*), respectively. Furthermore, the microbial sinks in the three sward types were some 4 to 5-fold larger than the plant sinks (Fig. 7.1), with proportionately higher rates of immobilization compared (on a per day basis) with plant uptake rates.

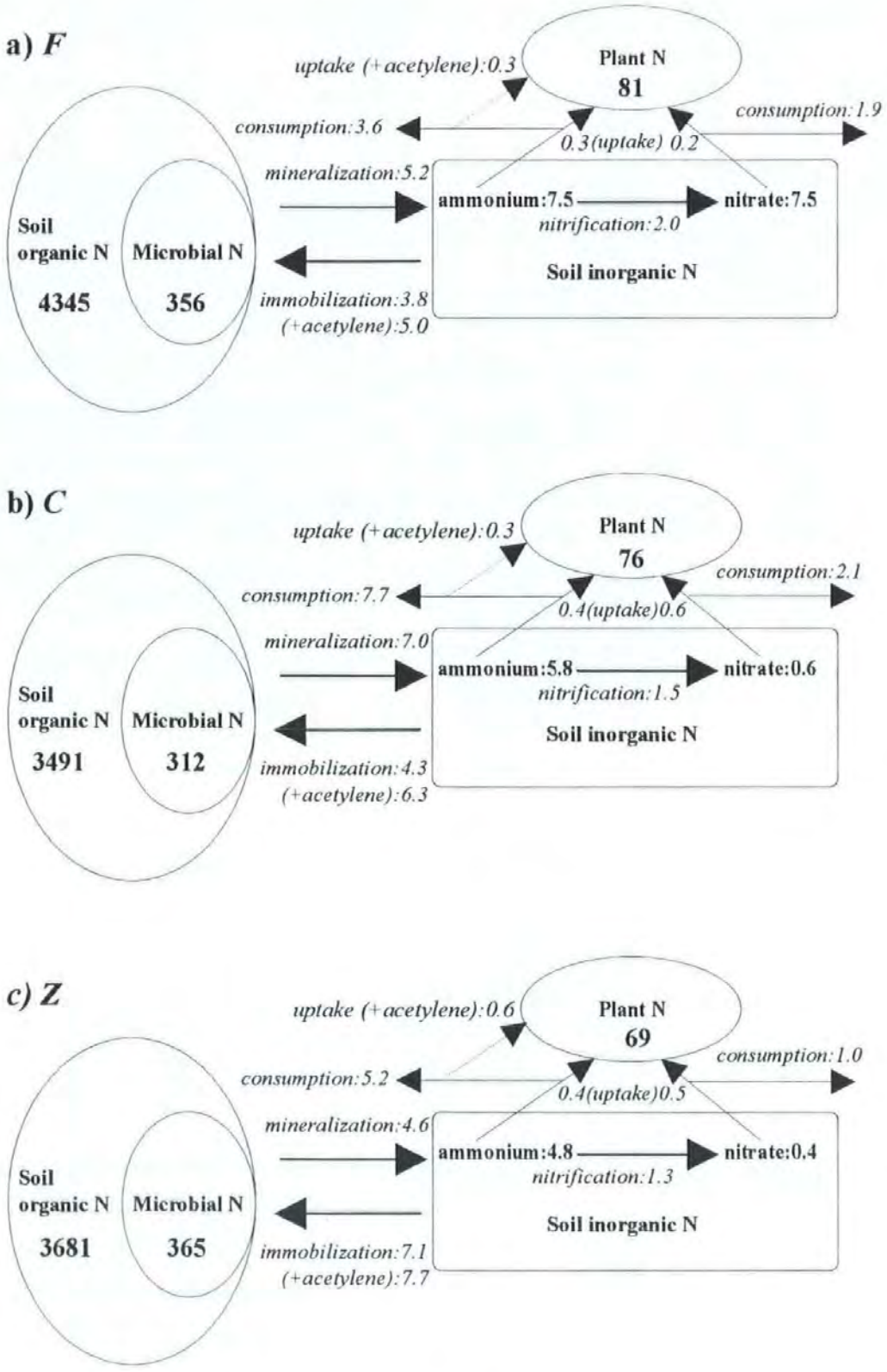


Figure 7.1 Diagram of N cycle processes (*italics*: kg N/ha/d) and N pools (**bold**: kg N/ha) for **a**) fertilized grass, **b**) grass/clover and **c**) unfertilized grass swards. Consumption processes (not determined separately), for nitrate include denitrification and leaching and for ammonium include volatilization

7.4.1 Microbial biomass

Where nitrification was inhibited (Table 6.3), immobilization rates increased in the *SMB* in *F*, C, and *Z*, demonstrating the affinity of microbial uptake for NH_4^+-N. The evidence was obtained from a microcosm study under natural field conditions (see Chapter 6) which overcame criticisms of extrapolation from purely laboratory-based incubations, or from field incubations where conditions are either unrepresentative or unrealistic. More subtle effects are indicated by this work and in various independent findings in the literature. For example, the conclusion that microbial uptake of N showed a preference for NH_4^+ over NO_3^- (Jackson *et al.*, 1989) would indicate strong competition between heterotrophic and autotrophic consumption of NH_4^+. The previous assumption that autotrophic nitrifiers are only poor competitors for NH_4^+ (Vitousek *et al.*, 1982) was challenged by more recent studies (Schimel *et al.*, 1989; Davidson *et al.*, 1990) and also by the present work. Hence the gross rate of nitrification in the fertilized (*F*) soil (2.0 kg N ha^{-1} d^{-1}, Fig. 7.1) was >50% of the ammonium immobilization rate, but <30% in the mixed sward (*C*) and only 15% in the unfertilized soil (*Z*).

7.4.2 Plant biomass

Whilst plants can absorb NH_4^+ and NO_3^- equally (Clarkson *et al.*, 1986), the dependence of roots on mass flow (mobile ions) and diffusion gradients (concentration dependent) to acquire N (Nye and Tinker, 1977), suggests that the form and concentration of inorganic N in soil solutions will influence uptake patterns. Hence, in the present study it was found that microbial uptake outstripped plant uptake, which confirmed previous work by Jackson *et al.*, (1989) and NO_3^- uptake by unfertilized swards was more efficient than that of NH_4^+, as previously proposed by Recous *et al.*, (1988a). The rapid immobilization of fertilizer

N (Bristow *et al.*, 1987) is likely, in the short-term, to limit the effficiency of fertilizer N use. In the longer-term, it can enhance N supply by saving N which might otherwise have been lost and ultimately increase the active organic N pool (Recous *et al.*, 1988b). The addition of a nitrification inhibitor (acetylene) made no significant difference to the pattern of NH_4^+ uptake in these swards (see Fig. 7.1); the reasons for which will be discussed later.

7.5 Limitations/drawbacks identified

Whilst the combination of measurements using the tube incubation technique and ^{15}N mean pool dilution technique provided important and independent comparisons, the effectiveness of the latter was decreased by sampling the soil profile down to 150 mm depth. Since the most biologically active region will be in the upper 50 mm layer, enrichment of the deeper layers will have had the effect of diluting the impact of process rates. More sensitive measurements of process rates could be achieved by confining the enrichment to the 0-100 mm depth, although the possibility of significant mineralization below this should not be overlooked and was further investigated as part of an associated study (Patra *et al.*, in press). Less disturbance of the plant processes could also have been achieved by cutting the herbage at least one week (rather than one day) before ^{15}N labelling, to avoid using plants which had been only recently perturbed. Another factor, over which less control can be exercised, is the extent of spatial variability which is encountered in these highly heterogeneous pasture soils. Greater replication could have been employed, but only at the expense of a severe restriction in the range of variables which could be investigated. It might be necessary in future work to be more selective in the choice of treatments, so that replication could be greatly increased, although this is unlikely to resolve the problem

of wide variation, since the numbers required could become unmanageable. For example, using data obtained for net mineralization by tube incubation (see Section 5.3.2) with 4 replicate soil cores to each treatment (CV = 52%), the number of cores which would be needed to obtain an estimate of the mean with a precision of 10%, can be found using the following formula (Goovaerts and Chiang, 1993):

$$sample\ number = \left[\frac{t_{\alpha} CV}{\varepsilon}\right]^2$$

Where: t is the Student's t statistic at the desired α level, CV is the coefficient of variation and ε is the degree of precision required (*viz.* 0.1). With 3 degrees of freedom, t = 3.17, where α = 0.05. For the example quoted, the number of incubated cores required to estimate the mean to within ±10% would be 272! Therefore, the information which can be obtained from field incubations is necessarily restricted to the comparison of trends. The resolution of temporal effects may not be possible without a large investment in extra resources of time and labour.

The mean pool dilution technique described in Section 6.1 evaluates changes in the enrichment of a labelled inorganic N pool. For example, measurements of gross mineralization are derived from the rate of addition of unlabelled native ammonium to the enriched inorganic $^{15}NH_4^+$-N pool, which results in the dilution of the ^{15}N label by sources of ^{14}N. However, further information may also be obtained from examination of the unlabelled inorganic N pools. Specifically, in the example quoted above, the nitrate pool

was not labelled, and where nitrification was measured, the ammonium N pool remained unlabelled. The inorganic N concentrations of the unlabelled N pools are shown in Table 7.1. It is clear that the presence of the nitrification inhibitor (I-data set) failed to prevent a rapid increase in NO_3^--N, following the injection of $^{15}NH_4^+$. The cause of this was due to the 24 h equilibration period allowed before the incubation over controlled-release capsules took place (*viz.* t0 to t1) during which C_2H_2 was not supplied. Effectiveness of the acetylene infusion was nevertheless confirmed, since no further increase in nitrate concentration occured between t1 and t5 (I-data set only). An explanation for the apparent lack of discrimination between inorganic N forms in plant uptake is therefore offered, as there is some uncertainty concerning the form of inorganic N which may have predominated during the measurement period. A possible future solution to overcome the difficulty of combining tube incubation with standard mean pool dilution techniques, whilst preserving the advantages of both methods, is discussed in the next section.

Table 7.1 Inorganic N contents (μg N g^{-1} dry soil ±SEM) of the unlabelled N pools *viz.* NH_4^+-N in the N treatments or NO_3^--N in the A/I treatments, before injection of 20 mg N kg^{-1} soil (t0) and at 24 h (t1) and 96 h (t5) after injection (n = 6)

Treatments (N pool)	t0		t1		t5	
FA (NO_3^--N)	5.9	(2.44)	18.1	(7.92)	16.8	(5.44)
FN (NH_4^+-N)	5.9	(0.52)	8.5	(1.52)	12.3	(4.48)
FI (NO_3^--N)	5.9	(2.44)	31.8	(10.31)	25.7	(8.68)
CA (NO_3^--N)	0.5	(0.15)	6.0	(8.20)	12.1	(1.66)
CN (NH_4^+-N)	4.5	(0.31)	9.9	(0.78)	10.3	(2.56)
CI (NO_3^--N)	0.5	(0.15)	7.1	(1.73)	6.5	(3.33)
ZA (NO_3^--N)	0.3	(0.07)	2.4	(0.68)	7.4	(2.83)
ZN (NH_4^+-N)	3.7	(0.44)	8.8	(1.51)	6.9	(0.76)
ZI (NO_3^--N)	0.3	(0.07)	3.2	(2.18)	1.0	(0.11)

In the labelled pools, A/I treatments received NH_4^+-N and the N treatment received NO_3^--N

7.6 Future priorities for investigation

The immediate stimulatory effect on nitrifying activity by the injection of $^{15}NH_4^+$ could be largely overcome by including acetylene in the enrichment solution. Other workers have used C_2H_2 dissolved in solution as a nitrification inhibitor (Kilham, 1987, Jackson *et al.*, 1989), but a novel approach would be to equilibrate the ^{15}N solution with C_2H_2 prior to injection. Nitrification of the newly added $^{15}NH_4^+$ would then be prevented and the effect could be sustained using the controlled-release capsules with tube incubation.

The role of heterotrophic nitrifiers in grassland soils has been overlooked and is deserving of attention, since low fertility soils tends to favour a higher representation of the fungal (heterotrophic) component over bacteria in the *SMB*. Labelling of the NH_4^+-N pool with ^{15}N and examination of the NO_3^--N pool for unlabelled NO_3^- would give a clearer indication of heterotrophic nitrification (i.e. organic N directly nitrified to nitrate N without ammonium substrate) as shown by Schimel *et al.* (1984) in acid forest soils. This information would be required before exercising control over autotrophic nitrification, since heterotrophs are immune to some physiological blocks which are effective against autotrophs (e.g. C_2H_2). It is also important that future studies are carried out over extended periods (*viz.* several years) to ascertain the persistence and activity of nitrification in soils from which the stimulatory effects of high N inputs have been withdrawn.

Future work should concentrate on resolving the specific nature of the interactions between nitrifying activity and N supply. For example, feedback systems could exist in which a decrease in N-containing substrates from plant decomposition will trigger greater immobilization (and hence less mineralization to NH_4^+ forms) so that nitrifiers will

decrease output. A more rapid response may be exhibited in the rhizosphere in response to cutting (often followed by fertilizer application), since the exudation of C-containing compounds from roots following removal of shoots could increase both mineralization and nitrification rates. Additionally, fertilizer N will speed up *MIT* with the result that surpluses not converted to plant/microbial biomass may be discharged into the wider environment. Nitrification will certainly be involved in determining how much of the N may succumb to this fate.

7.7 Options for control

Nitrification inhibitors have limited commercial benefit, but can provide invaluable comparisons and insights when used in research applications and serve as useful research tools for blocking selected pathways of N transformation reactions for comparative studies (see Section 4.1). In practical terms, however, their application would seem limited because of the expense of the formulations, their short-lived activity and concerns for the detrimental effects on the environment (nitrapyrin [*N-serve*] has now been withdrawn from commercial use). This approach to problems associated with the disadvantages of excessive rates of nitrification also addresses the symptom (i.e. excess nitrogen production), rather than the cause (i.e. excess N input). Of the options available, the more sustainable would seem to be better management of N inputs and to make fuller use of soil N supply in ways which avoid excesses and are more closely matched to plant requirements (Brown *et al.*, 1997).

7.8 Synopsis: acceptance/rejection of hypothesis

Superficially, the hypothesis may be deemed self-evident since the major loss processes

from fertilized grasslands (denitrification and leaching) both require nitrate as a precursor. Evidence for a direct coupling of nitrification with either of these processes has been limited to circumstantial, or inappropriate data and often obtained in conditions which were unrepresentative of field systems. The reason for this has been the difficulty in transferring established laboratory techniques to in-field measurements. The development of the new field incubation technique has now made this possible and has overcome many of the limitations of previous incubation methods. Testing of the hypothesis was based on data obtained from simultaneous measurements of the processes involved using the new incubation technique.

Acceptance of the proposed hypothesis would now seem to be justified, based on the major findings from the project, from which it has been deduced that:

- Nitrification activity increases markedly and persistently with inputs of N
- There are strong correlations between nitrification rates and denitrification losses
- Increased leaching losses may be associated with higher nitrifying activities
- Nitrification influences the outcome of competition between plants and microbes
- Net mineralization is determined by the balance between gross mineralization and immobilization (chiefly the latter, which in turn is influenced by nitrification)

Whilst verification of the correlation between leaching and nitrification requires additional investigation, the pivotal role attributed to nitrification as a controlling step in N losses, would seem justified, at least in grassland soils. In arable systems, the greater rates in turnover of plant residues and increased aeration when soils are cultivated means that

nitrification rates may not be the rate-limiting step. The wider role of nitrification in influencing the outcome of competition between plant and microbial biomass for available N sources has also been indicated.

7.9 Conclusions

That N inputs can stimulate nitrifying organisms and that such effects have been shown to persist into the next season, following the removal of external inputs, is of major importance in understanding the responses of biological systems in agricultural soils. This project has confirmed the original observations of Jarvis and Barraclough (1991), which were based on the distribution of mineral N between NH_4^+ and NO_3^- forms as indicators of nitrifying activity, by the use of more definitive laboratory assays of nitrification (Hatch *et al.*, 1998). This is especially important when considering the supplies of mineral N derived from mineralization of soil organic matter (SOM) and the fate of N determined by subsequent transformation into more mobile forms (e.g. NO_3^-). Nitrification will continue to feature as an important component of any investigation into N cycling in grasslands; not only is it clear that many of the N transformations within and between the lithosphere and the troposphere will be initiated by nitrifiers, but the complexity of these systems means that there is still much that is poorly understood. The dearth of field data examining the important process of nitrification ensures that the present study fills an identifiable gap in our current understanding. Whilst many other data exist in isolation, this study was able to draw together the interrelationships between major processes in the N cycle and overcame many of the limitations associated with existing methods.

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CHAPTER 8 References

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APPENDICES

Appendix 1

Preparation of ^{15}N enriched solutions

Stable isotopes of nitrogen, although expensive, are readily available in various formulations which are suitable for experiments which require labelling of plant, soil, animal excreta, etc. However, particular enrichments may not be obtainable, so that individual requirements are best catered for by dilution from a concentrated stock.

The following example requiring a nominal 20 At.% ^{15}N enrichment will serve to illustrate the calculations needed (taken from Hauck and Bremner, 1976):

Stock isotope source: Z g of 99.9 At.% $(^{15}NH_4)_2SO_4$

Required stock: X g of 20 At.% ^{15}N

The procedure is to 'dilute' the ^{15}N stock with a ^{14}N source from standard $(NH_4)_2SO_4$ (at natural abundance: 0.3663 At.% ^{15}N)

Then: $(X * 20) = (Z * 99.9) + ((X - Z) * 0.3663)$

$20X = 99.9Z + 0.3663X - 0.3663Z$

Rearranging:

$19.6337X = 99.534Z$

$Z / X = 19.6337 / 99.534$

Thus, Z needs to be 0.1973X

From these proportions an enrichment solution requiring 1.25 g N l^{-1} @ 20 At.%* (i.e. X = 1.25) would be prepared from:

Z = (1.25 * 0.1973)

Z = 0.246 g N @ 99.9 At.% ^{15}N

Plus: (X - Z)

or (1.25 - 0.246)

= 1.004 g N @ 0.3663 At.% ^{15}N

*Nb This is nominally 20 At.% based for convenience on proportions by mass (i.e. not atomic weights).

The calculation for the actual enrichment is shown below:

Using ^{12}C as the standard, atomic weights are 15.0001088 (^{15}N) and 14.0030732 (^{14}N)

The effective atomic mass (of N) when enriched to A% is:

[(A * 15.0001) + {(100-A) * 14.0031)}] / 100

= 0.0099704A + 14.003073

Therefore, g atoms N in 1.25 g enriched N = 1.25 / (0.0099704A + 14.003073)

Effective atomic mass of N in 99.9% enriched source is

[(14.0031 * 0.01) + (15.0001 * 99.9)] / 100 = 14.999111

Similarly, effective atomic mass of N in natural abundance source is

[(14.0031 * 96.63) + (15.0001 * 0.3663)] / 100 = 14.0067

g atoms in 0.246 g enriched source = 0.246 / 14.999111 = 0.0164009

g atoms in 1.004 g natural abundance source = 1.004/ 14.0067 = 0.0716799

Total g atoms in 1.25 g mixture = (0.0164009 + 0.0716799) = 0.0880808

Therefore,

1.25 / (0.0099704A + 14.003073) = 0.0880808

Actual enrichment (A) = 18.902 At.% ^{15}N.

Using the above equations the following range of standards were prepared, for the diffused and non-diffused standard (i.e. blank-correction) tests, giving nominal and actual enrichments achieved. Since the components are inter-dependent and pure forms of the sources of ^{15}N and ^{14}N are not readily available, this represents the most direct method of calculation:

Table 1 Weights required for 10 mls of each standard (containing 100 µg N/10µl) as $(NH_4)_2SO4$ or KNO_3 from stocks of unlabelled (^{14}N) @ 0.3663 At.% or labelled (^{15}N) @ 99.9 At.%

Standards	*a*	*b*	*c*	*d*
At. % ^{15}N	$^{15}NH_4^+$ (mg)	$^{14}NH_4^+$ (g)	$^{15}NO_3^-$ (mg)	$^{14}NO_3^-$ (g)
nominal (actual)				
2.5 (2.38)	10.142	0.4613	14.247	0.6508
5.0 (4.84)	21.951	0.4498	30.856	0.6337
7.5 (8.33)	33.775	0.4376	47.653	0.6169
10.0 (9.36)	45.624	0.4254	64.319	0.6001

Mix: (*a* + *b*) and (*c* + *d*) to produce range of $^{15}NH_4^+$ and $^{15}NO_3^-$standards as specified.

Appendix 2

Calculation of Microbial Respiration (CO_2 - C)

The amount of CO_2 - C respired is calculated from the titration values using 10 ml aliquots of the exposed 0.5 *M* NaOH, using the following formula:

$$\mu g\ C\ g^{-1}\ soil\ d^{-1} = [12000\ (A_1-A_2)\ M\ HCl\ V]\ /\ (2\ Wvd)$$

where:

A_1	=	volume of acid using to titrate controls (ml)
A_2	=	volume of acid used to titrate soil samples (ml)
*M*HCl	=	molarity of acid
W	=	weight of soil (dry)
V	=	volume of alkali in trap (ml)
v	=	volume of alkali titrated (ml)
2	=	molar ratio factor
12000	=	conversion from mMol to µg C
d	=	number of days incubated

Appendix 3

Blank-Correction of Samples for ^{15}N diffusion

Since the mass spectrometer (VG Micromass 602E) used in this work requires at least 100 μg N in each sample to generate a robust and reliable signal, it is clear that the evaluation of enrichments in control (blanks) samples (with inherently low amounts of contaminant ^{14}N and << 100 μg N) will be unreliable.

The method of blank-correction is taken from Kelly *et al.*, 1991, and the necessary information is obtained from comparisons between diffused and non-diffused standards in which the level of contamination of ^{14}N can be found from the differential effect on the dilution of known ^{15}N standards. The mass of N in the blank (M_b) can be found from the following istope dilution equation:

$$M_b = \frac{M_{std}\,(E_m - E_{std})}{(E_b - E_m)} \qquad (1)$$

where M_{std} is the mass of N in the standard, E_{std} is the ^{15}N enrichment of the non-diffused standards, E_m is the enrichment measured in the diffused standards and E_b is the enrichment of the blank (assumed to be 0.366%). The mass of N in the blank calculated using Equation (1), can then be used in the following equation (2) to calculate the blank-corrected ^{15}N enrichment of the sample:

$$E_s = E_m + \frac{M_b\,(E_m - E_b)}{M_s} \qquad (2)$$

where: E_s is the corected ^{15}N enrichment of the sample and M_s is the mass of N in the sample, prior to diffusion.

Appendix 4

Calculation of *MIT* Rates

Gross mineralization rates were calculated from the resultant dilutions of ^{15}N-labelled NH_4^+ between days 1 and 4, following the equation of Kirkham and Bartholomew (1954):

$$m = \frac{M_o - M_1}{t} \cdot \frac{\log (H_0 M_1 / H_1 M_0)}{\log (M_0 / M1)} \qquad (1)$$

Consumption of $^{15}NH_4^+$ includes the sum of immobilization (microbial assimilation, see later), autotrophic nitrification, volatilization and other possible fates:

$$c = \frac{M_0 - M_1}{t} \cdot \frac{\log (H_0 / H_1)}{\log (M_0 / M_1)} \qquad (2)$$

where:

Mo = initial $^{14+15}N$ pool (μg N g^{-1} dry soil)

M_1 = post-incubation $^{14+15}N$ pool (μg N g^{-1} dry soil)

H_0 = initial ^{15}N pool (μg N g^{-1} dry soil)

H_1 = post-incubation ^{15}N pool (μg N g^{-1} dry soil)

m = mineralization rate (μg N g^{-1} soil d^{-1})

c = consumption rate (μg N g^{-1} soil d^{-1})

t = time (4d for present study: t5 - t1)

The symbols M and H refer to the NH_4^+-N pool, where $^{15}NH_4^+$ was applied, but refer to the NO_3^--N pool where $^{15}NO_3^-$ was applied. For nitrification rates, the symbol m is replaced by n (μg N g^{-1} soil d^{-1}).

The rate at which ^{15}N enters the chloroform-labile pool (v) from F-E extraction of the soil microbial biomass can be found from the following equations of Davidson *et al.* (1991):

$$i = \frac{v_t}{\left(\frac{y_0}{x_0}\right) \cdot \left(\frac{1 - e^{-kt}}{k}\right)} \qquad (3)$$

where:

i = imobilization rate (μg N g^{-1} dry soil)

v_t = ^{15}N content of the choroform-labile pool (μg N g^{-1} dry soil) in excess of background ^{15}N at the end of incubation (4d for the present study, t5-t1)

(y_0/x_0) = initial enrichment of the NH_4^+ pool

k = rate constant, assuming the NH_4^+ pool declines exponentially as organic- ^{14}N is mineralized to $^{14}NH_4^+$

where:

$$k = \left[- \ln \left(\frac{H_1 M_0}{H_0 M_1} \right) \right] / t \qquad (4)$$

In this case, the chloroform-labile pool (microbial biomass-N) represents an infinite sink for immobilized ^{15}N, assuming that none of the ^{15}N is remineralized during the incubation period. A simpler method of obtaining K, assumes a linear decline in the inorganic N pool

enrichment during incubation (Barraclough *et al.*, 1985), which probably holds true for short incubations. Immobilization is then calculated as the ^{15}N flush from chloroform-fumigation divided by the average enrichment at the beginning and end of incubation.

List of abbreviations

CV	Coefficient of variation
DCD	Dicyandiamide
EC	Ethyl cellulose
FA	Fluorescent antibody (stain)
FIA	Fow-injection analyser
FE	Fumigation-extraction
HMPC	Hydroxypropylmethyl cellulose
I-E	Ion-exchange (resin)
MIT	Mobilization and immobilization turnover
MS	Mass spectrometer
MNP	Most probable number
OM	Organic matter
PTFE	Polytetrafluoroethylene
PVC	Polyvinyl chloride
SEM	Standard error of the mean
SIR	Substrate induced respiration
SMB	Soil microbial biomass
SOM	Soil organic matter
SWC	Soil water content