REGULATION OF DENITRIFICATION IN ORGANIC RIPARIAN SOILS

A thesis submitted in partial fulfilment

of the requirements for

the degree of

Doctor of Philosophy

in Biological Sciences

by

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Hamilton, New Zealand

1991

ABSTRACT

Riparian zones have been shown to remove nitrate from groundwater before it enters surface waters thus providing protection of surface water quality. Microbial denitrification is considered to be a mechanism which may be responsible for nitrate removal in riparian zones. The aim of the present study was to identify the rate at which denitrification occurred in riparian zones and to identify factors which regulated the rate. This was investigated using laboratory and field studies.

In laboratory studies, the addition of a range of single sources of carbon promoted denitrification in organic riparian soil during 10-day incubations. Denitrification lag times and rates were shown to be dependent on the broad groups of organic compounds tested. The denitrifier population responded most rapidly following the addition of a range of sugars, whereas the denitrification rate was highest following the addition of the fermentation endproducts lactate and ethanol. Cellulose and propionate did not stimulate denitrification above the control. Denitrification showed a diauxic response to xylan addition.

Volatile fatty acids (VFAs) were shown to inhibit denitrification for at least the first 100 hours of incubation, after which acetate and butyrate were used by denitrifiers; however, propionate continued to be inhibitory. It was thought that VFAs inhibited bacteria following acidification of the bacterial cytoplasm.

A longer-term incubation experiment (100 days) examined the suitability of three plant materials (fresh pine needles, senescent pine needles, and fresh watercress leaves) undergoing decomposition to act as electron donors for denitrification. Although denitrification was stimulated following the addition of all plant materials denitrification was greatest in those incubations where fresh rather than senescent plant material was added. Nitrous oxide, methane, and carbon dioxide production were shown to be dependent on the plant substrate added. Results demonstrated the interactive ecology of denitrifying, methanogenic, and fermentative bacteria in these anaerobic soils. Evidence suggested that denitrifying bacteria were dependent on fermentative bacteria making the added plant carbon more readily available. The long-term persistence (>100 days) of denitrifying activity in the absence of electron acceptors was also demonstrated.

Field studies were conducted in an organic riparian zone receiving nitrate from incoming groundwater. Mean and median denitrification rates were found to be 1.2 and 0.98 $gN.m^{-2}.day^{-1}$; while mean and median N_2O production rates were found to be 0.073 and 0.088 $gN.m^{-2}.day^{-1}$. These rates were 1 to 3 orders of magnitude greater than those reported in previous studies of upland soils. Up to 77% of the variation in onsite denitrification rate could be explained by nitrate concentration and denitrifying enzyme activity. Temperature may also

have regulated the rate at which denitrification occurred; however, too few sampling trips at different temperatures were conducted to fully establish a temperature effect.

Nitrate concentrations, and consequently denitrification rates, were highest at the upslope edge of the riparian zone and decreased towards the downslope end. The denitrification rate appeared to be high enough to be responsible for the major proportion of nitrate removal observed in this riparian zone.

ACKNOWLEDGEMENTS

Throughout this project there have been many people who have contributed to the work itself or to my enjoyment of time spent at the Forest Research Institute. I wish to thank them all.

I would like to thank my supervisory panel Drs Bryce Cooper, Bill Dyck, Chris Harfoot, Paul McFarlane, Warrick Silvester, and Peter Stevens not only for their encouragement and support during this project but also for their friendship. The Forest Research Institute is acknowledged for having sponsored this work. During the writing of this thesis the comments of Bryce Cooper, Chris Harfoot, and Shane McMahon were invaluable. Peter Beets, Christine Mees, Peter Stevens, David Whitehead are thanked for proofreading chapters of this thesis. Mark Kimberley is thanked for his assistance with the statistics used in this study. Thanks also to Alison Lowe and Carla Krutzsch who assisted me in the field and performed numerous laboratory analyses. Sheree Anderson, Hank Kroese, and Peter Dare are thanked for their assistance with gas chromatography and carbon analysis.

I have enjoyed my experiences at the Forest Research Institute largely due to the people I have had the pleasure to meet and I thank them all for contributing to the maintenance of my sanity. Particularly Christine, Bill, Paul, Shane, Tony, Alison, and my golfing partners come rain, hail or snow - Trevor, Richard, and Phil.

I would like to thank my family for their many years of support.

Most of all, thanks to Angela who has endured my ups and downs during this thesis with love and understanding, and without whom this thesis may never have happened.

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TABLE OF ABBREVIATIONS

ANOVA	Analysis of variance
CEC	Cation exchange capacity
DEA	Denitrifying enzyme activity
DNRA	Dissimilatory nitrate reduction to ammonium
ND	Not determined
VFA(s)	Volatile fatty acid(s)
WSC	Water soluble carbon

CHAPTER 1
INTRODUCTION

Nitrate pollution of surface waters and groundwaters has become of increasing concern in many countries. In New Zealand, concentrations of nitrate in groundwaters can exceed the World Health Organisation limit of 10 gN m^{-3} for drinking water (Burden 1982), and eutrophication of several nationally-important lakes has been attributed to increased N inputs from contaminated groundwater (Viner and White 1987). Similar problems exist to varying degrees in other parts of the world, including Europe (Fried in press; Croll and Hayes 1988) and the United States (Spalding and Exner in press). Excessive nitrate in groundwater can originate from a number of sources including fertilisation of forested or agricultural land, death and decomposition of nitrogen-fixing plants, and the application of wastes onto land.

One of the major difficulties in the protection of surface waters from nitrogen pollution is that nitrate does not enter as a point source. However, there is circumstantial evidence that nitrate removal occurs in the riparian zone soils of pastoral and forested watersheds. Nitrate concentrations in streamwaters (Wilcock 1986) are typically an order-of-magnitude less than nitrate concentrations reported in the groundwater which supplies the flow (Burden 1982). These riparian areas have also been noted in overseas studies as important for surface water quality protection from agricultural runoff, particularly the removal of nitrate (Peterjohn and Correll 1984; Lowrance *et al.* 1984a; Jacobs and Gilliam 1985). Cooper (1990) showed that a probable mechanism for nitrate removal in riparian zones was by microbial denitrification.

In addition to the potential of riparian zones to reduce the pollution of surface waters by nitrate pollution from pastures, their capacity to fulfil a similar role downslope of waste treatment sites is now becoming recognised in New Zealand (Cooper 1987; Schipper *et al.* 1989). The potential use of riparian zones below land treatment systems to protect surface water quality was the main driving force which initiated the present research project.

Treated effluent from the city of Rotorua (New Zealand) prior to 1991 was discharged into Lake Rotorua and nitrogen in the effluent was identified as contributing to the eutrophication of the lake (Rutherford and Pridmore 1987). Investigations were then made to identify alternatives for treating the effluent. Application of the effluent into nearby Whakarewarewa Forest was chosen. It was thought that nitrogen applied in effluent would leach to groundwater as nitrate and pass into riparian and wetland soils prior to discharge into surface waters. Preliminary work showed that the removal of nitrogen could be very high in these riparian and wetland soils (Cooper and Schipper unpublished data; Schipper *et al.* in press). Carbon content of the riparian soils was identified as one of the main regulators of the maximum rate at which denitrification could occur. It was shown that both the quality and quantity of the carbon present was important.

Questions were also raised about other factors which may regulate the rate of nitrate removal by denitrification in riparian soils. With this background of questions, the present study set out to investigate some of the factors which regulated denitrification in riparian zones.

Chapters 2 and 3 present a review of the literature and the common methodology used in the thesis, respectively. Results found during this study and their implications are discussed in Chapters 4 through 7. The specific investigations and their aims are outlined below.

Prior to any experimentation, a method for the storage of soil was selected. In chapter 4 the effect of this storage method on the denitrifying activity of the soil is described.

The effect of carbon quality on denitrification was examined in laboratory studies using two approaches. The first approach was to measure denitrification following the addition of sources of single carbon compounds (Chapter 5) in an attempt to identify the carbon compounds used by denitrifying bacteria. The second approach was to observe the denitrification response following the addition of a more complex carbon source: decaying plant material (Chapter 6). This was done in order to simulate processes likely to occur in riparian zones. In both of these studies, the interactions between denitrifiers, fermenters, and methanogens were also investigated.

It was recognised that results from laboratory studies have limitations for understanding processes which actually occur at a larger scale, for example at the subcatchment level. Although laboratory incubations had shown that the maximum rate of denitrification varied between riparian soils depending on the quality and quantity of carbon, this result had limited value in understanding the regulation of denitrification *in situ*. To fill this gap, a field study, described in Chapter 7, was designed to examine the factors controlling of denitrification at a riparian site below a forest which was used for the application of domestic effluent to land. As a consequence of this effluent application, groundwater entering the riparian zone contained high concentrations of nitrate.

There were thus 3 objectives to the research:

1. to compare the denitrification responses in organic riparian soil resulting from the addition of single sources of carbon and of plant material undergoing decomposition,

2. to investigate the relationship between denitrifiers and other anaerobic bacteria; and,

3. to determine the extent of nitrate removal by denitrification in an organic riparian zone and identify the factors which regulated this process *in situ*.

CHAPTER 2
A LITERATURE REVIEW OF DENITRIFICATION AND OF NITROGEN REMOVAL IN RIPARIAN ZONES.

2.1. INTRODUCTION

Research into denitrification dates back over 100 years (Maquenne 1882) but within the last decade the amount of information has expanded greatly. This is in part due to the relative ease with which denitrification can now be measured. Given the vast amount of literature available it is not the intention of the present literature review to cover all the past information. Rather this review will attempt to provide the reader with a general background to denitrification and its regulators in the soil environment. This review also examines what is known about nitrogen removal in riparian zones. One of the main aims of the present research was to investigate the role of organic carbon in regulation of denitrification, and this area is covered in greater depth. In appropriate sections, the reader is directed to pertinent reviews for further information. One recent review, Tiedje (1988), is particularly recommended to those interested in denitrification in general and more specifically in denitrifier ecology.

In Chapters 4 through to 7, research results are presented and discussed. In those chapters, the results are compared critically to findings in previous literature studies.

The present chapter is divided into three major sections. Initially, nitrogen removal in riparian zones is discussed. The second section deals with denitrification in general, and the last section discusses the regulation of denitrification.

2.2 NITROGEN REMOVAL IN RIPARIAN SOILS

In the following sections nitrogen removal in riparian zones is discussed. First, a definition of riparian zones is presented. Then evidence for nitrate removal in riparian zones is discussed along with the mechanisms by which nitrate removal might occur.

2.2.1 RIPARIAN ZONES

The term "riparian zone" is often loosely used to describe a variety of margins separating unsaturated soils from streams, rivers, lakes, and wetlands. Riparian zone soils are often high in clay and organic matter, and are saturated by laterally-moving groundwater which fluctuates in height; these zones may therefore be considered a special class of wetland (Lowrance *et al.* 1985). Since riparian strips are seldom water-limited, they support a wide

range of vegetation and animal life (Pase and Layser 1977). However, riparian zones do not generally maintain significant surface water.

For the purposes of this review, a distinction is made between two types of riparian zones and nitrate removal in each is discussed separately. This division is made because past research described in the literature has examined nitrogen removal in two morphologically different riparian zones (Figure 2.1). The differences in morphology are linked to differences in groundwater hydrology. In both these riparian types, nitrate is predominantly transported by groundwater to surface water, but the mechanisms and/or extent to which nitrate is removed may differ between the two riparian zones types. For simplicity these two riparian zones are referred to as type 1 and type 2. The differences between these types are discussed below and are summarised in Figure 2.1 and Table 2.1.

CHARACTERISTIC	TYPE 1 RIPARIAN ZONE	TYPE 2 RIPARIAN ZONE
Width	Order of metres	Order of tens of metres
Soil composition	Mosaic of organic and mineral patches	Mineral
Groundwater flow	Through organic and mineral soils up to surface	Subsurface before discharge to surface waters
Groundwater path length	Short	Long
Vegetation	Relatively little	Considerable, often forested

Table 2.1 Morphological differences between type 1 and type 2 riparian zones.

Type 1 riparian zones are characterised by short groundwater path lengths (metres) through a mosaic of organic and mineral soils to surface waters (Figure 2.1). Groundwater may rise to the surface of the riparian zone before discharge to surface water. The organic soils are water-saturated and anaerobic conditions are predominant; the mineral soils may also become anaerobic but probably to a lesser extent.

Type 2 riparian zones are characterised by their size (tens of metres) and consist predominantly of mineral soil, generally covered with riparian forest vegetation. In these riparian zones groundwater is shallow and rarely reaches the soil surface before discharge into surface waters (Figure 2.1).

Although the work presented in this thesis examines denitrification in type 1 riparian zones, a discussion of the evidence for nitrate removal in both these two riparian zone types is given for completeness. The possible mechanisms for nitrate removal are discussed.

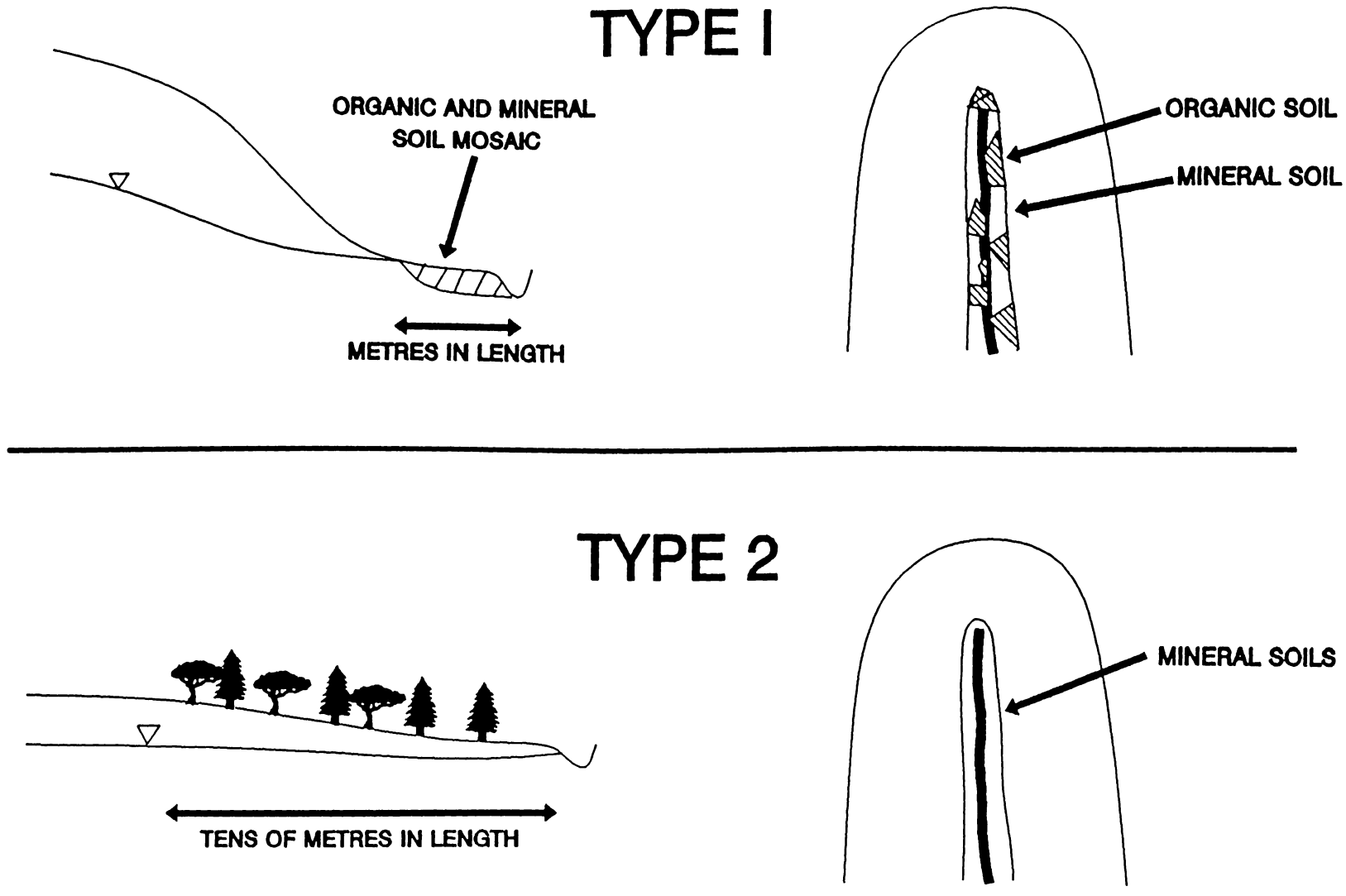


Figure 2.1 Profile and plan of type 1 and 2 riparian zones illustrating key differences.

2.2.2 TYPE 1 RIPARIAN ZONES

Varying degrees of nitrate removal have been shown to occur in type 1 riparian zones by a number of authors including Warwick and Hill (1988), Schipper *et al.* (1989), Cooper (1990), and Schipper *et al.* (in press). These studies generally considered nitrate removal to be primarily by denitrification rather than plant uptake. Although nitrogen uptake by vegetation on streambanks has been reported to remove nitrate from streams (e.g. Howard-Williams *et al.* 1986), plant uptake has not been reported to be a significant mechanism of nitrate removal from groundwater in type 1 riparian zones.

Cooper (1990) reported that the percentage of nitrate removed was dependent on the partitioning of groundwater flow between actively-denitrifying, organic soils of the mosaic and less-active mineral riparian soils. High denitrification rates in the organic soils were shown to remove nitrate; however, the nitrate removal rate was often below its maximum potential. It was suggested that denitrification was most likely nitrate-limited. Denitrification losses were estimated at between 28 and 183 kgN $ha^{-1}yr^{-1}$. In a study where extra nitrate was added to the groundwater entering a type 1 riparian zone, Schipper *et al.* (in press) found nitrate removal rates as high as 700 kgN $ha^{-1}yr^{-1}$. This value was considerably higher than those reported by Cooper (1990) and the difference was probably due to higher nitrate availability. Warwick and Hill (1988) found that little nitrate removal occurred in a riparian zone of a small woodland stream, even though relatively high denitrification potentials were observed in laboratory incubations. They concluded that this was due to a short residence time (less than 1 hour) of the nitrate in the riparian organic soils.

Nitrate removal in a riparian zone below spray-irrigated forest was reported to remove up to 98% of the incoming nitrate (Schipper *et al.* 1989). These measurements were made in an organic portion of the riparian zone and were unlikely to be representative of the nitrate removal along the entire length of the riparian zone. Schipper *et al.* (in press) compared denitrification rates in a range of riparian and wetland soils and found that the amount of available carbon limited the potential rate of denitrification whereas the nitrate concentration regulated the denitrification rate.

In summary, in type 1 riparian zones, nitrate removal appears to be predominantly by denitrification in the organic portions of the riparian zone. Denitrification would appear to be by concentration of nitrate and the extent of nitrate removal maybe limited by residence time. Very little is known about other environmental factors which regulate nitrate removal by denitrification in these riparian zones.

2.2.3 TYPE 2 RIPARIAN ZONES

Three groups of researchers along the east coast of the United States published results from independent watershed studies which examined the cycling of nutrients in type 2 riparian zones and reported remarkably similar nitrate removal rates of approximately 44 kgN $ha^{-1}yr^{-1}$ (Peterjohn and Correll (1984), 30 kgN $ha^{-1}yr^{-1}$ (Jacobs and Gilliam 1985), and 44.5 kgN $ha^{-1}yr^{-1}$ (Lowrance *et al.* 1984a). Nitrate removal occurred in the underlying groundwater; there are two possible mechanisms for the nitrate removal: plant uptake and denitrification.

Plant uptake of nitrogen in these zones has been estimated from increments in plant biomass and total nitrogen content, and the rate at which litter is cycled. Peterjohn and Correll (1984) estimated incremental uptake of nitrogen by riparian forest to be 15 kgN $ha^{-1}yr^{-1}$ (equivalent to one third of the nitrate loss in the riparian zone) but pointed out that the fine roots of this forest generally ended one metre above the groundwater level. They therefore suggested that plant uptake was not responsible for nitrate removal from the groundwater.

Denitrification in type 2 riparian zones was reported by Lowrance *et al.* (1984b) to be sufficient to remove the bulk of groundwater-nitrate coming from the upland soils. Gillham (in press) reported denitrification in groundwater less than 2-3 m below the surface. He suggested that denitrification was limited by the downward movement of labile organic matter. Evidence for this was shown by Starr and Gillham (1989) when comparing shallow and deep groundwater denitrification. Whereas denitrification occurred at the shallow groundwater site (less than 2-3 m), denitrification was only observed at the site with deeper groundwater (greater than 3.5 m) following the addition of a carbon source. These authors also analyzed the quantity and quality of organic matter with depth at both shallow and deep groundwater sites and found that although organic matter reached the shallow groundwater, it did not reach deeper groundwaters. Smith and Duff (1988) also concluded that denitrification was carbon-limited at their groundwater site. In these instances organic matter presumably acts not only as a carbon source for denitrifying bacteria but also increases the oxygen demand and creates local anaerobic conditions. It would seem logical that not only the quantity but also the quality of organic carbon influences the formation of anaerobic sites.

From the studies above there would appear to be good evidence to support the hypothesis that the nitrate removal observed in type 2 riparian zones is by denitrification since these sites generally have shallow groundwater. Whereas groundwater studies have measured denitrification at a local scale little is known about the rates at which nitrate is removed from groundwater at the catchment scale. These rates need to be measured in type 2 riparian zones in order to confirm that denitrification can account for nitrate loss. If this is found to be the case, then the movement of organic carbon from topsoils to groundwater, as

well as the contribution of dead roots to the carbon pool of subsurface soils, should also be further studied.

2.3 DENITRIFICATION

2.3.1 INTRODUCTION

Denitrification is an anaerobic bacterial process where the nitrogen oxides (nitrate (NO_3^-), nitrite (NO_2^-), and nitrous oxide (N_2O)) are used as electron acceptors and where organic matter is the electron donor resulting in the production of metabolic energy for the cell (Brock 1979).

Conversion of fixed nitrogen to nitrogen gases can also be driven by non-biological processes. Chemical transformation of nitrate to nitrogen gases is termed chemo-denitrification (for review see Chalk and Smith 1983) but is not thought to be of major significance on the global scale (Tiedje 1988). The term "denitrification" has also been used to also describe thermal production of nitrogen gases following burning of biomass such as wastes, e.g., slash remaining after forest harvesting. Neither of these non-biological processes are relevant to riparian zones and are not discussed in this review.

There have been a large number of reviews of biological denitrification in the recent literature to which the reader is referred for further information on specific aspects of denitrification. Some of the recent reviews have examined denitrification in general (Firestone 1982; Knowles 1982), denitrifier ecology (Tiedje 1988), denitrification in temperate forest soils (Davidson *et al.* 1990), denitrification in agricultural soils (von Rheinbaben 1990), denitrification at temporal and geographic scales (Groffman *et al.* 1988), interactions of denitrification with iron and sulphur cycling (Sorensen 1987), enzymes in denitrification (Ferguson 1987; Hochstein and Tomlinson 1988; Stouthamer 1988), carbon sources for denitrification (Beauchamp *et al.* 1989) and denitrification in sewage effluent (Christensen and Harremoes 1977) among others.

The following sections (2.3.2 - 2.3.6) examine the literature concerning biological denitrification in the following order; ecology of known denitrifying bacteria, methodology used to measure denitrification, rates of denitrification measured, implications of denitrification in the environment, and lastly dissimilatory nitrate reduction to ammonium (DNRA). Emphasis is placed on denitrification in soil and sediments, rather than on nitrogen removal in waste treatment plants.

2.3.2 DENITRIFYING BACTERIA

Denitrifying bacteria are thought to be present in nearly all soils (Tiedje *et al.* 1982) and come from a very wide range of genera which are generally facultative aerobes. Some of the main genera isolated from soil include *Pseudomonas*, *Alcaligenes*, *Flavobacterium*, *Paracoccus*, and *Bacillus* (Jeter and Ingraham 1981; Firestone 1982; Tiedje 1988). It is noteworthy that some of these genera are among the most metabolically-versatile bacteria known, particularly *Pseudomonas*.

As facultative aerobes, denitrifiers would be expected to survive in aerobic soils when conditions for denitrification did not occur. Expanding on this, Tiedje (1988) expressed the opinion that "most of the denitrifiers in nature exist because they are effective aerobic competitors for carbon and never use their denitrification capacity". Such an opinion would suggest that denitrifiers should be thought of as bacteria which are predominantly aerobic and which are opportunistically able to use nitrate as an alternative electron source under suitable anaerobic conditions. This might confer an advantage upon denitrifiers over obligate aerobes during brief periods when the soil becomes anaerobic and nitrate is present. It has been shown that denitrifiers tend to lose their ability to denitrify when maintained in aerobic laboratory cultures (Gamble *et al.* 1977). Evidence that this might also occur in the field was presented by Davidson *et al.* (1990) who suggested that some forest soils were devoid of denitrifying bacteria. They suggested that this was because, in these forest soils, conditions seldom arose under which denitrification would occur. It would seem likely that should conditions suitable for denitrification occur at these sites, bacteria with denitrifying ability would recolonise; although, the time required for this to occur is unknown.

Whereas denitrifiers are likely to have a competitive advantage over obligate aerobes in environments which fluctuate in oxygen status, denitrifying bacteria are also likely to be very competitive in anaerobic environments where nitrate is present. Koike and Hattori (1975) showed that denitrification in *Pseudomonas denitrificans* provided some 60% of the energy yield compared with aerobic growth in the same bacteria. Such energy yield is greater than that found in most other anaerobic forms of metabolism.

2.3.3 METHODS FOR MEASUREMENT OF DENITRIFICATION

In this section, a brief overview is given of the methods by which denitrification can be measured. There are a number of reviews to which the reader is directed towards for further detailed information (Hauck and Weaver 1986; Tiedje *et al.* 1989; Myrold 1991)

Generally, rate of denitrification is measured by quantifying the production of gaseous endproducts of denitrification. The major gas produced is generally N_2 gas, the formation of

which is hard to measure against the background nitrogen gas content in air. Currently, two main approaches to circumvent this problem are popularly used; acetylene blockage and the use of the mass-labelled isotope of nitrogen, ^{15}N. These are discussed in more detail below along with two other techniques which are used less often.

Acetylene blockage

Acetylene has been shown to block the conversion of N_2O to N_2 and was first shown to be useful in the measurement of denitrification by Yoshinari and Knowles (1976) and Balderston *et al.* (1976). The accumulation of nitrous oxide can be detected routinely using a gas chromatograph equipped with an electron capture detector (Kaspar and Tiedje 1980).

The acetylene-blockage technique has been applied to both field and laboratory studies. To obtain accurate measurements of denitrification rate, the even distribution of sufficient acetylene throughout the soil is essential, generally 10% of the headspace volume is recommended (Tiedje 1982). If even distribution of acetylene is not achieved then some of the nitrous oxide formed may be converted to nitrogen gas and the denitrification rate will be under-estimated.

Acetylene gas is water-soluble and in well-mixed laboratory incubations the distribution of acetylene is unlikely to be a major problem. It is somewhat more difficult to obtain uniform distribution of acetylene when making assessments of field rates of denitrification. Subsurface injection of acetylene into soil followed by measurements of nitrous oxide accumulation under soil covers have been made (e.g., Jury *et al.* 1982). These authors suggested that in some cases, it may be necessary to monitor soil covers for several weeks before accurate measures of denitrification rate can be made. Alternatively, soil cores can be removed, sealed, acetylene added, and incubated either *in situ* or in the laboratory at controlled temperatures (e.g., Parkin and Tiedje 1984; Groffman and Tiedje 1989a,b). Nitrous oxide production is then monitored over a set time period. Protocols for this method have been suggested by Tiedje *et al.* (1989). This method allows a large number of cores to be handled simultaneously and rapid assessments of denitrification rate can be made. However, taking soil cores does result in some soil disturbance which may increase the amount of carbon available for microbial activity thus increasing the denitrification rate.

A problem which can occur with the use of acetylene in aerobic soils is that acetylene also blocks nitrification (Oremland and Capone 1988). In some instances, denitrification is limited by the rate at which nitrate is formed by nitrification (discussed in more detail in section 2.4.3), and therefore actual rates of denitrification can be under-estimated.

Nitrogen isotopes

There are two nitrogen isotopes used in denitrification studies; a stable mass-labelled nitrogen isotope - ^{15}N, and a radio-active isotope - ^{13}N (with a half-life of less than 10 minutes).

The radio-active isotope ^{13}N is rarely used because of its very short half-life. Studies which use ^{13}N need to be conducted over a very short time frame and close to the site of the isotope's manufacture. Nevertheless, Smith *et al.* (1978) successfully used ^{13}N to confirm that acetylene blockage was an effective means for measuring denitrification rates in soil.

The mass isotope ^{15}N has been used in a number of different approaches in measurement of denitrification rate, and these are reviewed by Myrold (1991). The most common method is where ^{15}N is added as a substrate (e.g., as nitrate) for denitrifying bacteria and the evolution of ^{15}N-labelled nitrogen gases is monitored to determine denitrification rate. This has the added advantage of allowing measurements of other fates of nitrate to be examined, such as plant uptake. However, a disadvantage of adding ^{15}N as nitrate is that the rate of denitrification can be increased, particularly in soils where denitrification is nitrate-limited.

When using ^{15}N-nitrate in the assessment of denitrification it is necessary to ensure that the ^{15}N-labelled nitrate is evenly distributed throughout the native nitrate pool in the soil otherwise denitrification will not reflect the true rate of soil denitrification.

Nitrate loss

Measurement of the loss of nitrate from the medium studied has been most often used in confined incubations. This technique does not distinguish denitrification from other nitrate transformations such as microbial uptake and nitrate conversion to ammonium. In aerobic environments, nitrate formation by nitrification can confound the interpretation of results.

Conservative tracers

Chloride has been used as a conservative marker for nitrate in field studies. Two main assumptions are made; first, that nitrate and chloride travel at the same rate through soil and, second, that chloride is biologically and chemically inert. Changes in the nitrate to chloride ratio are interpreted to represent a loss of nitrate, presumably by denitrification but plant uptake may also be significant. There are a number of other assumptions associated with this technique and these have been outlined by Pratt *et al.* (1978). This technique can be useful in field situations to indicate where qualitative losses of nitrate occurs. Jacobs and Gilliam (1985) and Schipper *et al.* (1989) used this approach to show that nitrate loss occurred across a riparian zone.

2.3.4 RATES OF DENITRIFICATION

In this section different measures of the rate of soil denitrification are discussed. The focus is on a comparison of the rate of denitrification found in different landscapes.

Denitrifying enzyme activity

Measurement of denitrifying enzyme activity (DEA) (Smith and Tiedje 1979) has been used to indicate the potential denitrification rate of a soil. DEA rates are usually measured following additions of nitrate and carbon; the soil samples are then incubated under anaerobic conditions so that denitrification is non-limited. Rates obtained using this method are generally much higher than rates of denitrification measured *in situ*. Martin *et al.* (1988), in a study of DEA and other measurements of denitrifying bacteria numbers, concluded that "DEA is better interpreted as an estimate of the biomass of denitrifying bacteria in soil rather than as an index of actual denitrification rates". This certainly appears to be true for short-term estimates of denitrification rates measured *in situ* (such as hourly rates) which are subject to high spatial and temporal variability; however, Groffman and Tiedje (1989b) showed a strong correlation between DEA and annual rates of denitrification. In comparison to other soil environments, relatively high values of DEA have been found in permanently anaerobic environments such as sediments, organic wetlands, and some riparian soils (Table 2.2), although a few comparatively high values were reported by Davidson *et al.* (1990) in a summary of DEA in forest soils.

Although DEA values are high in anaerobic soil environments, fluxes of nitrate and oxygen are low and conditions suitable for denitrification or aerobic growth are seldom likely to occur. It is difficult to understand how denitrifying bacteria survive in these environments. Of the isolated denitrifiers (those which use nitrous oxides as electron acceptors during growth) none are capable of alternative forms of anaerobic energy production. Tiedje (1988) suggested that the most likely explanation for this would be that an unidentified group of denitrifiers exist which are capable of an alternative method for anaerobic growth or cell maintenance. Alternatively, denitrifiers may be able to remain dormant for long periods of time without energy supply and respond quickly to small increases in nitrate or oxygen.

Ecosystem	Denitrification enzyme activity ($ngN\ g^{-1}hr^{-1}$)	Reference
Forest soils (i) mineral soils (ii) forest floor	84 - 392 0 - 1,900 1 - 2,500	Tiedje (1988) Davidson *et al.* (1990) Davidson *et al.* (1990)
Agricultural soils	5.6 - 280	Tiedje (1988)
Eutrophic sediment	6,440	Tiedje (1988)
Wetland	200 - 3200	Cooke *et al.* (1990)
Organic riparian soils	170 - 3750	Schipper *et al.* (in press)
Mineral riparian soils	44	Cooper (1990)

Table 2.2 Denitrifying enzyme activity found in different soil ecosytems.

Landscape rate of denitrification

Research has now turned to the estimation of denitrification rate at the landscape scale. Direct measurement of landscape rates of denitrification are confounded by the high temporal (Sextone *et al.* 1985) and spatial (Folorunso and Rolston 1984; Parkin 1987) variability of denitrification rates. The problem of high variability may be overcome by large sample size (Parkin *et al.* 1987). Recently, protocols for the measurement of landscape rates by acetylene-blocked cores have been proposed (Tiedje *et al.* 1989). These methods have been used previously in a number of studies, and some of the results are presented in Table 2.3. As with the DEA measurements, landscape rates of denitrification are generally higher in wetland and riparian soils. There are a very limited number of reliable measurements of landscape rates of denitrification from different environments and this is an area which would benefit from further work.

Ecosystem	Field rate of denitrification ($kgN\ ha^{-1}yr^{-1}$)	Reference
Forest soils	<0.01 - 50	Summarised from Davidson *et al.* (1990)
Grassland	8 - 14	Bijay-Singh *et al.* (1989)
Arable land	50 - 87	Bijay-Singh *et al.* (1989)
Riparian soils low nitrate input high nitrate input	 26 - 183 700	 Cooper *et al.* (1990) Schipper *et al.* (in press)

Table 2.3 Landscape rates of denitrification measured from different ecosystems.

2.3.5 IMPLICATION OF DENITRIFICATION IN THE ENVIRONMENT

Biological denitrification is an important part of the total nitrogen cycle (Figure 2.2) and is thought to recycle between 52% and 110% of the total fixed nitrogen back to the atmosphere as nitrogen gases (Tiedje 1988). Denitrification converts a fixed form of nitrogen (e.g., nitrate) which is biologically and chemically active to a gaseous relatively-inert product (mostly nitrogen gas). In this way, denitrification can be both detrimental and beneficial to the environment. Some of the main effects are discussed below.

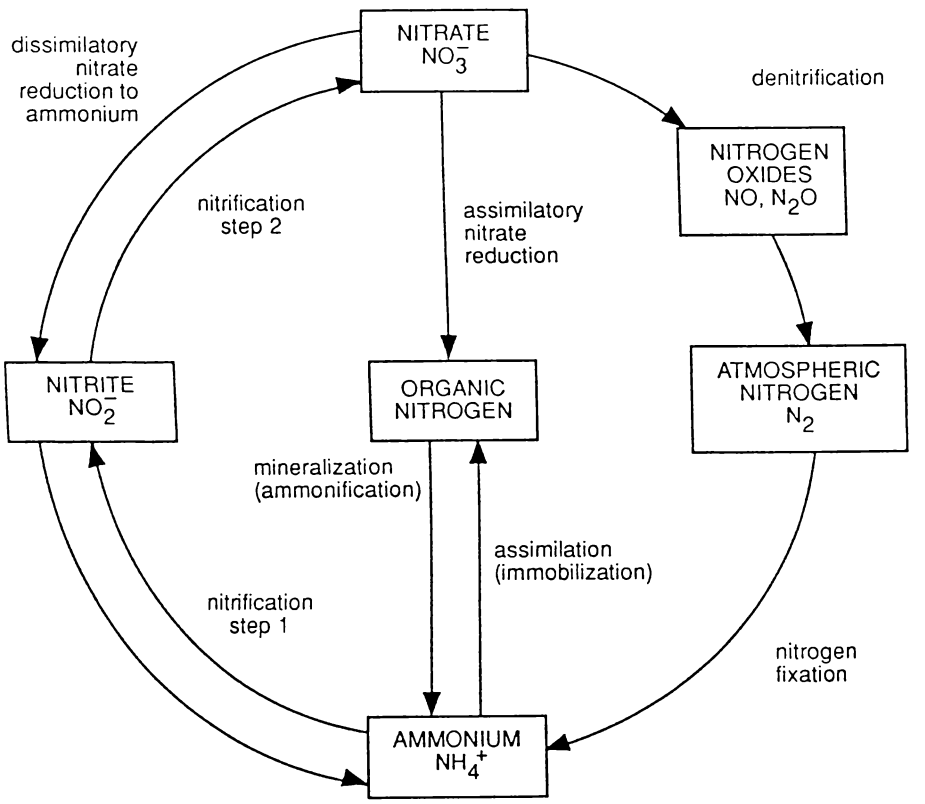


Figure 2.2 The nitrogen cycle showing the importance of denitrification in returning "fixed" nitrogen to the atmosphere. Adapted form Bowden (1987).

Loss of nitrogen from agricultural and forested land

Denitrification can remove substantial amounts of nitrogen from agricultural and forested soils and may thus reduce the crop yield. This nitrogen loss can be compensated for by adding nitrogen fertilizer; but this also increases the quantity of nitrate available for denitrifying bacteria. The loss of nitrogen fertiliser through denitrification has been estimated to be 10% (von Rheinbaben 1990), and between 20% and 30% (Firestone 1982) of that applied. These figures are likely to be highly dependent on the site, fertiliser application rate, and season. Continuing cultivation of agricultural and forested soil is likely to promote denitrification as these practices may increase the incorporation of organic matter in soil.

Nitrous oxide production and consumption

Nitrogen gas is generally the major product of denitrification; however, nitrous oxide can also be produced. Nitrous oxide has been suggested to account for approximately 5% of the expected global warming and the atmospheric concentration of nitrous oxide is increasing at approximately 0.2% per year (Rodhe 1990). Denitrification is only one of the sources by which nitrous oxide is produced; other sources of nitrous oxide result from nitrification and from chemical decomposition of nitrite and other nitrogen containing compounds (Sahrawat and Keeney 1986). The proportion of nitrous oxide to total nitrogen gas formed can vary widely depending on concentrations of carbon, nitrate, and oxygen, and on pH, temperature, and soil water content (Sahrawat and Keeney 1986).

Whereas denitrification can be seen as a source of N_2O, a number of denitrifying bacteria have been identified as capable of reducing N_2O to N_2 during growth (reviewed by Zumft and Kroneck 1990). Better understanding of the factors which regulate the proportion of N_2O produced by denitrification and the factors which regulate the rate of N_2O consumption may offer the possibility of managing total nitrous oxide flux from soil and water.

Nitrogen pollution management

Denitrification has been used as a means to remove nitrogen from wastewaters and research is continuing to improve the efficiency of nitrogen removal (Christensen and Harremoes 1977; Knowles 1982). In the natural environment, denitrification is important at mitigating the impact of excess nitrogen loading, particularly in groundwater and surface waters.

Increased nitrogen loading of land from fertilisation, nitrogen-fixing plants, or from the application of wastes to land, can elevate the nitrogen content of soil to a level above that of plant requirements. When this occurs there exists a potential for nitrate leaching to groundwater and finally into surface waters. Surface waters often also receive nitrogen directly following the discharge of wastewaters. Increased nitrogen loading to both groundwater and surface waters can have two main detrimental effects. Firstly, elevated concentrations of nitrate in drinking water has been implicated in a number of health-related problems, and second, increasing the concentration of nitrogen in surface waters can cause eutrophication.

A high nitrate concentration in drinking waters (the World Health Organisation suggested limit is 10 gNO_3-N m^{-3}) can cause methaemoglobinaemia in young infants and nitrate has also been considered to been linked to the occurrence of stomach cancer (Croll and Hayes 1988; Saull 1990). Much of the world's drinking water is derived from groundwater aquifers and many of these aquifers are becoming increasingly polluted by nitrate (Croll and Hayes 1988; Spalding and Exner in press). Denitrification in groundwater has been studied as a means

for reducing the nitrate concentration in groundwater (e.g, Smith and Duff 1988; Gillham in press), but to date no methods for enhancing the rate of nitrate removal appears to be successful at the aquifer scale. Another approach to the problem of high nitrate concentrations in drinking water is to pump groundwater out and remove nitrate by denitrification prior to distribution to water users (Rogalla *et al.* in press).

Elevated inputs of nitrogen, either from groundwater or from direct discharge of wastewaters can lead to the eutrophication of surface waters. Riparian zones have been shown to remove some of the nitrate from groundwater before it enters surface waters (Peterjohn and Correll 1984; Lowrance *et al.* 1984a; Jacobs and Gilliam 1985; Cooper 1990). Unfortunately, little is known about appropriate techniques by which riparian zones can be managed to elevate the degree of nitrate removal.

2.3.6 DISSIMILATORY NITRATE REDUCTION TO AMMONIUM

In anaerobic environments, nitrate can also be used as an electron acceptor and converted to ammonium rather than nitrogen gases (Tiedje 1988). This process is termed dissimilatory nitrate reduction to ammonium (DNRA). As this process utilises nitrate under anaerobic conditions it is likely to be in direct competition with denitrification. Much less is known about DNRA than denitrification. A comparison of DNRA and denitrification is shown in Table 2.4.

	DENITRIFICATION	DNRA
Carbon source	varied	varied
Endproducts	nitrogen gases	ammonium in solution
Organisms involved	facultative aerobes	facultative and obligate anaerobes
Other metabolic activity	aerobic	fermentation sulphate reduction
Electrons used per nitrate	5	8
$\triangle G^\circ$ kcal/mole nitrate	-133.9	-143.3

Table 2.4 Comparison of DNRA and denitrification processes. Summarised from Tiedje *et al.* (1982) and Tiedje *et al.* (1989).

From Table 2.4, it appears that the organisms involved in the two nitrate reduction pathways are from two distinct physiological groups. Denitrifiers tend to be facultative aerobes which use nitrate as an alternative electron sink to oxygen, whereas organisms responsible for DNRA tend to have other anaerobic metabolic pathways for growth when nitrate is absent. This suggests that organisms responsible for DNRA will be found mainly in permanently anaerobic environments whereas denitrifiers will be found in aerobic environments which

occasionally became anaerobic. While this may be true for DNRA, this may not hold for denitrifiers, since very high DEA's have been found in anaerobic environments. Some of the bacterial genera capable of DNRA which have been isolated include *Escherichia*, *Clostridium*, *Streptococcus*, *Klebsiella*, and *Achromobacter*, among others (Cole 1990)

Based on the distribution of where DNRA and denitrification predominate, Tiedje *et al.* (1982) hypothesised that the relative competition between these two groups of nitrate reducers was dependent on the ratio of carbon to e^- acceptor in the environment studied. They suggested that at high C:e^- acceptor ratios (e.g., the rumen and digested sludge) DNRA would dominate and, at relatively low C:e^- acceptor ratios (e.g., most upland soils) denitrification would predominate. This hypothesis was supported by the work of King and Nedwell (1985) who showed a shift from DNRA to denitrification as the nitrate concentration in soil increased. deCatanzaro *et al.* (1987) showed that DNRA was favoured over denitrification in more reduced environments, and again this may be a reflection of the C:e^- acceptor ratio.

Field studies of sediments may also be in agreement with these laboratory studies. Studies of sediments have shown that denitrification is most active at the surface and DNRA is more active at lower depths (Sorensen 1978; Jorgensen 1989). In sediments, nitrate diffusion from the overlying surface waters is likely to be one of the main limitations on nitrate reduction. As sediment depth increases nitrate concentration generally decreases as it is removed by denitrification; however, the carbon concentration is likely not to change as dramatically. These changes in concentration are likely to result in a increasing C:e^- acceptor ratio which makes the environment more favourable for DNRA.

Clearly, more work is necessary to understand DNRA and its impact in the environment. To date this has been hampered by the lack of simple methodology to assess DNRA in anaerobic environments.

2.4 REGULATION OF DENITRIFICATION IN THE ENVIRONMENT

2.4.1 INTRODUCTION

The rate of denitrification is regulated by a number of diverse environmental factors. These factors have been broadly divided into either proximate or distal regulators (Tiedje 1988) (Figure 2.3). Proximate regulators are those that affect the immediate environment of the bacterial cell, such as temperature, pH, oxygen, nitrate and carbon concentrations. Distal regulators control the proximate regulators on a larger scale such as rainfall, plant growth, soil structure and drainage pattern, and input of carbon by litterfall. Often a single distal factor influences more than one proximate factor. For example, rainfall may affect the diffusion of oxygen, carbon and nitrate. In most cases no individual proximate condition can be used to assess the denitrification rate in the landscape. It is important to distinguish between those factors which allow denitrification to occur and those factors which select the dominant denitrifying population (Figure 2.4). Denitrification will not generally occur in a soil microsite if O_2 is present or if either nitrate or carbon is absent. While extremes of temperature, pH, and probably nutrients (salts) can inhibit denitrification completely, these regulatory factors in most soils tend to select the dominant population of denitrifiers. The types of carbon compounds present may also place a selective pressure on the population of denitrifying bacteria.

Whereas many of the regulatory parameters have been investigated in laboratory studies, their effects at the landscape scale are poorly understood. One of the contributing reasons for the lack of information of landscape effects has been the difficulty and expense involved in using appropriate methodology. It is considerably easier to investigate the effect of proximate regulators as they are more suited to laboratory experimentation, whereas the investigation of distal effects is often confounded by on-site and between-site variability. In recent times, research has been directed towards identifying site or distal regulators which could be used to predict annual denitrification rates. In most studies of denitrification at the landscape scale, distal factors are examined but explanations for the regulation of denitrification are found by examining how these distal regulators influence some or all of the proximate regulators.

In the following sections (2.4.2 to 2.4.6), the proximate regulators (oxygen, nitrate, pH, temperature, and carbon) are discussed with emphasis on the influence of these regulators in the soil environment. The distal regulators are also included in this discussion. The influence of carbon is one of the main foci in this thesis and is dealt with in the most depth.

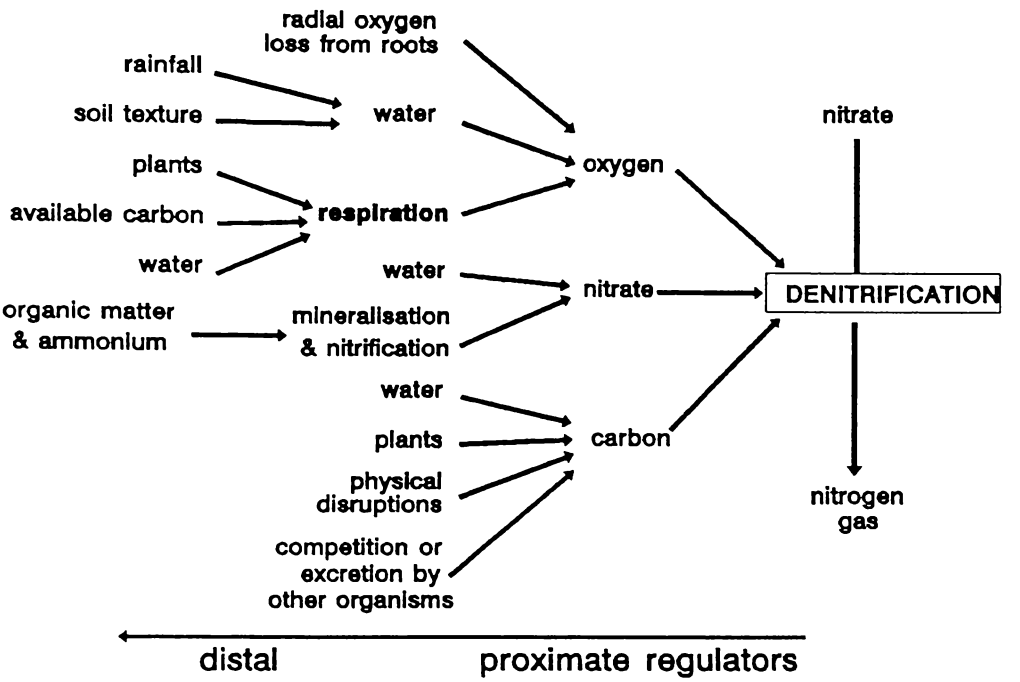


Figure 2.3 Proximate and distal regulators of denitrification (adapted from Tiedje 1988).

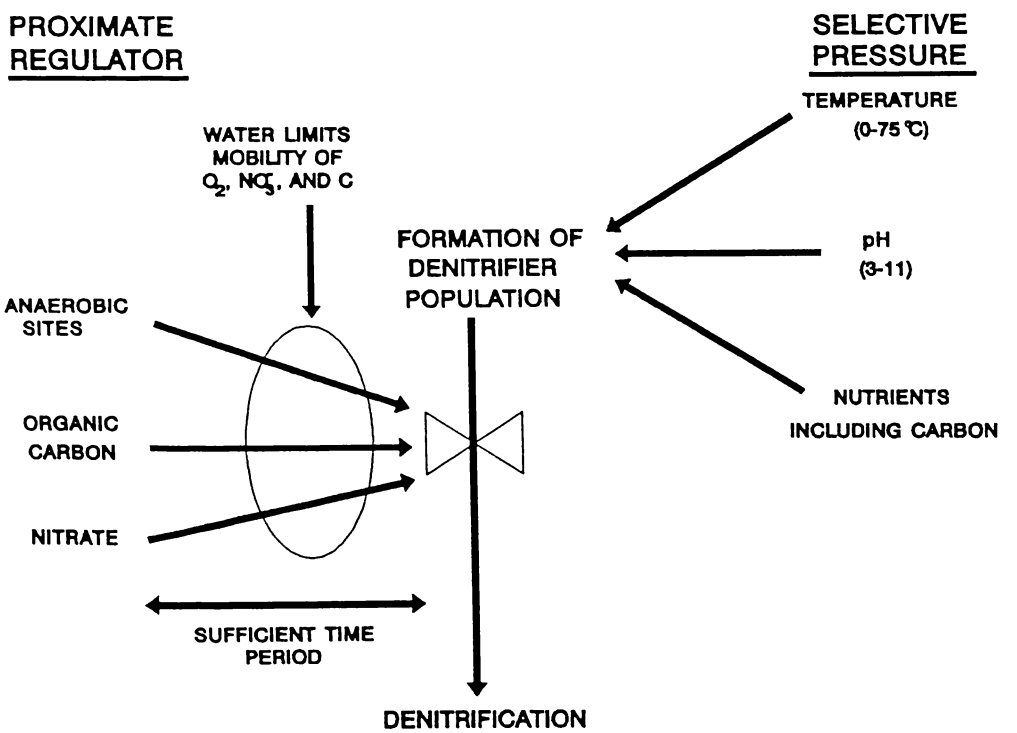


Figure 2.4 Proximate regulators and selective pressures which allow the formation of a denitrifying population and dictate the rate at which denitrification occurs.

2.4.2 OXYGEN

Introduction

Denitrifying bacteria are generally facultative aerobes (Jeter and Ingraham 1981) and under aerobic conditions these bacteria use oxygen instead of nitrate as an electron acceptor. The presence of oxygen causes a reversible inhibition of the bacterial enzymes involved in the denitrification process (Ferguson 1987). The mechanism by which oxygen inhibits denitrification is not known but discussion of possibilities is presented by Stouthamer (1988). Inhibition does not appear to be a passive preference of oxygen over nitrate as an electron acceptor because the introduction of oxygen causes a rapid cessation of nitrate reduction (Stouthamer 1988).

In soil, denitrification generally only occurs in sites which are anaerobic; this includes aerobic environments which have anaerobic microsites. Parkin and Tiedje (1984) demonstrated that the aerobic denitrification rate was typically 0.3-3% of anaerobic denitrification rates in soil cores. The oxygen concentration in soils is increased by diffusion from the atmosphere and decreases as oxygen is consumed by respiration, usually by aerobic soil bacteria. Hence any soil characteristic which influences either oxygen diffusion or consumption will directly affect the aerobic status of the soil and therefore the rate of denitrification. Two of the main distal regulators of oxygen concentration in soils are water content and organic matter.

Regulation of oxygen content in soil

Oxygen diffusion through water is considerably slower than through air and the addition of water (by rainfall or irrigation) to a site elevates denitrification rate (Sextone *et al.* 1985), presumably due to an increase in the number of anaerobic sites in the soil. The addition of organic matter such as straw (Aulakh and Rennie 1987) or manure (Guenzi *et al.* 1978) to aerobic soils increased denitrification and this was thought to be due to a decrease in oxygen concentration caused by the consumption of oxygen by aerobic bacteria. The spatial distribution of particulate organic matter (such as decomposing leaf material) has been shown to account for the high variability of denitrification in aerobic soils (Parkin 1987). Parkin argued that respiration was sufficiently high at the leaf surface to create anaerobic microsites for denitrification. For this mechanism to work, the organic matter needed to be coated by a film of water to slow inward oxygen diffusion (Parkin 1987). Rice *et al.* (1988) found increased rates of denitrification after injecting fermentation wastes into soil. Whereas the waste effluent increased nitrogen content of the soil, the respiration of carbon contained in the effluent created an anaerobic zone around the injection point and stimulated denitrification.

At a larger scale, the oxygen status of soil can be determined by soil drainage and texture. Soil texture and drainage were correlated to the denitrification rate at the landscape level by Groffman and Tiedje (1989a). Poorly drained soils were found to have higher denitrification rates than well drained sites. The higher water content of the poorly drained soils were expected to generate a higher proportion of anaerobic sites where denitrification could occur. These authors also showed that fine-textured soils had higher denitrification rates and suggested that this was because fine-textured soils have smaller pores that hold water more tightly and consequently become anaerobic more easily. Other authors (Davidson and Swank 1986; and Gilliam *et al.* 1979) have also found that the highest rates of denitrification occurred in soils where drainage was poor. A study by Groffman and Tiedje (1988) indicated that the rate of denitrification was strongly dependent on whether the soil was in a drying or wetting phase. They found that denitrification showed a hysteretic response to drying or rewetting of three different soils and suggested that this response was regulated by changes in oxygen concentration in soil pores.

Soil disturbance has also been shown to increase denitrification. This may be partly due to incorporation of organic matter and moisture, both of which may reduce soil oxygen concentration. Aulakh *et al.* (1984a,b) showed that nitrogen losses differed in fields that had been tilled using different methods. The variation in nitrogen loss was attributed to differences in the oxygen status of the soil following changes in soil moisture and the density of surface soil.

Riparian soils are often saturated by fluctuating groundwater levels and they can contain considerable amounts of organic matter. Such conditions ensure that a large proportion of these soils will be anaerobic; evidence for this was presented for type 1 riparian zones by Howard-Williams (1991). This suggests that oxygen may play less of an inhibitory role on denitrification in riparian soils than in many upland soils.

Aerobic denitrification

Denitrification has been reported to occur for short periods (5 hours) after oxygen was introduced to a previously anaerobically-grown bacterial culture (Lloyd *et al.* 1987), and some bacteria have been reported to denitrify under fully aerobic conditions in chemostat culture (Robertson *et al.* 1988a). When investigating the effect of oxygen on denitrification in sediments, Trevors and Starodub (1987) found that denitrification occurred in the presence of 0.33 to 10.1 μmol/ml oxygen; however, in this study the soil had been statically incubated and denitrification probably occurred in anoxic microsites. Groffman *et al.* (1988) suggested that anaerobic microsites could even form in aerobic liquid cultures, allowing denitrification to occur. While field studies to date indicate the rate of aerobic denitrification is considerably less than anaerobic rates, it should be remembered that the volume of aerobic soil is far

greater than the anaerobic soil volume. Thus even if aerobic denitrification is occurring at a much slower rate it may be significant at the landscape scale.

2.4.3 NITRATE

Introduction

Nitrate is used as the electron acceptor during the denitrification process, and hence nitrate concentration has a strong influence on denitrification rate. The availability of nitrate to denitrifying bacteria is dependent upon the rate of production (nitrification), the rate of nitrogen consumption by non-denitrifiers including plants and bacteria, and nitrate leaching and diffusion rates through the soil (Tiedje 1988).

Regulation of soil nitrate availability

Nitrate is produced from organic nitrogen by a series of microbial processes: ammonification converts organic nitrogen to ammonium which is then converted by nitrifying bacteria to nitrite and finally to nitrate. If the major form of nitrogen in an ecosystem is either organic nitrogen or ammonium, the rate at which mineralisation and nitrification occur may limit the rate of denitrification. Low denitrification rates in coniferous forests have been attributed to low rates of nitrate production by mineralisation and nitrification (Robertson and Tiedje 1984). Certainly, Cooper (1986) suggested that nitrification may be suppressed by the presence of live tree roots in an exotic conifer plantation. Davidson *et al.* (1990) showed that nitrate production increased following disturbance of forest soils; however, they did note exceptions such as forest with nitrogen fixing plants.

Nitrification is a strictly aerobic process and thus will be spatially separated from sites of active denitrification. Nitrate transport from aerobic sites to anaerobic sites where denitrification occurs may limit the rate of denitrification. A model developed by Myrold and Tiedje (1985a) suggested that nitrate diffusion became limiting in soil aggregates with a radius larger than 2 mm. In wetland soils where ammonium and organic nitrogen are the predominant nitrogen sources the rate of denitrification was thought to be limited by ammonium diffusion to aerobic sites and subsequent nitrification (Reddy and Graetz 1988). In organic riparian zones which are highly anaerobic it is unlikely that nitrate would be internally produced by nitrification and therefore that denitrification will be limited by nitrate influx in groundwater. Evidence for this comes from a study by Warwick and Hill (1988) who suggested that denitrification in type 1 riparian zones was not only limited by the concentration of nitrate but also by the residence time of groundwater containing nitrate.

Nitrate and denitrification kinetics

It is now generally accepted that the denitrification rate responds to nitrate concentration following Michaelis-Menten kinetics (Tiedje 1988; Firestone 1982). As such, the denitrification rate would be expected to follow first order kinetics (i.e. denitrification rate proportional to nitrate concentration) at low nitrate concentrations and zero order kinetics (i.e. denitrification rate independent of nitrate concentration) at high nitrate concentrations. In the past, it was speculated that the rate of denitrification was independent of the nitrate concentration and this reasoning may have been due to relatively high concentrations of nitrate used during the examination of denitrification kinetics (Firestone 1982). Reddy *et al.* (1980a) tested the effect of nitrate concentrations of 0.140, 0.35, 0.7 μmol N.l^{-1} on denitrification rate in an organic soil and found that the denitrification rate followed first order kinetics. These measurements were made in an organic soil and it was unlikely that carbon was limiting. Further investigation of a number of organic and mineral soils by Reddy *et al.* (1982) showed that the denitrification rate was dependent on both nitrate and available carbon concentrations.

In pure culture, denitrifying bacteria exhibit a very high affinity for nitrate, for example, the K_m values of three bacterial genera were reported as less than 15 μmol N.l^{-1} (Betlach and Tiedje 1981) which was in concurrence with the first order kinetics which Reddy *et al.* (1980a) observed at very low nitrate concentrations. Until recently, measurement of denitrifier affinity to nitrate in soil has been confounded by the difficulty of measuring very low nitrate concentrations. Murray *et al.* (1989) avoided this problem using soil which had previously been stripped of nitrate with an ion exchange resin and by denitrification. They then added known quantities of nitrate at very low concentrations and measured nitrous oxide accumulation. They were able to show that denitrifying bacteria in soil had a high affinity to nitrate (K_m ranging from 1.8 to 13.7 μM) which was similar to the K_m values found in pure cultures. The soil denitrifiers studied were shown to maintain this affinity in environments which received high nitrate inputs. These authors pointed out that these K_m values were similar in magnitude to the K_m values that plant roots have for nitrate suggesting that plants and denitrifiers compete for the same pool of nitrate in the soil.

2.4.4 pH

Given the large number of physiological groups and bacterial genera capable of denitrification perhaps it is not surprising that the pH range for denitrification is broad. In a review by Knowles (1982), denitrification was reported at a pH range of 3.5 to 11.0 and optimal soil pH for denitrification was considered to be between 7.0 and 8.0. It was suggested by Fillery (1983) that the pH optimum is slightly alkaline because this helps solubilise organic carbon.

In a study of denitrification in adjacent acidic and neutral soils, Parkin *et al.* (1985) showed that denitrification response was optimal at the pH of the respective soils. This suggested that denitrifying populations were adapted to pre-existing soil pH. Interestingly, Tiedje, who was one of the authors of this work, later commented that attempts at isolating acid-tolerant bacteria from the acidic soils had been unsuccessful (Tiedje 1988). The work by Parkin *et al.* (1985) made two other important points. First, denitrification rates were less in the soil with lower pH, which has previously been reported (Waring and Gilliam 1983). Second, denitrification was observed at pHs as low as 2.3, which raises questions concerning the limits of pH at which denitrification can occur.

2.4.5 TEMPERATURE

Denitrification, like most microbial processes, is dependent on temperature and has been recorded at temperatures between 0°C and 75°C (Knowles 1982). There is some debate concerning the lower range of temperature at which denitrification occurs; nevertheless, as the temperature approaches 0°C the denitrification rate also approaches zero.

It is thought that the response of denitrification to temperature follows the Arrhenius equation and evidence for this is discussed by Firestone (1982). The rate of most biochemical reactions approximately doubles when the temperature is increased by 10°C (Lehninger 1975), that is having a temperature correction factor (Q_{10}) of 2. A number of estimates of a temperature correction factor for denitrification rate have been reported: 1.9 (Jacobson and Alexander 1980); and between 1.4 and 2.5 (Reddy *et al.* 1982).

One of the major difficulties in measuring the effect of temperature on denitrification is that in the natural environment some of the other regulatory factors are often rate-limiting. Also, aside from its direct effect on enzymes involved in denitrification, temperature will affect a range of other biological and physical processes such as mineralisation and nitrification, diffusion rate of nitrate and carbon, and the solubility and diffusion rate of oxygen. Thus temperature may have complex synergistic effects on the rate of denitrification in soils.

Powlson *et al.* (1988) showed that denitrifying bacteria from temperate soils reduced nitrate at a lower temperature optimum than did bacterial populations from sub-tropical soils, indicating that denitrifying bacteria adapt to the soil temperature. In this way temperature acts as a selective pressure on denitrifier populations much as pH, and only in temperature extremes is denitrification likely to be completely inhibited.

2.4.6 CARBON

2.4.6.1 Introduction

The importance of the supply of organic matter in denitrification has been recognised for over a hundred years (Maquenne 1882). Yet in a recent review, Beauchamp *et al.* (1989) concluded that: "one of the most striking revelations of this review is the scarcity of information on the biodegradation of C [carbon] compounds and natural substrates in conjunction with denitrification under anaerobic conditions".

Carbon is the electron donor and provides the energy for the denitrification process (Tiedje 1988). During denitrification, electrons are transferred from reduced, organic carbon compounds to nitrate via an electron transport chain, resulting in the production of energy for the bacterial cell. Equation 1 shows a theoretical balance for this reaction

$$5CH_2O + 4NO_3^- + 4H^+ \text{ ---------> } 5CO_2 + 2N_2 + 7H_2O \text{ .....(1)}$$

Carbon compounds will also act as the main source of carbon for cell growth of denitrifying bacteria.

2.4.6.2 Carbon sources which stimulate soil denitrification

<u>Single sources of carbon</u>

Denitrifying bacteria are from diverse taxonomic groups which can utilise a broad range of organic compounds aerobically (Jeter and Ingraham 1981). Stanier *et al.* (1975) suggested that denitrifying bacteria could utilise the same compounds anaerobically (nitrate as electron acceptor) and aerobically with the exception of aromatic compounds. They considered that oxygen was required to initially break open the aromatic ring. However,a number of aromatic compounds have been shown to be utilised by denitrifying bacteria and support bacterial growth - eg., benzoate (Taylor *et al.* 1970), and phenol (Tschesch and Fuchs 1987). A range of sugars have also been shown to be used during denitrification (e.g. deCatanzaro and Beauchamp 1985). It is interesting to note that these compounds are the decomposition products of structural organic polymers in plants such as lignin, cellulose, and hemi-cellulose; however, pure cultures of denitrifiers have not been reported to degrade these larger structural compounds. Table 2.5 lists some of the main carbon compounds which have been identified to stimulate denitrification following their addition to soil.

Carbon group	Compound/material	Reference
PLANT MATTER	Straw	deCatanzaro and Beauchamp (1985) Aulakh *et al.* (1984b)
	Alfalfa	deCatanzaro and Beauchamp (1985) Myrold and Tiedje (1985b)
POLYMER	Cellulose	Bremner and Shaw (1958) Gok and Ottow (1988)
AROMATICS Reviewed by Evans and Fuchs (1988)	benzene	Major *et al.* (1988)
	toluene	Kuhn *et al.* (1988)
	naphthalene & acenaphthalene	Mihelcic and Luthy (1988)
	m-xylene	Kuhn *et al.* (1988)
Sugars	glucose	Bremner and Shaw (1958) Jacobson and Alexander (1980) deCatanzaro and Beauchamp (1985) Paul *et al.* (1989)
	sucrose	Bremner and Shaw (1958) deCatanzaro and Beauchamp (1985) Paul *et al.* (1989)
	mannitol	Bremner and Shaw (1958) deCatanzaro and Beauchamp (1985)
Fermentation endproducts	methanol	Jacobson and Alexander (1980) Yeomans and Bremner (1989)
	ethanol	Yeomans and Bremner (1989)
	acetone	Yeomans and Bremner (1989)
	succinate	Jacobson and Alexander (1980)
	butyrate	Paul *et al.* (1989)
	propionate	Paul *et al.* (1989)
	acetate	Paul *et al.* (1989)
	formate	Soares *et al.* (1991)
	citrate	Bremner and Shaw (1958)
	glycerol	deCatanzaro and Beauchamp (1985)

Table 2.5 Some of the studies which demonstrated stimulation of denitrification following addition of different carbon compounds to soil. This is not an all exhaustive list but highlights some of the variety of the carbon compounds used.

Complex sources of carbon

A number of studies have examined denitrification in soils following the addition of plant material. deCatanzaro and Beauchamp (1985) found that incorporation of alfalfa increased denitrification rate more than the incorporation of straw. It was suggested that the higher levels of water-extractable carbon and less lignin and cellulose in alfalfa compared to straw may have contributed to this phenomenon. Other studies have also shown increases in denitrification following the addition of wheat (Aulakh *et al.* 1984b; Aulakh and Rennie 1987) and alfalfa (Myrold and Tiedje 1985b). In these studies it is often difficult to establish whether

the addition of plant matter provided more carbon for denitrification or whether the addition of carbon stimulated respiration thus lowering oxygen concentrations in the soil.

Carbon sources other than plant material have also been shown to increase the denitrification rate. The addition of external carbon sources to nitrate-rich effluents has been attempted to increase nitrate removal by denitrification with mixed results (reviewed by Christensen and Harremoes 1977). Methanol was the most popularly used as a cheap source of carbon and appeared to give maximum denitrification rates. A number of industrial wastes with high chemical and biological oxygen demand were examined by Skrinde and Bhagat (1982) as alternatives to methanol. Yeast and spent sulphite liquor were found to result in optimum denitrification, equivalent to that with methanol. Kaplan *et al.* (1987) also tested a variety of industrial wastes high in total organic carbon. They ranked the wastes on their minimal $C:NO_3^-$ ratio required to remove 95% nitrate and 90% total carbon; methanol was ranked first closely followed by sweet whey and acid whey.

Essentially it appears that the addition of most carbon sources is likely to stimulate denitrification. However, the extent of this stimulation will be dependent on not only the quantity but also the quality of the carbon added. Most studies have not identified whether the carbon added was used by denitrifying bacteria directly and in many cases it is likely that the addition of a carbon source stimulated a number of different populations of soil bacteria including denitrifiers.

2.4.6.3 Availability of carbon

Accessibility of soil carbon

Whereas soil may contain considerable amounts of carbon, the accessibility of carbon supply to bacteria has a strong regulatory effect on denitrification. Increased carbon availability following soil disturbance has been shown to increase denitrification. In carbon-limited soils denitrification rates were found to be less in large soil aggregates than in smaller soil aggregates (Seech and Beauchamp 1988). Grinding the larger aggregates to a smaller size increased the denitrification potential above its antecedent potential and it was suggested that this was due to increased accessibility of carbon. Denitrification has been shown to be limited by the diffusion rate of organic compounds in some soils (Myrold and Tiedje 1985a). Following freezing and thawing of soil, carbon may be liberated and the denitrification rate has been shown to increase (Breitenbeck and Bremner 1987). These authors also found an increase in denitrification rate after air-drying of soil and attributed this to increased carbon accessibility.

Terry and Tate (1980) compared the denitrification rate of cropped soils to fallow soils and showed that the cropped soils had higher denitrification rates which may have been due to greater availability of organic carbon in cropped soils. Bakken (1988) found that soil denitrification was stimulated by plant growth and postulated that this was due not only to decreased oxygen in soil microsites but also due to increased organic carbon which was released by the plant roots.

Carbon quality

Many researchers have found increases in denitrification after incorporation of carbon into soil. However, only a fraction of the carbon is available for denitrification. In laboratory experiments, Burford and Bremner (1975) showed that the denitrification capacities of soils was most highly correlated to water soluble carbon ($r=0.99$, $n=17$) but less well correlated to the total carbon ($r=0.77$, $n=17$). Similar relationships were found in a number of other studies which examined a wide range of organic and mineral soils (Reddy *et al.* 1980b), water saturated organic wetland and riparian soils (Schipper *et al.* in press), and air-dried and field-moist mineral soils (Bijay-Singh *et al.* 1988). A possible reason for this correlation is found in pure culture studies of denitrifiers which have shown that denitrifiers tend only to use simpler rather than more complex carbon compounds.

Decomposition of plant matter

The quality of organic matter in riparian soils has been shown to have a major regulatory role on the maximum rate of denitrification and this is also true for other soils. Information on organic matter supply in riparian soils is limited; however, the availability of organic matter is likely to be dictated by the rate and mechanism by which dead plant matter decomposes.

Godshalk and Wetzel (1978a,b) identified three phases during the degradation of detritus: 1.) rapid leaching and autolytic release of dissolved organic matter which then may be rapidly metabolised; 2.) breakdown of polymers such as cellulose directly by bacteria at a much slower rate; 3.) degradation of highly refractory compounds at rates which may approach zero. A number of environmental factors regulate the degradation rate of plant material such as moisture (Sorenson 1974), oxygen (Godshalk and Wetzel 1978a,b), fibre content (Godshalk and Wetzel 1978a), C:N ratio (Howard-Williams 1983), and temperature (Godshalk and Wetzel 1978c).

Water-soluble carbon is produced during the decay of litter material by two main routes: 1.) partial decomposition of structural components and 2.) leaching of water soluble compounds (Polunin 1984). Under aerobic conditions organic substrates are often completely oxidized to CO_2, whereas under anaerobic conditions breakdown of organic substrates may be incomplete, such as the fermentation process resulting in soluble organic endproducts. A

study of anaerobic and aerobic plant degradation found more dissolved organic compounds formed in anaerobic incubations than aerobic incubations (Godshalk and Wetzel 1978a). It was postulated that dissolved organic compounds formed during aerobic incubations were completely metabolised to carbon dioxide.

2.4.6.4 Influence of non-denitrifying bacteria on carbon availability

As discussed in section 2.4.6.2, denitrifiers grown in pure culture are thought to only be capable of degrading relatively simple carbon compounds and have not been shown to degrade polymers such as cellulose. A number of studies have indicated that denitrifying bacteria are dependent on other soil bacteria (aerobic and anaerobic) to make carbon available.

In a review, Beauchamp *et al.* (1989) suggested that denitrifying bacteria may be involved in a syntrophic relationship with fermentative bacteria. Certainly, Reddy *et al.* (1980a) found evidence that the denitrification rate was limited by the rate at which the carbon was made available to denitrifiers. Fermentative bacteria degrade larger carbon compounds to fermentation end-products which may in turn be used as an energy source for denitrification. Many of the end-products formed during fermentation are used by denitrifying bacteria (Table 2.5).

Evidence for a relationship between fermenters and denitrifiers was presented by Paul and Beauchamp (1989a) who showed that fermentation and denitrification occurred simultaneously during the anaerobic degradation of plant material. In another series of experiments, Paul and Beauchamp (1989b) incubated manure in soil and monitored denitrification. They found that denitrification was most closely related to the disappearance of volatile fatty acids (VFAs) which were produced from readily available carbon by fermenters. There is evidence to suggest that low molecular weight fulvic acid-like compounds (these are low molecular weight polycarboxylic acids) are mineralised during denitrification (Katz *et al.* 1985). Fulvic acids are water soluble and therefore readily accessible to bacteria. Although Katz *et al.* (1985) did not show that denitrifying bacteria utilise these compounds directly, they concluded that denitrification would in part be dependent on the release of fulvic acid-like compounds by respiration.

A conceptual model of how the interaction between denitrifiers and fermenters might occur is shown in Figure 2.5. In this model denitrifiers in anaerobic environments are dependent on the initial breakdown of plant and structural compounds by other bacteria. Denitrifiers then compete directly for the sugars, aromatics, fermentation endproducts, and volatile fatty acids. Whereas fermenters may provide a supply of carbon for denitrifiers from decaying organic matter, fermenters may also be in direct competition with denitrifiers for simple carbon compounds such as sugars. Competition between denitrifying bacteria and other anaerobic bacteria is an area in which little research has been done.

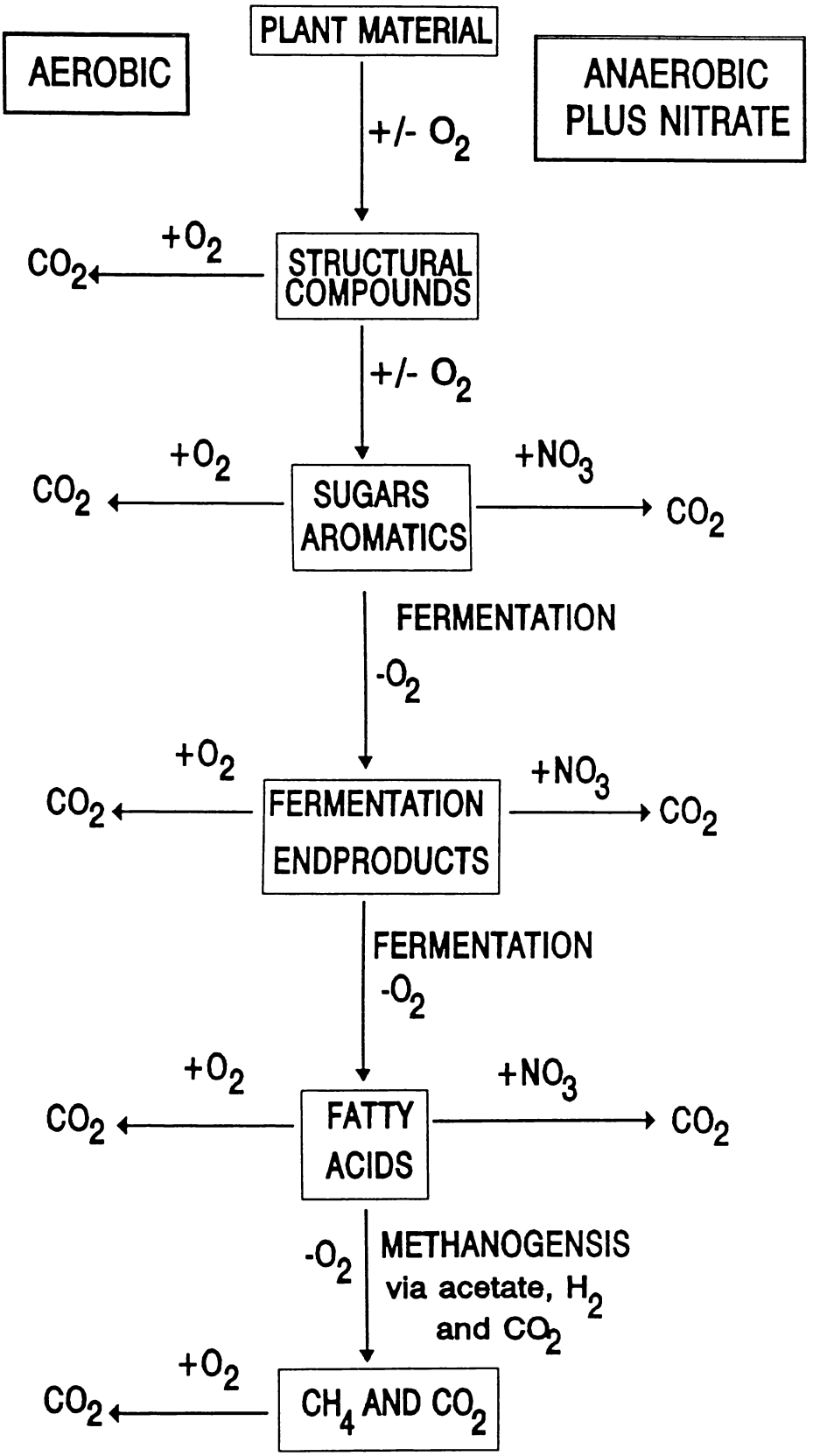


Figure 2.5 Degradation of plant material in aerobic and anaerobic environments showing the release of carbon compounds for which denitrifiers may compete.

2.5 FUTURE RESEARCH NEEDS

This literature review was divided into two major sections; nitrogen removal in riparian zones, and denitrification. There is a vast amount of information on wide ranging aspects of denitrification while there is considerably less on nitrogen removal in riparian zones. Both of these areas would benefit from further research and outlined below are some of the key areas.

2.5.1 DENITRIFICATION

Further research into denitrification needs to be directed into both larger (field and landscape) and smaller scales (soil microsite and bacterial cell). Denitrification rate and its regulation are often measured in laboratory incubations of soil samples. A better understanding of how the denitrification rate and its regulators can be managed at the landscape scale is needed.

Rates of denitrification and its regulation

There are only a limited number of measurements of denitrification at the landscape scale which have been measured using the same methodology. More such measurements are needed, along with the study of those regulatory factors which dictate the rate of denitrification at the landscape scale. Identification of these regulatory factors may lead to a better understanding of approaches which could be used to manage the rate of denitrification.

Management practices which reduce denitrification would be useful in reducing nitrogen losses from agricultural and forested soils and thus reduce the fertiliser requirement. It would also be useful to identify means by which DNRA could be stimulated in these soils; this would decrease nitrate leaching and make more nitrogen available for plant uptake.

Management practices which increase the rate of denitrification in riparian zones would result in less non-point source pollution of surface waters by nitrate. In most instances, agricultural and forestry practices do not include management of riparian zones at all. As application of wastes to land is becoming increasingly popular, techniques for enhancing onsite denitrification would be useful for reducing nitrogen load on the aquifers below.

Ecology of denitrifiers

Two main aspects of denitrifier ecology are of particular interest; how they survive in anaerobic environments in the absence of nitrate, and how they interact with other anaerobic and aerobic bacteria.

The denitrifying enzyme activity of many anaerobic soils is very high despite the fact that in anaerobic soils the rate of nitrate formation is very low. How these bacteria survive in these soils is unclear and further work needs to establish whether they survive using an alternative form of metabolism or whether denitrifiers are capable of long-term dormancy.

Being facultative aerobes, denitrifying bacteria will be in competition for nutrients and carbon with anaerobes when oxygen is absent and nitrate is present. The reliance of denitrifiers on other bacteria to release carbon compounds needs to be examined as does the competition between denitrifying bacteria and other anaerobes.

A specific regulatory factor of interest, in this thesis, is the effect of quality and source of carbon on denitrification in type I riparian soils and the competition for this carbon by other bacteria.

2.5.2 NITROGEN REMOVAL IN RIPARIAN ZONES

Although denitrification has been shown to occur in riparian zones, few measures of rates of nitrate removal have been made, particularly at the catchment scale. Also, little is understood about the factors which regulate denitrification in these soils.

Once nitrogen removal in riparian zones is quantified, appropriate management practices of riparian zones for protection of surface water quality can be developed and tested. Development of appropriate management practices for the protection of water quality are likely to be achieved by first obtaining a better understanding of the factors which regulate denitrification in riparian zones at the landscape scale.

One area of particular interest is the influence of riparian vegetation. The rates of productivity, death, and decomposition of this vegetation will contribute organic matter to the riparian zone. The quality and quantity of organic matter has been shown to be of critical importance to the rate at which denitrification proceeds.

Any management practices which are developed need to consider the ecological role of riparian zones such as provision of wildlife habitat.

CHAPTER 3
COMMON METHODOLOGY

3.1 INTRODUCTION

In this chapter the methodology common to Chapters 4 to 7 is described. Methodology specific to each chapter is described in that chapter. Similarly, any modification of the methods described in this chapter is detailed in the chapter in which the method is used.

3.2 SOIL COLLECTION AND STORAGE

In this section, brief discussion of the possible alternative methods of soil preservation and storage is given, followed by justification of the method finally used (section 3.2.1). The second section (3.2.2) discusses the method by which soil was collected, preserved and stored.

3.2.1 CHOICE OF STORAGE METHOD

Discussions on bacterial preservation can be found in Gherna (1981), Heckly (1978), and Stolp and Starr (1981). From these reviews it is clear that the majority of commonly-used preservation techniques were developed for the maintenance of bacterial pure cultures and that little is known about the preservation of mixed cultures found in soil. The five common preservation techniques identified in the reviews above were: storage in liquid nitrogen, freeze drying, air drying, storage above freezing, and storage below freezing. Some of these techniques, although very successful for the preservation of pure cultures of bacteria, would be very expensive for storage of large quantities of soil. This is particularly true for storage in liquid nitrogen and freeze-drying which also require specialised equipment. These methods were not considered practical for this study. The other methods are briefly discussed below, along with conclusions pertinent to this thesis.

Air-drying

Air-drying of soil is often used to preserve and store soil, although the effects of this technique on the populations and their activity are not well understood. The effect of air-drying soil on denitrification potential has been examined by Smith and Parsons (1985) who found a decrease of between 16% and 29% of the denitrification potential rate when compared to fresh soils. Similar changes in denitrification potential were found by Bijay-

Singh *et al.* (1988) following air-drying; they also showed that this treatment altered the availability of soil carbon to the denitrifiers present. Air-drying of water-saturated organic soil takes a long time, during which a considerable loss of organic matter might be expected. Also, a number of anaerobic bacteria (e.g. methanogens) are very sensitive to oxygen and may not survive air-drying.

Storage at 4°C

Studies by Breitenbeck and Bremner (1987) showed that soil storage for 30 days at 4°C did not affect denitrification rates compared to those from fresh soils. Although bacterial activity decreases as the temperature decreases, it was considered that some slow metabolism would continue at 4°C and that during long-term storage some bacterial populations would change. It was originally envisaged that for the present study the same soil stock would be needed for at least two years and that storage above freezing would not be satisfactory for this term.

Storage below 0°C

Early work by McGarity (1962) examined the effect of soil storage at a range of temperatures from 0° to -78°C and suggested that denitrification increased following freezing and thawing due to the release of organic matter. Breitenbeck and Bremner (1987) also found increases in denitrification rate following freezing and thawing. They found that storage of mineral soils at -4 °C in the presence of added organic carbon increased denitrification rates to above those in fresh soils with the same carbon addition; however, the increase in denitrification was not dependent on the length of time for which soil was frozen. Denitrification in these soils responded similarly, whether stored for 2 days or 80 days. Mackey (1984) suggested that during frozen storage, death of micro-organisms may initially be high, but after this initial period bacterial numbers remained unchanged.

In a series of experiments which examined the effect of fumigation on carbon dioxide evolution in soil, Jenkinson and Powlson (1976) also required a single soil source in order to allow comparisons to be made between experiments conducted over three years. These authors discussed methods for soil preservation and concluded that storage at -15°C was the best option. Storage by this method resulted in an slight increase of respiration rate over the three year study period.

Conclusion

There does not appear to be a universally-accepted method for the long-term preservation of soil bacterial populations. For the present study, freezing and storage was considered the best option for soil preservation. Soil storage by freezing would be likely to preserve oxygen-

sensitive bacteria and soil organic matter content. Although initial bacterial losses upon freezing would probably be high, the evidence from the literature suggests that once frozen, changes in bacterial numbers would be small. A -20°C freezer was the lowest storage temperature available and was used here. The effects of storage at -20 °C on soil bacterial populations is described in Chapter 4.

3.2.2 SOIL COLLECTION AND STORAGE

Organic soil for storage was collected on 26th January 1989 from the interface of an organic riparian zone and wetland in Whakarewarewa Forest about 10 km south of Rotorua. The soil was taken from a one metre square area of riparian zone where some surface water was present. This riparian zone was previously observed to have upland groundwater passing through to a wetland below. Approximately 90 kg of wet soil was collected, returned to the laboratory in large buckets, and sieved (1 cm mesh size) into a 200 litre container. The soil in the container was thoroughly mixed and left to stand for two hours to allow some removal of oxygen by bacteria. A small beaker was then used to remove approximately 100 ml aliquots of wet soil which were placed into aluminum-foil seed bags (17 cm by 27 cm) with an internal plastic lining (F. Cooper Limited, Auckland). The air was pressed out and the bag sealed by a hot iron which sealed the internal plastic lining. The sealed seed bags were then left at room temperature for 17 hours to allow resident bacteria to remove oxygen. The soil in these sealed bags was flattened to approximately 1 cm thickness to speed up freezing. All bags were then placed in a -20°C freezer room where they froze in I to 1½ hours.

3.3 PREPARATION OF SERUM BOTTLE INCUBATIONS

Soil was used either from stored stocks in seed bags or freshly collected from the organic riparian zone as described in section 3.2.2. Bags of frozen soil were removed from the freezer and thawed by placing in warm water where they thawed generally in five minutes. The contents of typically five to ten bags (each containing >100 g wet soil) were combined in a large beaker. Fresh soil was collected in a large bucket and immediately transported back to the laboratory (< ten minutes travel time). A soil subsample of approximately 30 g wet weight was rapidly transferred from the bucket or beaker into pre-weighed 160 ml serum bottles (Gibco NZ Ltd) using a small solids funnel. In later experiments, the soil slurry was transferred using an 10 ml autopipette with a plastic tip with the end cut off. Serum bottles were reweighed to determine the exact amount of wet soil added. The headspace was swept with welding grade argon at a rate of greater than 500 ml/min for 4 minutes in order to remove oxygen. Serum bottles were sealed using thick black butyl-rubber stoppers and crimped in place with a aluminium cap (Gibco NZ Ltd). A syringe needle was attached to a two-way air-tight stopcock (Cole Palmer, USA) and passed through the rubber stopper.

Additions of nitrate, carbon source, and acetylene were made as appropriate. Acetylene (16 ml) was added to each serum bottle to inhibit the final step of denitrification (Yoshinari and Knowles 1976). Acetylene was produced as described in section 3.8. All incubations were in the dark at 25°C in a orbital incubator shaking at 200 rev/min.

During incubation, headspace samples were periodically taken through the stopcock. A gas sample (3 ml) was then removed by syringe and placed under pressure in a vacutainer (2 ml, Becton Dickinson, New Jersey, USA) until analysis (section 3.6). Before samples of the headspace were taken, the headspace pressure was recorded by pressure transducer (similar to that described by Shelton and Tiedje 1984). The pressure transducer was calibrated by measuring the pressure within the serum bottles which had known volumes of air added.

At the end of the incubation the headspace volume was determined gravimetrically by the addition of water to fill the serum bottle. All bottles were briefly shaken by hand to suspend the soil and a liquid sample taken, passed through a 0.45μm filter and kept frozen until analyzed for nitrate and ammonium (section 3.5). On occasion, fermentation endproducts in the liquid sample were analyzed by flame ionisation gas chromatography (section 3.7). Serum bottles and their contents were then dried at 105°C in an oven to determine dry weight and moisture content of soil.

3.4 PHYSICAL AND CHEMICAL ANALYSIS OF SOIL

Many of the methods described here are standard procedures and only a brief description of each method follows with a reference for full details. Soil analyses were reported on an oven dry weight basis; however, oven drying at 105 °C can change some soil properties and therefore air-dried soil (40 °C) was used for analysis. The ratio of air-dried weight to oven-dry weight was used to express results on an oven-dry weight basis. Air-dried soil was ground through a sieve (2 mm mesh) before analysis.

Total soil carbon

Total carbon was analyzed by the colorimetric method described in Nicholson (1984) and sucrose was used to generate the standard curve.

Soil organic matter

Soil organic matter was determined by the loss of weight following ignition of air-dried soil at 500°C for one hour (Hesse 1971).

Total Kjeldahl nitrogen

A semi-micro Kjeldahl technique was used to determine total Kjeldahl nitrogen. The ammonium formed was measured by the method developed by Searle (1974) using a Technicon AutoAnalyser II.

Soil pH

Soil pH was measured in air-dried soil (5 g) suspended in 25 ml of water which was left to stand overnight before measurement using a PNM portable pH Meter (Watson Victor Ltd).

Cation exchange capacity

Cation exchange capacity was measured on air-dried soil using the method described by Nicholson (1984).

Particle size analysis

Particle size analysis (Piper 1950), as described by Nicholson (1984), was used to quantify the percentage of sand, silt, and clay in air-dried soil.

3.5 WATER ANALYSIS

Nitrate analysis

Two methods were used to determine nitrate concentrations. Both methods used a Technicon AutoAnalyser II (Technicon Instruments Corporation, New York) and both methods for nitrate measurement included the nitrite ion. In lysimeter samples and water extracts of soil, nitrate was analyzed using the hydrazine sulphate reduction method (APHA, AWWA, WPCF 1989). The hydrazine sulphate method was not used for analysis of KCl extracts of organic soils because of difficulties in obtaining a stable standard curve. Instead, nitrate was analyzed using the cadmium reduction method (United States Environmental Protection Agency 1979).

Ammonium analysis

Ammonium in water and KCl extracts was measured colorimetrically by the method of Searle (1974) using a Technicon AutoAnalyser II (Technicon Instruments Corporation, New York).

Chloride analysis

Chloride concentration was measured in lysimeter samples by the ferricyanide method (APHA, AWWA, WPCF 1989).

Water-soluble carbon analysis

The amount of water-soluble carbon was determined using the chemical oxygen demand method of Smith *et al.* (1982). Potassium hydrogen phthalate was used to generate the standard curve.

3.6 QUANTIFICATION OF NITROUS OXIDE, CARBON DIOXIDE AND METHANE

Gas subsamples were removed from the vacutainers using gas-tight gas chromatography syringes and analyzed for nitrous oxide, carbon dioxide, and methane

Nitrous oxide

Nitrous oxide was analyzed using gas chromatography (Kaspar and Tiedje 1980). Gas samples were separated on a 2 m column packed with Poropak QS. At the injector end of the column 10 cm of $MgSO_4$ (anhydrous) was packed in order to trap any injected water vapour. Nitrous oxide was quantified by an electron capture detector set at 300 °C (Hewlett Packard 5890 series II). The oven temperature was 70 °C and the injector port temperature was 140°C. Carrier gas consisted of argon with 10% (v/v) methane at a flow rate of 30 ml/minute. The carrier gas was first passed through a molecular sieve (5A 45/60 Mesh supplied by Hewlett Packard) to remove contaminants. When in use, the oven temperature was raised to 150 °C at least daily in order to drive off water trapped on the $MgSO_4$. Using standard gases, the practical detection limit was routinely 0.04 nmol N_2O. Duplicate injections of standard gas were generally within 3% of one another. Gas chromatograph conditions were such that clean separation of gases of interest occurred. Approximate retention times for the gases were: N_2 1.2 min, N_2O 2.6 min, acetylene 3.7 min. Exact retention time was dependent on the precise carrier gas flow rate.

Standard curves were generated using gas mixtures of known composition which were obtained from New Zealand Industrial Gases Ltd (Rotorua).

Carbon dioxide and methane

Carbon dioxide and methane were analyzed using an isothermal gas chromatograph (Carle Instruments Inc.) fitted with a thermal conductivity detector. The column was packed with

Poropak N and the carrier gas was helium (20 ml/min). The oven temperature was 85 °C. The detection limits for carbon dioxide and methane were both less than 0.5 μmol. Duplicate injections of standard gas were generally within 3% of one another. Approximate retention times for the gases were: N_2 0.7 min, CH_4 0.9 min, CO_2 1.8 min. Exact retention time was dependent on the precise carrier gas flow rate.

Standard curves were generated gases of known composition which were obtained from New Zealand Industrial Gases Ltd (Rotorua).

Calculation of gas concentration

Calculation of the amount of gas produced over time was done in the following manner. Following gas analysis by gas chromatography standard curves were used to calculate the number of moles of a gas in the injected volume. This was then adjusted for the headspace volume and pressure using Boyles law. Finally the number of moles of gas were adjusted for the amount of gas removed during previous samplings.

Nitrous oxide concentration was corrected for gas dissolved in water using Bunsen coefficients (Tiedje 1982).

3.7 VOLATILE FATTY ACID ANALYSIS

The concentrations of the volatile fatty acids: acetate, propionate, *i*-butyrate, and *n*-butyrate in water samples were determined using a modified method of Banfield *et al.* (1978). VFAs were quantified using a flame ionisation detector (FID) attached to a gas chromatograph (HP5840). VFAs were initially separated on a Chromosorb G (80-100 mesh) column. The carrier gas was nitrogen (30 ml/min) and was bubbled through 90% formic acid which aided separation of VFAs. FID gases were hydrogen (30 ml/min) and air (300 ml/min). Injector temperature was 200 °C, detector temperature was 240 °C, and the isothermal column temperature was 140°C. Prior to injection a water sample (2 ml) containing unknown VFAs had added 0.4 ml of i-valeric acid (1000 ppm) as an internal standard. Standard curves were generated using known concentrations of VFAs.

3.8 ACETYLENE PRODUCTION

Acetylene was used to block the conversion of N_2O to N_2 and was prepared in the following manner. Water was slowly added to calcium carbide in a sealed side-arm flask. The acetylene formed passed through the side-arm into the top of a second side arm flask and was bubbled through an acidified copper sulphate solution to remove gaseous contaminants. Acetylene then passed out of the second flask through its side-arm and into rubber tubing to which was attached a syringe needle leading into a serum bottle through its rubber stopper. The serum bottle was initially filled with distilled water and as acetylene gas was produced the water was displaced out through a second needle inserted in the rubber stopper. Using this method, acetylene could be prepared in the laboratory and stored before use.

CHAPTER 4

EFFECT OF SOIL STORAGE ON THE DENITRIFICATION ACTIVITY OF SOIL

4.1 INTRODUCTION

A major component of this thesis investigated the effect of different carbon sources on denitrification in organic riparian soils. To enable valid comparisons to be made between experiments conducted at different times, minimal change should occur in the soil bacterial population and activity. In an attempt to minimise such change a single collection of soil was made and stored at -20°C for subsequent experiments (described in section 3.2). The collected soil was sieved and thoroughly mixed in order to reduce the variability between subsamples taken for experiments. It was thought that the effect of differences between soil samples was likely to increase the longer the incubation period.

It was not known how soil storage would change the activity of resident bacteria. In this chapter the response of denitrifying and fermentative bacterial populations was compared following carbon addition to stored soil and to freshly-collected soil. The results obtained were used to assess the changes which occurred and to assess when it was appropriate to use freshly-collected or stored soil for further studies of the effect of carbon source addition on denitrification. The selected carbon sources used in the experiments were glucose and acetate. As a control, no carbon was added. The control treatments were used to assess changes in availability of endogenous soil carbon following -20°C storage. These preliminary experiments were also used to determine the best experimental design for use in later experiments.

A secondary objective of the work described in this chapter was to characterise the soil used in all the laboratory based studies.

4.2 METHODS

Stored soil was collected from a riparian zone in Whakarewarewa Forest. Soil was sieved, sealed in plastic-lined seed-bags, and stored at -20°C until use in serum bottle incubations. Fresh soil was collected just prior to preparation of serum bottle incubations. Full details can be found in section 3.3.

4.2.1 CHEMICAL AND PHYSICAL CHARACTERISATION OF SOIL

Soil properties were determined on samples taken from stored soil. Since the stored soil was collected from the same site as subsequent freshly-collected soil, the physical and chemical properties measured here were assumed to be representative of the freshly-collected soil.

Soil properties determined were: total carbon; organic matter; total Kjeldahl nitrogen; pH; cation exchange capacity; particle size; KCl-extractable nitrate. Methods used are described in section 3.4. The analysis of KCl-extractable nitrate in stored soil was conducted on nine replicate seed bags. Approximately 10 g dry weight of soil was added to 50 ml 2N KCl (with 0.04 mg/l $HgCl_2$ added as preservative), shaken for one minute and left to stand overnight. The following day the soil slurry was filtered through filter paper (Whatman 114) and then through a membrane filter (Millipore, 0.45 μm). The filtrate was stored frozen until analyzed for nitrate and ammonium using the methods described in section 3.5.

4.2.2 COMPARISON OF MICROBIAL ACTIVITIES IN FRESHLY COLLECTED AND STORED SOIL

Soil was prepared in serum bottles as described in section 3.3 and the following comments are made for clarification. The treatments imposed on both stored and freshly collected riparian soil were:

1. glucose and nitrate addition,

2. sodium acetate and nitrate addition,

3. control: nitrate addition only.

Carbon was added as 2.34 mmol C dissolved in 1 ml of water and nitrate was added as 0.5 mmol N dissolved in 1 ml of water. Acetylene (16 ml) was added at the start of incubation. Each treatment had three replicates. Soil used in the fresh soil incubations was collected from the same riparian zone from which the stored soil had been collected (section 3.2.2). The experiment was run at three time intervals: the frozen soil experiment which included glucose, acetate, and control treatments were run between 22/8/89 and 31/8/89; fresh soil incubations with acetate and control (hereafter called control 1) treatments between 4/10/89 and 14/10/89; fresh soil incubations with glucose and control (hereafter called control 2) treatments between 26/10/89 and 5/11/89.

All serum bottles were incubated for 10 days at 25 °C in a shaking incubator at 200 rpm. Gas samples were taken periodically through the stopcock and analyzed for nitrous oxide and carbon dioxide as described in section 3.6. At the end of the incubation period the

headspace volume and water content were determined gravimetrically following the addition of water. A liquid sample (10 ml) was taken, passed through a filter (Millipore, 0.45 μm), and analyzed for nitrate and a range of volatile fatty acids (section 3.7). Finally, the soil sample dry weight was determined following oven-drying at 105 °C.

4.2.3 DATA ANALYSIS

All significant differences are reported at the 95% confidence level. At the end of incubation, carbon dioxide and nitrous oxide production were expressed as per gram of oven-dried soil and were plotted against time. Using graphs, estimates were then made of total nitrous oxide at the end of incubation, denitrification lag time, and maximum denitrification rate for each replicate. Denitrification lag was the time taken before exponential nitrous oxide production commenced. Denitrification rate was determined as the highest denitrification rate observed during the exponential phase. In the control incubations where nitrous oxide production was linear, linear regression was used to determine this rate. Analysis of variance (ANOVA) was then used to determine significant differences. Prior to ANOVA, variance stabilisation was necessary in some cases using the criteria outlined in Box *et al.* (1978). Total nitrous oxide produced and denitrification rate were transformed by square root; lag data was untransformed.

For graphical presentation, 95% confidence intervals were displayed which were calculated in the following manner. A log transformation of the nitrous oxide and carbon dioxide data was required for variance stabilisation. The average nitrous oxide concentration was calculated irrespective of time for each replicate. The standard deviations were then determined for each treatment and 95% confidence intervals were calculated. In figures where two or more nitrous oxide or carbon dioxide production curves were compared, a pooled standard deviation was calculated from the standard deviations of each of the treatments before calculation of 95% confidence intervals.

4.3 RESULTS

4.3.1 CHEMICAL AND PHYSICAL CHARACTERISTICS OF SOIL

Physical and chemical characteristics of the stored soil are presented in Table 4.1.

CHARACTERISTIC	AVERAGE	STANDARD DEVIATION	n
Total Carbon (% by weight)	21.1	0.5	15
Organic Matter (% by weight)	36.6	0.8	15
Total Kjeldahl Nitrogen (% by weight)	0.98	0.14	15
Nitrate-N (μg/g oven dry soil)	2.6	0.8	9
pH	5.61	0.06	15
Cation Exchange Capacity (meq per 100 g dry soil)	48.5	2.4	3
% Sand	47.9	1.3	3
% Silt	42.0	1.3	3
% Clay	10.1	0.1	3

Table 4.1 Physical and chemical properties of test soil. Percentages are expressed on a oven dry weight basis. n = number of samples.

4.3.2 COMPARISON OF MICROBIAL ACTIVITY IN FRESH AND STORED SOIL

Figure 4.1 shows an example of how total nitrous oxide produced, denitrification rate and lag time were determined for a single replicate. Table 4.2 summarises the total nitrous oxide production, denitrification lag and rates for most treatments and shows significant differences. Much of the following presentation of results refers to this table. There was no discernable denitrification lag for control incubations of fresh soil. Denitrification rate was not determined for the control in the stored soil incubation due to its irregular curve shape (Figure 4.2).

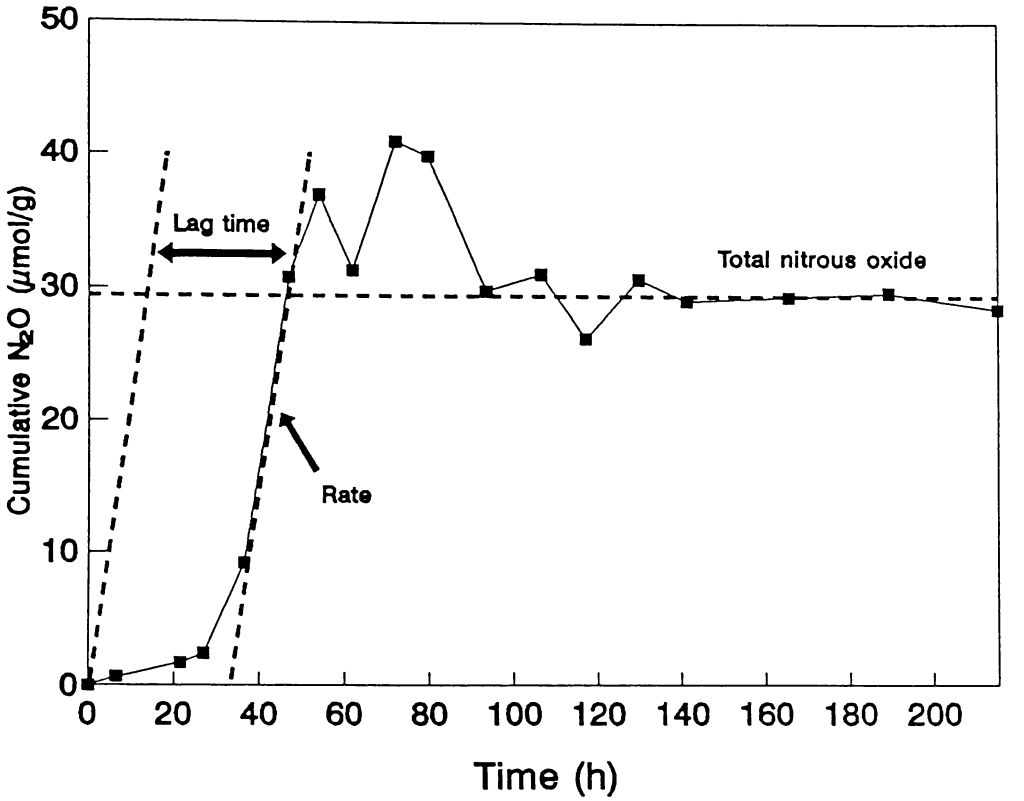


Figure 4.1 Assessment of denitrification lag time, rate, and total nitrous oxide production following the addition of glucose to fresh soil.

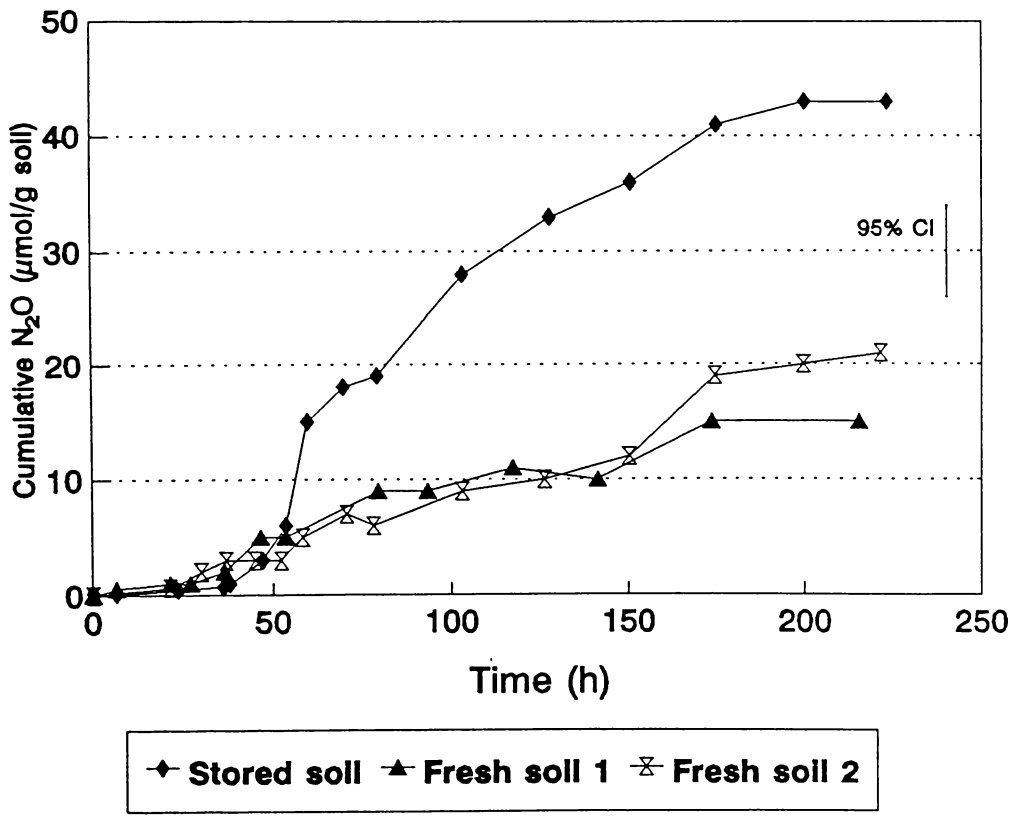


Figure 4.2 A comparison of the control incubations of both stored and fresh soil. The vertical bar denotes a 95% confidence interval.

Soil used	Treatment	Total N_2O produced ($\mu mol.g^{-1}$)	Maximum rate of N_2O production ($\mu mol.g^{-1}.h^{-1}$)	Lag Time (h)
Freshly collected	Control 1	24 a	0.90 a	ND
	Control 2	19 a	0.77 a	ND
	Glucose	32 b	17 b	29 a
	Acetate	34 b,c	5.7 c	141 b
Stored	Control	43 c	ND	39 c
	Acetate	39 b,c	7.8 c	79 d
	Glucose	61 d	16 b	75 d

Table 4.2 Total nitrous oxide production, and rate and lag of denitrification following carbon addition to stored and freshly collected soil. Values in the each column followed by the same letter are not significantly different at 95% confidence level. ND indicates not determined.

Control incubations

Nitrous oxide production in the fresh soil controls were generally linear over time and no significant difference was found in the nitrous oxide production rate between these incubations. Differences were observed in nitrous oxide production between fresh and stored soil control (Figure 4.2). After 50 h the nitrous oxide concentration in the stored soil control rapidly rose to exceed nitrous oxide concentration in the fresh soil control.

Total nitrous oxide production

In both fresh and stored soil, glucose addition increased total nitrous oxide production to exceed the control; whereas acetate addition increased nitrous oxide production above the control only in the fresh soil. Nitrous oxide production in the fresh soil controls were not significantly different from one another; however, both were significantly less than the nitrous oxide produced in the stored soil control.

Denitrification rate

The rate of denitrification was dependent on the carbon source added and not on whether the soil was stored or freshly collected. Glucose addition resulted in the same rate of denitrification whether added to fresh or stored soil; acetate addition also resulted in an equivalent denitrification rate independent of soil type. In fresh soil, the denitrification rate was higher in the presence of glucose and acetate than in the control. A similar comparison could not be made in the stored soil as the denitrification rate in the control was not determined.

Denitrification lag time

Denitrification lag time was dependent on both the carbon source added and the soil used. In fresh soil, glucose and acetate exhibited significantly different lag times; whereas, in the stored-soil incubations the lag times of nitrous oxide production following glucose and acetate addition were similar. Although the total amount of nitrous oxide produced following acetate addition to stored-soil was not significantly different than in the control treatment, the lag time of denitrification differed. Denitrification appeared to be initially suppressed by acetate addition in both stored and fresh soil (fresh soil is shown as an example in Figure 4.3).

Carbon dioxide production

Nitrous oxide and carbon dioxide production following glucose addition is shown in Figure 4.4. In the freshly collected soil, carbon dioxide production occurred simultaneously with nitrous oxide production. In the stored soil, two distinct phases of carbon dioxide production were observed. Initial carbon dioxide production was observed for the first 40 h of incubation but was not associated with nitrous oxide production. Carbon dioxide production then ceased for 30 h before a second phase of carbon dioxide production occurred, when nitrous oxide was also produced.

Nitrate and ammonium remaining

Results of nitrate and ammonium analysis in fresh soil incubations are shown in Table 4.3. At the end of the incubation, very little nitrate remained in the treatments which had an added carbon source, whereas nitrate remained in the control incubation. Ammonium concentrations in all treatments were not significantly different at 95% confidence level.

Treatment	Nitrate (μmol)	Ammonium (μmol)
Glucose	4.4 a	13 a
Acetate	1.8 a	10 a
Control 1	165 b	14 a
Control 2	145 b	14 a

TABLE 4.3 Total nitrate and ammonium concentrations remaining in the fresh soil at the end of the incubation period. Values in each column followed by the same letter are not significantly different at 95% confidence level.

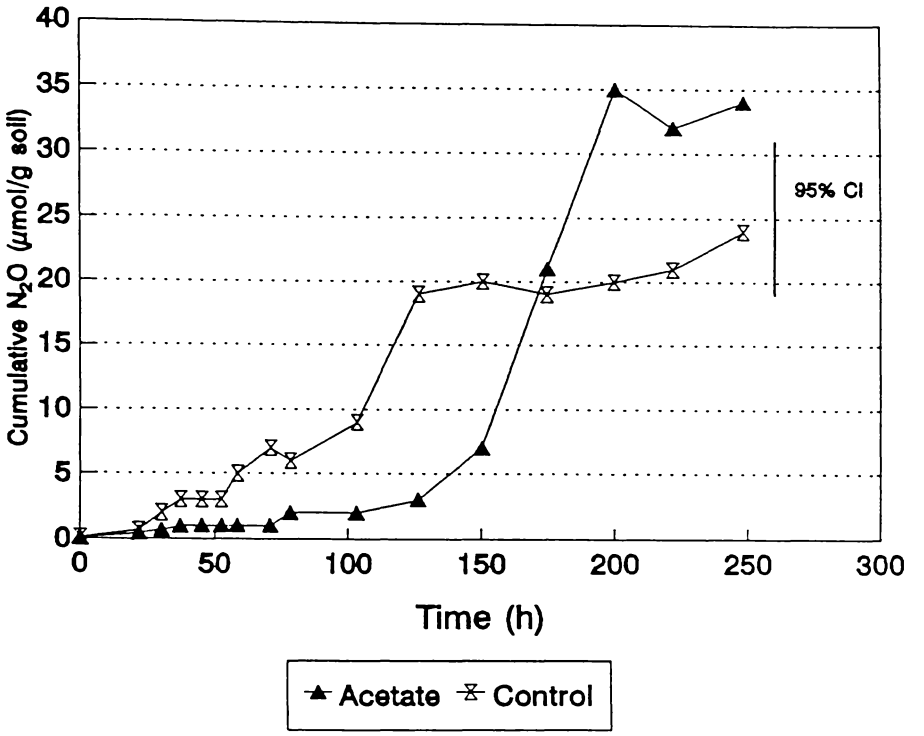


Figure 4.3 The initial inhibition of denitrification following the addition of acetate. Vertical bar denotes a 95% confidence interval.

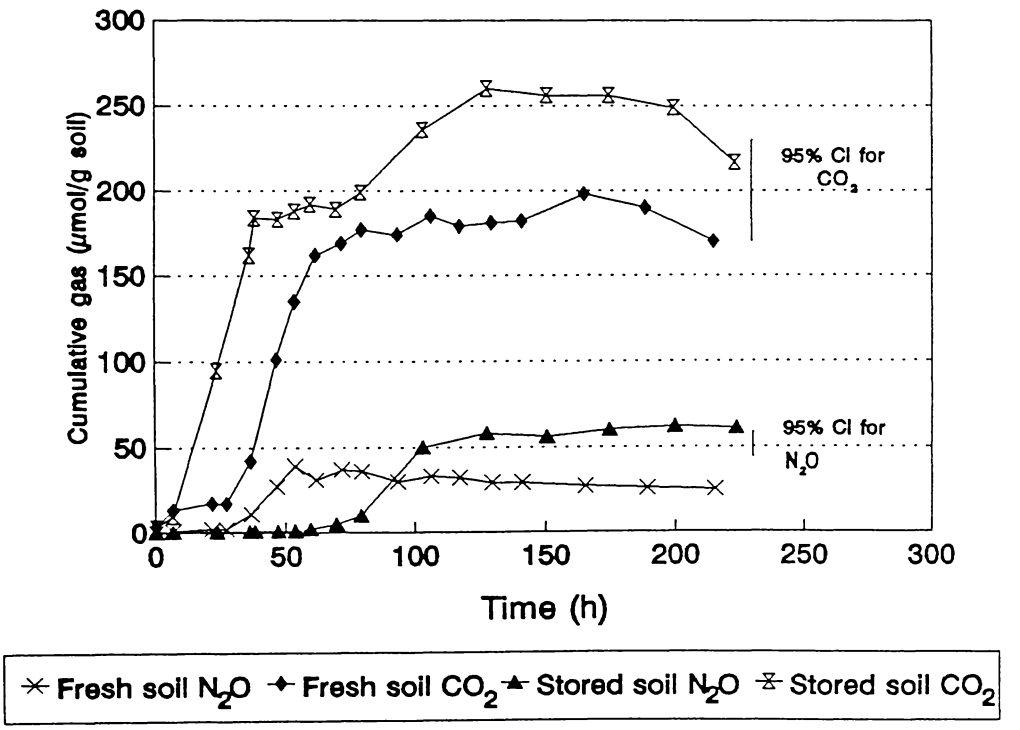


Figure 4.4 Gas production following glucose addition to fresh and stored soil. Vertical bars denote combined 95% confidence intervals.

4.4 DISCUSSION

4.4.1 CHEMICAL AND PHYSICAL CHARACTERISATION OF SOIL

Soil organic matter, soil carbon, and total kjeldahl nitrogen were high (Gibbs 1980). This was probably due to the high input of organic matter from endemic riparian vegetation and slow anaerobic decomposition in a water-saturated environment. The cation exchange capacity (CEC) was also high in comparison to other mineral soils and this was probably due to high organic matter content and high percentage of clay and silt (Brady 1974).

Extractable nitrate was very low which would be expected as nitrate removal by denitrification was likely to be high in these organic soils.

4.4.2 COMPARISON OF MICROBIAL ACTIVITY IN FRESH AND STORED SOIL

The discussion here is limited to a comparison of the denitrification response following carbon addition to fresh and stored soil. In Chapters 5 and 6, the denitrification response to different carbon compounds is explored more fully.

In fresh soil, nitrate limited denitrification in acetate and glucose treatments at the end of the incubation period, whereas the control incubations continued to produce nitrous oxide and nitrate remained at the end of incubation period. Control incubations were likely to have been limited by the availability of carbon.

Nitrous oxide production in stored controls was significantly higher than nitrous oxide production in the fresh soil controls (Table 4.2). This difference may have been due to differences in the amount of carbon available for denitrification. During the storage procedure, soil was thoroughly mixed, sieved, and then the soil frozen and stored. Before use in incubation studies the frozen soil was removed from the freezer and thawed. During the entire procedure many of the larger soil aggregates were broken up or crushed. Both physical disturbance and freezing/thawing have been shown to increase denitrification in soil: Seech and Beauchamp (1988) found that crushing soil aggregates increased denitrification and suggested that this was due to increased carbon availability. Early work by McGarity (1962) found that denitrification was stimulated by freezing and thawing of soil and it was suggested that this was also due to the release of organic matter. Similar conclusions were reached by Christensen and Tiedje (1990), who observed vigorous soil denitrification during the spring thaw; they suggested that denitrification was fuelled by carbon released either following death of microorganisms or from physical breakdown of detritus during the freeze/thaw cycle.

Stimulation of denitrification by glucose and acetate has been shown in a number of other studies (e.g. Paul *et al.* 1989). However, the differences in patterns of nitrous oxide response has not been previously reported. Differences in denitrification response were observed between stored and fresh soil following glucose or acetate addition. Acetate addition increased nitrous oxide production above the control in the fresh soil but not in stored soil. This may have been due to an increase in biologically-available organic carbon following the storage procedure, so that carbon became less limiting. However, acetate addition to the stored soil altered the onset of denitrification. Acetate also appeared to initially inhibit denitrification relative to the control in both soils; this phenomenon was further investigated in Chapter 5.

Differences in the production of nitrous oxide following glucose addition in fresh and stored soil were linked to carbon dioxide production (Figure 4.4). In these incubations, carbon dioxide may have been formed by two processes: denitrification and fermentation. Following the addition of glucose to fresh soil incubations, nitrous oxide production took place simultaneously with carbon dioxide production. Although fermentation may have occurred, it would appear that some of the carbon dioxide was produced from denitrification. In stored soil, initial carbon dioxide production was not concurrent with nitrous oxide production and presumably resulted from the fermentation of glucose. Carbon dioxide production halted at 30 hours presumably because glucose had been fully converted to fermentation endproducts. The second increase in carbon dioxide was probably due to denitrification, as it was observed that nitrous oxide production occurred simultaneously to this carbon dioxide production. Fermentation endproducts may have been the carbon sources used for denitrification at this time. It has been suggested that denitrifiers in some soils may be dependent on fermenting micro-organisms to carry out the initial decomposition of larger organic compounds (Beauchamp *et al.* 1989).

The total ammonium found at the end of incubation in the fresh soil was low, which indicated that dissimilatory nitrate reduction to ammonium (DNRA) had not occurred during incubation. This is somewhat at odds with current ideas in the literature: Tiedje *et al.* (1982) suggested that 10 to 40% of added nitrate would be expected to be reduced to ammonium in organic soils and soils with added carbon source, whereas in this instance, a maximum of only 2-3% of the added nitrate was converted to ammonium.

4.5 CONCLUSIONS

The use of either freshly collected or stored soil in studies which examine the effect of carbon compounds on denitrification should be carefully considered.

Soil storage at -20°C maintained fermentative and denitrifying bacteria but altered the response of these bacteria to additions of carbon compounds. The soil preparation and storage method may also have increased the amount of carbon available. If stored soils were used to assess the response of denitrifying bacteria to the addition of simple carbon compounds, the results would differ from those obtained from the natural environment. However, the soil storage technique used here provided a readily accessible supply of soil which had populations of denitrifying and fermenting soil bacteria. Stored soil was thoroughly mixed and sieved, which provided a soil supply with less variability between samples than would be expected in freshly collected soil. Low sample variability could be of particular importance in longer term studies, where differences between soil sample characteristics (such as organic matter content) would perhaps increase with increasing incubation time.

It was decided that for experimental work in this thesis, freshly collected soil would be used for the study of pure carbon compounds as electron donors during denitrification. These studies would be short-term (less than 10 days). Stored soil would be used in the long-term studies (100 days) using plant material, where it was considered important to keep differences between soil samples to a minimum.

The results obtained from this set of preliminary experiments showed that resident denitrifiers in riparian soils were able to utilise glucose more quickly than acetate. This suggested that in riparian soils, denitrifying bacteria may fulfil a specific niche in the degradation of organic matter and this was examined in greater detail in Chapter 5. The apparent inhibitory effect by acetate on denitrification was also examined in Chapter 5.

CHAPTER 5

THE STIMULATION AND INHIBITION OF DENITRIFICATION IN RIPARIAN SOIL FOLLOWING THE ADDITION OF SINGLE CARBON SOURCES.

5.1 INTRODUCTION

Denitrifying bacteria utilise carbon both for cell synthesis and as an electron source during energy production. Examination of the literature reveals that denitrifiers generally utilise simple compounds such as sugars (Bremner and Shaw 1958), aromatic compounds (Evans and Fuchs 1988), and fermentation endproducts (Paul *et al.* 1989). These compounds probably form a small fraction of the water soluble carbon present in soil. It has been hypothesised that denitrifiers are dependent upon other bacteria to degrade larger compounds such as cellulose (Beauchamp *et al.* 1989). There is some evidence to suggest that denitrification and fermentation occur simultaneously in soil, but the relationship between denitrifiers and fermenters has not been thoroughly examined.

A strong correlation has been shown between biologically available carbon and the denitrification potential in agricultural soils (Burford and Bremner 1975), and in wetland and riparian soils (Schipper *et al.* in press). In wetland and riparian soils much of the organic matter is derived from litter and its subsequent degradation. Litter decomposition in these water-saturated soils is likely to be by anaerobic degradation which probably follows the generalized sequence: 1. breakdown of plant material to large fragments/polymeric material (this step may be very slow under anaerobic conditions); 2. cleavage of polymers to monomers (e.g., sugars and aromatics); 3. monomers fermented to end products; 4. fermentation endproducts converted to carbon dioxide and methane via acetate, hydrogen and carbon dioxide (as depicted in Figure 2.5). Some anaerobic bacteria will be capable of more than one step in this sequence. Each of the steps in this breakdown pathway can lead to the release of carbon to the environment which is then available for other anaerobic bacteria. It is not known at which steps denitrifiers will compete for released organic carbon.

Preliminary studies (Chapter 4) showed that denitrification lag time and rate differed in response depending on whether glucose or acetate were added. In this chapter, measurements were made of denitrification response following addition of various carbon compounds. The carbon compounds selected represented those likely to be released during the anaerobic degradation of plant material. The object was to identify which carbon compounds were used by denitrifying bacteria in riparian soils and to examine the relationship between denitrifiers and fermenters. A second experiment was designed which examined the inhibitory effect of acetate on denitrifiers.

5.2 MATERIALS AND METHODS

5.2.1 EXPERIMENT 1: ADDITION OF SINGLE CARBON COMPOUNDS

The incubation procedure used is described in detail in section 3.3. Soil was collected from an organic riparian zone (section 3.2.2) directly before incubation commenced. All incubations had added 0.75 mmol of potassium nitrate (in 5 ml water). Also added to each bottle was 16 ml of acetylene in order to block the conversion of N_2O to N_2. N_2O production was measured as an index of denitrification.

The carbon sources used were -

Volatile fatty acids:	acetate, propionate, *n*-butyrate (all as sodium salts).
Fermentation endproducts:	lactate (as sodium salt), ethanol.
Monomeric sugars:	glucose, xylose, fructose.
Polymers:	cellulose, xylan.

The carbon sources were dissolved (2.34 mmol of carbon in 1 ml water) and added to serum bottles (three replicates) by syringe, at the same time as the nitrate was added. The non-water soluble carbon sources (cellulose and xylan) were added as a slurry in water. The different carbon source treatments were divided into two groups for incubation in order to overcome the logistical problems associated with handling a large number of serum bottles and gas samples. The first batch was incubated from the 5th to the 15th March 1990 in which the treatments were: control (no carbon added), acetate, propionate, butyrate, lactate, ethanol, and glucose. The second batch was incubated between the 15th to the 26th March 1990 in which the treatments were: control, acetate, glucose, xylose, fructose, cellulose, and xylan. Glucose, acetate, and control incubations were included in each experiment as overlapping treatments in order to assess whether soil biological properties changed between soil collections. Nitrate was added to all incubations and was labelled with 24.5% ^{15}N to allow a nitrogen balance to be calculated at the end of the incubation. During incubation, headspace samples were taken periodically through the stopcock, stored in vacutainers, and subsequently analyzed for nitrous oxide and carbon dioxide (section 3.6). At the end of the incubation, the headspace volume was determined gravimetrically following the addition of water. Following the addition of water, a 10 ml liquid sample was taken, passed through a filter (Millipore, 0.45 μm), and analyzed for nitrate and a range of volatile fatty acids (sections 3.5 and 3.7 respectively). Ammonium concentration was not measured in the water sample because dissimilatory nitrate reduction to ammonium was not considered to occur under these incubation conditions (section 4.4.2). Serum bottles were then dried in a 105°C oven to determine oven-dry soil weight and moisture content. Once headspace

volume and oven-dry soil weight had been determined, the soil was ground in a mortar and pestle before analysis for ^{15}N content.

^{15}N was analyzed by methods developed at the University of Waikato Stable Isotope Unit. Soil samples (approximately 5 mg) were accurately weighed into tin cups and combusted in the oxidation column of a CNS Elemental Analyzer (Carlo Erba NA 1500). In principle, nitrogen is oxidised to nitrogen oxide gases which then pass through carbon dioxide and water filters and into a copper reduction column where the nitrogen oxides are reduced to N_2. The nitrogen then passes into a stable isotope analyzer (Europa Tracermass) where nitrogen gas masses 28, 29, and 30 are detected and quantified. The elemental analyzer and mass detector are periodically calibrated using a reference sample of known isotopic enrichment.

At the end of incubation, nitrogen balances were calculated. The total nitrous oxide produced, the amount of nitrate in the 10 ml of removed water, and the amount of nitrogen remaining in soil (calculated from ^{15}N analysis) were summed for each replicate. ^{15}N natural abundance was allowed for in calculating the amount of nitrogen remaining in soil. 95% confidence intervals for nitrogen balance were then determined for each treatment.

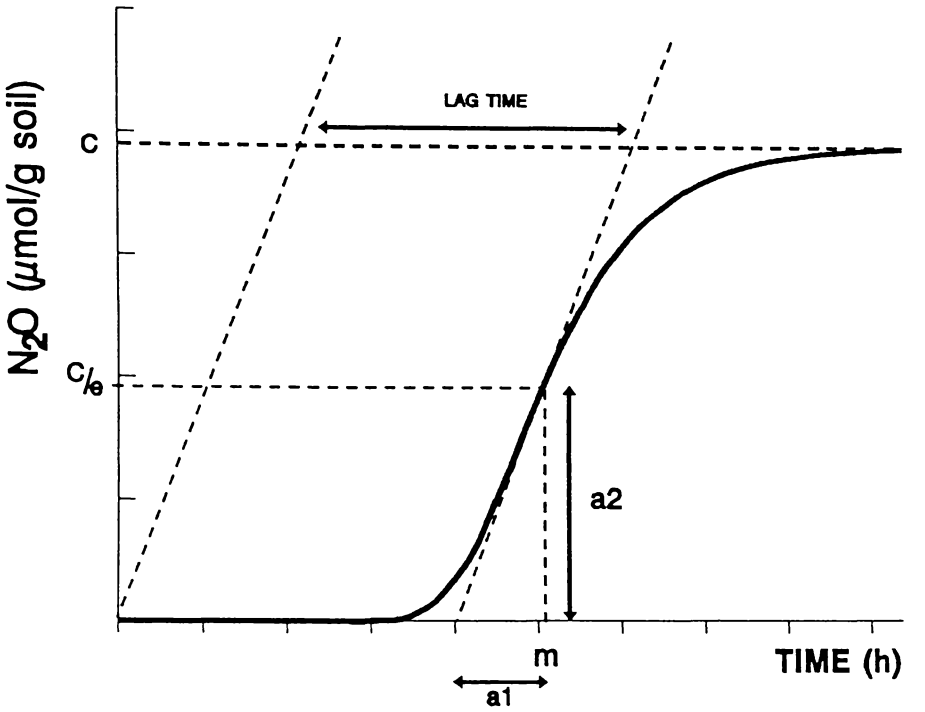
5.2.2 EXPERIMENT 2: INHIBITION OF DENITRIFICATION BY ACETATE

Nine incubations were done in serum bottles to which glucose (2.34 mmol C in 1 ml water) and nitrate (0.75 mmol nitrate-N in 5 ml of water) were added at the start of the incubation (as described in section 5.2.1). In this case nitrate was not ^{15}N labelled. Acetate (2.34 mmol carbon in 1 ml) was then added to three replicates at 0 h, 46 h, and 118 h after incubation commenced. These times corresponded to the lag, exponential, and stationary growth phases following glucose addition. At 118 h (stationary phase) denitrification was known to be limited by nitrate, and further nitrate (0.75 mmol nitrate-N in 5 ml water) was added with acetate. The overlapping treatments (glucose, acetate, and control) were also prepared and incubated as above. This experiment was run between 12th and 24th June 1990.

5.2.3 DATA ANALYSIS

Carbon dioxide and nitrous oxide production were expressed as per gram of oven dried soil. Once nitrous oxide data from each replicate were collated and was plotted against time, a Gompertz curve was fitted using Genstat (Genstat, A General Statistical Program, Rothamsted Experimental Station). The maximum denitrification rate (calculated at the point of inflexion) and lag time before denitrification onset were mathematically derived from the coefficients of the resultant equation for each replicate (Figure 5.1). All significant differences are reported at 95% confidence level.

Where the Gompertz equation was not an appropriate fit, the maximum denitrification rate and lag period of each replicate were determined either by linear regression (for propionate, cellulose and control incubations) or by graphical estimation (acetate incubations).



Gompertz equation

$$y = ce^{(-e^{(-b(x-m))})}$$

Derivative

$$y' = bce^{(-e^{(-b(x-m))}).e^{(-b(x-m))}}$$

Maximum denitrification = slope at point of inflexion, m

$$at \quad x = m, \quad y' = bce^{-1}$$

Lag time

$$lag = m - a_1$$

$$= m - \frac{a_2}{y'}$$

$$= m - \frac{1}{b}$$

Figure 5.1 Calculation of maximum rate and lag time of denitrification.

In experiment 1, in order to assess whether changes in soil biological processes had occurred between incubation periods the student T-test was used to test for differences in the denitrification rates and lags observed in the overlapping treatments (control, acetate, and glucose). Denitrification rates and lags observed in experiment 1 were then compared by analysis of variance ANOVA (SAS Institute Inc. 1985) using the least squares difference method. Prior to ANOVA, variance stabilisation was necessary; the lag data required an inverse square root transformation and the rate data required a log transformation (Box *et al.* 1978).

For graphical presentation, the average nitrous oxide concentration was calculated irrespective of time for each replicate. The standard deviation of these averages was then determined for each treatment and 95% confidence intervals determined. A log transformation of the nitrous oxide data was first required for variance stabilisation. In figures where two or more nitrous oxide or carbon dioxide production curves were compared, a pooled standard deviation was calculated from the standard deviations of each treatment before construction of 95% confidence intervals.

5.3 RESULTS

5.3.1 EXPERIMENT 1: ADDITION OF SINGLE CARBON SOURCES

5.3.1.1 Gas production

By the end of the incubation period, most carbon additions had shown increased nitrous oxide production above that of the control, with the exceptions of cellulose and propionate (detailed data for nitrous oxide production and carbon dioxide production data are presented in Appendix 9.1). Nitrous oxide production was well-described by Gompertz curves, generally accounting for greater than 90% of the variation. Figure 5.2 shows an example of nitrous oxide production obtained from selected replicates from glucose, butyrate, and control incubations along with the corresponding fitted regression curves.

In all cases, exponential carbon dioxide production occurred simultaneously with the exponential nitrous oxide production (e.g., Figure 5.3). Generally carbon dioxide production stopped at the same time as nitrous oxide production, with the exceptions being lactate and xylan (Figure 5.4) whereas carbon dioxide production continued after nitrous oxide production had reached the stationary phase.

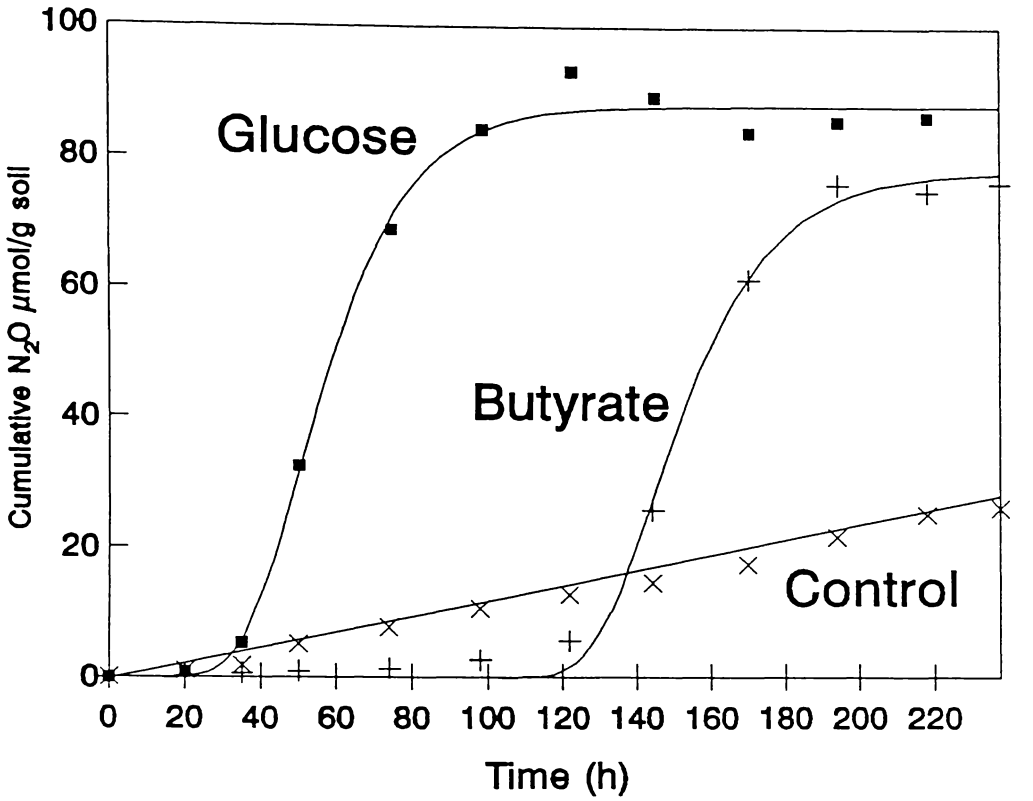


Figure 5.2 Selected replicates of nitrous oxide production in glucose, acetate, and control treatments with fitted curves.

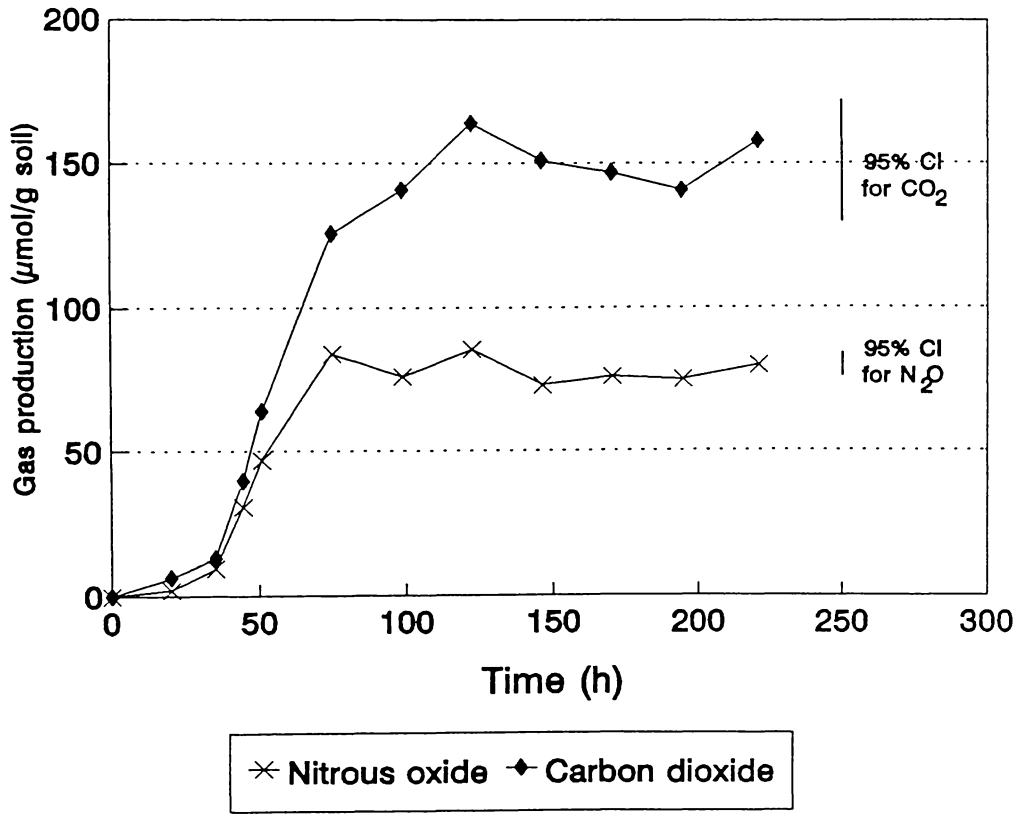


Figure 5.3 Average gas production following glucose addition. The vertical lines denote 95% confidence intervals.

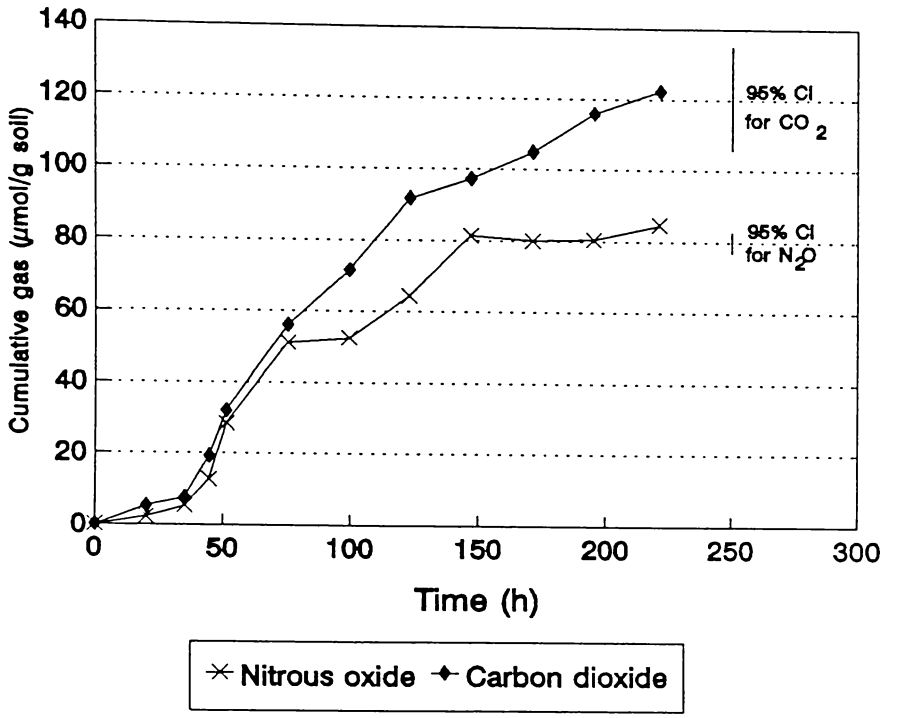


Figure 5.4 Gas production following xylan addition. Note the apparent diauxic response in nitrous oxide production. Vertical lines denote 95% confidence intervals.

5.3.1.2 Overlapping treatments

Differences in the rates and lags observed in the overlapping treatments in experiment 1 are shown in Table 5.1. A small but significant difference was found between the rates of control incubations; however, it was not thought that this difference indicated a major change of bacterial activity between soil collections. For subsequent analysis, data from the overlapping experiments were combined and treated as a single data set.

Carbon addition	Mean maximum rate of N_2O production ($\mu molN.g^{-1}.h^{-1}$)	Sig. diff.	Mean lag time (hours)	Sig. diff.
glucose 1	2.51	a	35.7	a
glucose 2	3.08	a	34.3	a
acetate 1	1.02	b	218.3	b
acetate 2	1.08	b	173.3	b
control 1	0.12	c		
control 2	0.087	d		

Table 5.1 Comparisons between lag and rates of overlapping treatments in experiment 1. Rates and lags followed by the same letter were not significantly different at the 95% confidence level.

5.3.1.3 Denitrification rates and lag times

The calculated lag times and rates for denitrification are shown in Tables 5.2 and 5.3 respectively. Analysis of variance of the lag times showed four significantly different groups: sugars (glucose, fructose, and xylose), lactate and ethanol, and butyrate, and acetate. Fewer significant differences were observed in a comparison of the maximum rates of nitrous oxide production. Whereas the fermentation endproducts, lactate and ethanol, gave the highest denitrification rates, these were not significantly different from denitrification rates following the addition of sugars or butyrate. There were no differences in denitrification rates between the cellulose and control treatments. The rate following propionate addition was significantly lower than found in the control. It was not possible to determine accurately a denitrification lag or rate following xylan addition because of the irregular two-step curve observed (Figure 5.4).

Carbon addition	Mean lag time (hours)	Significant difference
glucose	35.0	a
fructose	36.6	a
xylose	35.7	a
lactate	45.8	b
ethanol	48.5	b
butyrate	127.3	c
acetate	195.8	d

Table 5.2 The effect of treatment on the lag times of nitrous oxide production. Treatments followed by the same letter are not significantly different at 95% confidence level. Treatments which did not show any lag were cellulose, propionate, and controls.

Carbon addition	Mean maximum rate of N_2O production ($\mu molN.g^{-1}.h^{-1}$)	Significant differences
ethanol	4.06	a
lactate	3.43	a
fructose	2.94	a
xylose	2.87	a
glucose	2.79	a
butyrate	1.87	a,b
acetate	1.05	b
control	0.103	c
cellulose	0.091	c,d
propionate	0.035	d

Table 5.3 The effect of treatment on the maximum rate of nitrous oxide production. Treatments followed by the same letter are not significantly different at 95% confidence level. All treatments had three replicates except for the control, the glucose, and the acetate treatments each of which had six replicates.

5.3.1.4 Volatile fatty acid production

Volatile fatty acids (VFAs) were measured at the end of the incubations in order to indicate which of the added carbon compounds were fermented. The VFA data measured in each treatment is presented in Appendix 9.2. Acetate was the main VFA present, following additions of acetate, glucose, xylose, fructose, xylan, butyrate, lactate, and ethanol. Propionate was also a major endproduct in the lactate treatment. Trace amounts of *i*- and *n*- butyrate were also found in the glucose and fructose treatments. In the propionate incubation, an average of 85% (standard deviation 8.5%) of the added propionate was recovered as propionate. In the butyrate incubation an average of 56% (standard deviation 10%) was recovered as *n*-butyrate. No VFAs were found in the control and cellulose treatments.

5.3.1.5 Nitrogen balance

Nitrogen balances are shown in Figure 5.5. Very little of the 0.75 mmol N added as nitrate could be recovered as nitrate at the end of incubation, apart from in those treatments where denitrification was not stimulated by carbon addition (i.e., controls, propionate, and cellulose). Recoveries were generally lowest in treatments where denitrification had not been stimulated.

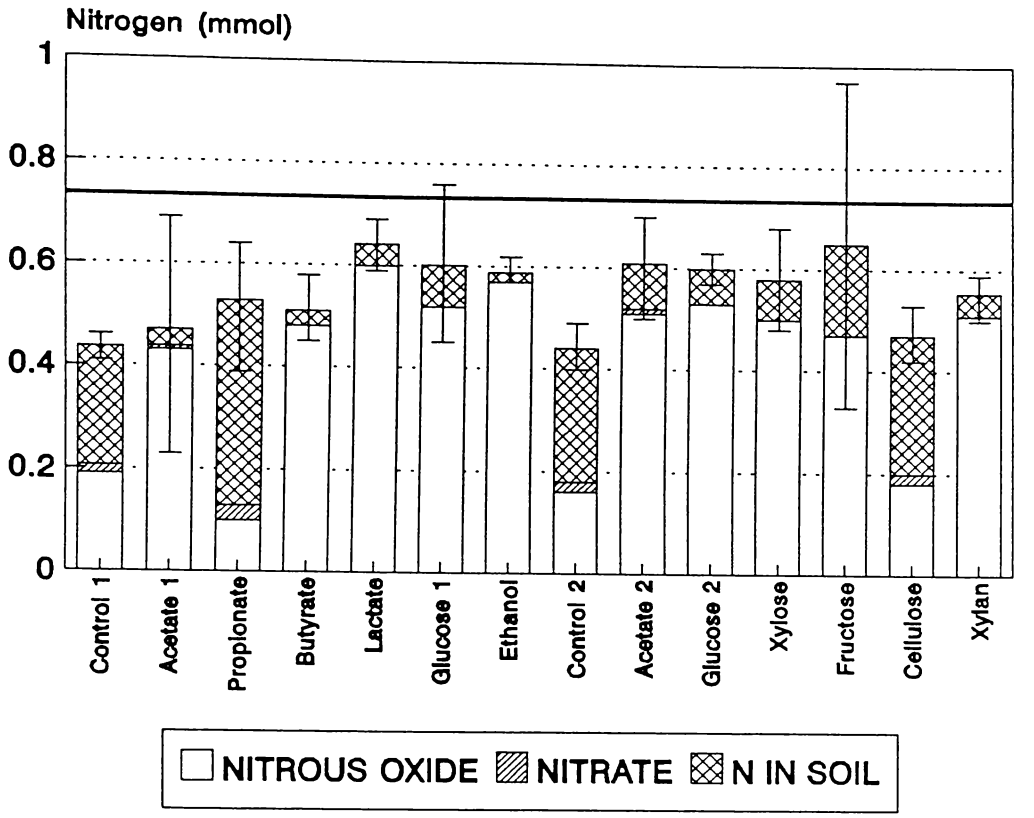


Figure 5.5 Nitrogen balance at the end of the incubation period. Initial nitrate addition was 0.75mmol NO_3^--N. 95% confidence intervals of the total nitrogen recovered are shown.

5.3.1.6 Inhibition of denitrification

A comparison of those incubations which had volatile fatty acids added and the control incubation indicated that nitrous oxide production was suppressed below that of the control for at least the first 100 h (Table 5.4). Butyrate inhibited denitrification for 100 hours, acetate for 150 h, and propionate inhibited denitrification during the entire incubation period (>240h).

TREATMENT	RATE OF N_2O PRODUCTION ($\mu molN.g^{-1}.h^{-1}$)	SIGNIFICANT DIFFERENCE
control	11.0	a
acetate	2.6	b
propionate	2.5	b
butyrate	4.6	b

Table 5.4 Nitrous oxide production rate during the first 100 hours of incubation in control and volatile fatty acid (VFA) treatments. Nitrous oxide production rate followed by the same letter are not significantly different at 95% confidence level. Standard error for comparison 1.03 (n=3).

5.3.2 EXPERIMENT 2: INHIBITION OF DENITRIFICATION BY ACETATE

The nitrous oxide lags and rates of the overlapping treatments behaved in a similar manner to the overlapping treatments in experiment 1 (Figure 5.6), except that the rate of nitrous oxide production following acetate addition which was higher than previously observed. Addition of acetate showed an initially inhibitory effect on nitrous oxide production. Effects of acetate addition on the different phases of denitrification (lag, exponential, and stationary) are shown in Figures 5.7 a, b, c.

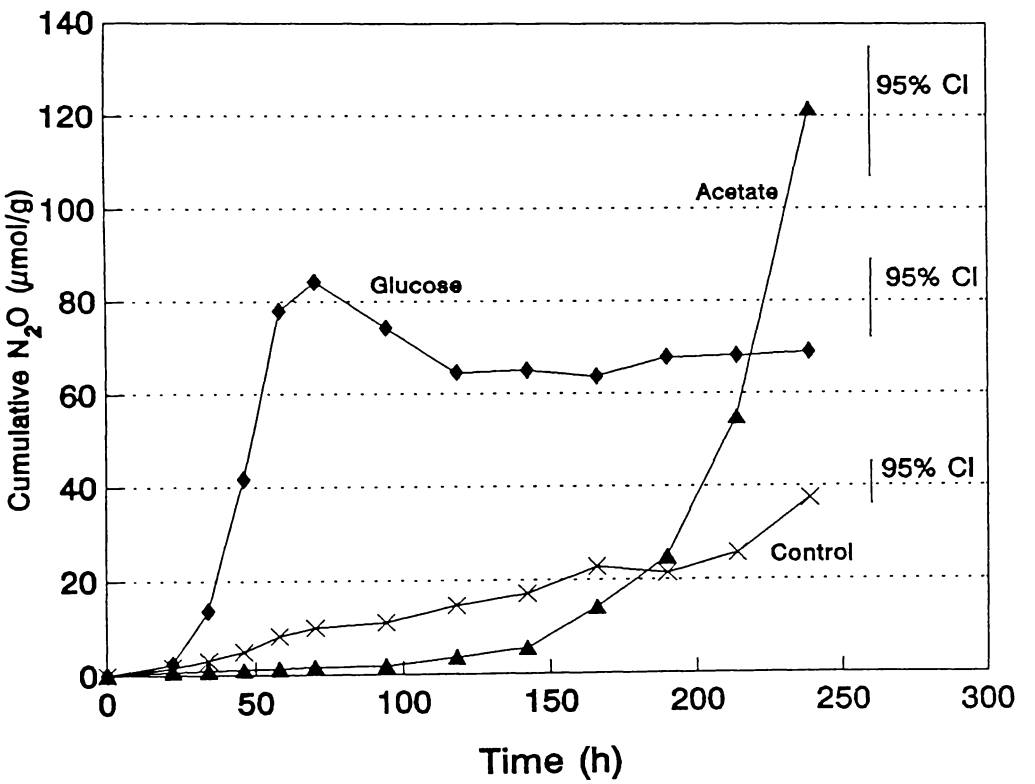


Figure 5.6 Nitrous oxide production in overlapping controls in acetate inhibition experiment. Vertical lines denote 95% confidence intervals.

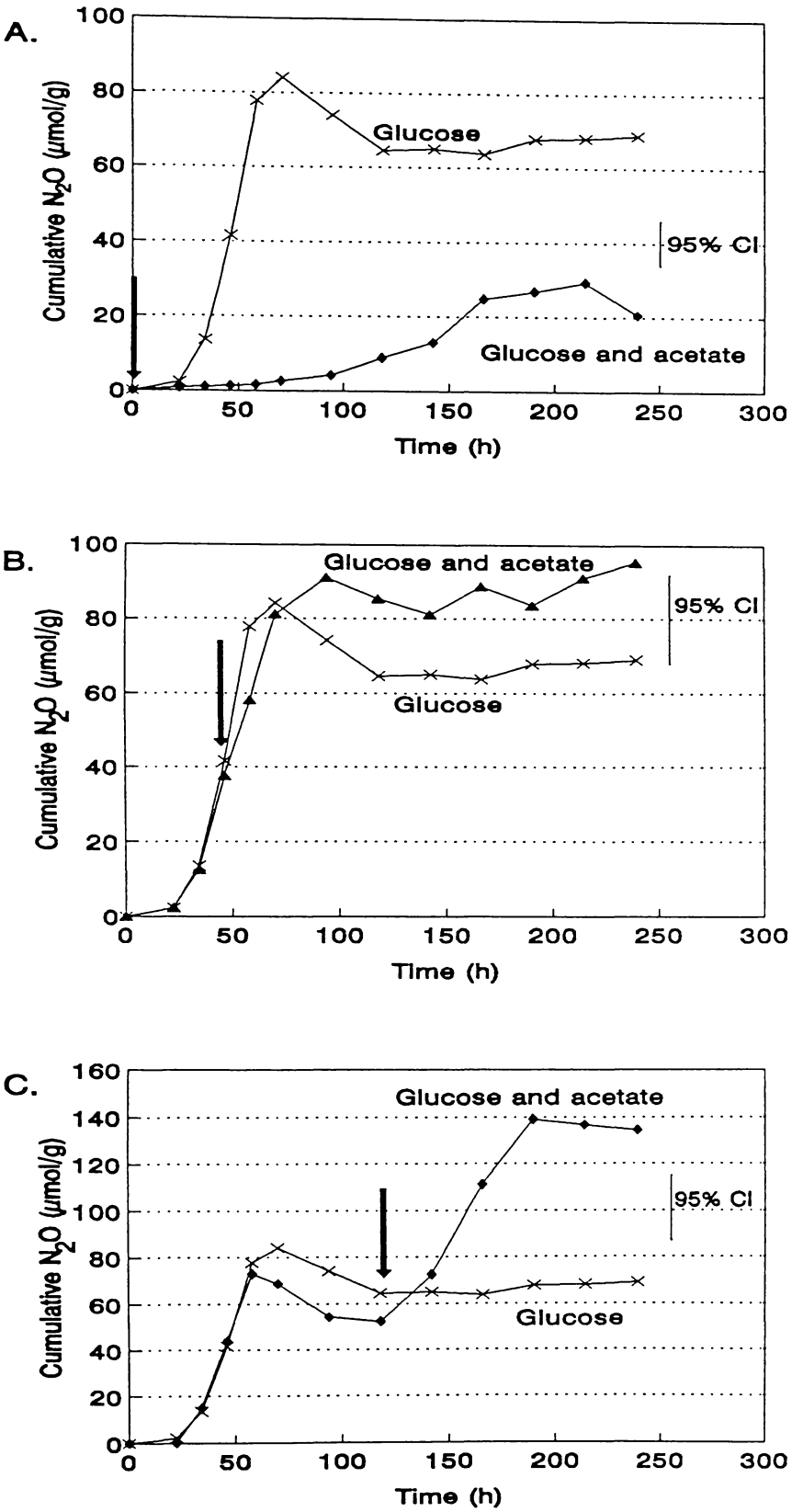


Figure 5.7 Nitrous oxide production in glucose incubations, arrows indicate when acetate was also added at (A) lag phase, (B) exponential phase, (C) stationary phase. Vertical lines denote 95% confidence intervals.

Acetate added to the glucose treatment at the start of incubation inhibited nitrous oxide production for the first 150 h and even after this time no exponential phase was observed (Figure 5.7A). Acetate addition to the exponential phase did not alter nitrous oxide production relative to the glucose control (Figure 5.7B). Acetate and nitrate added to the stationary phase resulted in further denitrification (Figure 5.7C).

The average nitrate concentration remaining in the control treatment was 1.2 mmol NO_3-N.l^{-1} (standard deviation 1.1), and in the treatment where acetate and glucose were added at the start of incubation the nitrate concentration was 2.6 mmol NO_3-N.l^{-1} (standard deviation 0.53). In all other treatments nitrate concentration was less than 0.001 mmol NO_3-N/l.

5.4 DISCUSSION

5.4.1 EFFECTIVENESS OF ACETYLENE INHIBITION

Nitrous oxide production

A possible criticism of the methodology employed here was that the acetylene blockage may not have been effective during the incubation period, resulting in some nitrous oxide conversion to nitrogen gas. In experiments 1 and 2, acetylene was initially added at greater than 10% of the headspace volume which should have been sufficient for effective blockage (Knowles 1982; Tiedje *et al.* 1989). Acetylene can be utilised by aerobic and anaerobic bacteria (Tiedje 1988), although Terry and Duxbury (1985) found a delay of greater than seven days before acetylene degradation occurred in anaerobic soils.

If the acetylene blockage was ineffective, any nitrous oxide in the headspace would have been used by denitrifying bacteria as an electron acceptor and converted to nitrogen gas. This would be particularly true if nitrate had become limiting at the end of the incubation period (Slater and Capone 1989; Tiedje *et al.* 1989). However, during the stationary phase, the nitrous oxide concentration remained constant which suggested that the acetylene block was effective. A possible exception to this was noted in the glucose treatment of experiment 2, where a decrease in nitrous oxide concentration occurred after the exponential phase (Figure 5.6). However, nitrous oxide concentration decreased for only a short period. Had the acetylene blockage failed, nitrous oxide would be expected to be completely used (Slater and Capone 1989).

Nitrogen balance

Whereas the nitrous oxide data suggested that the acetylene blockage was effective during incubation, the nitrogen balance data do not necessarily support this conclusion.

Nitrogen recoveries were uniformly low and the variation observed could not account for incomplete recovery. ^{15}N content in the gas phase was not measured and whether nitrogen gas had been produced could not be determined. Nitrogen recovery was generally lowest in those treatments where denitrification had not been stimulated (i.e., control, cellulose, acetate, and propionate). This suggests that the recovery of ^{15}N-labelled nitrate in the soil may not have been complete. Drying soil at 100 °C may also have led to some losses of volatile ^{15}N-labelled nitrogen. However, it is not thought that these reasons account for all of the poor recovery of nitrogen.

Unfortunately due to demands on equipment, ^{15}N analysis was done at the end of the thesis. Time and cost of ^{15}N analysis did not allow further work to be done to establish whether a more stringent ^{15}N methodology would confirm if acetylene blockage had been effective.

The stability of the stationary phase of nitrous oxide concentration supported the assumption that acetylene blockage had been effective throughout the course of the experiment. However, the nitrogen balance procedure used could not confirm this conclusion. Due to time and cost constraints the results of the ^{15}N balance procedure could not be validated. For the purposes of the following discussion it was assumed that the acetylene blockage was effective throughout the incubation period.

5.4.2 STIMULATION OF DENITRIFICATION FOLLOWING ADDITION OF CARBON

Denitrification was stimulated by the majority of carbon sources tested and the incubated soil samples generally became nitrate-limited before the end of the incubation period. Nitrous oxide and carbon dioxide production probably resulted from the growth of denitrifiers and fermenters during incubation. Many of the carbon compounds tested here have previously been shown to stimulate denitrification in soil (Table 2.5). However, there do not appear to be any reports describing the stimulation of soil denitrification following the addition of lactate, fructose, xylose, or xylan. Of the carbon sources added, only cellulose and propionate did not stimulate denitrification and possible explanations for this are discussed below. Propionate has previously been shown to stimulate denitrification in agricultural soils (Paul *et al.* 1989), and so has cellulose (Bremner and Shaw 1958; Gok and Ottow 1988).

5.4.3 DENITRIFICATION LAG TIME AND RATE

Addition of sugars resulted in the shortest lag time of approximately 35 h before exponential nitrous oxide production, whereas the addition of the fermentation endproducts ethanol and lactate delayed the lag time by a further ten hours. Wiggins *et al.* (1987) outlined a number of factors that may account for such a lag time before mineralisation of carbon compounds occurred in soil including 1, induction of necessary enzymes; 2, required genetic mutation; 3, limitation of inorganic nutrients; 4, use of other carbon compounds; and 5, time required for a small bacterial population to grow to an extent where a response above the control was evident. Since bacterial enzyme induction generally occurs within minutes (Richmond 1968), it is unlikely to have accounted for the longer lag times observed here. The observed lag times were reproducible and therefore it is also unlikely that genetic mutation was involved. Inorganic nutrients were unlikely to be limiting in the organic soil. Denitrifiers were able to use carbon compounds present in the soil in the control incubations and part of the lag time may have been due to these bacteria preferentially utilising this soil carbon before switching to added carbon sources. However, this does not explain the differences in the length of lag phases observed following the addition of different carbon compounds. The lag time observed following the addition of sugars and fermentation endproducts (lactate and ethanol) may have been due to the time required for the growth of an initially small bacterial population which utilised the added carbon. If this were the case, the difference between the lag times of these two groups of compounds would seem to indicate that the initial population of sugar-utilising denitrifiers was larger than the population of denitrifiers which utilised lactate and ethanol.

The lag time before response to addition of VFAs was considerably longer than that before response to the other carbon sources. While the reasoning above would also apply to the lags before VFA utilisation, this explanation would be confounded by the initial inhibition of denitrification by VFAs (discussed in section 5.4.5).

Other studies have also found differences in denitrification lag time in soil following additions of carbon. deCatanzaro and Beauchamp (1985) found that soil denitrification responded more quickly to addition of plant material than to addition of sugars or glycerol and suggested that this was due to an adaptation period for the soil denitrifiers. Mihelcic and Luthy (1988) found that an acclimation period of 12 to 36 days elapsed before denitrification was observed following addition of acenaphthene or naphthalene. They speculated that the lag was due to the growth of a small population of denitrifiers capable of using the added substrate.

The maximum rate of denitrification observed was highest with the lactate and ethanol treatments followed by sugar addition. While these rates were not significantly different, the general order suggests that ethanol and lactate stimulated faster growth than did sugars.

Acetate and butyrate addition resulted in slower denitrification rates; however, this effect may have been confounded by the initial inhibition of denitrification.

5.4.4 FERMENTATION AND DENITRIFICATION

Accumulation of acetate and other VFAs in most treatments provided evidence that fermentation occurred during incubation. Carbon dioxide is often produced during fermentation of carbon compounds (Hamilton 1988) and, since carbon dioxide and nitrous oxide production occurred simultaneously, it is likely that fermentation occurred concurrently with denitrification. This suggests that fermenters and denitrifiers were competing for added carbon compounds. Alternatively, denitrifiers may have used the organic endproducts of fermentation.

Beauchamp *et al.* (1989) suggested that denitrifiers may rely on initial degradation of larger organic compounds by other soil bacteria. This may have been the case in the xylan (xylose polymer) and cellulose (glucose polymer) treatments. In the xylan incubations, diauxie was observed and nitrous oxide production temporarily ceased between 75 and 100 h indicating that denitrifiers had exhausted the initially available carbon. Denitrification resumed at about 100 h when, presumably, a new carbon source had become available, possibly through xylan decomposition by other anaerobic bacteria. In these soils, the addition of cellulose did not promote denitrification or carbon dioxide production above that of the rates for the controls and no fermentation endproducts were seen at the end of the incubation. The particle size of the cellulose added (average diameter 50 μm) may have been too large for significant hydrolysis to occur during the incubation period. Bremner and Shaw (1958) demonstrated that denitrification rate increased following cellulose addition to soil over a 30 days incubation. They did not report the particle size of cellulose used and therefore it is difficult to compare their results to those in the current study. Gok and Ottow (1988) found that, following the addition of cellulose to an anaerobically incubated sandy loam, denitrification decreased due to an increase in nitrate immobilisation. They repeated this experiment under aerobic conditions and found that denitrification increased and suggested that this was due to accelerated cellulose decomposition which had increased the amount of carbon available for denitrification. Increased decomposition of cellulose would have also created anaerobic microsites where denitrification could occur.

5.4.5 VOLATILE FATTY ACID INHIBITION OF DENITRIFICATION

All three VFAs, acetate, propionate, and butyrate initially inhibited denitrification. Propionate was inhibitory throughout incubation, whereas acetate and butyrate were both eventually used in denitrification. There have been a number of previous studies which have shown inhibitory and/or lethal effects of short-chain fatty acids on bacterial growth.

Weiner and Draskóczy (1961) found that the addition of acetate inhibited the oxidative metabolism of *Escherichia coli*. They showed that inhibition increased as the concentration of unionised acid increased. The concentration of unionised acid is dependent on acid concentration, the acid pK_a, and solution pH (Brady and Humiston 1980). Luli and Strohl (1990) also investigated the inhibitory effect of VFAs on the growth of *E.coli*. They suggested that the protonated form of acetate was lipid soluble and moved though the cell membrane into the cytoplasm where it dissociated at the higher internal pH. The internal pH would decrease due to an increase of H^+ ions, while the external pH would not significantly change. The net result is a partial de-energization of proton motive force (PMF) across the cell membrane due to the decrease in proton gradient. A decrease in PMF reduces the ability of cells to produce energy and could lead to cell death or inhibition. It is not improbable that this might have occurred in the present study; the denitrifiers produce energy following the establishment of a transmembrane proton motive force in a manner analogous to that in aerobic bacteria (Moodie and Ingledew 1990)

Cherrington *et al.* (1991a) found that the addition of 500 to 700 $mmol.l^{-1}$ of formic or propionic acid to *E. coli* caused cell death. These authors suggested that lowering of cytoplasmic pH caused cell death by irreversible denaturation of DNA and acid-labile proteins. A second study by Cherrington *et al.* (1991b) showed that at lower concentrations of formic ($10mmol.^{-1}$) and propionic acid ($5mmol.l^{-1}$) bacterial growth was inhibited and they suggested that at these concentrations organic acids inhibited DNA synthesis without damaging the DNA structure.

The fatty acids used as carbon sources in experiments 1 and 2 were added as the sodium salts and would have dissociated in soil water, establishing an equilibrium of ionised and unionised acid. In experiment 1, the concentrations of acetate, propionate, and butyrate were approximately 41, 27, and 24 $mmol.l^{-1}$ respectively, i.e., between those VFA concentrations at which Cherrington *et al.* (1991a,b) observed bacterial inhibition and death. All of the inhibition studies discussed above were conducted for less than 24h and the possible adaptation of bacteria to VFAs after this time was not investigated. In experiment 1, soil denitrifiers were able to adapt to and utilise the added acetate and butyrate. The concentrations of the added VFAs is likely to have varied throughout the soil due to the soil's particulate nature which may have buffered the effect of the added acids on bacterial viability. Cell growth may have been slowed until soil bacteria were able to establish mechanisms to maintain their cytoplasmic pH and utilise the added VFA.

In experiment 2, addition of acetate at the start of incubation inhibited denitrification even in the presence of a utilisable carbon compound - glucose. However, the addition of acetate to growing (exponential phase) or recently active (stationary phase) bacteria did not cause any observed changes in nitrous oxide production. In the latter instances, the bacteria may have been able to maintain internal pH and avoid cell death. The maintenance of internal pH

by homeostatic mechanisms generally relies on an active cell metabolism (Booth 1985). When acetate was added at the start of the incubation the soil denitrifiers present may not have been recently-active and may not have been able to maintain their cytoplasmic pH.

Paul *et al.* (1989) found nitrite accumulation in soil following addition of propionate and butyrate but not acetate. They suggested that this indicated some inhibition of denitrification; however, no inhibition of nitrous oxide production was shown. Their study was conducted at a soil-water pH of 7.5 (the soil-water pH in experiment 1 was 5.6; see section 4.3.1) and at lower carbon concentrations than in experiment 1. Both these factors would have resulted in a lower concentration of unionised acid in comparison to experiment 1. Since the unionised form of acid was probably lower in incubations by Paul *et al.* (1989) a lesser inhibition might be expected.

5.5 CONCLUSIONS

Denitrifiers in riparian soils were able to use a wide range of carbon compounds, although in some cases, partial decomposition by other anaerobic bacteria may have been initially required. Lag times before the onset of denitrification were dependent on the carbon compound added. This may have been indicative of the initial populations of denitrifiers capable of utilizing the substrate added.

The addition of VFAs initially inhibited denitrification, even in the presence of other readily degradable carbon compounds. After an extended lag time, acetate and butyrate, but not propionate, were used as a carbon source for denitrification. The mechanism by which VFAs inhibited denitrification was not elucidated. However, studies described in the literature suggest that the addition of VFAs can acidify the cytoplasmic pH resulting in partial decrease in proton-motive force, inhibition of DNA replication, and/or damage of acid-labile proteins and DNA. Further investigations suggested that inhibition did not occur in active or recently-active denitrifying bacteria; these bacteria may have been able to maintain their internal pH by homeostatic mechanisms.

CHAPTER 6
DECOMPOSING PLANT MATTER AS A CARBON SOURCE FOR DENITRIFYING BACTERIA

6.1 INTRODUCTION

In the preceding chapters (4 and 5) the effect of adding single carbon compounds on denitrification in anaerobic soils was examined. Those chapters showed that denitrification can be stimulated by a wide range of pure carbon compounds and that denitrifiers were, to some extent, reliant on fermentative bacteria to initially degrade larger organic compounds. In the soil environment it is unlikely that high concentrations of a singe carbon compound would suddenly become available to denitrifying bacteria. A more likely scenario is that a range of carbon compounds would be released more or less continuously during the decay of plant organic matter and that they would be used at the same rate as they were produced. Previous studies have shown that the addition of plant matter can stimulate denitrification in agricultural soils (Aulakh *et al.* 1984b; deCatanzaro and Beauchamp 1985; Myrold and Tiedje 1985b; Paul and Beauchamp 1989a).

In riparian soils, the concentration of nitrate at the surface of decaying organic matter is likely to vary considerably depending on nitrate diffusion rates, the local hydrology of the riparian zone, and the extent of nitrate removal by neighbouring soil (some of these aspects are examined in Chapter 7). Since organic riparian zones tend to be devoid of oxygen, decomposition of organic matter is likely to be primarily by anaerobic processes. Periodically, when the nitrate concentration becomes high enough, denitrification will also occur. Previous studies have not examined in detail how plant matter undergoing decomposition behaves as a electron source for denitrification.

In this chapter, the role of decomposing organic matter as a carbon source for denitrification was examined. Three plant materials were examined, two of which were freshly-collected live tissue and the third was senescent material. Plant matter was anaerobically incubated with organic soils and nitrate additions were made periodically.

The primary objective was to compare the extent to which different decomposing plant matter provided carbon for the denitrification process. It was also hoped to examine the interactions and competition of anaerobic bacteria (fermenters, methanogens and denitrifiers) during the decomposition of plant matter. A third objective was to examine the long-term persistence of denitrifying bacteria in the absence of an electron acceptor.

6.2 MATERIALS AND METHODS

6.2.1 PLANT SELECTION, PREPARATION, AND STORAGE

Three plant materials were selected as potential sources of carbon for denitrification. These were: fresh pine needles (*Pinus radiata*), senescent pine needles (*Pinus radiata*), and fresh watercress leaves (*Nasturtium officinale*). A control treatment (no plant addition) was also included. These plants were chosen to represent a range of likely decomposition rates; watercress was expected to have the highest rate of decomposition, followed by freshly collected pine needles and senescent pine needles the slowest rate of decomposition (Howard-Williams *et al.* 1983; Baker *et al.* 1989).

Plant material was obtained and prepared in the following manner. Watercress leaves and fresh pine needles were collected from living plant material and immediately returned to the laboratory. Senescent needles were collected from Kaiangaroa Forest compartment 69 by placing plastic sheets on the ground and fallen needles were collected from these sheets within a week. Plant matter was placed in a mortar, frozen in liquid nitrogen, and ground to pass through 0.25 and 0.5 mm mesh sieves. Particle sizes of between 0.25 and 0.50 mm were used for plant additions. Grinding was done in a -20°C walk-in freezer to avoid plant matter thawing and smearing on the sieve. Ground plant matter was stored at -20°C until used.

6.2.2 PREPARATION AND INCUBATION OF SERUM BOTTLES

Sixteen sequentially-numbered serum bottles were prepared for each of the following treatments: watercress, fresh pine needles, senescent pine needles, and control (a total of 64 bottles).

The weight of wet plant material added to each serum bottle was calculated in the following manner. The moles of carbon per gram of plant dry weight was calculated on the assumption that the dry plant matter had the molecular formula of CH_2O. The ratio of plant wet to dry weight was determined following oven-drying at 105°C. The required addition of wet material was then calculated in order to add 2.34 mmol of plant carbon per bottle. The following wet weights of plant material were added to appropriate serum bottles; fresh pine needles, 219 mg; senescent pine needles, 70 mg and watercress, 585 mg.

Soil which had been stored at -20°C was used in this experiment (see Chapter 4). Thawed soil slurry (20 ml) was added to each serum bottle by autopipette; the exact amount of soil added was determined gravimetrically. The headspace was flushed with argon to remove oxygen, sealed with black butyl rubber stoppers, and crimped closed with aluminium seals (Bellco). All 64 bottles were then incubated in the dark at 25°C shaking at 200 rpm. Periodically headspace samples were taken for gas analysis (section 6.2.3).

At nine day intervals, 1 serum bottle from each treatment was removed from the incubator and nitrate (2 ml of 50.5 g/l potassium nitrate i.e., 1 mmol N) and acetylene (16 ml into a 160 ml bottle) were added. These bottles were then incubated for a further 9 days during which time gas samples were taken (section 6.2.3). After 9 days of incubation the acetylene-inhibited serum bottles were removed from the incubator. The headspace volume was determined gravimetrically following the addition of water. The bottles were then shaken briefly and a water aliquot was taken. The water aliquot was passed through a Whatman filter number 114 and then a Millipore 0.45μm filter and subsequently analyzed for nitrate and ammonium concentration (section 3.5). The dry weight of soil in the serum bottle was then determined following oven drying at 105°C.

Simultaneous to the termination of the first set of bottles, nitrate and acetylene were added to the second bottle in each treatment which had by then been incubated for 9 days in the absence of nitrate and acetylene. As with the first set of serum bottles, gas samples were taken periodically for a nine day period before the second set of serum bottles were analyzed for headspace volume, nitrate and ammonium concentration, and soil dry weight. Nitrate and acetylene were then added to the third set of serum bottles and this cycle was continued up to and including the twelfth set of serum bottles.

6.2.3 MEASUREMENT OF GAS PRODUCTION

Gas production was measured in two separate phases of incubations: prior to and following nitrate addition.

Prior to nitrate addition

Measurements of CO_2 and CH_4 were made prior to nitrate addition. This was done to observe the extent to which fermentation and methanogenesis occurred in the absence of nitrate.

At days 0, 10, 38, 64, and 91 gas samples were taken from all serum bottles to which nitrate had not been added. Prior to gas sampling headspace pressure was recorded (section 3.3). Gas samples and pressures were taken through a stopcock valve attached to a syringe needle which was passed through the rubber stopper (section 3.3). Gas samples were stored in 2 ml vacutainers until analyzed for CO_2 and CH_4 (section 3.6).

Following nitrate addition

In some respects this part of the experiment can be considered as an assay of the amount of carbon available for denitrification during the decomposition of plant material.

Gas samples were taken from bottles immediately following nitrate addition, and at 2 days (48 h), 4 days (96 h), 6 days (144 h), and 9 days (216 h). Gas samples were analyzed for N_2O, CH_4, and CO_2 (section 3.6). Prior to gas sampling, headspace pressure was recorded (section 3.3).

6.2.4 LONG-TERM PERSISTENCE OF DENITRIFYING ACTIVITY

When bottle 12 of all treatments was completed, the persistence of denitrifying activity was examined in the following manner.

Four fresh anaerobic serum bottles were prepared with the addition of stored soil as previously described (section 6.2.2). To these bottles and to the remaining control bottles (numbers 13, 14, 15, and 16) the following additions were made - fresh pine needles (2.34 mmol C), nitrate (1 mmol N dissolved in 2 ml distilled water), and acetylene (16 ml). These bottles were then incubated at 25°C at 200 rpm, and gas samples and headspace pressures were taken at regular intervals for nine days. Gas samples were analyzed as previously described and after nine days of incubation headspace volume, nitrate and ammonium concentrations, and soil dry weight was determined as described above (section 6.2.3).

6.2.5 WATER SOLUBLE CARBON CONTENT OF PLANT MATERIALS

Denitrification potential rates in soil have been correlated to water soluble carbon (WSC) concentration (section 2.4.6.3). Differences observed in long-term production of gas may also be related to the initial WSC content of the plant materials and WSC of added plant material was measured in the following manner. Approximately 0.3 g frozen ground plant material (0.25-0.5 mm particle size) was weighed accurately into a centrifuge tube and 50 ml of distilled water was added. A blank control (i.e., no plant material added) was also prepared and treated in the same manner as plant material throughout extraction. The blank control was done to ensure that carbon was not extracted during the workup procedure. The centrifuge tubes were shaken for 5 minutes at 200 rpm and then centrifuged at 3000 rpm for 5 minutes to sediment the plant material. The supernatant was decanted out of the tube and forced through a Millipore 0.45μm filter. The amount of carbon in the filtered liquid was determined using the Hach proprietary method (Hach Co. Colorado) which is based on the method described by Jirka and Carter (1975).

6.2.6 DATA ANALYSIS

Gas production, nitrate and ammonium concentrations were expressed as per gram of dry soil in order to account for differences in initial soil weight and organic matter content. Confidence limits shown on graphs were determined as described in section 5.2.3.

6.3 RESULTS

Results are presented in the following sequence: first, gas production before and after nitrate addition; second, the concentration of nitrate and ammonium found at the end of each incubation; third, the long term viability of denitrifiers; and lastly, the amount of water soluble carbon extracted from plant material.

The gas production was examined from two perspectives: 1. the amount of CO_2 and CH_4 prior to nitrate addition (this will be referred to as gas produced during the "fermentative phase"); 2. CO_2, CH_4, and N_2O production during the 9 day incubation following nitrate addition (this will be referred to as gas produced during the "denitrification phase").

6.3.1 GAS PRODUCTION DURING THE FERMENTATIVE PHASE

The amounts of CO_2 and CH_4 produced during the fermentative phase are shown in Figure 6.1a,b. Both CO_2 and CH_4 production were highest from the watercress, followed by fresh pine needles, senescent pine needles, and control treatments. Both CO_2 and CH_4 production continued throughout the incubation prior to nitrate addition. The coefficient of variation of the CO_2 and CH_4 concentrations in the serum bottles were generally less than 10% and no obvious outliers were observed.

The molar ratio of CH_4:CO_2 (Figure 6.1c) was initially zero, as methane was not produced. Once methane production began, the ratio increased and was dependent on the treatment imposed, watercress gave the highest ratio of 1, senescent and fresh pine needles gave a ratio of 0.8, and the control ratio was 0.6.

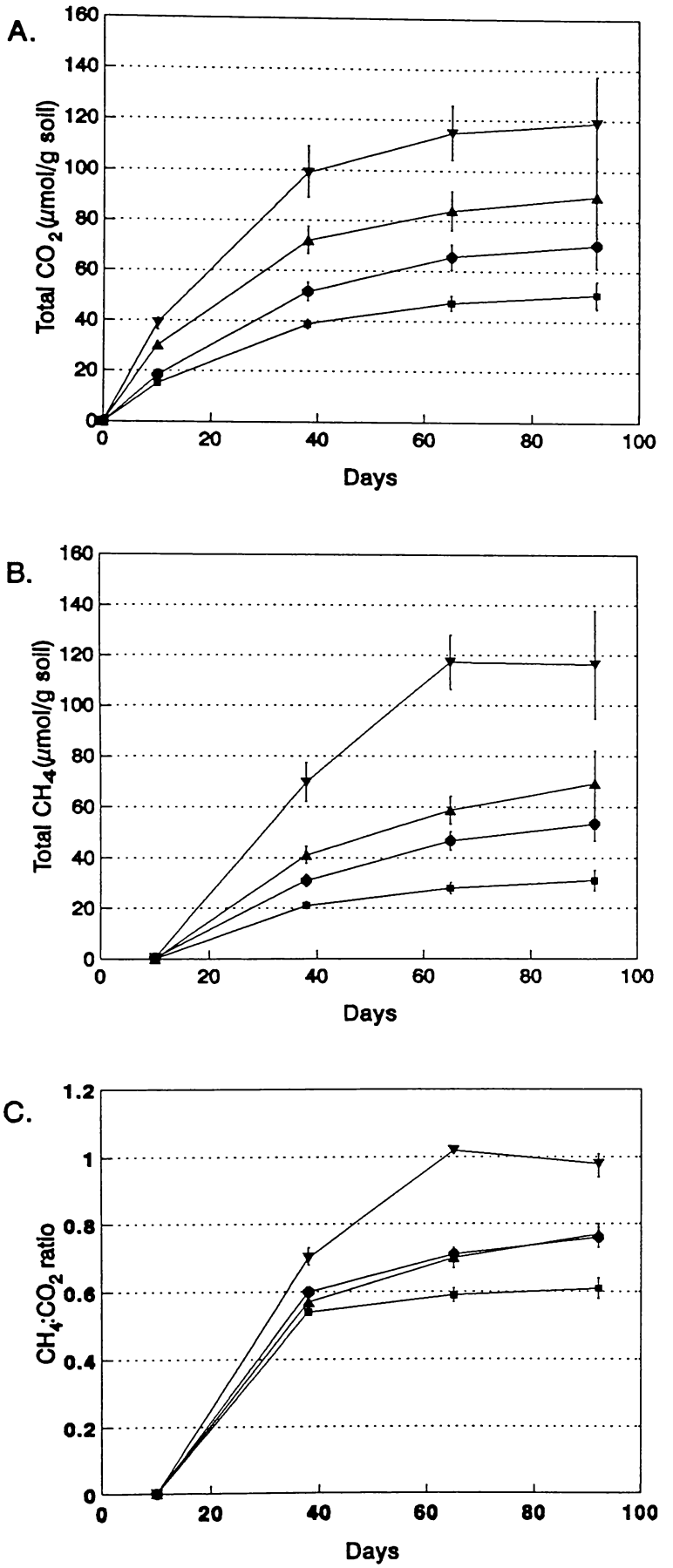


Figure 6.1 Gas production during the fermentative phase (A) CO_2, (B) CH_4, (C) CH_4:CO_2 ratio. ▾ - watercress, ▲ - fresh pine, ● - senescent pine, ■ - control. Error bars denote 95% confidence limits.

6.3.2 GAS PRODUCTION DURING THE DENITRIFICATION PHASE

Nitrous oxide production generally began within two days of incubation following nitrate addition and continued to increase throughout the 9 day incubation in all treatments (Figure 6.2)

Figure 6.3a,b summarises the total N_2O and CO_2 produced during the 9 day incubations from all treatments. All additions of plant material increased N_2O and CO_2 production above the control. Addition of fresh pine and watercress resulted in similar N_2O and CO_2 production and both gave greater N_2O and CO_2 production following nitrate addition than did the addition of senescent pine. The ratio of N_2O to CO_2 produced during each of the 9 day incubations was effectively constant for the first 50 days (Figure 6.4), after which the errors associated with measuring the small amounts of CO_2 and N_2O produced resulted in greater variation of the N_2O:CO_2 ratio.

Once nitrate was added to each incubation, no further methane production was detected (data not shown).

6.3.3 NITRATE AND AMMONIUM REMAINING

At the end of the incubation period the remaining nitrate and ammonium concentrations were measured and results are shown in Figure 6.5a,b. Nitrate recovered in serum bottles increased as the incubation period prior to nitrate addition increased and in later incubations recovery of nitrate approached 100% of the 1 mmol nitrate added. Ammonium concentrations also increased over time and were generally an order of magnitude less than nitrate concentrations.

6.3.4 LONG-TERM DENITRIFIER VIABILITY

Denitrification in both sets of serum bottles started between 24 and 48 h (Figure 6.6). Total nitrous oxide production in the pre-incubated serum bottles was less than that found in the freshly-prepared serum bottles. Carbon dioxide production (not shown) followed the same pattern as nitrous oxide production. Methane was not detected.

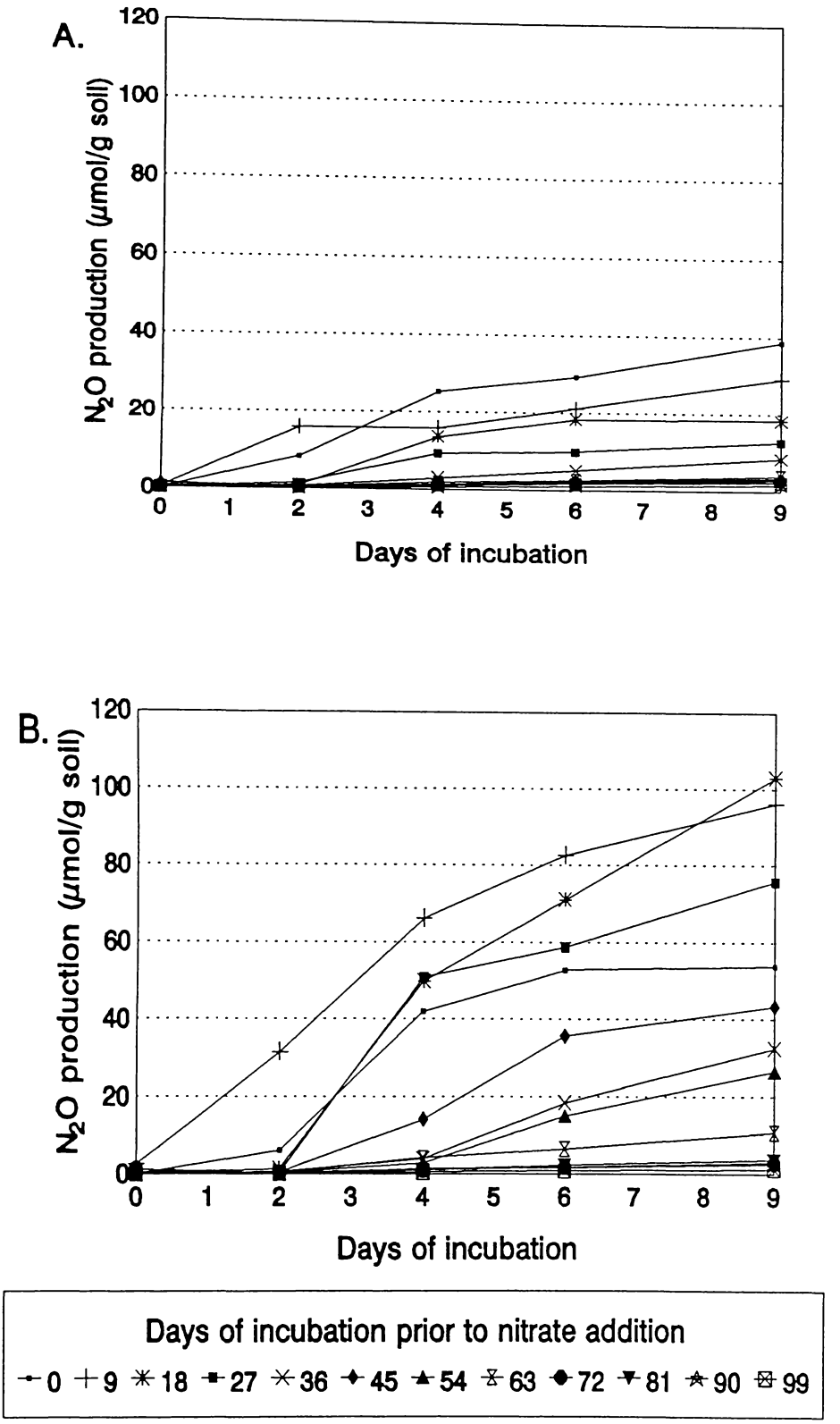


Figure 6.2 Nitrous oxide production during 9 day incubations following nitrate addition (A) control, (B) watercress.

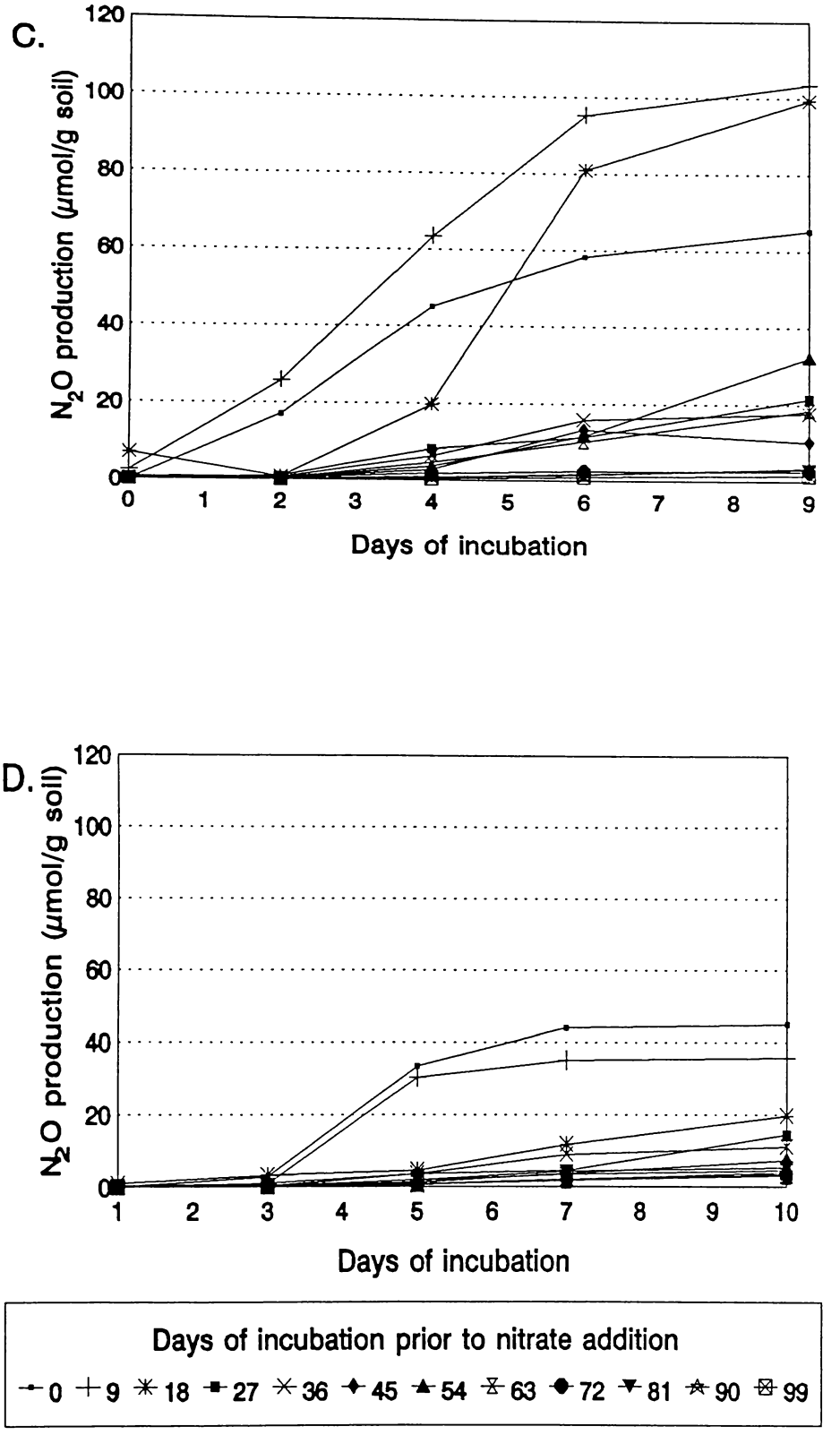


Figure 6.2 cont. Nitrous oxide production during 9 day incubations following ni trate addition (C) fresh pine, (D) senescent pine.

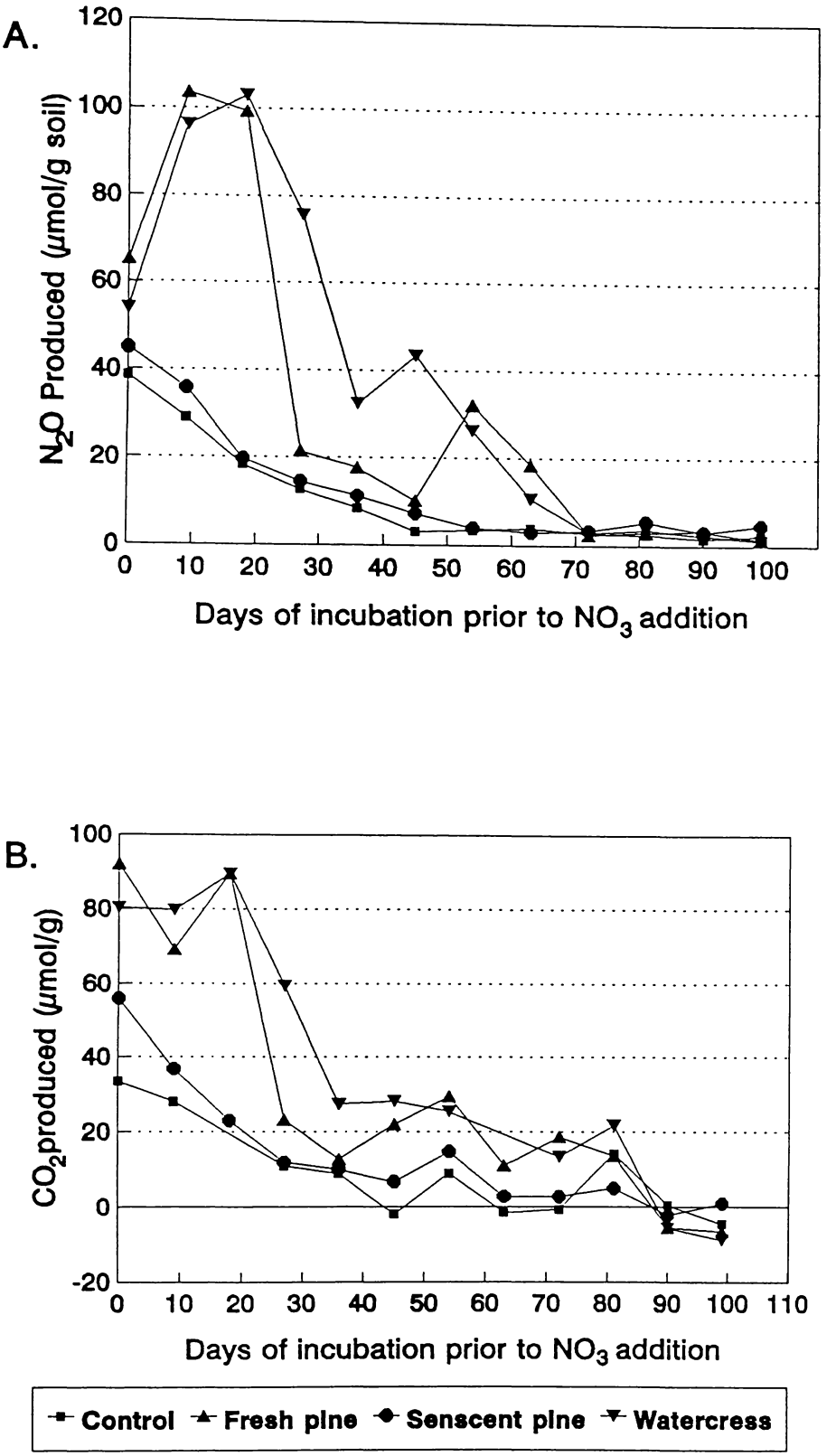


Figure 6.3 Total (A) N_2O and (B) CO_2 produced during nine day incubations shown as a function of days of incubation prior to nitrate addition.

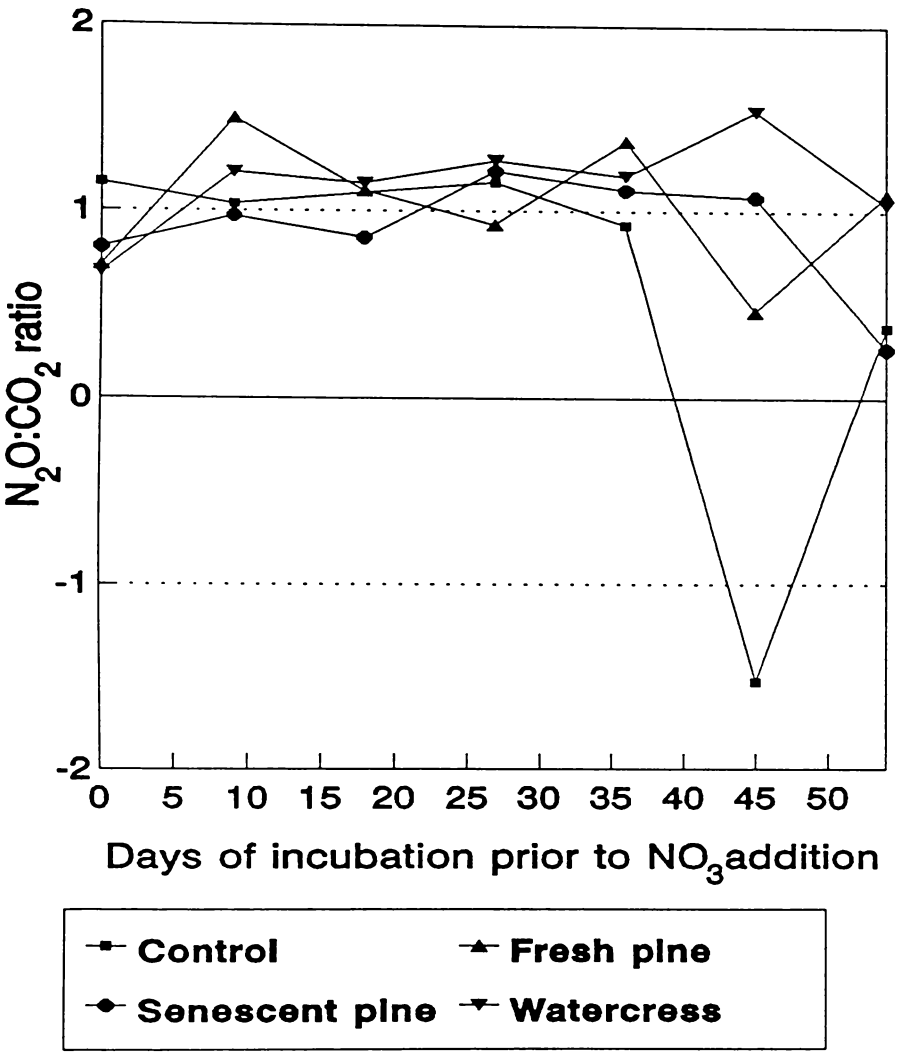


Figure 6.4 $N_2O:CO_2$ ratio observed during denitrification phase.

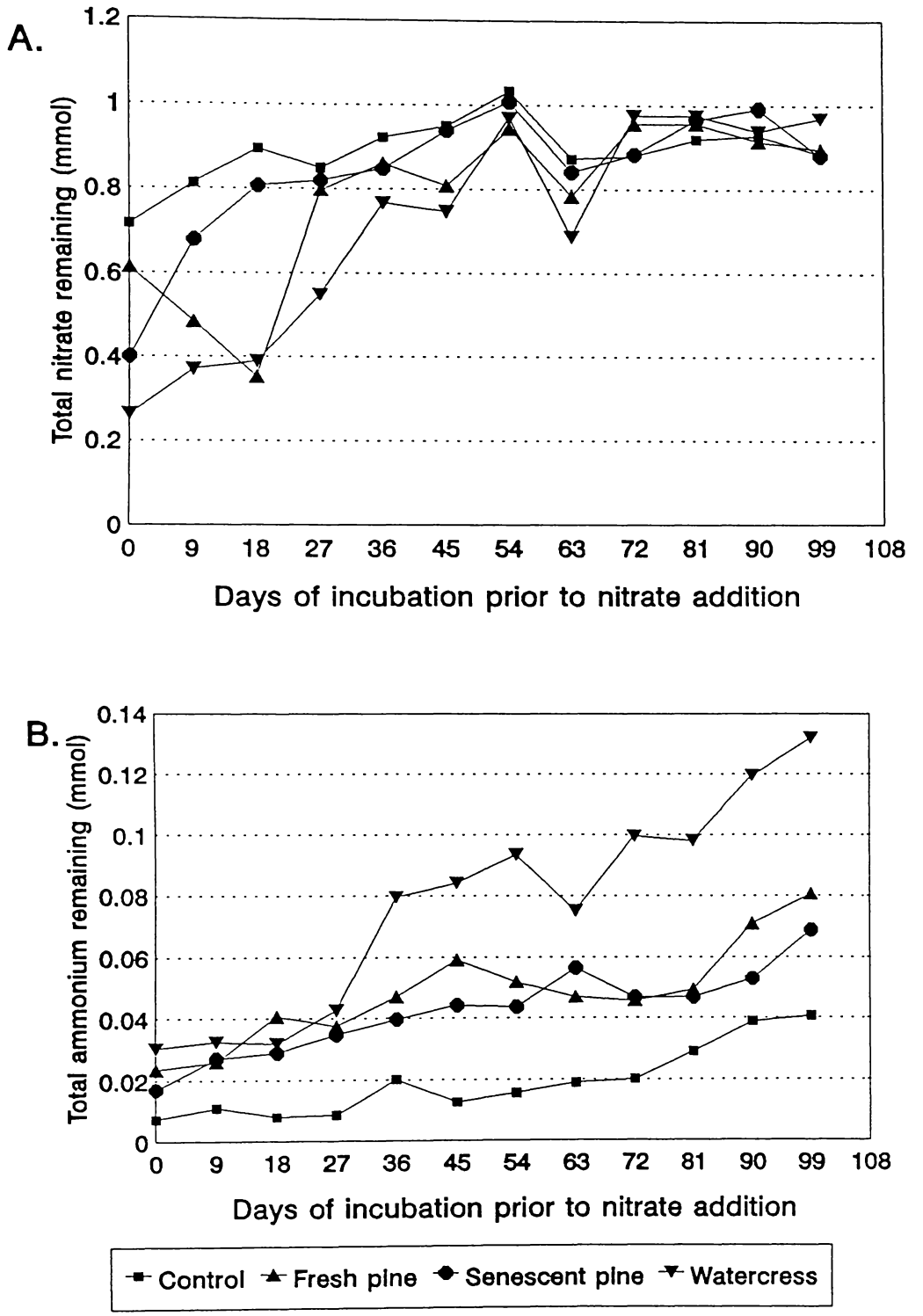


Figure 6.5 (A) Nitrate and (B) ammonium concentrations found at the end of the incubation period. Note 1 mmol of nitrate was initially added to all incubations.

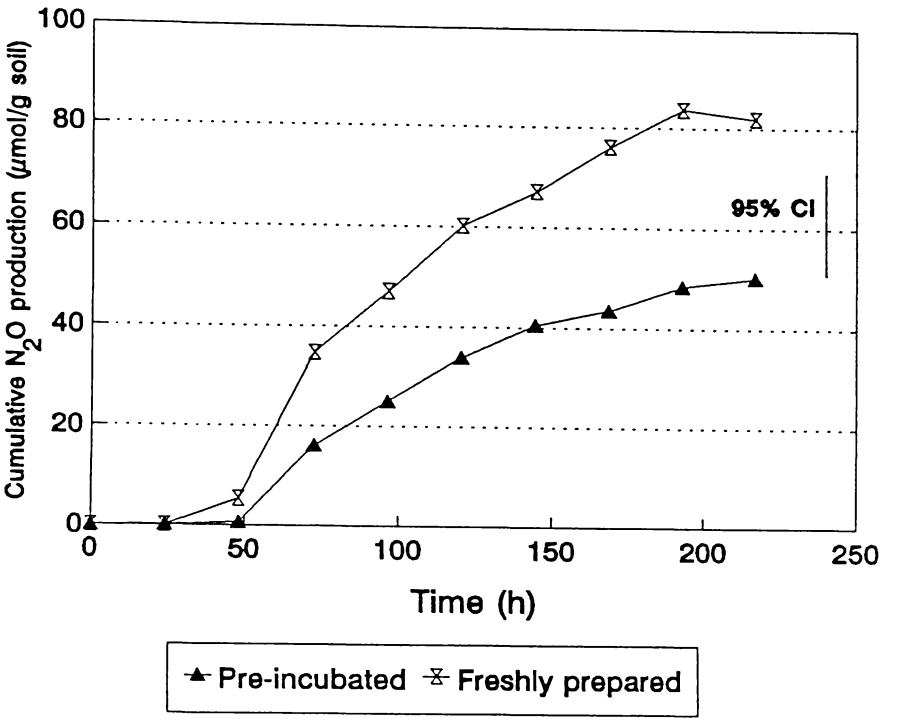


Figure 6.6 N_2O production in pre-incubated and freshly-prepared soil following nitrate and carbon additions.

6.3.5 WATER SOLUBLE CARBON CONTENT OF PLANT MATERIALS

Results of WSC determination are shown in Table 6.1. Fresh pine needles had the highest WSC followed by watercress and senescent pine needles. Control experiments showed that no WSC was produced during the work-up procedure for the assay of WSC.

PLANT	AVERAGE WATER SOLUBLE CARBON ($mg.g^{-1}$ plant dry weight)
FRESH PINE NEEDLES	388
SENESCENT PINE NEEDLES	47
WATERCRESS	226

Table 6.1 Water soluble carbon concentration from ground plant materials used in long-term decomposition incubations. n=7, except in fresh pine needles where n=6. All values were significantly different at 95% confidence determined by One Way ANOVA.

6.4 DISCUSSION

The above results are discussed in the following four sections. The first section examines the interactions of fermentative and methanogenic bacteria prior to nitrate addition in the "fermentative phase". The second section discusses how these bacteria adapted following the addition of nitrate and also their interaction with denitrifying bacteria in the "denitrification phase". The third section compares the microbial responses to different plant additions and this also includes discussion on the results of the carbon extracted from the plants. Lastly, the long-term viability of denitrifying bacteria in the absence of nitrate is discussed.

6.4.1 BACTERIAL ACTIVITY DURING THE FERMENTATIVE PHASE

Before nitrate addition, CO_2 and CH_4 were presumably produced by fermentation and methanogenesis. As the incubation progressed, the rate of CO_2 and CH_4 production decreased (Figure 6.1a,b), most likely as a result of a decrease in degradable organic matter. The organic matter remaining presumably became progressively more recalcitrant to anaerobic degradation during the course of incubation. The initial delay in methane production was probably due to the slow growth rate and/or poor recovery of methanogens following freezing and thawing.

The CH_4:CO_2 ratio became constant after about 40 days incubation (Figure 6.1c), which implied that CO_2 and CH_4 production were linked. There were two possible explanations for this linkage. The fermentation endproducts acetate and hydrogen are the major electron donors used by methanogens and the formation rate of these endproducts may have dictated the rate of CH_4 production. Conversely, the rate of hydrogen removal by methanogens may have limited the rate of fermentation. Some forms of fermentation are only energetically possible when the hydrogen concentration is maintained at very low partial pressures and this can be accomplished by interspecies hydrogen transfer (Zehnder and Stumm 1988). Both these scenarios for anaerobic decomposition have been previously presented by Zehnder and Colberg (1986), and Zehnder and Stumm (1988). In the present experiment, the first explanation is the most likely for the following reasons. Carbon dioxide production occurred prior to methane production suggesting that, at least initially, fermentation was independent of methanogenesis. Also, interspecies hydrogen transfer only occurs when highly reduced compounds (such as propionate and butyrate) are fermented to carbon dioxide and hydrogen, and this form of metabolism is likely to be only a small fraction of the total fermentation of the organic compounds produced by the degradation of plant polymers in this experiment. A schematic diagram of the bacterial interactions prior to nitrate addition is shown in Figure 6.7a.

The magnitude of the CH_4:CO_2 ratio was dependent on the treatment imposed. The addition of watercress gave the highest ratio followed by pine needle addition and finally the control treatment.

Other previous studies have also shown the CH_4:CO_2 ratio to be dependent on the substrate's oxidation state (Buswell and Mueller 1952) or its biodegradability (Jerger *et al.* 1982).

6.4.2 BACTERIAL ACTIVITY DURING THE DENITRIFICATION PHASE

After nitrate and acetylene addition, nitrous oxide concentration increased in all incubations (Figure 6.2). It was unlikely that denitrification was limited by nitrate in any of these incubations as nitrate was present in the soil at the end of each 9 day incubation. CO_2 was produced simultaneously to N_2O production. While N_2O was presumably produced by denitrification, CO_2 may have been produced by either denitrification or fermentation. The ratio of N_2O:CO_2 was constant for the first five batch incubations (Figure 6.4), which implied that N_2O and CO_2 production were linked. There are two possible explanations for this constant ratio. The first is that CO_2 production was predominantly formed via denitrification and thus CO_2 production would necessarily be related to N_2O production. If so, it would suggest that following nitrate addition, fermenting bacteria were inhibited or out-competed by denitrifiers. An alternative explanation for the constant N_2O:CO_2 ratio was that denitrification was dependent on the release of carbon from larger organic compounds by fermentation. Thus, the rate at which fermentation released CO_2 would be related to the rate at which denitrification occurred. The dependence of denitrifying bacteria on fermentation has previously been postulated by Beauchamp *et al.* (1989) and some supporting evidence for this hypothesis was also found in Chapter 5 as well as by Reddy *et al.* (1982), deCatanzaro and Beauchamp (1985), and Paul and Beauchamp (1989a).

Methane production stopped following the addition of nitrate and acetylene. This was most likely due to the inhibition of methanogenesis by acetylene which has previously been shown in a number of studies (reviewed by Oremland and Capone 1988). However, addition of nitrate to anaerobic soils has also been shown to inhibit methane production in the absence of acetylene (Gok and Ottow 1988; Westermann and Ahring 1987). In these previous studies nitrate may have inhibited methanogens directly or growth of denitrifiers may have out-competed methanogens for available nutrients and electron donors.

Figure 6.7b presents a schematic diagram which summarises the interactions of soil bacteria following nitrate addition based on observations in the present study.

The N_2O-N:CO_2-C ratio was between 1.8 and 2.2 for all incubations. This value is considerably higher than the theoretical N_2O-N:CO_2-C ratio of 1 using glucose as the carbon source.

$$6C(H)_2O + 6NO_3^- + 6H^+ \text{ --------> } 6CO_2 + 3N_2O + 9H_2O$$

In a study of mineral and organic soils, Reddy *et al.* (1982) found a molar ratio of nitrate-N consumed to CO_2 formed of between 0.6 and 1.8, the higher figure being greater than the theoretical ratio of 1.

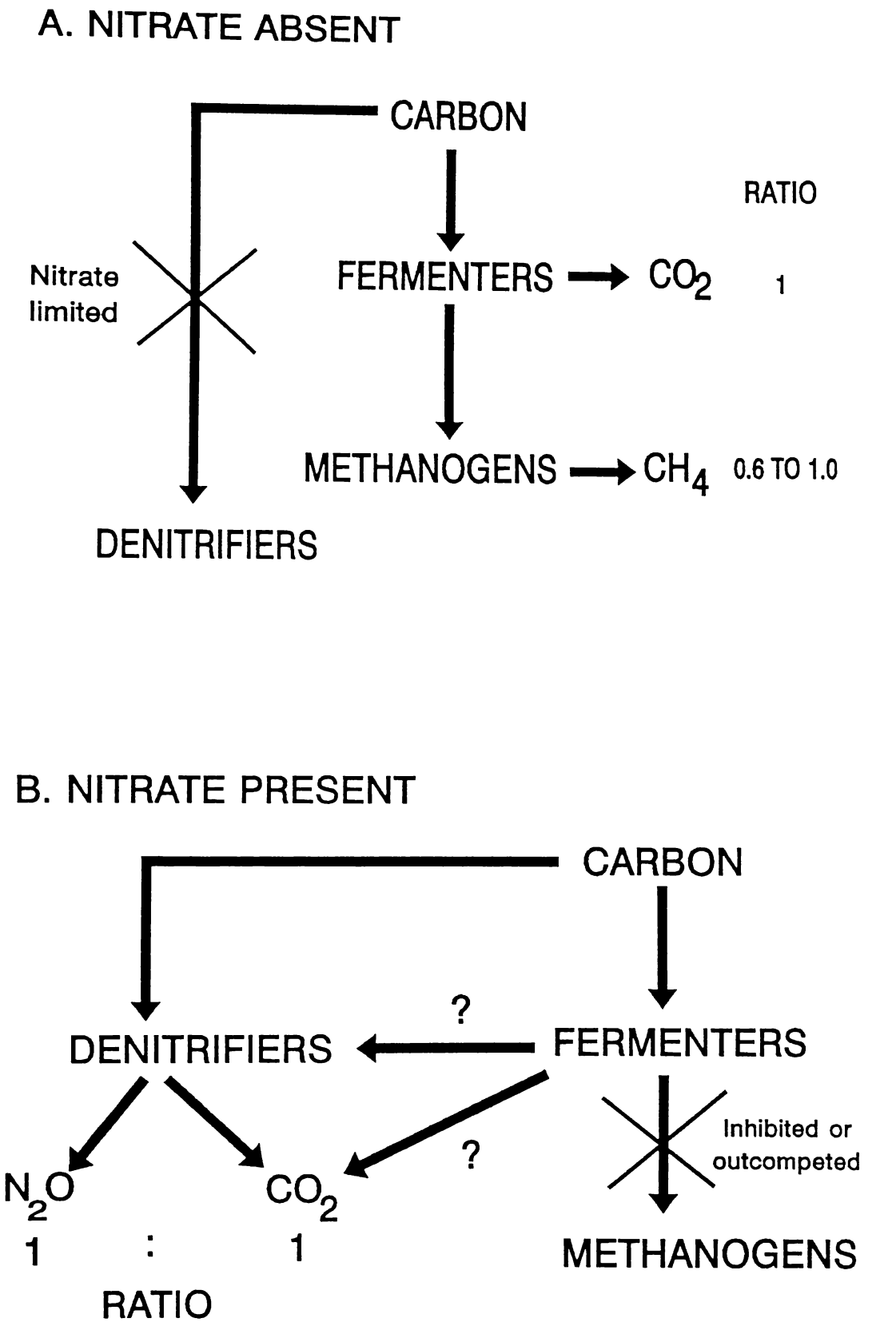


Figure 6.7 Schematic diagram of anaerobic bacterial interactions in (A) the absence of nitrate, (B) the presence of nitrate.

6.4.3 EFFECT OF PLANT ADDITIONS

N_2O, CO_2, and CH_4 production in the fermentative and denitrification phases were dependent on which plant material was added. Nitrous oxide production suggested that addition of the fresh plant-material (watercress and fresh pine needles) made the most carbon available for denitrification. To a much lesser extent, senescent pine needles also increased denitrification above the control (Figure 6.3). Fresh plant material contained 5 to 8 times the amount of WSC as senescent pine needles (Table 6.1). If WSC is considered as a measure of available carbon then the observed differences in WSC may have accounted for the increased gas production following the addition of fresh plant material when compared to senescent plant matter. However, initial WSC was not the only factor which determined the extent of denitrification. Fresh pine needles were shown to have approximately twice the WSC of that found in watercress and yet N_2O and CO_2 were similar in both of these treatments (Figure 6.3). In fact, watercress addition stimulated more CO_2 and CH_4 production during the fermentative phase than did the addition of fresh pine needles. This would suggest that the WSC from watercress was somewhat more biologically-available to the bacteria in soil than the WSC found in fresh pine needles. Alternatively, a greater amount of WSC may have been physically released from watercress than from fresh pine needles during the course of incubation.

Previous studies have also found that the addition of dried plant matter stimulated denitrification (Aulakh *et al.* 1984b; deCatanzaro and Beauchamp 1985; Paul and Beauchamp 1989a; Myrold and Tiedje 1985b); however, those studies did not examine the effect of fresh plant material on denitrification. Results from the present study clearly highlighted that the quality of organic carbon can be of greater importance than the quantity of carbon in determining the extent of microbiological response.

In all treatments, the amount of CO_2 and N_2O produced during the denitrification phase generally decreased as the length of pre-incubation increased. This was probably due to decreasing amounts of available carbon to support denitrification. After 70 days of pre-incubation, the N_2O production was the same in all treatments, suggesting that the remaining plant carbon was recalcitrant to decomposition.

6.4.4 LONG-TERM PERSISTENCE OF DENITRIFIER ACTIVITY

When examining the long-term persistence of denitrifier activity, the production of N_2O started simultaneously in the pre-incubated and in the freshly prepared soil treatments (Figure 6.6). However, the amount of N_2O formed differed and this probably reflected differences in the amount of carbon initially available in the two sets of incubations. Carbon was previously lost from the pre-incubated soil due to conversion to CO_2 and CH_4 by fermentation and methanogenesis. Carbon may also have been tied up as microbial biomass.

It is important to note that numbers of denitrifying bacteria were not measured but rather an assessment of the recovery of denitrifying activity was made. That N_2O production was similar in both sets of incubations, indicated that at least some of the denitrifying population was able to survive long periods in the absence of an electron acceptor (nitrate or oxygen). However, it is not known whether these bacteria survive by becoming dormant or whether they are capable of an alternative form of anaerobic metabolism. Other possible forms of anaerobic metabolism have not been identified in denitrifiers. This long-term survival of denitrifying bacteria might account for high denitrification potentials found in anaerobic soils (Tiedje *et al.* 1982; Tiedje 1988) where nitrate is limiting or only periodically present.

6.5 CONCLUSIONS

In this chapter, decomposing plant material was examined as a potential carbon source for denitrification. Due to the experimental design, some indication of bacterial interactions was possible prior and subsequent to nitrate addition. Three different plant additions were compared to a control incubation: fresh watercress leaves, fresh pine needles, and senescent pine needles.

Prior to nitrate addition, watercress was shown to promote the greatest production of CO_2 and CH_4, followed by fresh pine senescent pine and the control incubations. In all treatments the CH_4:CO_2 ratio became constant indicating a relationship between fermentative and methanogenic bacteria. It is postulated that methanogens were dependent on the release of electron donors from complex carbon compounds by fermentative bacteria. This suggestion has been made in the literature previously. The magnitude of the CH_4:CO_2 ratio was dependent on the plant matter added and previous studies have suggested that this might be related to the oxidation state of the carbon in the added substrate.

Following nitrate addition, denitrification and CO_2 production were highest in the fresh watercress and fresh pine needle additions. Addition of senescent pine needle also increased denitrification above the control but to a much lesser extent. This suggested that the quality of added carbon may be of greater importance than quantity with respect to microbial response. After 70 days of incubation prior to nitrate addition, the denitrification response was similar in all treatments, presumably because the remaining plant carbon was recalcitrant to further decomposition. Water-extracted carbon from the three plants examined were in general agreement with denitrification response; although WSC did not account for all of the differences in gas production observed. The ratio of N_2O:CO_2 produced following nitrate addition was constant for all treatments and a number of different explanations are presented. First, that in the presence of nitrate, denitrifying bacteria out-competed fermenters for organic carbon and nutrients and as a result little fermentation occurred. Second, that denitrification was dependent on the production of organic carbon by fermenters.

Finally, the long-term viability of denitrifying bacteria in the absence of nitrate or oxygen was examined. Nitrate and carbon additions were made to freshly prepared incubations of soil and to soil which had been incubated for over 99 days in the absence of nitrate and oxygen. Denitrification started simultaneously in both incubation sets indicating that denitrifying bacteria were well equipped to survive long periods in the absence of oxygen and nitrate. This observation was in agreement with the general observation that high denitrifying enzyme activities are often found in anaerobic environments where nitrate is at low concentrations or infrequently present. It was not determined whether survival was by use of an alternative mechanism of metabolism or whether it was by the bacteria becoming dormant.

CHAPTER 7
REGULATION OF RIPARIAN DENITRIFICATION - A FIELD STUDY

7.1 INTRODUCTION

A preliminary study of nitrate movement below a land effluent treatment system showed that nitrate in draining groundwater was removed as it passed through a narrow organic riparian zone (Schipper *et al.* 1989). It was suggested that bacterial denitrification was the mechanism responsible. Nitrate removal has been observed in a number of other studies of broader mineral riparian zones where denitrification and plant uptake were considered to be the mechanisms for nitrogen removal (Peterjohn and Correll 1984; Jacobs and Gilliam 1985; Lowrance *et al.* 1984b). However, few studies to date have measured denitrification in either type of riparian zone. Cooper (1990) showed that denitrification rates in riparian zones could be very high relative to rates of denitrification in other landscapes and that maximum nitrate removal and denitrification occurred in organic riparian soils.

While there is some understanding of the factors which regulate denitrification in upland soils, such as forested and agricultural land, little is known about the regulatory factors of denitrification in riparian zones. An understanding of these regulatory factors might define appropriate management practices for enhancing riparian zone denitrification, resulting in the protection of surface waters from excess nitrate contamination. The immediate or proximate regulators of soil denitrification are considered to be carbon, nitrate, and oxygen (Tiedje 1988) and their influence on denitrification has been demonstrated in a number of studies of upland soils (e.g. Robertson and Tiedje 1984; Myrold 1988; Parsons *et al.* 1991). There are a number of key differences between organic riparian soils and upland soils which might affect the regulation of denitrification rate. Riparian soils are often more water-saturated and contain higher quantities of organic matter than do upland soils; this can lead to a highly anaerobic soil environment. Also, in aerobic upland soils, nitrate tends to be generated following nitrification at the site whereas nitrate is unlikely to be formed in anaerobic riparian soils. The supply of nitrate for riparian denitrification might therefore be dependent on nitrate movement from upland soils by groundwater flow.

There were two main objectives in the present study. The first was to confirm that nitrate removal occurred in an organic riparian zone receiving nitrate in groundwater. The second was to measure denitrification rates in the riparian zone along with other biological and soil properties which might indicate how denitrification was regulated in organic riparian soils.

7.2 METHODS

7.2.1 STUDY SITE

The study site was situated in a subcatchment of the Whangamata sewage irrigation scheme in Tairua Forest, Coramandel Peninsula, North Island, New Zealand. The Wharekawa soils present were a subgroup of dystrohrept soils and consist of Whangamata volcanic ash overlying an older Waihi ash weathered to allophane clay. The upslope vegetation at the site consisted of radiata pine (*Pinus radiata*) planted in 1975 with a final stocking of 220 stems/ha, with a native and exotic understorey. Sewage was applied into the forest by spray irrigation at a rate of not more than 55 $mm.wk^{-1}$ since October 1986. The effluent was pre-treated in an extended aerated lagoon followed by an oxidation pond and was high in nitrogen which varied between 20 and 40 $gN.m^{-3}$ (Schipper *et al.* 1989; unpublished data). This high nitrogen loading resulted in high nitrate concentrations in the groundwater leaching away from the spray site to surface water. The subcatchment studied was drained by a small perennial stream which was surrounded by a riparian zone. The riparian zone consisted of a mosaic of organic and mineral soil patches of varying width, generally less than five meters between the stream and forest edge.

7.2.2 LYSIMETER STUDY

7.2.2.1 Transect establishment

A transect of suction cup lysimeters (Grover and Lamborn 1970) was established in July 1988. The transect consisted of five sets of five lysimeters. The placement of lysimeter sets is shown in Figure 7.1. Holes were augured to the appropriate depth, lysimeters placed in the holes, and backfilled around the ceramic cup with a slurry of fine pumice and distilled water. The remainder of the hole was backfilled using augured soil. The lysimeter depths in centimetres were: set 1 - 20, 60, 60, 100, 100; set 2 - 20, 60, 60, 100, 100; set 3 - 60, 60, 100, 100, 120, 120; set 4 - 130, 140, 140, 200, 200; set 5 - 60, 60, 100, 100, 100. Lysimeters in sets 1 to 4 were all installed into groundwater whereas lysimeters in set 5 were not installed into groundwater. Lysimeter sets 1 and 2 were installed in the organic riparian zone, sets 3 and 4 above the organic zone, and set 5 in the effluent irrigation zone. For the first three months the lysimeters were periodically emptied and collected water was discarded. This was to allow the lysimeters to equilibrate with the soil-water chemistry following disturbance during installation. This lysimeter transect lay parallel to the original lysimeter transect described in Schipper *et al.* (1989).

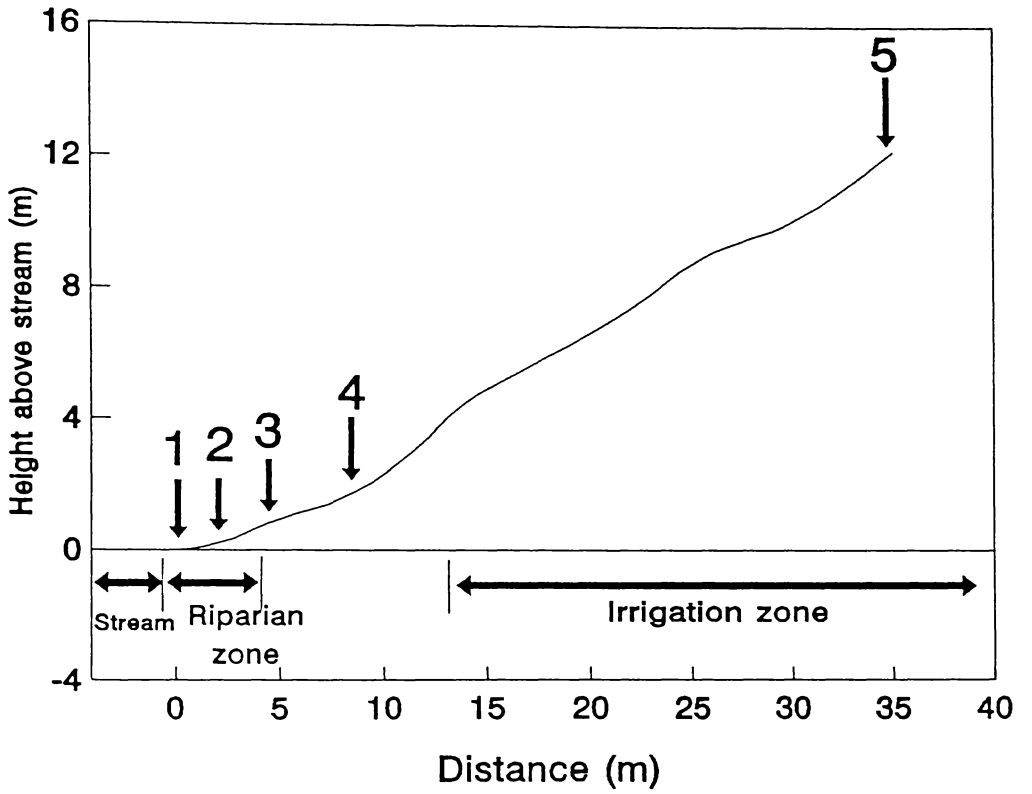


Figure 7.1 Slope profile showing location of the 5 sets of lysimeters.

7.2.2.2 Routine lysimeter and stream sampling

Eleven lysimeter sampling visits were made over a three year period. First, a handheld vacuum pump was used to empty the lysimeters of water which had accumulated between sampling visits, and then a vacuum was placed on each lysimeter. The following day water samples were taken from the lysimeters and transported back to the laboratory where the samples were stored at 4°C until analyzed for nitrate and chloride as described in section 3.5.

The ratio of nitrate to chloride was calculated for each lysimeter; these ratios were then averaged for each lysimeter set for each sampling visit and plotted against time. Chloride was used as a conservative tracer for nitrate as changes in the nitrate:chloride ratio reflect changes in total nitrate movement. The main assumptions were that chloride and nitrate have similar movements through soil and also that chloride is not substantially removed below the root zone (Pratt *et al.* 1978). Here the aims of the lysimeter monitoring were to assess qualitative changes in nitrate concentration in order to confirm that the riparian zone studied was the site of substantial nitrate removal.

At the time of each lysimeter sampling, a stream water sample was also collected at the base of the subcatchment. The stream sample was analyzed for nitrate and chloride concentration within seven days (section 3.5).

7.2.3 RIPARIAN ZONE SAMPLING

7.2.3.1 Riparian study site

The riparian zone chosen for intensive sampling lay between lysimeter sites 1 and 3 (Figure 7.1). The soil present was highly organic and water-saturated with occasional patches of surface water. The sparse plant cover initially present at the site was removed by hand and any plant cover that returned between samplings was also removed. Wooden planks were laid across the site to allow access while minimising compaction of the site.

Five visits were made to this riparian zone during a two year period on the following dates - 24/1/90, 12/7/90, 27/11/90, 11/2/91, 2/5/91. Initially a 1.85 m by 1.85 m section of the riparian zone was divided into a grid of 32 sampling sites (Figure 7.2). Each site was marked with a thin plastic rod so that it was easily identified during subsequent visits. The grid could not be evenly spaced because of large particulate debris which could not be removed without having a substantial impact on the site. The contour lines were determined at the end of the study by driving four stakes into the ground surrounding the riparian zone and building a level plane above the soil. Vertical height measurements were made from the plane to the soil; the lowest point was assigned a zero relative height.

7.2.3.2 Soil sampling

A soil core was taken within a 2 cm radius from each plastic rod to a depth of 30 cm using a hoffer tube. It was not possible to remove an intact soil core as the riparian soil was water-saturated, homogeneous, rich in organic matter and tended to compact easily. The lip of the hoffer tube which runs the length of the corer was used to remove a soil sample from the inside of the initial hole made. The soil was removed from the hoffer tube and mixed by hand in a plastic tray. Soil subsamples were then used in all subsequent analyses detailed in sections 7.2.3.3 to 7.2.3.7. A schematic diagram which shows the soil analyses completed for each soil sample is given in Figure 7.3.

Between sampling visits the core holes generally filled up, presumably by the movement of water and organic matter through the riparian zone.

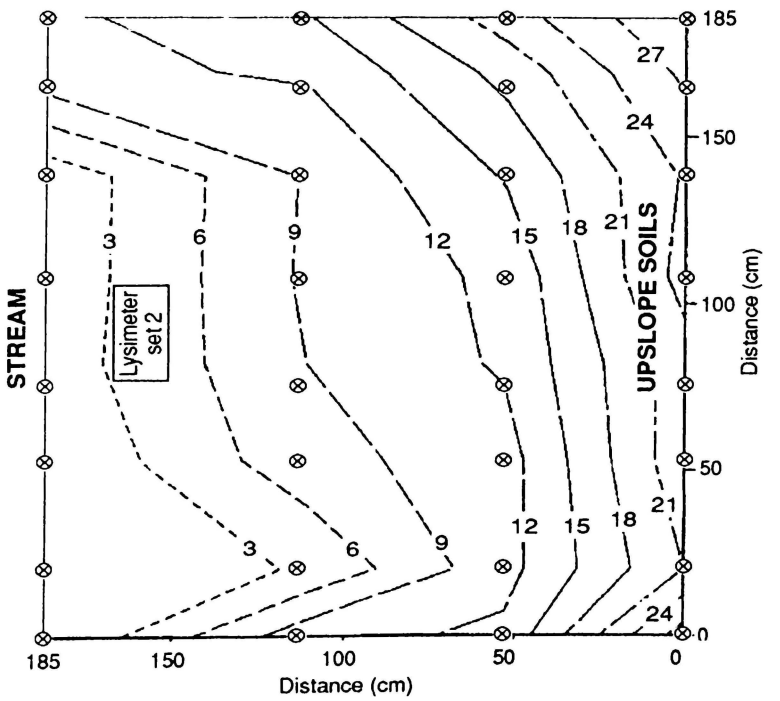


Figure 7.2 Photograph and plan of riparian zone study site. Contour lines show relative height (cm), circles indicate soil sampling sites.

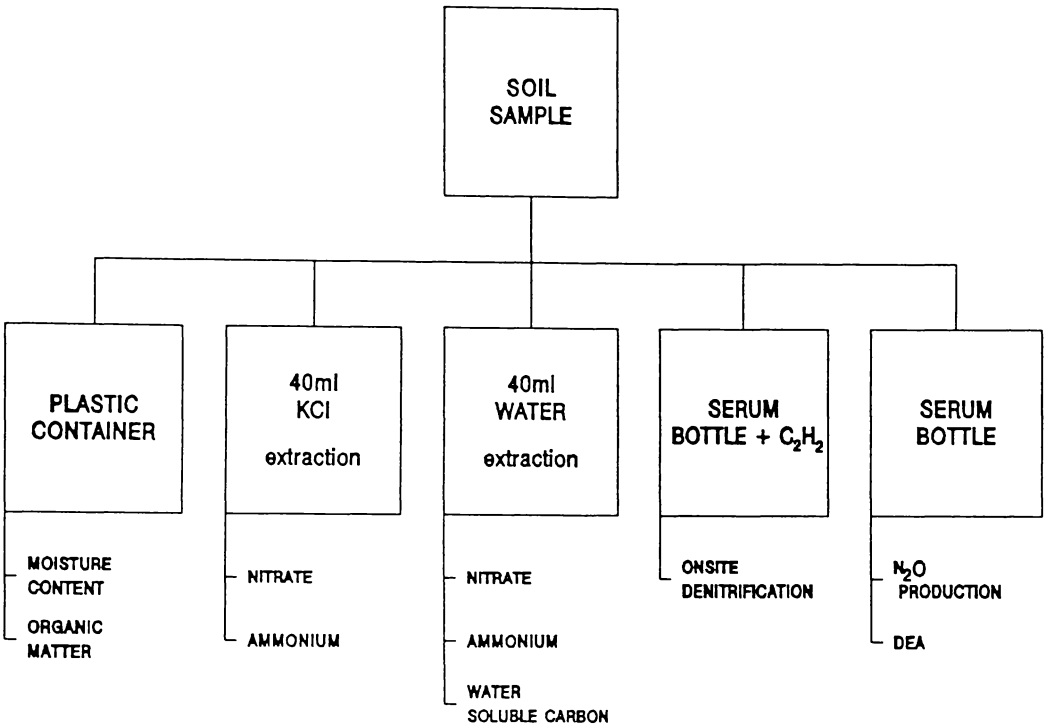


Figure 7.3 Schematic diagram of analysis performed on each soil core sample.

7.2.3.3 Temperature measurement

Soil temperature recordings were made at each core site several times during each sampling visit using a electronic thermometer (Type K Thermocouple Thermometer, Portec Instrumentation Ltd). Soil temperature values were averaged throughout the sampling period to give a single temperature value for each sampling visit.

7.2.3.4 Moisture content and percentage organic matter

A soil subsample of approximately 20 to 30g wet soil weight was sealed in a pre-weighed plastic tube. Once back in the laboratory, the tubes were reweighed to determine soil wet weight, the soil in the tubes was then oven-dried at 105°C and reweighed for oven dry weight determination. A known weight of oven-dried soil was then transferred to a pre-weighed crucible which was placed in the furnace at 500°C for four hours. Crucibles were removed, cooled, and weighed to determine loss of organic matter (section 3.4).

Moisture content was the amount of water expressed as a proportion of soil oven-dry weight. Organic matter was expressed as a percentage of soil oven-dry weight.

7.2.3.5 Water and KCl extracts

Soil subsamples were transferred into two pre-weighed plastic bottles. One contained 40 ml of KCl(2M) and the other contained 40 ml of distilled water. Both solutions included 0.1 $g.l^{-1}$ $HgCl_2$ as a preservative. Bottles were closed with lids and briefly shaken by hand. Bottles were then placed on ice for transport back to the laboratory where they were weighed to determine the wet weight of soil added. Both the KCl- and water-extracts were then filtered through filter paper (Whatman 114). The filter paper was prewashed with distilled water to remove any nitrate or ammonium contaminants in the filter paper. The water-extracted filtrate was then run through a 0.45 μm filter (Millipore) to remove particulates. Both filtrates were then stored at 4°C until analyzed for nitrate and ammonium (described in section 3.5), usually within seven days. Water-soluble carbon in the water-extracted filtrate was determined within two days of returning to the laboratory (section 3.5).

The wet soil weight was converted to an oven-dry weight equivalent using the calculated moisture content. Nitrate, ammonium, and water soluble carbon were then expressed as weight per gram of oven-dry soil.

7.2.3.6 Onsite denitrification and natural nitrous oxide production

Onsite denitrification was measured by a similar method to that described by Cooper (1990). As Cooper (1990) comments, the method may appear somewhat crude in comparison to other methods used to estimate onsite denitrification; however, it was appropriate for the soil system studied.

A subsample of approximately 20 g of wet soil was quickly transferred into a pre-weighed serum bottle (60 ml nominal volume) using a small solids funnel and a syringe plunger. The serum bottle was then sealed with a rubber stopper which was crimped in place with an aluminium seal (Gibco NZ Ltd). The serum bottle headspace was replaced with argon by repeated evacuation and flushing using a large 50 ml syringe. Argon was obtained via a stopcock (Cole-Palmer) attached to a large, airtight butyl-rubber bag. Five ml of deoxygenated distilled water and 5 ml of acetylene were added to the serum bottle. The bottle was then vigorously shaken by hand for two minutes to distribute acetylene throughout the soil and release trapped N_2O to the headspace. A 3 ml gas sample was taken by syringe and stored in a 2 ml vacutainer (Becton Dickinson, New Jersey, USA) until analysis for N_2O. The bottle was then incubated on-site for one hour when the bottle was once again vigorously shaken for two minutes and a further 3 ml gas sample taken and stored in a vacutainer until analysis for N_2O (section 3.6). The serum bottle was returned to the laboratory, filled with water, and weighed to determine headspace volume. The bottle was then oven-dried at 100°C and reweighed to determine oven-dry weight of soil added.

Denitrification rate was expressed as the amount of N_2O formed during the incubation period (one hour) per g of oven-dry soil weight.

Natural N_2O production was estimated concurrently with and in the same manner as the measurement of onsite denitrification rate with the exception that acetylene was not added to the headspace. At the end of the incubation this serum bottle was transported to the laboratory on ice for subsequent estimation of denitrifying enzyme activity (section 7.2.3.7). Natural N_2O production was also expressed as per g soil oven-dry weight.

7.2.3.7 Denitrifying enzyme activity

The serum bottles used for natural N_2O production (see section 7.2.3.6) were used to estimate the denitrifying enzyme activity (DEA). The serum bottles were stored at 4°C after return from the field site until the following day when DEA was measured. The method used was similar to that described by Tiedje *et al.* (1989). The headspace of the serum bottles was flushed with argon. Five ml of distilled water containing 0.5 mg of KNO_3, and 5 ml of acetylene was added to each serum bottle. The bottles were then incubated in the dark at 25°C shaking at 200 rpm. Headspace samples were taken at 15 and 75 minutes and analyzed for N_2O (section 3.6). DEA was expressed N_2O formed per g of oven-dry soil weight.

For the assessment of DEA, Tiedje *et al.* (1989) also suggested adding chloramphenicol to inhibit protein synthesis and adding glucose to ensure that denitrification was not limited by carbon. Both these additions are made to extend the time during which N_2O production was linear. In the present study, preliminary work, in which headspace samples were taken four times during the incubation period, showed that N_2O production was linear without these additions (data not shown). As N_2O production was linear, and to reduce handling of serum bottles, carbon and chloramphenicol additions were not made.

7.2.3.8 Cation exchange capacity

During the 4th sampling visit, a bulked soil sample was taken from the riparian zone and transported back to the laboratory. Cation exchange capacity was determined using three duplicate samples as described in section 3.4.

7.2.3.9 Data analysis

All significant differences are shown at the 95% confidence limit unless stated otherwise. Stepwise multiple regression (Statistix V3.5 Analytical Software, MN, USA) was used to identify the soil and biological parameters which were correlated to onsite denitrification rate and to natural N_2O production. Wilk-Shapiro plots and statistics were used to test for normality and, where necessary, the data were transformed by $\log_{10}$ or by reciprocal before applying multiple regression. Addition of a constant (0.1) to all data was necessary before transformation to avoid loss of zero values.

Temperature readings could not be directly incorporated into the multiple regression because only one independent measurement was made per field visit. To examine the differences in onsite denitrification rate between samplings (which may be due to a temperature effect) the F-statistic was used to determine whether there was significant difference between the slopes determined by multiple regression for each visit. Similarly, the F-statistic was used to determine significant differences in intercept.

Three dimensional plots of soil and biological data were produced using SAS (SAS Institute Inc. 1985).

7.3 RESULTS

7.3.1 LYSIMETER SAMPLING

7.3.1.1 Nitrate and chloride concentrations

Lysimeter

Figure 7.4 a and b show the changes in nitrate concentration and in nitrate:chloride ratios over time and lysimeter location. There was considerable variation in the nitrate concentration and in the ratio at sites 3, 4, and 5. A consistent pattern was evident between sites 2 and 3 where high nitrate removal appeared to have occurred across the riparian zone.

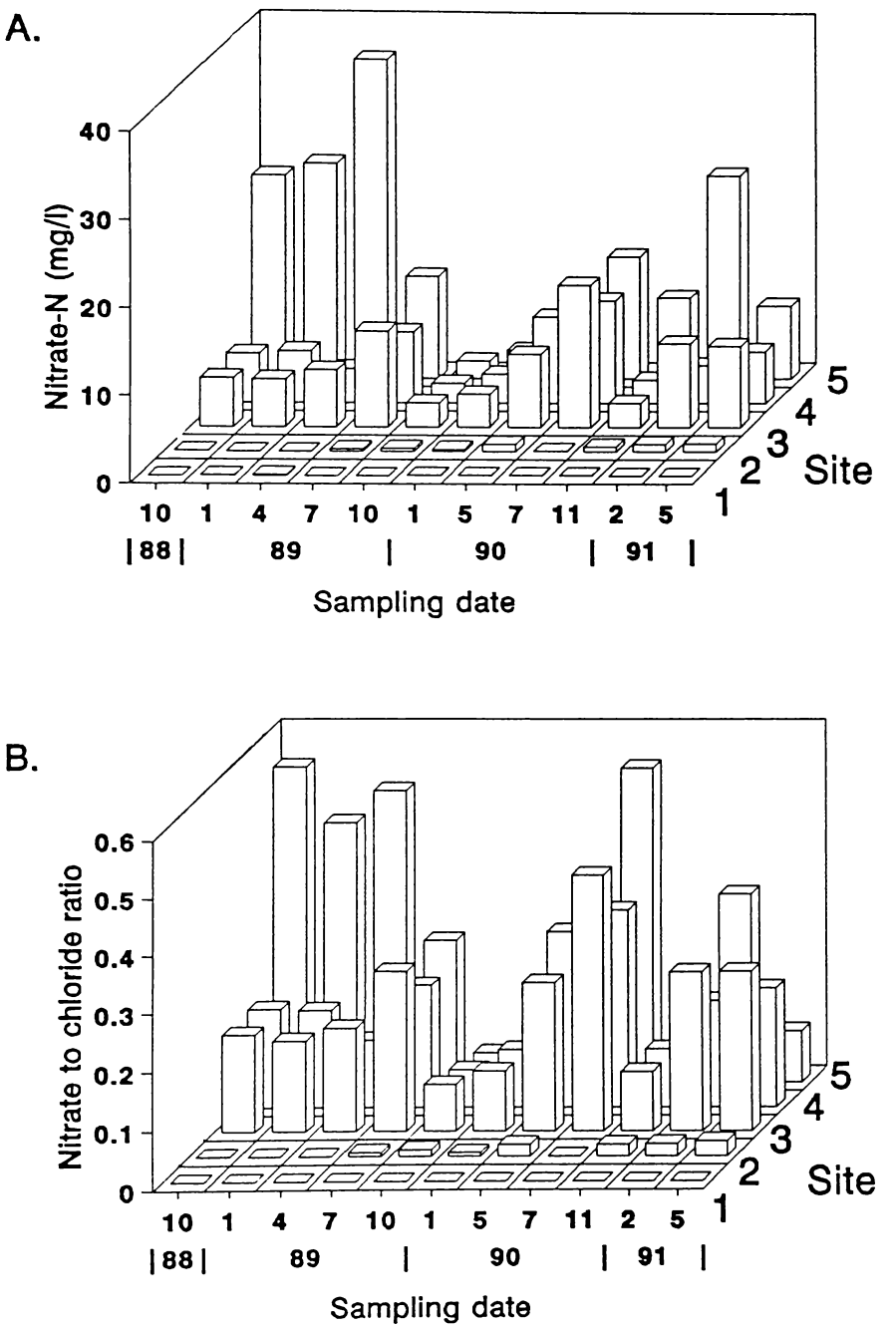


Figure 7.4 Plots of (a) nitrate concentration, and (b) the nitrate:chloride ratio observed in lysimeters. Results shown through time and sampling site.

Stream

Nitrate and chloride concentrations detected in the stream water are shown in Table 7.1. Nitrate concentrations fluctuated with time, whereas chloride concentrations remained relatively constant.

DATE	10/88	1/89	4/89	7/89	10/89	1/90	5/90	7/90	11/90	2/91	5/91
NO_3-N (mg/l)	2.3	10.1	0.3	12.2	9.2	4.1	10.5	ND	1.7	9.3	4.1
Cl (mg/l)	40.1	38.8	34.1	43.8	37.8	43.9	41.7	ND	39.2	40.1	40.4

Table 7.1 Nitrate and chloride concentrations in the stream draining the studied subcatchment. ND - not determined.

7.3.2 RIPARIAN ZONE SAMPLING

Results from this study are presented in the following order. First, the soil and biological data are summarised in terms of their frequency distribution; second, correlations within the data are shown; and third, the spatial patterns of nitrate, ammonium, onsite denitrification and DEA in the riparian zone are shown. All data are presented in Appendix 9.3. In the following sections several data sets are discussed. Individual data sets pertain to a data set which summarises data from each sampling visit to the site, the merged data set combines the data from all the sampling visits into one large data set, the averaged data set is one where data from individual data sets were averaged at each sampling location (1 to 32) through five time periods.

7.3.2.1 Summary and distribution of measured parameters

In this section the frequency distribution of all the soil and biological parameters are shown. The frequency distributions are drawn from the merged data set. Also presented are changes observed in measured soil parameters between sampling trips.

Figures 7.5 a to h show histograms of the measured soil and biological parameters. Onsite denitrification rate, DEA, natural N_2O production, and KCl-extracted nitrate and ammonium showed skewed distributions. The distribution of water-extracted nitrate and ammonium is not shown but was similar to the distribution observed for KCl-extracted nitrate and ammonium, respectively. The remaining soil parameters appeared to be normally distributed although some degree of skewness was apparent.

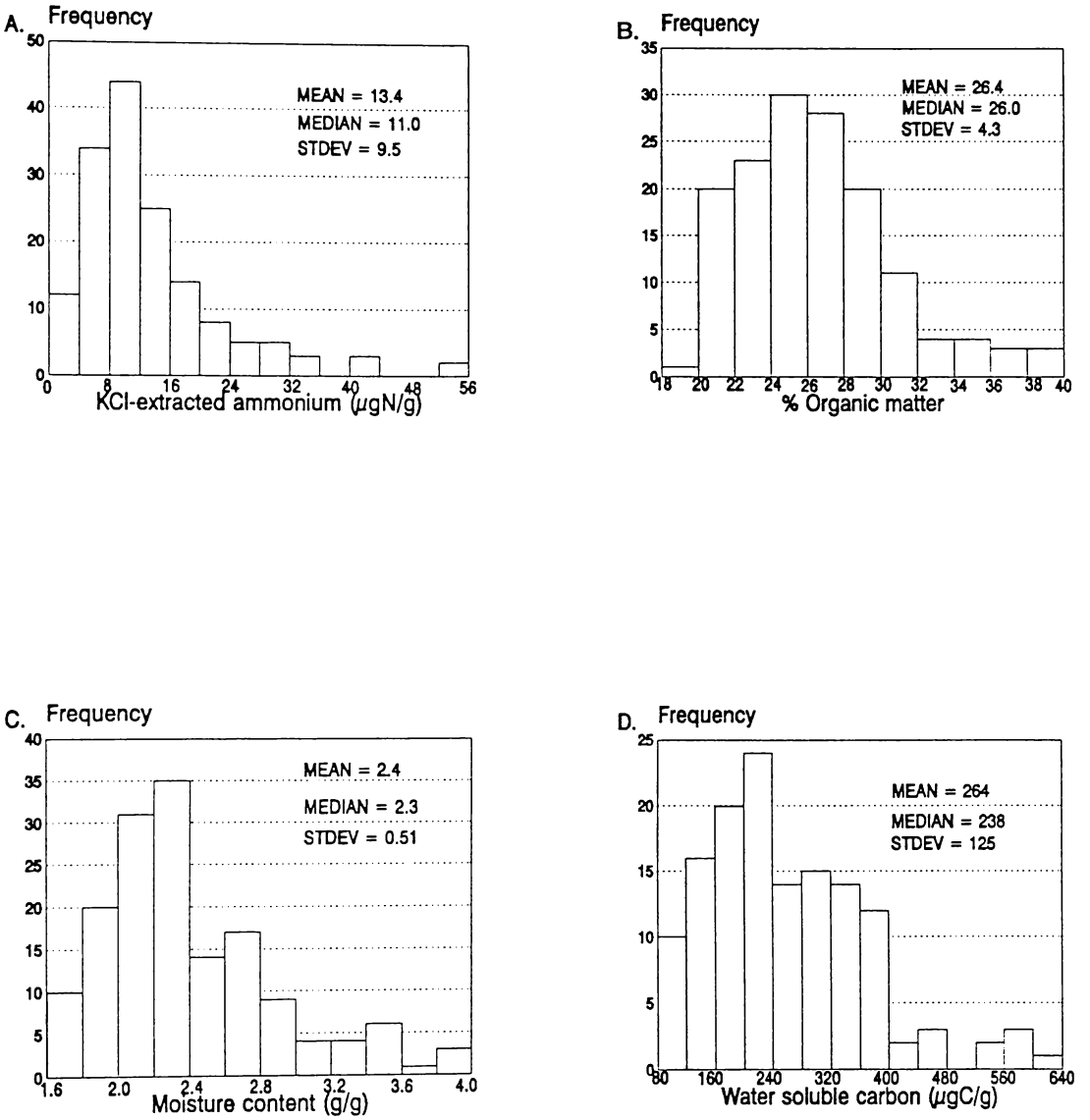


Figure 7.5 a,b,c,d Histograms of soil and biological properties measured during 5 riparian sampling trips.

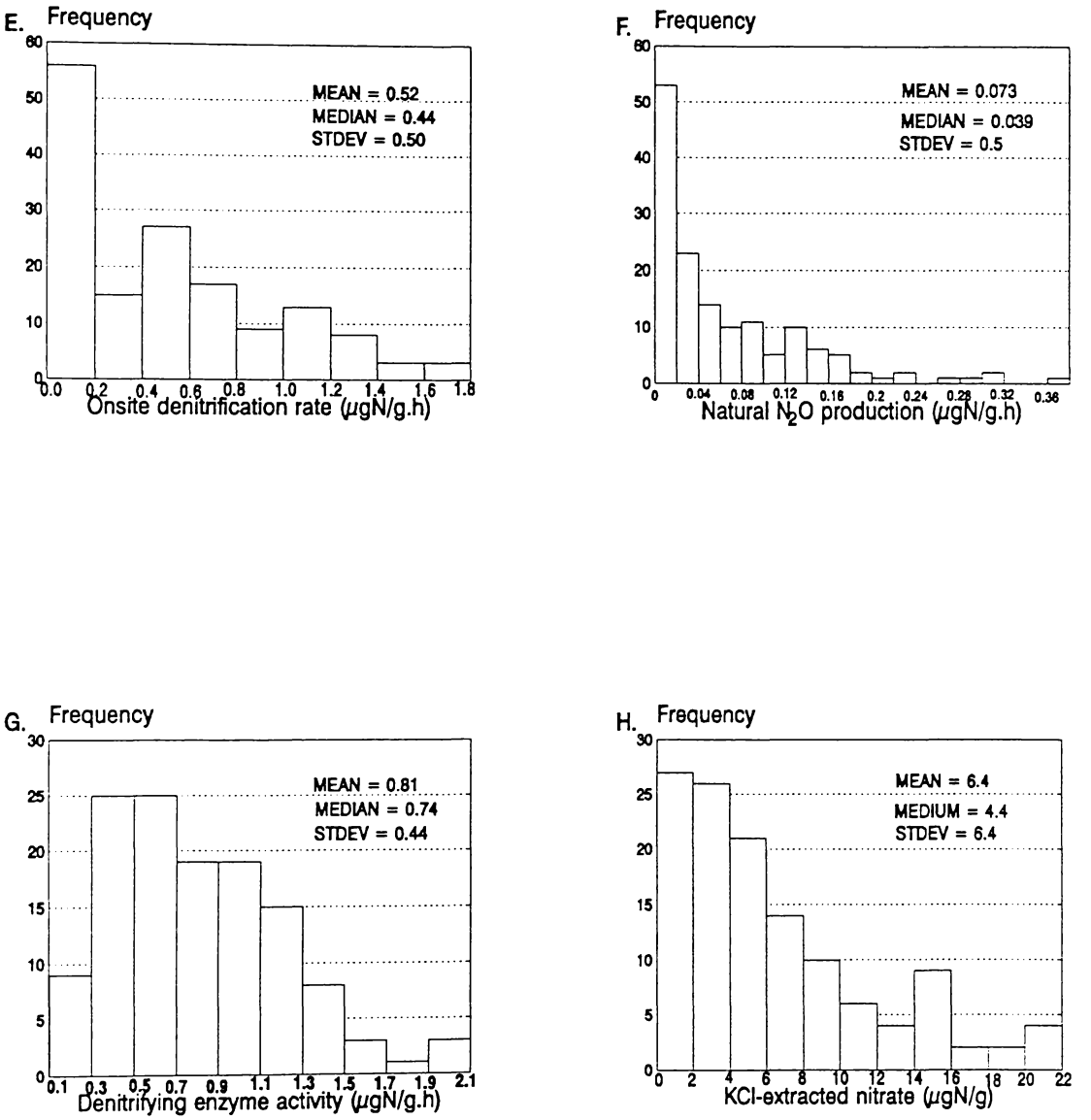


Figure 7.5 e,f,g,h Histograms of soil and biological properties measured during 5 riparian sampling trips.

Table 7.2 shows the variability between the five consecutive riparian sampling visits with regard to temperature, % organic matter, water soluble carbon concentration, and moisture content of the soil. Arithmetic averages are shown, significant differences were calculated using ANOVA (Statistix, Analytical Software, St Paul, USA).

SAMPLING Visit	TEMPERATURE (°C)	ORGANIC MATTER (%)	WATER SOLUBLE CARBON (μgC/g soil)	MOISTURE CONTENT (g/g soil)
1	20.9 a	28.0 a	201 a	2.14 a
2	12.1 b	24.5 b	236 a	2.44 b
3	17.1 c	26.5 a,b	221 a	2.45 b
4	21.2 d	26.7 a	312 b	2.31 a,b
5	13.1 e	26.3 a,b	326 b	2.66 c

Table 7.2 Temperature, % organic matter, water soluble carbon, moisture content of the soil from five riparian sampling visits. Values in the each column followed by the same letter are not significantly different at 95% confidence level.

Temperature was significantly different for each visit, water soluble carbon was significantly higher in the last two visits than in the first three visits, there may have been some significant differences in % organic matter, and there were some significant differences in moisture content.

7.3.2.2 Cation exchange capacity

The average cation exchange capacity was 50.3 milliequivalents per 100 grams oven-dried soil, with a standard deviation of 2.4. This CEC was high in comparison to that found in many soils (Brady 1974).

7.3.2.3 Onsite denitrification rate

Correlations with biological and soil parameters

In the individual field visits and in the merged data set onsite denitrification, DEA, and, KCl- and water-extracted nitrate data required $\log_{10}$ transformation to normalise the data before multiple regression was applied. Other soil variables were untransformed. These variables included %organic matter, moisture content, and water soluble carbon. Temperature, extracted ammonium, and natural N_2O production were not included in the multiple

regression. In the multiple regression of the averaged data set neither onsite denitrification nor DEA required transformation.

Onsite rate of denitrification was best predicted by DEA and nitrate extracted (either KCl or water) as shown in Table 7.3. Other soil parameters were not significant explanatory variables at the 5% probability level.

DATA SET Extracted nitrate	CONSTANT	NITRATE	DENITRIFYING ENZYME ACTIVITY	ADJUSTED r^2
Visit 1 - KCl extract	-0.44 **	0.37 *	1.12 **	0.67 **
- water extract	-0.17 **	0.31 **	1.18 **	0.73 **
Visit 2 - KCl extract	-0.46 **	0.19 *	0.91 **	0.47 **
- water extract	-0.50 **	0.22 *	0.96 **	0.46 **
Visit 3 - KCl extract	-0.59 **	0.67 **	0.45	0.59 **
- water extract	-0.38 **	0.51 **	0.52	0.55 **
Visit 4 - KCl extract	-0.45 **	0.45 **	0.76 **	0.77 **
- water extract	-0.69 **	0.75 **	0.91 *	0.53 **
Visit 5 - KCl extract	-0.62 **	0.38 **	0.82 *	0.41 **
- water extract	-0.54 **	0.34 **	0.64 *	0.45 **
COMBINED DATA - KCl extract	-0.49 **	0.35 **	0.96 **	0.56 **
- water extract	-0.39 **	0.24 **	0.98 **	0.45 **
AVERAGED DATA - KCl extract	-0.29 *	0.47 **	0.62 **	0.74 **
- water extract	-0.18 *	0.51 **	0.51 **	0.77 **

Table 7.3 Coefficients of significant independent variables explaining onsite denitrification rate fitted by multiple regression. Values for KCl and water extract were first $\log_{10}$ transformed in all regressions, onsite rate and DEA were $\log_{10}$ transformed in visit 1 to 5 and in combined data. Transformation was not necessary in the averaged data set. * and ** indicate statistical significance at probability levels of 0.05 and 0.01, respectively.

Effect of temperature

There was no significant difference ($p<0.05$) between the slopes of the regression equations for the individual sampling visits and from the merged data set. However, there was a significant difference ($p<0.01$) in the intercepts. To examine the differences between onsite denitrification rates from individual sampling visits, predicted values were determined from the regression with common slope but with separate intercept for average values of nitrate

and DEA. Figure 7.6 shows the relationship between temperature and these predictions, the fitted linear regression had a r^2=0.47, p=0.12.. A Q_{10} of 1.8 was calculated from the fitted line.

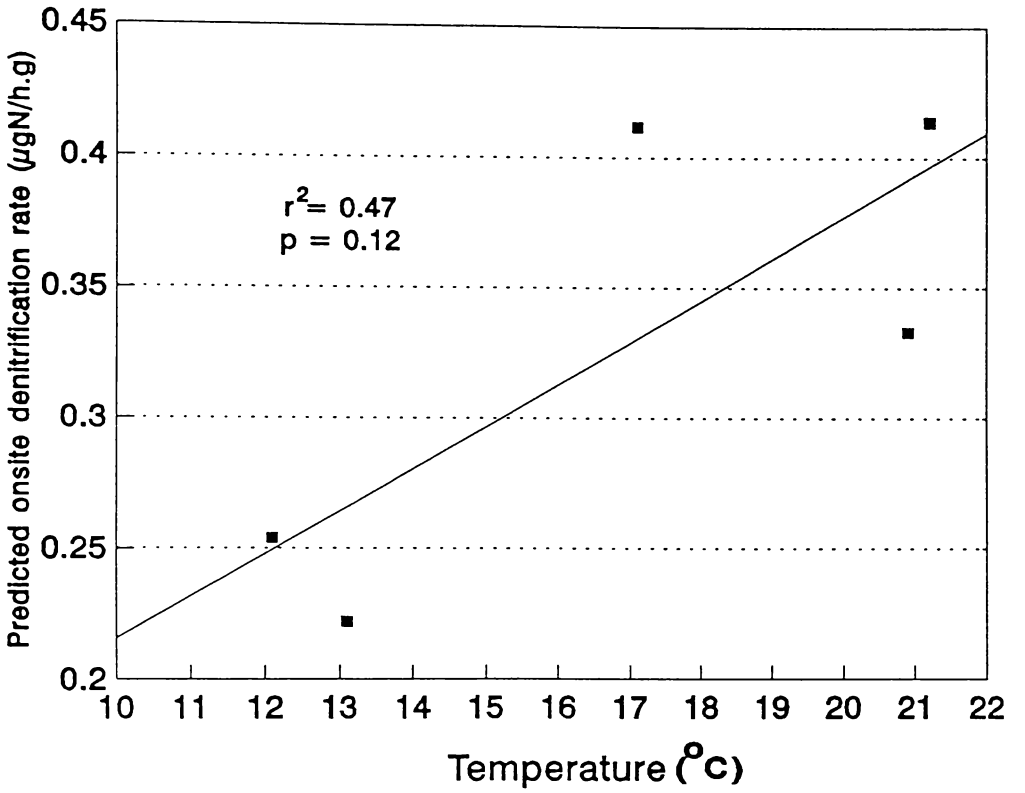


Figure 7.6 Relationship between temperature and the predicted onsite denitrification rates.

Averaged data set

Soil and biological data from each of 32 sampling points were averaged for each of the five sampling visits irrespective of time to produce an averaged data set. Using the averaged data, multiple regression was repeated. Extracted nitrate and DEA were shown to be the best predictors of onsite denitrification (Table 7.3). However, in this analysis onsite denitrification and DEA no longer required $\log_{10}$ transformation as averaging of the replicates had normalised the data (as expected with the central limit theorem); however, water- and KCl-extracted nitrate still required $\log_{10}$ transformation. Nitrate concentration was the dominant predictor of denitrification. Figure 7.7 shows the relationship between onsite denitrification rate and nitrate concentration.

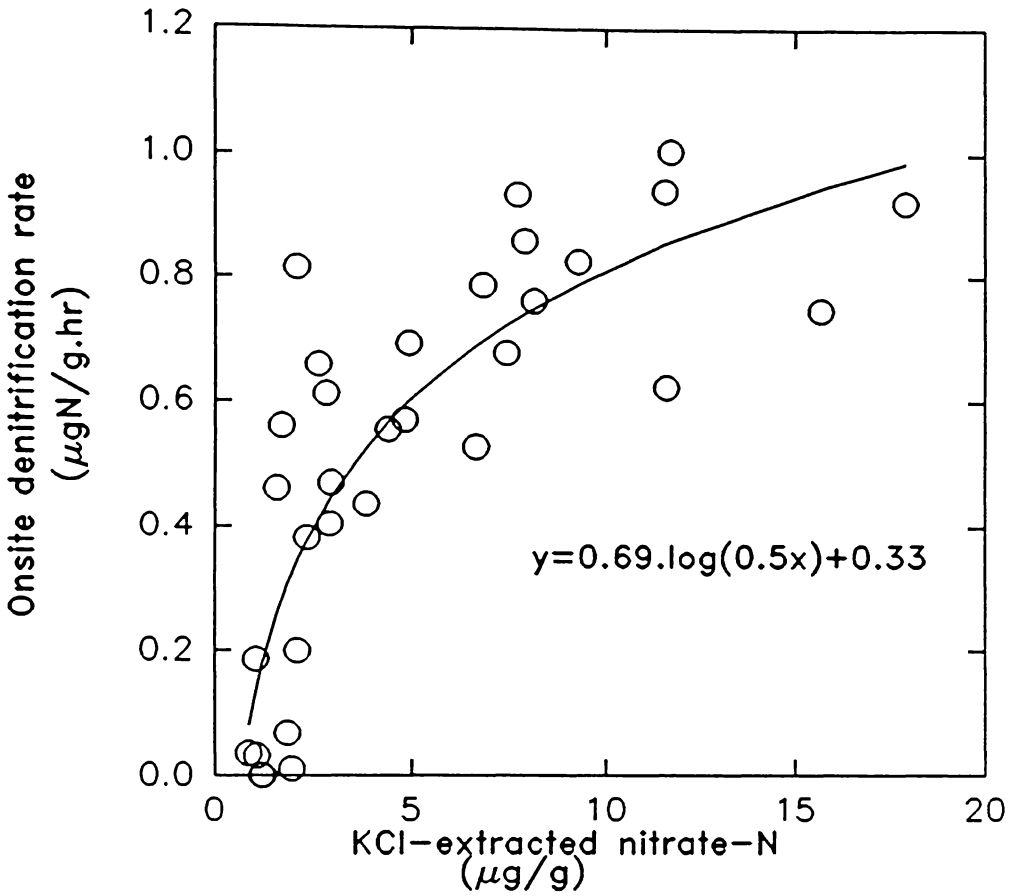


Figure 7.7 Relationship between nitrate and onsite denitrification, data taken from merged data set.

7.3.2.4 Regression analysis of natural N_2O production

The rate of natural N_2O production was analyzed by the same statistical approach used for onsite denitrification rate (section 7.3.2.3). Variables in the stepwise multiple regression were; % organic matter, moisture content, water soluble carbon, onsite denitrification, DEA, and water- or KCl-extracted nitrate. A reciprocal transformation of the natural N_2O production was used to normalise the data and a $\log_{10}$ transformation was used for onsite denitrification, DEA, and water- or KCl-extracted nitrate. Log_{10} transformation was not required in the averaged data set as outlined in section 7.3.2.3. In all cases stepwise multiple regression showed that natural N_2O production was best predicted by onsite denitrification rate (Table 7.4), no other measured parameter improved the fit.

No significant differences were found between the regressions for each sampling visit and the combined data set for either slope or intercept ($p<0.05$) suggesting that natural N_2O production was independent of sampling time.

DATA SET	CONSTANT	ONSITE DENITRIFICATION RATE	ADJUSTED r^2
Visit 1	-4.48	6.11	0.68
Visit 2	-4.03	6.49	0.75
Visit 3	-5.73	4.25	0.61
Visit 4	-5.38	4.54	0.77
Visit 5	-5.23	5.03	0.64
COMBINED DATA	-5.17	4.94	0.67
AVERAGED DATA	-41.8	8.95	0.45

Table 7.4 Relationship between natural N_2O production and onsite denitrification rate. Natural N_2O production rate was transformed by reciprocal and the onsite denitrification rate for visits 1-5 and the merged data set was $\log_{10}$ transformed; however, onsite denitrification was not transformed in the averaged data set. All coefficients and r^2 were significant at the 95% confidence limit.

7.3.2.5 Regression analysis of extracted nitrate and ammonium

A linear relationship was observed between KCl- and water-extracted nitrate in all data sets. Similarly a linear relationship was also observed between ammonium extracted using KCl and water. Results from the averaged data set are shown in Figures 7.8 a and b (the data were untransformed).

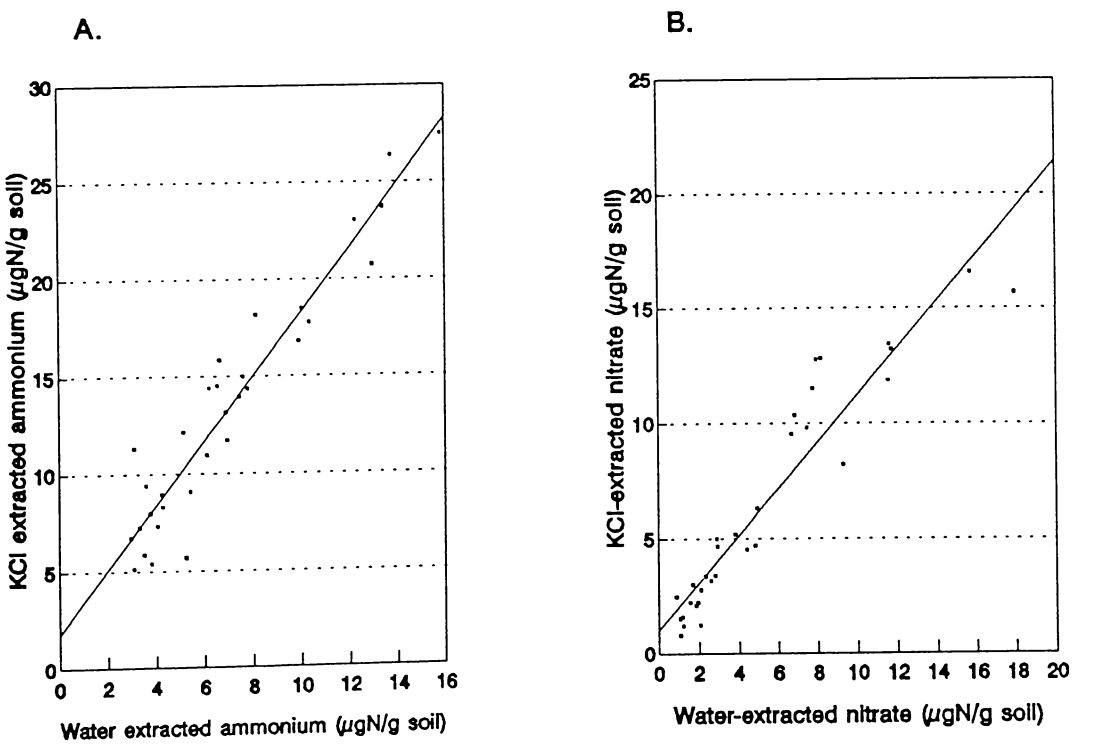


Figure 7.8 Comparison of extraction of soil (a) nitrate and (b) ammonium using water and KCl as an extractant.

An inverse relationship was also found between nitrate and ammonium (extracted by either water or KCl); the KCl-extracted example is shown in Figure 7.9 using untransformed data from the averaged data set.

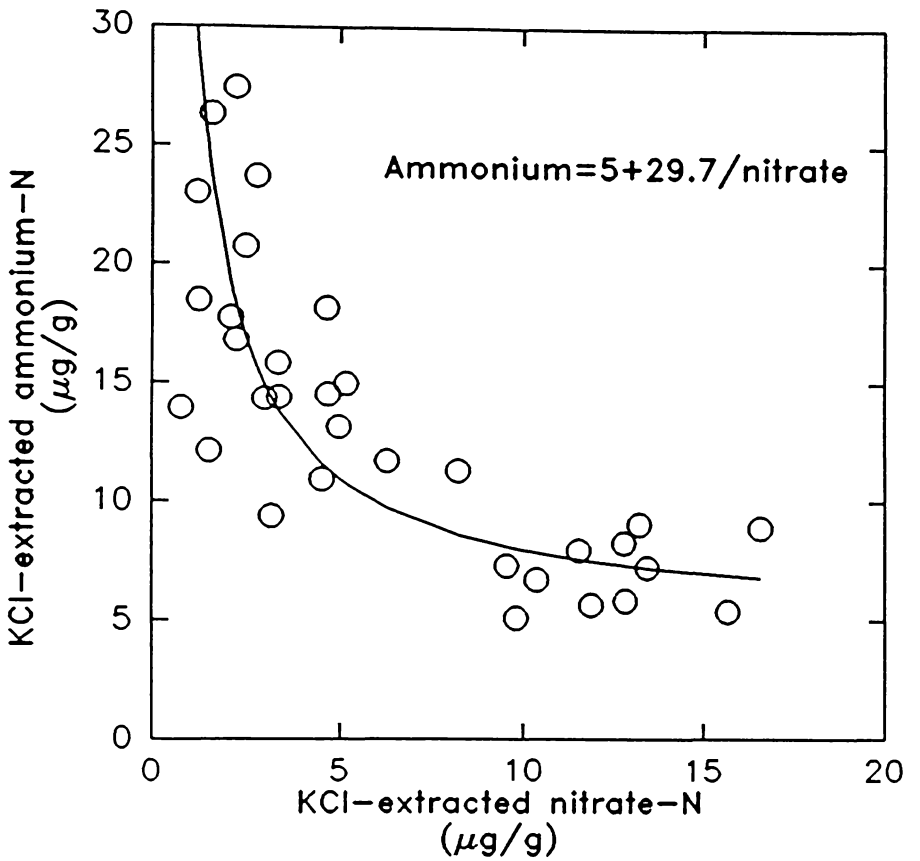


Figure 7.9 Relationship between nitrate and ammonium extracted using KCl from the same locations in the riparian zone.

7.3.2.6 Patterns in the riparian zone

The averaged data set was used to generate Figures 7.10 a,b,c,d, which show the spatial distribution of onsite denitrification rate, KCl-extracted nitrate and ammonium, and DEA. Although contour plots for water-extracted nitrate and ammonium are not shown they were similar to the KCl-extracted plots. Nitrate, onsite denitrification, and DEA were all high at the upslope edge of the riparian zone and generally low at the downslope edge. However, there was a small area at the downslope edge next to the stream which was also high in nitrate, onsite denitrification, and DEA. The spatial distribution of ammonium appeared to be opposite to the previous distributions and was high at the downslope edge and low at the upslope edge. Natural N_2O production (not shown) exhibited similar spatial trends to those observed for onsite denitrification rates but were less clear. All other measured soil parameters did not shown any obvious spatial trends.

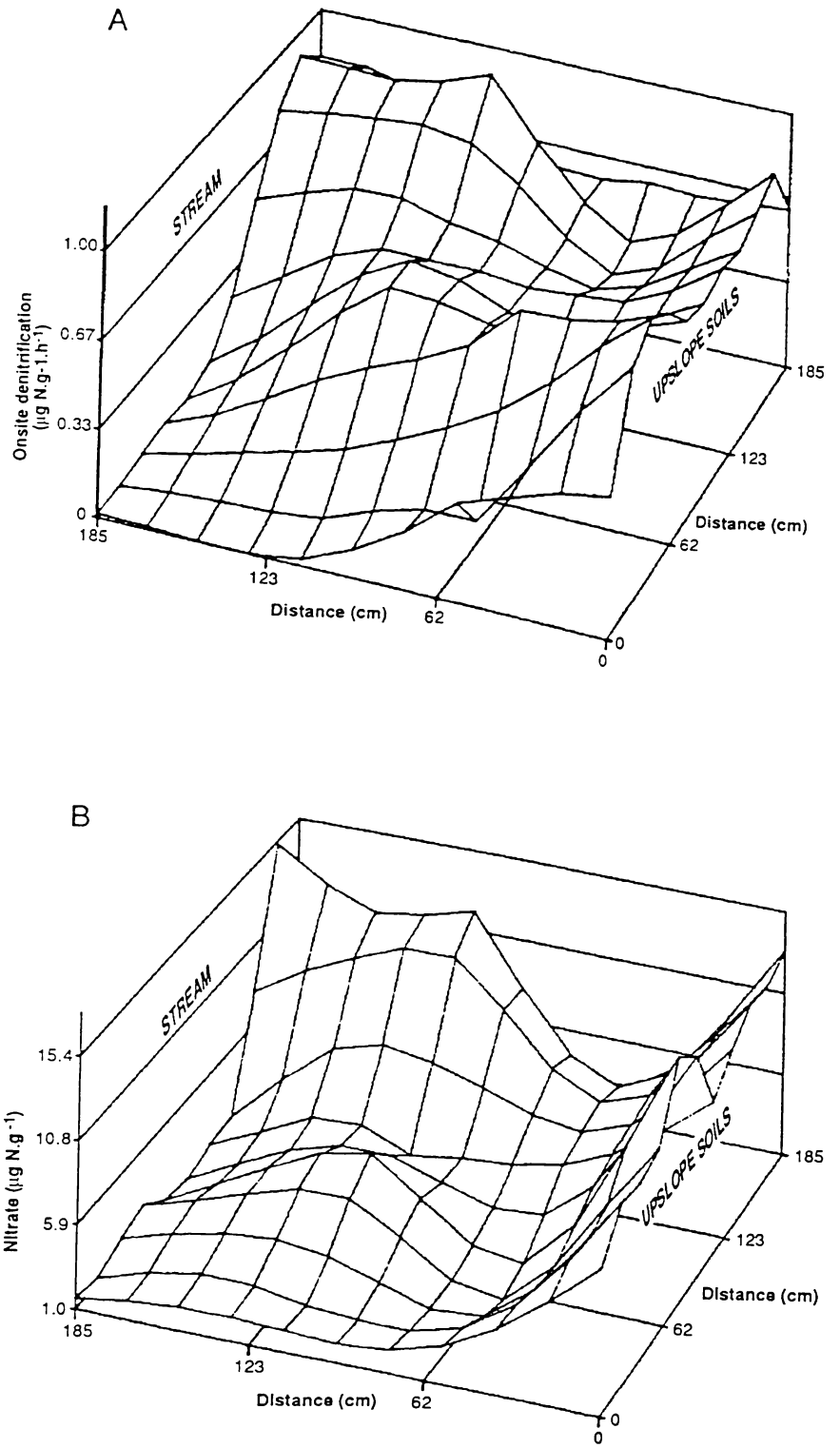


Figure 7.10 Spatial distribution of (a) onsite denitrification and (b) KCl-extracted nitrate, in the riparian zone studied.

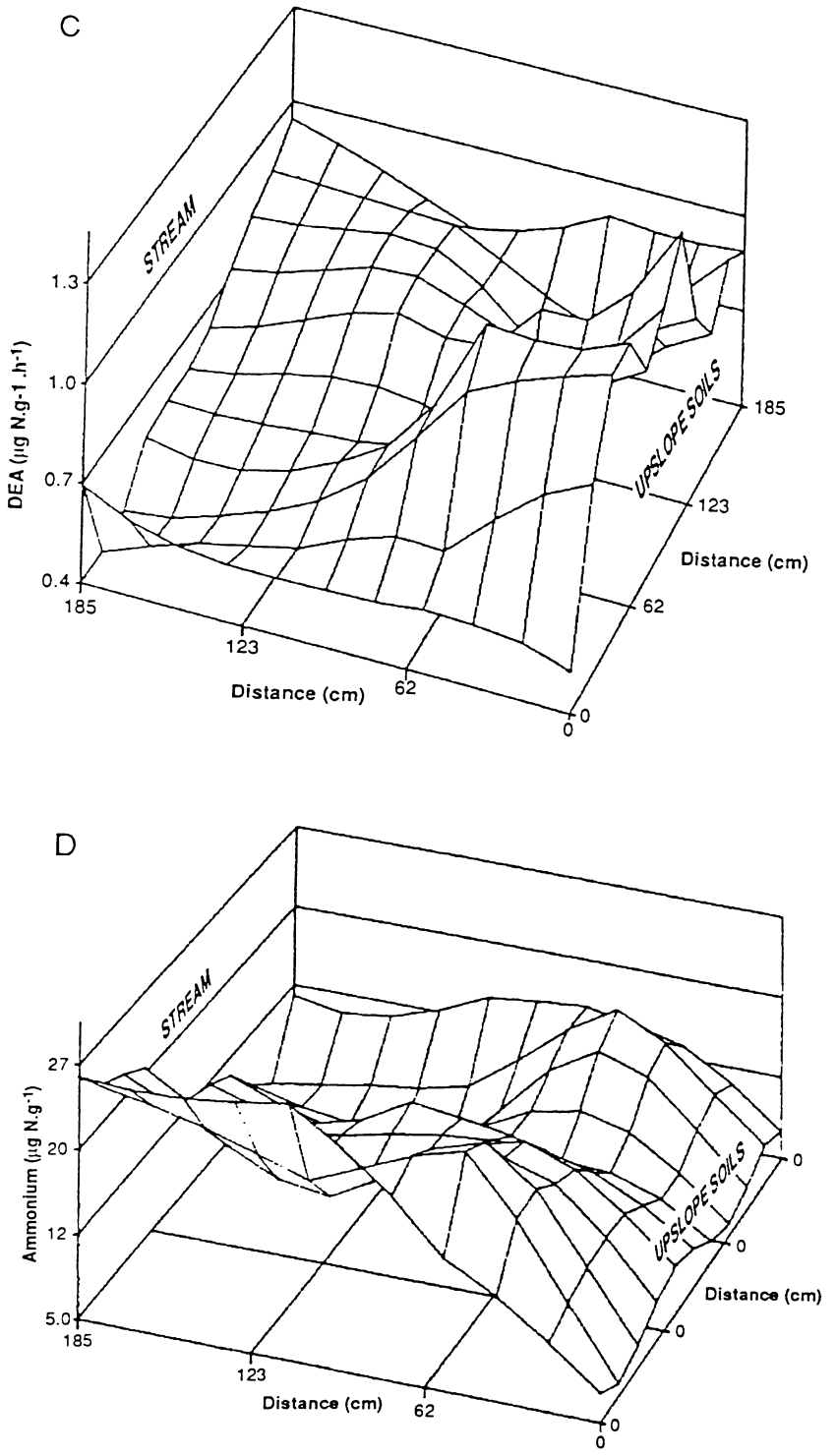


Figure 7.10 cont. Spatial distribution of (c) denitrifying enzyme activity and (d) KCl-extracted ammonium, in the riparian zone studied.

7.4 DISCUSSION

The results are discussed in two main sections. The first section discusses results from the lysimeter and stream sampling. The second main section examines the results from the riparian soil sampling. The second section is divided into several subsections. These subsections are discussed in the following order: frequency distributions of all collected soil and biological data; factors which were correlated to denitrification rate and natural N_2O production; areal rates of denitrification and natural N_2O in the riparian zone; the observed spatial patterns of soil and biological parameters; and lastly, the relationship between nitrate and ammonium concentrations.

7.4.1 LYSIMETER TRANSECT AND STREAM DATA

The lysimeter data indicated that high nitrate removal was occurring in the riparian zone between lysimeter sites 2 and 3. These results were similar to those observed by Schipper *et al.* (1989) in an adjacent transect. The nitrate concentration in stream water samples was high and similar to the nitrate concentrations found upslope of the riparian zone at lysimeter site 3. This suggested that some groundwater nitrate enters into the stream bypassing zones of high denitrification. The portion of the riparian zone studied was rich in organic matter whereas other adjacent riparian sites in the same subcatchment were of a mineral soil nature. Cooper (1990) showed that, at the subcatchment scale, nitrate removal was greatest when nitrate-laden groundwater passed through organic riparian soils rather than mineral riparian soils. It is likely that the high nitrate found in the stream water had passed through predominantly mineral riparian soils where denitrification rate was lower and less nitrate removal occurred.

The lysimeter results showed that a high degree of nitrate removal occurred in the studied riparian zone. Denitrification was a possible mechanism by which this nitrate removal could have occurred and this is discussed in the following sections.

7.4.2 RIPARIAN ZONE SAMPLING

7.4.2.1 Frequency distributions

Onsite denitrification, natural N_2O production, DEA, and extracted nitrate and ammonium all appeared to be log-normally distributed. Onsite denitrification has been generally reported as log-normally distributed in agricultural soils (Parkin 1987; Myrold 1988; Parkin and Robinson 1989) and forest soils (Groffman and Tiedje 1989a), as has nitrate and ammonium

concentration in agricultural soils (White *et al.* 1987), and as has DEA in agricultural soils (Parkin 1987).

Parkin (1987) speculated that the heterogeneous distribution of particulate organic matter was responsible for the log-normal distribution of onsite denitrification rates. This suggestion has been supported with findings by Parkin and Robinson (1989), and Christensen *et al.* (1990a). Groffman and Tiedje (1989a) found that denitrification rates can be normally distributed in spring and fall and suggested this was due to high moisture content and plentiful homogeneously-distributed carbon. In the riparian zone studied here, the soil was permanently saturated and high in organic matter. However, normally-distributed denitrification rates were not observed. Onsite denitrification rate showed a high correlation to nitrate concentration ($r^2=0.60$ $p<0.001$ for the averaged data set) and thus the denitrification rate may have been log-normally distributed due to the log-normal distribution of nitrate concentration.

7.4.2.2 Factors correlated to onsite denitrification

Conceptually, carbon, oxygen, and nitrate are the primary proximate regulators which dictate whether denitrification will occur (Tiedje 1988). The instantaneous denitrification rate will be related to these regulators but also to the number of active denitrifying bacteria present in the soil and to soil temperature (Davidson *et al.* 1990). In the present study, regression analysis showed that onsite denitrification was most highly correlated with DEA and nitrate concentration. Temperature may also have been correlated with onsite denitrification rate; however, this relation was weak.

The correlation between point measurements of onsite denitrification rate and nitrate and DEA was highly significant ($p<0.01$) for each individual sampling visit and the r^2 ranged from 0.41 to 0.77. This predictive ability was similar to, and in many cases greater than that found in studies on agricultural soils (Myrold 1988; Parsons *et al.* 1991) and forest soils (Robertson and Tiedje 1984; Davidson and Swank 1986; Groffman and Tiedje 1991). In most of these published studies, the primary predictors of denitrification have been measures which dictate oxygen status of soil; factors such as water content, respiration rate, and soil porosity. In the riparian zone studied here, it was likely that the bulk of the soil was anaerobic due to the high organic matter content and water-saturated nature of the soil. Therefore, it would seem unlikely that denitrification in these soils would be inhibited by the presence of oxygen. Likewise, because there was considerable organic matter and water-soluble carbon present it was unlikely that denitrification was carbon-limited. The high correlation between onsite denitrification and nitrate and DEA is therefore not surprising from a process perspective.

Nitrate and DEA limitation

Previous studies in upland soils have also found correlations between denitrification rate and nitrate concentration (Myrold 1988); and, between denitrification and nitrate production rate (Robertson and Tiedje 1984; Robertson *et al.* 1988b). In the above studies the soils were generally aerobic and nitrate was probably formed by nitrification at the site where denitrification was measured. In the present study, nitrification was unlikely to have occurred in the riparian soils as they were predominately anaerobic, suggesting that the source of nitrate was external, coming either from upslope drainage or from the stream. Figure 7.7 showed that the relationship between denitrification rate and nitrate concentration was non-linear; the fitted equation was logarithmic (fitted by SigmaPlot, Jandel Scientific, California, USA). However, the basic trend of the points suggests that other curve fits were also possible. One of the possibilities would include a curve fit using Michaelis-Menton kinetics.

DEA is considered to be an approximation of the number of active denitrifying bacteria (Martin *et al.* 1988) and is thought to reflect the environmental history of the site (Tiedje *et al.* 1989). Due to the method by which DEA is measured (i.e. non-limiting conditions for denitrification), DEA is likely to represent the maximum rate at which onsite denitrification could occur at the time of sampling. A previous study which compared bulked soils from a number of riparian and wetland sites found a strong linear relationship between DEA and available carbon (Schipper *et al.* in press). Other studies have shown similar results for different soil types (Burford and Bremner 1975; Reddy *et al.* 1980b). This would appear to indicate that the development of DEA in a soil is, in some cases, dictated by the amount of carbon available. So that although onsite denitrification may not be directly limited by available carbon at this riparian zone, the maximum denitrification rate may be dictated by the concentration of available carbon in any one riparian zone.

In most upland soils, the measured onsite rate of denitrification has been estimated to be a small fraction of the DEA measured (Martin *et al.* 1988; Groffman 1987); whereas, in this study onsite rates were often as high as DEA. This was probably due to the highly favourable conditions for the expression of DEA as on-site denitrification, i.e. low oxygen concentration and high nitrate and carbon concentrations.

Temperature

The correlation between temperature and denitrification rate suggested a Q_{10} of approximately 1.8. The correlation was weak and significant only at $P<0.12$; however, the Q_{10} was similar to the value of 1.9 reported by Jacobson and Alexander (1980); and, was within the range of 1.4 to 2.5 observed by Reddy *et al.* (1980a). Similar weak correlations between temperature and denitrification were found by Davidson and Swank (1986), Myrold (1988), and Parsons *et al.* (1991). Each of these studies suggested that the correlation between

denitrification rate and temperature was poor because of a confounding negative correlation between temperature and water content. In these studies water content was presumably an indication of the degree of anaerobic conditions. However, this explanation is unlikely to be the reason for the poor correlation between denitrification rate and temperature in the present study as the soils were in all cases water-saturated. One limitation of this study was that onsite denitrification rates were measured at only five different temperatures.

Temporal averaging

Stronger correlation between regulatory factors and denitrification can be found by averaging data either temporally or spatially. In this study, a stronger correlation between onsite denitrification rates and nitrate concentration and DEA (r^2=0.77) was found by averaging all the data at each sampling site irrespective of time. Other studies have also improved the degree of predictability by temporal or spatial averaging. Parsons *et al.* (1991) averaged their data over a sampling day and greatly improved their observed correlation between denitrification and measured soil parameters. Groffman and Tiedje (1989b) were able to obtain better correlation between regulators and denitrification rate in different forests by using annual denitrification rates which were calculated by extrapolating measured rates for periods between sampling times. Thus by averaging data, either spatially or temporally, the effect of extremely high or low denitrification rates on the regression analysis may be reduced, thus improving the overall fit of the applied model.

7.4.2.3 Natural N_2O production

Nitrous oxide and nitrogen gas are the major nitrogen gases formed by denitrification. Of these end-products, nitrous oxide generally forms the smaller fraction; although exceptions have been observed in highly acidic soils (Christensen *et al.* 1990a,b). Nitrous oxide can also be formed by other processes such as nitrification (Sahrawat and Keeney 1986). However, since nitrification only occurs in aerobic environments it was likely that the predominant source of N_2O production in the riparian soils examined here arose through denitrification.

Natural N_2O production was best correlated to the onsite rate of denitrification (r^2 = 0.45 to 0.77). None of the other factors could account for the remaining variation. Other studies have shown that factors such as temperature, nitrate and carbon concentration, pH, and oxygen concentration can alter the ratio of N_2O to total denitrification (reviewed by Firestone 1982; Sahrawat and Keeney 1986). Given that the soil sampling was from a very small area, the factors listed above may not have varied enough to cause major changes in the N_2O proportion of total denitrification.

7.4.2.4 Areal rates of denitrification and N_2O production

Often it is of interest to establish the rate at which denitrification occurs on an areal basis. Generally, this involves making a large number of localised denitrification measurements through time and space and obtaining an average denitrification rate throughout the year. Such measurements must be treated with care, as intense denitrification can occur in very short time periods, such as those following rain (Sextone *et al.* 1985). Unless sufficient measurements are made, landscape denitrification rates may be poorly estimated.

Here the areal rates of denitrification in the riparian zone were calculated to allow comparison of riparian zone denitrification rates with those found in other landscapes. The riparian zone was assumed to be 30 cm deep and the soil bulk density was 0.3 gcm^{-3} (an average value for the riparian soil). The average and median denitrification rate from the merged data set was 0.52 and 0.44 $\mu gN.g^{-1}.hr^{-1}$ respectively. Areal denitrification rates were then calculated to be 1.2 and 0.98 $gN.m^{-2}.day^{-1}$ using the average and median rates respectively. These areal denitrification rates were one to three orders of magnitude greater than those described for most other upland landscapes such as forests (Robertson and Tiedje, 1984; Davidson *et al.* 1990; Groffman and Tiedje 1989a) and agricultural land (Myrold 1988; Bijay-Singh *et al.* 1989; von Rheinbaben 1990) and sediments (Seitzinger 1990). However they are similar to denitrification rates found by Cooper (1990) in the upslope edge of other organic riparian zones which received high nitrate inputs. Although these rates were extremely high, they only occur in small patches of organic riparian zone and careful assessment of the extent of organic and mineral patches of riparian soils and the pathway of groundwater movement would have to be made in order to determine the overall nitrate removal within a catchment. Rates of denitrification have been shown to be much less in mineral riparian zones than in organic riparian zones (Cooper 1990).

Making the same assumptions as above and using the average and median natural N_2O production rates which were 0.073 and 0.039 $\mu gN.g^{-1}.hr^{-1}$, the areal rate of natural N_2O production was calculated to be 0.16 and 0.088 $gN.m^{-2}.day^{-1}$ respectively. These rates are also 1 to 3 orders of magnitude greater than those found in fertilised soils (Eichner 1990) and sediments (Seitzinger 1990). The N_2O concentration in the atmosphere is estimated to be increasing and as a consequence may contribute to the greenhouse effect (Rodhe 1990). Although the rates of N_2O production from riparian zones appears to be very high on an areal basis, the total active riparian area needs to be known before the contribution these soils make to atmospheric N_2O concentration can be assessed.

The reasons for the much higher rates of denitrification and natural N_2O production measured in riparian zones in comparison to other landscapes may be found by considering the unique environment provided by organic riparian soils. These soils are generally anaerobic due to the water and organic matter content and can receive a continuous flux of

nitrate by drainage from above. Many other water-saturated sediments can also have a high organic matter content, but nitrate diffusion from overlying water or the rate of *in situ* nitrification may limit denitrification.

The continual influx of nitrate to this anaerobic environment would create a high selection pressure in favour of bacteria capable of nitrate respiration. The energy yield using nitrate as an electron acceptor has been shown to be about 60% that for aerobic respiration in pure culture (Koike and Hattori 1975). Thus denitrifying bacteria are likely to be very competitive with other anaerobic bacteria for carbon and nutrients in the environment.

7.4.2.5 Spatial patterns

In the riparian zone studied, both nitrate and onsite denitrification rate appeared to be spatially dependent. The spatial distribution of denitrification rates was similar to that of nitrate concentration and this was probably a reflection of the dependence of denitrification on nitrate concentration. Nitrate was unlikely to be formed in the riparian soils by nitrification since these soils were predominately anaerobic. Nitrate was probably transported from upslope soils to the riparian zone by groundwater flow. As nitrate passed through the riparian zone it would have been removed by denitrification and consequently the nitrate concentration would decrease. This mechanism may have generated the observed spatial distribution of nitrate concentration and the spatial distribution of denitrification rate.

Spatial dependence of denitrification rates has also been demonstrated by Robertson *et al.* (1988b) in a study of a 69 m by 69 m study site, where denitrification rates were assessed at 1 m intervals. Conversely, Folorunso and Rolston (1984) were only able to demonstrate spatial dependence in one of twelve studied transects and Parkin (1990) was unable to demonstrate any spatial dependence of denitrification. Parkin (1990) suggested that the spatial dependence of denitrification was at the microsite scale (distances of <20 cm) and that the high spatial variability of denitrification rate was due to the heterogenous distribution of factors which regulated denitrification.

High nitrate values were also observed at the lower end of the riparian zone near the stream. Nitrate concentrations in the stream were high and it was thought that some of the nitrate diffused or was carried back up into the riparian zone. At sampling locations at the downslope edge of the riparian zones where nitrate concentrations were high, denitrification was also observed to be high. These data strengthen the suggestion of Cooper (1990) that further understanding of the processes in riparian soils is reliant on a better understanding of stream, riparian zone, and upslope hydrology.

7.4.2.6 Nitrate and ammonium concentrations

It is not surprising that nitrate extracted by water and KCl were highly correlated. The slope of the fitted line was close to 1 indicating that KCl and water extracted the same fraction of nitrate from the soil. This was not the case in the extraction of ammonium; although the concentrations extracted by KCl and water were highly correlated, KCl extracted more ammonium than did water extraction. This was probably a reflection of the high cation exchange capacity of the soil so that KCl was required to displace ammonium on the soil exchange sites.

A possible relationship between extracted nitrate and ammonium was initially observed in an examination of the nitrate and ammonium contour maps. Where nitrate was high, ammonium was low and this relationship was confirmed in a graphical comparison of extracted nitrate and ammonium (Figure 7.9). A possible explanation might be found when examining the partitioning of anaerobic nitrate reduction to nitrogen gases (by denitrification) and to ammonium (by dissimilatory nitrate reduction to ammonium (DNRA)). Tiedje *et al.* (1982) hypothesised that the partitioning between DNRA and denitrification was dependent on the carbon:nitrate ratio in the environment studied. When the ratio was relatively high, DNRA was favoured and when the ratio was low, denitrification was favoured. To date this hypothesis still stands (Tiedje 1988; Cole 1990) and supporting evidence has been presented by King and Nedwell (1985).

In the studied riparian zone, organic matter and WSC were evenly distributed spatially, and the carbon:nitrate ratio would therefore be dependent on nitrate concentration alone. Thus where nitrate was low, the ratio would be high and DNRA would be promoted relative to denitrification, resulting in the increased formation of ammonium. Where nitrate was high, denitrification would be favoured and relatively less ammonium would be formed. Due to the high cation exchange capacity of the riparian soil, ammonium would be retained and, over time, spatial patterns of ammonium would form within the riparian zone. If the above argument is correct, then at sites where nitrate was low, ammonium might be expected to be high and the converse would also be true as can be see in Figure 7.9. Similar findings were reported by Nedwell (1975), who found that denitrification was predominant in estuarine sediments where nitrate concentration was high, and that DNRA was dominant when nitrate concentration was low.

The relationship between nitrate and ammonium concentration found in this study may have been created by denitrification and DNRA. However, the evidence is circumstantial and further work is required to establish the competing roles of these nitrate reduction processes. Of particular importance is the measurement of DNRA rate in these soils.

7.5 CONCLUSIONS

Lysimeter data provided evidence that large amounts of nitrate were removed in the organic riparian zone and denitrification appeared to be a possible mechanism. Denitrification was shown to be highly active in organic riparian soils, as much as one to three orders of magnitude greater than rates found in upland soils. The high rates were thought to be due to almost ideal conditions for denitrification; i.e., anaerobic water-saturated soils with a high percentage of organic matter and a continual supply of nitrate from incoming groundwater.

In this riparian zone, the denitrification rate was shown to be predominately limited by nitrate concentration and the denitrifying enzyme activity. Nitrate was thought to have entered the riparian zone at the upslope edge in groundwater draining from the land treatment system, but was also observed to enter the riparian downslope edge possibly from the stream. It was likely that as nitrate moved through the riparian zone its concentration decreased following removal by denitrification. This removal and the dependence of denitrification on nitrate concentration may have been responsible for the spatial patterns observed.

An inverse relationship was found between nitrate and ammonium concentration in soil extracts. Denitrification appeared to be favoured where nitrate concentration was high and nitrate reduction to ammonium appeared to be favoured where nitrate concentration was low. It was thought that this relationship was due to competition between bacteria which reduced nitrate to nitrogen gases and bacteria which reduced nitrate to ammonium.

The organic riparian zone studied appeared to be highly efficient at nitrate removal; however, nitrate concentrations in the draining stream were nonetheless high. It was suggested that nitrate draining in groundwater had passed through mineral riparian zones where denitrification and nitrate removal was less. Whereas riparian zone denitrification might be an attractive solution to non-point nitrate pollution of surface waters, further work is required to assess the extent to which reduction of nitrate in these zones releases N_2O and contributes to the greenhouse effect.

CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

8.1 CONCLUSIONS

8.1.1 INTRODUCTION

A number of objectives were outlined in the introduction of this thesis. In this chapter the objectives will be addressed in the light of results found during the course of the present study. Other findings were made which were outside the original objectives and these are also discussed.

8.1.2 EFFECT OF CARBON ADDITIONS ON DENITRIFICATION

The objectives of this work were:

1. to compare the denitrification responses in organic riparian soil resulting from the addition of single sources of carbon and of plant material undergoing decomposition, and,

2. to investigate the relationship between denitrifiers and other anaerobic bacteria.

Denitrification in riparian soils was stimulated following the addition of a wide range of simple carbon compounds and plant materials. The response of denitrifiers varied depending on the carbon source added.

Single sources of carbon

When examining the influence of single sources of carbon, the denitrifier population responded quickest following the addition of a range of sugars, whereas the rate of denitrification was highest following the addition of the fermentation endproducts lactate and ethanol. Denitrifiers were initially inhibited following the addition of volatile fatty acids (VFAs), possibly due to acidification of the bacterial cytoplasm. The effects of acidification of the cytoplasm would include a partial decrease in the proton-motive force and a detrimental effect on acid-labile proteins and DNA. The addition of VFAs did not inhibit actively-growing denitrifiers and it was thought that these bacteria were able to maintain their internal pH by homeostasis. Whether denitrifying bacteria in riparian soils are inhibited by VFAs is unknown

and would depend on the formation rate and concentration of VFAs in riparian soils. However, it is probable the VFA concentrations in these soils are low.

Decomposing plant material

The carbon supply from the decomposition of fresh plant material appeared to increase denitrification more than did the addition of senescent plant material; this was presumably due to the availability of carbon. Water-extractable carbon was higher in the fresh plant material than in the senescent plant material and this may have accounted for differences in denitrification response.

Relationship between denitrifiers and fermenters

Denitrifiers may require the initial breakdown of more complex carbon compounds by other anaerobic bacteria. This was supported by evidence found when investigating the role of decomposing plant matter as a carbon source for denitrification. In incubations with decomposing plant material, the ratio of CO_2 and N_2O formed was constant following the addition of nitrate. This was thought to be due either to a relationship between fermenters and denitrifiers, whereby denitrifiers either used fermentation endproducts as a carbon source, and/or due to denitrifiers outcompeting the fermenters for carbon. It was shown when examining the effect of cellulose and xylan addition on denitrification that denitrifiers were dependent on the breakdown of complex carbon sources by other bacteria. Denitrification was not stimulated following the addition of cellulose, although denitrification was stimulated following addition of glucose - the monomer of cellulose. Denitrification showed a diauxic response following the addition of xylan and this was thought to be due to denitrifiers using fermentation endproducts released from xylan.

Long-term persistence of denitrifier activity

In studies of the effects of organic matter on denitrification, it was shown that denitrifying activity persisted for long periods (up to 99 days) in the absence of an electron acceptor. It was not known how these bacteria survived. However, this observation is in agreement with the fact that high potential rates of denitrification are frequently observed in anaerobic soils where nitrate and oxygen are probably rarely present.

8.1.3 NITRATE REMOVAL IN RIPARIAN SOILS

The objective of the field study was:

to determine the extent of nitrate removal by denitrification in an organic riparian zone and identify the factors which regulated this process *in situ*.

Nitrate removal in riparian zones

Lysimeter studies indicated that nitrate removal occurred in the riparian zone studied. In this riparian zone, denitrification rates were observed to be at least 1 to 3 orders of magnitude greater than those found in most upland soils and this denitrification was thought to account for the majority of nitrate removal. The nitrate concentration observed in the stream water was similar to nitrate concentrations found in the groundwater (compare figure7.4, site 4 and table 7.1) and this was attributed to the portion of groundwater that passed through mineral rather than organic riparian soils. In this study, denitrification was not measured in mineral riparian soils; however, in other studies it has been shown that denitrification is less in mineral soils than in organic soils (Cooper 1990; Schipper *et al.* in press). These observations highlighted the importance of the quantity and quality of organic carbon in riparian soils for the promotion of high rates of denitrification.

Concurrent with the high rates of denitrification observed, it was shown that N_2O was also produced at rates 1 to 3 orders of magnitude greater than found in upland soils. So although riparian zones may be able to provide some protection of water quality, they may also contribute to the greenhouse effect.

Regulation of denitrification

Within the riparian zone, nitrate and denitrifying enzyme activity (an index of the number of denitrifying bacteria present) appeared to be the main regulators of the onsite rate of denitrification. Temperature was also thought to regulate the denitrification rate; however, insufficient field trips were conducted at different temperatures to fully test this effect.

Spatial patterns in the riparian zone

Onsite denitrification, denitrifying enzyme activity, and KCl-extracted nitrate all exhibited spatial patterns within the riparian zone. These soil parameters were highest at the upslope edge of the riparian zone and decreased closer to the stream. High levels of denitrification, denitrifying enzyme activity, and nitrate were also observed at one point near the stream and it was thought that this was due to nitrate moving back into the riparian zone from the stream.

Denitrification and DNRA

Results from the field study indicated that there was an inverse relationship between nitrate and ammonium concentration in soil extracts. This suggested that dissimilatory nitrate reduction to ammonium (DNRA) and denitrification both occurred in the riparian zone. Whether these processes were in direct competition for nitrate was not determined. Direct measurements of DNRA were not made; however, it appeared that DNRA was favoured at sites where the ratio of nitrate to e^- donor was lowest. This finding was in accordance with previous theory on competition between denitrifiers and bacteria carrying out DNRA (Tiedje *et al.* 1982).

8.2 FUTURE WORK

In this section, some possibilities for further work are discussed. It is not suggested that this is the only further work that should be conducted; rather it represents some of the key areas of interest that arose from the present study.

8.2.1 CARBON SUPPLY FOR DENITRIFICATION IN RIPARIAN SOILS

Carbon was thought to regulate the maximum denitrification rate that could occur in a riparian zone. The source of this carbon is likely to be predominantly from decaying plant matter. An examination of the rates of production, death, and subsequent decomposition of different species of riparian vegetation might indicate which species might supply carbon at the most appropriate rate in order to support the denitrification process.

Although the present study has shown that denitrifiers are capable of using a number of different carbon compounds, it is still not clear which carbon sources are used in the soil environment. It would be useful to understand the nature of the competition which exists between denitrifiers and other anaerobic bacteria for carbon compounds. It needs to be established whether these bacteria are in competition for the same carbon sources or whether denitrifiers tend to use the endproducts of fermentation. It is possible that both of these relationships occur concurrently. It would also be interesting to establish the extent to which DNRA occurs in riparian soils and whether the bacteria carrying out DNRA compete with denitrifiers for nitrate.

8.2.2 DENITRIFICATION IN RIPARIAN SOILS

Nitrate removal from groundwater

Work in the present study examined the regulators of denitrification in a small organic patch of riparian zone. Since the maximum rate of denitrification has been shown to limited by the amount of available carbon, the methodology developed during this study should be repeated at a number of riparian zones which contain a wide range of organic matter contents, including mineral riparian zones. The number of sampling trips should be increased in order to better define the effect of temperature; however, the number of soil samples within each riparian zone could probably be reduced. A model of nitrate removal by denitrification in riparian zones could then be developed. Such a model should take into account the concentration of nitrate entering the riparian zone, and the amount of organic matter available for denitrification. Some understanding of hydrology throughout a catchment would also be required in order to understand the contact time that nitrate has with riparian organic matter. The model might also include the effects of temperature. Preliminary evidence found in the present study suggested that the response of denitrification would follow Michaelis-Menton kinetics. This implies that the development of a model could be based on experimental data as well as theoretical considerations.

Successful development of such a model hopefully would allow prediction of the breadth and type of riparian zone required to remove incoming nitrate to concentrations which were environmentally acceptable to the receiving surface waters. A successful model would also be able to predict the outcome of different management strategies of riparian zones. Two of the key parameters in the model would be the available organic matter and groundwater nitrate concentrations, and these may be open to management in the landscape. The amount of available organic matter in riparian soils will be dependent on rates of production, death, and subsequent decomposition of vegetation present. The groundwater concentrations of nitrate are dependent on the management of the upslope land e.g., fertilisation and application of wastes to land and the pattern of movement of nitrate-rich groundwater through the soil.

Nitrate removal in stream water

In the field study, a high concentration of nitrate and high rates of denitrification were observed in one corner of the downslope edge of the riparian zone. It was thought that nitrate in stream water moved back up into the riparian zone where some nitrate was removed by denitrification. This observation suggested that riparian soils may have a role to play in the removal of nitrate from stream water and such a mechanism could be of importance in lowland streams where flow velocities are low. Further work is necessary to assess the extent

to which stream nitrate is removed by riparian zones and to identify the structural and hydrological features which regulate this process.

Nitrous oxide production

Finally a note of caution. Whereas denitrification in riparian zones may be of benefit to surface water quality, the production of N_2O from riparian zones needs to be examined to investigate the contribution it makes to the greenhouse effect.

CHAPTER 9

APPENDICES

APPENDIX 9.1

Nitrous oxide and carbon dioxide production during incubation following the addition of a range of single sources of carbon (section 5.3.1.1). M denotes a missing value.

CARBON SOURCE	TIME (h)	CO2 production (umol C/g soil) Replicate			N2O production (umol N/g soil) Replicate		
		1	2	3	1	2	3
CONTROL 1	20	8.5	7.4	8.7	1.0	1.0	1.1
	35	9.8	8.8	14.2	1.8	2.0	2.0
	50	13.6	12.1	12.3	5.2	4.6	5.0
	74	17.8	15.7	16.1	7.7	7.3	6.9
	98	18.5 M	M		10.6	12.2	9.0
	122	22.2	22.2	21.6	12.8	13.8	16.2
	145	23.9	22.9	26.9	14.6	15.0	15.0
	170	27.4	23.7	30.1	17.5	18.4	17.6
	194	28.5	26.3	24.9	21.7	23.3	19.2
	218	30.3	28.8	29.8	25.3	26.2	23.5
	238	27.4	30.5	38.7	26.2	27.5	29.1
ACETATE 1	20	6.8	7.4	7.2	0.2	0.4	0.4
	35	6.7	7.6	8.5	0.5	0.6	0.6
	50	6.6	0.0	9.8	1.4	0.9	1.0
	74	8.6	9.5	9.9	1.3	1.2	1.4
	98	9.0	0.0	12.1	2.7	2.3	2.9
	122	9.2	14.1	10.7	4.0	2.9	4.6
	145	13.6	16.6	15.8	5.4	4.1	6.6
	170	24.6	21.2	17.1	8.0	6.3	12.1
	194	25.8	54.1	19.6	11.9	11.8	44.8
	218	68.2	56.9	50.6	38.9	30.1	66.7
	238	89.9	59.3	58.3	74.0	36.8	71.7
PROPIONATE	20	7.2	6.6	7.0	0.3	0.3	0.7
	35	8.0	8.2	7.0	0.5	0.6	1.1
	50	7.6	8.9	0.0	0.9	1.1	1.8
	74	8.6	9.8	8.8	1.1	1.4	2.2
	98	10.1	10.6	10.5	1.7	2.4	3.3
	122	8.3	9.8	9.6	1.9	2.8	3.9
	145	11.3	12.2	12.2	2.1	3.7	4.8
	170	11.4	11.7	13.4	3.0	4.1	5.8
	194	12.3	17.6	15.2	4.0	5.5 M	
	218	16.7	14.0	15.9	4.5	7.1	9.8
	238	15.5	18.0	16.1	6.8	9.7	24.5

CARBON SOURCE	TIME (h)	CO_2 production (umol C/g soil) Replicate			N_2O production (umol N/g soil) Replicate		
		1	2	3	1	2	3
BUTYRATE	20	7.6	7.4	7.4	0.2	0.3	0.2
	35	7.6	8.4	7.5	0.5	0.6	0.6
	50	8.8	0.0	9.9	1.0	1.1	0.8
	74	11.7	8.7	11.3	1.9	2.8	1.3
	98	15.3	8.0	0.0	5.0	6.2	2.6
	122	18.4	10.5	16.3	10.5	13.1	5.6
	145	35.4	30.8	27.7	23.7	33.5	25.7
	170	50.4	52.1	57.8	67.5	67.5	61.3
	194	53.3	54.3	53.5	82.1	78.9	76.0
	218	61.2	60.8	60.1	75.7	79.6	74.9
	238	55.3	58.9	54.1	77.8	80.2	76.2
LACTATE	20	8.0	6.6	8.1	0.6	0.4	0.5
	35	10.2	9.4	9.6	1.7	2.6	2.6
	50	24.8	24.5	19.1	10.1	16.0	16.1
	74	90.4	68.9	78.4	84.3	75.3	64.2
	98	97.1	79.8	78.2	80.5	88.1	71.8
	122	98.4	84.1	82.2	72.8	89.8	62.8
	145	113.8	100.4	112.2	70.0	78.4	53.6
	170	98.5	98.5	104.1	64.2	80.8	49.9
	194	120.0	92.5	107.6	67.7	83.5	50.4
	218	141.3	114.0	108.7	64.9	93.1	55.8
	238	126.9	98.6	109.9	79.7	94.2	80.8
GLUCOSE 1	20	8.1	7.9	7.7	0.8	1.2	1.0
	35	20.5	12.9	14.3	5.3	7.5	3.4
	50	45.1	51.2	43.3	32.4	37.3	35.1
	74	105.0	126.1	100.3	68.8	74.6	73.7
	98	135.0	135.7	153.2	84.2	84.9	78.8
	122	115.8	126.6	141.2	93.2	70.4	79.7
	145	139.7	155.8	154.9	89.3	76.2	83.0
	170	118.6	143.0	149.8	83.9	68.3	83.3
	194	135.7	139.9	156.2	85.7	67.6	71.9
	218	137.4	138.8	152.5	86.4	71.9	79.2
	238	126.6	141.3	136.1	52.7	78.1	86.4

CARBON SOURCE	TIME (h)	CO2 production (umol C/g soil) Replicate			N2O production (umol N/g soil) Replicate		
		1	2	3	1	2	3
ETHANOL	20	7.3	8.8	8.0	0.0	0.9	1.0
	35	8.0	8.1	8.1	2.4	2.3	2.1
	50	16.1	16.3	14.5	9.1	9.7	5.0
	74	51.2	55.3	47.1	81.4	89.3	72.2
	98	56.9	65.8	54.5	82.6	87.1	98.4
	122	57.6	62.9	53.2	89.9	87.7	96.5
	145	59.6	65.5	55.6	71.5	75.7	72.5
	170	66.7	75.8	54.9	66.0	82.8	81.9
	194	67.3	69.9	51.1	65.5	81.7	75.0
	218	54.7	64.8	53.8	64.1	72.6	72.3
	238	57.7	58.2	44.2	79.6	91.6	77.6
CONTROL 2	20	0.0	8.9	7.1	2.1	2.7	1.4
	35	10.9	9.2	11.3	2.9	2.9	3.4
	45	0.0	10.4	11.8	3.4	4.2	4.4
	51	0.0	12.3	13.2	4.9	4.4	4.5
	75	16.9	14.4	15.9	8.1	6.7	6.7
	99	19.9	16.2	18.9	8.0	8.2	8.4
	123	23.7	18.5	20.6	12.1	11.9	11.2
	147 M		15.6	24.1	12.6	12.6	12.7
	171	24.4	20.7	22.8	14.5	15.5	15.1
	195	30.4	23.1	32.2	16.7	17.0	16.4
	221	27.3	26.7	31.0	17.9	21.2	17.9
	263	30.3	33.0	37.4	23.8	26.5	20.5
ACETATE 2	20	3.4	7.4	8.1	1.4	1.1	1.2
	35	9.3	0.0	0.0	1.7	1.6	1.5
	45	9.3	8.4	9.4	2.2	1.6	1.8
	51	15.4	8.8	9.8	5.9	2.4	2.0
	75	19.2	12.8	9.3	10.7	3.8	2.5
	99	12.0	14.8	22.7	9.6	3.8	2.3
	123	15.2	14.8	15.7	4.8	4.4	4.1
	147	18.1	0.0	16.7	5.9	7.1	5.4
	171	17.4	22.5	20.7	8.1	10.5	8.1
	195	21.8	29.5	29.5	10.6	18.8	15.2
	221	30.8	60.7	57.2	19.8	54.9	27.3
	263	57.4	71.9	83.6	79.2	100.3	59.3

CARBON SOURCE	TIME (h)	CO2 production (umol C/g soil) Replicate			N2O production (umol N/g soil) Replicate		
		1	2	3	1	2	3
GLUCOSE 2	20	10.1	0.0	8.7	2.4	1.9	1.7
	35	19.6	0.0	19.1	9.9	8.7	9.9
	45	45.5	33.6	39.9	35.3	27.3	29.7
	51	81.3	60.2	50.5	54.6	45.3	40.6
	75	144.9	119.4	113.1	78.1	83.6	90.5
	99	143.3	147.6	132.0	72.1	75.5	80.6
	123	167.4	162.9	161.0	79.2	80.7	97.1
	147	161.2	156.2	135.7	68.3	77.5	73.3
	171	153.3	152.6	133.7	72.1	78.3	77.6
	195	125.0	148.7	148.3	66.8	76.5	81.4
	221	159.1	160.9	154.1	74.7	81.7	83.6
	263	153.6	167.6	159.3	71.3	81.8	88.9
XYLOSE	20	9.3	11.8	0.0	2.1	2.5	2.3
	35	14.2	18.6	2.5	7.0	9.5	10.1
	45	20.8	32.4	40.1	18.8	30.3	30.6
	51	27.1	51.0	57.6	22.9	39.9	45.1
	75	132.6	179.4	163.9	62.0	113.9	81.0
	99	126.2	212.4	175.0	47.1	96.1	79.6
	123	142.7	204.7	183.1	56.5	78.8	65.3
	147	117.2	232.7	170.9	44.6	90.1	73.0
	171	124.1	229.6	163.5	43.3	89.2	70.8
	195	128.1	214.4	169.4	43.2	92.4	69.9
	221	128.2	211.5	155.9	44.5	90.4	63.3
	263	131.6	212.8	160.6	45.0	91.5	72.8
FRUCTOSE	20	3.2	8.3	0.0	2.2	1.9	2.1
	35	4.4	10.9	16.3	6.6	4.1	7.1
	45	29.3	21.8	41.6	26.2	12.5	28.5
	51	53.4	37.8	69.5	42.9	35.6	44.3
	75	152.0	84.7	154.2	94.5	83.4	65.9
	99	206.9	133.1	141.0	80.0	101.5	59.7
	123	206.0	135.9	139.7	87.9	109.3	53.8
	147	210.6	126.5	156.6	89.7	97.5	58.2
	171	225.2	178.2	165.1	91.7	108.0	59.4
	195	247.8	155.8	149.1	89.1	98.6	55.6
	221	246.4	167.5	153.1	95.4	116.5	61.6
	263	258.1	176.2	140.0	97.5	116.5	56.8

CARBON SOURCE	TIME (h)	CO2 production (umol C/g soil) Replicate			N2O production (umol N/g soil) Replicate		
		1	2	3	1	2	3
CELLULOSE	20	8.6	9.1	9.8	1.6	1.7	2.0
	35	11.9	0.0	10.9	3.0	3.3	3.3
	45	11.7	13.9	11.6	4.2	5.0	4.3
	51	14.0	13.1	12.7	5.2	5.1	5.7
	75	16.5	17.3	20.0	7.2	7.1	11.3
	99	17.9	20.1	19.1	8.8	10.2	10.5
	123	22.5	23.0	21.6	12.0	12.1	12.8
	147	30.6	23.2	22.3	17.2	15.4	15.0
	171	33.2	26.1	24.2	20.0	17.1	17.5
	195	26.1	39.6	26.0	18.6	22.7	20.3
	221	25.2	27.2	26.7	20.1	17.1	21.4
	263	32.2	30.3	31.5	22.2	20.8	24.7
XYLAN	20	0.0	8.0	7.5	2.3	2.4	1.9
	35	0.0	12.3	10.5	5.2	6.1	4.4
	45	19.7	19.6	18.5	18.3	10.1	9.5
	51	32.8	28.4	34.7	28.0	27.2	29.9
	75	55.5	59.0	54.3	53.5	51.4	48.6
	99	68.2	72.0	75.1	50.4	51.5	55.8
	123	94.7	75.2	105.7	66.9	55.4	71.4
	147	97.2	91.6	103.4	69.1	86.4	88.9
	171	109.4	95.0	111.0	71.5	80.9	88.0
	195	114.7	105.0	128.5	74.1	77.6	89.9
	221	115.5	132.0	119.9	76.9	86.8	90.5
	263	120.6	134.8	147.5	69.8	88.7	92.7

APPENDIX 9.2

Total volatile fatty acids remaining at the end of the incubation period (section 4.3.1.4).

CARBON SOURCE	REP	VOLATILE FATTY ACID REMAINING			
		ACETATE mmol C	PROPIONATE mmol C	i-BUTYRATE mmol C	n-BUTYRATE mmol C
CONTROL 1	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
ACETATE 1	1	0.49	0	0	0
	2	0.16	0	0	0
	3	0.46	0	0	0
PROPIONATE	1	0	2.17	0	0
	2	0	1.77	0	0
	3	0	1.98	0	0
BUTYRATE	1	0.39	0	0.07	0.91
	2	0.65	0	0	0.58
	3	0.52	0	0.11	0.68
LACTATE	1	0.37	0.69	0	0
	2	0.79	0.27	0	0
	3	0.64	0	0	0
GLUCOSE 1	1	0.32	0.54	0	0
	2	0.59	0	0	0
	3	0.57	0.08	0.06	0.07
ETHANOL	1	0.63	0	0	0
	2	0.42	0	0	0
	3	0.40	0	0	0
CONTROL 2	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
ACETATE 2	1	0.41	0	0	0
	2	0.35	0	0	0
	3	0.52	0	0	0
GLUCOSE 2	1	0.53	0.07	0.05	0.09
	2	0.71	0.06	0.05	0.14
	3	0.71	0.13	0.04	0.13
XYLOSE	1	0.51	0.10	0.05	0.19
	2	0.78	0.15	0	0
	3	0.70	0.20	0	0.07
FRUCTOSE	1	0.63	0.31	0	0.39
	2	0.50	0	0	0
	3	0.72	0.21	0	0.10
CELLULOSE	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
XYLAN	1	0.37	0.21	0	0
	2	0.30	0.10	0	0
	3	0.30	0.08	0	0

APPENDIX 9.3

Results from five sampling visits to the Whangamata riparian zone (section 7.3.2). M denotes a missing value.

Sampling date	site	height cm	Distance row cm	Distance column cm	Temperature Celcius	Onsite denitrificat ugN/g/hr	Potential denitrificat ugN/g/hr	Natural N2O production ugN/g/hr	Water-extract nitrate ugN/g soil	KCl-extract nitrate ugN/g soil	% Organic matter	WSC ugC/g	Moisture content %	Water-extract ammonium ugN/g soil	KCl-extract ammonium ugN/g soil
24/1/90	1	29.9	0	0	20.9	m	m	m	m	m	m	m	m	m	m
	2	27.7	0	16	20.9	0.44	1.08	0.03	0.31	9.45	22.9	185	164	10.11	11.94
	3	24.4	0	47	20.9	1.18	0.65	0.38	2.11	10.24	29.6	198	215	5.74	4.26
	4	25.3	0	77	20.9	0.80	0.82	0.16	3.24	9.84	24.6	124	176	5.78	11.72
	5	22.7	0	103	20.9	0.77	0.93	0.72	5.13	16.34	26.7	217	202	7.38	12.05
	6	23.1	0	132	20.9	m	0.51	0.10	5.97	11.81	25.6	189	195	4.97	7.22
	7	21	0	165	20.9	0.70	0.45	0.02	6.79	4.65	32.4	265	235	6.43	7.36
	8	28.4	0	185	20.9	0.88	m	m	3.27	6.38	26.5	m	171	5.22	3.76
	9	22.5	53	0	20.9	m	m	m	m	m	m	m	m	m	m
	10	19	53	16	20.9	0.49	0.90	0.09	0.36	5.84	30.2	208	207	18.82	32.20
	11	15.3	53	47	20.9	0.50	0.97	0.09	1.02	7.25	27.5	300	211	23.17	30.29
	12	12.7	53	77	20.9	0.86	0.45	0.19	1.78	3.33	25.9	218	205	16.58	17.77
	13	12.4	53	103	20.9	0.10	0.22	0.00	0.00	0.74	27.8	204	202	23.08	33.36
	14	10.7	53	132	20.9	0.45	1.35	0.04	0.00	1.20	30.6	170	265	21.88	31.31
	15	10.9	53	165	20.9	0.53	1.10	0.04	0.49	1.84	28.4	150	213	27.71	33.98
	16	12.6	53	185	20.9	0.50	m	m	2.19	4.17	31.3	m	225	10.30	15.00
	17	14.8	111	0	20.9	m	m	m	m	m	m	m	m	m	m
	18	12.4	111	16	20.9	0.44	0.60	0.12	0.87	4.59	24.4	193	188	16.61	23.56
	19	9.3	111	47	20.9	0.32	0.71	0.08	0.93	2.52	24.3	150	189	6.94	9.59
	20	9.5	111	77	20.9	0.12	0.47	0.00	1.13	1.40	29.2	189	235	16.09	24.80
	21	9	111	103	20.9	0.04	0.25	0.00	0.48	2.73	24.6	167	197	13.27	22.61
	22	7.9	111	132	20.9	0.00	0.30	0.00	0.49	6.21	34.2	244	268	17.96	27.36
	23	3.3	111	165	20.9	0.00	0.23	0.00	0.00	3.25	29.4	236	226	28.92	42.11
	24	10.8	111	185	20.9	0.00	m	m	1.34	3.67	28.5	m	227	37.62	53.11
	25	11.3	185	0	20.9	m	m	m	m	m	m	m	m	m	m
	26	11.3	185	16	20.9	1.23	1.16	0.12	1.09	12.79	31.5	329	261	10.45	10.43
	27	1.1	185	47	20.9	0.28	0.43	0.00	0.72	3.64	27.2	127	222	7.21	12.43
	28	1.1	185	77	20.9	0.00	0.30	0.00	0.39	1.38	23.9	147	200	9.18	16.46
	29	1.5	185	103	20.9	0.00	0.39	0.00	0.55	0.63	24.3	128	198	18.38	23.78
	30	0.4	185	132	20.9	0.00	0.36	0.00	0.00	1.13	26.4	221	219	35.16	42.76
	31	0.4	185	165	20.9	0.00	0.19	0.00	0.00	1.65	26.3	265	209	30.37	40.15
	32	0	185	185	20.9	0.00	m	m	0.90	5.04	39.8	m	286	36.69	54.59

Sampling date	site	height cm	Distance row cm	Distance column cm	Temperature Celcius	Onsite denitrificat ugN/g/hr	Potential denitrificat ugN/g/hr	Natural N2O production ugN/g/hr	Water-extract nitrate ugN/g soil	KCl-extract nitrate ugN/g soil	% Organic matter	WSC ugC/g	Moisture content %	Water-extract ammonium ugN/g soil	KCl-extract ammonium ugN/g soil
12/7/91	1	29.9	0	0	12.1	0.48	1.28	0.08	16.11	17.22	25.6	271	204	3.87	7.40
	2	27.7	0	16	12.1	0.25	0.51	0.02	12.64	15.63	17.6	190	174	2.38	5.73
	3	24.4	0	47	12.1	0.57	0.96	0.08	14.67	21.19	26.9	175	236	3.99	6.71
	4	25.3	0	77	12.1	0.11	0.57	0.02	12.13	14.30	22.4	165	178	2.58	5.47
	5	22.7	0	103	12.1	0.44	0.80	0.14	34.01	35.45	26.6	325	272	3.20	10.47
	6	23.1	0	132	12.1	0.36	0.50	0.03	15.74	20.08	26.8	281	231	2.43	8.12
	7	21	0	165	12.1	0.20	1.00	0.02	21.38	21.83	28.2	281	232	5.47	7.19
	8	28.4	0	185	12.1	0.08	0.27	0.00	15.01	18.12	21.7	307	197	2.51	4.13
	9	22.5	53	0	12.1	0.14	0.30	0.00	4.03	6.12	22.8	m	186	6.13	9.95
	10	19	53	16	12.1	0.18	0.29	0.01	4.94	5.10	22.5	152	206	7.16	10.75
	11	15.3	53	47	12.1	0.11	0.30	0.02	7.36	5.86	20.8	m	192	2.77	7.76
	12	12.7	53	77	12.1	1.02	1.35	0.16	8.64	8.99	m	295	271	4.01	10.97
	13	12.4	53	103	12.1	0.50	0.64	0.21	2.90	2.58	24.1	152	259	9.37	13.64
	14	10.7	53	132	12.1	1.38	1.93	0.29	1.39	1.47	28.9	149	264	4.93	10.99
	15	10.9	53	165	12.1	0.09	0.52	0.01	6.03	0.44	22.9	446	206	7.61	11.67
	16	12.6	53	185	12.1	0.20	0.41	0.02	5.28	7.84	27.6	94	255	6.71	17.00
	17	14.8	111	0	12.1	0.09	0.46	0.01	6.56	3.35	22.9	198	219	7.00	10.97
	18	12.4	111	16	12.1	0.04	0.37	0.03	4.44	2.77	23.2	91	212	4.08	7.10
	19	9.3	111	47	12.1	0.44	0.86	0.10	8.36	6.59	20.8	59	182	1.61	4.97
	20	9.5	111	77	12.1	0.33	0.74	0.04	1.17	0.92	25.8	238	233	4.36	9.66
	21	9	111	103	12.1	0.34	0.63	0.04	6.69	4.94	25.0	172	282	6.69	9.03
	22	7.9	111	132	12.1	0.10	0.23	0.01	5.01	3.54	20.8	m	195	4.38	7.78
	23	3.3	111	165	12.1	0.13	0.40	0.06	2.59	0.71	22.4	116	206	7.08	8.18
	24	10.8	111	185	12.1	0.05	0.37	0.00	3.17	0.23	25.4	244	297	10.35	11.13
	25	11.3	185	0	12.1	0.27	0.50	0.06	9.70	4.07	20.2	372	200	3.09	10.97
	26	11.3	185	16	12.1	0.65	0.78	0.31	36.30	24.10	30.9	m	381	3.22	6.05
	27	1.1	185	47	12.1	1.26	0.87	0.13	6.04	3.97	28.6	729	353	4.35	11.34
	28	1.1	185	77	12.1	0.74	0.94	0.15	2.44	1.93	21.8	m	269	5.41	9.67
	29	1.5	185	103	12.1	0.13	0.75	0.03	2.33	0.74	24.7	82	226	5.04	9.38
	30	0.4	185	132	12.1	0.00	1.37	0.00	0.87	0.70	33.0	236	365	12.03	13.44
	31	0.4	185	165	12.1	0.00	0.62	0.00	1.70	0.78	29.7	390	389	9.39	14.77
	32	0	185	185	12.1	0.00	0.24	0.00	0.50	0.33	25.4	157	223	6.98	9.45

Sampling date	site	height cm	Distance row cm	Distance column cm	Temperature Celcius	Onsite denitrificat ugN/g/hr	Potential denitrificat ugN/g/hr	Natural N2O production ugN/g/hr	Water-extract nitrate ugN/g soil	KCl-extract nitrate ugN/g soil	% Organic matter	WSC ugC/g	Moisture content %	Water-extract ammonium ugN/g soil	KCl-extract ammonium ugN/g soil
27/11/90	1	29.9	0	0	17.2	0.42	0.84	0.05	2.94	4.35	20.6	221	165	4.28	11.38
	2	27.7	0	16	17.2	1.68	1.77	0.08	2.53	9.47	26.5	234	210	3.04	16.76
	3	24.4	0	47	17.2	0.58	0.68	0.03	2.68	5.17	25.5	189	186	3.93	11.99
	4	25.3	0	77	17.2	1.13	1.04	0.10	6.31	8.56	23.1	110	211	2.00	9.18
	5	22.7	0	103	17.2	1.06	1.17	0.14	7.93	11.53	27.6	219	258	2.78	7.58
	6	23.1	0	132	17.2	1.04	1.28	0.09	2.55	6.81	28.3	223	227	2.47	9.28
	7	21	0	165	17.2	2.05	1.24	0.19	5.73	9.92	34.2	244	269	6.26	4.69
	8	28.4	0	185	17.2	0.91	0.79	0.15	2.51	6.59	26.1	387	192	4.61	12.20
	9	22.5	53	0	17.2	0.60	1.16	0.02	0.32	0.93	29.2	361	246	10.80	23.21
	10	19	53	16	17.2	0.70	m	0.02	4.00	8.06	20.4	m	m	5.83	17.81
	11	15.3	53	47	17.2	0.52	0.55	0.07	3.36	4.60	20.7	m	197	5.11	13.85
	12	12.7	53	77	17.2	0.00	1.35	0.03	2.20	2.95	30.1	m	284	3.72	10.26
	13	12.4	53	103	17.2	1.13	0.48	0.16	1.96	3.83	22.4	m	230	5.99	13.32
	14	10.7	53	132	17.2	1.12	0.47	0.13	5.87	2.20	26.2	m	235	7.75	14.87
	15	10.9	53	165	17.2	0.20	0.46	0.02	1.09	1.40	28.0	m	229	14.93	30.31
	16	12.6	53	185	17.2	0.48	0.99	0.05	0.29	1.04	31.4	392	235	8.47	25.02
	17	14.8	111	0	17.2	0.44	0.57	0.01	2.51	3.85	23.8	389	234	8.35	18.91
	18	12.4	111	16	17.2	0.60	0.93	0.05	13.74	13.08	21.4	135	217	1.99	6.10
	19	9.3	111	47	17.2	0.72	0.90	0.05	4.66	7.60	22.0	96	208	1.92	3.59
	20	9.5	111	77	17.2	1.45	1.67	0.04	1.04	4.17	33.3	177	344	11.47	20.00
	21	9	111	103	17.2	1.25	1.49	0.05	8.58	9.39	23.5	211	278	4.51	14.32
	22	7.9	111	132	17.2	0.59	1.26	0.05	2.59	3.34	26.1	215	245	3.83	8.47
	23	3.3	111	165	17.2	0.20	0.35	0.00	0.65	2.64	27.1	m	237	5.82	13.52
	24	10.8	111	185	17.2	0.00	0.68	0.01	0.11	0.36	26.8	174	235	11.39	21.10
	25	11.3	185	0	17.2	1.18	1.05	0.17	8.32	8.48	25.3	119	266	2.92	7.97
	26	11.3	185	16	17.2	1.62	0.97	0.70	13.85	10.02	31.0	223	323	1.60	3.69
	27	1.1	185	47	17.2	m	1.07	m	1.39	3.04	m	122	262	1.05	5.54
	28	1.1	185	77	17.2	0.14	1.33	0.00	0.48	0.66	29.0	117	320	3.12	9.40
	29	1.5	185	103	17.2	0.00	0.67	0.01	0.11	1.00	20.8	129	210	3.29	11.43
	30	0.4	185	132	17.2	0.00	0.32	0.01	0.09	1.69	26.9	328	237	5.97	18.84
	31	0.4	185	165	17.2	0.00	m	0.00	0.51	0.47	32.1	222	355	7.25	26.79
	32	0	185	185	17.2	0.00	0.67	0.00	0.34	0.20	30.5	294	262	8.60	18.68

Sampling date	site	height cm	Distance row cm	Distance column cm	Temperature Celcius	Onsite denitrificat ugN/g/hr	Potential denitrificat ugN/g/hr	Natural N2O production ugN/g/hr	Water-extract nitrate ugN/g soil	KCl-extract nitrate ugN/g soil	% Organic matter	WSC ugC/g	Moisture content %	Water-extract ammonium ugN/g soil	KCl-extract ammonium ugN/g soil
11/2/91	1	29.9	0	0	21.2	1.16	m	m	8.33	10.58	21.4	385	170	3.59	8.44
	2	27.7	0	16	21.2	1.51	0.65	0.22	12.00	15.01	m	276	178	3.30	4.32
	3	24.4	0	47	21.2	0.94	1.25	0.05	6.85	11.52	26.6	328	180	1.93	5.81
	4	25.3	0	77	21.2	0.95	1.34	0.14	6.20	9.22	21.0	305	168	2.49	5.39
	5	22.7	0	103	21.2	1.00	1.51	0.15	m	13.90	25.3	m	199	m	5.88
	6	23.1	0	132	21.2	1.34	2.02	0.13	1.38	4.77	31.2	572	224	3.97	10.47
	7	21	0	165	21.2	1.32	1.40	0.08	7.20	8.11	31.7	450	226	3.83	7.53
	8	28.4	0	185	21.2	0.56	0.68	0.03	8.83	9.57	23.8	446	201	2.69	3.71
	9	22.5	53	0	21.2	0.84	1.20	0.12	2.40	2.04	24.6	373	210	4.57	16.77
	10	19	53	16	21.2	0.61	1.10	0.08	2.67	2.21	24.4	564	184	4.11	14.10
	11	15.3	53	47	21.2	0.76	0.76	0.05	3.02	3.82	24.2	301	201	2.63	12.06
	12	12.7	53	77	21.2	0.13	0.88	0.03	1.47	0.00	22.4	273	188	2.83	10.95
	13	12.4	53	103	21.2	0.38	m	0.08	1.25	0.81	27.7	226	214	3.64	13.00
	14	10.7	53	132	21.2	0.50	m	0.04	0.92	0.00	29.0	222	223	3.41	11.55
	15	10.9	53	165	21.2	0.18	m	0.00	1.08	0.00	m	194	225	3.95	16.14
	16	12.6	53	185	21.2	m	m	0.14	2.68	2.18	25.9	299	235	2.34	6.45
	17	14.8	111	0	21.2	0.42	0.50	0.07	5.00	5.43	22.2	303	185	3.61	11.68
	18	12.4	111	16	21.2	2.35	1.38	0.07	14.17	15.60	34.7	113	250	1.60	5.15
	19	9.3	111	47	21.2	1.28	1.21	0.10	15.78	18.78	24.6	325	253	1.87	3.27
	20	9.5	111	77	21.2	0.71	0.89	0.09	1.40	2.14	27.3	82	247	2.90	8.26
	21	9	111	103	21.2	1.78	0.68	0.13	8.98	9.14	29.0	m	287	3.16	5.79
	22	7.9	111	132	21.2	1.18	1.29	0.10	1.91	3.33	27.6	325	283	2.66	6.34
	23	3.3	111	165	21.2	0.01	1.27	0.00	3.00	0.00	28.3	344	282	4.84	15.51
	24	10.8	111	185	21.2	0.00	1.15	0.00	1.81	0.00	m	355	381	12.24	30.51
	25	11.3	185	0	21.2	0.72	1.28	0.17	4.38	5.07	27.4	156	240	2.74	18.02
	26	11.3	185	16	21.2	0.50	0.80	0.11	5.98	6.38	24.4	221	229	1.82	5.25
	27	1.1	185	47	21.2	0.15	0.50	0.00	1.23	2.01	m	258	260	3.53	8.26
	28	1.1	185	77	21.2	0.00	0.16	0.01	0.79	0.00	27.5	311	181	3.39	10.67
	29	1.5	185	103	21.2	0.00	0.65	0.00	1.34	0.00	21.3	358	248	5.19	13.17
	30	0.4	185	132	21.2	0.00	0.44	0.00	1.82	4.50	28.4	332	315	5.45	15.48
	31	0.4	185	165	21.2	0.00	0.60	0.00	1.93	0.62	39.8	259	287	8.57	15.65
	32	0	185	185	21.2	0.00	0.67	0.00	1.49	0.10	26.7	410	233	9.43	29.80

Sampling date	site	height cm	Distance row cm	Distance column cm	Temperature Celcius	Onsite denitrificat ugN/g/hr	Potential denitrificat ugN/g/hr	Natural N2O production ugN/g/hr	Water-extract nitrate ugN/g soil	KCl-extract nitrate ugN/g soil	% Organic matter	WSC ugC/g	Moisture content %	Water-extract ammonium ugN/g soil	KCl-extract ammonium ugN/g soil
2/5/91	1	29.9	0	0	13.1	0.44	0.60	0.03	18.97	21.54	21.0	239	217	1.50	1.70
	2	27.7	0	16	13.1	0.42	0.87	0.04	12.07	14.28	19.5	375	195	2.40	2.61
	3	24.4	0	47	13.1	0.55	0.60	0.06	14.44	15.97	25.4	180	241	1.94	0.43
	4	25.3	0	77	13.1	0.94	2.83	0.17	6.30	9.82	25.0	331	222	1.89	1.85
	5	22.7	0	103	13.1	0.47	0.80	0.04	m	5.63	29.2	691	224	3.48	8.60
	6	23.1	0	132	13.1	1.00	1.93	0.11	12.98	14.08	36.5	587	302	4.86	4.75
	7	21	0	165	13.1	0.44	1.06	0.07	16.65	14.81	36.1	552	326	4.15	1.54
	8	28.4	0	185	13.1	0.22	0.50	0.02	3.67	6.98	23.9	274	205	5.20	12.73
	9	22.5	53	0	13.1	0.87	1.01	0.11	4.52	4.42	25.7	271	257	3.20	7.68
	10	19	53	16	13.1	0.37	0.45	0.02	2.76	2.04	23.1	391	230	4.58	15.95
	11	15.3	53	47	13.1	0.29	0.48	0.03	4.49	4.35	21.8	281	210	4.10	10.93
	12	12.7	53	77	13.1	0.77	0.84	0.06	7.92	7.29	25.4	347	272	3.26	4.76
	13	12.4	53	103	13.1	0.20	0.38	0.02	1.74	3.20	20.5	286	197	7.31	10.74
	14	10.7	53	132	13.1	0.63	1.24	0.00	2.12	1.30	35.7	610	341	12.05	23.69
	15	10.9	53	165	13.1	0.00	0.81	0.00	1.79	10.15	38.9	537	352	12.70	26.52
	16	12.6	53	185	13.1	0.35	0.48	m	1.24	1.49	25.1	337	225	5.18	15.73
	17	14.8	111	0	13.1	1.34	1.53	0.05	5.27	6.12	29.1	375	297	7.08	16.56
	18	12.4	111	16	13.1	1.60	0.98	0.27	25.34	29.94	29.8	121	351	2.71	3.43
	19	9.3	111	47	13.1	0.64	0.67	0.08	7.43	13.50	20.5	298	217	3.02	4.14
	20	9.5	111	77	13.1	0.20	0.41	0.07	3.74	6.32	22.2	354	232	4.00	9.02
	21	9	111	103	13.1	0.06	0.21	0.00	0.00	5.18	23.8	m	220	m	6.78
	22	7.9	111	132	13.1	0.16	0.28	0.02	4.57	8.47	26.0	434	238	5.43	15.79
	23	3.3	111	165	13.1	0.00	0.76	0.00	2.96	3.81	22.0	271	254	4.91	9.42
	24	10.8	111	185	13.1	0.00	0.21	0.00	3.34	6.88	21.0	286	212	7.40	21.35
	25	11.3	185	0	13.1	1.13	1.01	0.30	14.71	15.16	25.7	172	279	3.61	8.28
	26	11.3	185	16	13.1	0.61	0.75	0.23	32.33	25.05	23.1	129	277	1.85	1.54
	27	1.1	185	47	13.1	0.95	1.09	0.13	3.68	m	23.9	213	310	1.70	m
	28	1.1	185	77	13.1	0.05	0.51	0.00	1.17	3.63	m	184	322	4.43	14.42
	29	1.5	185	103	13.1	0.02	0.63	0.03	1.03	1.51	m	208	249	5.14	11.95
	30	0.4	185	132	13.1	0.18	0.44	0.00	1.56	4.37	24.7	161	279	6.06	13.24
	31	0.4	185	165	13.1	0.00	0.30	0.00	1.93	2.43	29.1	266	330	5.52	17.82
	32	0	185	185	13.1	0.00	1.30	0.00	2.68	2.26	36.4	372	432	6.92	19.15

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