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THE INFLUENCE OF SECONDARY TREATED EFFLUENT ON DENITRIFICATION IN A NATURAL WETLAND

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SUMMARY

The influence of effluent addition on denitrification potential in the Thredbo Wetland was observed by comparing an area of the wetland receiving secondary treated effluent with another area receiving no effluent addition.

Physico-chemical measurements (Eh, pH and temperature) of the soil were conducted in both sampling areas to characterise the denitrifying environment. Levels of nitrate plus nitrite and ammonium ion in the soil from 0-30cm depth were recorded on a seasonal basis to identify the role of effluent addition and vertical distribution of inorganic nitrogen species in controlling the distribution of denitrification potential in the soil.

Denitrification potentials of soils and decaying plant material were evaluated by the acetylene blockage technique. This involved laboratory incubations under optimum conditions of pH, temperature, nitrate concentration, carbon supply, and diffusion. The influence of these physico-chemical factors on denitrification was also investigated.

It was found that the effluent addition caused higher denitrification potential in soils and surface decaying plant material by raising soil temperature, lowering Eh, and increasing concentrations of nitrate plus nitrite and ammonium ions. The highest denitrification potential was recorded in the decaying plant material on the soil surface. The highest soil denitrification potential occurred in the 0-6cm depth segment. Carbon supply and pH had no influence on denitrification potential whilst low temperature (5°C), and restricted diffusion limited denitrification.

In terms of tertiary water treatment denitrification in Thredbo Wetland makes a significant contribution to the removal of nitrogen year-round. However, total nitrogen removal could be increased by increasing the residence time of water in the wetland thereby encouraging greater spatial and temporal interaction between the denitrifiers and the wastewater nitrogen.

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ABBREVIATIONS

A.E.I.	Above Effluent Inflow
A.W.R.C.	Australian Water Resources Council
B.E.I.	Below Effluent Inflow
B.O.D.	Biological Oxygen Demand
B.S.T.I.D.	Board on Science and Technology for International Development
C.E.C.	Cation Exchange Capacity
C.O.D.	Chemical Oxygen Demand
C.S.I.R.O.	Commonwealth Scientific and Industrial Research Organisation
D.S.V.	Decaying Surface Vegetation
F.R.S.	Frog Ringers Solution
M.P.I.S.	Max Planck Institute System
P.S.	Pig Slurry

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CHAPTER 1 INTRODUCTION

1.1 THE IMPORTANCE OF DENITRIFICATION IN WASTEWATER TREATMENT - THE REMOVAL OF NITROGEN

Nitrogen occurs in water as organic and inorganic species. Anthropogenic inputs of organic and inorganic nitrogen to aquatic systems can lead to the degradation of water quality. Often this degradation takes the form of accelerated plant and algal growth in a process known as eutrophication. The seasonal death and decay of large masses of plants and algae in eutrophicated waters can lead to the reduction of dissolved oxygen and consequent odour problems.

Whilst stimulating growth in some organisms, high levels of nitrate and ammonium ion can be toxic to others (Alabaster and Lloyd, 1980). For example high levels of nitrate in public water supply can lead to the onset of methaemoglobinaemia in humans (Barnes and Bliss, 1983). An abundance of ammonium ion can reduce the amount of free oxygen available for respiration of aquatic organisms by stimulating nitrification whereby ammonium ion is converted to nitrate (Hynes, 1970).

If water quality is to be protected, agricultural, industrial and community inputs of nitrogen may have to be controlled. Biological processes provide a means of controlling nitrogen in wastewater. Of the biological processes involved in nitrogen transformations (see Chapter 3) denitrification can protect aquatic ecosystems faced with eutrophication by converting nitrate to nitrogen. By removing nitrate in this way (nitrogen 'lost' to the atmosphere) denitrification may limit the supply of nutrients available to algae and so prevent excessive growth. Although denitrifying bacteria exist in water they are usually more abundant in sediments and submerged soils (Bavor et al., 1981). As a result soils and sediments have been used in wastewater treatment due to their ability to 'remove' nitrogen from water by adsorption and biological transformations.

The most desirable nitrogen removal system is one by which nitrogen is transformed into a relatively stable form that will not contaminate ground waters or contribute to eutrophication in surface waters. The reduction of nitrate to nitrogen by biological denitrification meets this requirement.

1.2 THE WETLAND - A SUITABLE ENVIRONMENT FOR DENITRIFICATION

A wetland may be defined as any area where sediment accumulation supports the establishment and growth of rooted emergent vegetation, and where a throughput of water occurs (Gosselink and Turner, 1978). Therefore a broad range of vegetatively distinct areas are included ranging from sub-alpine swamps, like that under consideration in this study, to tidal marshes.

Efficient wastewater treatment is becoming increasingly important as the multiple use and reuse of water increases. Industrial installations and smaller communities like that at Thredbo are faced with a considerable financial outlay for sewage treatment. In most cases primary (screening, filtration) and secondary (activated sludge) treatment must still be carried out in conventional treatment plants, but the wetland may prove economic in tertiary treatment. This final stage of the treatment process involves the removal of organic and inorganic forms of nutrients, particularly nitrogen and phosphorus which have been shown to be responsible for eutrophication problems in both lentic and lotic freshwater environments (Moss, 1980).

Potential rate studies of denitrification in wetland sediments (incubation under optimum physico-chemical conditions) suggest that the microbial populations are capable of removing treated effluent concentrations of nitrate (Tilton and Kadlec, 1979). Measured rates of denitrification in wetlands range from 105 kgN/ha/month (Sloey *et al.*, 1978) to 700 kgN/ha/month (Bouwer and Chaney, 1974). Specific nitrogen loadings for these results were not recorded by Sloey *et al.* (1978), but the concentration of organic nitrogen and ammonium ion in the wastewater, applied at a rate of 2 cm^3/day^{-1} by Bouwer and Chaney, was 310 to 660 mg/liter. Rates of denitrification within the sediments will

be controlled by a wide range of factors. Those factors which stimulate denitrification such as abundant nitrate, organic carbon and optimum pH seem to be pre-existent in wetlands or can be encouraged to develop by wise management. Gersberg et al. (1983) have stated that wetlands can be termed selective for nitrate, exhibiting high removal efficiency by denitrification and therefore demonstrating their suitability as sites for microbial reduction of nitrate.

1.3 THREDBO WATER TREATMENT WORKS - WATER QUALITY DEMANDS

The treatment works serving Thredbo Village and restaurants on the slopes of the Ramshead Ranges discharges secondary treated effluent into the Crackenback River. The river has its source in the Ramshead Ranges with its main valley starting at Dead Horse Gap 5 km upstream of the village. The entire area lies within the Kosciusko National Park.

Water quality criteria for the river have been outlined in the Kosciusko National Park Plan of Management 1982. The main management objectives are

- to maintain the waters of the mountain catchments in as natural and unpolluted a state as possible.

- to endeavour to treat any effluents discharged within the park to the highest possible levels including the reduction of nutrient loads consistent with an assessment of the impact that this may have on other park values.

In addition to the above, the treatment works are presently licensed under provision of the Clean Waters Act 1970 to discharge water into the Crackenback River with the following specifications:

- volume of waste not to exceed 900 kilolitres/day (under dry weather conditions)

- wastes shall not

  - cause >20 mg/l Biological Oxygen Demand (B.O.D.)
  - contain >30 mg/l non-filterable residues
  - have a pH value <6.5 or >8.5
  - contain visible oil or grease and their concentration in the wastes shall not exceed 25 mg/l.

Although the village is predominantly a winter resort it also receives a considerable influx of tourists over the summer. In the winter of 1983 3000 beds were provided for overnight visitors and daily numbers averaged 2000 but reached 3000 when snow cover was high. Upgrading of the treatment works is proposed to cater for 4800 overnight visitors and 7000 day visitors.

Concern by the National Parks and Wildlife Service as to the present and future impact of village growth and development led to the initiation of a water quality monitoring program in 1980 . The river was found to be relatively unchanged from its natural state, although minor changes in numbers of invertebrates and seasonal growth of algae were recorded downstream of the effluent outflow (Cullen, 1983). However further development of the village could endanger the natural river system, the quality of water for human consumption downstream, and the nutrient status of Lake Jindabyne.

The major threat to water quality downstream of Thredbo Sewage Treatment Works appears to be eutrophication related to the high levels of nitrogen and phosphorus discharged . It has been proposed (Hogg, 1984) that the treatment works be upgraded to include tertiary treatment of wastewater in order to reduce nutrient levels. The swamp presently in use at Thredbo as a 'wetland filter' trapping certain suspended and dissolved material is seen as an integral part of this development.

1.4 THE EFFECTIVENESS OF THREDBO WETLAND IN NITROGEN REMOVAL

Input-output investigations of nitrate plus nitrite and ammonium ion have shown that the wetland is effective in immobilization and removal nutrients following the application of wastewater (Finlayson et al., in prep.). It was found that over a two year study period the annual input of nitrogen was 4601 KgN and the output was 3883 KgN giving a retention of 16%.

Separation of the results according to season gave the following results.

Table 1.1 Seasonal loads of nitrogen through the wetland (Finlayson et al., in prep.).

	Nitrogen Mean Load $g\ day^{-1}$		
	IN	OUT	% Retention & immobilization
Summer 1982	6419	2220	65
Winter 1982	14101	12171	14
Summer 1983	3528	2351	33
Winter 1983	18079	16701	8

Despite low temperatures in winter the wetland continued to retain nitrogen. This is important because peak nitrogen loadings occur during the ski season when biological removal processes are limited by low temperatures.It appears that the wetland is capable of contributing to nitrogen removal year-round.

1.5 OBJECTIVES OF THE STUDY

Previous studies of the wetland at Thredbo have shown it to be effective in removing and immobilizing wastewater nitrogen (Cullen, 1983; Finlayson et al., in prep.). This study aims to advance from the 'black box' (input-output) understanding of the system by examining some aspects of the role of denitrification in the nitrogen removal process.

The objectives are:

(1) To investigate the influence of the effluent inflow on physico-chemical properties of the wetland soils to determine the controls operating in wetlands that effect the distribution of denitrification potential.

(2) To quantify the amount, and observe the distribution of nitrate plus nitrite and ammonium ions in the soil in relation to season, depth and effluent loading to determine the relationships between nitrogen species and denitrification potential in the wetland soil.

(3) To monitor the influence of some physico-chemical factors on denitrification to determine the areas in which environmental conditions in the soil at Thredbo could be improved to enhance and/or preserve denitrification.

(4) To suggest some possible management options for the wetland to enhance and/or preserve denitrification as a nutrient removal process.

CHAPTER 2 WETLAND SYSTEMS

This chapter examines the role of natural and artificial wetlands systems in wastewater treatment (nutrient removal), and examines some of the system components that contribute to the treatment of wastewater.

2.1 THE PRESENT STATE OF RESEARCH

The majority of studies on wetlands have taken a systems approach. This method of monitoring the inputs and outputs of complex systems was introduced in the sixties to gain an understanding of their response to inputs without detailing the processes operating within them to produce the resultant outputs.

The input-output approach monitoring the nutrient concentration and physico-chemical characteristics of added effluent and outflowing water has been applied to two major types of wetland systems in use as treatment facilities. These are:

- natural wetlands
- artificial wetlands

In addition to input - output study, more detailed analyses of the processes operating within each system have been undertaken (eg. hydrology, nutrient uptake by plants etc.). Although information in these areas is accruing, detail of processes, particularly in the sediment - microbial compartment, is lacking.

2.2 NATURAL WETLANDS

Wetlands are found in a wide variety of locations and may be available for exploitation as water treatment sites. Many natural wetlands are already in use for commercial and municipal wastewater treatment. The following examples illustrate the importance of the hydrology, aquatic macrophytes, seasonal effects (temperature and precipitation), and the sediment-microbial compartment in providing efficient nutrient removal from water.

Boyt et al. (1977) reported on the efficiency of a wetland in Florida that had been receiving primary treated effluent for twenty years. They found that nutrient uptake by plants and adsorption onto the sediments led to a reduction in nutrient concentration to values equalling those in an adjacent wetland where there was no effluent addition. A large proportion of nitrogen and phosphorus was stored in the upper 5 cm of the sediment and most of the nutrient removal was achieved in an area representing 3% of the wetland (≈6 ha). It was estimated that the use of the wetland in tertiary treatment saved $US 79,500/yr.

Nichols (1983) investigated the importance of loading rates and retention times in the removal of nitrogen from a wetland in Minnesota receiving secondary treated sewage. Nitrogen removal decreased rapidly as wastewater loading rates were increased . The conclusion drawn was that the major mechanism for removing nitrogen was denitrification, and that the capacity of this process was such that if the supply of nitrate was maintained it would continue to remove nitrogen. The decline in nitrogen removal efficiency observed was therefore a result of factors limiting denitrification. Nichols identified the major limiting factor as being diffusion. He incubated sediment samples under anaerobic conditions with continuous stirring and found that 90% of added nitrate at typical wastewater concentrations was denitrified. The controls on nitrogen removal in this wetland were therefore closely linked to the hydrology, being dependent on turbulence, retention time, and water depth which all influence diffusion rates.

Uptake of nutrients and subsequent release via decomposition can lead to a distinct seasonal influence on nutrient removal by the aquatic macrophytes. Any seasonal changes in the performance of wetlands as sites for nutrient removal would have implications for their use in wastewater treatment. Some studies have been restricted to the growing season alone (Tilton and Kadlec, 1979) but others demonstrate that the ability of wetlands to store nutrients varies according to season and concurrent changes in water flow regimes (Lee et al., 1975). There may be a greater export of nutrients at particular times of the year, but these usually occur in times of higher flow rate and thus the impact is reduced by dilution (Peverly, 1982). Changes in temperature may influence the activity of the microbial compartment and thus nutrient

removal, although in the case of nitrogen removal denitrification has been seen to continue through the winter (Sorensen, 1978 c.)

Natural wetlands presently in use as treatment facilities remove nutrients, bacteria, suspended solids, heavy metals etc. and reduce the Biological and Chemical Oxygen Demand of water. Therefore any wetland situated near an effluent outflow could be manipulated to treat water. However long term benefits would only be achieved if the assimilative capacity of the wetland was understood. Effluent discharge rates must be controlled to ensure that the natural retention processes of the wetland are not overloaded.

The majority of wetlands in use as treatment facilities are attached to small communities and agricultural/industrial installations where loadings in relation to wetland area are not excessive. It is at this small scale where per-capita costs of commercial treatment systems are high that the cost effectiveness of natural wetland filters is most evident.

2.3 ARTIFICIAL WETLANDS

In areas where natural wetlands are not present or protected from wastewater discharge by environmental, legal or aesthetic restraints, artificial wetlands offer an alternative for low-cost wastewater treatment. The majority of systems presently in operation are based on a number of vegetated trenches through which wastewater is passed. This method is very versatile and can be scaled for villages, farms, hotels, factories, dairies, piggeries, and small municipalities. Although raw sewage can be treated directly (De Jong, 1976) most workers have found that some degree of dilution or pre-treatment is required to allow the plants to establish and flourish (Board on Science and Technology for International Development, 1976; Finlayson and Mitchell, 1983).

Free-floating, submerged and emergent species have been used in experimental wetlands. Results favour emergent species such as Typha sp. and Phragmites sp. which encourage the development of an active rhizosphere population of micro-organisms aiding water treatment by

utilising nutrients (De Jong, 1976; Howard-Williams, 1979). The efficiency of a wide variety of plant species in wastewater treatment has been monitored. Finlayson and Mitchell (1983) selected plants on the following criteria: rapid and constant growth, ease of propagation, capacity to absorb pollutants, tolerance of hyper-eutrophic conditions, ease of harvesting, usefulness of harvested material, and status as non-noxious species.

One of the first commercially available treatment 'packages' was developed at the Max Planck Institute in West Germany and patented as M.P.I.S. (Seidel, 1976). Several considerations guide the installation of M.P.I.S. to ensure that the only energy sources required are solar and gravitational thus creating an energy-efficient treatment system. The plants are prevented from obtaining their required nutrients from the substrate by being rooted in inert material (gravel or sand). Thus they are forced to rely on the nutrients available in the applied water which is enriched with oxygen by a cascade system. Drainage is provided within the substrate to prevent the development of extremely anaerobic conditions. There is some debate as to the viability of harvesting plant biomass but the M.P.I.S. advocates the use of trenches for accessibility during management. Some pretreatment of the water is also advised.

An operational artificial wetland system has been running for several years at the Disney World Complex in America. Gersberg et al. (1983) have monitored the nitrogen removal efficiency at the complex and have reported that the use of an inert substrate material causes a deficiency of organic material for denitrifiers. Trenches with no carbon supplement removed only 25% of added nitrogen as opposed to 86% removed by those where harvested plant material was mulched and returned to the system. If carbon was not added the effluent application rates had to be significantly reduced. It is therefore important to know the capacity of the vegetation and substrate to assimilate or adsorb nutrients so that loading rates and residence time of water can be controlled to maximise nutrient removal (B.S.T.I.D., 1976).

Finlayson and Mitchell (1983) discuss the application of artificial wetlands to the treatment of wastes from three different agricultural installations in Australia. In each case the system consisted of a number of plastic-lined gravel trenches. They confined the plant species used to the emergents Typha sp, Phragmites australis and Schoenoplectus validus which had been shown to be tolerant of effluent loadings in glasshouse experiments. One study was conducted at a piggery where effluent was diluted to avoid killing the plants. Flow was adjusted to provide theoretical retention times of five days in each of two trenches. The results in Table 2.1. demonstrate the effectiveness of nutrient removal and improvement in water quality achieved by these artificial wetlands (Finlayson and Mitchell, 1983).

Table 2.1. Comparison of the mean values for the inflow and outflow analyses from two aquatic plant filters at a piggery. (Finlayson and Mitchell,1983).

	FILTER I		FILTER II	
	IN	OUT	IN	OUT
Suspended solids mg/l	220	88	210	80
Turbidity (NTU)	68	36	45	39
pH	7.9	7.8	8.9	7.9
Conductivity 25°C uS/cm	2665	2100	2634	2146
COD mg/l	567	349	538	354
NH_3-N mg/l	991	797	961	753

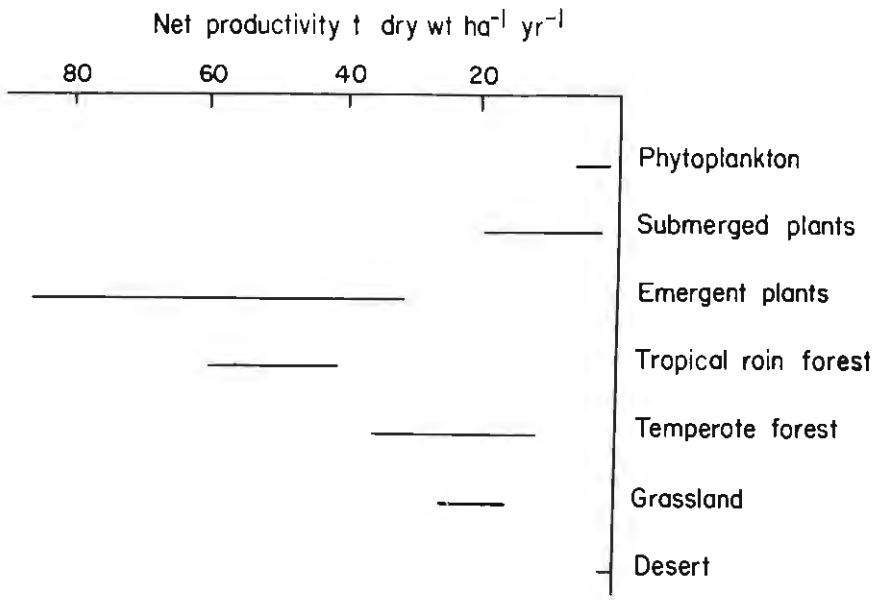
Input-output studies of artificial wetlands as described above have shown them to be an efficient means of treating wastewater (DeJong, 1976; Finlayson and Mitchell, 1983; Gersberg et al., 1983). Their versatility allows their application to a wide variety of effluent types. The B.S.T.I.D. (1976) stressed their potential for use in developing countries, the advantages lying in the lack of complicated technology, low cost, and use of local weeds and equipment to meet specific local needs.

In conclusion artificial wetlands provide an alternative to natural wetlands for the treatment of wastewater although a lack of organic material in the inert substrate may inhibit microbial activity (eg. denitrification). The advantage of artificial wetlands over natural wetlands is the inert subsrate which ensures that plants and micro-organisms will draw nutrients from the added water and not from the sediment.

2.4 AQUATIC PLANTS

Initial interest in wetlands as nutrient sinks was stimulated by their extremely high productivity evidenced by plant biomass. Moss (1980) constructed a diagram contrasting the net productivity of various vegetative communities (Figure 2.1.). Emergent aquatic plant communities are shown to be among the most productive, per unit area, of the vegetation types. Whilst water and nutrients are limiting in terrestrial ecosystems they are abundant in wetland soils and sediments, and therefore productivity is higher in aquatic systems.The higher productivity of emergent species over submerged aquatic plants is related to nutrient supplies from the sediment and greater exposure to sunlight.

FIGURE 2.1



NET PRIMARY PRODUCTIVITY OF AQUATIC COMMUNITIES IN COMPARISON WITH THOSE OF PHYTOPLANKTON AND TERRESTRIAL VEGETATION. MOSS,1980

An investigation into the efficiency of different types of aquatic plants in nutrient removal was conducted by Howard-Williams (1979). He compared free-floaters (eg. Eichhornia sp.), submerged species (Potamogeton sp.) and emergents (Phragmites sp.). Emergents proved most efficient in wastewater treatment as they provide a substantial above and below-ground biomass for the storage of nutrients, and support large populations of bacteria in their rhizosphere.

In addition to being highly productive, emergent wetland vegetation can act as a regulator dissipating the affect of flooding on downstream areas by modifying and easing the flow rate (Nicholls, 1983). Therefore the biological importance of aquatic macrophytes is matched by the physical aspects of buffering hydrologic variation and maintaining a firm substrate within the wetland.

In regions with a defined growing season the wetland plants capture, store and release nutrients in a pulse-like fashion (Lee et al., 1975). Although biologically productive for only part of the year the input and output of water, nutrients and organic matter is perennial and therefore wetlands can act as a source of nutrients in certain seasons.

A further control of seasonal variation on nutrient removal by plants is in changes of nutrient assimilation and tissue nutrient levels with a general decline from spring to autumn (Prentki et al., 1978). Uptake of nitrogen by plants peaks at the start of the growing season. Phragmites sp. was shown to obtain 60% of its nitrogen standing stock during the first 45 days of this maximum growth period with translocation rates of 34-750 $mgN.m^{-2}.d^{-1}$ (Prentki et al., 1978). Throughout the growing season wetland plants are acting as nutrient pumps, rapidly assimilating nutrients from the sediment and, to a limited extent, from the surrounding water (Klopatek, 1978).

The nutrients assimilated during the growing season are then released by the decomposition of above and below-ground plant structures (leaves, roots etc.). The maximum concentration of nutrients in above-ground plant tissues occurs at the beginning of the growing season and can peak at any time in below-ground tissues. As a result of these seasonal fluxes it has been advocated that plant material be harvested prior to translocation below ground and senescence in order to remove nutrients from the system (Klopatek, 1978). Material that is released to the decomposer organisms is usually broken-down fairly slowly due to its resistance in terms of cellulolytic composition, and the depressed rates of decomposition in the anoxic wetland environment.

Two other mechanisms of nutrient release are leaching of above ground tissue into surrounding water, and decomposition of detritus falling into the water. Decomposition of plant material within the wetland sediment leads to most of the nutrients being 'buried' or lost from the system by microbial activity. Burial involves incorporation into the microbial biomass or immobilization within the sediment on the cation exchange system. Steward and Ornes (1975) demonstrated that 43% of the nutrients applied to a wetland were retained in the bottom sediments by

chemical and biochemical pathways. They gave no estimate of the proportion of this that would have been permanent retention.

The role of plants in this process is to convert nutrients, such as the inorganic species of nitrogen, from available to less-available forms thus reducing their concentration in surface waters. Due to seasonal releases of nutrients the major role of aquatic plants in water treatment is to temporarily store nutrients. This softens the impact on downstream water quality, and allows natural purification mechanisms to operate without being overloaded.

The response of wetland plants to wastewater loading of nutrients is generally seen as changes in growth, morphology, yield and species composition. In general, yield is increased by wastewater input but some species may not be tolerant of hypertonic conditions (Mudroch and Capobianco, 1979). Seidel (1976) has demonstrated that plants exposed to a variety of effluents are highly adaptable and often exhibit characteristics which enable them to 'treat' the effluent more efficiently. An emergent species (Alisma plantago) grew to gigantic size and formed a large storage bulb when grown in faecal sewage. Thus above and below-ground storage of nutrients was increased.

Aquatic macrophytes also have inherent characteristics which aid wastewater treatment. For example, the root exudates of Juncus efusus and Phragmites communis have been shown to kill disease bacteria in contaminated water (Seidel, 1976). The root exudates protect the plant from decomposer micro-organisms and help the rhizosphere bacteria, such as denitrifers, to survive when conditions in the sediment may be toxic or harbouring disease bacteria. Many aquatic species have nitrate reductase enzyme in their roots enabling them to assimilate nitrate from the sediment (Nichols, 1983). The majority of plants transport air down into their roots by diffusion which then passes out into the rhizosphere possibly aiding nutrient uptake and encouraging nitrification adjacent to the roots. In this way plants can influence the redox potential of the soil and thus effect nitrogen transformations. Microbial activity is further enhanced by the exudation of organic compounds from the roots which act as a carbon source and influence soil pH (Boyd, 1978).

Studies of the plant communities discussed earlier have demonstrated that a large proportion of the nutrients are assimilated in one growing season and released at the end of that season, thus providing only temporary storage. Prentki et al. (1978) presented three options to increase the removal of nutrients from a wetland. The first is to harvest plants at times of peak nutrient concentration in the tissues. Spangler et al. (1978) determined that plants had to be harvested bi-weekly in order to remove enough nutrients from the system. Harvesting can therefore prove costly and the disposal of vegetative material can remain a problem. In addition only 5-20% of the nutrients detained by wetlands, are stored in harvestable plant tissue the majority being associated with root-rhizome and microflora substrate complexes (Sloey et al. 1978). The second method of removing nutrients from solution is to immobilise them within the sediment. Long term storage and slow release of nutrients can be achieved by these mechanisms but most of the plant nutrients, such as nitrogen, are moved through more rapid transformations (eg. nitrification, denitrification, etc.). These transformations form the third method of removal and are the most favoured for removing nutrients from a system. Many of the biological transformations involve loss of elements as gaseous compounds, for example nitrogen is lost following denitrification of nitrate.

It is now recognised that the major role of plants in wastewater treatment is in providing an area within the rhizosphere and sediment where aerobic and anaerobic micro-organisms can survive and metabolise concurrently to remove nutrients, for example nitrogen by denitrification (Valiela et al., 1977).

2.5 THE SEDIMENT

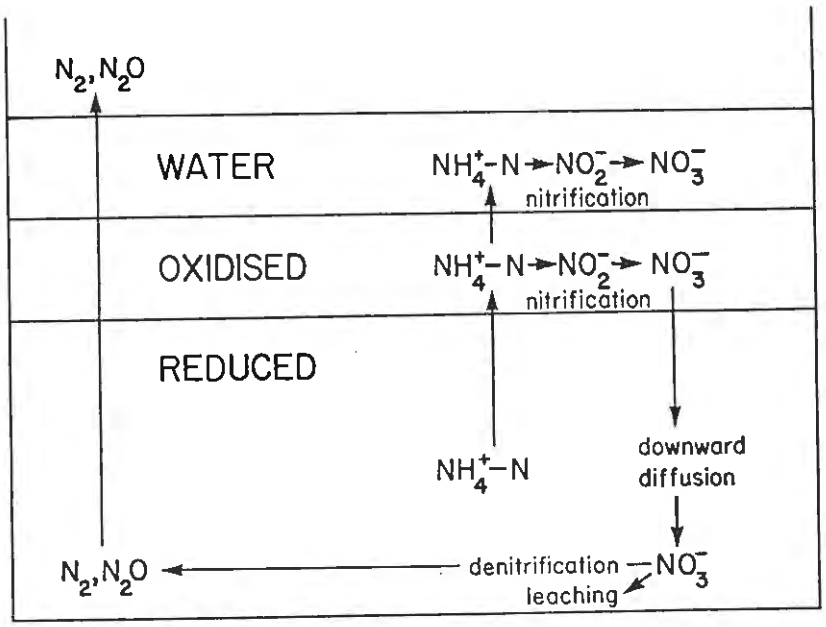
Flooded soils and sediments are a matrix of solid, liquid and gaseous phases receiving inputs of water, nutrients and organic material (plant residues). The conditions within the soil may vary markedly in space and time due to variations in the inputs. Concentration gradients of gases and nutrients exist on a scale of micrometers. Therefore the sediment environment is highly heterogeneous.

Inundation usually causes an absence of oxygen within the sediment although overlying floodwater may have a high dissolved oxygen content (Reddy and Patrick, 1984). The oxygen demand in the water is usually low in contrast to that of the sediment, especially those with large amounts of organic carbon supporting a high level of microbial activity. Oxygen reaching the sediment surface is consumed during biochemical processes : heterotrophic microbial respiration using oxygen as an electron acceptor; chemical oxidation of reduced iron, manganese and sulfides; and nitrification (biological autotrophic oxidation of ammonium ion). The greater consumption of oxygen within the sediment profile compared to its renewal rate through water results in the development of two distinctly different soil layers (Klopatek, 1978; Reddy and Patrick, 1984). At the surface an oxidized or aerobic layer develops varying in thickness from a few millimeters in sediments of high biological activity to 1 or 2 cm in less active soils. Underlying this is a reduced or anaerobic zone where little or no free oxygen is present. Within this reduced layer many microbial processes can occur, reflecting the development of increasingly reduced condition. First, following the onset of anaerobiosis , nitrate is used as an electron donor in denitrification. As nitrate levels decrease, sulfate reduction begins, and finally methanogenesis occurs in highly reduced sediments (Bender et al., 1977). The presence of these layers has been confirmed by redox measurements (Sorensen, 1978 a.; Reddy and Patrick, 1984).

The development of two distinct zones (aerobic and anaerobic) allows the concurrent activity within the sediment of facultative aerobes and anaerobes. An example of this is the nitrification-denitrification reaction which leads to the removal of nitrogen from the sediment (see Figure 2.2). However in terms of this reaction the two-layer model observed in many sediments and flooded soils does not always explain observed nutient profiles particularly where benthic infauna influence vertical zonation, and where microsites allow aerobic and anaerobic processes to coexist in close proximity (Jenkins and Kemp, 1984). In either case the sediments can support large and diverse populations of micro-organisms (Jones, 1979) which consume large amounts of energy and materials with subsequent release of dissolved organic and inorganic

material (Pomeroy et al., 1977). Micro-organisms in the sediment therefore represent a major means of removing nutrients from percolating wastewater.

FIGURE 2.2



DIAGRAMATIC REPRESENTATION OF THE AEROBIC--ANAEROBIC LAYERS IN SEDIMENTS AND FLOODED SOILS AND THEIR INFLUENCE ON NITRIFICATION--DENITRIFICATION REACTIONS (adapted from Reddy and Patrick, 1984)

Micro-organisms require an energy source for their metabolic activity. Heterotrophs use organic carbon derived from decaying plant and animal material within the sediment. Many of the micro-organisms involved in nitrogen transformations can be affected by the availability of carbon (Haines et al., 1977; Blackburn and Henriksen, 1983). Other micro-organisms (chemolithotrophs) are dependent on a supply of inorganic ions for their energy supply. The soil water is a ready source of these nutrients such as nitrate and ammonium ion (Steward and Ornes, 1975; Klopatek, 1978). The high surface-volume ratio of micro-organisms allows rapid and efficient uptake of materials from solution.

Cations are also removed from the soil solution by being fixed by cation exchange within the soil (Richardson et al., 1978). In terms of nitrogen removal from wastewater the cation exchange capacity (C.E.C.) is a very important parameter in determining the fate of surplus ammonium ions in the sediment. The capacity is often increased when fresh detritus is degraded (Blackburn and Henriksen, 1983). Increasing organic matter content of sediments therefore tends to increase the efficiency with which exchangeable ammonium ions are held within the soil and removed from percolating water. The ammonium ions remain available to denitrifiers whose activity is also increased by a greater abundance of organic carbon.

Many wetland sediments are organic rich supporting a wide variety of vegetation types. Plant growth is encouraged by the availability of nutrients in the interstitial water. The plants can aid the removal of nutrients in a variety of ways (see Section 2.4). Most importantly they maintain a seasonal input of organic material to the sediment.

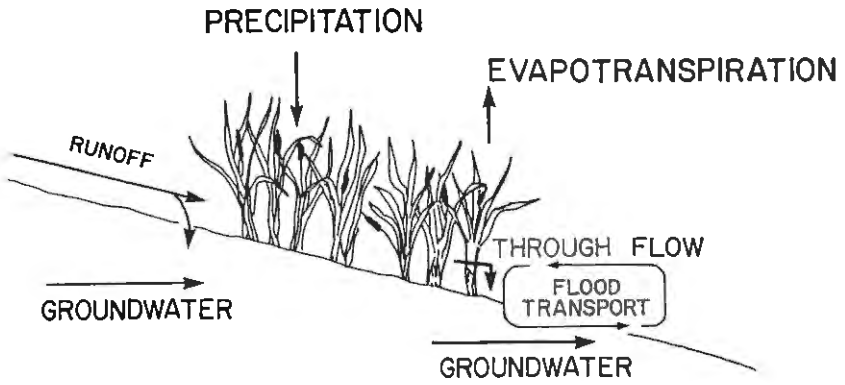
In addition to acting as an energy source for micro-organisms and increasing the cation exchange capacity, organic carbon influences soil permeability (structure, texture, etc.). This is important in wastewater treatment where water must infiltrate to undergo biological and chemical transformations within the sediment. Following infiltration the sediment structure also controls the length of time that wastewater stays in contact with micro-organisms (Lind, 1977). Concurrent aerobic and anaerobic microbial activity is facilitated by an aggregate sediment structure creating microsites (Jones, 1979).

Land infiltration of wastewater under experimental conditions has repeatedly demonstrated that sediments, especially those high in organic matter, are very effective in water treatment by removing nutrients from solution (Wells, 1973; Bouwer and Chaney, 1974; Farnham, 1974; Lind, 1977).

2.6 HYDROLOGY

Throughput of water is one of the factors which determines whether a land-water ecosystem can be classified as a wetland (Gosselink and Turner, 1978). The inputs and outputs of water in wetlands are depicted in Figure 2.3 (Prentki et al., 1978). The source, velocity, renewal rate and timing of these inputs and subsequent outputs directly control spatial heterogeneity of vegetation (species composition, productivity etc.) and soils (structure, organic content etc.) within and between wetlands. The supplies and exports of organic and inorganic nutrients are a result of water movements, and are an integral part of nutrient cycling.

FIGURE 2.3



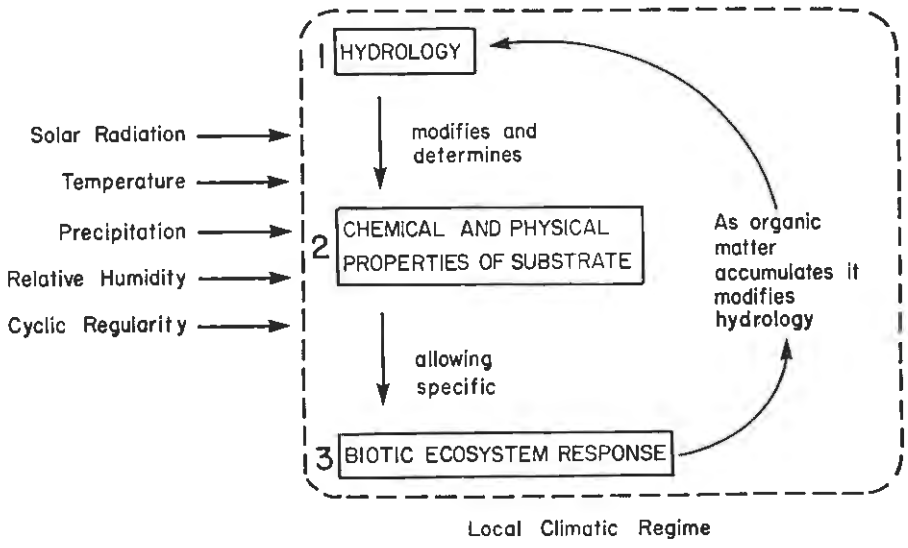
A CONCEPTUAL INPUT-OUTPUT FOR WETLAND HYDROLOGY ADAPTED FROM PRENTKI et al, 1978.

Water is slowed as it enters a wetland and therefore sediments are deposited and stabilised by plant growth.This removes large amounts of particulate matter from the water as it passes through. The sedimented material is a source of nutrients which if released downstream could be detrimental to water quality.

In addition to trapping sediments the wetland can reduce the fluctuation in streamflow by dissipating and storing water. This leveling of flow tends to reduce erosion of sediments and leaching of organic and inorganic nutrients (Lee et al., 1975).

Gosselink and Turner (1978) have developed a general conceptual model depicting the role of hydrology in the wetland ecosystem. Climatic influences such as temperature and solar radiation are those which determine spatial heterogeneity in wetlands, but the hydrology of individual wetlands will determine their effect on water quality at the local scale (leaching and erosion).

FIGURE 2.4



GENERAL CONCEPTUAL MODEL OF THE ROLE OF HYDROLOGY IN WETLAND ECOSYSTEMS. (GOSSELINK AND TURNER, 1978).

The hydrologic inputs (Figure 2.4) represented by compartment 1 include rainfall, runoff, groundwater and flooding. In terms of nutrient loading rainfall is generally not significant. Prentki et al. (1978) measured annual loadings in a Wisconsin marsh of only 0.8 - 1.6 gN m^{-2}. yr^{-1}. The major sources of nutrients are runoff and groundwater with specific influences from flood events. The source of the water also determines oxygen saturation and toxin load (heavy metals, pesticides etc.).

The erosional capability (turbulence) and the ability of the water to carry suspended particulate matter is controlled by water velocity. During flooding velocity may be increased thus increasing supplies and exports by erosion and suspension. The renewal rate of water in the wetland will determine the speed with which water, nutrients and suspended particles are flushed through the system. Seasonal changes in the morphology of wetland plants will alter the degree of resistance offered to incoming water. Thus the timing of peak runoff and flood events is important.

The flooded condition of wetland soils results in an anoxic environment. Many micronutrients are more soluble in anaerobic sediments making them mobile for export and plant uptake. The latter can be limited by the lack of oxygen in the soil so many plants facilitate uptake by releasing oxygen out into the rhizosphere (Richardson, et al., 1978; Klopatek, 1978). Anaerobic conditions often encourage the production of natural toxins such as hydrogen sulfide which is thought to inhibit denitrification in some systems (Sorensen et al., 1980; Tam and Knowles, 1979).

To manipulate the hydrology of a wetland to increase nutrient removal one must be prepared to understand a very complex system. Steward and Ornes (1975) stress that any addition of wastewater must not increase nutrient loadings, nutrient dynamics and physical hydraulics to an extent which overloads the natural capacity for nutrient removal. The suspended solids concentration of the wastewater should be low to avoid clogging sediment infiltration capacity (Bouwer and Chaney, 1974). If infiltration capacity is lowered spatial and temporal contact of the water with natural nutrient removal and retention mechanisms is reduced.

Thus the advantage of dissipating the wastewater point source over a large absorptive area is lost (Tilton and Kadlec, 1979).

Draining of wetlands for short periods can increase nutrient removal by increasing mineralization of organic matter and thus promoting greater microbial activity on the return of anaerobic conditions. Long term drainage of wetland soils can be detrimental as the activity of aerobic decomposers leads to the release of large quantities of soluble organic and inorganic nutrients into the water. Richardson et al. (1978) recorded elevated concentrations of ammonium ion and nitrate in the outflow of Houghton Lake Fen due to mineralization of organic matter and reduced denitrification following drainage.

Bott (1976) and Krone (1982) recommend that wetland hydrology should be manipulated to reduce the amount of standing water. The water in stagnant channels and pools is usually high in nutrients and encourages the growth of nitrogen-fixing bacteria such as Anabena sp. These species increase the nitrate and nitrite concentrations of the water. In contrast to this Magette et al. (1982) suggests that water residence time in the root zone should be maximised to allow greater nutrient assimilation. It is therefore necessary to control the wetland hydrology very carefully.

The hydrology of a wetland system is extremely complex and it is often difficult to quantify volumes and nutrient loadings especially if groundwater and evaporation are significant. A complete hydrologic model of a wetland would prove costly and therefore exact mass-balance calculations of the chemicals transported have not been made. As a result any manipulation of wetland hydraulics must be done with care to avoid disturbing the natural system while still exploiting to the full those conditions which aid wastewater treatment.

2.7 THE NEED TO EXAMINE PROCESS

This chapter has outlined the complexity of the wetland system and has detailed the potential of the wetland environment as a site for wastewater treatment by nutrient removal. It is clear that the combination of features found within the wetland (plant communities, hydrologic regimes etc.), and particularly those within the sediment - microbial compartment, enhance the removal of a wide variety of pollutants.

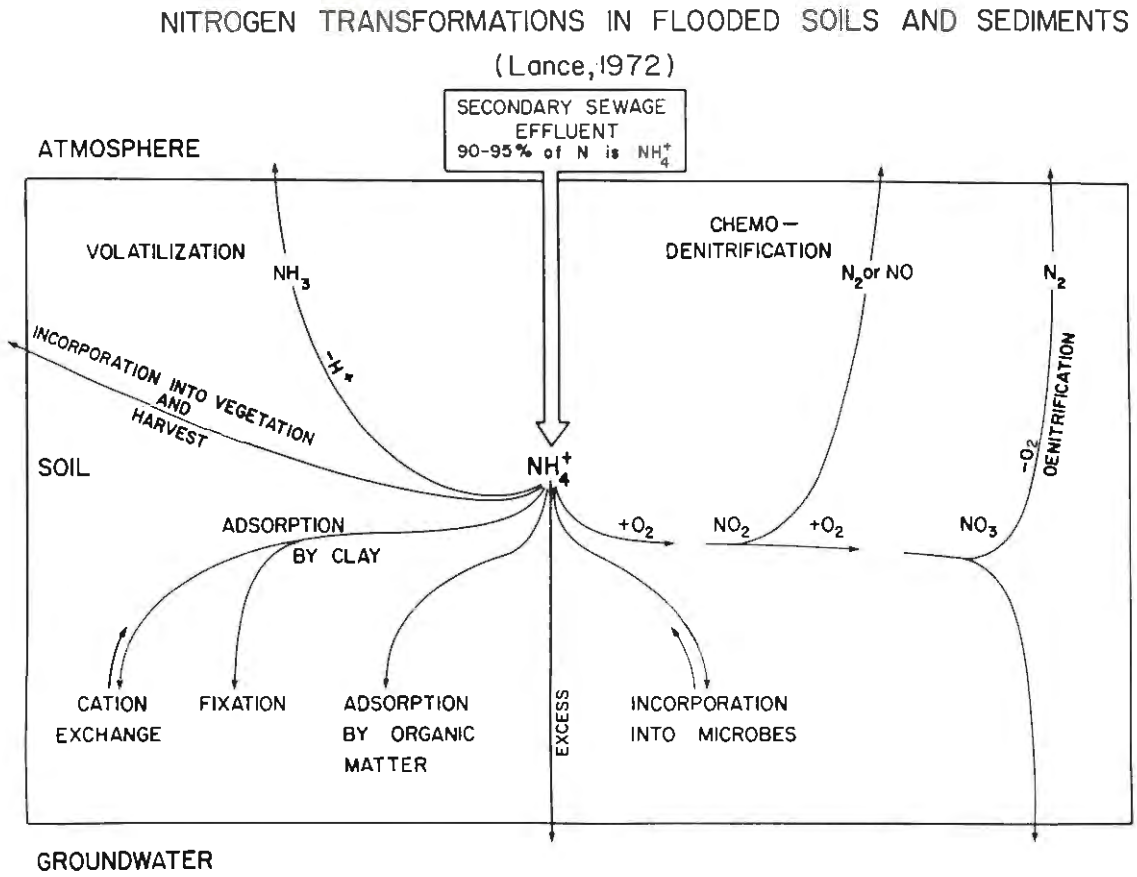
If management of wetlands for nutrient removal is to be successfully undertaken it is important that the capacity of the wetland to accept change to its various compartments is known. In addition the interrelationships that are developed to increase nutrient removal, such as turbulence of the sediment surface aiding diffusion, must be exploited without reducing efficiency in other areas. To manage wetlands for nutrient removal detail about process rates and influencing factors must be known. This study hopes to contribute to the understanding of denitrification in wetland soils so that they can be more effectively managed to remove nitrogen from wastewater.

CHAPTER 3 THE NITROGEN CYCLE IN SEDIMENTS

Having looked at the components of wetland systems that are relevant to wastewater treatment in Chapter 2 this chapter will focus on the role of sediment, and the biological and chemical mechanisms that are involved in the cycling of nitrogen therein. It is by exploiting the nitrogen cycle in sediments that nitrogen removal from wastewater can be achieved.

Biogeochemical cycles can be used to explain the sources, sinks, and transfers between compartments of nutrients. Figure 3.1 depicts the biological and chemical processes involved in that part of the global nitrogen cycle which occurs in flooded sediment systems. Available nitrogen occurs in sediments in four major pools: organic nitrogen, exchangeable ammonium ion, dissolved nitrate, and nitrogen gas (Blackburn and Henriksen, 1983).

FIGURE 3.1



In terms of wastewater treatment the most desirable nitrogen removal mechanisms are those by which it is removed from rapid cycling between water and the sediment into longer-term storage. This immobilization and/or removal of nitrogen can be achieved by physico-chemical adsorption within the sediment, biological uptake or dentrification (loss of gaseous nitrogen to the atmosphere.

3.1 ASSIMILATION AND MINERALIZATION

Assimilation involves the incorporation of molecular nitrogen, ammonium ions, nitrite and nitrate ions or organic nitrogen into organisms (plants, animals and micro-organisms). The assimilated nitrogen is used to form cell constituents such as amino acids and thus the majority of organisms selectively assimilate ammonium ions and ammonia for this purpose. When nitrate is taken up it is reduced to ammonia and the oxygen released then becomes available for oxidative purposes (Painter, 1970). Lance (1972) estimates that 5-10% of the nitrogen in secondary effluent could be immobilized in microbial tissues following assimilation. Utimately this nitrogen is released by decomposition of dead tissue but remains in a stable form that is readily assimilated by other micro-organisms.

Mineralization is the release of nitrogen from tissues on decomposition. It occurs simultaneously with assimilation in the sediment but at a faster rate (Painter, 1970).

In addition to the inorganic nitrogen species discussed organic nitrogen is also involved in these two processes. The sediment organic nitrogen content is influenced by the rate of addition of erodable materials, decomposition of plant and animal tissues, and sedimentation. The organic material in the sediments is less prone to decomposition than that in living organisms as reactions with organic compounds occur within the sediment forming complex hetero-cyclic materials resistant to decomposition (Keeney, 1973).

Wetland environments contain a large amount of organic material which is a potential source of nitrogen via decomposition. However the release

of nitrogen from organic material is slowed by the lack of oxygen in sediments and thus the effects of seasonal patterns of growth and decay on nutrient releases is reduced.

3.2 NITRIFICATION

Nitrification can be defined as the biological conversion of nitrogen in organic and inorganic compounds from a reduced to a more oxidised form. It has been determined (Tate, 1980) that heterotrophic bacteria contribute a negligible proportion of the oxidised nitrogen, and therefore most investigations of the process consider autotrophic bacteria alone (Painter, 1970; Keeney, 1973).

The net reaction during autotrophic nitrification is:

$$\underset{\text{Ammonium ion}}{NH_4^+} + \underset{\text{Oxygen}}{2O_2} \longrightarrow \underset{\text{Nitrate}}{NO_3^-} + \underset{\text{Water}}{H_2O} + \underset{\text{Hydrogen ion}}{2H^+}$$

Nitrification is a key process in the nitrogen budget of the sediment system releasing nitrate from ammonium ion. The nitrate released becomes available to organisms such as denitifiers. Conversely nitrifiers can be in direct competition with organisms which preferentially assimilate ammonium ion.

The reaction is an exothermic one with energy being generated by the two major genera of micro-organisms involved, Nitrosomonas and Nitrobacter. The nitrification reaction has been shown to be a Q_{10} reaction, its rate doubling with every $10^{o}C$ rise in temperature (Keeney, 1973). Its optimum temperature is around $30^{o}C$ and it has an optimum pH of 7. Under field conditions rates of 1.2 - 1.5 gN m^{-2} yr^{-1} have been recorded (Haines et al., 1977).

Adequate oxygen must be present although nitrification will occur down to 0.3 mg/l dissolved O_2 (Reddy and Patrick, 1984). The availability of oxygen is therefore a major limiting factor in the predominantly anaerobic sediment environment. Significant rates of nitrification have only been recorded in the upper 0-6 cm of submerged sediments (Preul and

Schroepfer, 1968; Kemp and Mudrochova, 1972; Starr et al., 1974; Henriksen, 1980). It is thought that aerobic microsites harbouring active nitrifier populations occur within the sediment in association with plant roots (Haines et al., 1977; Reddy and Patrick, 1984) and faunal burrows (Henriksen, 1980; Blackburn and Henriksen, 1983). However significant populations of nitrifying organisms have been found deep in the sediment and were immediately activated on exposure to oxygen (Henriksen, 1980).

As anaerobic conditions dominate the sediment environment nitrification is restricted to the sediment-water interface where an oxidised micro-layer exists, and to aerobic microsites within the sediment. The result is a negligible release of nitrate to the overlying water because the nitrate formed rapidly diffuses down concentration gradients to sites of assimilation and denitrification.

3.3 DENITRIFICATION

Denitrification can be defined as the biochemical reduction of nitrate or nitrite to gaseous nitrogen. In the majority of systems studied the availability of inorganic nitrogen is not the limiting factor and instead denitrification is controlled by a wide range of factors including oxygen supply and the availability of organic carbon.

Within the denitrification reaction nitrate serves as the terminal exogenous hydrogen ion acceptor for the oxidation of an organic substrate. The bacteria involved are facultative anaerobes, for example the genera Pseudomonas, Achromobacter, Bacillus and Micrococcus. Heterotrophic denitrification predominates over autotrophic in submerged soil systems (Painter, 1970), the net reaction being:

$$\underset{\text{Nitrate}}{NO_3^-} \text{ ------> } \underset{\text{Nitrite}}{NO_2^-} \text{ ------> } \underset{\text{Nitrous Oxide}}{N_2O} \text{ ------> } \underset{\text{Nitrogen Gas}}{N_2^{\uparrow}}$$

Denitrification will occur in any environment which is essentially anaerobic. It is therefore widespread in sediments although it often occurs at microsites (Knowles, 1982).

Carbon supply has been found to be a major limiting factor in denitrifying systems that are being exploited for wastewater treatment (Gersberg et al., 1983). The organic matter performs a dual function: it provides a substrate for the energetic reactions and subsequent growth of micro-organisms which consume oxygen, and acts as the hydrogen donor for the electron transport system (Keeney, 1973).

The denitrification reaction has been shown to be a first order reaction (Terry and Tate, 1980). The rate of the reaction declines as the nitrate substrate is depleted. However most field investigations show that the nitrate supply is rarely limiting in sediment systems especially where secondary treated effluent is applied.

In general measured rates of denitrification within the sediment from 0-60 cm depth are high ,an Australian example being 670 mg N m^{-2} d^{-1} recorded by Bavor (1981) in McLeod Morass.

3.4 NITROGEN FIXATION

Nitrogen fixation involves the synthesis of cellular nitrogen from elemental nitrogen by aerobic and anaerobic phototrophic and heterotrophic bacteria. It has been postulated that a considerable proportion of the nitrogen gas released by denitrification in the sediments would be immediately fixed by bacteria thus preventing its loss from the system. As this would be undesirable in terms of wastewater treatment the process has been studied in detail.

Painter (1970) states that the rate of fixation was reduced as oxygen concentration declined in a sediment system. His conclusion was that nitrogen fixation would be insignificant except in a well-aerated (stirred) sediment. Reddy and Patrick (1979) demonstrated that carbon addition and the increased moisture content of a completely flooded as opposed to moist soil both increased nitrogen fixation but their general conclusion was that rates of fixation remained so low as to be inadequate to supply the growth requirements of aquatic macrophytes. In systems enriched with ammonium the activity of nitrogen fixers was halted (Valiela et al., 1977).

The conclusion is that nitrogen fixation is of limited importance in systems with adequate supplies of organic and inorganic nitrogen, such as those receiving secondary treated effluent.

3.5 THE ROLE OF PLANTS

Nitrogen can be removed from the sediment into storage within plant tissues during the growing season. The length of time that nitrogen is stored is a function of the growth and decay pattern of the plant species concerned. As discussed in Section 3.1 the rate at which nitrogen is released from decaying material is a function of its resistance to decomposition. This resistance is often dependent on species, for example large plants such as Phragmites sp. require extensive supporting tissue and therefore have tough cellulolytic cell wall material surrounding the protoplasmic constituents (amino groups). Following death and the onset of decay the nitrogen is protected from rapid release by complexing with the structural materials (Boyd, 1978).

Many of the nitrogenous compounds are translocated to storage organs or perennial tissue prior to senescence. This maintains the valuable proteins within the plant creating a longer term storage.

Klopatek (1978) described rooted aquatic macrophytes as nutrient pumps which rapidly remove nutrients from the sediment during the growing season and return them slowly to the soil and water following senescence and decay. The plants are also important in creating an environment suitable for a wide variety of aerobic and anaerobic microbial reactions within the rhizosphere (see Section 2.4).

3.6 PHYSICO-CHEMICAL PROCESSES

Physico-chemical processes involve the interaction of inorganic and organic forms of nitrogen with other parts of the sediment compartment. Each sediment varies in its physico-chemical characteristics the environment within the sediment being very heterogeneous too. As a result the degree to which each of the mechanisms to be discussed occurs can vary both within and between areas of wetland sediment.

The ammonium ion in wastewater can be adsorbed by negatively charged clays and organic colloids of the soil. The cation exchange capacity (C.E.C.) of different soils is very variable and depends on the amount of organic matter, and the amount and type of clay minerals in the soil (Lance, 1972). Other ions in the water, particularly the divalents calcium and magnesium, compete for these sites. The ammonium ions adsorbed are not necessarily stable as they can be oxidised biologically to nitrate when oxygen is available. Only the ammonium ions adsorbed in a zone that remains anaerobic are stable.

Physical adsorption is important for ammonium ions but relatively unimportant for nitrate which is readily leached out of the soil by percolating water (Preul and Schroepfer, 1968).

Kemp and Mudrochova (1972) working with sediments from Lake Ontario found that fixed ammonium ion was the dominant form of inorganic nitrogen, its concentration increasing with depth. The ammonium ion is fixed in the soil by entrapment in the intermicellar layers of clay minerals such as montmorillonite and vermiculite. Nitrogen immobilised in this way is quite stable because it is resistant to nitrification and removal by plants.

Both ammonium ion and nitrate can be immobilised by the organic material in soils forming complexes resistant to leaching and decomposition (Lance, 1972). Immobilisation of ammonium ions by this mechanism increases rapidly as the pH is raised. Burge and Broadbent (1961) demonstrated that the immobilisation of ammonium ions was correlated with the carbon content of soils and would therefore be an important long-term storage process in the organic rich sediment of wetlands. Organic immobilisation could account for the removal of a considerable amount of nitrogen from wastewater applied to land because the pH of secondary effluent is usually around 7 and most of the nitrogen exists as ammonium ion.

Other less important mechanisms (in terms of total nitrogen losses) are ammonia volatilization (Lance, 1972); chemical denitrification (Lance, 1972).

CHAPTER 4 FACTORS INFLUENCING DENITRIFICATION

Having considered the nitrogen cycle in flooded soils and sediments in Chapter 3, this chapter reviews the factors influencing denitrification, a component of the nitrogen cycle that is directly involved in nitrogen removal.

4.1 SOIL MOISTURE AND OXYGEN STATUS

Bremner and Shaw (1958) demonstrated that the rate of denitrification in soil was affected by water content when other conditions such as pH, temperature and organic carbon concentration were favourable. The soil moisture content interacts with soil structure to control the supply of oxygen diffusing into and through the soil therefore influencing the redox potential of the soil , and thus denitrification. Pilot and Patrick (1972) demonstrated that each soil has a critical moisture content at which the environment changes from a reducing to an oxidizing one. The effect of decreasing soil moisture content in their laboratory experiments was to increase the volume of airfilled pores , and thus to increase aeration. The finer the soil texture the higher the number of airfilled pores required to prevent denitrification as anaerobic microsites remained within the soil matrix because oxygen diffusion was limited.

Anaerobic zones within which denitrification can occur are therefore determined by the rates of diffusion of oxygen through water films and unfilled pores. The rate of denitrification in these zones appears to be controlled by the diffusion of nitrate (Knowles, 1982). It is thought that water saturated aggregates with diameters > 3 mm may have anaerobic centres and thus act as microsites for denitrification (Craswell and Martin, 1974). The relationship between soil moisture and oxygen status is determined by the relative amounts of moisture and the degree of aeration, plus the extant structural conditions of the soil or sediment. Therefore the influence of moisture content on

denitrification is largely in its effect on aeration, although it is also important in terms of providing readily available soluble fractions of compounds such as carbon and nitrogen (Stanford et al., 1975), and through removing the products of decomposition by leaching (Bremner and Shaw, 1958).

Terry and Tate (1980) investigated the effect of flooding on an organic soil. The denitrification rate increased immediately when the soil was flooded, with a maximum rate of 18 ug cm^{-3} d^{-1}. The microbial denitrifier population increased in activity causing an 80% reduction in soil nitrate concentration within the first few days of flooding. Investigations like these have demonstrated the importance of wetting and drying periods on denitrification rates. This can be used as a way to control the aeration status of the soil (Lance et al., 1976).

The greatest nitrogen losses in a study by Reddy and Patrick (1975) resulted from alternate aerobic - anaerobic incubation. They determined that alternating aerobic and anaerobic conditions affected a number of important soil processes. First the rate and end products of organic matter decomposition were different. In general decomposition was faster under continuous aerobic conditions and stimulated in soils by alternate wetting and drying. The predominance of nitrification versus denitrification also changed between aerobic and anaerobic periods. Patten et al. (1980) also identified the importance of aeration status and soil moisture in controlling the quantity of organic matter available to micro-organisms. They found that air drying resulted in a 45-197% increase in denitrifying capacity and even partial drying of moist soils led to a significant increase in denitrification under anaerobic conditions. Therefore although denitrification predominantly occurs in waterlogged soils (Stanford et al., 1975) it may also be significant in drier soils in the range from field capacity to saturation.

There has been a long standing controversy over the role of oxygen in denitrification stemming from conflicting results where denitrification rates are measureable or insignificant in soils of low water content.

Expete and Cornfield (1954) published the results of denitrification assays in soils ranging from 20-133% soil moisture content. They found that denitrification continued at moisture levels near wilting point, and in coarse-textured soils with low moisture contents.

Terry and Nelson (1975) compared denitrification rates between helium purged soils (anaerobic) and non-purged soils (aerobic). No statistically significant differences were observed between denitrification rates of aerobic and anaerobic incubations, in fact some non-purged samples had more active denitrifier populations than purged samples. These experiments were conducted in waterlogged sediments. As the samples were not shaken the upper 1 mm of sediment in the non-purged incubations became yellow during incubation (oxidized), whilst the underlying sediment retained a dark, reduced colouration. This indicates that dissolved oxygen in the water (3-5 mg/l non-purged) was rapidly consumed as it diffused into the sediment. Large amounts of organic matter in sediments lead to a high oxygen consumption and a subsequent oxygen demand (Reddy and Patrick, 1984). The combination of a diffusion gradient into the sediment and rapid use of oxygen by micro-organisms leads to the development of a thin oxidized microlayer at the sediment surface. Terry and Nelson (1975) therefore concluded that denitrification proceeds under aerated conditions as long as the micro-organisms consume oxygen at a faster rate than it is being added. This was supported by Oren and Blackburn (1979) who observed the highest rates of denitrification in the upper 1 cm of sediment from Kysing Fjord, Denmark, where concurrent nitrification - denitrification reactions were occurring.

Other workers claim that denitrification cannot be observed at all in aerobic sediments, or only occuring at negligible rates. Wijler and Delwiche (1954) demonstrated that denitrification was very dependent on moisture content. Moisture content controlled the rate of oxygen diffusion into the soil. In soils which were anaerobic, decreasing the moisture content did not affect denitrification. To demonstrate the importance of diffusion rates of oxygen into the soil in controlling denitrification, Bremner and Shaw (1958) compared shaken with non-shaken soils. Denitrification was depressed in flasks containing oxygen. Unshaken waterlogged soils with oxygen amended atmospheres had the same

nitrogen loss as those incubated under anaerobic conditions. It appears that the diffusion of atmospheric oxygen into the waterlogged soil was so slow that it had no significant effect on denitrification if other conditions (pH, temperature, etc.) were favourable.

In a further experiment Bremner and Shaw (1958) passed air throughout the soil at a rate of 40 ml/min. for seven days at 20°C. No loss of nitrogen could be detected at a wide range of moisture contents. This also supports the theory that it is the diffusion rate of oxygen to the sites of denitrification that is important. Pilot and Patrick (1972) found that only a very small amount of oxygen completely penetrating a soil core sample was sufficient to prevent denitrification, but the lowering of soil moisture content increased nitrate reduction.

It is thought that the affect of oxygen on denitrification results from a number of mechanisms including competition as an electron acceptor, preferential inhibition of nitrous oxide reductase enzyme, and the overall slowing of the denitrification reaction allowing the nitrous oxide time to diffuse away from active sites thus preventing its reduction to molecular nitrogen (Firestone et al., 1979).

Craswell and Martin (1974) have noted that many of the experiments recording denitrification in well-drained or aerobic soils have used carbon amendments. This could have increased oxygen uptake by aerobic microbes and depleted the soil solution of oxygen at lower moisture contents than in unamended soils. It is also thought that nitrification and denitrification can occur concurrently in aerobic soils due to anaerobic microsites (Patrick and Reddy, 1976). Jones (1979) recorded denitrification in the oxidized surface layer and identified anaerobic microsites associated with sediment particles.

Soil moisture and subsequent oxygen status of the sediment has been shown to influence denitrification. The soil moisture status of any soil will be a function of its own structure both interaggregate (particle size, shape, etc.) and intraaggregate (chemical composition). In fine textured organic soils like those at Thredbo wetland, anaerobic conditions are often more persistant than they are in coarser textured soils because a higher air filled porosity is required to raise the

oxygen status of the soil to a point where denitrification is limited (Pilot and Patrick, 1972). In addition to structure the oxygen status will be controlled by the amount of drying that occurs on a temporal scale, and the amount of oxygen contained in the overlying or infiltrating water. The interaction of these to influence denitrification is still not fully understood but it is thought that alternating wet and dry periods could be a useful tool in management to increase nitrogen removal by denitrification from effluent amended sediments (Reddy and Patrick, 1975; Lance et al., 1976).

4.2 pH

Similarly to the dispute over the influence of oxygen on the denitrification process the role of pH is also important. Bremner and Shaw (1958) observed that denitrification was slow in acid soils (pH 3.6 to 4.8) and rapid in soils of high pH (pH 8.0 to 8.6). They concluded that significant losses of nitrogen by denitrification would not occur if the pH was < 5. Expete and Cornfield (1965) published work which disagreed with these results. They found that high losses of nitrogen occured even at pH 4.7 and concluded that denitrification involving significant nitrogen loss could occur over a wide range of pH.

Stanford, Vander Pol and Dzienia (1975) incubated several sub-samples of a soil each at a different pH ranging from pH 4-8.5. During the course of a 72 hour incubation soils initially low in pH tended to become less acidic whereas those initially alkaline (pH >7) tended to decline in pH. Before the incubations the pH range was 5.3 to 8.1 and afterwards it was 5.7 to 7.5 demonstrating a narrowing of pH range during incubations. This led to the conclusion that the effect of pH over the 72 hour period was negligible and probably a result of most soils being in the range pH 6 to 7.5 which would maintain conditions suitable for denitrification. Initially 60% of soils were in this range and afterwards 96% were in this range where pH exerts only a moderate affect on denitrification.

More recent work (Müller et al., 1980) has supported the findings of Bremner and Shaw. A series of spodosolized soils of different pH were analyzed for denitrification rates. There was a strong correlation

between denitrification rate and pH. It appears that denitrifying bacteria were sensitive to pH and that their ability to denitrify was depressed by low pH. In soils of pH < 4.5 only 3-10% of added nitrate was reduced during incubations. Denitrification rates in the spodosolized soils were higher at depth than at the soil surface. This reflected the higher pH values of the deeper soil.

The influence of pH on denitrification is not clear but it is generally agreed that optimum pH for denitrification is pH 7 to 8 (Brezonik, 1977; Knowles, 1982), and that the denitrification rate is slowed below pH 5. Many wetland soils lie in the range pH 6 to 7.5 where pH is thought to exert only a moderate effect on denitrification and is generally not influential if other factors such as the supply of organic carbon are limiting (Stanford, Vander Pol and Dzienia, 1975).

4.3 TEMPERATURE

As denitrification is a biological reaction the affect of changes in temperature can be assessed by monitoring the Q_{10} (reaction rate response to consecutive 10°C rises in temperature) of the denitrifier population in a soil or sediment sample. Stanford, Dzienia and Vander Pol (1975) monitored the biological reaction rates within a limited temperature range (5-35°C) and found that rates increased by a factor of two or three for each 10 degree rise in temperature. Between 15-35°C the Q_{10} was 2 and from 10°C down to 5°C, the rate declined abruptly. Denitrification was barely detectable at 0°C but was measurable in the range 2-5°C after long incubation.

Reddy, Sacco and Graetz (1980) observed maximum reaction rates at 28°C and minimum rates at 8°C in flooded soils. In the range 8-18°C the Q_{10} was 2.0-2.5 and in the range 18-28°C the Q_{10} was 1.4-2.1 demonstrating increased rates with increased temperature. Between 18 and 28°C nitrate was rapidly removed by anaerobic respirers whereas below 18°C the nitrate remained in the soil longer and diffused to greater depth. Although lower temperatures slowed the reaction rate there was a greater temporal and spatial interaction of nitrate with the soil organisms and micro-organisms. The Q_{10} values were not influenced by the diffusion

rate of nitrate from the overlying water but instead temperature had the dominant control on reaction rate in agreement with previous investigations (Stanford, Dzienia and Vander Pol, 1975; Bailey, 1976).

Work on maximum, minimum and optimum temperature ranges for denitrification has produced a wide variety of results. Bremner and Shaw (1958) observed that denitrification rate increased rapidly with a rise in temperature from 2-25^{o}C but was not significantly effected by rises beyond 25^{o}C until a temperature of 70^{o}C inhibited denitrification. Rates were negligible at 2^{o}C in organic soils and at 10^{o}C in soils of low carbon content. Their results suggested an optimum temperature of 60^{o}C. In contrast Terry and Nelson (1975) working with Indiana lake sediments recorded an optimum temperature of 10-15^{o}C and significant denitrification at temperatures as low as 5^{o}C. These differences are probably a result of different microbial populations adapted to different environments.

The widespread use of soil and sediment systems for nitrogen removal from wastewaters by denitrification requires adequate knowledge of the affect of temperature on the denitrification process. Results which suggest that denitrification is severely inhibited below 5^{o}C (Bremner and Shaw, 1958; Bailey, 1973) point to cessation of denitrification and subsequent nitrogen removal over winter. However significant rates of denitrification have been observed year-round in sediments ranging from 3^{o}C in winter to 18^{o}C in summer (Sorensen et al., 1979) and 2^{o}C to 15^{o}C (Seitzinger et al., 1984). Denitrifiers in a coastal marine sediment showed considerable activity in winter at 2.5^{o}C giving a maximum rate of denitrification of 35nmol N.cm^{3}.d^{-1} (Sorensen, 1978a; Sorensen, 1978b). Bowman and Focht (1974) also suggest that freezing and thawing of soils can stimulate denitrification by increasing the availability of organic matter although denitrification rates may be depressed while the ground is frozen.

Halmø and Eimhjellen (1981) conducted laboratory experiments on waterlogged sediments to examine the influence of low temperature (5^{o}C) and high temperature (20^{o}C) on the activity of different populations of denitrifying bacteria (Psychrophiles and Mesophiles). In the temperature range 0-17^{o}C the denitrification rate in the low temperature

sediments was 1.5 to 4 times that in the high temperature sediments. Temperatures below 8°C appeared to be most favourable. The responses of the sediments to temperature changes were variations in the reaction rate, and changes in the composition of the microbial population (Psycrophiles or Mesophiles predominating). Ninety percent of the microbial population in the low temperature sample were a single species of gram-negative, rod-shaped bacteria growing at 0°C or below with an optimum temperature of 15°C and a maximum of 20-25°C. The high temperature sludge was also dominated by a single species. These mesophilic gram-positive, rod-shaped bacteria had a temperature range of 5-40°C and an optimum of 25°C. Halmø and Eimhjellen (1981) concluded that efficient year-round biological treatment of wastewaters could be possible through the selection of low temperature optimum organisms.

There is still much controversy as to the role of temperature in controlling denitrification but it appears that species of denitrifiers exist which are adapted to a broad range of temperatures. The monitoring of psychrophilic species is important for biological treatment of wastewaters in temperate and sub-alpine areas where temperatures within the sediment may fall below 4-5°C for part of the day during winter.

4.4 ORGANIC CARBON

Denitrification by heterotrophic organisms cannot occur unless a substrate is available which contains an organic compound able to support the growth of the organisms and act as an electron donor (Payne, 1981). Organic carbon is readily metabolised by micro-organisms and its presence can create an oxygen demand within the sediment (Stefanson, 1972; Stanford, Vander Pol and Dzienia, 1975). Therefore in addition to acting as a substrate and electron donor, organic carbon can ameliorate the environment for denitrifiers by causing the onset of reduced (anaerobic) conditions following its use in microbial respiration. It is probable that the denitrification rate in a soil or sediment will depend on the amount and type of organic matter available.

Experiments involving the addition of different quantities and types

(soluble, cellulitic etc.) of organic carbon to sediments have demonstrated its influence on denitrificiation. Bowman and Focht (1974) recorded that high glucose concentrations of 1.8% retarded denitrification because fungal growth was promoted and the pH dropped below the level suitable to maintain the denitrification rate. In studying the capacity of soil cores to improve the quality of infiltrating sewage water, Lance (1977) monitored the influence of organic carbon additions. Nitrogen removal in the soil columns was increased by 90% on addition of 150 mg/l dextrose and dropped to 60% with the use of 80 mg/l. The nitrate loadings in the water were so high (150-200 mg/l) that there was a large carbon demand which tended to occur in peaks. To combat this Lance applied carbon in a pulse but found that this didn't provide a long enough interaction time with the micro-organisms involved. He then added 200 mg/l carbon continuously and discovered that denitrification was maximised as the denitrifier population was constantly maintained at high numbers. In contrast other soils have been shown to have sufficient available carbon to maintain significant rates of denitrification without additions. Terry and Nelson (1975) contrasted carbon amended with non-amended sediments and found little difference in the rates of denitrification in each.

In addition to the quantity of organic matter its availability to micro-organisms is also important. Expete and Cornfield (1965) found that the addition of organic materials to soils increased the extent of nitrogen losses. Greater loss occurred where straw rather than compost was added. They concluded that the higher content of readily decomposable substances in straw supplied a more available energy source to denitrifiers. In a comparison of a variety of organic carbon sources, Bremner and Shaw (1958) found that the more readily decomposable compounds such as glucose and mannitol induced more rapid denitrification compared to lignin and sawdust. As leaching and microbial decomposition are important in acting on resistant materials in soils, their effect on denitrification was analysed by pre-leaching straw and adding it to soil samples. Those samples amended with pre-leached straw had slower rates of denitrification than those with unleached straw. Bremner and Shaw (1958) concluded that it is the water soluble and readily decomposable constituents of organic materials which are most effective in promoting denitrification. The quantity of these

soluble fractions in decaying organic material and their accessibility to micro-organisms will influence denitrification rates in soils and sediments. The results of Burford and Bremner (1975) showed a significant correlation (r=0.77) between denitrification and total organic carbon and a high correlation (r=0.99) of denitrification with water-soluble organic carbon. Other workers agree (Stanford, Vander Pol and Dzienia, 1975) that the amount of water-soluble organic carbon in a soil is a good index of its capacity for denitrification.

In terms of wastewater treatment via denitrification in soils and sediments some studies have demonstrated that organic carbon can be a rate limiting factor (Terry and Tate, 1980a; Van Kessel, 1978). Although organic carbon is present in these soils it may be in a form that is not readily biodegradable. Terry and Tate (1980b) stressed the importance of seasonal input of organic carbon in controlling the quantity of substrate available to denitrifiers. The quantity of this material could be manipulated by the type of plant species grown in soils used for water treatment. The quantity and biodegradability of organic carbon available is therefore an important factor in the management of soil and sediment systems for wastewater treatment due to its influence on denitrification.

4.5 ORGANISMS AND MICRO-ORGANISMS

The efficiency of nitrogen removal from soil and sediment systems is dependent on the activity of two major groups of micro-organisms. Nitrate is removed from the soil system by denitrification whilst nitrification in the oxidized surface microlayer and at aerobic microsites within the profile may provide a large proportion of the nitrate required. Organisms living in the sediment affect these two microbial processes by aiding movement and diffusion of gases and solutes by bioturbation.

Organisms disrupt the physical structure of the sediment and therefore enhance the rate of denitrification by increasing nitrate availability (Kaspar, 1982). Sorensen (1978a) observed that the presence of nitrate was restricted to the upper 5-6 cm in marine sediment except where

migrations of bacteria and bioturbation by burrowing animals transported bacteria and nitrogen oxides down to the deeper, nitrate deficient layers. Nitrification occuring in the aerobic zones can provide nitrate which is transported to anaerobic areas.

The presence of secondary zones of denitrification at depth in coastal sediments was thought to be associated with oxidized patches introduced by the burrowing activity of macrofauna (Sorensen, 1978c; Sorensen et al., 1979). Nitrification in these areas and translocation of nitrogen oxides to them provides nitrate for denitrifier activity. The benthic infauna involved include chironomids, tubificid and polychaete worms, and crustacea. The burrowing and water-pumping activity of such animals increases the rate of exchange of oxygen and nitrate allowing their penetration to greater depth, and stimulating both nitrification and denitrification (Knowles, 1982). In addition, Stefanson (1972) demonstrated that the disturbance of soil samples increased denitrification rates by increasing the accessibility of soil organic carbon and changing the pore size distribution in favour of smaller pores. Smaller pores would lead to the development of anaerobic microsites creating a more heterogeneous sediment environment (Sorensen, 1978b). Within wetland soils a similar function could be performed by worms.

A detailed study on the effects of tubificid worms on denitrification in laboratory sediment columns was conducted by Chatarpaul et al. (1980). The tubificids accelerated the movement of nitrate from an overlying solution into the sediment, and increased denitrification by 80% in comparison to a control. The worms also increased carbon loss from the sediment and their ability to do this was enhanced by the presence of added nitrate. Observing the behaviour of the worms showed that the mechanical transfer of nitrate into the sediment was achieved by the worms drawing currents of nitrate rich water into their network of tunnels. The additional surface area for diffusion provided by the tunnels allowed greater access of nitrate to denitrification sites. Burrowing could also increase the movement of ammonium ion upwards to

aerobic areas near the surface where it could be nitrified. Ammonia excretion by the worms and its subsequent oxidation to nitrate can also contribute to increased concentration of nitrate in sediments containing tubificids.

Further investigation involved the observation of worms in a column of inert glass beads. Chatarpaul et al. (1980) found that denitrification rates in these systems remained high and concluded that denitrification was occurring in or on the worms. They isolated denitrifying bacteria of the genera Pseudomonas and Flavobacterium from the exterior and guts of the worms. It was also found that bacteria of the genera Pseudomonas, Bacillus, Micrococcus and Flavobacterium, which contain denitrifying forms, could survive passage through the tubificids guts, and were grazed on selectively by the worms. It is still not known whether denitrification occurs mostly in the gut or on the outside of the tubificids.

Pasteurization and autoclaving of soil samples and subsequent cessation of denitrification have demonstrated that it is a biological process (Payne, 1981; Kaspar et al., 1981). Many species of denitrifying bacteria have been isolated from a wide variety of environments. Some of these bacteria are very specific to particular environments and/or substrate characteristics. The most commonly isolated genera are the heterotrophs Pseudomonas and Bacillus, and representatives of these are commonly found in soils and aquatic sediments. In freshwater sediments denitrifying bacteria usually occur in numbers ranging from 10^5 to 10^{10} g (dry wt) of sediment^{-1} (Knowles, 1982). Studies in Australia by Bavor et al. (1981) recorded populations of denitrifiers at 10^7 - 10^9 per gram dry wt. of sediment in Westernport Bay sediments. This indicates a considerable potential for denitrification in the environments which harbour these bacteria.

4.6 THE CONCENTRATION AND MOVEMENT OF NITRATE AND AMMONIUM IONS

In many sediment systems the highest rates of denitrification have been associated with that part of the profile which has the highest nitrate concentrations (Sorensen, 1978c; Oren and Blackburn, 1979). It seems that denitrification is directly dependent on nitrate concentration

particularly in soils where organic carbon is not limiting (Reddy et al., 1980).

Bowman and Focht (1974) demonstrated that denitrification rates in soils with nitrate applications wrose rapidly until an application of 1300 ug/ml NO_3^--N above which rates remained constant. Similarly Terry and Nelson (1975) found that lower levels of nitrogen were denitrified at nitrate applications of 1940 ug NO_3^--N/g sediment compared to 970 ug NO_3^--N/g which achieved optimum rates of denitrification. These experimental results show that denitrifiers may be inhibited by extremely high nitrate concentrations. Nitrate can accumulate to high levels in soils. Terry and Tate (1980a) recorded levels of 100-2000 ug NO_3^--N/g in a soil. Nitrate can leave the soil by plant uptake, in drainage waters (leaching) or by conversion to nitrous oxide and nitrogen gas by denitrification. Although a large proportion of the nitrate in the surface layers of the soil is used by plants the majority remains in the soil profile. The Everglades were shown to have an annual nitrate mineralization of 1400 kg NO_3^--N ha^{-1} yr^{-1}, but only 20-40 kg NO_3^-N ha^{-1} yr^{-1} left the soil in drainage waters (Terry and Tate, 1980a). It was concluded that the majority of the mineralized nitrate was lost from the system by denitrification.

The supply of nitrate within the sediment is dependent on the availability of ammonium ion and its subsequent nitrification to provide nitrate (Sorensen et al., 1979). Starr et al. (1974) could find no measurable quantities of ammonium ion below 40 cm in a soil which was well aerated, as nitrification was occurring rapidly and converting ammonium ion to nitrate. The nitrate concentration decreased with depth from 0-60 cm indicating active denitrification in the profile. The effect of ammonium ion concentration on nitrifiers was examined by Jones and Hood (1980). A freshwater bacterium had an optimum rate of nitrification at concentrations of ammonium ion of 0.5 g/litre. Freshwater sediments commonly contain such high levels of ammonium ion explaining why nitrifiers are tolerant to high concentrations. Nitrate concentrations were monitored up to 2.0 g/l NO_3^--N with no inhibition of nitrifier activity recorded.

Other factors influencing nitrifiers and denitrifiers are the rates of

diffusion and infiltration of nitrate and ammonium ions into the soil.

Lance et al. (1976) observed that infiltration rate rather than nitrogen loading rate was the primary factor controlling nitrogen removal during soil filtration treatment of effluent. The infiltration rate was controlled by the physical structure of the soil and the suspended solids content of the fluid. The rate of water movement through the soil profile is also important in terms of influencing the amount of nitrate and ammonium ion which is leached out of the soil system and exported to the local base level (Lance, 1977).

In flooded systems there are diffusion gradients operating across the sediment-water interface. The direction of movement of a solute (in or out of the sediment) will be dependent on its concentration or the rate at which it is being used in biological reactions. In nitrate amended floodwater systems nitrate diffuses into the sediment and is converted to nitrogen gas by denitrifying bacteria (Van Kessel, 1977). In other systems production of nitrate in the soil nitrification may exceed its removal by denitrification and therefore nitrate can be exported from the sediment into the overlying water (Sorensen et al., 1977; Nishio et al., 1982).

In soils of high carbon content, diffusion rates are increased as there is a great demand for electron acceptors by active populations of micro-organisms. As the denitrification reaction is a biological one it is also temperature dependent, therefore nitrate diffusion can be reduced at low temperatures. Reddy and Patrick (1984) observed that nitrate diffused more slowly but also penetrated to greater depth in low temperature sediments ($8^{o}C$) compared to high temperature sediments ($28^{o}C$). Increased concentrations at depth were a result of depressed denitrification rates using less nitrate throughout the profile. Nedwell (1982) compared nitrate amended to non-amended soil cores and found that levels of nitrate were enhanced in the amended core compared to the control for only the top 2 cm. This demonstrates the rapid use of nitrate by denitrifiers in the uppermost layers of the soil when other factors (carbon supply, temperature, etc) are favourable.

Concentrations of nitrate within the soil will also be controlled by

nitrification of ammonium ion. The movement of ammonium ion from the anaerobic layers of the sediment to the surface aerobic layer is necessary to allow large losses of nitrogen in flooded systems. Removal of ammonium ion by nitrification in the aerobic layer creates a concentration gradient causing ammonium ion to diffuse upward (Patrick and Reddy, 1976). The nitrate formed then diffuses down the concentration gradient to sites of active denitrification (Kaspar, 1982).

There are still questions concerning the dependence of denitrification on nitrate concentration. Keeney (1973) and Chan and Knowles (1979) considered the denitrification rate to be independent of nitrate concentration, while other investigators have reported that denitrification follows first order kinetics being limited by nitrate concentration (Bowman and Focht, 1974; Reddy et al., 1980; Terry and Tate, 1980a&b). The problem with reaction rate experiments is that they are conducted in flooded sediment systems where diffusion is a limiting factor. Reddy et al. (1978) designed an experiment contrasting reaction rates in sediment systems with and without the influence of diffusion. Both were amended with nitrate and it was found that the samples incubated with no excess floodwater had higher rates of nitrogen removal than those with 3 cm of overlying water. In the soil with excess floodwater two independent processes were controlling the denitrification reaction. Firstly diffusion of nitrate from the floodwater to the soil which was dependent on the relative concentrations of nitrate between sediment and the overlying water. The second process was denitrification increasing the steepnessof the nitrate gradient from the water into the soil.

In conclusion if carbon is non-limiting denitrification appears to be independent of nitrate concentration. However, when the two processes outlined previously (nitrate diffusion gradients and microbial demand for nitrate) are combined the overall denitrification rate in the soil system becomes dependent on nitrate concentration. The discrepencies in the literature can therefore be explained by considering whether the sediments under observation are carbon limiting, nitrate limiting, or affected by both.

4.7 OTHER BIOLOGICAL PROCESSES

The biochemistry of the sediment is complicated by the concurrent activity of numerous micro-organisms with differing trophic demands (requiring different substrates for metabolism). The activity of one trophic group will often influence that of another in terms of the availability of substrates and the types of end products released.

4.7.1 SULFATE REDUCTION

Sorensen (1978c) observed that significant accumulation of the denitrification intermediates nitric oxide and nitrous oxide occurred in the redox transition zone close to the sulfide-rich lower layers of the sediment. He suggested that these accumulations were caused by either low redox potential or the presence of sulfide in the transition zone. To investigate this Sorensen *et al.* (1980) incubated sediment slurries containing the denitrifying bacteria *Pseudomonas fluorescens* and observed the effect of the reducing agents Thallium (III) and Hydrogen sulfide compared to a control with no reductant. In the hydrogen sulfide treatment the production of nitrous oxide following denitrification was much greater than that in the two other treatments. Despite this, the total gas production between treatments was similar indicating that there was no inhibition by hydrogen sulfide at the level of nitrate and nitrite reduction. Gas composition in the presence of Thallium (III) reductant was similar to that in the absence of a reducing agent. This suggests that the inhibitions were not caused by low redox potential but were induced by some specific action of the sulfide compound preventing the further reduction of nitrous oxide to nitrogen gas in denitrification. This inhibition has been shown to be a biological reduction rather than a chemical one by the absence of nitrous oxide production and reduction in autocalve-sterilized soil samples amended with nitrous oxide and sulfide (Tam and Knowles, 1979).

The inhibitory affect of sulfide on the reduction of nitrous oxide was also observed in soil and pure cultures of *Pseudomonas aeruginosa* (Tam and Knowles, 1979), *Alcaligenes faecalis* and *Flavobacterium* (Sorensen *et al.*, 1980). It seems that the effect of sulfate reduction on nitrous

oxide reduction is common to all denitrifiers.

The importance of this inhibition in terms of wastewater treatment has been demonstrated by Kaspar et al. (1981). They incubated sludge from the secondary digestor of a wastewater plant and found that a greater proportion of nitrous oxide was released compared to nitrogen gas following denitrification of nitrate. The sludge contained substantially high concentrations of sulfide which inhibited nitrous oxide reduction. Many of the sediment environments chosen for wastewater treatment could contain high concentrations of sulfide. Denitrification of wastewater nitrate may result in accumulations of nitrous oxide which may be lost into the atmosphere or used in other microbial processes. The latter may prevent loss of nitrogen from the system which occurs when nitrogen gas is the final product of denitrification. Although denitrification and sulfate reduction have been shown to occur concurrently within sediments they had a mutually exclusive pattern in regard to spatial distribution (Sorensen et al., 1979). This may result in a lowered overall denitrification rate for a sediment system if bacterial activity is inhibited in nitrate rich areas by the presence of sulfide.

Further studies are required to determine in which environments the effect of sulfate reduction is significant and to define what concentrations of sulfide will produce elevated concentrations of nitrous oxide.

4.7.2 NITRIFICATION

Nitrification and denitrification occur simultaneously in flooded soils and water bottoms when an aerobic surface layer (up to 1 cm thick) develops over an anaerobic layer. In addition aerobic conditions exist within the deeper anaerobic sediments associated with animal tunnels and the root rhizosphere of wetland plants (Kaspar, 1982). Nitrifier and denitrifier activity is therefore unevenly distributed in the sediment profile.

Ammonium ion is the predominant inorganic form of nitrogen in oxygen-deficient flooded systems. The activity of denityrifying bacteria is therefore dependent on the nitrification of ammonium ion occurring in the aerobic surface microlayer and microsites within the anaerobic sediment below. The nitrate formed in the aerobic layer diffuses down a concentration gradient to the anaerobic layer where it is denitrified. Experiments have shown that denitrifiers utilize the nitrate so quickly that it rarely builds up to high concentrations in areas of active denitrification (Van Kessel, 1977).

Halmø and Eimhjellen (1981) found that nitrification was the critical, rate-limiting step in a three stage (carbon oxidation, nitrification, denitrification) biological process receiving domestic sewage. The effluent contained high concentrations of ammonium ion which had to be nitrified (oxidation) before denitrification could occur and efficiently remove nitrogen from the water. If flooded sediment systems are to be used for the removal of nitrogen from wastewater by denitrification it is necessary to ensure that conditions conducive to nitrification of effluent ammonium ion are maintained.

4.8 PLANTS

As discussed in previous sections the availability of organic matter as a carbon source is necessary for nitrate reduction to occur. Plants act as a major source of organic material in soils and sediments and this has led to investigation of their influence on denitrification.

Woldendorp (1962) compared the percentage of added nitrate and ammonium ion lost from soil samples containing living and dead root systems. Losses were more than doubled in the living root systems compared to the dead, showing that plants have a quantitative influence on nitrogen loss.

Considerable differences in nitrogen removal have been observed between vegetated and unvegetated plots (Bailey, 1976; Lance, 1977; Sherr and Payne, 1978). Sherr and Payne (1978) monitored denitrification in plots with and without Spartina alterniflora. There was a significant reduction in soil denitrification in plots without Spartina over an eighteen month period. The potentials remained similar for the first 5 months demonstrating the important buffering effect of the standing stock of soil organic matter. A difference in potential was also observed between plots of tall and short plants. The growth forms of these plants were thought to control denitrification : tall plants put down a few deep roots whereas the short plants showed a horizontal root and rhizome pattern resulting in a dense mat of material at 10-20 cm depth. The short plants therefore released a seasonal input of organic material into the zone identified as that of peak denitrification activity (decay of roots in 10-20 cm zone).

Large populations of denitrifiers have been recorded in the rhizosphere (Woldendorp, 1962; Sherr and Payne, 1978). Using a split plate technique Smith and Tiedje (1979) were able to separate rhizosphere and non-rhizosphere soil. The soil from the root zone denitrified at more than twice the rate of non-rhizosphere samples (greater than 5 mm from the root). It has also been observed that detached root tips stimulate denitrification (Bailey, 1976). In addition to containing sloughed-off root cap cells (Lance, 1977) the rhizosphere also contained organic substances exuded by the roots, the release of which was aided by decapitation.

Conditions for denitrification in the rhizosphere would be futher improved by the reduction of oxygen concentration in the soil around roots. This would result from active uptake of oxygen by root cells, and oxygen consumption by micro-organisms in the rhizosphere utilizing the abundant organic material during decomposition (Bailey, 1976).

Woldendorp (1962) compared the oxygen consumption of living and dead roots and found that living roots consumed oxygen at a rate twenty times that of decaying roots. In anaerobic, water-saturated soils, roots may stimulate nitrification-denitrification reactions by oxidizing the rhizosphere. Oxygen is transported to the roots down a concentration gradient by a system of lacunae (Knowles, 1982). The nitrate formed following nitrification of ammonium ion can then diffuse to anaerobic areas where it is denitrified to nitrogen gas (Reddy and Patrick, 1984).

In some instances plants may lower the denitrification rate, for example in nitrate limited sediments where they compete with denitrifiers (Chan and Knowles, 1979). Significant uptake of nitrate by plants in such environments could decrease the amount of substrate available for denitrification. Smith and Tiedje (1979) showed that when soil nitrate concentration was high denitrification rates were increased in the rhizosphere. In contrast low nitrate concentrations resulted in a dramatic reduction of rhizosphere denitrification due to plant uptake of nitrate.

In addition to a subsurface influence aquatic macrophytes have been shown to aid denitrification by providing a substrate for the development of periphyton communities. In a lotic environment considerable denitrification occured in association with a periphyton mat of Cladophora sp. (Triska and Oremland, 1981). However activity was limited during daylight hours due to photosynthetic oxygen production.

CHAPTER 5 THE WETLAND AT THREDBO

5.1 LOCATION

Thredbo Village is a subalpine resort located on the Crackenback River in Kosciusko National Park (see Figure 5.1). It is served by a sewage treatment works located about one kilometer downstream from the resort.

FIGURE 5.1

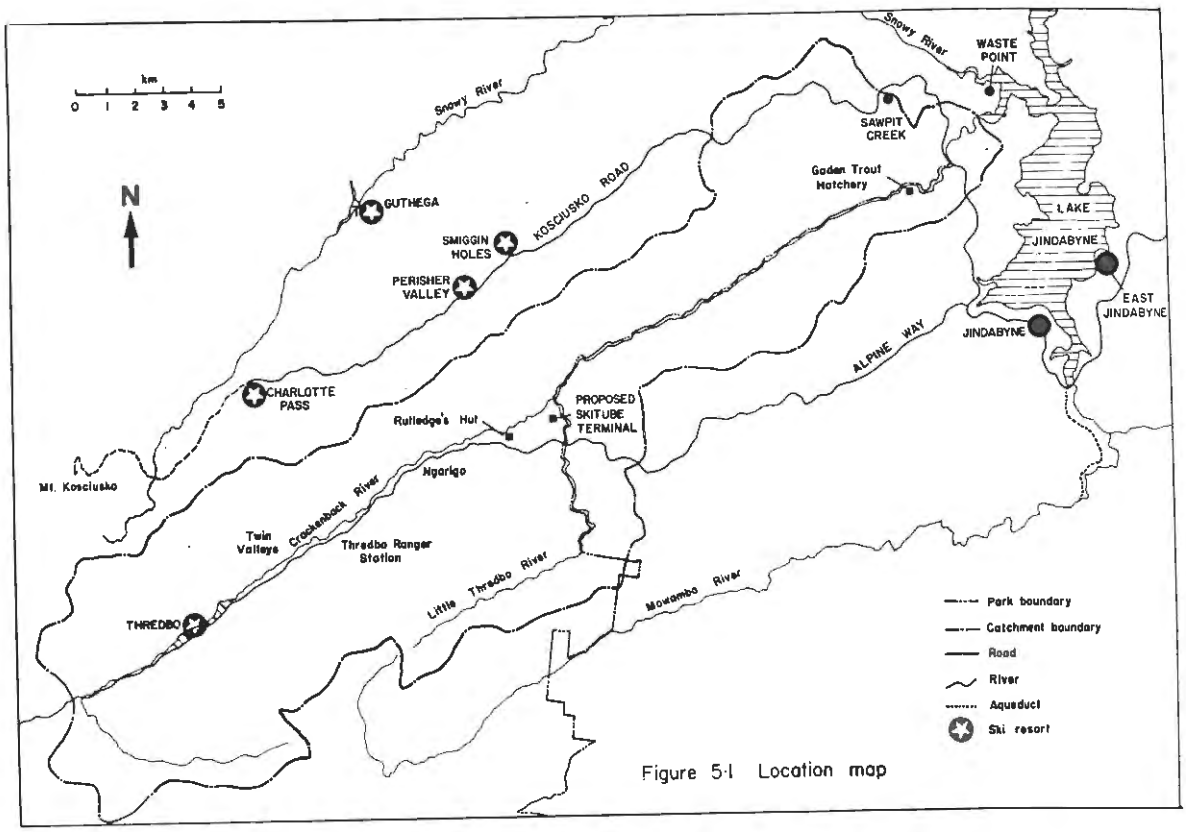


Figure 5.1 Location map

The sewage treatment works is an activated sludge plant with secondary treatment being completed by a stage of effluent maturation in ponds (die-off of enteric micro-organisms and oxidation). The effluent is then discharged into a wetland of approximately 3.3 ha. in area located on a minor tributary of the Crackenback River, and adjacent to the treatment works. The wetland discharges into the river which then flows a further 18 km to enter Lake Jindabyne.

5.2 THE PLACE OF THE THREDBO WETLAND IN THE WATER TREATMENT SYSTEM

Sewage flows by gravity from Thredbo Village to the sewage treatment works. It consists of household sanitary wastes, kitchen and laundry wastes with an input of surface and groundwater during wet periods. The sewage treatment works operates on a principle of aerobic digestion whereby organic material is degraded by the action of micro-organisms naturally occuring in the sewage. The organic material is converted into water, gas and inorganic salts during the process of metabolism. A small amount of indigestable material remains as sludge which must be periodically removed from the plant for separate disposal.

The initial phase of the process includes mechanical raking and sedimentation to remove scum, grit, sand, miscellaneous solids, paper and rags. The total volume of materials removed is small and can be disposed of by burial in the refuse tip adjacent to the treatment works.

Following this the sewage passes into an equalisation tank. Sewage entering during peak flow periods is held here and released when the inflow diminishes. The introduction of this tank in 1977 led to an increase in plant capacity and a more uniform flow through the plant.

The next phase is aerobic digestion. Effluent from the equalisation tank is pumped into an aeration compartment where air is pumped through the liquid. The micro-organisms present in the sewage respire aerobically deriving energy from the organic material and converting it

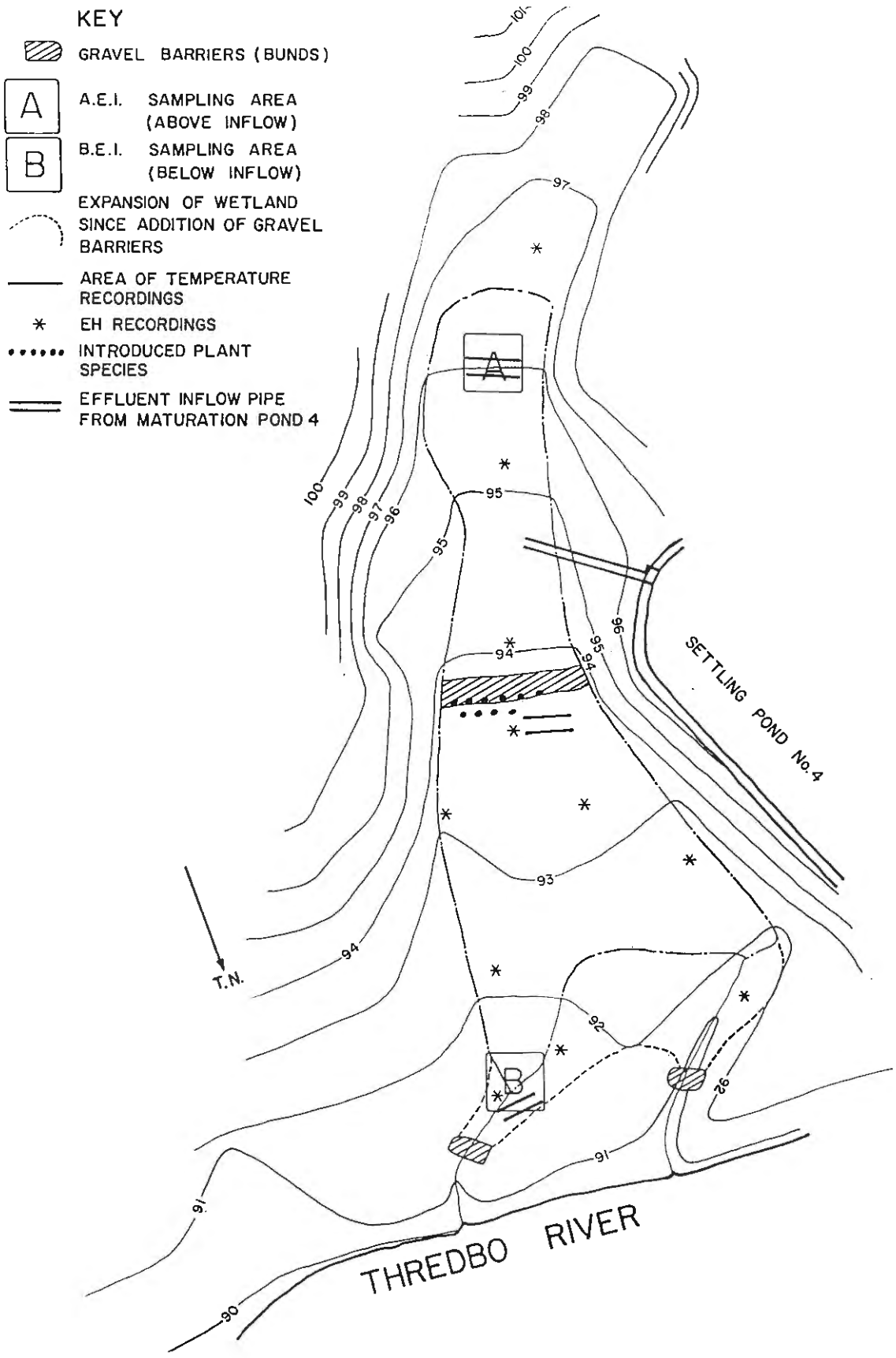
to carbon dioxide and water. The nutient concentration is lowered to some extent following uptake by micro-organisms. The effluent remains in the tank for at least twelve hours during which time some ammonia and ammonium ion may be oxidised to nitrate.

The next stage is clarification which involves the separation of the biomass (living micro-organisms), and remaining solids from the liquid effluent. The scum and settled sludge are recycled continuously to the aeration compartment to maintain the biomass during aerobic digestion. As the biomass increases excess sludge is removed to a separate sludge digestion lagoon.

The clarified effluent is passed into maturation ponds. Here enteric micro-organisms harmful to man are killed by starvation, predation and high levels of ultraviolet radiation. Further precipitation of fine solids occurs. Oxidation of organic matter continues and nutrients are removed by algal uptake. Ideally the four maturation ponds should achieve a total residence time of at least ten days. Flow data show that theoretical residence time of the ponds is about 9.5 days during winter and 20 days during off-peak periods. However, effluent may short circuit during high flows and therefore an air-baffle was installed in the final maturation pond to reduce channelized flow within the water body.

The water from the final maturation pond flows continuously by subsurface pipe into the wetland. Since 1980 input-output measurements of nitrogen species have been taken at the final maturation pond outflow and at the discharge point of the wetland to the river (see Figure 5.2). Cullen (1983) reports that the wetland removed 30% of the overall nitrogen load in the summer months and 8% in the winter months from January 1982 to September 1983.

FIGURE 5.2


KEY
GRAVEL BARRIERS (BUNDS)
A
A.E.I. SAMPLING AREA (ABOVE INFLOW)
B
B.E.I. SAMPLING AREA (BELOW INFLOW)
EXPANSION OF WETLAND SINCE ADDITION OF GRAVEL BARRIERS
AREA OF TEMPERATURE RECORDINGS
EH RECORDINGS
INTRODUCED PLANT SPECIES
EFFLUENT INFLOW PIPE FROM MATURATION POND 4
101
100
99
98
97
96
95
94
93
92
91
90
SETTLING POND No.4
T.N.
THREDBO RIVER

To increase the residence time of the water in the wetland, bunds (gravel barriers) were installed in 1983 in order to retard flow, facilitate greater uptake of nutrients by plants, and promote movement of organic and inorganic material into the soil.

The wetland therefore makes a considerable contribution to the treatment process in the form of tertiary treatment removing nitrogen and phosphorus from the water (Cullen 1983). However seasonal conditions of temperature and rainfall may influence its nutrient removal capabilities. Low temperatures retard plant and microbial uptake whilst high rainfall may stimulate leaching following infiltration. Nutrient releases are often combated by dilution from natural waters of the creek which enter the wetland from the south. The contribution of groundwater to the hydrology of the wetland is not known.

As a cheap and effective form of tertiary treatment the wetland is an important component in the structure, operation and water quality control of the sewage treatment plant at Thredbo.

5.3 CLIMATE

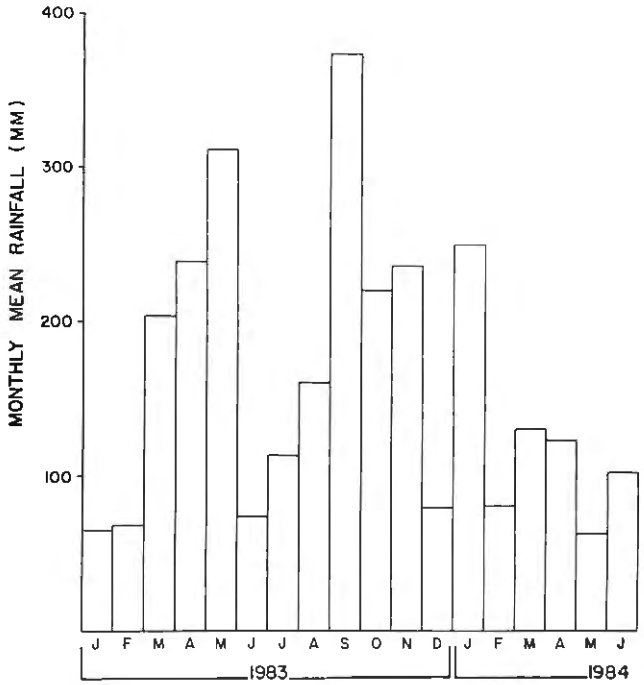
The climate at Thredbo is characterised by low winter temperatures (minimum - 4^{o}C, maximum 12^{o}C) with mild conditions in summer (minimum 5^{o}C, maximum 20^{o}C). Low night time temperatures can occur year round due to local conditions in the valley.

Precipitation is usually high (average annual rainfall 1975 mm) and falls mainly as snow in winter. Peak rainfall occurs in August and October (Hogg 1984). Rainfall and temperature data are depicted in Figure 5.3.

FIGURE 5.3

MONTHLY MEAN RAINFALL (MM) FOR THREDBO VILLAGE JAN. 1983 TO JUNE 1984.

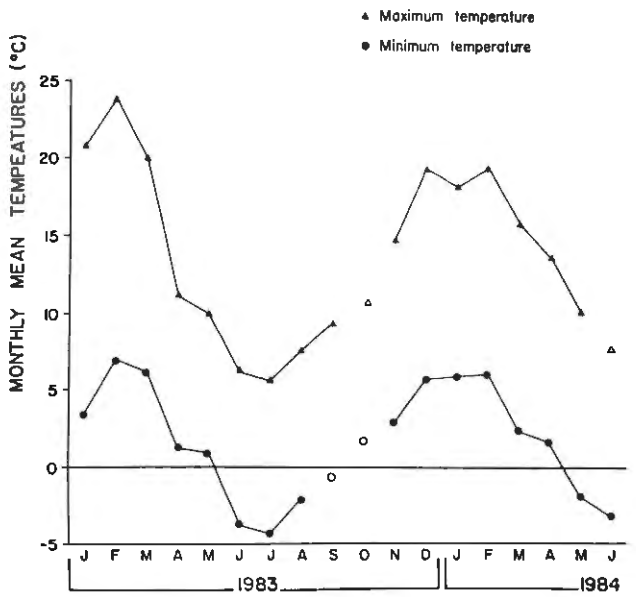
Prepared from data supplied by the Bureau of Meteorology



MONTHLY MEAN MAXIMUM AND MINIMUM TEMPERATURES (°C) FOR THREDBO VILLAGE JAN 1983 TO JUNE 1984.

Prepared from data supplied by the Bureau of Meteorology

Open characters = substitute 1982 data



5.4 VEGETATION

An investigation of vegetation in the wetland at Thredbo was conducted between January 1981 and January 1983 by D.S. Mitchell, C.M. Finlayson and A.J. Chick of the CSIRO Division of Irrigation Research, Griffith. Differences in species dominance, levels of tissue nitrogen and phosphorus, soil seed bank size and % germination were found between areas above and below the point of effluent inflow. Levels of tissue nitrogen and phosphorus, seed bank size and % germination of the dominant species were all higher below the effluent inflow compared to the upstream area.

The dominant plant species in the area above the effluent inflow are Carex gaudichaudiana, Juncus sp. and Baeckea gunniana.

C. gaudichaudiana is a perennial alpine sedge with creeping rhizomes and helps to form compact peats in alpine regions (Costin et al., 1979).

J. sp. is a perennial rush with short rhizomes.

B. gunniana is an aromatic shrub growing at the margins of the wetland where drier conditions prevail.

Below the point of effluent inflow Epilobium sarmentaceum and Rumex crispus dominate. Both of these species are senescent for the majority of the winter.

E. sarmentaceum, known as mountain willow-herb, has a loosely rhizomatous base (Costin et al., 1979).

R. crispus is an introduced species with the common name of curled dock. The plant starts growing in winter and spring and flowers in response to increasing daylength. The top dies off in summer and remains as a large brown inflorescence (Sainty and Jacobs, 1981).

The wetland area below the point of effluent entry is dominanted by opportunist weeds. Very few of the native species appear to have adapted to the changes in nutrient and water levels imposed by the effluent input.

In an attempt to reduce the dominance of opportunist weeds the CSIRO transplanted the native species Phragmites australis and Schoenoplectus validus from areas around Jindabyne. P. australis and S. validus have been used in tertiary treatment systems where they aid microbial populations around their roots and increase nutrient accumulation in their tissues and the substrate (Finlayson and Mitchell, 1983). It was hoped that they would perform the same function in the Thredbo wetland by increasing the below-ground biomass.This was combined with the construction of bunds within the wetland to reduce channelized flow, raise the water level and increase retention time so that hydrologic conditions would favour the transplanted emergents over the opportunist weeds.

Although raising of the water level has been achieved the transplanted species are confined to the area where they were originally introduced. (see Figure 5.2). The growth of individual plants at this point is healthy although only a small percentage of those transplanted remain. The plants have not been able to encroach into the remainder of the wetland where E. sarmentaceum and R. crispus still dominate. The reasons behind this have still to be investigated.

5.5 SOILS

The soil in many areas of the wetland is submerged for most of the year. The character of the soil (structure and texture) in submerged and non-submerged areas does not differ significantly and the following profile description applies to all areas of the wetland sampled.

The soil can be described as a uniform fine organic clay/loam soil (Johnson pers. comm.). The profile consists of a thick (0-50 cm), dark A horizon composed largely of organic matter with a fine to medium structure (peds and aggregates). At the top of the profile is a varying

thickness of partially decomposed organic material. There is no gradation in texture, structure, mineral content or colour with increasing depth. The clay content of the A horizon is in the order of 45-50% which would influence the cation exchange capacity of the soil.

The presence of muscovite (mica) through the entire profile showing no evidence of oxidation demonstrates the strength of the reducing environment. Mica being a relatively unstable mineral under most weathering conditions is normally one of the first minerals to be weathered thus indicating that the reducing environment is preserving both minerals and organic matter with only gradual long term breakdown.

The depth of the soil profile is in the order of 1 metre, below which fragments of weathered granite bedrock are encountered. However, there is no textural change leading up to this intersection of the regolith. At the base of the profile fine quartz is present again indicating a granite bedrock, and associated with a sharp decline in clay content. Much of the mica and high clay content above this point may be a result of depositional processes related to the dissipation of stream waters as they enter the wetland area.

The groundwater table in areas adjacent to flooded sections is intercepted at a depth of approximately 40-50 cm with no obvious lateral flow through the profile. The profile between 0-30 cm even below submerged areas can only be described as moist with very little free pore water observed.

5.6 NITRATE AND AMMONIUM ION LOADINGS TO THE WETLAND FROM THREDBO WATER TREATMENT WORKS

The monthly loadings of (nitrate + nitrite) and ammonium ion entering the wetland in the effluent from the final maturation pond (see Figure 5.2.) are contained in Table 5.1. The data covers the period from January 1983 to March 1985.

The mean effluent volume entering the wetland is 0.008 cumecs, with flow in the winter averaging 0.012 cumecs (Finlayson et al., in prep.). Once the effluent has entered the wetland it is subject to dilution. The degree to which dilution occurs will be dependent on season (snow melt etc.).

Table 5.1 Loadings of (nitrate + nitrite) and ammonium nitrogen (Kg/month) entering the wetland in the effluent (Cullen, pers.comm.)

Date	$(NO_3^- + NO_2^-)$-N Kg/mth	NH_4^+-N Kg/mth
18 Jan 83	50.7	88.74
27 Jan 83	-	0.9
17 Feb 83	-	1.74
9 Mar 83	10.44	2.64
20 Apr 83	155.94	72.72
22 May 83	328.44	72.78
16 Jun 83	286.14	291.12
17 Jul 83	25.26	774.48
25 Aug 83	2.34	1091.76
20 Sep 83	1.38	836.7
2 Dec 83	2.94	105.66
21 Mar 84	642.84	36.48
25 Jun 84	44.76	4.02
25 Jul 84	15.24	1194.36
30 Aug 84	37.32	982.86
18 Dec 84	0.78	0.3
5 Mar 85	1.08	1.26

The results exhibit an increase in loadings in winter coincident with the peak of the ski season. However despite this general trend there is a great variability in the results. In January 1983 two samples were taken on separate days and analysed for ammonium ion. The difference in loading is quite significant and highlights the variablility that may be occuring from day to day depending on how the treatment works is operating. Values for (nitrate + nitrite) also demonstrate large variation with a loading of 642.84 Kg/mth occuring in March 1984 compared to 10.44 and 1.08 Kg/mth in March 1983 and 1985 respectively. This suggests that the calculated monthly loading (single monthly result multiplied by 30) could be incorrect by several orders of magnitude. Despite this the data is of use in highlighting the major seasonal trends in loading and the anomalies that can occur due to changes in the operation of the treatment works.

The level of (nitrate + nitrite) in the effluent is dependent on the length of time that the wastewater has been in the maturation ponds. From March to the beginning of the ski season in June the residence time can be up to twenty days, whilst from June to August it can drop to nine days. This explains the higher loadings of (nitrate + nitrite) in autumn compared to winter where the majority of the nitrogen in the effluent is contained in ammonium ions.

The intermediate levels of (nitrate + nitrite) and ammonium ions in January reflect the secondary tourist season that occurs over Christmas and the New Year.

The continuous flow of secondary treated wastewater into the wetland provides a significant loading of (nitrate + nitrite) and ammonium ions. The character of the effluent in relation to these nitrogen species changes seasonally and, on occasions, daily in response to changes in the operation of the treatment works.

CHAPTER 6 METHODS

6.1 FIELD WORK

The locations of the sampling areas described are detailed in Figure 5.2.

6.1.1 CORE SAMPLING

Cores were taken from areas of the wetland above and below the point of effluent entry. Electrical conduit pipe (external diameter 5 cm) cut to 80 cm length was securely stoppered at one end and lowered through the overlying water to enter the surface soil. The bung was then removed and the core hammered into the soil to obtain a continuous core of about 30 cm. Before extraction the bung was replaced and after a single twist the core was pulled from the soil, immediately stoppered at the bottom end, and returned to the lab on ice.

Prior to analysis complete cores were pushed out from the tube and sectioned according to depth.

6.1.2 PHYSICO-CHEMICAL MEASUREMENTS

Eh, pH and temperature of the soil was measured seasonally at points across the wetland.

The meters used for Eh and pH readings were calibrated in the laboratory just prior to use in the field and the calibration rechecked immediately after use. Eh and pH readings were taken at points across the 3.3 ha of the wetland in association with the existing CSIRO transect posts. Probes were inserted approximately 1 cm and 6 cm into the soil surface, and left to equilibrate for 30 minutes before reading.

Redox potentials (Eh) were taken according to the method described by Willet (1983). Eh was measured as the potential between an inert platinum electrode and a standard reference electrode (calomel). Measurements were taken by pH meter using the millivolt scale. To obtain Eh values the potential of the calomel electrode relative to that

of the standard hydrogen electrode at ambient temperature was added to the millivolt reading. In this case the standard figure for addition was 245 mV.

pH in the top 0-5 cm of the soil was measured directly using a field meter and electrode.

Immersion thermometers were used to take temperature readings in the top 5-10 cm of the soil. Once distributed on a 1 metre grid system above and below the effluent inflow the thermometers were left to equilibrate for 30 minutes before reading.

6.1.3 FIELD SITE OBSERVATIONS

The weather conditions and time of each sampling trip were recorded. In addition the water flow, algal growth and general condition of the wetland vegetation were observed. A visual assessment was made of turbidity in the final maturation pond, and the amount of effluent in the equalisation tank was observed.

6.2 LABORATORY PRACTICE

6.2.1 GENERAL

Laboratory procedures were based on those of Fritz and Schenk (1979).

All glassware was soaked in 2% Decon detergent for at least 24 hours prior to dilute sulfuric acid washing. Glassware for use in the acetylene blockage assay was heat sterilized.

Reagents and stock standards were stored in dark glass bottles at 4^{o}C. Ultra-pure water from a SYBRON/Barnstead NANOpure machine was used for all the reagents and standards.

6.2.2 USE OF THE TECHNICON A.A.II FOR THE ANALYSIS OF EXCHANGEABLE NITRATE PLUS NITRITE, AND AMMONIUM IONS

The Technicon Auto-analyser II was used to measure ammonium ion and (nitrate + nitrite) concentration in standards and one gram soil samples following extraction with 2M KCL.

Ammonium ion concentration was measured by the phenolhypochlorite method (Solorzano, 1969). The ammonium ion manifold was assembled in the laboratory according to the automated phenate method (A.P.H.A. Standard Methods, 1980). Reagents and standards were made up according to this method. It was found necessary to sample at a slower rate of 30 samples/hr 2:1 (two portions of sample to one of washwater) in order to obtain adequate peak separation.

The cadmium column reduction method was used to measure (nitrate + nitrite) (Brewer and Riley, 1965; Armstrong et al., 1967). The manifold was operated according to A.P.H.A. Standard Methods (1980). Again it was found that a slower sampling rate of 20 samples/hr 1:1 was required. Additional information on copper-cadmium column preparation and storage was obtained from Technicon Industrial Systems Data Release (Dec. 1972).

6.2.3 USE OF THE GAS CHROMATOGRAPH AND INTEGRATED RECORDER FOR THE ANALYSIS OF NITROUS OXIDE

The Packard Model 420 Gas Chromatograph was operated under the following conditions:

Injector Temperature	60°C
Detector Temperature	120°C
Oven Temperature	30°C (cooling port open)
Filament Setting	250 mA
Attenuation	1

Although identification of soil denitrification as a nitrous oxide peak has been achieved by Flame Ionisation Detection (Zimmerman and Rasmussen, 1975), and Electron Capture Detection (Hall and Dowdell, 1981), the method used involved a Thermal Conductivity Detector (Van Cleemput, 1969; Bavor, et al., 1981).

A stainless steel column of 2 mm internal diameter and 2 metre length was packed in the laboratory according to Fritz and Schenk (1979). Poropak Q and Poropak R have been identified as a good stationary phases for the separation of gases emanating from saturated soils (Bailey and Beauchamp, 1973). One metre of Poropak R (mesh size 80-100) was followed by one metre of Poropak Q (mesh size 100-120). The column was conditioned at 200^{o}C for five hours prior to use in order to eliminate impurities.

Helium was used as the carrier gas at a flow rate of 30 ml/min. The gas was passed through a drying tube packed with granular magnesium perchlorate (Smith and Dowdell, 1973) prior to passage through the column and reference column (3% QF on Chrom - W(H), mesh 80-100).

The only problems shown to be associated with this method are the proximity of the carbon dioxide and nitrous oxide peaks (Delwiche and Rolston, 1976), and the sensitivity of the thermal conductivity detector filaments to oxygen at higher temperatures (Blackmer and Bremner, 1977). It was found that good separation of nitrous oxide and carbon dioxide was achieved by using Poropak R followed by Poropak Q in the column. Nitrous oxide could be detected down to 0.001 mg/10 g of soil. As the incubations used were anaerobic with only small concentrations of oxygen and low detector temperature there was no sign of interference with the thermal conductivity detector filaments.

Quantitative analysis was achieved by controlling experimental variables such as gas sample size (0.5 ml), sample temperature, flow rate, etc. Each sample received identical treatment. Sampling precision was aided by the use of gas tight syringes (Bavor, et al., 1981).

Peak areas were calculated by a normalisation program using a Shimadzu C-RIB Chromotapac Integrator under the following operating conditions:

Width	5
Slope	Variable
Minimum Area	0
Stop Time	6
Attenuation	3
Speed	8

Nitrous oxide standards were made daily using a calibration gas cannister. The method by which N_2O-N in mg/soil sample was calculated is contained in Appendix I.

6.3 PROFILES OF EXCHANGEABLE NITROGEN SPECIES

6.3.1 SOIL EXTRACTION TECHNIQUE

Exchangeable pools of ammonium ion and (nitrate + nitrite) in the soil are those available to nitrifier and denitrifier micro-organisms which compete with plants for inorganic nitrogen. The importance of these pools to agriculture led to the development of a wide range of techniques for analysing levels of inorganic nitrogen in soil samples.

Bengtsson (1924) reviewed the methods in use at that time and found that potassium chloride (KCl) solution was the most effective of a range of extractants. His findings were supported by Harper (1924) who found that KCl was the most effective extracting agent in soils representing a wide range of pH values. In the sixties the use of KCl for extracting exchangeable nitrogen was again favoured (Bremner, 1965). Contemporary studies (Sahrawat, 1979) have demonstrated that KCl proves a better extractant than a range of other solutions commonly in use.

Following the selection of KCl as the extracting solution a number of variables are involved in its use. These include: molarity of the solution; length of the extraction period; number of extractions and the method of agitation during extraction.

The main molarities used are 0.5M (Sorensen, 1978b); 1M (Blackburn and Henriken, 1983) and 2M (Smith and Patrick, 1983). Solutions stronger than 2M often encounter depositional problems. Sahrawat (1979) concluded that the molarity of the solution was not critical when he observed that different molarities of KCl extracted the same amount of ammonium ion from identical samples.

The majority of workers favour a single extraction period although the length of extraction ranges from 30 minutes (Jenkins and Kemp, 1984) to 24 hours (Klingensmith and Alexander, 1983). The most commonly used periods are of one to two hours duration.

Various soil:solvent ratios have been employed ranging from 1:1 (Henriksen, 1980) to 1:10 (Burford and Bremner, 1975).

A great variety of agitation methods have been used for extraction. Some workers favour an initial period of extraction on a wrist-action shaker followed by centrifugation (Terry and Nelson, 1975; Haines, et al., 1977). Others have found that centrifugation alone for periods of 10-15 minutes at 2000 x g are sufficient to achieve complete extraction (Henriksen, 1980; Sorensen, 1978b). In some cases centrifugation is not used (Keeney and Bremner, 1967) although this has the danger of not including porewater inorganic nitrogen which can be extracted by centrifugation (Reynolds, 1984). In addition the use of the centrifuge creates a clear supernatant which is suitable for analysis by automated methods avoiding lengthy sedimentation or filtration of samples.

The variety of techniques employed throughout the literature undoubtedly represent the differences in amount and availability of exchangeable inorganic nitrogen between soils. In this study the available information was assimilated and used to develop a suitable methodology by experimentation. The developmental procedure examined the variables outlined above and is contained in Section 7.2.

6.3.2 LABORATORY PROCEDURE

Equipment — 50 ml polypropylene centrifuge tubes and lids
Sample cups and lids (A.A. II)
Wrist-action shaker
Sorval centrifuge and SS34 rota
2M KCl

Method — The experimental procedure which led to the development of this technique is outlined in Section 7.2.

(i) Place 1 g of soil from core sample at the bottom of a 50 ml polypropylene centrifuge tube.

(ii) Add 25 ml of 2M KCl and check pH, adjust to pH7.

(iii) Seal tubes with O-rings and caps. Invert three times.

(iv) Place in rack on wrist-action shaker and shake for 2 hours at ambient temperature.

(v) At the end of this period remove tubes in batches of eight and centrifuge for 10 minutes at 15000 x g using a chamber temperature of 4°C.

(vi) Analyse the supernatant for exchangeable (nitrate + nitrite) and ammonium ions using the Technicon Autoanalyser II. If dilutions are required use 2M KCl to dilute by 1 in 10.

(vii) When necessary store decanted supernatant in air tight sample cups at room temperature.

6.4 ACETYLENE BLOCKAGE TECHNIQUE

6.4.1 REVIEW

The Acetylene Blockage Technique is based on the observation that acetylene inhibits the reduction of nitrous oxide to nitrogen gas during denitrification (Federova et al., 1973). Following this discovery the inhibition was monitored in pure cultures of denitrifying bacteria. Balderston et al. (1976) observed that Pseudomonas perfectomarinus exposed to 0.01 atmospheres of acetylene exhibited inhibition of nitrous oxide reductase enzyme evidenced by a build-up of nitrous oxide as the end product of denitrification. It is therefore possible to quantify denitrification by measuring the progressive accumulation of nitrous oxide in acetylene amended systems. The technique has now been employed in a variety of field and laboratory systems analysing denitrification in soils (Müller et al., 1980); freshwater sediments (Chan and Knowles, 1979); deep-sea sediments (Sorensen et al., 1984); periphyton mats (Triska and Oremland, 1981); rhizosphere soils (Smith and Tiedje, 1979); and coastal marine sediments (Sorensen et al.,1979).

Prior to the discovery of this technique the methods used included labelling of nitrogen compounds and gas chromatography. The problems encountered with ^{15}N and ^{13}N tracers include insensitivity (Yoshinari et al., 1977; Sorensen, 1978a); expense (Sorensen, 1978b; Ryden et al., 1979a); necessity to add substrate (Sorensen, 1978b; Aulakh et al., 1984; Smith et al., 1978); and the length of time required to conduct experiments (Smith et al., 1978). Other problems include the possible non-uniform distribution of tracers between nitrogen species and within soils, also leaching of tracers from the sampling area (Aulakh et al., 1984). Despite these problems nitrous oxide emissions recorded in similar samples by acetylene blockage and labelled nitrogen compounds have been shown to be similar (Smith and Delaune, 1983; Aulakh et al., 1984). Direct measurements of molecular nitrogen also require long incubation periods (Sorensen, 1978b). Perhaps the greatest problem with this technique is the risk of contamination by atmospheric nitrogen during assays (Yoshinari et al, 1977; Smith et al., 1978).

Following the initial discovery of acetylene inhibition, pure culture experiments were designed to study the effect of various concentrations of acetylene on nitrous oxide reduction. Balderston et al. (1976) observed that acetylene blockage was complete in Pseudomonas perfectomarinus over a range of acetylene concentrations. The blockage of nitrate reduction by a wide range of acetylene concentrations (0.01 - 80% headspace) has been repeatedly verified in laboratory and field studies (Yoshinari et al., 1977; Chan and Knowles, 1979; Oremland et al., 1984). Comparison of denitrification rates in acetylene amended and non-amended incubations have demonstrated that the presence of acetylene has no effect on the rate and extent of denitrification (Ryden et al., 1979a; Kaspar et al., 1981).

On occasions incomplete inhibition has been observed leading to the gradual disappearance of accumulated nitrous oxide. For example Kaspar et al. (1981) observed that at nitrate concentrations below 10 uM the reduction of nitrous oxide to molecular nitrogen was not inhibited by acetylene concentrations of 80%. The efficiency of the acetylene block is therefore a function of nitrate and acetylene concentrations. Although low nitrate concentrations have prevented a complete inhibition in some samples (Kaspar, 1982; Oremland et al., 1984) successful inhibitions have also been recorded at low nitrate concentrations (Ryden et al., 1979a).

Lack of inhibition in prolonged incubations has also been related to high sulfide concentration. Tam and Knowles (1979) combined acetylene at normally inhibitory concentrations and sodium sulfide (8 umol S^{2-}ml), and found that the sulfide relieved the acetylene inhibition after three days. Addition of sulfide to autoclaved soils did not relieve the acetylene inhibition. They concluded that a biological mechanism involving sulfide was responsible for the relief of the acetylene inhibition. The mechanism by which this proceeds and the bacteria involved have not been identified. Smith and DeLaune (1983) conclude that the effect would not be a serious problem over short-term incubations although over prolonged periods components of the bacterial flora may produce sulfide when anaerobiosis intensifies.

It has been observed that acetylene can inhibit the growth of sulfate-respiring bacteria thus complicating the sulfide affect. Acetylene at 20% completely inhibited the growth of Desulfovibrio desulfuricans (Payne and Grant, 1982). In contrast to this Culbertson et al. (1981) report that acetylene stimulated sulfate reduction. It appears that acetylene may have different effects on different species of sulfate-respiring bacteria.

Acetylene also inhibits nitrogen fixation (Culbertson et al., 1981); methanogenesis (Knowles, 1979; Chan and Knowles, 1979); and nitrification (Bremner and Blackmer, 1979; Smith and DeLaune, 1983).

Monitoring of acetylene levels during incubations has demonstrated that it can be used as a substrate by anaerobic bacteria. Culbertson et al. (1981) observed gradual loss of acetylene occurring after six days of incubation. In some samples the loss can be correlated to ethylene formation following the complete denitrification of added nitrate (Yoshinari et al., 1977; Knowles, 1979; Chan and Knowles, 1979). However, on occasion acetylene has been removed and ethylene not produced (Balderston et al., 1976). The acetylene may be used as a substrate in denitrification which could complicate the results of acetylene blockage studies. Yeomans and Beauchamp (1982) monitored acetylene concentrations in incubations lasting up to 600 hours. They found that acetylene did enhance denitrification over very long incubations but were unable to show conclusively that denitrifiers were using it as a substrate. It appears that acetylene loss would not be a problem in short term anaerobic incubations prior to the exhaustion of nitrate supply.

The data obtained by acetylene blockage can be used for evaluating the extent of denitrification, the distribution of denitrification products, and the effect of environmental factors on denitrification. The equipment required is generally available with gas chromatographic methods being rapid and sensitive (Sorensen et al., 1978). The high solubility of acetylene makes it ideal for penetrating aggregates where active denitrification is occuring at anaerobic microsites (Smith et al., 1978; Ryden et al., 1979b). In addition acetylene has no affect on anaerobic respirers as evidenced by similar carbon dioxide production

in acetylene amended and non-amended samples (Balderston, 1976; Ryden et al., 1979a). Quantitative results can be obtained by measuring nitrous oxide as no other biological source of the gas is known.

Potential problems with the technique include the tendency of acetylene to accelerate denitrification reaction kinetics (Lensi and Chalamet, 1982), and the breakdown of inhibition at low nitrate and high sulfide levels. The latter may disturb in situ measurements of denitrification over the long term. In addition a fairly labour-intensive sampling program is required to monitor the build-up of nitrous oxide over time (Aulakh et al., 1984).

Acetylene is a barrier which can be used, relieved (Ryden et al., 1979a), and reapplied repeatedly in a range of natural systems. Quantifiable amounts of nitrous oxide can be recorded over short or prolonged periods allowing the dynamics of nitrogen loss to be related to environmental changes. The Acetylene Blockage Technique has the potential to become a direct, sensitive and routine measurement of denitrification in laboratory and field systems.

6.4.2 LABORATORY PROCEDURE

The following laboratory procedure was adapted from Bavor et al. (1981):

Equipment
- 120 ml glass serum vials
- Rubber 'suba seal' serum caps and crimp seals
- 100 mg/l nitrate solution
- TYNL Media (see below)
- 10 ml and 1 ml Precision Sampling Corporation 'pressure lok' syringes
- Acetylene and Helium gases
- Nitrous Oxide calibration gas

Method

Changes were made to this method in order to monitor the influence of various factors on denitrification (see Chapter 7).

(i) 10 g of wetland soil from a core sample is added to the 120 ml serum vial.

(ii) 10 ml of TYNL media (Balderston et al.,1976; Bavor, pers. comm.) is added. The constituents in 1 litre of distilled water are as follows:

Tryptone	5.0 g
Yeast Extract	1.5 g
Sodium nitrate	1.0 g
Sodium lactate	10.0 g

pH is adjusted to 7.6 prior to autoclaving.

(iii) The sample vial is capped with a 'suba seal' and crimp sealed.

(iv) The vial is flushed with helium for 10 minutes whilst shaken on a rotary shaker. Flushing is achieved by attaching a hollow needle to a rubber tube which is connected to a cylinder of helium. This needle and a second hollow needle are inserted through the septum into the vial so that continuous flushing is achieved.

(v) Both needles are removed simultaneously.

(vi) 10 ml of gas is removed from the vial using the appropriate gas tight syringe.

(vii) 10 ml of acetylene is injected into the vial which is then shaken for 10 minutes in a rotary shaker. This allows the acetylene to mix with the sediment.

(viii) 1 ml of 100 mg/l nitrate is injected into the bottle using a syringe.

(ix) A 0.5 ml sample of gas (time zero) is taken from the vial using the 1 ml gas tight syringe and is analysed immediately by gas chromatograph. The area of the nitrous oxide peak is noted. The serum vials are then incubated at the desired temperature in a rotary shaker.

(x) Every 2-3 hours in the first 30 hours the shaker is stopped and a 0.5 ml sample of gas is taken from the vial and analysed. The area of the nitrous oxide peak for each sample is noted. Sampling is continued at 4-5 hourly intervals until no more nitrous oxide is being produced in the serum vial.

(xi) At each sampling time 0.5 ml of the nitrous oxide standard (2 mls of nitrous oxide calibration gas in 120 ml 'suba'sealed serum vial) is analysed to give a standard nitrous oxide peak area.

(xii) Following computation of nitrous oxide in mg (see Appendix I) a graph can be drawn of mg nitrous oxide liberated into the serum vial headspace versus time of incubation. The maximum rate of denitrification is then calculated from the area of the curve where the gradient is steepest (see Appendix II). A rate estimate is obtained in mg N_2O-N/hr for 10 g of soil (division by 10 to give mg N_2O-N/hr/g soil).

CHAPTER 7 RESULTS AND DISCUSSION

7.1 FIELD MEASUREMENTS

7.1.1 REDOX POTENTIAL (Eh)

Redox reactions are reactions which involve the transfer of electrons, an example being transfer from organic matter to other soil components such as inorganic cations. Under waterlogged conditions as found in the wetland at Thredbo, the diffusion of oxygen into and through the soil is reduced. Aerobic micro-organisms reduce the oxygen that is in the soil faster than it can be replaced by diffusion and thus an anaerobic environment develops. At this stage, facultative anaerobes then turn to oxidised soil components to replace oxygen as an electron acceptor so initiating soil reduction. Denitrification is one of these processes whereby anaerobic bacteria use nitrate as the terminal exogenous electron acceptor resulting in the production of nitrogen gas.

Measured redox potential (Eh) can be used as an indication of the oxidation status of the elements in solution (Whisler et al., 1974). It provides a measure of the ratio of oxidised to reduced forms in the system (Whitfield, 1969), and therefore has the potential to be a quantitative measure of the tendency of that system to accept or donate electrons. However redox potentials in soils as yet defy quantitative measurement due to the complexity of soil systems (Graetz et al., 1973). The problem lies in the importance of microsites where Eh can differ significantly within a millimeter (Willett, 1983). Eh values are generally used as a qualitative measure of the reduction status of the environment (Whitfield, 1969; Mortimer, 1971).

In order to standardise qualitative determinations of Eh various systems have been developed relating Eh values to stages in the reduction of an environment. The progression is also seen within sediments, and related to depth (Bender et al., 1977). Schemes formulated by Graetz et al. (1978) and Patrick and Mahapatra (1968) are shown below.

Graetz *et al.* (1973)		Patrick and Mahapatra (1968)	
mV		V	
+300 to +350	First stages of anaerobic respiration	0.4 to 0.2	MODERATELY REDUCED
+200 to +100	Oxygen depleted. NO_3^- used as electron acceptor		
<+100 to 0	Mn and Fe used as electron acceptors	0.1 to -0.1	REDUCED
0 to -150	SO_4^{2-} reduced to S^{2-}	-0.1 to -0.3	HIGHLY REDUCED
<-150	Methane production		

The table shows that denitrification is associated with Eh values of +200 to +100 mV. This has been supported by Vanderborght and Billen (1975), Lance *et al.* (1976), and Jones (1979), although denitrification has been observed at higher (Reddy and Patrick, 1984) and lower (Smith *et al.*, 1983) Eh values.

Jones (1979) used nitrate reductase activity as an indicator of denitrification and found that the maximum activity coincided with +210 mV. It seems that there is a close relationship between nitrate reduction and redox potential. This conclusion has been come to on the observation that when nitrate levels fall the redox potential drops rapidly (Pilot and Patrick, 1972). A shortage of oxygen in the soil causes this affect.

Other factors in this relationship between denitrification and Eh include the stabilisation of Eh values at around +200 mV when nitrate is fairly abundant (Reddy and Patrick, 1975). In addition Eh varies with depth in soils and can be used to characterise the aerobic and anaerobic zones within the sediment (see Section 2.5).

The following results are averaged from twenty readings taken at both 1 cm and 6 cm depths in the soil across the wetland.

Eh above the effluent inflow +197.5 ± 6.8

Eh below the effluent inflow +111.6 ± 7.4

Eh was depressed below the point where effluent from the final maturation pond enters the wetland. This difference was significant ($p>0.05$) and is probably related to the greater degree of waterlogging below the inflow. However both areas demonstrate a moderately reducing environment (Patrick and Mahapatra, 1968) lying within the range where nitrate is being used as an electron acceptor in denitrification (Graetz et al., 1973; Whisler et al., 1974). The millivolt values obtained correspond to approximately 0.2 volts indicating that nitrate, manganese, and ferrous iron are being reduced (Willett, 1983).

Eh values at 1 cm and 6 cm depth were not significantly different ($p>0.05$). Redox potentials ranged from -5 mV to +365 mV. The Eh of +365 mV demonstrates the importance of aerobic micro-environments within a soil which is generally reduced in character.

Smith et al. (1983) observed that the critical Eh for the onset of denitrification was +250 mV in soils of pH 6. Readings taken at the same time as redox potentials showed that pH ranged from 6.4 to 6.7 in the wetland soil. The onset of denitrification would probably occur at around +200 to +250 mV in this soil.

It seems that the soil across the 3.3 ha of the wetland has a moderately reducing environment from 1 to 6 cm depth where denitrification could occur. These conditions may persist to greater depth as Eh >+100 mV has been recorded to 12 cm depth in lake sediments (Klingensmith and Alexander, 1983). High redox potentials (up to +365 mV) in the top 1 cm indicate an aerobic surface layer where nitrification could occur.

Redox potentials from the wetland were used qualitatively to characterise the oxidation-reduction status of the soil, and to ensure that representative sampling sites were chosen where denitrification would be occuring. Values from above and below the effluent inflow suggest that redox potentials encouraging denitrification are widespread throughout the wetland soil.

7.1.2 pH

Readings within the top 0-5 cm at ten sites across the wetland ranged from pH 6.4 to 6.7 giving an average value of pH 6.63 $\pm$ 0.01. This lies well above the point (pH 5 and below) where denitrification is slowed or halted in some soils (Bremner and Shaw, 1958; Müller *et al.*, 1980). The optimum pH for denitrification in laboratory studies has been shown to be pH 7 to 8. Although the values at Thredbo are below this they lie in the range where pH exerts only a moderate affect on denitrification (pH 6 to 7.5), and where other limiting factors such as lack of organic carbon usually dominate (Stanford, Vander Pol and Dzienia, 1975).

The pH values recorded across the 3.3 ha of the wetland suggest that conditions conducive to denitrification are widespread. Values of pH 6.4 to 6.7 at the surface (0-5 cm) would allow denitrification to occur. It is likely that pH would increase with depth (Klingensmith and Alexander, 1983) so that pH below 5 cm depth may be in the region of pH 7 to 8 which is optimum for denitrification.

It is unlikely that pH is hindering denitrification in the soil. Some soils around pH 6.3 have been shown to greatly favour denitrification (Broadbent, 1951).

7.1.3 TEMPERATURE

Temperatures were recorded on four occasions in 5-10 cm depth soil at the above and below effluent inflow sampling areas (see Figure 5.2.). This depth was chosen as it coincides with the area where the highest rates of denitrification have been observed in flooded soils and sediments (Sorensen, 1978c; Oren and Blackburn, 1979).

The results are contained in Table 7.1 and represent winter, spring, summer and autumn.

Table 7.1 Temperature data and conditions at time of sampling for four months.

	June	September	January	March
Temp. above inflow $^{o}C \pm$ std. error	1.83±0.02	5.21±0.07	14.96±0.06	9.43±0.11
Temp. below inflow $^{o}C \pm$ std. error	3.67±0.05	9.37±0.08	19.11±0.09	12.5±0.09
Time	09.30	15.30	17.00	14.30
Approx. air temp ^{o}C	0	4	26	13
n (no. of samples)	14	9	10	12

Temperatures at 5-10 cm depth in the soil were higher than the air temperature in winter and spring, but lower in summer and autumn. Temperatures below the point of effluent inflow were consistently higher than those recorded above the inflow on the same day. This difference was significant ($p>0.05$)

Figure 5.3. shows that the mean monthly maximum and minimum air temperatures were as follows:

Table 7.2 Maximum and minimum air temperatures at Thredbo Village (data from the Bureau of Meteorology).

	June	September	January	March
Minimum °C	-3	-1	6	3
Maximum °C	6	10	18	16

These figures also represent the diurnal variation in air temperature which would be influencing temperatures in the top 0-10 cm of the soil and thus affecting *in situ* denitrification rate. Although the inflow of effluent increases the soil temperature year-round, temperatures overnight in spring and autumn, and for a large proportion of each day in mid-winter, would be below 5°C.

In Section 4.3 the influences of temperature on denitrification were discussed. The controversy over the role of temperature in controlling denitrification was highlighted. In some cases significant denitrification has been recorded at temperatures of 5°C and below (Terry and Nelson, 1975; Sorensen *et al.*, 1979; Seitzinger *et al.*, 1984). In others denitrification has been severely inhibited below 5°C (Bailey, 1976; Reddy *et al.*, 1980). Bremner and Shaw (1958) demonstrated that populations of denitrifiers were able to denitrify (at sub-optimum rates) at temperatures down to 2°C. This was dependent on the soil being organic with high levels of available carbon. In soils of low carbon content denitrification rates were negligible once the temperature was lowered to 10°C. The availability of carbon in the form of decaying plant material could therefore be of importance to the influence of temperature on denitrification in the soil at Thredbo

wetland. Increased availability of carbon may promote a continuation of denitrification, all be it at sub-optimal rates, even at temperatures below 5°C.

The role of temperature in controlling denitrification and therefore nitrogen removal at Thredbo wetland is very important as the peak loadings of ammonium ion and nitrate occur during winter (see Section 5.6). Nitrate diffusion can be reduced at temperatures below 8°C (Reddy and Patrick, 1984). However as the majority of nitrogen in the effluent occurs as ammonium ion the influence of reduced nitrate diffusion on denitrification would probably be negligible.

In some sub-alpine regions and lake sediments psychrophilic denitrifying bacteria have been identified. These populations demonstrate higher rates of denitrification at temperatures of 5°C than at higher temperatures (Halmø and Eimhjellen). Experimental results to be described later in this report (see Section 7.5.2.) have shown that *in situ* bacteria did support a significant level of denitrification at 5°C in laboratory incubations.These same bacteria were shown to have a significantly higher rate of denitrification at 35°C (i.e. not psychrophilic).

The use of wetland systems as a means of removing nitrogen from wastewaters by denitrification requires adequate information on the effect of temperature. Results which suggest that denitrification is severely inhibited below 5°C (Bailey, 1976; Reddy *et al.*, 1980) point to the cessation of denitrification and subsequent nitrogen removal over winter. At Thredbo, peak nitrogen species loadings occur in mid-winter at the height of the ski season. The temperature data shows that in June soil temperature may fall below 5°C for a large proportion of the day. In such cases the ability of the wetland soil and plants to entrap ammonium ions and nitrate may become precedent until such time as denitrification activity increases later in the day, month or season.

7.2 SOIL EXTRACTION TECHNIQUE

As outlined in Section 6.3.1. a wide variety of techniques have been used to estimate exchangeable inorganic nitrogen. This section deals with the approach taken to develop a technique suitable for use in this laboratory.

7.2.1 PRELIMINARY SOIL EXTRACTION EXPERIMENT

The technique initially employed was based on that of Henriksen (1980). One gram soil samples were placed in centrifuge tubes with 20ml of 2M KCL. After capping and inverting the tubes three times the samples were centrifuged at 2000 x g (≃ 4500 r.p.m.) for ten minutes.

To monitor the efficiency of this centrifugation technique in extracting inorganic nitrogen, four one gram sub-samples were taken from a mixed soil sample and the effect of four consecutive extractions was examined. After each centrifugation the original supernatant was poured-off and another 25ml of 2M KCL added prior to the next extraction.

Yields of (nitrate + nitrite)-nitrogen [$(NO_3^- + NO_2^-)$-N] for each of the four consecutive extractions are contained in Table 7.3. The yields for each sub-sample were very similar for the first three extractions but declined in the fourth.

Table 7.3 Yields of (NO_3^- + NO_2^-)-N ug from consecutive extractions.

	10 minute extraction periods			
Sub-sample	1	2	3	4
1	9.25	12.30	11.00	10.20
2	13.05	13.05	13.10	9.55
3	13.80	12.30	13.10	10.25
4	13.80	12.20	12.40	10.25

It was clear that this method was not an effective way to extract exchangeable (NO_3^- + NO_2^-)-N from one gram soil samples. This may have been caused by the lack of spatial and temporal interaction between the 2M KCL and the soil in ten minutes of centrifugation.

7.2.2 THE EFFECT OF TIME AND AGITATION ON YIELD

Following the findings of the preliminary experiment the extraction period was extended, and the means of agitation was altered in an attempt to increase interaction between the extractant and the soil.

An experiment was conducted using the same soil sample as that in the preliminary experiment. One gram soil samples were placed in centrifuge tubes with 25ml of 2M KCL and shaken on a wrist-action shaker for two hours. The samples were then centrifuged for ten minutes at 2000 x g and the supernatant analysed for (NO_3^- + NO_2^-)-N.

Yields of (NO_3^- + NO_2^-)-N from four one gram sub-samples shaken for two hours and then centrifuged are contained in Table 7.4.

Table 7.4 Yield of (NO_3^- + NO_2^-)-N following shaking

Sub-sample	(NO_3^- + NO_2^-)-N ug
1	17.4
2	23.87
3	24.59
4	17.43

The new method achieved a higher yield per volume of KCL than the method involving centrifugation alone.

7.2.3 DETERMINATION OF THE OPTIMUM EXTRACTION PERIOD

Using the method of shaking and centrifugation (above) an experiment was designed to determine the extraction period required to extract all the exchangeable (NO_3^- + NO_2^-)-N from a one gram soil sample.

Sub-samples were extracxted with 25ml 2M KCL and were shaken for a total of five hours. At each hour the shaker was stopped and the samples centrifuged under the normal conditions to achieve a clear superantant. A small amount of the supernatant was then analysed for (NO_3^- + NO_2^-)-N. The samples were returned to the wrist-action shaker for a further hour.

The results of (NO_3^- + NO_2^-)-N yields for each hourly period (Table 7.5) show that there was no increase in yield after two hours.

Table 7.5 Yields of (NO_3^- + NO_2^-)-N ug after various periods of shaking.

	Hourly shaking periods				
Sub-sample	1	2	3	4	5
1	29.6	25.0	14.7	12.9	18.8
2	12.4	20.8	9.4	17.0	17.2
3	11.7	20.1	11.5	12.5	15.0
4	13.8	13.4	14.0	11.0	12.8
5	-	17.3	14.0	12.4	14.0
Average	16.9±2.1	19.3±0.86	12.72±0.44	13.16±0.45	15.56±0.48

A two hour shaking period was determined as being necessary to extract most of the obtainable exchangeable (NO_3^- + NO_2^-)-N from a one gram soil sample using one aliquot of KCL.

7.2.4 THE EFFECT OF VOLUME OF 2M KCL ON YIELD

An experiment was designed to determine the effect of the soil:extractant ratio on the yield of (NO_3^- + NO_2^-)-N from one gram soil samples. Four different aliquots of 2M KCL were compared, with at least four one gram soil sub-samples being assigned to each aliquot. The sub-samples were shaken for two hours and then centrifuged under the normal conditions prior to analysis of supernatant.

The yields from one gram soil sub-samples extracted with 20ml, 25ml, 40ml and 50ml of 2M KCL are contained in Table 7.6.

Table 7.6 The average yield of (NO_3^- + NO_2^-)-N in ug/g of soil with different quantities of 2M KCL

Volume of 2M KCL (ml)	Average Yield (NO_3^- + NO_2^-)-N ug
50	19.73 ± 0.39
40	7.76 ± 0.63
25	19.32 ± 0.86
20	20.83 ± 0.98

There was very little difference in yield between the 20ml, 25ml and 50ml aliquots whilst the 40ml aliquot showed a lower yield. Despite the anomalie in the 40ml sub-samples the other results suggested that volumes of 20ml, 25ml and 50ml 2M KCL caused little difference in the yield of exchangeable (NO_3^- + NO_2^-)-N from one gram soil samples.

As volume of extractant from 20ml to 50ml has little affect on yield a 25ml aliquot was selected as being a convenient volume.

7.2.5 THE EFFECT OF RE-EXTRACTION

One gram soil sub-samples were used to determine the influence of consecutive two hour extraction periods on yields of (NO_3^- + NO_2^-)-N and ammonium ion nitrogen (NH_4^+-N). Four sub-samples in the case of (NO_3^- + NO_2^-)-N, and five in the case of NH_4^+-N were taken and shaken with 25ml 2M KCL. After each two hour shaking period the samples were centrifuged at 15000 r.p.m. to achieve a clear supernatant. The supernatant was

poured-off and analysed for $(NO_3^- + NO_2^-)$-N or NH_4^+-N. A further 25ml aliquot of 2M KCL was then added to each sub-sample and returned to the wrist-action shaker for a further two hours. A total of three extraction periods were monitored.

Yields for all nitrogen species are contained in Table 7.7.

Table 7.7 Yields of inorganic nitrogen from one gram soil samples re-extracted over two-hourly periods.

Sub-samples	Extraction Periods		
	First	Second	Third
		ugN	
$(NO_3^- + NO_2^-)$-N			
1	10.9	7.5	9.5
2	8.3	6.9	7.5
3	6.9	6.9	8.9
4	5.0	10.2	10.9
NH_4^+-N			
1	383.0	231.0	259.0
2	379.0	242.0	275.0
3	371.0	253.0	256.0
4	371.0	229.0	269.0
5	340.0	235.0	244.0

In the case of $(NO_3^- + NO_2^-)$-N each extraction period yielded about the same amount whilst NH_4^+-N showed an abrupt decline in yield after the first extraction period, followed by continued yield at a lower level.

The continued yields of inorganic nitrogen on re-extraction placed the method in doubt as a quantitative means of determining exchangeable $(NO_3^- + NO_2^-)$-N and NH_4^+-N.

7.2.6 REPEATED EXTRACTIONS TO DETERMINE YIELD END-POINT

An experiment was designed to see if an end-point of NH_4^+-N yield from a one gram sample could be reached. Five sub-samples were established with 25ml 2M KCL and shaken for consecutive two-hourly periods. After each two hours the samples were centrifuged and the supernatant poured-off and analysed for NH_4^+-N. A further 25ml aliquot of 2M KCL was then added to each sample, and a further extraction period begun.

The yields of NH_4^+-N were highest after the initial two hour extraction period but exchangeable NH_4^+-N was still being extracted after the seventh period. These results are contained in Table 7.8.

Table 7.8 The effect of two-hourly extractions over fourteen hours on yields of NH_4^+-N from one gram soil samples.

	Extraction Period						
Sub-sample	1st	2nd	3rd	4th	5th	6th	7th
	NH_4^+-N ug						
1	383	231	259	238	251	320	241
2	279	242	275	200	238	334	223
3	371	253	256	238	259	320	267
4	371	229	269	267	238	300	241
5	340	235	244	200	245	300	218

A quantitative total extraction of exchangeable NH_4^+-N/gram of soil could not be achieved by seven extraction periods.

7.2.7 SPIKING EXPERIMENTS

The influences of biological and chemical reactions on yields within a single extraction period were monitored by two spiking experiments.

Both experiments involved eight one gram sub-samples from a mixed soil samle. One experiment monitored NH_4^+-N, the other (NO_3^- + NO_2^-)-N. In each experiment four sub-samples had 40ml of mid-standard added (either 0.6mg/litre NH_4^+-N or 42 ug/litre NO_3^--N). The remaining four sub-samples had 40ml of nanopure water added. All eight sub-samples were then shaken for two hours to equilibrate and then KCL was added to all tubes to make a 2M solution. At this stage the pH was checked and adjusted to pH7 where necessary. This was to ensure that acidity would not influence the extraction by leaching fixed inorganic nitrogen. Following a further two hours of shaking the pH was rechecked to ensure it remained around pH7. After centrifugation the supernatant was analysed.

The recovery of NH_4^+-N at 0.6mg/litre ranged from 70 to 100% and averaged 81.3 $\pm$ 3.43%.

The recovery of NO_3^--N at 42 ug/litre ranged from 78 to 89% and averaged 84.643 $\pm$ 1.18%.

The pH of the NH_4^+-N sub-samples remained stable throughout but fell slightly during extraction for (NO_3^- + NO_2^-)-N.

It appears that the yields of (NO_3^- + NO_2^-)-N and NH_4^+-N were being affected by biological and/or physico-chemical activity either changing and utilising the nitrogen species during extraction, or influencing chemical uptake and release of exchangeable and fixed ammonium and (nitrate plus nitrite). This may also explain the continued yield on re-extraction observed in some experiments.

7.2.8. GENERAL CONCLUSIONS FROM SOIL EXTRACTION EXPERIMENTS

These experiments showed that the technique commonly employed could not be used to quantify exchangeable inorganic nitrogen in these soil samples.

It is noted that workers using these extraction techniques do not detail their efficiencies in quantifying exchangeable inorganic nitrogen. It appears that a great deal of work needs to be done in this area despite the apparent acceptance of 'standard' techniques.

This observation that quantitative recovery of added NH_4^+ and (NO_3^- + NO_2^-) appears to be impossible using the technique described can be related to the fixation of inorganic nitrogen by chemical and biological processes in the soil. Ammonia can be trapped in the intramicellar layers of clay minerals (Sahrawat, 1979b.). Muscovite (Mica) is present throughout the entire soil profile at Thredbo (see Section 5.5). If other clay minerals are as abundant such fixation could be influential in these samples.

Both ammonium ion and nitrate can be adsorbed by the organic fraction of the soil (Lind, 1977). Studies on ammonia have not revealed the mechanism of the reactions involved, or the chemical nature of the complexes produced although lignin fractions are thought to be involved (Burge and Broadbent, 1961). Despite this oxygen is known to have a significant influence on fixation. Lance (1972) observed that although substantial quantities of NH_4^+ were fixed under anaerobic conditions, far more was fixed in the presence of oxygen. This is of significance when one considers that the Thredbo samples are being removed from an anaerobic environment to be exposed to oxygen during extraction.

Ammonimum ions can also be fixed by absorption on amorphous soil materials such as colloidal hydrated oxides of Aluminium, Iron etc. (Sahrawat, 1979b.).

The processes involved in fixation are complicated and not well understood. It is possible that slow release of fixed nitrogen species may be causing the continued yields on re-extraction in these experiments. Losses of (NH_4^+)-N and $(NO_3^- + NO_2^-)$-N in spiking experiments may be explained by their fixation within the soil or biological mechanisms transforming them to species undetectable by the analytical method.

It was decided that seasonal analysis of inorganic nitrogen species in the soil profile at Thredbo should be continued but with the following limitations acknowledged. The levels of inorganic nitrogen could not be evaluated quantitatively but could possibly be used in comparative terms to identify major differences in distribution due to season, depth within the soil, and site in relation to effluent loading (either above or below the point of effluent inflow). Therefore results were used on a relative rather than an absolute basis to expose major differences.

7.2.9 SAMPLE STORAGE

Changes which take place in samples may be a result of chemical or biological processes. As a result preservation methods are based on the retardation of these processes.

The conditions advised for storage of samples for (NH_4^+)-N and $(NO_3^- + NO_2^-)$-N usually involve lowering of temperature to slow reactions in the sample (Freshwater Biology Investigation Unit N.I., 1980) and are as follows:

1. with 40 mg Hg Cl_2/litre at 4°C (lasts 7 days)
2. at 4°C
3. frozen

It was not possible to use Mercuric chloride as a preservative as it interferes with the copper coated cadmium column on the A.A.II (Major *et al.*, 1972). Storage at 4°C is advised for periods ranging from no

greater than six hours (Env. Protection Authority VIC., 1973) to twenty four hours (Inland Waters Directorate Canada, 1979). Freezing is advised for periods of 24 hours or more (Env. Protection Authority VIC., 1976).

Due to the need to change manifolds on the autoanalyser it was apparent that storage of supernatant would be necessary. An initial observation where samples were frozen for fourteen hours showed a +34 $\pm$ 1.71% difference levels of $(NO_3^- + NO_2^-)$-N before and after freezing.

Following this an experiment was conducted where supernatant was analysed for $(NO_3^- + NO_2^-)$-N and (NH_4^+)-N and then stored in air-tight sample cups for 18 hours. The conditions were as follows:

1. 4^oC
2. room temperature (23^oC)
3. frozen

All samples were allowed to reach ambient temperature and were then analysed. The percentage difference between 'before' and 'after' results was calculated.

Results for $(NO_3^- + NO_2^-)$-N

Treatment	Number of Replicates	$\bar{X}$ % Difference
4^oC	21	15.04 $\pm$ 0.64
Room Temp	22	6.42 $\pm$ 0.34
Frozen	16	59.62 $\pm$ 2.9

Results for (NH_4^+)-N

Treatment	Number of Replicates	$\bar{X}$ % Difference
4^oC	17	14.31 $\pm$ 0.52
Room Temp	18	13.45 $\pm$ 0.35
Frozen	20	37.35 $\pm$ 0.95

These results demonstrate that storage at room temperature for 18 hours produced the least difference in results.On the whole the 'difference' involved an increase in $(NO_3^- + NO_2^-)$-N and (NH_4^+)-N over the storage period. Where possible analysis for (NH_4^+)-N was conducted first (immediately after soil extraction) and samples for $(NO_3^- + NO_2^-)$-N were stored in air-tight cups at room temperature for no longer than 14 hours.

7.3 NITROGEN SPECIES PROFILES : THE INFLUENCE OF DEPTH, EFFLUENT LOADING AND SEASON ON THE DISTRIBUTION OF NITRATE PLUS NITRITE, AND AMMONIUM IONS IN SOILS

The method by which these results were obtained is discussed in Section 7.2. soil cores from the above effluent inflow sampling site and the below effluent inflow sampling site (see Figure 5.2) were taken in each season and analysed for $(NO_3^- + NO_2^-)$-N and NH_4^+-N.

Due to the proximity of the above and below effluent inflow sampling sites both with identical geology and soil formation mechanisms (Johnson, pers. comm.) it has been assumed that the above effluent site is representative of the character of the wetland prior to effluent addition.

The seasonal results of nitrogen species in 30cm cores from above and below the effluent inflow are shown as profiles in Figure 7.1 $(NH_4^+$-N) and Figure 7.2 $[(NO_3^- + NO_2^-)$-N]. Error bars are not recorded on the

graphs because typical standard errors were less than 1.5% (e.g. 0.0147±0.0001, n=3). Tables of these results are contained in Appendix III.

FIGURE 7.1

PROFILES OF NH_4^+-N FOR ABOVE (▲) AND BELOW (●) EFFLUENT INFLOW CORES AT DIFFERENT TIMES OF YEAR

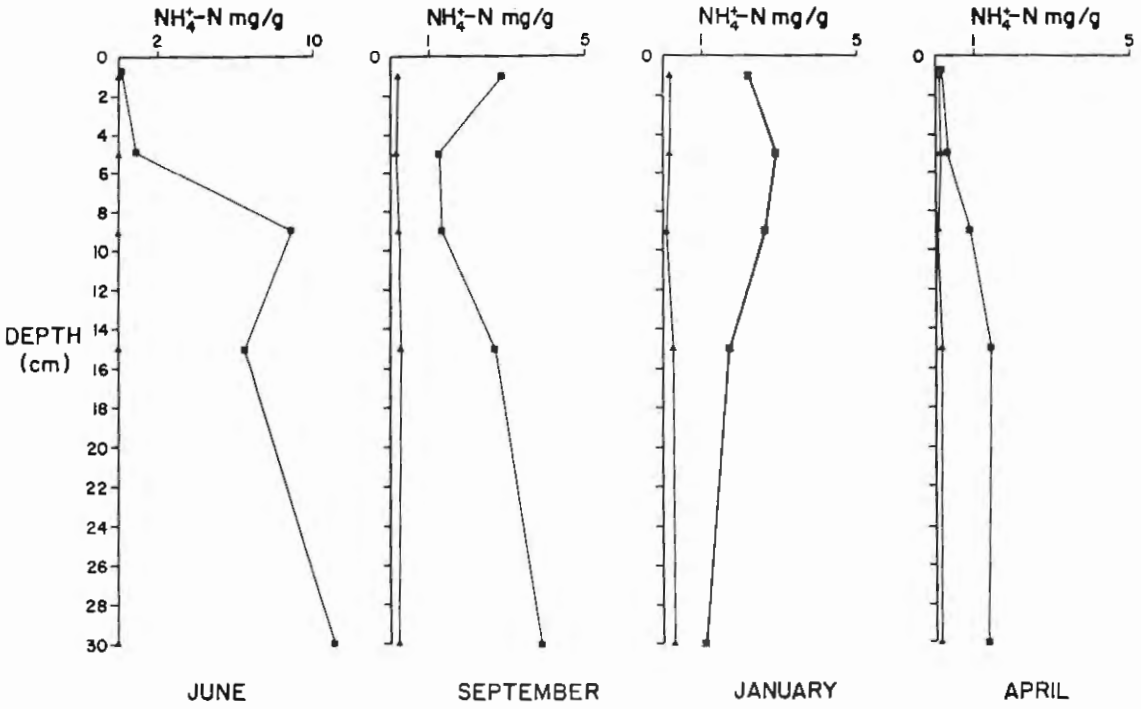
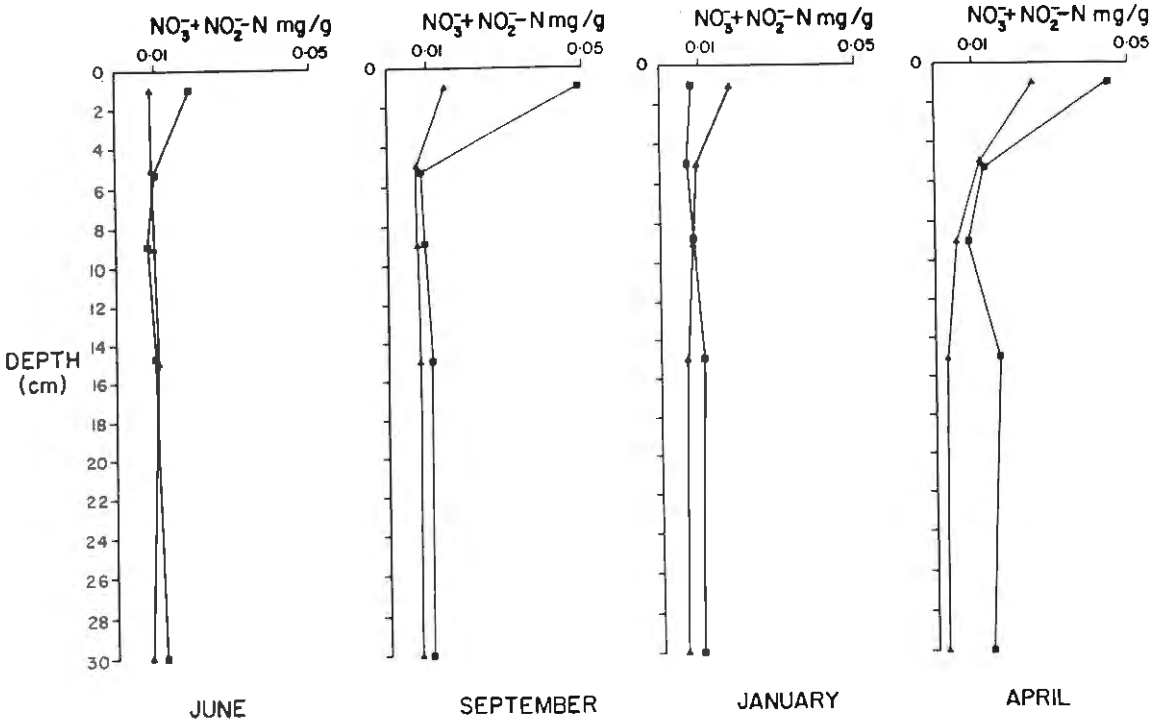


FIGURE 7.2

PROFILES OF $NO_3^-+NO_2^-$-N FOR ABOVE (▲) AND BELOW (■) EFFLUENT INFLOW CORES AT DIFFERENT TIMES OF YEAR



7.3.1 AMMONIUM ION (FIGURE 7.1)

The levels of NH_4^+-N extracted from the above effluent inflow core varied significantly with the season ($p>0.05$). Highest levels were recorded in September and January with intermediate levels in April, and the lowest level in June. As the above effluent inflow site receives no sewage nutrient input the changes in NH_4^+-N concentration with season are attributed to biological activity (micro-organisms and plants), soil chemical processes (fixation and release of NH_4^+-N), and leaching due to increased precipitation in winter, and snowmelt in spring. There is no pattern in the relationship between NH_4^+-N and depth within season although in September and January there appears to be some accumulation at depth.

The levels of NH_4^+-N extracted from the below effluent inflow core were significantly higher than those in cores from above the effluent inflow for all seasons ($p>0.05$). The higher levels at all depths in the below effluent inflow core are probably a function of the effluent loading. A similar response to effluent loading was observed by King (1982).

The levels of NH_4^+-N also vary significantly according to season at each depth ($p>0.05$). Levels below the inflow were highest in June from (8-10cm) depth to 30cm depth but at that time levels from (0-2cm) depth to (4-6cm) depth were some of the lowest recorded. This coupled with the lowest loading of NH_4^+-N recorded in the effluent on the same day as the core was analysed (Section 5.6), and the fact that there was a net export of NH_4^+-N from the wetland on that day (Cullen, unpub. data) suggests that there may have been a concentration gradient operating moving NH_4^+-N from the sediments into the overlying water. The loading of NH_4^+-N was 0.130 mg/ml while levels in the 0-2cm soil were 0.169 mg/g and increased consistently below this point in the profile (see Figure 7.1).

Although there is a significant difference in levels of NH_4^+-N between depths within each season ($p>0.05$), and between each season ($p>0.05$) no obvious trends can be discerned. There is an apparent accumulation of NH_4^+-N with depth below 4-6cm in June, September and April.

7.3.2 THE USE OF AMMONIUM PROFILES IN PREDICTING PATTERNS OF NITRIFICATION

It has been suggested that a drop in NH_4^+-N concentration in the first few centimetres of the soil is indicative of nitrification (Kemp and Mudrochova, 1972). Such a pattern was seen below the effluent inflow in September. In contrast to this other workers have interpreted low NH_4^+-N at the surface with higher levels at depth as being indicative of nitrification (Reddy and Patrick, 1984). Such a pattern was seen in June, January and April below the inflow. The conclusion to be drawn is that the concentration of NH_4^+-N and its distribution in the soil is conditioned by such a wide range of factors that the 'shape' of the profile cannot be used as an indication of nitrifier activity unless

there is other supporting evidence (populations studies etc.). Some of the factors involved are: the amount and type of clay minerals affecting cation exchange; fixation and release of NH_4^+-N by clays and organic material; uptake and release by micro-organisms and plants; and seasonal changes in the saturation of the soil. In the case of the Thredbo soil the explicit reasons for the significant effect of depth and season on NH_4^+-N levels cannot be explained but the increased levels of NH_4^+-N below the effluent inflow are significant in all seasons ($p>0.05$).

7.3.3 NITRATE PLUS NITRITE (FIGURE 7.2)

Levels of $(NO_3^- + NO_2^-)$-N in the 0-2cm segment of above effluent inflow and below effluent inflow cores were significantly different in all seasons ($p>0.05$). The loading of $(NO_3^- + NO_2^-)$-N in the effluent raised the level of these inorganic nitrogen species in the 0-2cm segment of below effluent inflow cores in June, September and April compared to levels in above effluent inflow cores. The lower level in the 0-2cm segment of the B.E.I. core in January may be a result of greater uptake by plants (Chan and Knowles, 1979; Terry and Tate, 1980b), or higher microbiol activity (Jenkins and Kemp, 1984) in comparison to the A.E.I. core.

At depths from 4-6cm down to 30cm there is no significant difference in the levels of $(NO_3^- + NO_2^-)$-N between A.E.I. and B.E.I. cores in all seasons ($p>0.05$). The fact that $(NO_3^- + NO_2^-)$-N persists at a detectable level both upstream and downstream of the effluent inflow suggests that there is a moderately oxidising environment in soils from 0-30cm depth at both sampling areas.

7.3.4 THE USE OF NITRATE PLUS NITRITE PROFILES IN PREDICTING PATTERNS OF DENITRIFICATION

A sharp drop in $(NO_3^- + NO_2^-)$-N concentration with depth has been used as an indication of denitrification (Sorensen, 1978c; Oren and Blackburn, 1979; Nishio et al., 1982; Jenkins and Kemp, 1984). Both A.E.I. and B.E.I. cores from Thredbo wetland demonstrate this pattern of high $(NO_3^- + NO_2^-)$-N at the surface with a rapid decline in levels over 0-6cm. It is probable that this 'loss' of $(NO_3^- + NO_2^-)$-N is a result of denitrification as the highest denitrification potential (activity under optimum physico-chemical conditions) was recorded in the 0-6cm segment for both A.E.I. and B.E.I. cores (see Section 7.6.).

High levels of $(NO_3^- + NO_2^-)$-N at the surface have also been used as an indicator of nitrification occuring in the oxidised surface zone (Sorensen et al., 1979; Reddy and Patrick, 1984; Kemp and Mudrochova, 1972). As no effluent is added above the inflow, nitrification of NH_4^+-N in September, January and April could well explain the observed profile pattern. Low temperatures in winter may depress nitrification and lead to the relatively 'shapeless' profile that is observed above the inflow in June. Nitrification is probably as important in the below inflow soil and would add to the nitrate that is diffusing into the soil from the overlying, effluent amended water.

7.3.5 NITROGEN SPECIES PROFILES: VERTICAL OR HORIZONTAL CONDITIONING OF DISTRIBUTION

That the patterns of NH_4^+-N and $(NO_3^- + NO_2^-)$-N distribution observed here were related to vertical rather than horizontal movements of nitrogen species was confirmed by the digging of pits in the areas of the wetland concerned at the end of the study. No lateral movement of water could be observed from 0-30cm and therefore it was assumed that the patterns observed were related to vertical movements controlled by diffusion gradients, biological activity, and the structure of the soil (organic matter content, clay content).

7.3.6 CONCLUSION

Profiles of nitrogen species were a useful tool in determining the relationship between their distribution and depth, season and effluent loading in Thredbo wetland as discussed above. In addition they provided some information on the probable distribution of nitrifier and denitrifier activity of micro-organisms according to depth.

7.4 VALIDATING THE ACETYLENE BLOCKAGE TECHNIQUE

The following incubations were conducted to demonstrate the efficacy of the acetylene blockage technique as a means of determining the potential rate of denitrification. The experiments were conducted during the period in which the technique was being established at this laboratory. To ensure adequate denitrifier activity pig slurry was used in these and other early experiments.

Optimum conditions in terms of nitrate (1 ml of 1000 mgNO_3^-/l) and temperature (25^oC) were used. Each 10 ml sample of pig slurry had 4 ml of quarter-strength Frog Ringers Solution added to act as a source of nutrients for the bacteria. The sample and additions occupied 15 ml of the sample vial.

Five separate incubations of pig slurry (P.S.) were established:

1. Sterile P.S. (chloroform added) + nitrate (NO_3^-) + 10 ml acetylene (C_2H_2)

2. P.S. + NO_3^- + 10 ml C_2H_2

3. P.S. + NO_3^-

4. P.S. + nitrous oxide (N_2O) + 10 ml C_2H_2

5. P.S. + N_2O

7.4.1 INCUBATION OF STERILE SUBSTRATE

This was included to demonstrate that the denitrification reaction is a biological one carried out by organisms living in the slurry. Addition of chloroform killed the denitrifying bacteria and thus prevented N_2O production. No accumulation of N_2O was recorded over 6.25 hours despite the presence of added NO_3^- and C_2H_2.

7.4.2 INCUBATION WITH ACETYLENE

This incubation demonstrated that acetylene can be used to block the denitrification reaction leading to a build-up of nitrous oxide over time. Following the establishment of this incubation the accumulation of N_2O-N was observed as follows:

Time from start (hrs)	N_2O-N mg/1ml P.S.
0	-
1.39	0.0146
5.37	0.0330
7.14	0.0570

The accumulation can be used to calculate the potential rate of denitrification in 1 ml of sample (see Appendix II). In this incubation period of 7.14 hours the maximum rate of denitrification under optimum conditions was 0.0175 mg N_2O-N/hr/1 ml of pig slurry.

7.4.3 INCUBATION WITHOUT ACETYLENE

If acetylene is not present in the incubation then the denitrification reaction should continue to its normal end-point with the production of nitrogen gas. If this is occurring no N_2O accumulation will be observed.

The incubation was maintained for 6.25 hours. After 1.08 hours there was some build-up of N_2O-N with a level of 0.222 mg being recorded. However, within another 1.20 hours all this N_2O-N had disappeared. Without acetylene to block the reaction at the N_2O stage denitrification continued to a final end-point where all N_2O was converted to nitrogen gas.

7.4.4 INCUBATION WITH NITROUS OXIDE AND ACETYLENE

This incubation also demonstrates the blocking effect of acetylene. N_2O was added to the incubation in the presence of C_2H_2. Very little N_2O was lost (converted to nitrogen gas). An incubation for 5.15 hours gave the following results:

Time from start (hrs)	N_2O-N mg/1 ml P.S.
1.30	0.0998
5.15	0.0977

7.4.5 INCUBATION WITH NITROUS OXIDE IN THE ABSENCE OF ACETYLENE

In support of the previous two incubations N_2O was added to the sample in the absence of C_2H_2. As a result there was a gradual disappearance of N_2O-N over time. It is assumed that the N_2O was converted to nitrogen gas by denitrifying bacteria.

Time from start (hrs)	N_2O-N mg/1 ml P.S.
1.08	0.4124
2.28	0.2803
4.00	0.2053

These results demonstrate that denitrification is a biological reaction which can be blocked by acetylene gas leading to the build-up of N_2O as opposed to nitrogen gas. Monitoring the production of N_2O into the headspace of a serum vial is a means of determining the maximum potential rate of denitrification under optimum conditions of temperature and substrate.

7.5 DEVELOPMENT OF THE INCUBATION PROCEDURE FOR THE ACETYLENE BLOCKAGE TECHNIQUE

In order to measure maximum potential rates of denitrification it was necessary to determine the optimum conditions to be used in the laboratory technique.

Temperatures for the incubations were set at 25°C or 35°C. In addition some incubations were shaken continuously to aid diffusion.

Substrate additions included nitrate (100 or 1000 mg/l), carbon (glucose and lactate), and 25% vol/vol Frog Ringers Solution (contains potassium chloride, sodium chloride, calcium chloride and water).

Several different types of material were examined : pond sediment, soil, lake sediment, pig slurry and wetland soil from Thredbo. Despite the optimum temperatures and additions of nitrate, carbon and Frog Ringers Solution (F.R.S.) no activity, in the form of N_2O accumulation, was observed except in the pig slurry. Different combinations of 1000 mg/l and 100 mg/l NO_3^-; 0.1% and 1% glucose; and F.R.S. were established to try and identify the reason for the lack of denitrification. No accumulation of N_2O-N was observed in any of the incubations other than those involving pig slurry.

As it was winter and low temperatures may have reduced denitrifier populations, pre-incubations were attempted to see if the bacteria required a period of optimum conditions to become active. Anaerobic preincubations at 25°C and 35°C of 0, 24, 48 and 76 hours duration were examined. Various combinations of nitrate, carbon and F.R.S. were included with the samples. Some were completely saturated with F.R.S.

to determine if moisture content was influencing denitrification. After preincubation each vial was reflushed with helium and then a further 1 ml of nitrate was added to start the incubation. Analysis continued up to a maximum of twelve hours but at no point was any quantifiable N_2O measured.

Despite the wide variety of incubations no trace of N_2O could be found in over 100 assays. Although the instrumentation had been proven in its ability to record levels of N_2O down to 0.01 mg from 10 ml of pig slurry (see Section 7.4), a spiking experiment was conducted to ensure that N_2O could be detected. Three concentrations of 0.2 ml, 0.05 ml and 0.03 ml N_2O in 120 ml all gave quantifiable peaks following separation on the Poropak R and Q column of the gas chromatograph.

To see if low pH could be inhibiting denitrification the pH of samples before and after incubation were recorded. Prior to incubation pH was around pH 6.4 and afterwards ranged from pH 5.95 to 6.35 with a mean of 6.2 $\pm$ 0.005. Although pH was depressed following preincubation and incubation it did not fall below the range where pH exerts only a moderate affect on denitrification (pH 6 to 7.5).

The next stage in development was to monitor the effect of using TYNL media which successfully supported populations of the denitrifier Pseudomonas perfectomarinus (Balderston et al., 1976). The media contained yeast extract (nicotinic acid and riboflavin), tryptone (pancreatic digest of casein), potassium nitrate and sodium lactate.

An experiment comparing different concentrations, forms and amounts of carbon and media was designed. Five incubations of 0-6 cm depth soil from Thredbo wetland were established as follows:

	1/4 str FRS +7% Glucose	1/4 str FRS +70% Na lactate	1/4 str FRS +70% Na lactate	TYNL 3 ml	TYNL 10 ml
Soil (g)	10	10	10	10	10
F.R.S. (ml)	10	10	10	-	-
Carbon Source (ml)	1	1	1	-	-
TYNL (ml)	-	-	-	3	10
100 mg/l NO_3^- (ml)	1	1	1	1	1
TOTAL (ml)	22	22	22	14	21

The incubation temperature was 35°C and the samples remained unshaken.

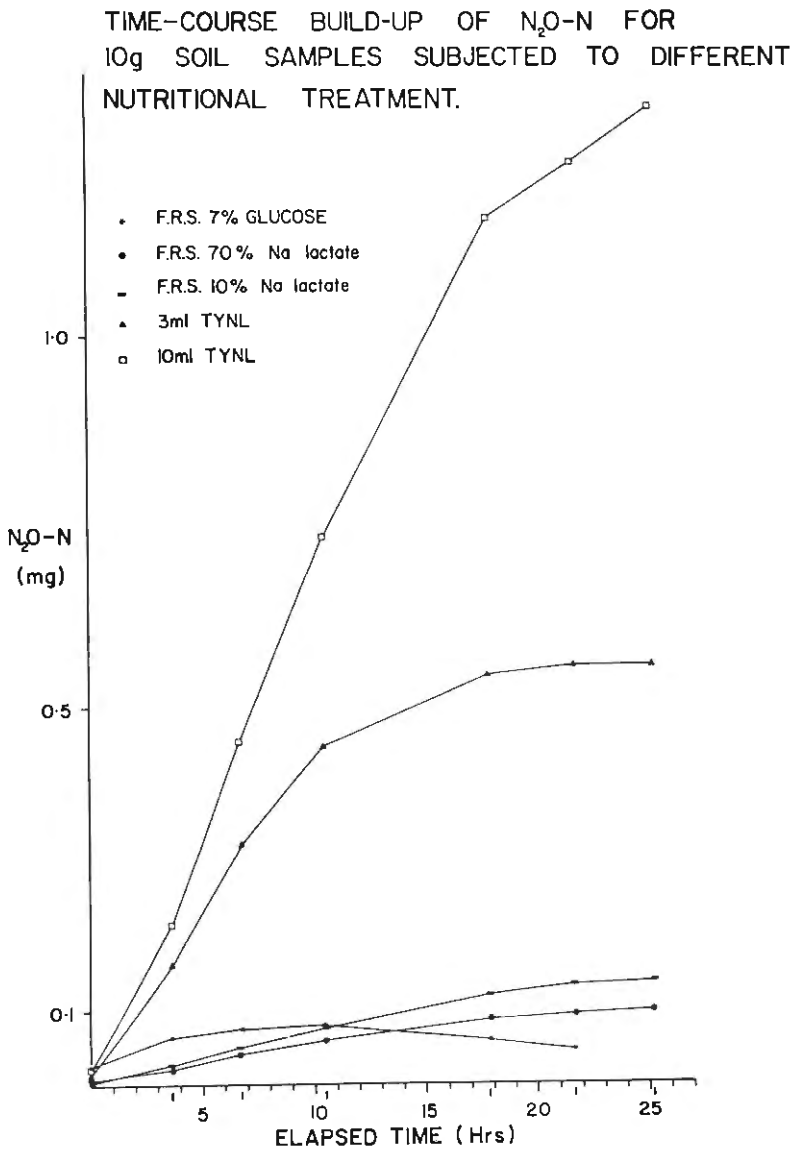
The time-course build-up of N_2O-N for each 10 g sample of 0-6 cm soil can be seen in Figure 7.3. Figures representing maximum measured rates of denitrification per gram of soil, and the total amount of N_2O-N liberated in the experimental period are contained in the following table (Table 7.9).

Table 7.9. Maximum hourly rates of denitrification per gram of soil, and total N_2O-N liberated by 10 g soil samples receiving different nutritional treatments.

Sample	Max. Hourly Rate of Denitrification N_2O-N mg/g	Max. Accumulation of N_2O-N mg
FRS + 7% glucose	.0012	0.081
FRS + 70% Na lactate	.0007	0.099
FRS + 70% Na lactate	.0008	0.137
3 ml TYNL	.0052	0.556
10 ml TYNL	.0079	1.309

The Na lactate replicate was included to expose any major differences in denitrification rates between replicate 10 g samples from a mixed soil sample.

FIGURE 7.3



The results demonstrate that the TYNL medium provides a more efficient substrate for achieving maximum rates of denitrification in laboratory incubations of wetland soil. Despite additions of carbon to the FRS incubations they were unable to support a rate or amount of denitrification equal to that of the TYNL.

The observation that 7% glucose gave a slightly higher rate of denitrification than 70% Na lactate replicates may be a result of different starting populations of denitrifiers between samples, or a carbon over-loading in the Na lactate incubations. Although

denitrification rate was lowest in the Na lactate incubations, denitrification was sustained for a longer period than that of the 7% glucose incubation where activity began to decline after ten and a half hours.

Despite the difference in denitrification rate between F.R.S. 7% glucose and F.R.S. 70% Na lactate it was obvious that some nutritional factor in addition to carbon was limiting denitrification in the F.R.S. incubations but was provided by the TYNL. The TYNL contains 10 g of 70% Na lactate in 1 litre and can therefore be compared with the 7% glucose incubation. Such a comparison demonstrates that despite having the same carbon loading the F.R.S. 7% glucose incubation had a lower potential rate of denitrification , and lower total accumulation indicating that nutritional factors other than carbon were limiting denitrification. The denitrifiers responded readily to the TYNL additions of proteins , vitamins , and nitrate (1.650 mg NO_3^--N per 10g of soil) which appear to provide a better "all-round" substrate than the F.R.S. with carbon amendments. It is likely that the cause of the difference in potential rates of denitrification between TYNL and (F.R.S. plus carbon) incubations is the different loadings of nitrate (1.650 mg NO_3^--N versus 0.0014 mg NO_3^--N) although this could be coupled with the availability of proteins and vitamins for enzyme synthesis and growth in the TYNL incubations. The influence of carbon and nitrate on denitrification will be dealt with in Sections 7.7.3 and 7.7.4 respectively.

10 ml of TYNL permitted the denitrification of 0.75 mg more N_2O-N than 3 ml of TYNL within the same time period. Observation of the 3 ml TYNL incubation demonstrated that it was only moistened by the additions in comparison to 10 ml of TYNL which saturated the soil. In addition to a greater abundance of carbon, nitrate and other nutritional elements in the 10 ml TYNL incubation, diffusion of materials to sites of active denitrification would be facilitated by saturated conditions.

TYNL media (10ml) was chosen as the standard addition for laboratory incubations of wetland soil during the acetylene blockage technique.

7.6 DENITRIFICATION PROFILES

Acetylene blockage experiments under optimum conditions of nitrate concentration, carbon supply, aeration status, pH, diffusion and temperature were conducted to examine the role of depth in the soil profile (0-25cm) in controlling denitrifier activity, and to determine any influence of effluent loading on populations of soil denitrifiers. The latter was achieved by comparing soils from within the wetland (identical geology and soil formation processes, Johnson, pers comm.) that had no effluent inflow with those that had a continuous effluent inflow.

Cores from above and below the point of effluent inflow (see Figure 5.2) were sectioned at the following depths:

0 - 6 cm
7 - 14 cm
18 - 25 cm

For the below effluent inflow core (B.E.I.) three replicate 10g samples of mixed soil were taken from each depth interval. Problems were encountered with analysing this number of samples because of the length of time required to process each one. As a result only two replicate 10g samples from each depth interval were taken from the above effluent core (A.E.I.) so reducing the number of samples from nine to six. This greatly increased the precision and ease of analysis over the forty hour period. Differences in denitrifiction activity were still clear in the A.E.I. core despite the reduction in replicates (see Figure 7.6.e.).

The analysis of the B.E.I. core was conducted in September 1984. Due to instrument problems the analysis of the A.E.I core was delayed until February 1985. Any differences between denitrification in A.E.I. and B.E.I. soil will therefore be discussed in relation to the possible influence of season.

In addition to the analysis of core material from three depth intervals an analysis was made of denitrification in surface decaying organic matter (partially decomposed plant material). The analysis was conducted one month prior to core analysis (August 1984). Some of the material was been preincubated for several weeks with carbon and nitrate additions. Despite the nutritional additions the preincubated samples exhibited lowered denitrification activity compared to fresh material analysed at the same time (see Figure 7.4).

7.6.1 GENERAL DESCRIPTION OF CORES

All samples taken from below 7cm depth in the A.E.I. core were very black in colour and contained considerable quantities of un-oxidised mica in comparison to the B.E.I. core. In addition the complete absence of any small segmented worms (1-2cm) from all A.E.I samples pointed to a highly reduced environment.

In contrast the B.E.I. core samples were predominantly brown in colour down to 14cm. The 18-25cm samples were black and had high quantities of unoxidised mica. The 0-6cm and 7-14cm depth samples consistently contained up to four small segmented worms per 10g of soil. These invertebrates could be influential in the distribution of solutes and gases to and from sites of active denitrification within the soil profile (Knowles, 1982). In addition populations of denitrifiers may exist in or on the worms thus contributing directly to nitrogen removal (Chatarpaul et al., 1980).

These qualitative differences related to the oxidation-reduction status of the soil may be influenced by the seasonal difference between the core samples. The apparent higher reduction status in the A.E.I. core may caused by higher rates of microbial activity in summer depleting oxygen within the soil. Conversely the B.E.I. core may be less reduced because of the continuous input of nitrate in the effluent. The different sampling times according to season may explain why the A.E.I. core appears more reduced than the B.E.I. core despite the observation that Eh in autumn above the inflow was higher than that below (see Section 7.1.1.).

7.6.2 RESULTS OF ACETYLENE BLOCKAGE INCUBATIONS

The results of maximum denitrification rate and N_2O-N accumulation (total activity) over the experimental period for all the soils are contained in Table 7.10. Time-course accumulations of N_2O-N for decaying surface vegetation (D.S.V.) versus 0-6cm soil are contained in Figure 7.4. Accumulations over time for B.E.I. core samples are contained in Figure 7.5 to 7.7, and for A.E.I. core samples in Figure 7.8.

FIGURE 7.4

TIME-COURSE ACCUMULATION OF N_2O-N FOR 10g SOIL SAMPLES OF 0-6 cm CORE MATERIAL (▵,▵) AND DECAYING SURFACE VEGETATION (▪,▫)

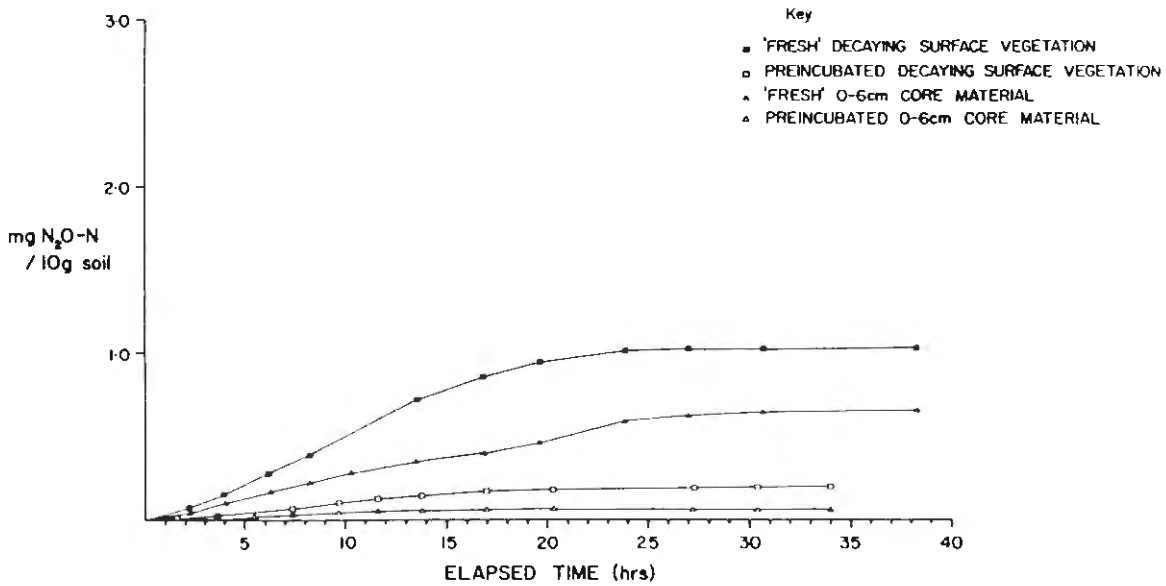


FIGURE 7.5

TIME-COURSE ACCUMULATION OF N_2O-N FOR 10g SOIL SAMPLES FROM BELOW EFFLUENT INFLOW CORE (SEPTEMBER 1984). THREE REPLICATE SAMPLES FROM 0-6cm DEPTH IN THE PROFILE

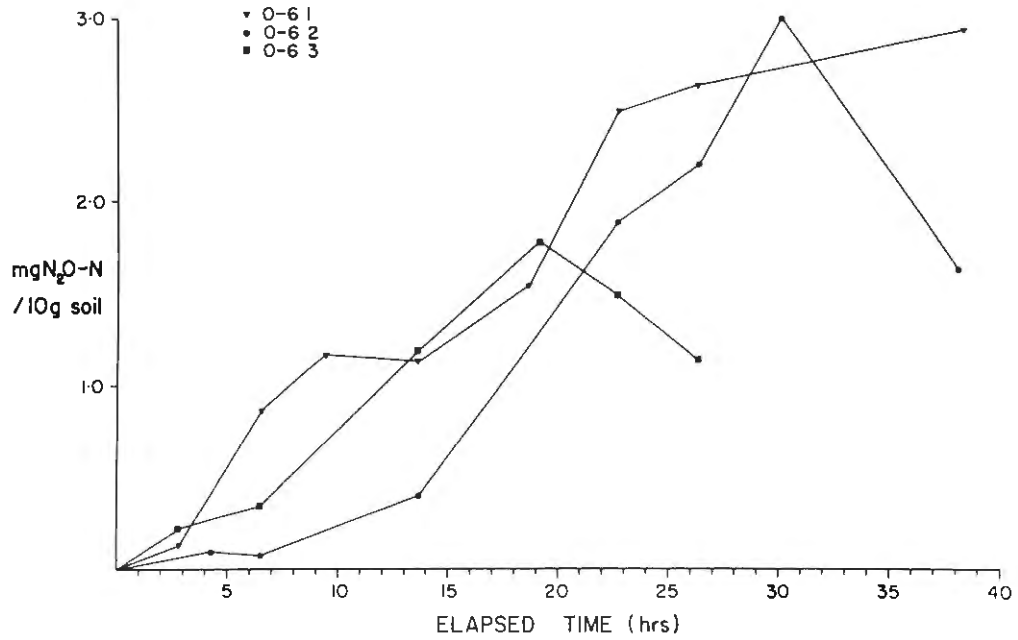


FIGURE 7.6

TIME-COURSE ACCUMULATION OF N_2O-N FOR 10g SOIL SAMPLES FROM A BELOW EFFLUENT INFLOW CORE (SEPTEMBER 1984). THREE REPLICATE SAMPLES FROM 7-14cm DEPTH IN THE PROFILE

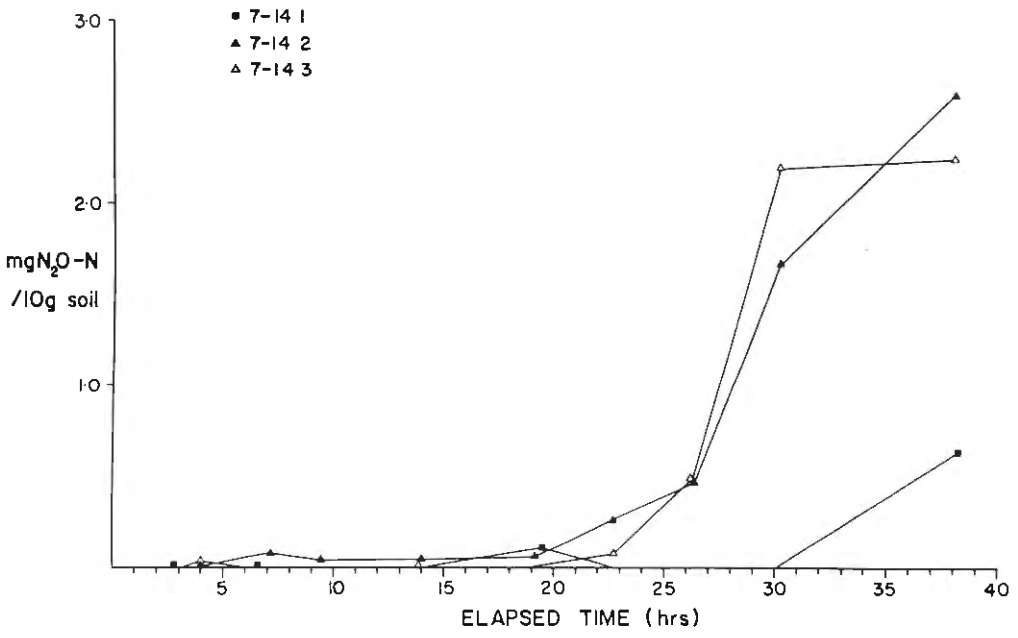


FIGURE 7.7

TIME-COURSE ACCUMULATION OF N_2O-N FOR 10g SOIL SAMPLES FROM A BELOW EFFLUENT INFLOW CORE (SEPTEMBER 1984). THREE REPLICATE SAMPLES FROM 18-25cm DEPTH IN THE PROFILE

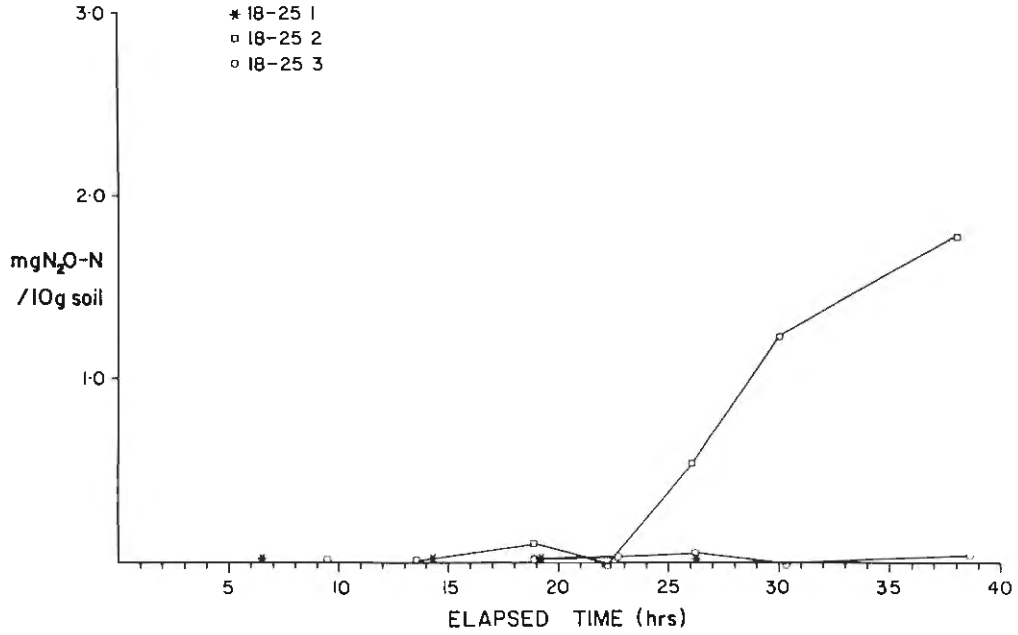


FIGURE 7.8

TIME-COURSE ACCUMULATION OF N_2O-N FOR 10g SOIL SAMPLES FROM ABOVE EFFLUENT INFLOW CORE (FEB. 1985.) SAMPLES TAKEN FROM THREE DEPTHS IN THE PROFILE

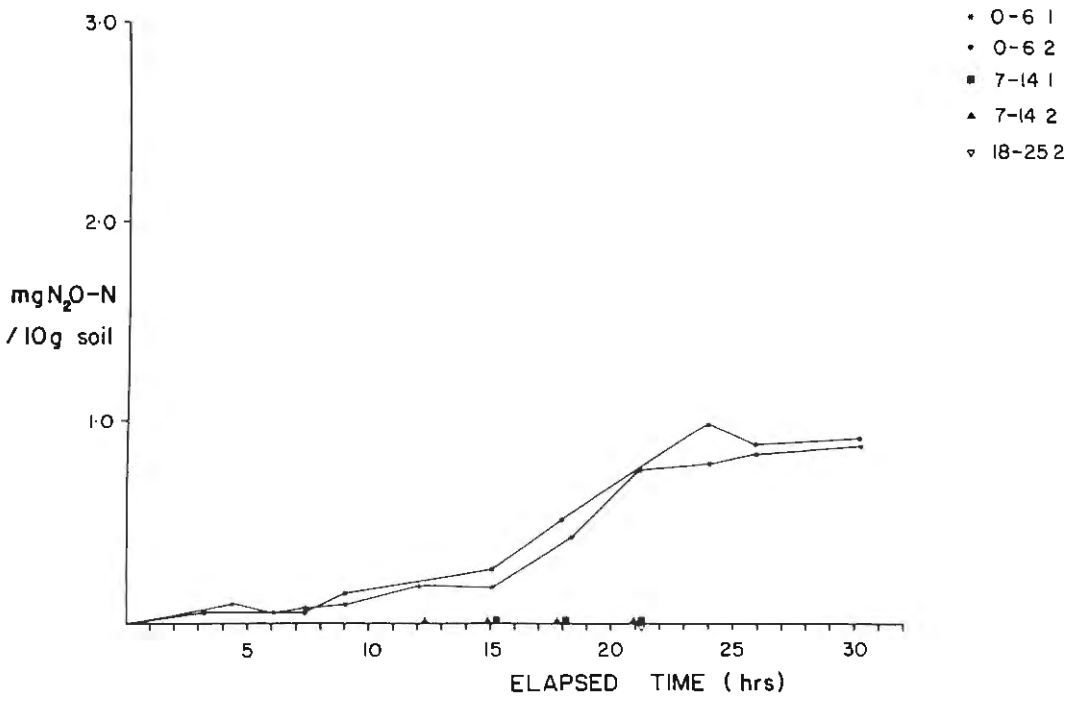


Table 7.10 Maximum denitrification rate per gram of soil and total nitrous oxide-N liberated by each 10g soil sample

Sample	Max. rate of denitrification mgN_2O-N/hr/g	1st Observation of denitrification hrs + mins	Max. accumulation of N_2O-N mg
Fresh D.S.V.	0.0056	Time 0	1.071
Preinc.D.S.V.	0.0014	Time 0	0.187
Fresh 0-6cm	0.0033	Time 0	0.650
Preinc.0-6cm	0.0008	1.35	0.055
A.E.I. 0-6 1	0.00134	4.23	0.871
A.E.I. 0-6 2	0.01	3.13	0.988
B.E.I. 0-6 1	0.0235	2.50	2.957
B.E.I. 0-6 2	0.025	4.19	3.07
B.E.I. 0-6 3	0.0121	2.48	1.785
A.E.I. 7-14 1	-	15.16	<0.0003
A.E.I. 7-14 2	-	12.16	<0.0003
B.E.I. 7-14 1	0.008	2.48	0.64
B.E.I. 7-14 2	0.0333	4.02	2.594
B.E.I. 7-14 3	0.0484	4.02	2.245
A.E.I. 18-25 1	-	-	-
A.E.I. 18-25 2	-	21.17	<0.0003
B.E.I. 18-25 1	-	6.30	<0.0003
B.E.I. 18-25 2	0.192	13.35	1.762
B.E.I. 18-25 3	0.003	9.30	0.003

7.6.3 DECAYING SURFACE VEGETATION VERSUS 0-6CM SOIL

The graphical (Figure 7.4) and tabled results show that levels of denitrification in the decaying surface vegetation appear to be higher than those in 0-6cm soil. This may be because the supply of nitrate is more accessible to the decaying surface vegetation (D.S.V.) denitrifiers compared to those in the soil because diffusion of nitrate directly from the overlying water can occur. In addition the D.S.V. denitrifiers have immediate access to a large supply of organic carbon being decomposed by aerobes.

Denitrifier populations in the D.S.V. are probably located at anaerobic microsites. These could quite easily form as the material is composed of closely tangled strands forming a dense mat over the soil surface.

Decaying plant material not yet incorporated into the soil profile is a potential source of nutrients via leaching (Klopatek, 1978). The large quantities of such material within Thredbo wetland could be a source of increased nutrient loading to the surface waters. It is encouraging to see from these results that active denitrifier populations are located within the material, and so involved with the removal of nitrogen from the surface water as it is released by leaching and decomposition.

In terms of the general status of the wetland as a means of nitrogen removal it appears that denitrification by bacteria in decaying vegetation across the wetland is of considerable importance.

7.6.4 ABOVE AND BELOW EFFLUENT INFLOW SOIL

A similar pattern of denitrification activity with depth was observed in both the above and below effluent inflow cores. In agreement with other studies the highest denitrifier activity was located in the top segment (0-6cm) of the soil profile. Jones (1979) examined denitrification in freshwater lake sediments and found that activity was restricted to the 0-8cm depth segment. In Danish coastal sediments 90% of the measurable denitrification was attributed to the top 0-6cm (Kasper, 1982), and in

another study of estuarine sediments no denitrification was observed below 7cm (Sorensen et al., 1979). It has been shown consistently that this location of peak denitrifier activity in the upper soil segment coincides with the area of peak NO_3^- concentration (Oren and Blackburn, 1979; Klingensmith and Alexander, 1983). The result of (NO_3^- + NO_2^-)-N profiles in 0-30cm soil (Section 7.3) suggest that a similar relationship between denitrifier activity and inorganic nitrogen concentration is operating in the 0-6cm segment of the soil at Thredbo.

Below the 0-6cm segment there was a considerable reduction in denitrifier activity. In many soils this has been linked to the depletion of NO_3^- with depth (Nishio et al., 1982; Nedwell, 1982; Klingensmith and Alexander, 1983; Sorensen, et al., 1979). However results of (NO_3^- + NO_2^-)-N distribution with depth in the soil (Section 7.3) show that they are present down to 30cm. Therefore it is unlikely that NO_3^--N concentration is limiting denitrification at depths below 6cm.

7.6.5 THE PATTERN OF ACTIVITY IN INCUBATIONS

The Figures 7.4 to 7.8 showing accumulation of N_2O-N over time, exhibit curves which are typical of microbial growth and activity (Brock, 1979). There is an initial lag phase which is brief or extended depending on conditions within each incubation. In terms of the denitrifier populations in 0-6cm samples the lag period probably represents the time required to generate the enzymes and cell constituents necessary for denitrification and growth under optimum conditions. In samples from below 6cm depth the lag may represent the time required for a very small starting population to multiply to a size where detectable rates of denitrification are achieved.

The lag phase is followed in the majority of samples by a period of exponential growth/activity. Under optimum conditions differences in activity at this stage are caused by inherent genetic limitations within the sample populations, and/or different environmental conditions within each incubation caused by differences between the soil samples (Brock, 1979).

In a closed culture vessel the populations cannot grow indefinately at an exponential rate because either essential nutrients are exhausted or toxic metabolic products accumulate. Activity therefore ceases due to self-crowding and so a stationary phase is achieved which is followed by the death phase.

Within this type of incubation under optimum conditions there are three factors associated with denitrifier activity which can be used to distinguish between samples. These are the length of the lag period, the rate of activity, and the total activity. The length of the lag period gives an indication of the size of the population originally present in the soil (small populations lead to a longer lag), the rate of activity may not differ between samples but the total activity may differ due to factors related to the nature of the sampling populations, and the development of specific environmental conditions within each sample (Brock, 1979).

7.6.6 DENITRIFICATION IN 0-6CM DEPTH SOIL SAMPLES

A comparison of maximum denitrification rates in 0-6cm samples from above and below the effluent inflow found no significant difference ($p>0.05$). However there was a difference in total activity measured in terms of total N_2O-N accumulation over the experimental period ($p>0.05$). As conditions of nitrate concentration, carbon supply, pH, temperature, diffusion and aeration status were optimum the difference in total activity must be a function of other limiting factors such as: trace element supply; growth factors; or the toxicity of, and inhibition by organic or inorganic chemicals (Barnes and Bliss, 1983). This would result from some inherent difference in the soils and/or the denitrifier populations at the time of sampling.

The above effluent inflow core was sampled in summer when biological activity would have been high. The immediate onset of denitrification on incubation indicates that there was a physiologically active population of denitrifiers in the soil sample (Oremland et al., 1984). However despite this the A.E.I. samples were unable to achieve total activity of the order of that in 0-6cm B.E.I. soil which was sampled in winter when one would expect denitrifier populations to be restricted in activity due to the low temperatures. The lower rates in A.E.I soil may be a function of in situ aeration status limiting the size and activity of the denitrifier population.

The major trace elements required by denitrifiers are molybdenum, iron, copper and magnesium (Bryan, 1981). As both above and below inflow areas of the wetland have the same geology and soil type (see Section 5.5) it is expected that supplies of these elements would be similar and thus if they were limiting in one area they would be limiting in another. This would depend to a certain extent on the aeration status of the soils but both areas have been shown to have a similar aeration status (Section 7.1.1.).

The other factor which may be limiting total activity in the above inflow soil is some form of inhibition by an organic or inorganic substrate. Very few naturally occuring inhibitors of denitrification in the soil are known (Bryan, 1981) but inhibition by sulfide has been observed in soils (Myers, 1972), sediments (Sorensen et al., 1979) and pure cultures of denitrifying bacteria (Tam and Knowles, 1979; Sorensen et al., 1980). Denitrification and sulfate reduction to sulfide can occur concurrently within sediments but they have been shown to have a mutually exclusive pattern in regard to their spatial distribution (Sorensen et al., 1979). It may be that the presence of sulfide in the 0-6cm soil above the effluent inflow is restricting the size of denitrifier populations and limiting them to specific microsites where conditions are conducive to denitrification. This could well explain

why similar rates of denitrification are achieved in the laboratory whilst total activity between above and below effluent inflow samples differ significantly.

Greater total denitrification activity in below effluent inflow soils may be caused by the addition of wastewater. Nishio et al. (1982) observed a similar higher denitrification activity in the eutrophicated Tama estuary compared to the sediments in oligotrophic Odawa Bay and Tokyo Bay.

7.6.7 DENITRIFICATION IN SAMPLES FROM 7CM TO 25CM

Soils from below 7cm in A.E.I. and B.E.I. soils show a very different pattern of nitrous oxide accumulation during the experimental period compared to the 0-6cm soil samples (see Figures 7.5 to 7.8).

In the above inflow soils below 7cm denitrification activity was very low with isolated traces of nitrous oxide (<0.0003 mg/g) being observed during the experimental period following a lag of at least 12-16 hours. This indicates that although bacteria capable of denitrification existed at these depths they were not actively denitrifying at the time of core sampling. Such bacteria may be utilising an alternate electron acceptor but are capable of switching to NO_3^--N (Oremland et al., 1984). The fact that (NO_3^- + NO_2^-)-N is fairly abundant in the soils below 7cm (see Section 7.3), and the black colouration of the samples suggests that denitrification in 7-25cm soil may be inhibited by sulfide. On incubation under optimum conditions as described, some bacteria were able to become active within microsites whilst the majority of the soil remained 'inaccessible' due to the presence of sulfide. In addition a low carbon supply may be limiting dentrification below 7cm depth.

This also appears to be the case for 18-25cm soil samples from below the effluent inflow (see Figure 7.7).

The pattern of N_2O-N accumulation exhibited by samples 7-14 (1 to 3) and 18-25 (3) in the below effluent inflow core is significantly different ($p>0.05$) from that of samples at the same depth but above the inflow.

In B.E.I. 7-14cm and 18-25cm (3) samples exponential denitrification activity not significantly different to that in 0-6cm samples ($p>0.05$) occured after an eighteen hour lag. It appears that in the first 18-22 hours of incubation the existing populations of denitrifiers are being restricted to a low rate of activity. This inhibitor may be sulfide which keeps denitrification activity low until sulfate reduction ceases. Alternatively the 18-22 hour lag prior to exponential denitrifier activity may represent the time required for the bacteria using a substrate other than nitrate to generate the enzyme system necessary for denitrification. Following this the denitrifiers are able to increase in activity and reproduction, moving out from microsites to take advantage of the optimum conditions created by the incubation procedure.

7.6.8 POPULATION DYNAMICS AND ACTIVITY *IN SITU*

It has been suggested that incubations of the kind employed here may accurately mirror the *in situ* population dynamics and patterns of activity through time. Smith *et al.* (1978) describe the field situation as exhibiting bursts of denitrification against a background of slow yet continuous activity year round. In the short-term these results may mirror the response of populations located in microsites which are able to expand spatially and increase in activity when conditions for denitrification in the surrounding soil improve (Kasper, 1982; Firestone and Tiedje, 1979). It appears that in both A.E.I. and B.E.I. soils denitrifier populations become smaller and more restricted to microsites with depth in the soil profile. In B.E.I. soil below 7cm the populations are better able, and/or the environment is more suitable to achieve the type of denitrification response described by Smith *et al.* (1978) than the A.E.I. soil. It is suggested that this is a function of effluent loading.

7.6.9 CONCLUSION

The response of denitrifiers to optimum conditions (total denitrification activity) in laboratory incubations differs significantly according to site and depth ($p>0.05$). It appears that denitrification activity at all depths in soil from below the effluent inflow is higher than that of soil from above the effluent inflow. It is suggested that this is a function of the effluent ameliorating conditions in terms of $(NO_3^- + NO_2^-)$-N concentration (Section 7.3.), aeration status of the soil (Section 7.1.1.), soil temperature (Section 7.1.3.), seasonal carbon supply from readily decomposable plant material (Section 5.4.), burrowing activity of invertebrates (Section 7.6.), and the large annual input of NH_4^+-N which would be available for nitrification to NO_3^--N (Section 7.3.).

Despite these differences denitrifier activity shows a similar decline with depth in the soil profile of both A.E.I. and B.E.I. soil possibly due to inhibition by sulfate respiring bacteria limiting denitrification to small populations within microsites.

7.7 FACTORS INFLUENCING DENITRIFICATION IN 0-6CM DEPTH SOIL FROM BELOW THE EFFLUENT INFLOW

A series of experiments were conducted to identify the physico-chemical factors which limit denitrification in the soil at Thredbo wetland.

7.7.1 pH

Values of pH recorded in the field at the Thredbo wetland ranged from 6.4 to 6.7 in the top 0-5 cm of the soil. To determine the affect of pH, 10 g samples of 0-6 cm depth soil were incubated with TYNL adjusted to a pH at the lower end of the range recorded in the soil at Thredbo (pH 6.35).

Incubations were established according to the general procedure (Section 6.4.2). Conditions of carbon, nitrate and general nutrition were held at optimum during the experiment so that the influence of pH could be observed.

Results of maximum denitrification rate and the total amount of N_2O-N liberated in the experimental period are contained in the following table.

Table 7.11 Maximum hourly rates of denitrification per gram of soil, and total N_2O-N liberated by 10 g soil samples at pH 6.35.

Sample	Max. rate of denitrification mg N_2O-N/hr/g	Max. accumulation of N_2O-N (mg)
pH 6.35 1	.0240	2.957
pH 6.35 2	.0150	3.066

It appears that the denitrifiers in the 0-6 cm depth samples operate quite effectively at pH 6.35. Denitrification was not depressed as it was in incubations monitoring the influence of other limiting factors such as temperature (see Section 7.7.2.).

As outlined in Section 4.2 there is a dispute as to the influence of pH on denitrification. Some workers have observed depressed rates of denitrification at low pH, with optimum rates achieved at pH 7-8 (Bremner and Shaw, 1958). Others have observed significant losses of nitrogen down to pH 4.7 (Expete and Cornfield, 1965).It seems that the in situ denitrifier populations at Thredbo were active in both samples prior to incubation and were stimulated by the additions of carbon and nitrate, the influence of pH being negligible.
It appears that the pH of the soil at Thredbo is having little influence

on denitrification in the field. This is demonstrated by the high activity of bacteria in laboratory incubations adjusted to pH 6.35. Other soils around pH 6.3 have been shown to support denitrification in this way (Broadbent, 1951).

7.7.2 TEMPERATURE

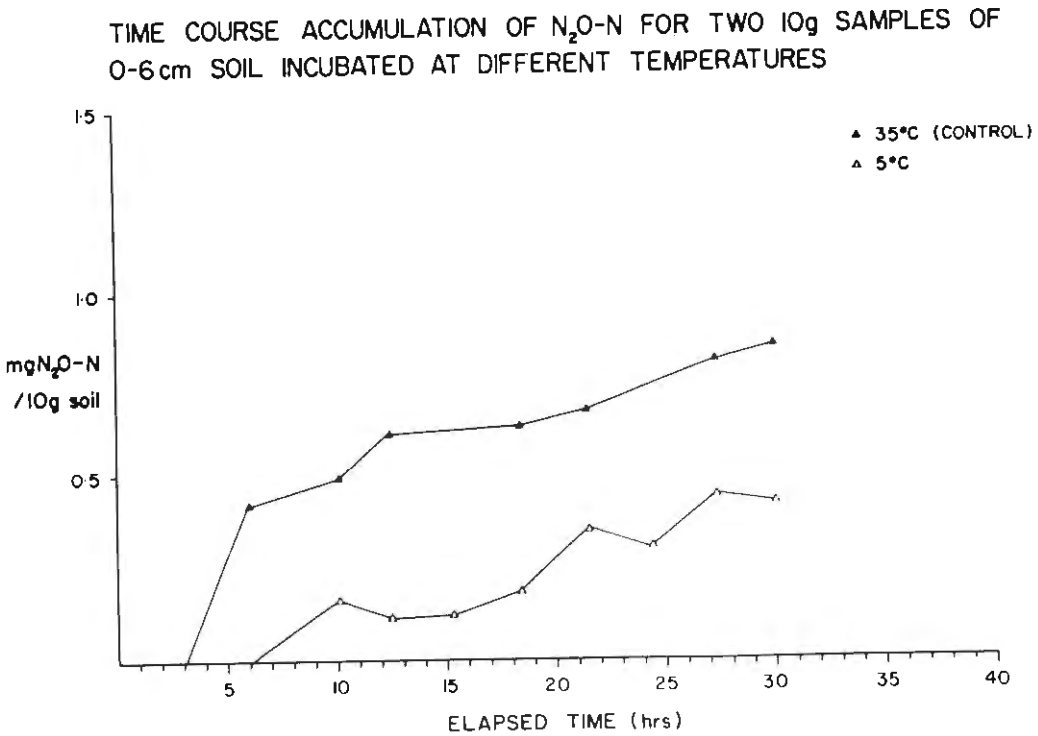
Analysis of the influence of temperature on denitrification was conducted on 10g samples of 0-6 cm core material . One sample was incubated at 35°C and the other at 5°C. Both samples had a 10ml addition of TYNL pH 6.35 and remained unshaken.

Maximum rates of denitrification for each sample, and total amounts of N_2O-N liberated in each experimental period are contained in Table 7.12. The accumulation of N_2O-N during the experimental period is shown graphically in Figure 7.9.

Table 7.12 Maximum hourly rates of denitrification per gram of soil and total N_2O-N liberated by 10g of 0-6 cm soil incubated at different temperatures.

Sample	Max. rate of denitrification mg N_2O-N/hr/g	Max. accumulation of N_2O-N mg
35°C	0.0136	0.850
5°C	0.0054	0.443

FIGURE 7.9



In Section 7.1.3. information on seasonal changes in soil and air temperatures were discussed. It was concluded that in mid-winter temperatures in the top 0-6 cm of the soil below the effluent inflow could remain at 5°C or below for a large proportion of the day. Even the temperature amelioration caused by the inflowing effluent may not be sufficient to raise soil temperatures above 5°C. The importance of this has been shown by investigations in which denitrification has been severely depressed or halted by temperatures of 5°C and below (Bremner and Shaw, 1958; Bailey, 1976). Such an effect at Thredbo wetland may effect its usefulness as a means of nitrogen removal by denitrification. Denitrification in soil from Thredbo wetland was not halted at 5°C but

showed a maximum hourly rate of 0.0054 mg N_2O-N/hr/g soil when loaded with 0.165 mg NO_3^--N/g soil. This was approximately half the rate achieved by denitrifiers incubated under identical conditions but at a temperature of 35°C. When one considers the levels of (NO_3^- + NO_2^-)-N recorded in 0-6 cm soil over the winter period [0.0094 to 0.0189 mg (NO_3^-+ NO_2^-)-N/g] the rate of denitrification achieved at 5oC would permit a considerable removal of nitrogen to occur over winter.

It appears that denitrification continues at 5°C in 0-6 cm soil but at a reduced rate compared to higher temperatures up to 35°C. The combined influence of other factors such as nitrate concentration and diffusion at low temperatures in situ is not known, although the samples were incubated unshaken (diffusion not facilitated) and yet maintained reasonable rates of denitrification. The nitrogen removal efficiency of the wetland may be dependent on the ability of the soil and plants to store nitrogen species until soil temperatures rise and higher rates of denitrification can be achieved.

7.7.3 CARBON SUPPLY

Two 10g soil samples were incubated with (TYNL) and without (TYN) a carbon source although some carbon was available to both soil samples in the form of tryptone and yeast. The material used was 0-6 cm depth soil incubated according to the procedure in Section 6.4.2.

This experiment was designed following the observations made during the development of the incubation procedure for acetylene blockage (Section 7.5). It was found that Frog Ringers Solution (F.R.S.) plus a carbon source in the form of glucose or lactate did not promote rates of denitrification as high as those samples incubated with TYNL media. It appeared that some nutritional factor in addition to carbon was limiting denitrification in the F.R.S. incubations. As conditions of pH, diffusion and temperature were optimum it was assumed that the presence of extra nitrate, proteins and vitamins in the TYNL media was causing the difference in denitrification rate between the samples. By comparing TYNL with TYN (no lactate) it was hoped to identify the role

of the carbon source in denitrification. If it were found that the soil was not dependent on an external carbon source this would also suggest that the 0-6 cm soils below the effluent inflow at Thredbo have an adequate supply of organic carbon to maintain denitrification under optimum conditions of nitrate concentration, temperature and diffusion.

The time-course accumulation of N_2O-N for two 10g samples can be seen in Figure 7.10. Results representing maximum hourly rates of denitrification and the total amount of N_2O-N liberated in the experimental period are contained in Table 7.13.

FIGURE 7.10

TIME-COURSE ACCUMULATION OF N_2O-N FOR 10g SOIL SAMPLES (0-6cm) INCUBATED WITH (TYNL) AND WITHOUT (TYN) A CARBON AMENDMENT

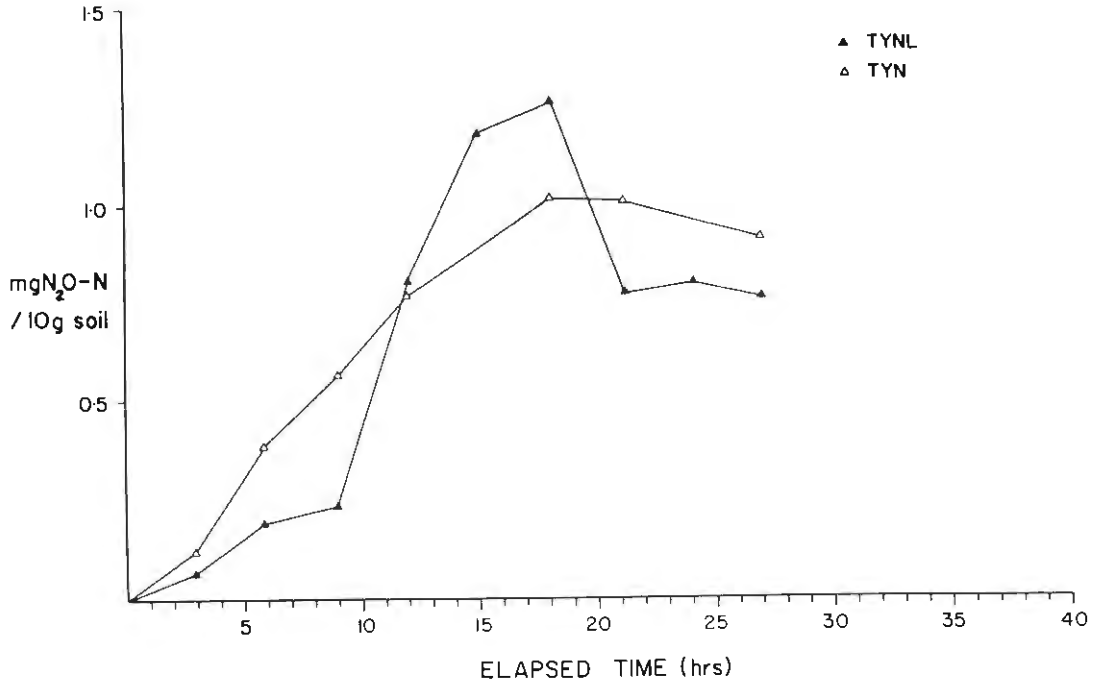


Table 7.13 Maximum hourly rates of denitrification per gram of soil, and total N_2O-N liberated by 10g soil samples receiving different nutritional treatments.

Sample	Max. rate of denitrification N_2O-Nmg/hr/g	Max. accumulation of N_2O-N mg
TYNL	0.0189	1.263
TYN	0.0103	1.017

Although some reduction in maximum hourly rate and accumulation of N_2O-N is seen in the TYN sample there is not a large reduction in rate as compared to that caused by incubation at low temperature (see Section 7.7.2). Also the maximum hourly rate of denitrification in the TYN sample is higher than those recorded on previous occasions in samples incubated with TYNL (see Section 7.5), or those with TYNL where another influencing factor was being monitored (see Section 7.7.5). Therefore it appears that the availablility of carbon is not limiting denitrification in the field. This may be a function of the growth form of the major plant species in the below effluent inflow sampling area. The seasonal input of readily decomposable organic material by R. Crispus and E. Sarmentaceum would appear to provide adequate organic carbon for the denitrifiers in the top 0-6 cm of the soil.

Coupling these results with those of the decreased activity in the [F.R.S. plus carbon] as opposed to TYNL samples it is possible to conclude that the denitrifiers are more limited by the availablility of protein (Tryptone), vitamins (yeast extract), and nitrate than by carbon when other conditions of pH, diffusion and temperature are optimum.

Organic carbon supply does not limit denitrification in 0-6 cm soil from below the effluent inflow at Thredbo wetland when it is incubated under optimum conditions of temperature, diffusion, aeration status, pH and nitrate supply. This suggests that it is also non-limiting *in situ*.

7.7.4. NITRATE SUPPLY

Ten gram soil samples were taken from the 0-6 cm segment of a below-effluent inflow (B.E.I.) core. Incubations were established according to the procedure in Section 6.4.2. Two different types of media were used to analyse for any influence that nitrate concentration was having on denitrification. The samples were established as follows:

1. 10 ml TYL (no added NO_3^--N)
2. 10 ml TYL + 1 ml 100 mg/l NO_3^- (0.0014 mg NO_3^--N)
3. 10 ml TYNL (1.650 mg NO_3^--N)

The incubations were maintained for seven hours after which a failure in the gas chromatograph prevented further study being conducted.

Table 7.14 shows the time-course accumulation of N_2O-N for each gram of soil in the samples. Table 7.15 gives the maximum hourly rate of denitrification for each sample.

Table 7.14 Time-course accumulation of N_2O-N over a seven hour incubation

Time hrs	TYL mgN_2O-N/g	Time hrs	TYL+0.0014mgNO_3^--N mgN_2O-N/g	Time hrs	TYNL mgN_2O-N/g
0.00	-	0.00	-	0.00	-
3.03	0.0054	3.31	0.0115	3.09	0.0123
6.20	0.0120	5.56	0.0064	6.56	0.0117

Table 7.15 Maximum hourly rates of denitrification for each gram of soil in the samples

Sample	NO_3^--N addition mg/g	Max. hourly rate mgN_2O-N/hr/g
TYL	-	0.0021
TYL	0.00014	0.0035
TYNL	0.1650	0.0040

Both the rate of denitrification and the total activity recorded over seven hours was very similar in all samples regardless of NO_3^--N addition. Smith et al.(1978) have predicted that in incubations under optimum conditions activity within the first two to eight hours is more

representative of indigenous denitrification compared to rates and activity in the following hours where population size has been 'artificially' increased. They supported their theory by the addition of a protein synthesis inhibitor (chloramphenical) to their incubations. The inhibitor had no effect on the initial phase of denitrification but markedly decreased subsequent rate increases. The findings of Smith *et al.* are important because they suggest that *in situ* levels of nitrate in the soils at Thredbo wetland are supporting denitrification at rates very similar to those observed in these seven hour incubations.

Major differences in denitrification rates between samples may have occurred later in the incubations if NO_3^--N became limiting in those with low NO_3^--N loadings (Smith *et al.*, 1978). This would be a function of the capacity of the *in situ* populations of denitrifiers to multiply in response to the ameliorated conditions. Unfortunately this could not be observed due to instrument failure.

These results suggest that *in situ* levels of NO_3^--N below the effluent inflow are supporting denitrification. Other results have shown that pH (Section 7.7.1) and carbon (Section 7.7.3) are not limiting denitrification *in situ* but that temperature (Section 7.7.2) and diffusion (Section 7.7.5) could be. *In situ* it could well be the interaction of these factors which may be controlling the rate of denitrification.

7.7.5 DIFFUSION

Incubations of 0-6 cm soil were established according to the general procedure (Section 6.4.2).

The samples may only be compared in pairs as the two experiments were conducted at different times on 0-6 cm soil from different cores. The comparisons are as follows:

10ml TYNL shaken v. 10ml TYNL unshaken
10ml TYNL shaken 25°C v. 10ml TYNL unshaken 35°C

The time-course accumulations of N_2O-N in the samples are shown in Figures 7.11 and 7.12. Results of maximum rate of denitrification per gram of soil, and the total amount of N_2O-N liberated in the experimental period are contained in Table 7.16.

FIGURE 7.11

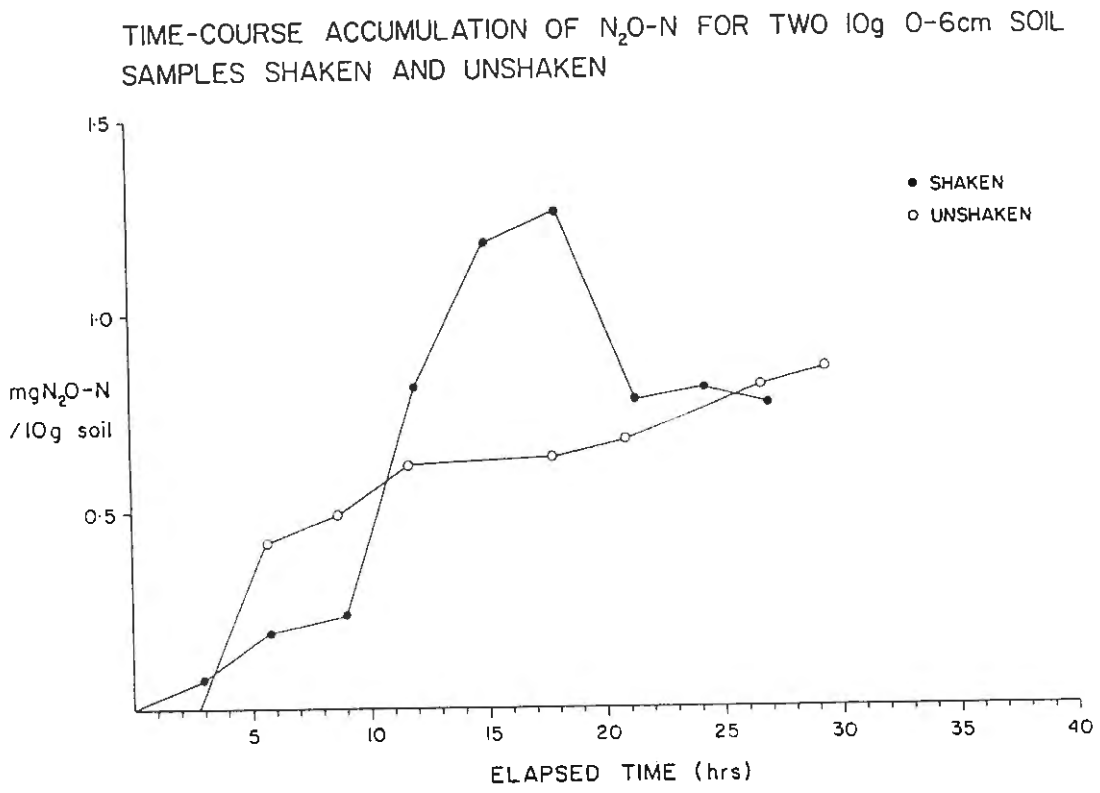


FIGURE 7.12

TIME-COURSE ACCUMULATION OF N_2O-N FOR TWO 10g SAMPLES OF 0-6cm DEPTH SOIL INCUBATED AT TWO DIFFERENT TEMPERATURES AND UNDER DIFFERENT CONDITIONS FOR DIFFUSION

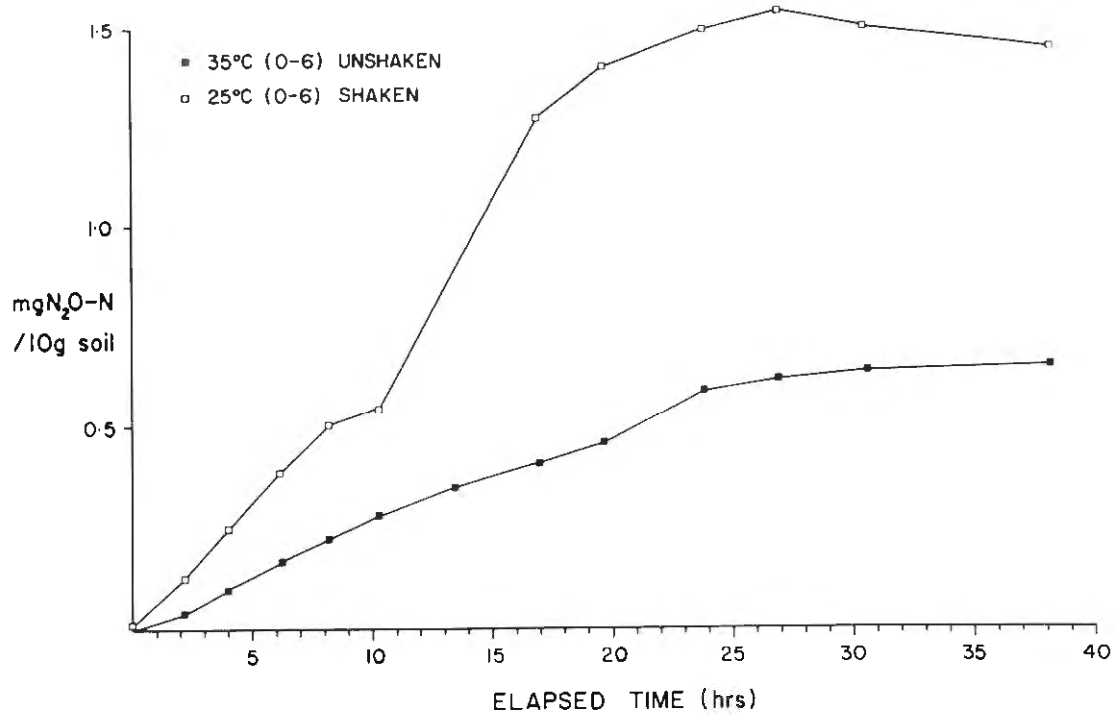


Table 7.16 Maximum hourly rates of denitrification per gram of soil, and total N_2O-N liberated by 10g soil samples incubated to monitor the influence of diffusion.

Sample	Max. rate of denitrification mgN_2O-N/hr/g	Max. accumulation of N_2O-N mg
10ml TYNL Shaken 35°C	0.0189	1.263
10ml TYNL Unshaken 35°C	0.0136	0.850
10ml TYNL Shaken 25°C	0.0115	1.541
10ml TYNL Unshaken 35°C	0.0033	0.650

Figure 7.11 and Figure 7.12 show higher maximum rates of denitrification and greater accumulation of N_2O-N in shaken samples compared to unshaken. Shaking the sample may reduce the influence of soil structure which can inhibit the diffusion of NO_3^--N even under conditions of maximum loading (Lance et al., 1976). The soil at Thredbo is very fine textured (see Section 5.5) and very cohesive, both factors which would reduce the ease of diffusion.

Figure 7.12 shows a higher maximum accumulation of nitrous oxide occurring at 25°C than at 35°C. One would expect an increase in denitrification rate from 25°C to 35°C but this did not occur.

The results of the shaken versus unshaken experiments suggest that even under optimum conditions of NO_3^--N loading (0.1640 mg/g) diffusion still influences denitrification. This demonstrates that the influence of shaking (facilitated diffusion) on denitrification activity exceeded that of temperature.

In relation to this study we can review the influence of diffusion operating on two scales in the wetland: first moving NO_3^- from the surface water in the wetland across the soil-water interface and into the soil; second from the soil water to sites of active denitrification within the soil (interaggregate or intraaggregate microsites). It is important to note that diffusion of NO_3^- has been seen to be retarded by low temperature (Reddy and Patrick, 1984). This may influence denitrification in winter by controlling the availablity of NO_3^-. It may also help to explain why NO_3^- + NO_2^- appear to accumulate below 15cm in the below effluent inflow soil. In terms of structure the compact nature of the organic soil may be inhibiting diffusion in situ as the shaking of laboratory incubations increased the rate of denitrification.

CHAPTER 8 CONCLUSION

Physico-chemical measurements in soils from above the effluent inflow and below the effluent inflow at Thredbo Wetland have shown that the effluent ameliorates the soil environment for denitrification by lowering Eh, raising the soil temperature, and increasing loadings of $(NO_3^- + NO_2^-)$-N and NH_4^+-N. As a result denitrification potential was highest in samples from below the effluent inflow, the greatest potential for denitrification being located in areas of decaying surface vegetation.

The relative distribution of $(NO_3^- + NO_2^-)$-N and NH_4^+-N in relation to effluent loading, depth and season showed that:

- Ammonium ion was significantly increased throughout the soil profile below the effluent inflow. Nitrification of this ammonium ion to nitrate could enhance denitrification in soil below the point of effluent inflow.

- Nitrate plus Nitrite were increased in the top 0-2cm of the soil from below the effluent inflow providing a larger supply of these ions for denitrifiers in the soil.

- The rapid decline in nitrate plus nitrite from 2cm to 6cm depth in the soil is indicative of denitrification. This was supported by the coincidence of this depth segment with the area of peak denitrification potential during acetylene blockage experiments.

- Seasonal influences were obscure in the case of ammonium ion but the concentration of nitrate plus nitrite in the top 0-6cm of soil cores from above and below the effluent inflow increased or decreased according to season.

It was also found that the exact concentrations of $(NO_3^- + NO_2^-)$-N and NH_4^+-N could not be obtained because the 'standard' soil extraction technique employed by many workers was not an effective means of acheiving quantifiable results. The widespread use of this technique brings into question the validity of many published results.

The factors with the greatest influence on denitrification appear to be temperature, diffusion and depth in the soil profile as these caused a reduction in the rate of denitrification in laboratory incubations. Carbon supply and pH had little influence on denitrification. Denitrification potential could not be related to the depth distribution of NH_4^+-N and (NO_3^- + NO_2^-)-N except in 0-6cm depth soil where it appeared to be related to the high nitrate plus nitrite concentrations acting as a substrate for denitrifiers. It was thought that sulfide inhibition may be restricting denitrification at depth.

In terms of management the hydrology of the wetland should be manipulated to increase the temporal interaction of the water and soil so aiding the diffusion of solutes into the soil. At present channelized flow occurs which results in much of the wastewater nitrogen by-passing the natural removal processes of the wetland. By encouraging sheet flow, for example by raising the water level in the wetland (gravel barriers), a longer residence time for water in the wetland could be achieved, and greater diffusion and subsequent nitrogen removal promoted. Management to increase residence time would also aid diffusion in winter when denitrification rate is reduced by low temperature. If the wastewater nitrogen can stay in contact with the denitrifiers for a longer period at this time of year then a higher total nitrogen removal should be achieved. In addition the highly active denitrifiers in the decaying surface vegetation should be exploited by increasing temporal interaction with this material and the wastewater.

A more diffuse sewage inflow across the width of the wetland would be preferable to the present point source in terms of distributing wastewater nitrogen through the soil to achieve greater interaction with the soil denitrifiers.

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APPENDIX I

Calculating nitrous oxide gas concentrations using pure nitrous oxide gas for calibration

The nitrous oxide (N_2O) standard was made by serum capping and crimp sealing a 120 ml serum vial and removing 2 ml of air by gas tight syringe. 2 ml of N_2O from the calibration gas cannister were then injected into the serum vial to form the standard which was incubated at the appropriate temperature.

The basis of the calculation lies in the Standard Gas Equation which can be used to determine the molecular weight of N_2O at standard temperature and pressure. The equation is as follows:

$$PV = n R T$$

where P = pressure
V = volume
n = Avogadro's Number
R = constant
T = Temperature

For our N_2O standards the values of the above are as follows:

P = 1 atmosphere (atmospheric) at A.C.T.
V = 2 ml (volume of N_2O in standard)
n = unknown (moles)
R = 82.057 ml.atm/mole K
T = Temperature (^oC) + 273

Rearranging the equation allows n to be found:

$$n = \frac{PV}{RT}$$

Therefore for a standard containing 2 mls of N_2O and incubated at 35°C the calculation is as follows:

$$n = \frac{1}{[82.057 \times (35+273)]} \times 2$$

$$n = 7.913 \times 10^{-5} \text{ moles}$$

$$n = 7.913 \times 10^{-2} \text{ mmoles}$$

1 mmole of N_2O (2N+10) = 28+16 = 44 mg

We can now multiply our n value by the molecular weight to find the number of mg of N_2O in 2 mls of N_2O standard at S.T.P.

$$(7.913 \times 10^{-2}) \times 44 = 3.482 \text{ mg } N_2O$$

However, we are interested in knowing how much Nitrogen this represents as it is the potential for nitrogen removal from the wetland system that we are looking at.

To find the weight of nitrogen we must divide the molecular weight of N_2O by that of 2N:

$$1 \text{ mg of } N_2O\text{-}N = \frac{44}{28} \text{ mg } N_2O$$

By reversal,

$$1 \text{ mg of } N_2O = \frac{28}{44} = 0.6369 \text{ mg } N_2O\text{-}N$$

Therefore the standard at 35°C and atmospheric pressure had

$$3.482 \times 0.6369 \text{ mg } N_2O\text{-}N/120 \text{ ml vial}$$

$$= 2.218 \text{ mg}$$

By knowing the weight of N_2O-N in the standard and the equivalent peak area produced on the gas chromatograph, it is possible to calculate the weight of N_2O-N in each sample given their peak areas.

As we are working in a fixed volume of 120 mls we must remember to adjust for the fact that each sample has a reduced headspace compared to the standard. This is caused by the addition of soil, 100 mg/l nitrate and TYNL media. Therefore, we must multiply by the appropriate factor:

$$\frac{\text{Sample Headspace ml}}{120 \text{ ml}}$$

To calculate the amount of N_2O-N in any sample the following equation is used:

$$\frac{N_2O\text{-N in Standard at STP} \times \text{Peak Area of Sample}}{\text{Peak Area of Standard}} \times \frac{\text{Sample Headspace}}{120}$$

Using the Standard Gas Equation as described the following amounts of N_2O-N have been calculated for standards at different temperatures:

Temperature	mg N_2O-N
35°C	2.218
25°C	2.291
5°C	2.457

An example of this calculation for a sample at 25°C

Peak Area of Sample	1535200
Peak Area of Standard	5766000
N_2O-N in Standard (25°C)	2.291
Sample headspace (ie. 21 ml of additions)	99 ml

$$N_2O\text{-N in sample} = \frac{2.291 \times 1535200}{5766000} \times \frac{99}{120} = 0.503 \text{ mg}$$

APPENDIX II

Calculating the maximum potential rate of denitrification.

During the acetylene blockage experimental period the amount of N_2O-N in the headspace above 10g of soil was recorded approximately every three hours. From this a graph of N_2O-N concentration versus elapsed time could be drawn.

To calculate the maximum potential rate of denitrification the point of steepest slope on the graph was identified and the time period over which it occurred was calculated. The amount of N_2O-N liberated in one hour was calculated using the following equation:

$$\frac{\text{one hour}}{\text{Time period over which steepest gradient occured.}} \times \text{Amount of } N_2O\text{-N liberated within the time period of maximum gradient.}$$

Example

Amount of N_2O-N liberated over period of maximum gradient = 0.16 mg

Length of time period = 3.09 hours

Maximum Hourly Rate $\frac{1.00}{3.09} \times 0.16$ = 0.052 mg N_2O-N/hr for 10g of soil.

The maximum hourly rate for a one gram sample can then be calculated by division i.e. 0.0052 mg N_2O-N/hr/g.

APPENDIX III

Results of ammonium ion and nitrate plus nitrite concentration in soil cores from above and below the effluent inflow.

Table A. Levels of NH_4^+-N in soils of above and below effluent inflow cores according to depth and season (results in mg/g soil).

Depth (cm)	Site	Winter June	Spring September	Summer January	Autumn April
0-2	Above	0.072±0.001	0.181±0.007	0.154±0.007	0.100±0.01
	Below	0.169±0.004	2.856±0.164	2.187±0.184	0.149±0.009
4-6	Above	0.080±0.025	0.147±0.001	0.143±0.002	0.117±0.002
	Below	0.903±0.084	1.246±0.033	2.900±0.1	0.291±0.021
8-10	Above	0.061±0.006	0.196±0.015	0.113±0.005	0.080±0.003
	Below	8.940±0.968	1.327±0.023	2.633±0.126	0.865±0.005
15	Above	0.029±0.013	0.242±0.032	0.247±0.005	0.161±0.007
	Below	6.526±0.533	2.696±0.038	1.697±0.178	1.383±0.001
30	Above	0.092±0.02	0.229±0.006	0.260±0.003	0.126±0.007
	Below	11.11±0.792	3.886±0.143	1.043±0.018	1.339±0.040

Table B. Levels of $(NO_3^- + NO_2^-)$-N in soils of above and below effluent inflow cores according to depth and season (results in mg/g soil).

Depth (cm)	Site	Winter June	Spring September	Summer January	Autumn April
0-2	Above	0.0087±0.0002	0.0147±0.0001	0.0176±0.0012	0.0254±0.0021
	Below	0.0189±0.0014	0.0491±0.0009	0.0078±0.0002	0.0448±0.0002
4-6	Above	0.0091±0.0002	0.0072±0.0003	0.0093±0.0001	0.0119±0.0025
	Below	0.0094±0.0003	0.0082±0.0004	0.0066±0.0002	0.0127±0.0012
8-10	Above	0.0098±0.0009	0.0072±0	0.0082±0.0006	0.0058±0.0006
	Below	0.0081±0.0002	0.0093±0.0009	0.0082±0.0002	0.0084±0.0010
15	Above	0.0106±0.0005	0.0079±0.0001	0.0067±0	0.0033±0.0003
	Below	0.0102±0.0004	0.0111±0.0007	0.0107±0.0002	0.0164±0.0007
30	Above	0.0088±0.0003	0.0081±0.0002	0.0061±0.0004	0.0027±0.0002
	Below	0.0125±0.0015	0.0108±0.0002	0.0098±0.0004	0.0139±0.0021