SOIL MICROBIAL BIOMASS AND ACTIVITY AND PLANT NUTRITION IN SEMI-NATURAL ECOSYSTEMS SUBJECTED TO POLLUTANT NITROGEN DEPOSITION

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Soil microbial biomass and activity and plant nutrition in semi-natural ecosystems subjected to pollutant nitrogen deposition

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SUMMARY

The response of UK semi-natural soil and plant communities to long-term inputs of pollutant nitrogen (N) is presently poorly understood. This study aims to investigate the effects of up to 8 years simulated pollutant N additions on plant nutrition and key below ground nutrient cycling processes in an upland heath, an acid grassland and a calcareous grassland. An additional series of plots was established in the grasslands that received short-term N and phosphorus (P) inputs which enabled investigation of N/P interactions and the determination of plant biomass.

Soil inorganic N concentrations indicated that all of the sites had become 'N saturated', and that P limitation may be of increasing importance, particularly in the heathland and calcareous grassland. This was confirmed in the calcareous grassland where significant increases in above ground biomass were seen in only P treated plots, while in the acid grassland, there were no growth responses to nutrient additions. A field-based bioassay was developed which demonstrated that both short and long-term inputs of N significantly increased root surface phosphomonoesterase (PME) activity of *Plantago lanceolata* seedlings. A parallel microcosm-based bioassay revealed a significant relationship between root surface PME activity of *Agrostis capillaris* and extractable NH_4 concentrations (r^2 = 0.86) in the long-term acid grassland plots, indicating that this site may have reached or be approaching N saturation.

In response to long-term N additions, soil microbial biomass carbon increased in the heathland, decreased in the acid grassland and remained constant in the calcareous grassland. Chloroform fumigation indicated that microbial biomass P decreased in the acid and calcareous grasslands, while microbial biomass N increased in the heathland and acid grassland in the N treated plots. Soil PME activity was highly sensitive to the N treatments. It increased at all sites and was significantly correlated with extractable inorganic N concentrations (r^2 = 0.71) in the calcareous grassland, indicating close coupling between N saturation and P limitation. This relationship was also seen in the heathland where there was increased utilisation of monoester P sources in standard and customised BIOLOG plates and increased respiration rates in soils amended with organic P compounds.

The implications of these results are discussed, with reference to the critical loads concept and to recent research by other workers in similar and contrasting environments.

CHAPTER 1

GENERAL INTRODUCTION

1.1 NITROGEN EMISSIONS AND DEPOSITION IN THE UK

The previous decades have seen a considerable number of ecological studies focusing on the impacts of the major group of acidifying airborne pollutants, sulphur oxides (SO_x). The advent of legislation requiring the fitting of flue-gas desulphurisation units to coal burning power stations in the 1980's led to a substantial reduction in emissions of SO_x (Fig. 1.1). Attention is now beginning to focus on other major groups of airborne pollutants, in particular oxidised and reduced forms of nitrogen (N) including nitrous oxides (NO_x) and ammonium (NH_4), since emissions of nitrogenous compounds have increased steadily throughout the latter half of this century (DoE, 1994). In contrast to sulphur, the major source of anthropogenic emissions of NO_x is not limited to fossil fuel combustion. Although NO_x emissions arise largely from this source, emissions of the other major nitrogenous pollutant, ammonia, result from the use of organic manure's by the agricultural industry. Volatilisation of ammonia from the spreading of animal wastes accounts for between 34 % (Jarvis and Pain, 1990) and 52 % (Asman, 1992) of UK livestock emissions.

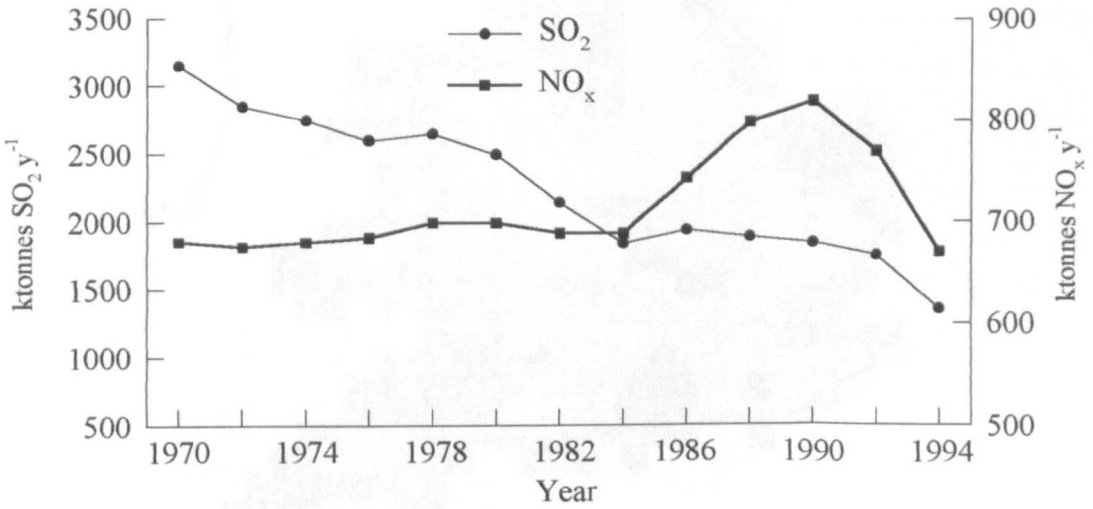


Fig. 1.1. Emissions of SO_2 and NO_x (ktonne y^{-1}) in the UK (UKRGAR, 1997).

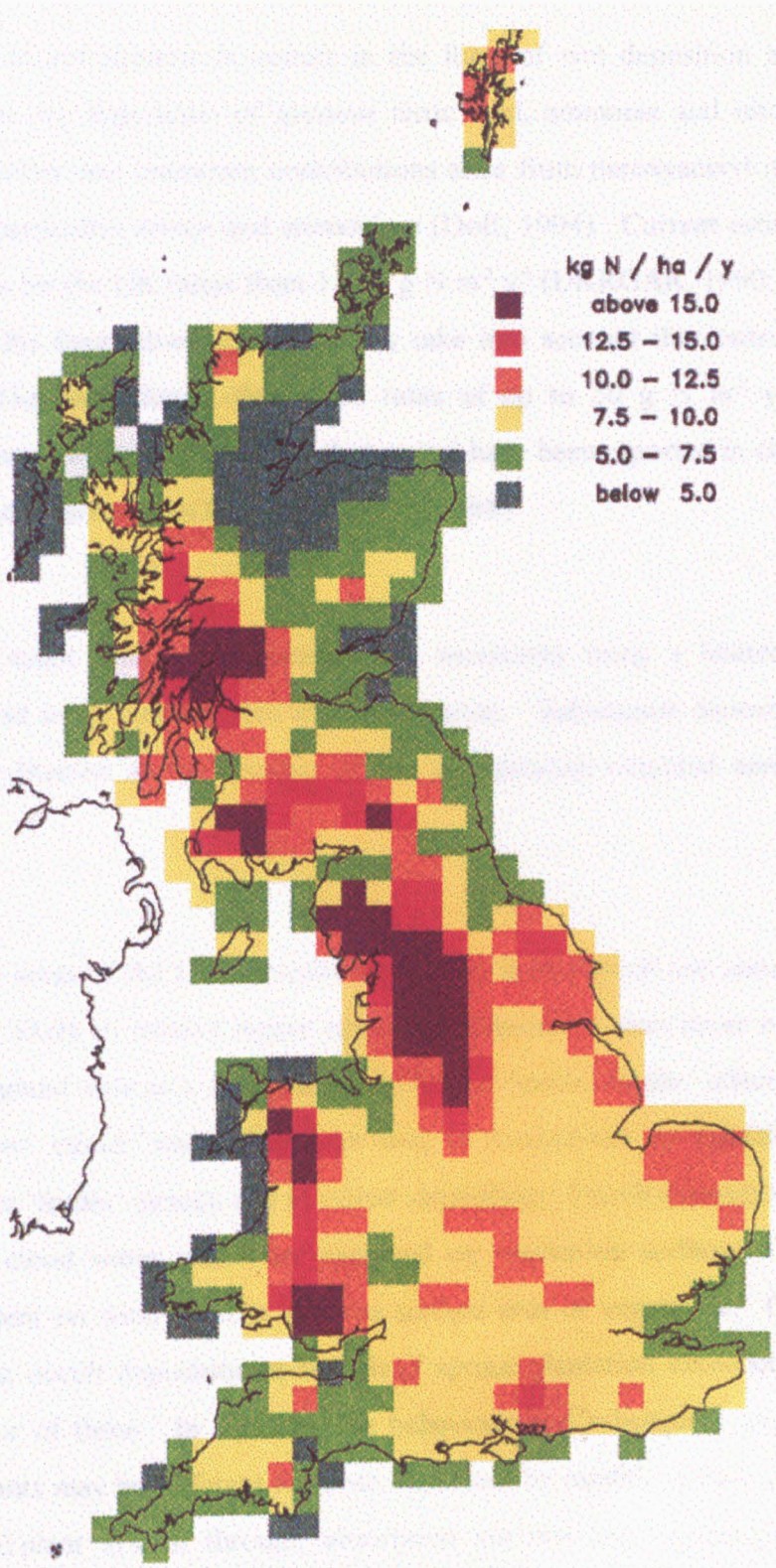


Plate 1.1. Total wet deposition of N (kg N ha^{-1} y^{-1}) for the UK, 1989-1992 (DoE, 1994).

The main inputs of atmospheric N occurs in the form of wet deposition as nitrate and ammonium and as dry deposition of gaseous nitric acid, ammonia and nitrogen dioxide (DoE, 1994). Further less important contributions arise from peroxyacetyl nitrate (PAN), nitrous acid and particulate nitrate and ammonium (DoE, 1994). Current estimates of total N deposition rates for the UK range from 1 to 8 g N m^{-2} y^{-1} (UKRGAR, 1990; Sutton *et al.*, 1993), although this figure does not adequately take into account the contribution of dry deposition. In The Netherlands, deposition rates of up to 50 g N m^{-2} y^{-1} comprising primarily of gaseous ammonia in localised 'hot-spots' have been reported in close proximity to intensive livestock farms (Van Dijk and Roelofs, 1988).

In the UK, the major ions in precipitation are monitored using a limited network of collectors that tend to be concentrated in lowland areas. Subsequent deposition estimates for the UK are obtained as the product of the precipitation-weighted annual mean ion concentration.

However, upland areas of the UK, typically supporting semi-natural and natural vegetation communities, are likely to receive higher rates of N deposition than those estimated from precipitation-weighted data as a consequence of i), the 'seeder-feeder' effect, in which hill cloud (the 'seeder' cloud) rising over high land is washed-out by rainfall from higher altitude cloud (the 'feeder' cloud), and ii) occult deposition. Occult deposition occurs from fine droplets of cloud water which are captured on vegetation surfaces. Deposition is therefore dependent on wind velocity and the surface area of vegetation. Crossley *et al.* (1992) found that occult deposition at an upland spruce plantation exceeded precipitation inputs by a factor of three. In addition, the behaviour and subsequent impact of occult deposited pollutants may be different to those deposited by rainfall as they are more likely to enter the soil:plant system through absorption via standing vegetation, rather than directly onto surface soil horizons. The total wet deposition of N over Great Britain, corrected for enhanced levels in upland areas, is shown in Plate 1.1 (DoE, 1994). The areas that receive the highest concentrations of N are found in the Pennines, Lake District and central Wales.

1.2 SEMI-NATURAL ECOSYSTEMS TYPICAL TO THE UK

Many of the upland areas of the UK that receive high inputs of N support stands of *Calluna vulgaris* (L.) Hull[1]. *Calluna* is widespread and forms stands of many thousands of hectares in both upland and lowland areas (Perring and Walters, 1962). Acid heathlands, found in most upland areas of the UK, have considerable conservation value since they are an ideal habitat for rearing red grouse. Since about 1850, large tracts of land have been managed specifically for this purpose by controlled burning which is undertaken on a cyclical basis every 10-15 years. The result is often an almost pure monoculture of *Calluna* which leads to increased production of lignin-rich litter promoting the development of mor humus and eventually hastening podsolisation (Gimingham, 1972). Grasslands can be divided into mesotrophic, calcicolous and calcifugous systems. Mesotrophic communities are often located on neutral or slightly acidic soils. Any agricultural improvements on such systems usually take the form of mowing once or twice a year for hay, grazing and occasional fertilisation. Unimproved mesotrophic communities are often dominated by *Arrhenatherum elatius* (L.) P. Beauv. grassland, while improved areas comprise *Lolium perenne* L. leys and related grasslands (Rodwell, 1992). Calcicolous grassland are dominant on calcareous areas of the UK, such as the Derbyshire White Peak, Yorkshire Dales and chalk Downs, and are often underlain by rendziniform soils. The composition of these communities is governed to a large extent by climatic factors which affect the community composition and distribution both directly (e.g. influencing sexual reproduction) or indirectly (e.g. influencing the development of rendzinas). In addition, grazing helps maintain these systems as grassy swards (Rodwell, 1992). Calcareous grasslands typically have a high conservation value due to their species richness (Bobbink and Willems, 1987). Calcifugous communities are located on acidic soils, such as humic rankers and peaty and gleyed stagnopodsols, often in upland areas of the UK. Species typical of these areas include *Agrostis capillaris* L., *Festuca ovina* L., *Galium saxatile* L. and *Nardus stricta* L. (Rodwell, 1992).

The present study utilises two grasslands in close proximity which share the same Carboniferous limestone bedrock, but paradoxically, their soil properties and vegetation communities differ markedly. This feature is not uncommon in the Peak National Park and results from a combination of the topographical and geological characteristics of the area.

[1] Subsequently referred to as *Calluna* since it is a monospecific genus.

The acid grassland soil is a brown earth and occurs where loess (fine wind blown silty material, approximately 10-80 μm diameter) has been deposited into depressions in the limestone and onto plateaux at the end of the last glaciation. The source of the loess is thought to be the surrounding escarpments of acidic Millstone grit (Pigott, 1962). Soil formation and vegetation succession are therefore thought to be proceeding on two separate and distinct fronts, viz (Grime, 1963):

Surface soil pH					
8	7	6	5	4	3
Unstable rendzinas (*F. ovina*)	→ Stable rendzinas (*F. ovina, F. rubra*)	→ Limestone rankers (*D. flexuosa* (L.) Trin.)			
		Brown earths (*A. capillaris*)	→ Incipient podsols (*A. capillaris*)	→ Podsols (*D. flexuosa*)	

Based on this diagram, the calcareous grassland is likely to be in the stable rendzina stage, and the acid grassland in the latter half of the brown earth stage.

1.3 NUTRIENT CYCLING IN SEMI-NATURAL ECOSYSTEMS

1.3.1 Nitrogen

In many plant communities, N is recognised as being the main nutrient limiting plant productivity (Ellenberg, 1988). Nitrogenous fertilisers are used to overcome this limitation in agricultural systems, but in semi-natural and natural ecosystems, which receive no fertiliser additions, the most important external source of N is from atmospheric deposition. The "natural" cycling of C, N and P therefore has particular significance for the maintenance of unmanaged or partially managed terrestrial ecosystems. The principal transformations of N within these ecosystems (Fig. 1.2) are mediated by microbially driven processes, which are dependent on both abiotic and edaphic factors. Plants assimilate N largely in the form of nitrate (NO_3^-) and ammonium (NH_4^+), depending on the plant species and availability of each ion (Marschner, 1995), and therefore rely on the microbial biomass for the provision of adequate mineral N. The mineralisation of organic N compounds to ammonia (ammonification) is driven by the need of microorganisms for carbon (C), and release of ammonium eventually follows (Sprent, 1987). Subsequent conversion of ammonium to nitrite (NO_2^-) and from nitrite to nitrate (nitrification) is most commonly performed by the

gram-negative, chemoautotrophic genera of bacteria *Nitrosomonas* and *Nitrobacter*. Nitrification may also be performed by heterotrophic microorganisms whereby organic N is converted to nitrate or nitrite. Nitrogen transformation and plant uptake of mineral N involve the production and consumption of protons and can, therefore, have the potential to cause soil acidification. Nitrification produces 2 moles protons for each mole of NH_4^+ oxidised:

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+$$

This mechanism can be the main sources of acidity in some semi-natural ecosystems (Makarov and Kiseleva, 1995; Nambu *et al.*, 1994; Wilson and Skeffington, 1994). However, the production of protons by this process is often balanced by proton removal or OH^- production by NO_3^- uptake and ammonification (DoE, 1994).

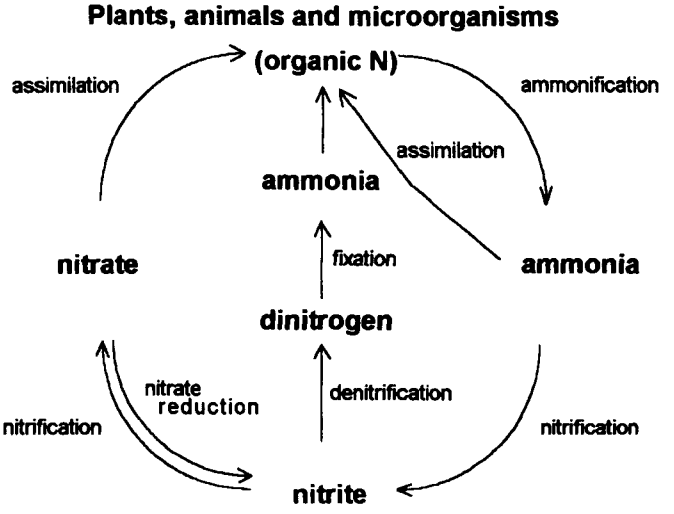


Fig. 1.2. Generalised N cycle (The Royal Society, 1983).

1.3.2 Phosphorus

The turnover of phosphorus (P) in semi-natural ecosystems forms an almost completely closed cycle (Fig. 1.3). The soil and shoot/root P contents in the systems used in the present study are likely to differ markedly from the example given in Fig. 1.3. However, the figure gives an indication of the importance of the organic component and the role of microbial biomass, even in soil with low organic matter content. Almost 100 % of mineral P originates from the mineralisation of organic matter, the remainder either being deposited

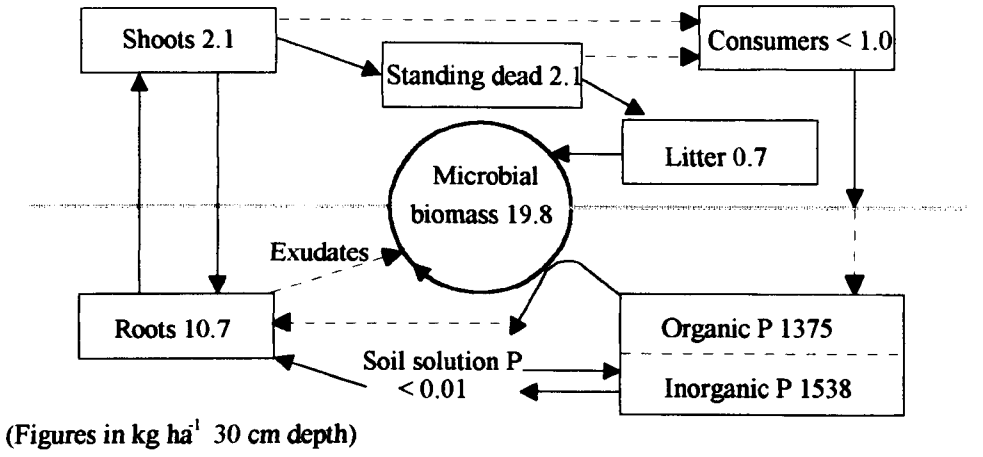


Fig. 1.3. P cycle in a prarie grassland in Western Canada (adapted from Halm *et al.*, 1972).

from the atmosphere or weathered from primary P minerals (Stewart and Tiessen, 1987). In many semi-natural soils, organic P can constitute up to 90 % of the total soil P (Dalal, 1977). However, the chemical nature of only about 50 % of soil organic P is known (Anderson and Malcolm, 1974). The major groups identified to date include the inositol phosphates, phospholipids and nucleic acids (Dalal, 1977). The greatest proportion of organic P is in the monoester form and can account for between 25 and 58 % of the total organic P (Dalal, 1977). Although nucleic acid P only accounts for up to about 3 % of total organic P, the greater lability of phosphodiesters indicates that these compounds may have an important role in plant nutrition (Adams and Pate, 1992; Myers and Leake, 1996). Central to the organic P cycle is the microbial biomass P component which can be taken up directly by saprotrophic and predatory (e.g. protozoa) organisms and incorporated into new biomass. This is the most rapid process in the organic P cycle and is often accompanied by rapid rates of C and N transformations (Stewart and Tiessen, 1987). Phosphatase enzymes in the soil, of either microbial, plant-root or mycorrhizal origin, are capable of catalysing the mineralisation of organic P. They are found in soil solution, attached to soil colloids, in the microbial biomass or associated with plant roots. The phosphatase group comprises of five principal enzymes (Florkin and Stotz, 1964), although the most ecologically significant and most widely studied are the phosphomonoesterases (PME) and phosphodiesterases (PDE; Eivazi and Tabatabai, 1977). PME degrades the most common soil phosphomonoesters,

such as inositol P, while PDE catalyses the mineralisation of phosphodiesters, including DNA, RNA and phospholipids.

In many semi-natural and natural systems the rate of N and P mineralisation is extremely slow. This is largely as a consequence of the acidity of many upland soils which results in relatively inactive microbial populations. Furthermore, in acidic soils, the microbial biomass is often dominated by fungi and comprises few nitrifying bacteria, which are primarily responsible for the nitrification process. Binding of $-PO_4$ onto colloidal material has been shown to be a major mechanism in reducing the availability of P in organic soils (Marschner, 1995). Paradoxically, soils with a relatively high pH derived from material with a high content of calcium carbonates (such as limestone and chalk) may also support vegetation that is limited or co-limited by N and P. High concentrations of Ca^{2+} ions often results in the formation of insoluble calcium phosphates (Cresser *et al.*, 1993). The ability of carbonates and calcites to bind P may also be influenced by other cations. Hamad *et al.* (1992) reported that separate-phase Fe oxide contributed to between 30 and 40 % of P sorption in calcite and calcareous soils. When inorganic P is tightly fixed, movement of P in calcareous soils is often dominated by organic forms (Hannapel *et al.*, 1964a). As a result, the importance of the microbial biomass in the cycling of P in calcareous soils is considerable (Hannapel *et al.*, 1964b).

1.3.3 Characteristics of microbial biomass in semi-natural ecosystems

Almost all semi-natural ecosystems support vegetation communities adapted to low rates of inorganic N and P production by the soil microbial biomass and which are often characterised by low rates of primary productivity. One of the most ecologically significant adaptations found in most plant species in oligotrophic environments is the colonisation of their root systems by mycorrhizal fungi. The importance of this symbiotic relationship in the acquisition of nutrients can be most readily illustrated in upland acidic soils, such as the *Calluna* dominated heaths of the UK. Loss of base cations with an associated drop in soil pH and an accumulation of recalcitrant organic matter as a result of inactive microbial populations are characteristics of heathland environments. The ericoid mycorrhizal association provides both direct nutritional benefits through the acquisition of N and P, and

indirect benefits through the detoxification of metal ions and organic acids (Read, 1991). The production of proteinases (Leake and Read, 1989, 1990a, 1991), chitinases (Leake and Read, 1990b) and acid phosphatases (Pearson and Read, 1975) by the ericoid mycorrhizal edophyte, *Hymenoscyphus ericae* (Read) Korf & Kernan, and its ability to use organic N (Bajwa and Read, 1985) and P compounds (Mitchell and Read, 1981; Leake and Miles, 1996) as sole N and P sources illustrates a significant adaptation to oligotrophic environments with characteristically low rates of nutrient mineralisation. Similar chitinolytic capabilities for free-living microfungi isolated from acid soils have also been reported (Bååth and Söderström, 1980). Free-living microorganisms provide a further vital role in nutrient cycling since the amount of N and P held in the microbial biomass is substantial. The relatively high lability of this nutrient pool forms a potentially accessible supply of N and P for plant nutrition.

The interactions occurring within the soil microbial and mycorrhizal biomass are very complex and are often thought to be tightly integrated (Janos, 1983). Breakdown of complex C skeletons, primarily by saprotrophic decomposer fungi such as *Phanaerochaete chrysosporium* (DC. :Pers.), enables less specialist microorganisms to mineralise simpler compounds which may lead to release of inorganic N or P (Swift *et al.*, 1979). For example, the release of mineral N in coniferous forest soils has been shown to correlate strongly with leaf litter polyphenolic content (Northup *et al.*, 1995). This indicates that soil microbial activity is likely to be limited by the quality of C entering the soil system. Little information is available detailing the interactions occurring between mycorrhizas and free living soil microorganisms. Both antagonistic (Dighton *et al.*, 1987; Gadgil and Gadgil, 1971, 1975) and synergistic (Dighton *et al.*, 1987) effects on decomposition processes have been reported in controlled communities of ectomycorrhizal and saprotrophic fungi.

The soil microbial community within natural and semi-natural ecosystems often comprises different species and functional groups and is dependent on many factors including the soil type, the plant community and climatic conditions. Soils with a low pH tend to support a more limited number of microorganisms than soils with a neutral/alkaline pH. In addition, the composition of the microbial population is often quite different. Latter *et al.* (1967) used the dilution plate technique in conjunction with selective inhibitors in order to calculate

relative numbers of groups of bacteria and fungi in a limestone grassland, *Juncus* moor, mixed moor and bare peat systems. The greatest number of nitrifying, denitrifying and gelatin liquifying bacteria were found in the limestone grassland soil, while the mixed moor and bare peat systems did not appear to support any nitrifying bacteria. Total counts of bacteria were almost two orders of magnitude greater in the limestone grassland than in the bare peat systems while the fungal population was found to be greatest in the limestone grassland and mixed moor. The authors attributed the lack of microbial activity in the peat systems to low pH, occasional water logging and low nutrient supply.

1.4 PLANT RESPONSES TO NUTRIENT INPUTS

The response of plant communities to nutrient manipulations has been under investigation since the mid 19th Century (e.g. Lawes and Gilbert, 1863). The Park Grass Experiment (established in 1856) undertaken at Rothamsted Experimental Station, and others thereafter, have resulted in a considerable wealth of information on effects of nutrient additions on the species composition and productivity of agricultural plant communities. A recent analysis of 60-years (1920-1979) visual surveys of the plant communities of the Park Grass Experiment by Dodd *et al.* (1995) revealed significant long-term trends and outbreaks of a number of species in what has traditionally been considered a stable grassland community. Less information is available relating to nutrient manipulations to ecologically important plant communities, such as those found in many calcareous systems. The vegetation ecology of Braunton Burrows, North Devon, has been extensively studied in relation to soil macro- and micro-nutrient supply by Willis *et al.* (1959), Willis and Yemm (1961) and Willis (1963). The authors concluded that the vegetation communities of the exposed dune soils were highly dependent on the supply of mineral nutrients, in particular N and P, and to a lesser extent K, while other factors, such as exposure and water supply had little effect. Using a similar approach, Jeffrey and Pigott (1973) reported that the addition of 10 g N m^{-2} to a species rich calcareous grassland soil in Teesdale had little effect on the species composition but the addition of 5 g P m^{-2} caused a substantial increase in numbers of the grasses *Festuca ovina, F. rubra* L. and *Agrostis stolonifera* L., and a corresponding decrease in the quantity of *Kobresia simpliciuscula* (Wahlenb.) Mackenzie. The authors noted further increases in the abundance of *F. ovina, F. rubra* and *A. stolonifera* following addition of N and P in combination indicating the vegetation was N and P co-limited. More

recently, Mountford *et al.* (1993) observed a loss of species diversity coupled with an increase in abundance of grasses in a lowland heath following fertilisation with between 2.5 and 20 g N m^{-2} y^{-1}.

Interpretation of fertilisation experiments with regard to atmospheric N pollution must be done with caution. Pollutant N contrasts markedly to fertiliser N since it is deposited as a chronic dose (i.e. low concentrations for long periods) where it may be able to gradually accumulate in soil and vegetation with no apparent immediate effects. It has only recently been realised that chronic inputs of atmospheric N can have significant impacts on vegetation. In the Netherlands, increased pollutant N deposition has resulted in widespread conversion of heathland into grassland, through competitive invasion of grasses such as *Molinia caerulea* (L.) Moench, *Deschampsia flexuosa* and *Festuca ovina* (Heil and Diemont, 1983; Pearson and Stewart, 1993). This dramatic loss of heathland has yet to be seen in the UK. However, Caporn *et al.* (1998) suggested N enriched *Calluna* may be more susceptible to frost damage. It has also been recognised that species rich calcareous grasslands may be under threat as a result of increased dominance of the grass *Brachypodium pinnatum* (L.) P. Beauv., which is highly responsive to elevated inputs of N (Bobbink, 1991). In addition, there is growing evidence that N deposition may be a major factor involved in large scale forest die-back. Analysis of pine stands unfertilised for 25 years has revealed a clear increase in needle N concentrations (Hüttl and Schaaf, 1995). Although increased N uptake often lead to initial growth improvements, in the longer term, tree health can be affected as a consequence of imbalances in elemental foliar concentrations, causing chlorosis, premature needle fall and declining tree vigour and ultimately death (Aber, 1992). Furthermore, soil acidification and eutrophication may also reduce the number of ectomycorrhizal fruit bodies (Van Breeman and Van Dijk, 1988), although the impact of N on ectomycorrhizal mycelium is unclear (Wallenda and Kottke, 1998). In many ecosystems, the result of sustained N deposition is often 'N saturation' (Skeffington and Wilson, 1988; Aber *et al.*, 1989) which implies stages of declining ability of an ecosystem to retain added N (Aber, 1992). Plant growth in N saturated ecosystems may then become limited by one or more other nutrients, such as P, as seen in ombrotrophic bogs (Aerts *et al.*, 1992), fens (Verhoeven and Schmitz, 1991), coniferous forests (Emmett *et al.*, 1995) and heathlands (Aerts and Berendse, 1988). It is therefore clear that

significant interactions between N deposition and the cycling of P could occur in ecosystems that have become or are close to becoming N saturated.

1.5 BELOW GROUND RESPONSES TO NUTRIENT INPUTS

Fertiliser inputs of N and P have been shown to have significant effects on many important components of agricultural and forest soils including microbial biomass C (Kowalenko *et al.*, 1978; Witter *et al.*, 1993), N (Witter *et al.*, 1993) and P (Brookes *et al.*, 1984), soil PME activity (Lovell *et al.*, 1995), root surface PME activity (Kieliszewska-Rokicka, 1992), litter decomposition (Fog, 1988), amino acid utilisation (Hopkins *et al.*, 1997) and N mineralisation (Glendinings *et al.*, 1996). Söderström *et al.* (1983) found N addition to reduce rates of microbial activity and mineralisation in a Swedish pine forest.

In comparison, little information is available focusing on the effects of increased N supply to semi-natural and natural soil systems. This is surprising given the importance of the soil microbial biomass in nutrient cycling processes. N fertilisation of a lowland heath in The Netherlands was shown to increase rates of mineralisation of both N and especially P (Berendse *et al.*, 1987). Caporn *et al.* (1995) applied sustained chronic doses of N to a heathland, used in the present study, for five years but reported no apparent change in mycorrhizal infection of *Calluna*. In contrast, Johansson (1992) found greater levels of infection in roots of *Calluna* that received 3 g N m^{-2} y^{-1} than in roots of *Calluna* that received 1 g N m^{-2} y^{-1}. However, Caporn *et al.* (1995) used ergosterol as a biochemical index of infection which is not mycorrhizal specific, and thus measurements are likely to have included contributions from non-mycorrhizal fungi. Sanger *et al.* (1994) reported decreases in decomposition of *Eriophorum angustifolium* Honck. and *Calluna* litter along a natural atmospheric pollution gradient from the north-west of Scotland to central England. Clearly, factors other than N are likely to be involved including differences in deposition other pollutants such as SO_x. The comprehensive review by Fog (1988) on the effects of N on decomposition rates indicated the importance of litter C:N ratios. The addition of N to a poor quality substrate (C:N ratio >50; high lignin content) causes inhibition of lignin decomposers to the advantage of cellulose decomposers. The resultant population shift, and the depletion of carbohydrate normally required for lignin decomposition results in

long-term negative effects. Accumulation of litter in forests in north-east Scotland between 1949/50 and 1989 is thought to be associated with increased atmospheric deposited NO_x and SO_x (Billett *et al.*, 1990). Enchytraeid worm populations of *Calluna* dominated ecosystems, which are thought to have an important role in nutrient turnover and comminution of litter, have been shown to decline along a natural N pollution gradient (Yesmin *et al.*, 1995). Few studies have investigated changes in microbial biomass and enzyme activities in response to long-term inputs of N. Lovell *et al.* (1995) applied 200 kg N ha^{-1} y^{-1} for 10 years to a long-term grassland. The authors found that microbial biomass C and N and soil PME activity decreased in response to the treatments.

These contrasting responses indicate that the effects of pollutants on microbial communities and microbial activities are complex. Since many of these studies have involved applications of relatively large amounts of N to soil they may not reliably indicate the effects of chronic deposition of pollutant N which occurs principally as wet-deposition, much of which is intercepted by plant canopies (Pearson and Stewart, 1993).

1.6 CRITICAL LOADS OF N

The increased emissions of airborne pollutants, such as SO_x and NO_x, has led to concerns regarding the sustainability of both terrestrial and aquatic ecosystems. The concept of critical loads was developed in an attempt to safeguard ecosystems from damage by imposing empirically derived limits on atmospheric emissions. The critical load of a pollutant element or compound can be defined as the input "below which empirical detectable changes in ecosystem structure and function do not occur according to present knowledge" (Grennfelt and Thornelof, 1992). Although this approach has been successfully adopted with respect to both SO_2 and total acidic deposition to vegetation, soils and water (Bull *et al.*, 1991), its application for N deposition is more difficult since N is both an acidifying ion and an essential plant nutrient. The situation is further confounded since there are few data available regarding the effects of long-term, field-based additions of N on semi-natural vegetation communities, and virtually nothing is known about impacts on the important processes occurring below-ground in these systems. Nevertheless, critical loads of N have been developed for a number of ecosystems including lowland wet heaths, species

rich calcareous grasslands and species poor acidic grasslands based on vegetation type (Table 1.1; Grennfelt and Thornelof, 1992).

Table 1.1. Current critical loads of N (g N m^{-2} y^{-1}) and level of uncertainty for semi-natural and natural terrestrial and wetland ecosystems. ** = reliable, * = quite reliable, (*) = best guess. (Grennfelt and Thornelof, 1992).

Habitat	Critical load	Level of uncertainty
Acid (managed) coniferous forests	1.5-2	*
Acid (managed) deciduous forests	<1.5-2	*
Calcareous forests	unknown	
Acidic (unmanaged) forests	unknown	
Lowland dry heaths	1.5-2	**
Lowland wet heaths	1.7-2.2	**
Species rich lowland heaths/acid grasslands	0.7-2	*
Arctic and Alpine heaths	0.5-1.5	(*)
Calcareous species rich grasslands	1.4-2.5	**
Neutral-acid species rich grasslands	2-3	*
Montane subalpine grasslands	1-1.5	(*)
Shallow soft water bodies	0.5-1	**
Mesotrophic fens	2-3.5	*
Ombrotrophic bogs	0.5-1	*

It can be seen that critical loads have only been calculated for a limited number of ecosystems and that the reliability of some of the values are poor. Despite the sensitivity of *Calluna* in Dutch systems to increased N deposition,critical loads have not been developed for wet upland heaths characteristic of the UK. It must also be stressed that virtually all of the critical loads are based on aboveground vegetation responses. The exception is the value for acidic (managed) coniferous forests, which took into account effects on mycorrhiza. Given the importance of soil processes in all of the ecosystem categories in Table 1.1, it is crucial that data are available which may be taken into consideration when

calculating future critical loads. For many ecosystems, this problem is twofold in that little is known concerning either the functioning of soil microbial biomass or its response to sustained N inputs.

When applied to the UK, many of the critical loads are exceeded by current N deposition rates (Plate 1.2; BES, 1994). The exceedance map shows a similar pattern to the N deposition data (Plate 1.1) with the areas most at risk concentrated in upland areas of England and Wales. This reinforces the need for information relating to the functioning and response of upland semi-natural soil and plant systems to chronic N deposition.

1.7 CONCLUSIONS

N deposition is of increasing importance, particularly in semi-natural plant communities which are dependent on rainfall-derived N for their nutrition. Although the response of many semi-natural vegetation and soil systems to fertiliser inputs of N is well documented, little information is available about the potential impacts of chronic N inputs of these systems. In particular, virtually nothing is known about the impacts of long-term N deposition on soil microbial biomass and key nutrient cycling activities, and the interactions between increased N supply and cycling of other nutrients such as P.

Therefore, the fundamental aims of this research are to take an integrated standpoint by considering the effects of incremental additions of N on three semi-natural ecosystems typical to the UK. In light of the lack of data relating to soil processes, particular attention is paid to the size and functioning of the soil microbial biomass. Chapter 2 provides soil chemical data for the N and N and P treated plots. Chapter 3 focuses on the above ground responses of grassland vegetation communities to enhanced N pollution while root morphological and physiological responses of selected species are considered in Chapter 4. Soil microbial processes are investigated in the second section; Chapter 5 quantifies the microbial biomass using substrate induced respiration and fumigation extraction and the amount of N and P held in microbial biomass are assessed. Soil phosphatase activities are presented in Chapter 6 and finally, Chapter 7 focuses on the utilisation of C, N and P compounds by both extractable soil bacteria and the soil microbial biomass as a whole.

Plate 1.2. Exceedance of critical loads of N for the UK (BES, 1994).

CHAPTER 2

DESCRIPTION OF FIELD SITES, GENERAL MATERIALS AND METHODS AND SOIL CHEMICAL PROPERTIES IN LONG-TERM N TREATED AND SHORT-TERM N AND P TREATED PLOTS

2.1 DESCRIPTION OF FIELD SITES

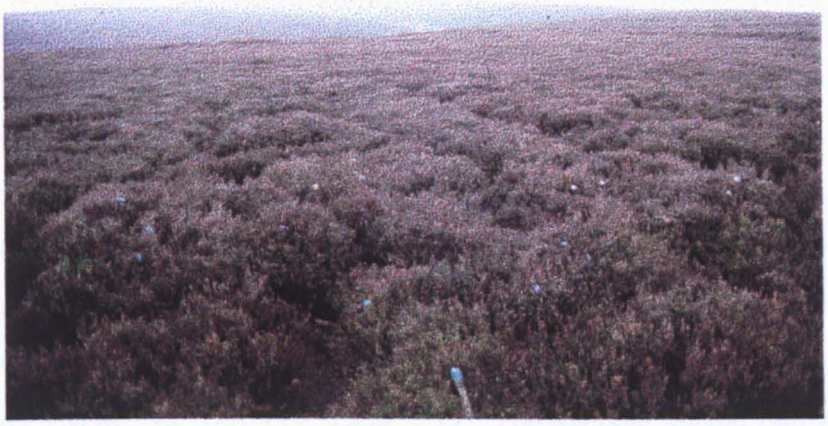
Although a number of investigations has extended our knowledge of nutrient cycling processes and vegetation responses in semi-natural and natural ecosystems (particularly forests) in response to short-term fertiliser additions of N (e.g. Bååth and Söderström, 1980), very few have adequately addressed the specific problems of N deposition. A thorough understanding of N deposition effects and the formulation of meaningful pollution control strategies (e.g. critical loads) for UK semi-natural ecosystems must at least in part be obtained from empirical field-based approaches utilising long-term chronic inputs of N. Virtually no experimental sites in the UK have a history of controlled long-term regular additions of N applied in concentrations designed to simulate increased atmospheric deposition. This study makes use of experimental plots that are unique in that they have received incremental chronic additions of N for up to eight years.

The recognition that N saturated ecosystems can become limited by other nutrients such as P (Aerts and Berendse, 1988; Aerts *et al.*, 1992; Verhoeven and Schmitz, 1991; Emmett *et al.*, 1995) implies potentially important links between N deposition and the key processes involved in P cycling. Interactions between N deposition and P cycling can be addressed by artificially increasing the P supply to plots that receive different levels of N addition. This study therefore makes use of additional plots that have received factorial combinations of N and P for 18 months. This approach has been routinely applied in agricultural systems where both N and P are applied as a fertiliser in order to provide short-term improvements in crop yields, but has rarely been used when investigating the effects of chronic N inputs to semi-natural ecosystems. Although the main focus of this research is on long-term N deposition effects, it is recognised that short-term inputs may or may not mirror long-term inputs, as seen for some agricultural grasslands (Lovell *et al.*, 1995). The additional plots will also enable comparisons between the effects of short-term and long-term N treatments.

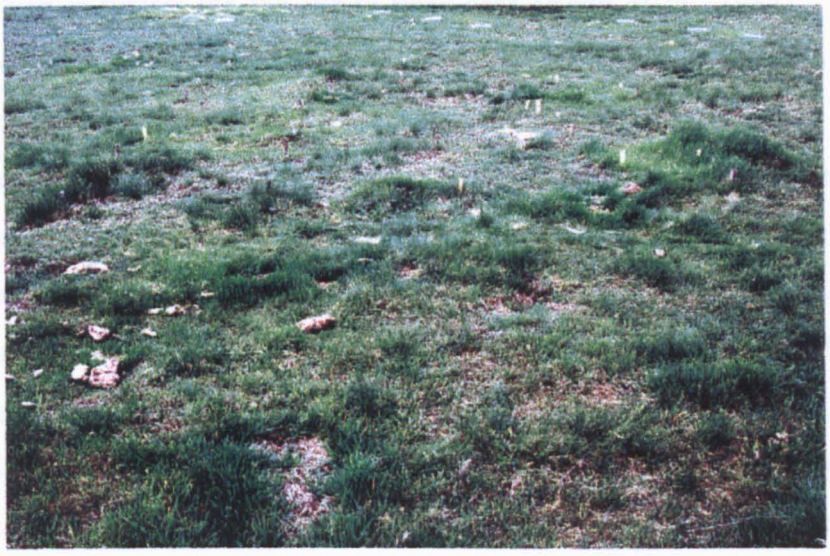
2.1.1 Location

The long-term plots (1 m^{-2}) are located in semi-natural ecosystems typical of the UK: an upland heath, an acid grassland and a calcareous grassland (Plate 2.1). The heathland site, established in 1989, is located on the slopes of Cyrn-y-Brain, near Wrexham in North Wales

(a)



(b)



(c)

Plate 2.1. The three field sites used in the present study. (a) heathland, (b) acid grassland, and (c) calcareous grassland (showing the backpack sprayer used for treatment applications).

(NGR: SJ 225 490; Table 2.1). The topography of the surrounding 50 km^2 is mountainous and the plots are at an altitude of 470 m on a gentle south-easterly slope (approximately 5 °). Between June 1996 and May 1997, air temperatures ranged from -7 to 34 °C while frost events were recorded in September and during November to May (L. Cawley, pers. comm.). The average annual rainfall for the plots is 900 mm, which is less than for much of Wales which typically receives between 1000 and 2000 mm (Rudeforth *et al.*, 1984). The acid and calcareous grassland sites, established in 1990, are located in close proximity on Wardlow Hay Cop National Nature Reserve (managed by English Nature), near Bakewell in the Peak District National Park (NGR: SK 180 739; Table 2.1). The average annual rainfall for the plots is 893 mm which is typical for the White Peak area (Ragg *et al.*, 1984). The acid grassland plots are on an 8 ° East facing slope while the calcareous grassland plots are on a steep 40 ° South facing slope. Consequently, the soil in these plots is very shallow (1-7 cm) with frequent exposure of the underlying limestone. The calcareous site is therefore likely to receive high concentrations of solar irradiance resulting in higher air temperatures than the acid site, as reported for North and South facing slopes in nearby Lathkill Dale (Rorison *et al.*, 1986). Additional 3 m × 3 m ('short-term') plots were established in July 1995 on both the acid and calcareous grassland sites and these receive factorial combinations of N and P. The short-term acid grassland plots are located on similar topography to the long-term plots, but the short-term calcareous grassland plots are located on a 15 ° South facing slope.

2.1.2 Vegetation characteristics

The heathland site is dominated by late building phase *Calluna* (approximately 15 years old). *Vaccinium myrtillus* L. occurs evenly but sparsely throughout the site (Table 2.2). *Calluna* is characterised by a dense woody canopy which results in a high litter C:N ratio (120:1; Read, 1991) and an abundance of fine roots. The roots are concentrated in the surface soil horizons and are host to the ericoid mycorrhizal endophyte *Hymenoscyphus ericae* (Smith and Read, 1997). The acid grassland is classified as U4e, *Festuca-Agrostis-Galium*, under the British National Vegetation Classification (NVC; Rodwell, 1992), and occurs where loess has been deposited on to the limestone bedrock (Pigott, 1962). The vegetation is dominated by *Agrostis capillaris*, *Festuca rubra*, *F. ovina* and *Potentilla erecta* (L.) Raeusch. (Table 2.2), all of which are host to VA-mycorrhizal fungi (Harley and Harley, 1987) and have typical litter C:N ratios of 30:1 (Read, 1991). Mature hawthorn trees occur throughout the

acid grassland, but do not occur within any of the plots. The height of the vegetation canopy typically ranges from 5-15 cm. Mosses form a dense layer immediately above the litter horizon. The calcareous grassland is NVC class CG2d, *Festuca-Avenula* grassland and contrasts markedly with the adjacent acid grassland, being species rich rather than species poor (Table 2.2). The site supports a predominantly VA-mycorrhizal community and is host to a considerable number of forbs. Other non-VA-mycorrhizal species present include *Orchis mascula* and *Helianthemum nummularium*, which is thought to be colonised by the ectomycorrhizal fungus *Cenococcum graniforme* (Read *et al.*, 1977). The canopy is very short, typically ranging from 1-5 cm. Both grassland sites are subjected to light grazing by cattle and sheep during the Summer.

2.1.3 Nutrient manipulations

The N treatments are applied to all of the field sites in addition to background atmospheric N deposition (Table 2.3). The heathland plots receive the following treatments: control (water only), 4, 8 and 12 g N m^{-2} y^{-1} (as NH_4NO_3). Until 1992, applications were made approximately every two weeks (1.4 l of water m^{-2}) and thereafter on a monthly basis (2 l of water m^{-2}). The acidic and calcareous grassland sites receive: control (water only), 3.5, 7 and 14 g N m^{-2} y^{-1} (as NH_4NO_3) and 14 g N m^{-2} y^{-1} as $(NH_4)_2SO_4$. For the first year, these applications were made every two weeks (1 l of water m^{-2}) but since 1991 they have been made on a monthly basis (2 l of water m^{-2}). All of the long-term experimental sites are arranged in a completely randomised design with four blocks at each site (Figs. 2.1 and 2.2).

The short-term field plots established in 1995 received factorial applications of N and P in the following regime: control (water only), 3.5 and 14 g N m^{-2} y^{-1} repeated in combination with 3.5 g P g m^{-2} y^{-1} to give a total of 6 treatments. The plots are arranged in a completely randomised block design comprising of three blocks. Applications were made quarterly for the first year and monthly thereafter (2 l water $plot^{-1}$), using a backpack sprayer (Bastion 15, Application Techniques Ltd.) enabling precise and even coverage. The P additions to the calcareous grassland were terminated after 12 months since the response of the vegetation was considerable (Chapter 3). The 3.5 P treatments now receive water, while the 3.5 N+P and 14 N+P receive only respective additions of N.

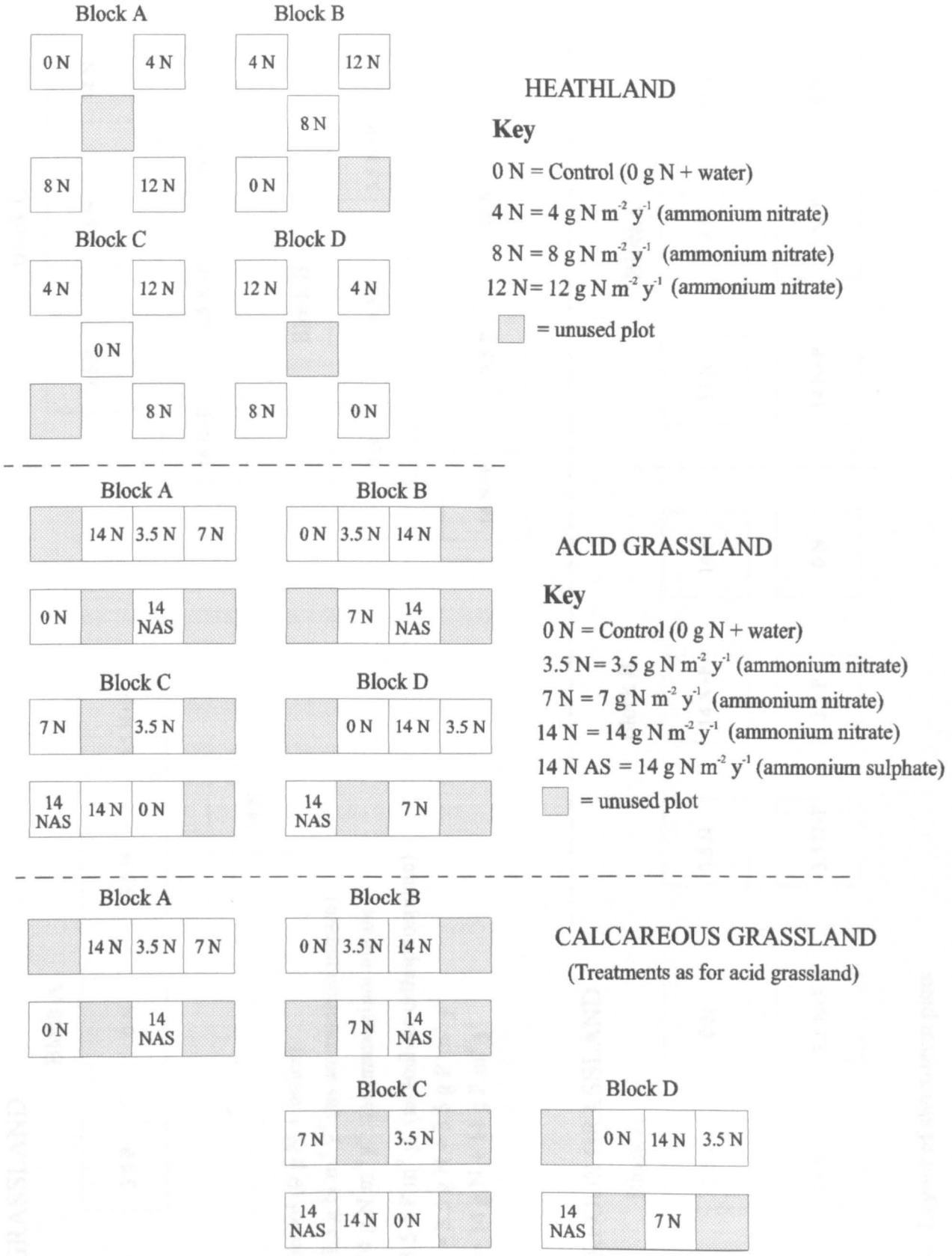


Fig. 2.1. Experimental layout of long-term plots.

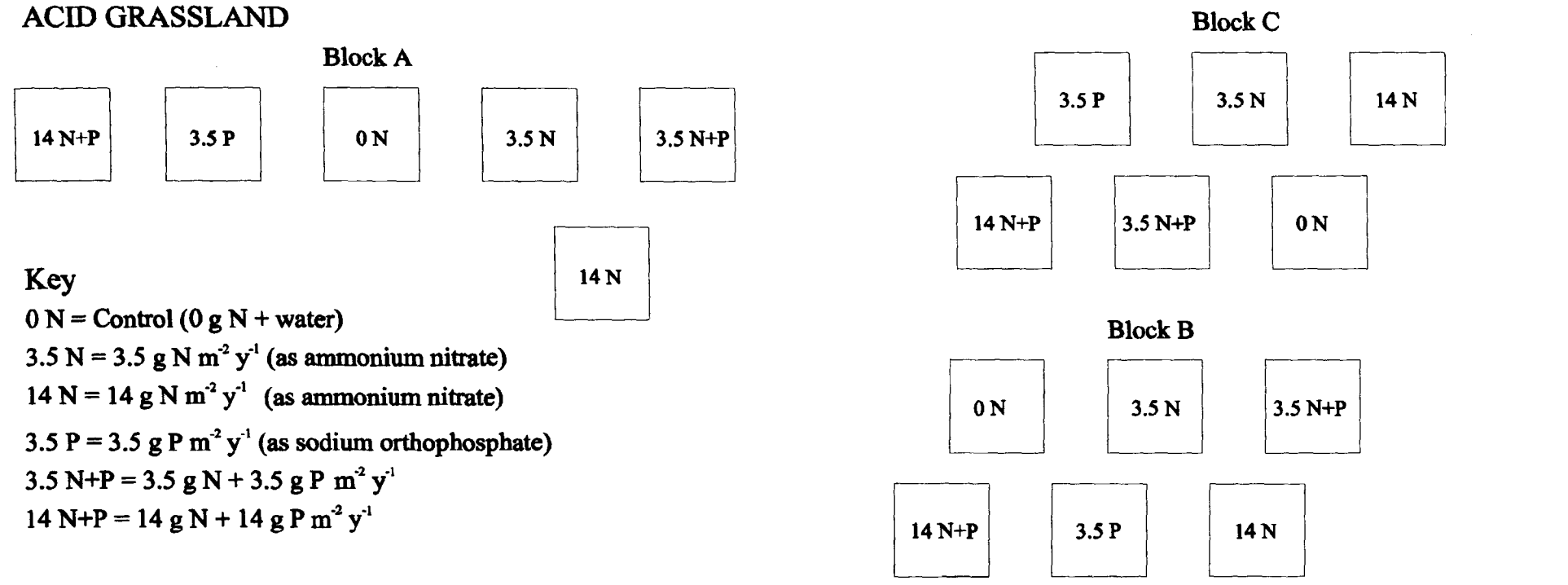


Fig. 2.3. Layout of short-term plots

2.2 GENERAL MATERIALS AND METHODS

2.2.1 Soil sampling and preparation

Many standard procedures characterising soil chemical and biological properties have been developed to use relatively large quantities of soil which has resulted in the adoption of sampling procedures that remove suitably large samples. However, removal of large quantities of soil from the long-term grassland and heathland plots is likely to result in considerable disturbance and damage to the vegetation owing to their small area (1 m^2). Consequently, the methodologies that are established for many soil analyses were adapted to permit removal of small soil samples so as to minimise the degree of vegetation disturbance. Two sizes of soil corers were used: a 1 cm diameter cork borer when only one or two measurements were required and a custom built 4 cm diameter corer when multiple measurements were required or when sampling from the larger short-term plots. Sampling usually involved removal of three cores during the two weeks prior to treatment applications from each plot to a maximum depth of 9 cm and were kept as separate samples (i.e. giving 12 replicate cores per treatment from the long-term plots and 9 replicate cores per treatment from the short-term plots). The cores were subsequently divided into individual horizons (F and H for heathland, F and Ah for acid grassland and Ah for calcareous grassland), sieved (2 mm) and incubated at room temperature for seven days. The incubation period allows microbial activity to stabilise following sieving. After this stabilisation period, the soils were either used immediately or stored at 4 °C and reincubated for 24 h at room temperature before use. Moist soil was used for determination of extractable N and P, pH, microbial biomass C, N and P, enzyme assays and substrate utilisation rates. Samples used for determination of soil cations, loss-on-ignition and moisture contents were dried (105 °C) for 48 h immediately after sieving.

2.2.2 Nutrient analysis of vegetation

All vegetation samples analysed for N and P were initially dried for 48 h (80 °C). Sample sizes were dependent on the age and species but typically ranged from 1-50 mg dwt. Samples were digested at 370 °C for 4 hours in a salicylic acid/sulphuric acid mix (Bremner and Mulvaney, 1982) and diluted with deionised water (1:10). The salicylic acid is added to minimise the volatility of the digest at high temperatures, while the addition of approximately

500 mg lithium sulphate/copper sulphate (10:1 ratio) to the digest catalyses the conversion of all NO_3-N to NH_4. Such a large range of sample weights necessitated adjustment of the amounts of reagent used in the acid digestion. In general, 0.5 ml of acid was used for samples <2 mg dwt, 1 ml for 2-10 mg dwt and 2 ml for 10-25 mg dwt. Samples were ground (mortar and pestle) only when a sub-sample was used. Determination of N and P was undertaken colorimetrically by auto-analysis (Tecator FIAStar 5010 auto-analyser) or using the ammonium molybdate method for P (John, 1970) and the indophenol blue method for NH_4 (Scheiner, 1976). The ammonium molybdate and indophenol blue methods proved particularly sensitive for very small samples (e.g. ca. <1 mg dwt) since the pH of the acid digest mix is neutralised by addition of 3.44 M NaOH rather than by dilution.

2.2.3 Loss-on-ignition and pH of soils

Loss-on-ignition was calculated by igniting approximately 1 g dry weight soil at 850 °C for 5 h and measuring weight loss (Allen, 1974). The pH of sieved soil (2 mm), which had not been stored, was mixed with water (1:2 ratio) and measured using a digital meter fitted with a glass electrode.

2.2.4 Extractable soil N and P

Nitrate (NO_3^-) and ammonium (NH_4^+) were extracted from the H horizon of the heathland and Ah horizon of the grassland soils by shaking (side-to-side) 1 g soil dwt (prepared as for Section 2.2.1) with 10 ml 2 M KCl for 45 minutes. The soil suspensions were either centrifuged (3000 rpm) or vacuum filtered through a Whatman No. 1 filter. P (PO_4) was extracted with 0.5 M $NaHCO_3$ adjusted to pH 8.5 with 0.5 M NaOH (Olsen *et al.*, 1954). For all the soils, an extractant:soil ratio of 10:1 was found to be optimal for determination of the low concentrations of P typically found in semi-natural ecosystems. All extracts were stored at -20 °C prior to colorimetric analysis (Tecator FIAStar 5010 auto-analyser).

2.2.5 Soil cations

The concentration of total soil Ca^{2+}, Al^{3+}, Fe^{2+}, Na^+ and K^+ were determined in the long-term calcareous grassland plots. 1.5 g OD soil (prepared as for Section 2.2.1) was digested in 30

ml aqua-regia mix ($HCl:HNO_3$ ratio of 1:4) for 12 h at 80 °C prior to filtering through a Whatman No. 1 filter. 10 ml of supernatant were made up to 50 ml with de-ionised water and 1 ml 0.5 M $LiSO_4$ (to minimise interference when measuring Ca) for the determination of the metal concentrations by flame AAS (Perkin-Elmer 2100).

2.2.6 Statistical analysis

Main treatment effects were analysed by analysis of variance (ANOVA) using Minitab (Release 9 or 11), unless otherwise stated. Multiple factor experiments or experiments with missing values were analysed using the Generalised Linear Model. Within treatment effects were assessed using the Tukey honestly significantly different multiple comparison test, as this is generally regarded as being more conservative and reliable than other tests, such as the LSD (Zar, 1996). Data that could not be normalised or have variance equalised following Log_{10} or square root transformations were analysed using non-parametric ANOVA (Kruskall-Wallis). All percentage data were arc-sine transformed prior to analysis. Mean values are presented as arithmetic means throughout the thesis.

2.3 SOIL CHEMICAL PROPERTIES FOLLOWING LONG-TERM N AND SHORT-TERM N AND P TREATMENTS

The concentrations of extractable inorganic N and P are of fundamental importance in semi-natural ecosystems, largely because plant growth in these systems is primarily limited by either one or both of these nutrients (Ellenberg, 1988). The vegetation in most semi-natural ecosystems is dependent upon rainfall to provide an adequate supply of N and therefore enhanced aerial N deposition has the potential to increase the supply of plant-available N. Alterations in nutrient supply may affect the growth of vegetation and subsequent rates of nutrient turnover. In some semi-natural ecosystems, nutrients other than N may exert the main control on plant productivity. In ombrotrophic bogs (Aerts *et al.*, 1992), fens (Verhoeven and Schmitz, 1991), coniferous forests (Emmett *et al.*, 1995) and heathlands (Aerts and Berendse, 1988), N enrichment has led to P being the main nutrient limiting plant productivity. Ecosystems which are naturally P limited are often characterised by low soil concentrations of available P. The lack of any significant change in the vegetation in the calcareous grassland used in the present study in response to three years N treatments

provides strong evidence that this site is naturally P limited (Morecroft *et al.*, 1994). Although the authors did not measure soil P concentrations, it is likely that much of the inorganic P is made unavailable through formation of insoluble Ca phosphates.

The degree of N saturation at the three sites used in this study may differ markedly given their contrasting soil and vegetation characteristics. Consequently, the demand for nutrients other than N (i.e. P) may also differ between each site.

2.3.1 Aims

This section aims to highlight the contrasting soil characteristics of the heathland, acid grassland and calcareous grassland and to quantify the soil inorganic N and P concentrations in the three field sites in response to long-term inputs of N. In addition, the N and P concentrations in the acid and calcareous grasslands will be quantified in response to short-term inputs of N and P.

2.3.2 Results

2.3.2.1 Inter-site differences in soil characteristics

The contrasting vegetation systems supported by the three field sites is reflected by marked differences in the soil chemical properties. The heathland is formed on a shallow layer (8-10 cm) of peat overlying shale and is classified as an ironpan stagnopodsol of the Hiraethog series (Rudeforth *et al.*, 1984; Table 2.1). The soil is enriched with organic matter which is reflected by a loss-on-ignition (LOI) value of 58 % (H horizon; Table 2.1). The high organic matter content is largely due to the low pH of this soil (3.5). The low pH and high organic matter content results in low concentrations of extractable inorganic N and P (1.02 and 15.22 $\mu g\ g^{-1}$ soil dwt respectively; Table 2.4). Ammonium was not found in any of the soil extracts.

The acid grassland soil overlays limestone to a depth of 70 cm and is classified as a Paleo-argillic brown earth of the Nordrach series (Ragg *et al.*, 1984; Table 2.1). The Ah horizon is enriched with organic matter, although not as strongly as the heathland, resulting in a high LOI value (40 %) and low pH (4.8). The extractable inorganic N was 6.1 $\mu g\ g^{-1}$ soil

dwt, which comprised 1.75 μg g^{-1} soil dwt nitrate and 4.34 μg g^{-1} soil dwt ammonium (Table 2.4). The extractable inorganic P concentration is 22 μg g^{-1} soil dwt, which is 45 % higher than the heathland.

The calcareous grassland soil is a shallow (5-10 cm) humic rendzina of the Marian series overlying limestone (Ragg *et al.*, 1984). The soil contrasts markedly with the other sites, having a lower organic matter content (LOI 32 %) and higher pH (pH 6.8; Tables 2.1 and 2.4). The concentration of extractable inorganic N was 10.8 μg g^{-1} soil dwt, which comprised 7.6 μg g^{-1} soil dwt nitrate and 3.2 μg g^{-1} soil dwt ammonium (Table 2.4). The close proximity of the limestone bedrock results in a low concentration of extractable inorganic P (7.9 μg g^{-1} soil dwt) probably due to the formation of insoluble calcium phosphates.

In the short-term acid grassland control plots, extractable inorganic N was 17.0 μg g^{-1} soil dwt, which comprised 0.58 μg g^{-1} soil dwt nitrate and 16.4 μg g^{-1} soil dwt ammonium (Table 2.6). These data differ markedly to the concentrations measured in the long-term control plots. In contrast, the concentration of extractable P was 24.3 μg g^{-1} soil dwt, a value similar to the long-term control plots.

In the short-term calcareous grassland control plots, extractable inorganic N was 16.3 μg g^{-1} soil dwt, and comprised 13.4 μg g^{-1} soil dwt nitrate and 2.92 μg g^{-1} soil dwt ammonium (Table 2.8). The concentration of extractable P was 3.4 μg g^{-1} soil dwt. The marked differences from the long-term control plots are reflected in the N:P ratios, which were 4.8 for the short-term plots and 1.4 for the long-term plots.

2.3.2.2 Effect of long-term N treatments

In the heathland, the N treatments resulted in significant changes in extractable inorganic N and P concentrations but did not affect soil pH. The concentration of nitrate increased significantly ($P<0.05$) from 1.02 μg N g^{-1} soil dwt in the control to 3.15 μg N g^{-1} soil dwt in the 8 N treatment and 4.84 μg N g^{-1} soil dwt in the 12 N treatment (Table 2.4). No

significant changes occurred in the 4 N treatment. A similar effect was seen for ammonium which increased significantly ($P<0.05$) to 4.17 and 6.79 μg N g^{-1} soil dwt in the 8 and 12 N treatments respectively. This resulted in an overall significant ($P<0.05$) increase in the total extractable N (nitrate + ammonium) in the 8 and 12 N treatments. In contrast, extractable inorganic P concentrations decreased significantly ($P<0.05$) from 15.2 μg P g^{-1} soil dwt in the control plots to 11.3 μg P g^{-1} soil dwt in the 4 N treatment, 9.8 μg P g^{-1} soil dwt in the 8 N treatment and 10.3 μg P g^{-1} soil dwt in the 12 N treatment (Table 2.4).

In the long-term acid grassland plots, the N additions had no effect on nitrate concentrations, which ranged from 2.9 μg N g^{-1} soil dwt in the 3.5 N treatment to 4.3 μg N g^{-1} soil dwt in the control, but caused a significant ($P<0.01$) increase in extractable inorganic ammonium at all treatment levels (Table 2.4). The ammonium concentrations extended over a 180-fold variation (0.67 to 124 μg N g^{-1} soil dwt). The concentrations reached a plateaux in the 7 and 14 N treatments, but increased further by over 110 % in the 14 N AS treatment. The dominance of ammonium over nitrate resulted in a similar pattern for total extractable inorganic N which increased significantly ($P<0.01$) at all treatment levels from 6.1 μg g^{-1} soil dwt in the control to 113 μg g^{-1} soil dwt. Extractable inorganic P was not significantly affected by the N treatments when compared to the control. However, the concentrations increased significantly ($P<0.05$) from 18.3 and 20.0 μg P g^{-1} soil dwt in the 3.5 N and 14 N treatments to 29.1 μg P g^{-1} soil dwt in the 14 N AS treatment (Table 2.4).

In the calcareous grassland, the N treatments had the greatest effect on extractable nitrate and ammonium concentrations. Here, nitrate concentrations increased progressively with each increment of ammonium nitrate from 7.6 μg N g^{-1} soil dwt in the control to 45.8 μg N g^{-1} soil dwt in the 14 N treatment. The concentration of nitrate in the 14 N AS treated plots was 38.2 μg N g^{-1} soil dwt which was also significantly ($P<0.05$) higher than in the control, but significantly ($P<0.05$) lower than the 14 N treatment. The concentration of ammonium increased significantly ($P<0.05$) from 3.2 μg N g^{-1} soil dwt in the control to 9.2 μg N g^{-1} soil dwt in the 14 N treatment and 45.3 μg N g^{-1} soil dwt in the 14 N AS treatment (Table 2.4). The changes in concentrations of nitrate and ammonium resulted in progressive increases in total extractable inorganic N in response to the treatments (Table 2.4) which extended over a 35-fold variation (3.8-136 μg N g^{-1} soil dwt). In contrast, extractable inorganic P

concentrations progressively decreased from 7.9 μg P g^{-1} soil dwt in the control to 4.7 μg P g^{-1} soil dwt in the 14 N AS treatment (Table 2.4).

Considerable soil acidification was seen at the calcareous grassland where the pH decreased significantly ($P<0.05$) from 6.8 in the control to 6.1 in the 14 N treatment and 5.2 in the 14 N AS treatment (Table 2.4). There was strong evidence suggesting that the soil acidification has resulted in decalcification in the 14 N AS treated plots, where the concentration of total Ca decreased significantly ($P<0.05$) from 8.7 to 5.5 mg g^{-1} dwt (Table 2.5). No changes were seen in the concentration of total Al, Fe, Na or K (Table 2.5).

2.3.2.3 Effect of short-term N and P treatments

Short-term (18 months) additions of N to the acid grassland significantly ($P<0.05$) increased extractable nitrate concentrations from 0.58 μg N g^{-1} soil dwt in the control to 2.94 μg N g^{-1} soil dwt in the 14 N treatment (Table 2.6). When P was added in combination, the concentrations fell to control levels. No significant effects of the combined N and P treatments were seen on ammonium or total extractable inorganic N concentrations. However, there was a significant ($P<0.05$) overall N effect which increased ammonium concentrations from 14.7 μg N g^{-1} soil dwt in the no added N treatments (i.e. control and 3.5 P) to 33.1 μg N g^{-1} soil dwt in the +14 N treatments (i.e. 14 N and 14 N+P; Table 2.7). Similarly, total extractable inorganic N concentrations increased significantly ($P<0.05$) from 15.2 μg N g^{-1} soil dwt in the no added N treatment to 35.8 μg N g^{-1} soil dwt in the +14 N treatment (Table 2.7). Concentrations of nitrate were typically thirty times lower than ammonium.

Concentrations of inorganic P ranged from 22.0 to 50.1 μg g^{-1} soil dwt in the N and P treated plots (Table 2.6). There was a strong suggestion of a progressive increase in P concentrations following additions of N and P, where they increased from 24.3 μg g^{-1} soil dwt in the control to 31.8 μg g^{-1} soil dwt in the 3.5 P treatment, 45.4 μg g^{-1} soil dwt in the 3.5 N+P treatment and 50.1 μg g^{-1} soil dwt in the 14 N+P treatment (Table 2.6). However, the P concentration was significantly ($P<0.05$) higher only in plots that received P (i.e. 3.5 P,

3.5 N+P and 14 N+P treatments), where it increased from 26.0 μg P g^{-1} soil dwt in the no added P treatments to 44.7 μg P g^{-1} soil dwt in the +P treatments (Table 2.7).

In the short-term calcareous grassland plots, the concentrations of nitrate and ammonium ranged from 1.8 to 13.4 and 2.9 to 10.5 μg N g^{-1} soil dwt respectively while extractable inorganic P ranged from 3.1 to 5.4 μg P g^{-1} soil dwt (Table 2.8). The response to the N and P applications at this site contrasted markedly with the long-term plots. Only a 15-fold variation (1.9-29.9 μg N g^{-1} soil dwt) was seen in the concentration of total extractable inorganic N. More importantly, no changes in nitrate or total inorganic N concentrations were seen when N was applied without P. However, in plots receiving P, the concentrations of nitrate and total inorganic N decreased significantly ($P<0.05$) and inorganic P concentrations increased significantly (Table 2.9).

Table 2.1. General characteristics of the field sites used. Letters in parentheses refer to relevant soil horizon.

Field site	Location (NGR)	Altitude (m)	Precipitation (mm)	Soil series	Soil type	Soil texture	Soil horizons and depths (cm)	pH (H_2O)	Loss-on-ignition (%)
Heathland	SJ 225 490	470	900[a]	Hiraethog[c]	Ironpan stagnopodsol[c]	Silty clay loam[c] (H)	L (0-2) F (2-5) H (5-15)	3.7 (H)	58 (H)
Acid grassland	SK 180 739	350	893[b]	Nordrach[d]	Paleo-argillic brown earth[d]	Silt loam[d] (Ah)	L (0-1) F (1-3) Ah (3-15)	4.8 (Ah)	40 (Ah)
Calcareous grassland	SK 178 738	350	893[b]	Marian[d]	Humic rendzina[d]	Sandy loam[d] (Ah)	L (0-1) Ah (1-7)	6.8 (Ah)	32 (Ah)

[a]S. J. M. Caporn (pers. comm.)

[b]UKRGAR (1997)

[c]Rudeforth *et al.* (1984)

[d]Ragg *et al.* (1984)

Table 2.2. Mean % cover of higher and lower plant species found at the field sites (J. Carroll, pers. comm.)

Field site					
Heathland		Acid grassland		Calcareous grassland	
Calluna vulgaris	96	*Agrostis capillaris*	75.7	*Festuca ovina*	80.5
Vaccinium myrtillus	<1	*Festuca ovina, F. rubra*	72.4	*Carex caryophyllea* Latour	44.2
Juncus effusus L.	<1	*Potentilla erecta*	62.4	*Thymus praecox* Auct. non Opiz	36.5
Moss and lichens †	<1	Moss‡	54.1	*Briza media* L.	31.8
		Galium saxatile	46.7	*Avenula pratensis* (L.) Dumort.	27.8
		Nardus stricta	30.6	*Carex flacca* Schreber	26.0
		Lathyrus montanus Bernh.	16.0	*Sanguisorba minor* Scop.	25.2
		Trifolium pratense L.	10.3	*Helianthemum nummularium* (L.) Miller	25.0
		Deschampsia flexuosa	7.6	*Koeleria cristata* auct. non (L.) Pers.	14.3
		Anenome nemorosa L.	7	*Lotus corniculatus* L.	10.6
				Moss †‡	10.0
				Hieracium pilosella L.	8.6
				Plantago lanceolata L.	5
				Scabiosa columbaria L.	5
				Orchis mascula (L.) L.	<1
				Gentianella amarella Ssp. *amarella* (L.) Boerner	<1
Total higher plant species:	3		10		16

† Includes: *Hypnum commutatum* Hedw., *Pleurozium schreberi* (Brid.) Mitt., *Dicranum scoparium* Hedw., *Cladonia arbuscula* (Wallr.) Rabenh., *C. portentosa* (Dufour) Coem., *Hypogymnia physodes* (L.) Nyl..

‡ Includes: *Rhytidiadelphus squarrosus* (Hedw.) Warnst., *Hypnum cupressiforme* Hedw., *Pleurozium schreberi, Mnium hornum* Hedw., *Pseudoscleropodium purum* (Hedw.) Fleisch., *Plagiothecium denticulatum* (Hedw.) B., S. & G., *Dicranum bonjeanii* DeNot..

†‡ Includes: *Ctenidium molluscum* (Hedw.) Mitt., *Dicranum scoparium* Hedw., *Fissidens taxifolius* Hedw., *P. denticulatum*, *Pseudoscleropodium purum*, *Thuidium tamariscinum* (Hedw.) B., S. & G.

Table 2.3. Atmospheric inputs of wet deposited N to the field sites and atmospheric concentrations of NO_2 and NH_3. NA = not available.

Field site	Atmospheric inputs				
	Wet deposition (g N m^{-2} y^{-1})			Gas concentration (µg m^{-3})	
	NO_3	NH_4	Total	NO_2	NH_3
Heathland†	3.981	NA	3.981	132	0.62
Acid and calcareous grasslands	0.413‡	0.550‡	0.963‡	21.6‡	1.52

† L. Cawley, pers. comm.

‡ UKRGAR (1997)

Table 2.4. Extractable NO_3^--N, NH_4^+-N and P (μg g^{-1} soil dwt) and pH in plots that have received N for up to 8 years (± SEM). Values sharing a letter are not significantly different (P>0.05). NA = not analysed.

Treatment	NO_3	NH_4	$NO_3 + NH_4$	P	pH
	Heathland				
Control	1.02^a (± 0.35)	0.00^a (± 0.00)	1.02^a (± 0.35)	15.22^a (± 1.00)	3.67^a (± 0.03)
4 N	0.86^a (± 0.35)	0.35^a (± 0.20)	1.10^a (± 0.43)	11.30^b (± 1.76)	NA
8 N	3.15^b (± 0.59)	1.02^b (± 0.25)	4.17^b (± 0.79)	9.79^b (± 1.78)	3.67^a (± 0.03)
12 N	4.84^b (± 0.66)	3.68^c (± 1.33)	6.79^b (± 1.93)	10.25^b (± 1.56)	3.72^a (± 0.05)
	Acid grassland				
Control	4.34^a (± 0.61)	1.75^a (± 0.61)	6.09^a (± 1.16)	22.13^{ab} (± 1.10)	4.80^a (± 0.21)
3.5 N	2.89^a (± 0.38)	24.60^b (± 1.73)	27.49^b (± 1.57)	18.29^b (± 1.92)	4.58^a (± 0.12)
7 N	4.00^a (± 0.39)	60.52^{bc} (± 19.86)	64.52^{bc} (± 19.60)	21.95^{ab} (± 1.71)	4.33^a (± 0.20)
14 N	3.45^a (± 0.69)	51.19^{bc} (± 19.10)	54.63^{bc} (± 18.43)	20.01^b (± 2.59)	4.39^a (± 0.17)
14 N AS	3.17^a (± 0.57)	110.1^c (± 5.6)	113.25^c (± 5.86)	29.14^a (± 4.57)	4.58^a (± 0.11)
	Calcareous grassland				
Control	7.55^a (± 1.65)	3.19^a (± 0.19)	10.75^a (± 1.60)	7.91^a (± 1.04)	6.77^a (± 0.08)
3.5 N	11.26^b (± 0.75)	2.82^a (± 0.11)	14.08^a (± 0.75)	7.54^a (± 0.64)	6.56^{ab} (± 0.06)
7 N	20.55^{bc} (± 2.09)	4.10^{ab} (± 0.30)	24.64^b (± 2.12)	NA	6.27^{bc} (± 0.04)
14 N	45.77^d (± 5.71)	9.16^b (± 1.78)	54.93^c (± 6.12)	6.27^{ab} (± 0.21)	6.10^c (± 0.08)
14 N AS	38.22^c (± 7.37)	45.31^c (± 9.69)	83.52^c (± 9.83)	4.67^b (± 0.54)	5.19^d (± 0.11)

Table 2.5. Total soil Ca, Al, Fe, Na and K concentrations in calcareous grassland plots that have received 7 years of N treatments (± SEM). Values sharing a letter are not significantly different (P>0.05).

Treatment	Ca (mg g^{-1} dwt)	Al (mg g^{-1} dwt)	Fe (mg g^{-1} dwt)	Na (µg g^{-1} dwt)	K (µg g^{-1} dwt)
Control	8.71^{a} (± 0.26)	9.36^{a} (± 0.22)	16.61^{a} (± 0.15)	65^{a} (± 2.3)	668^{a} (± 15.8)
3.5 N	9.23^{a} (± 0.10)	9.88^{a} (± 0.15)	17.15^{a} (± 0.15)	57^{a} (± 3.5)	667^{a} (± 26.2)
14 N	7.39^{ab} (± 0.20)	9.80^{a} (± 0.13)	17.39^{a} (± 0.13)	43^{a} (± 1.1)	655^{a} (± 11.0)
14 N AS	5.51^{b} (± 0.19)	9.64^{a} (± 0.18)	17.13^{a} (± 0.20)	47^{a} (± 1.5)	661^{a} (± 21.7)

Table 2.6. Extractable NO_3^--N, NH_4^+-N and P (μg g^{-1} soil dwt) in an acid grassland soil that has received 18 months N and P treatments (± SEM). Values sharing a letter are not significantly different (P>0.05).

Treatment	NO_3	†NH_4	†NO_3 + NH_4	‡P
Control	0.58^a (± 0.07)	16.4^a (± 3.9)	17.0^a (± 3.9)	24.3^a (± 3.5)
3.5 N	0.60^a (± 0.15)	21.2^a (± 7.1)	21.8^a (± 7.1)	26.7^a (± 4.1)
14 N	2.94^b (± 0.64)	46.7^a (± 6.3)	49.3^a (± 6.6)	22.0^a (± 4.3)
3.5 P	0.39^a (± 0.05)	13.0^a (± 5.2)	13.3^a (± 5.2)	31.8^a (± 10.0)
3.5 N+P	1.14^a (± 0.39)	24.2^a (± 4.2)	25.2^a (± 4.4)	45.4^a (± 9.7)
14 N+P	0.74^a (± 0.11)	21.6^a (± 2.2)	22.2^a (± 2.2)	50.1^a (± 7.3)

† Overall N effect; see Table 2.7.

‡ Overall P effect; see Table 2.7.

Table 2.7. Overall effect of 18 months N additions on extractable NH_4^+-N and NO_3^--N + NH_4^+-N concentrations and 18 months P additions on extractable P concentrations in acid grassland plots (μg g^{-1} dwt; ± SEM). Values sharing a letter are not significantly different (P>0.05).

Treatment	NH_4	NO_3 + NH_4	Treatment	P
No added N	14.7^a (± 4.5)	15.2^a (± 4.6)	No added P	26.0^a (± 4.1)
3.5 g N m^{-2} y^{-1}	22.7^{ab} (± 5.6)	23.5^{ab} (± 5.7)	3.5 g P m^{-2} y^{-1}	44.7^b (± 10.3)
14 g N m^{-2} y^{-1}	33.1^b (± 4.3)	35.8^b (± 4.4)		

Table 2.8. Extractable NO_3^--N, NH_4^+-N and P (μg g^{-1} soil dwt) in a calcareous grassland soil that has received 18 months N and 12 months P treatments (± SEM). Values sharing a letter are not significantly different (P>0.05).

Treatment	†NO_3	NH_4	†NO_3 + NH_4	†P
Control	13.35^a (± 1.55)	2.92^a (± 0.53)	16.28^a (± 1.87)	3.40^a (± 0.34)
3.5 N	9.44^a (± 1.85)	10.45^b (± 2.50)	18.73^a (± 2.08)	3.88^a (± 0.27)
14 N	10.91^a (± 2.46)	7.93^{ab} (± 2.09)	18.84^a (± 1.86)	3.12^a (± 0.40)
3.5 P	3.02^a (± 0.87)	5.32^{ab} (± 2.00)	7.67^a (± 2.70)	5.37^a (± 0.74)
3.5 N+P	1.82^a (± 0.49)	4.11^{ab} (± 0.71)	5.94^a (± 0.70)	4.85^a (± 0.39)
14 N+P	4.49^a (± 1.71)	3.87^{ab} (± 0.97)	8.36^a (± 1.74)	4.39^a (± 0.80)

† Overall P effect; see Table 2.9.

Table 2.9. Overall effect of 12 months P additions on extractable NO_3^--N, NO_3^--N + NH_4^+-N and P concentrations in calcareous grassland plots (μg g^{-1} dwt; ± SEM). Values sharing a letter are not significantly different (P>0.05).

Treatment	NO_3	NO_3 + NH_4	P
No added P	11.24^a (± 1.95)	17.95^a (± 1.94)	3.47^a (± 0.34)
3.5 g P m^{-2} y^{-1}	3.11^b (± 1.02)	7.32^b (± 1.71)	4.88^b (± 0.65)

2.4 DISCUSSION

2.4.1 Inter site characteristics

The field sites used for this study are unique in that they enable the effects of both long-term (eight years) and short-term (18 months) chronic N additions to be investigated in three contrasting semi-natural ecosystems. In addition, the field sites form a gradient of increasing species richness (Table 2.2). The *Calluna* dominated heathland plots supports just 3 higher plant species, the acid grassland 10 while the calcareous grassland plots supports 16. The response of the heathland vegetation to increased N supply may therefore differ from many of the Dutch heaths which often contain considerably higher proportions of grasses, such as *Molinia caerulea*. The gradient in species richness is also reflected by the soil pH and extractable inorganic N concentrations. Soil pH increased from 3.7 in the heathland to 4.8 in the acid grassland and 6.8 in the calcareous grassland, while extractable inorganic N increased from 1.02 μg g^{-1} soil dwt in the heathland to 6.1 μg N g^{-1} soil dwt in the acid grassland and 10.8 μg N g^{-1} soil dwt in the calcareous grassland. The extractable inorganic P concentrations were, however, greatest in the acid grassland and lowest in the calcareous grassland. This is expected since the high Ca content of calcareous soils leads to formation of insoluble Ca phosphates. The relative abundance of P in relation to N at the three sites is reflected by the N:P ratios. In the heathland control plots, the N:P ratio was 0.067, in the acid grassland 0.276 and in the calcareous grassland 1.36. This ratio may thus provide a useful index of P limitation.

The N and P concentrations reported here are generally similar to published values relating to similar ecosystems. In many heathland soils, ammonium is found in greater concentrations than nitrate. For example, De Boer *et al.* (1990) reported nitrate concentrations in the range 0 and 12.4 μg N g^{-1} soil dwt and ammonium concentrations in the range 9.3 and 211 μg N g^{-1} soil dwt in the F and H horizons of 12 *Calluna* dominated lowland heaths. A similar pattern was reported by Troelstra *et al.* (1995) who measured up to 4 μg N g^{-1} soil dwt nitrate and between 1 and 10 μg N g^{-1} soil dwt ammonium in two *Deschampsia flexuosa* dominated heaths. It is therefore surprising that ammonium was not found to be the dominant form of inorganic N in the present study since nitrification is often inhibited in acidic soils due to the high proportion of fungal biomass and reduced activity of

nitrifying bacteria. In addition, nitrate is more mobile than ammonium, which can be tightly adsorbed onto humic material or made relatively unavailable by fixation between inter-layer spaces of clay minerals (Paul and Clarke, 1996). The concentrations of nitrate and ammonium in the acid and calcareous grasslands are low, but are comparable to other similar semi-natural grasslands. Birch (1988) reported between 8 and 40 μg N g^{-1} dwt as nitrate and 5 and 9 μg N g^{-1} dwt ammonium in a rendzinaform soil on the slopes of Lathkill Dale, Derbyshire (8 km South of Wardlow Hay Cop).

Few studies have investigated the concentrations of extractable inorganic P in upland heathland soils. Troelstra *et al.* (1995) measured only 6.2 and 6.6 μg P g^{-1} soil dwt in two *D. flexuosa* dominated lowland heaths in The Netherlands, but considerably higher values of between 50 and 89 μg P g^{-1} soil dwt have been reported for deciduous woodland soils with comparable pH (3.1-4.5) and organic matter contents (% organic matter 28-39; Harrison, 1975, 1983). Adams (1986) measured 11 μg g^{-1} soil dwt inorganic P in an upland heather moor in Glen Tanar, Scotland, a value not dissimilar from the present study. In the A_o horizon of a loessic brown earth on the plateau of Lathkill Dale, Gupta and Rorison (1974) measured only 1.5 μg g^{-1} soil dwt extractable inorganic P. This value is considerably lower than those presented in this thesis, despite the Lathkill Dale soil sharing many similarities to the acid grassland used in the present study. In contrast, a concentration of 7.2 μg P g^{-1} soil dwt was measured in the rendzina soil, found on the steep slopes of Lathkill Dale, which is almost identical to the concentrations reported for the calcareous grassland in the present study.

Substantial differences in extractable inorganic N and P concentrations were seen between the short-term and long-term control plots in both the acid and calcareous grasslands, but was particularly apparent for the ammonium and nitrate concentrations in the acid grassland. Although the plots are in close proximity, the soil depths differ which may imply disparities in physicochemical properties. Storage of soils for more than 1 week has been shown to lead to significant release of inorganic P in rendzina and brown earth soils, although this effect is minimised when soils are stored at 5 °C (Gupta and Rorison, 1974). Of further importance, however, is the sample date. Seasonal changes in the nutrient status of soils is common. Shand *et al.* (1994) reported that inorganic P concentrations fluctuated

from 2.0 to 12.1, 3.2 to 9.9 and 2.6 to 7.2 μg dm^{-3} in three Scottish upland grasslands between May and October. The greatest increases or decreases appeared to occur in August. In the present study, the soils from the long-term and short-term plots were only sampled on one occasion between June and August and, therefore, seasonal effects could account for the differences in N and P concentrations. Future work at the three field sites will involve quantification of inorganic N and P concentrations throughout the season.

2.4.2 Effect of N and P treatments on soil chemical properties

2.4.2.1 Long-term plots

The accumulation of inorganic N in the soil solution of the heathland provides strong evidence that this site has become, or is becoming N saturated. This is supported by the decreases in P concentrations which may indicate that the N saturation has led to an increase in the demand for this nutrient. The lack of any change in soil pH at this site indicates i) equal uptake of nitrate and ammonium ions, ii) no uptake of ammonium and nitrate, or iii) the system is capable of buffering any additional release of H^+ or OH^- ions as a consequence of preferential uptake of ammonium or nitrate. It seems likely that some of the additional N is being used since both substantial growth responses and increases in shoot N concentrations have been reported at this site (S. J. M. Caporn, pers. comm.). In addition, *Calluna* has been shown to preferentially use ammonium rather than nitrate as a N source (Read and Bajwa, 1985). Exclusive utilisation of ammonium has the potential to cause soil acidification since plants release a proton for each ammonium ion taken up, but also because nitrate leaching is often associated with loss of base cations. Although it is presently not known if nitrate leaching and associated loss of base cations is occurring, it would be expected that plants are preferentially taking up ammonium and releasing H^+ ions. This would support point iii) that the system is presently capable of buffering changes in the ionic balance of the soil.

Although the range of ammonium concentrations in the acid grassland plots was substantial, extending over a 180-fold variation, the increases were not in proportion to the amount of N added. This contrasted markedly with nitrate concentrations which did not differ significantly in any of the treatments. The lack of any effect of the N additions on

extractable nitrate concentrations suggests increases in either nitrate leaching or denitrification. The latter process has been shown to be an important pathway for nitrate losses in acid soils (Killham and Hulm, 1988). This may also help to explain the lack of any acidification at this site since the reduction of 1 mol NO_3^- to N_2 involves the consumption of up to 6 protons (Paul and Clarke, 1996):

$$2NO_3^- + 12H^+ + 10e^- \rightarrow 6H_2O + N_2$$

The increase in ammonium concentrations but no change in P indicates that this site is clearly N saturated and may be moving towards P limitation. A number of factors are likely to be influencing the pattern of ammonium accumulation. It is certainly probable that ammonium ions are being adsorbed onto colloidal organic material at this site, since this is one of the most important mechanisms for ammonium retention in acid organic soils (Paul and Clarke, 1996). However, N additions to the 7 N, 14 N and 14 N AS treatments are resulting in a 'priming effect' (Bingeman *et al.*, 1953) or an 'added nitrogen interaction' (Jenkinson *et al.*, 1985) whereby inputs of inorganic N leads to more rapid mineralisation of residual organic N (J. Carroll pers. comm.) and so this process is also likely to contribute to the considerable increases in ammonium concentrations reported here. The N treatments have led to progressive reductions in vegetation cover in the acid grassland plots (Table 5.4; J. Carroll, pers. comm.), which was most apparent in the 14 N AS treatment. Clearly, this large-scale loss of vegetation will lead to lower levels of N uptake while commensurate increases in senescing root material may stimulate N mineralisation. The loss of bryophytes (Chapter 3) in particular may also have a significant role in N accumulation in soil. Mosses rely almost exclusively on rainfall derived nutrients for their nutrition and so the deep mat normally present is likely to have been an important sink for any added N.

In the calcareous grassland, concentrations of both extractable nitrate and ammonium increased in the plots receiving N additions. The accumulation of nitrate in the ammonium nitrate treatments is surprising given the mobility of this ion and the shallow soil at this site. Of further interest is the accumulation of nitrate in the 14 N AS treatment, since the only N source in this treatment is ammonium. This indicates that substantial nitrification is occurring. Autotrophic nitrification is dependent on an adequate supply of ammonium which is then oxidised via nitrite to nitrate. Significant increases in the nitrification rate in response to the 7 N, 14 N and 14 N AS treatments have been reported (Morecroft *et al.*,

1994). A disproportionate increase in the concentration of ammonium was seen between the 14 N and 14 N AS treatment (Table 2.4). It would be expected that the concentrations in the 14 N AS treatment would be approximately double those in the 14 N treatment, since twice as much ammonium is applied. This may, in part, be explained by the higher nitrification rates observed in the 14 N treatments and the higher ammonification rates reported during Winter and Spring in the 14 N AS treatment (J. Carroll, pers. comm.).

All three sites appear to be accumulating relatively large amounts of inorganic N, although the extent differs depending on the soil type and form of N considered. This provides some evidence that these sites are becoming or have become N saturated. This may imply that the systems are likely to become limited by nutrients other than N, such as P. The N:P ratio may provide an indication of the strength of P limitation. The ratio increased in the heathland from 0.067 in the control to 0.66 in the 12 N treatment, in the acid grassland from 0.276 to 3.89 in the 14 N AS treatment and in the calcareous grassland from 1.36 to 17.8 in the 14 N AS treatment. These potential increases in P limitation will be investigated in future chapters by quantifying phosphatase activities (Chapters 4 and 6) and microbial respiratory responses to additions of organic P compounds (Chapter 7).

The high rates of nitrification are likely to be the cause of the reduction in soil pH, as reported for other N enriched ecosystems (Makarov and Kiseleva, 1995; Nambu *et al.*, 1994; Wilson and Skeffington, 1994), since the production of nitrate by this process releases two H^+ ions. The considerable soil acidification at this site might be expected to increase P availability through the dissolution of dicalcium and tricalcium phosphates. However, Faurie and Fardeau (1990) have examined the effects of acidification arising from nitrification in neutral and calcareous soils and showed that although Ca and Mg ions are brought into solution by this process, these ions originate from dissolution of carbonates and the Ca phosphates are not dissolved and remain unavailable to plants. This is consistent with the results presented in this thesis which indicate reduced rather than increased P availability in the calcareous grassland soil, despite the considerable acidification.

2.4.2.2 Short-term plots

In the short-term acid grassland plots, concentrations of ammonium and total extractable inorganic N increased in response to the addition of N (Table 2.7). The concentrations of total extractable inorganic N in the 14 N treatment were similar to the 14 N treatment in the long-term plots which, at first sight, may indicate that N saturation is occurring after just 18 months of N additions. However, the percentage increase from the control was only 190 % which contrasts markedly to the long-term plots where an 800 % increase was seen. Extractable inorganic P concentrations increased in plots that had received additions of P (i.e. 3.5 P, 3.5 N+P and 14 N+P treatments; Table 2.7). Despite the greater P availability, no increases in above ground biomass or significant alterations in the vegetation structure in the P treated plots were seen (Chapter 3). Alterations to the vegetation structure in response to the N additions, as reflected by a significant decrease in the dicotyledon:monocotyledon ratio, provides evidence that the acid grassland remains N limited after 18 months of treatments.

Only a 15-fold variation (1.9 to 29.9 μg N g^{-1} soil dwt) was seen in the concentrations of total extractable inorganic N in the short-term calcareous grassland plots. However, total extractable N concentrations were significantly reduced in plots that received additions of P. It is likely that this decrease may at least in part be explained by the increases in above ground biomass that were recorded for these plots (120 to 180 g m^{-2}; Fig 3.6a).

CHAPTER 3

ABOVE GROUND VEGETATION RESPONSES TO LONG-TERM INPUTS OF N AND SHORT-TERM INPUTS OF N AND P

3.1 INTRODUCTION

Recognition that increased aerial deposition of N may lead to adverse effects on vegetation systems was first reported in The Netherlands. Heil and Diemont (1983) showed that raised inputs of N to plant communities adapted to low nutrient levels may alter the competitive relationships between species resulting in a loss of diversity. Many Dutch lowland heaths have been lost through the invasion of grasses such as *Molinia caerulea, Deschampsia flexuosa* and *Festuca ovina* (Pearson and Stewart, 1993). In a greenhouse experiment, *Deschampsia flexuosa* was found to be more competitive in the use of added N than *Calluna vulgaris* (Mickel *et al.*, 1991). Interactions between N supply and other factors, such as drought resistance and susceptibility to attack by heather beetle (*Lochmaea suturalis* (L.) Thomson) appear to be crucial. Heil and Diemont (1983) observed that the *Calluna* canopy opened-out following a heather beetle infestation enabling the more competitive *Festuca ovina* to invade. The subsequent decay of *Calluna* shoots and heather beetle corpses and faeces provides an additional source of N to these soils. Species rich grasslands, particularly those associated with shallow, nutrient-poor calcareous soils, have also been found to be sensitive to N pollution. Bobbink (1991) reported reduced species diversity as a consequence of increased dominance by the nitrophilous grass *Brachypodium pinnatum* in Dutch calcareous grasslands in response to raised loads of N pollution. The mechanism responsible for the success of *Brachypodium pinnatum* has not been established but an increase in biomass and litter production may limit the amount of light available to forbs (Wilson *et al.*, 1994).

In the UK, such dramatic vegetation responses have yet to be observed, although this does not mean that vegetation communities are not under threat. A number of differences exist between UK and Dutch systems, notably; i) deposition rates in the UK are lower than those in The Netherlands, ii) ammonia is a more important component of total deposited N in The Netherlands, iii) many of the chalk grasslands in the UK are traditionally grazed, while in The Netherlands they are unmanaged or mown (Wilson *et al.*, 1994), iv) *Brachypodium pinnatum* is less common in many UK grasslands which are dominated instead by grasses such as *Festuca* spp., v) management of heathlands and grasslands in each country differs. Upland heaths and heather moors are burnt regularly in the UK and by this process can loose substantial amounts of N accumulated in biomass (Allen *et al.*, 1969) so as to form

monocultures of *Calluna*, while the lowland Dutch heaths tend to be interspersed with the more competitive grasses, and vi) the climate of UK upland heathlands may prevent population explosions of the heather beetle.

There is growing evidence that increased N deposition may affect the frost tolerance of *Calluna*. For example, Caporn *et al.* (1998) observed a 75 % increase in frost damage to the leading shoots of *Calluna* treated regularly with 12 g N m^{-2} for seven years. However, these effects could in part be due to the taller canopy of the N treated plots giving greater exposure to wind desiccation and low temperature stress. Many of the factors outlined above may lead to more subtle changes in UK plant communities. Bryophytes in particular have been shown to be affected by increased atmospheric pollutants, including deposition NO_x (Baddeley *et al.*, 1994; Pitcairn *et al.*, 1995). Their sensitivity arises from them lacking cuticles and their direct foliar absorption of a high proportion of their nutrient supply which originates from atmospheric sources. The dominant species of moss in ombrotrophic mires belong to the genus *Sphagnum*. Experimentally elevated levels of N supply have been shown to lead to increased uptake of inorganic N and subsequent release of organic N indicating the importance of these species in the cycling of N in ombrotrophic mires (Silcock and Williams, 1995; Baxter *et al.*, 1992). Deposition of SO_x has caused changes in the abundance and diversity of *Sphagnum* species and although this form of pollution has decreased, raised levels of atmospheric N affecting the growth and physiology of *Sphagnum* species may have prevented re-establishment (Lee *et al.*, 1993).

There are few data in the literature from field-based experimental investigations into the effects of simulated N deposition on UK semi-natural grasslands. However, Morecroft *et al.* (1994) revealed a decrease in the abundance of the moss *Rhytidiadelphus squarrosus* in response to 14 g N m^{-2} y^{-1} (as ammonium sulphate) in an acid grassland. Incremental additions of N from 3.5 to 14 g N m^{-2} y^{-1} (as ammonium nitrate) also led to a decrease in nitrate reductase activity in shoots of *R. squarrosus*. No effects were observed on the abundance of bryophytes or vascular plants present in the calcareous grassland, which were assumed to be co-limited by N and P, or in vascular plants present in the acid grassland. The compounding effects of N and P co-limitation in some ecosystems make interpretation of N deposition effects difficult. There is increasing evidence that some ecosystems

receiving increased supplies of N deposition have now become 'N saturated' and no longer show increased biomass production after addition of N (Aber *et al.*, 1989). However, the absence of growth stimulation in response to increasing N deposition should not be viewed as evidence that N enrichment has no significant effects; indeed in many cases N saturation has resulted in devastating declines in 'ecosystem health' in both forests (Aber, 1992) and raised mires (Aerts *et al.*, 1992).

Ecosystems vulnerable to the change from N limitation to N saturation include those which are extremely oligotrophic, with historically very low rates of N mineralization and a high dependence upon rainfall-nutrient inputs as seen in raised bogs (Aerts *et al.*, 1992), some heathlands (Aerts and Berendse, 1988) and some coniferous forests (Aber *et al.*, 1989). In each of these cases, the plant communities are adapted to low rates of N supply by slow growth rates and low nutrient demand and so can quickly reach the point of N saturation. Plant communities which are approaching, or have reached N saturation, show strong parallels with ecosystems which are naturally co-limited by N and P. The availability and rates of recycling of nutrients other than N is of increasing importance, since these may then exert the main control on plant productivity. In ombrotrophic bogs (Aerts *et al.*, 1992), fens (Verhoeven and Schmitz, 1991), coniferous forests (Emmett *et al.*, 1995) and heathlands (Aerts and Berendse, 1988) N enrichment has led to P being the main nutrient limiting plant productivity. There is also increasing evidence of strong interrelationships between the cycling of N and P in oligotrophic ecosystems with low rates of productivity (Pastor *et al.*, 1984; Van Oorschot *et al.*, 1997). The change from N to P as the nutrient limiting plant productivity in some ecosystems is likely to lead to more rapid rates of organic P mineralization by the soil biota which may be reflected above ground by changes in shoot N:P ratios and P concentrations.

3.1.1 Aims

The principal aims of the experiments described in this chapter were to investigate the interactions that may be occurring between atmospheric N deposition and P availability in terms of plant species abundance and biomass. A comprehensive survey of vegetation in both the long-term grassland sites has been reported elsewhere using non-destructive

harvests to estimate changes in species composition (Lee and Caporn, 1998). The first experiment (Section 3.2) investigated the effect of long-term inputs of N to the acid grassland plots on (a) the shoot biomass and nutrient status of *Anemone nemorosa* and (b) the occurrence of bryophytes[1]. The second group of experiments investigated short-term N and P additions on species composition and aboveground biomass production at the calcareous grassland (Section 3.3.1) and the acid grassland (Section 3.3.2). The short-term plots, each 3 m × 3m, were established in 1995. The third group of experimental results (Section 3.3.3-4) relate to field observations on the occurrence of *Carex flacca* and *Gentianella amarella* in the short-term calcareous grassland plots.

3.2 ABOVE GROUND VEGETATION RESPONSES TO LONG-TERM INPUTS OF N

3.2.1 Materials and methods

3.2.1.1 Abundance and nutrient status of *Anemone nemorosa*

Visual observations indicated changes to both leaf size and leaf coloration of *A. nemorosa* growing in the long-term acid grassland plots (Plate 3.1). Total shoots were harvested from all of the treated plots in May 1996 from which leaf dry weights and shoot N and P concentrations were determined (Section 2.2.2).

3.2.1.2 Abundance and nutrient status of bryophytes

An investigation on the effects of the N treatments on the bryophyte population at the acidic grassland site was undertaken in May 1995. The investigation took the form of an initial preliminary survey which provided data (not presented) on the frequency of occurrence of bryophytes in order to establish the dominant species. The frequency data for each plot were obtained on a presence/absence basis, using two 25 cm × 25 cm grids subdivided into 25 quadrats, and converted to percentages. The assessment of treatment effects on shoot density data was restricted to the dominant species *Rhytidiadelphus squarrosus* and *Pleurozium schreberi*. The data were obtained by counting the number of shoots present within five of the quadrats in each 25 cm grid, therefore providing a total of ten observations per plot.

[1] The survey was undertaken with Dr. A. F. S. Taylor whose help is gratefully acknowledged.

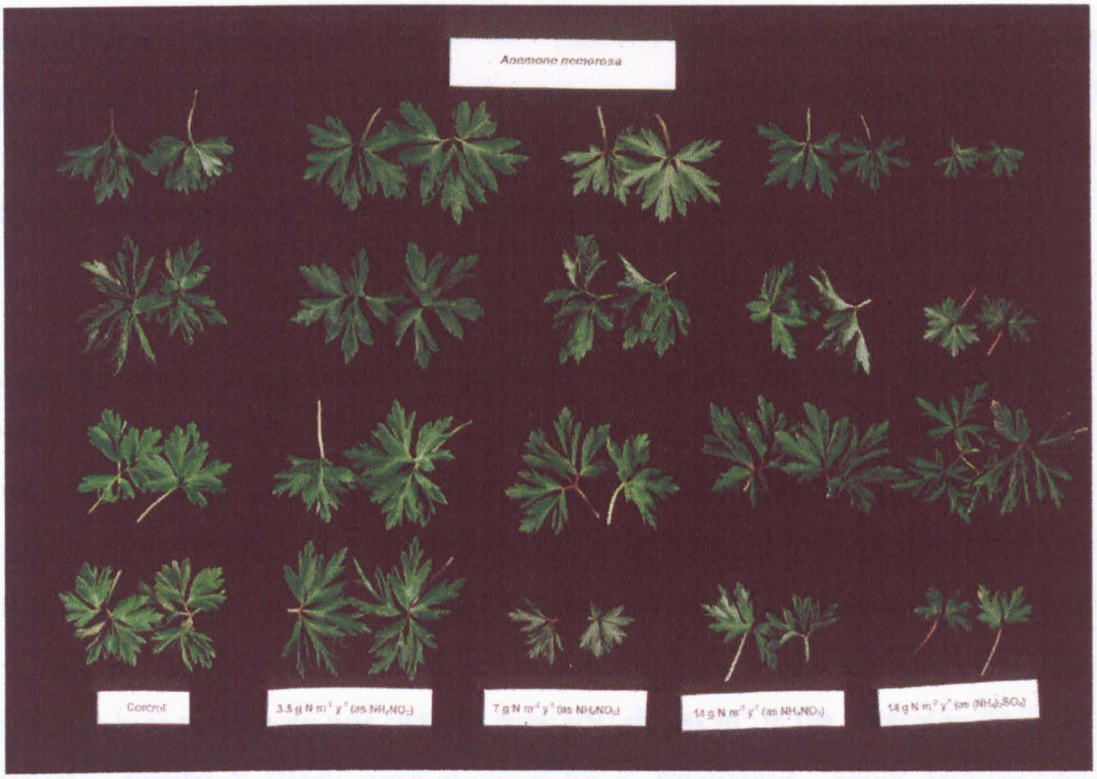


Plate 3.1. Decrease in the size of *Anemone nemorosa* leaflets in response to seven years N treatments to the acid grassland plots. Treatments are (from left to right in columns): Control, 3.5 N, 7 N, 14 N, 14 N AS.

3.2.2 Results

The mean dry weight of *Anemone nemorosa* leaf clusters ranged from 12 mg in the 14 AS treatment to 30 mg in the 3.5 AN treatment (Fig. 3.1a) while the mean weight in the control plots was 25.3 mg. Significant differences between the control were only observed in the 14 AN and 14 AS treatments ($P<0.05$). The initial stimulation in leaf dry weight from the 3.5 AN treatment followed by a progressive decrease resulting from additional inputs of N was supported by visual observations of leaf size (Plate 3.1). In contrast, all of the N treatments led to significant ($P<0.05$) increases in tissue N concentration and significant decreases in tissue P concentration (Fig. 3.1b). Shoot N concentration rose from 23.7 mg g^{-1} in the control to 36.3 mg g^{-1} in the 14 AS treatment while P concentrations fell from 8.9 mg g^{-1} to 5.2 mg g^{-1}. This was reflected more strongly in the shoot N:P ratios which rose from 2.7 in the control to 7.1 in the 14 AS treatment (Fig. 3.1c).

Increasing increments of N tended to progressively reduce the shoot densities of *P. schreberi* and *R. squarrosus* shoots in the acid grassland plots (Fig. 3.2a). The density of *P. schreberi* and *R. squarrosus* shoots in the controls were approximately 3700 and 950 m^{-2} respectively. *P. schreberi* was most sensitive to the N treatments where even the lowest increment of N (3.5 g N m^{-2} y^{-1}) significantly reduced ($P<0.01$) the density by a factor of three to 300 shoots m^{-2}. The greatest effect was observed in the ammonium sulphate treated plots where the density was reduced from 950 shoots m^{-2} in the control to 23 shoots m^{-2} ($P<0.01$). Similar N effects were observed for *R. squarrosus*. Shoot densities were progressively reduced by 10, 17 and 30 % in the ammonium nitrate treatments and by 75 % in the 14 AS treatment. The decreases were significant ($P<0.05$) in all except the lowest application rate (3.5 N). Shoot N concentrations, which were only measured for *R. squarrosus*, progressively increased from 14 mg N g^{-1} dwt in the control to 21 mg N g^{-1} dwt in the 14 N treatment ($P<0.05$; Fig. 3.2b). However, the 14 AS treatment only increased the shoot N concentration by a further 5 % to 22 mg N g^{-1} dwt.

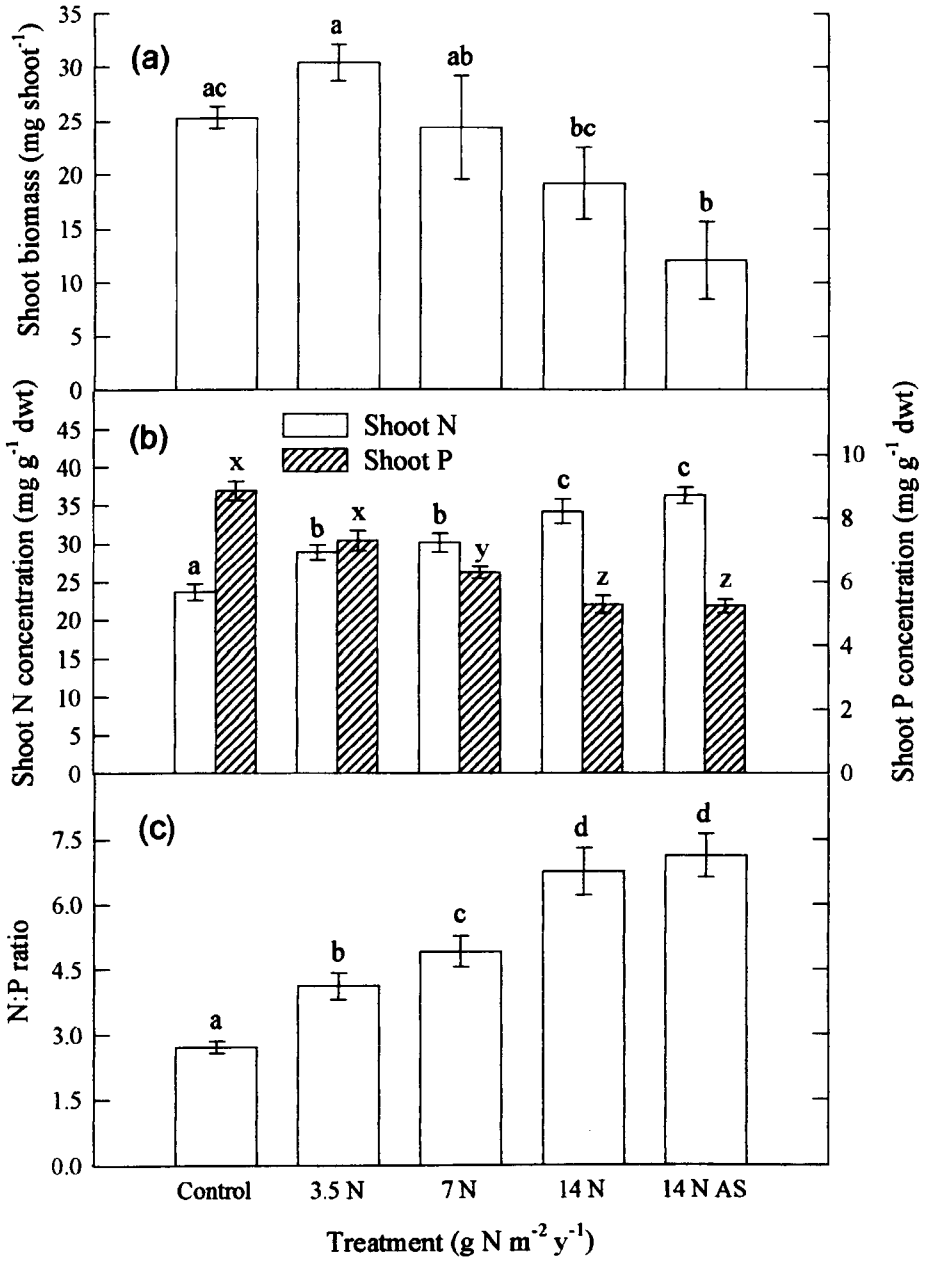


Fig. 3.1. Changes in (a) shoot biomass, (b) N and P concentrations, and (c) shoot N:P ratio (mg mg^{-1} dwt) of *Anemone nemorosa* in acid grassland plots that have received seven years N treatments (± SEM). Treatment codes (g N m^{-2} y^{-1}): N = ammonium nitrate, N AS = ammonium sulphate. Bars sharing a letter are not significantly different (P>0.01). Shoot N and P concentrations analysed independently.

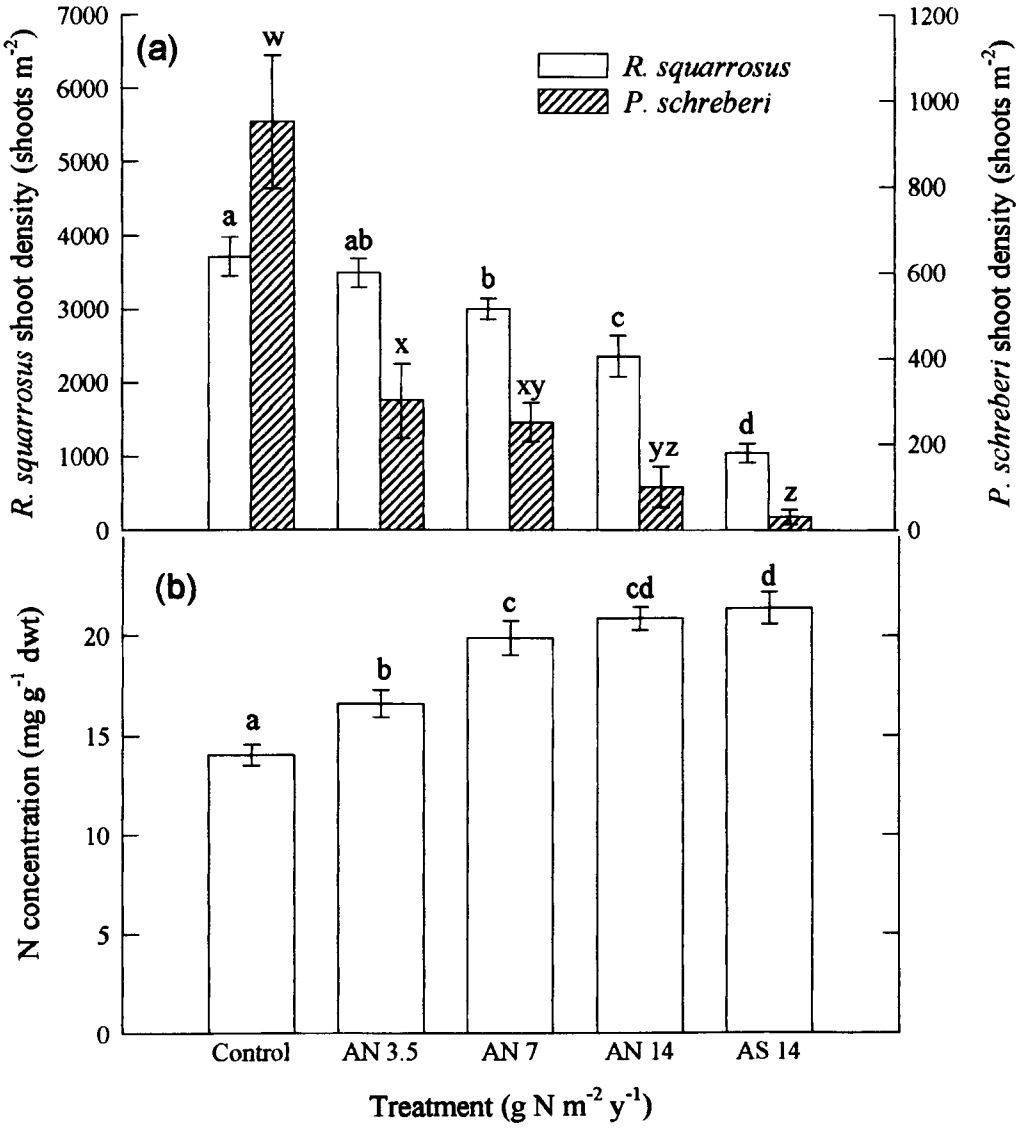


Fig. 3.2. Changes in (a) shoot density of *Rhytidiadelphus squarrosus* and *Pleurozium schreberi* and (b) shoot N concentration of *R. squarrosus* in acid grassland plots that have received seven years of N treatments (± SEM). Treatment codes as for Fig. 3.1. Bars sharing a letter are not significantly different ($P>0.05$). *R. squarrosus* and *P. schreberi* shoot density data analysed independently.

3.3 ABOVE GROUND VEGETATION RESPONSES TO SHORT-TERM INPUTS OF N AND P

3.3.1 Materials and methods

3.3.1.1 Species diversity and above ground biomass

i) Calcareous grassland

The treatments applied to the new grassland plots have been described in Section 2.1.3. Visual observations concluded that the treatments in the calcareous grassland were having a significant impact on species diversity and productivity (Plate 3.2). The nature reserve on which the experimental plots are located are subjected to sporadic grazing by both cattle and sheep during a limited period in the Summer. Grazing animals are known to have significant impacts on low productivity grassland ecosystems (Hill *et al.*, 1992). Furthermore, since one of the main aims of the experiment was to calculate aboveground biomass, a series of small grazing exclosures was constructed. A total of 54 exclosures approximately 40 cm × 30 cm × 15 cm were constructed using 1" galvanised steel mesh. Three frames were used in each plot which were secured to the ground with metal tent pegs. The grassland vegetation under the frames was harvested flush with the litter layer using scissors in July 1996. Each sample was air dried, weighed and the individual species sorted (except for grass (primarily: *Festuca ovina, Briza media, Avenula pratensis* and *Koelaria cristata*) and moss species which were recorded as a single categories) and weighed, including the number of flowering grasses.

ii) Acid grassland

In contrast to the calcareous system, dramatic alterations in the vegetation community of the acid grassland plots were not apparent from visual observations. A preliminary harvest of vegetation from three 10 cm x 10 cm patches in each plot was therefore undertaken. from which the total aboveground biomass was recorded.

(a)

(b)

(c)

(d)

Plate 3.2. Growth of vegetation in short-term calcareous grassland plots (showing mesh grazing exclosures) that have received (a) no nutrient additions, (b) 14 g N m^{-2} y^{-1} for 18 months, (c) 3.5 g P m^{-2} y^{-1} for 12 months, and (d) 3.5 g P m^{-2} y^{-1} for 12 months + 14 g N m^{-2} y^{-1} for 18 months.

3.3.1.2 Abundance and nutrient status of *Carex flacca*

Visual observations suggested that the abundance of *Carex flacca* shoots in the short-term calcareous grassland was declining following 18 months of N and P applications. An investigation was initiated which would enable measurement of shoot density and biomass, standing biomass and shoot N and P concentrations. Triplicate counts of the number of shoots in each of the 25 squares (each 5 cm × 5 cm) in a 50 cm × 50 cm strung quadrat were obtained. All of the shoots from between five and ten 5 cm × 5 cm squares were harvested and subsequently dried and weighed for the determination of average shoot biomass. Standing biomass estimates could then be obtained by combining the average shoot biomass and shoot density data. Tissue N and P concentrations were determined on *C. flacca* shoots removed using a 4 cm diameter soil corer from the control, 14 N, 3.5 P and 14 N+P treatments.

3.3.1.3 Abundance of *Gentianella amarella*

Previous surveys using the existing calcareous grassland plots and the grazing exclusion data from the new plots may have failed to identify some species due to either the time of flowering or their sparse distribution. Changes in the abundance of rooted *Gentianella amarella* Ssp. *amarella* shoots, which is both sparsely distributed and flowers late in the season, were observed in the short-term calcareous grassland in response to N and P treatments. A visual survey was initiated in which the total number of shoots in each plot were counted.

3.3.2 Results

3.3.2.1 Species diversity and above ground biomass

i) Calcareous grassland

The effects of the nutrient treatments on above ground biomass and species abundance were considerable, although the most marked changes occurred in plots that had received P either with or without N (Plate 3.2). Combined inputs of N and P had no significant effect on the live, monocotyledon or dicotyledon above ground biomass within grazing exclosures, although the number of flowering grasses increased substantially in the plots receiving N+P

(Fig. 3.3). Both the 3.5 and 14 N treatments resulted in a decrease of 33 % from 40 to 27 flowers m^{-2}, while an increase of approximately 350 % to 184 flowers m^{-2} resulted following addition of both 3.5 g P and 3.5 g N+P, and a further 80 % increase to 332 flowers m^{-2} in the 14 N+P treatment (Fig. 3.3). In contrast, the overall effect of P addition was to significantly increase live above ground biomass by 60 % from 116 to 185 g m^{-2} ($P<0.001$; Fig. 3.4a). This was largely mirrored by parallel changes in the productivity of monocotyledons which increased by 88 % in response to additions of P ($P<0.001$; Fig. 3.4a). N applications did not significantly affect live and dicotyledonous above ground biomass and the number of flowering grasses. However, monocotyledon above ground biomass increased by 25 % from 92 to 115 g m^{-2} in the plots receiving 3.5 g N m^{-2} ($P<0.05$) and by 21 % to 111 g m^{-2} ($P>0.05$) in the plots receiving 14 g N m^{-2} (Fig. 3.4b).

Combined inputs of N and P had no significant effect on the above ground biomass of any dicotyledonous species (Fig. 3.5). Although the above ground biomass of the dicotyledon group was not affected by either addition of N or P (Fig. 3.4a and b), significant treatment effects were observed for the individual species. The application of P had greatest impact on the occasional species (*Plantago lanceolata, Scabiosa columbaria* and *Hieracium pilosella*) and significantly increased above ground biomass by 230 % from 2.1 to 6.9 g m^{-2} ($P<0.001$; Fig. 3.6a) while inputs of N had no significant effect (Fig. 3.6b). The only dicotyledon that responded positively to both levels of N addition was *Lotus corniculatus* where above ground biomass increased significantly ($P<0.05$) by 73 % in the plots receiving 3.5 g N m^{-2} and by 30 % in the 14 g N m^{-2} treatments (Fig. 3.6b). Above ground biomass of *Helianthemum nummularium* was stimulated only in the plots receiving combinations of 3.5 g N m^{-2} where it increased by 47 % (Fig. 3.6b). Changes in the proportions of dicotyledons and monocotyledons in response to the treatments are reflected in the dicotyledon:monocotyledon ratio (Fig. 3.7). The 3.5 N treatment significantly reduced ($P<0.05$) the ratio by 57 % from 0.73 to 0.32 while the 14 N treatment only led to a 34 % reduction to 0.48, which was not significantly different from the control ($P>0.05$). However, significant decreases occurred in the plots treated with P, even when in combination with the 14 g N m^{-2} application rate ($P<0.05$).

The above ground biomass of moss in the calcareous grassland plots was not significantly affected by factorial additions of N and P despite reductions arising from the N, P and N+P treatments (Fig. 3.8a). N additions had the greatest impact on moss productivity, even when applied in combination with P, and progressively decreased the above ground biomass by 35 % from 13.2 to 8.7 g m^{-2} in plots receiving 3.5 g N m^{-2}, and by 50 % to 6.6 m^{-2} in plots receiving 14 g N m^{-2} ($P<0.05$; Fig. 3.8b).

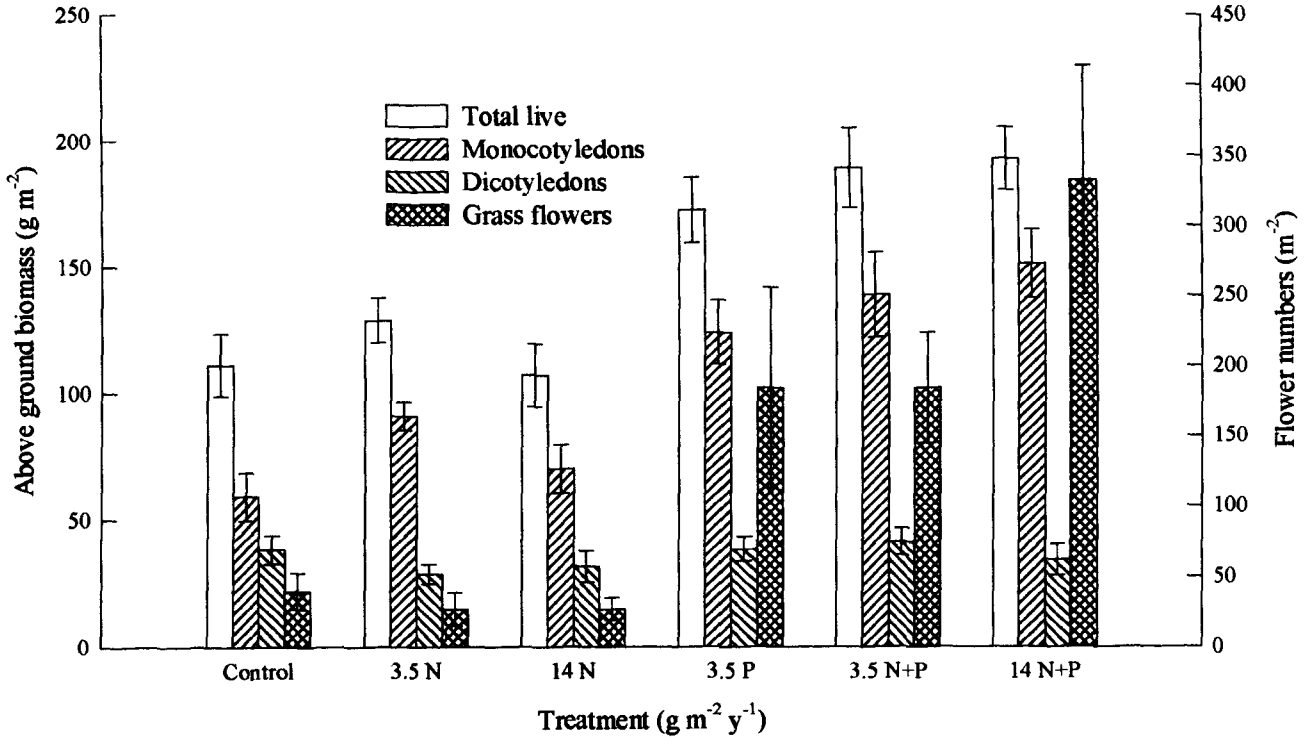


Fig. 3.3. Total live, monocotlyledon and dicotyledon above ground biomass and number of grass flowers in calcareous grassland plots that have received 12 months P and 18 months N additions (± SEM). Treatment codes (m^{-2} y^{-1}): Control = 0 g N, 3.5 N = 3.5 g N, 14 N = 14 g N, 3.5 P = 3.5 g P, 3.5 N+P = 3.5 g N + 3.5 g P, 14 g N+P = 14 g N + 3.5 g P. Bars are not significantly different (P>0.05).

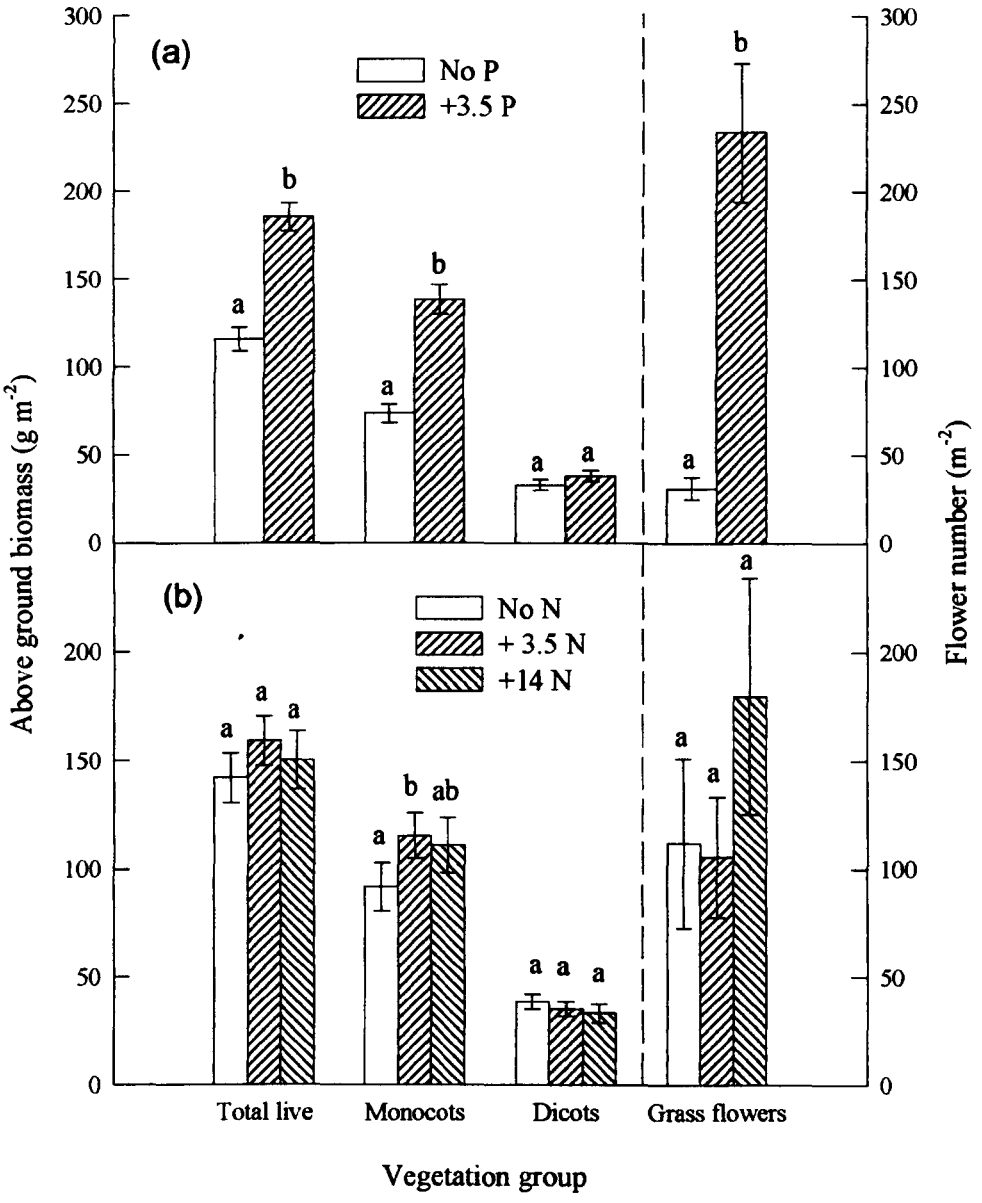


Fig. 3.4. Total live, monocotyledon and dicotyledon above ground biomass and number of grass flowers in calcareous grassland plots that have received factorial combinations of N and P. Overall effect of (a) 12 months P (0 or 3.5 g m^{-2} y^{-1}) and (b) 18 months N additions (0, 3.5 or 14 g m^{-2} y^{-1}; ± SEM). Bars sharing a letter are not significantly different (P>0.05). Vegetation groups are analysed independently.

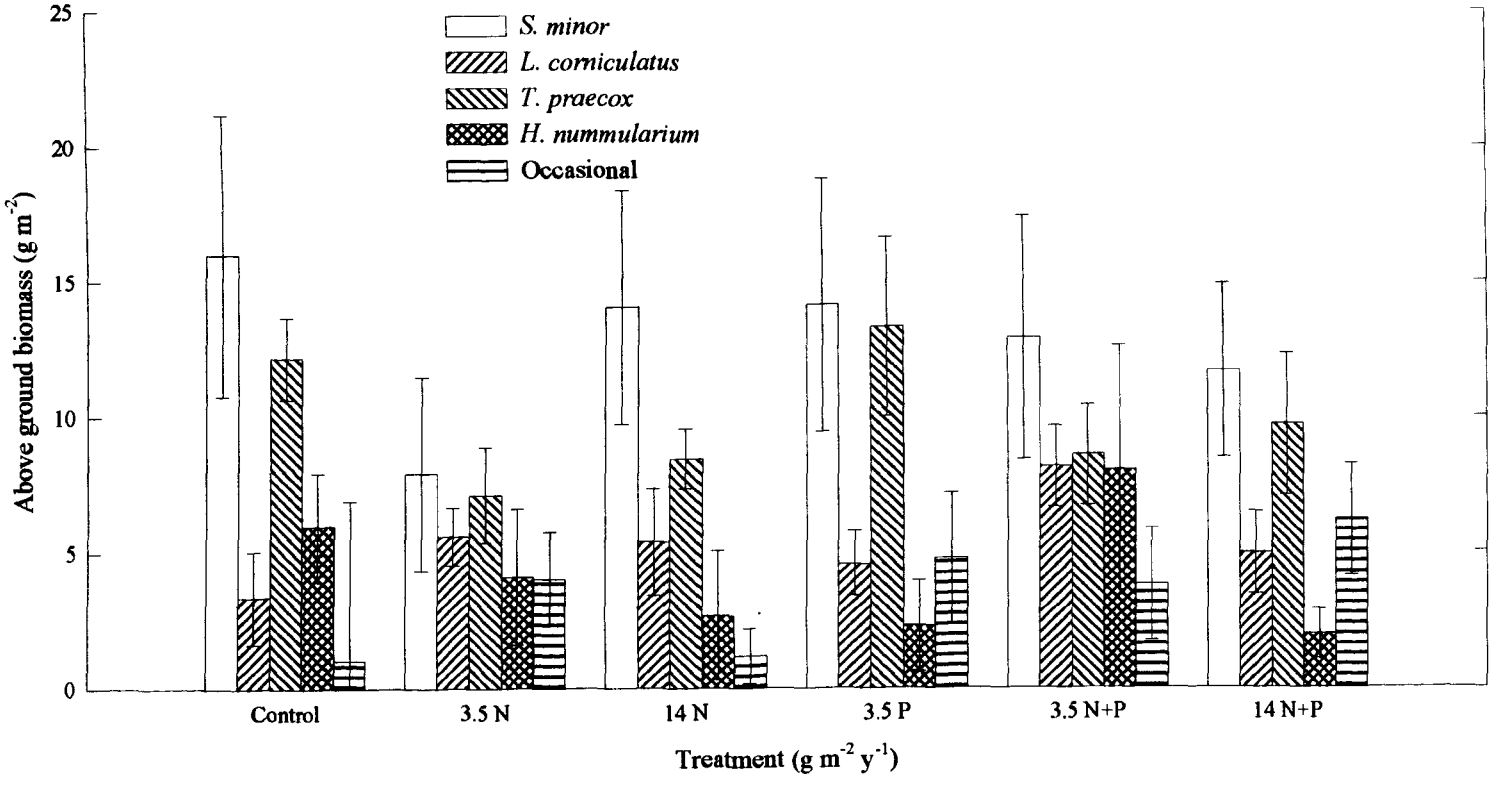


Fig. 3.5. Above ground biomass of *Sanguisorba minor*, *Lotus corniculatus*, *Thymus praecox*, *Helianthemum nummularium* and Occasional species (*Plantago lanceolata*, *Scabiosa columbaria* and *Hieracium pilosella)* in calcareous grassland plots that have received 12 months P and 18 months N additions (± SEM). Treatment codes as for Fig. 3.3. Bars are not significantly different.

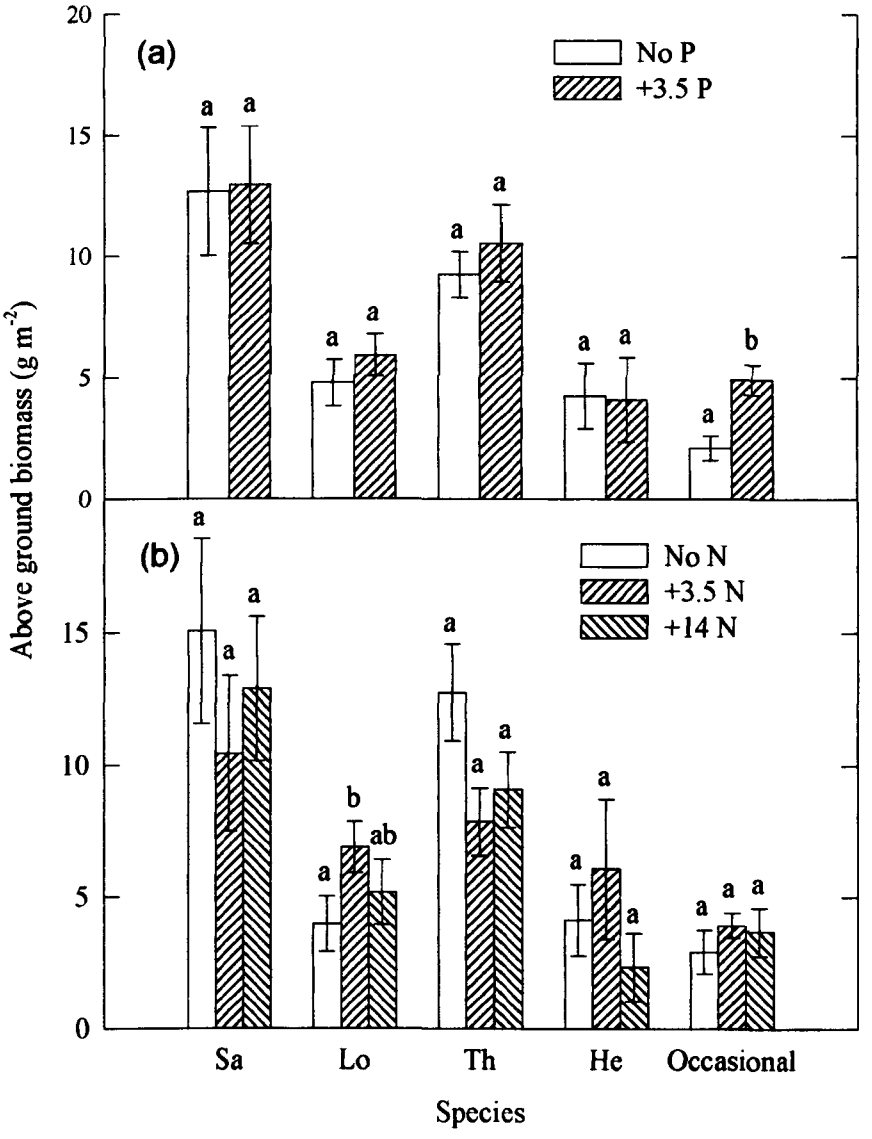


Fig. 3.6. Above ground biomass of dicotyledonous species in calcareous grassland plots that have received factorial combinations of N and P. Overall effect of (a) 12 months P (0 or 3.5 g m^{-2} y^{-1}) and (b) 18 months N additions (0, 3.5 or 14 g m^{-2} y^{-1}; ± SEM). Sa = *Sanguisorba minor*, Lo = *Lotus corniculatus*, Th = *Thymus praecox*, He = *Helianthemum nummularium*, Occasional = *Plantago lanceolata*, *Scabiosa columbaria* and *Hieracium pilosella*. Bars sharing a letter are not significantly different (P>0.05). Each species is analysed independently.

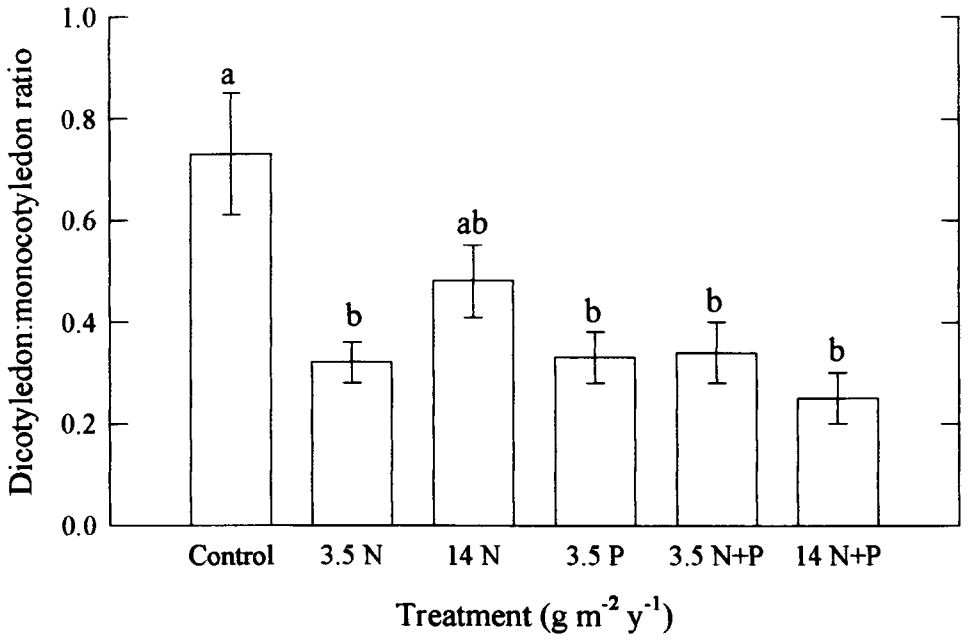


Fig. 3.7. Dicotyledon:monocotyledon ratio (g g^{-1} dwt) in calcareous grassland plots that have received 12 months P and 18 months N additions (± SEM). Treatment codes as for Fig. 3.3. Bars sharing a letter are not significantly different (P>0.05).

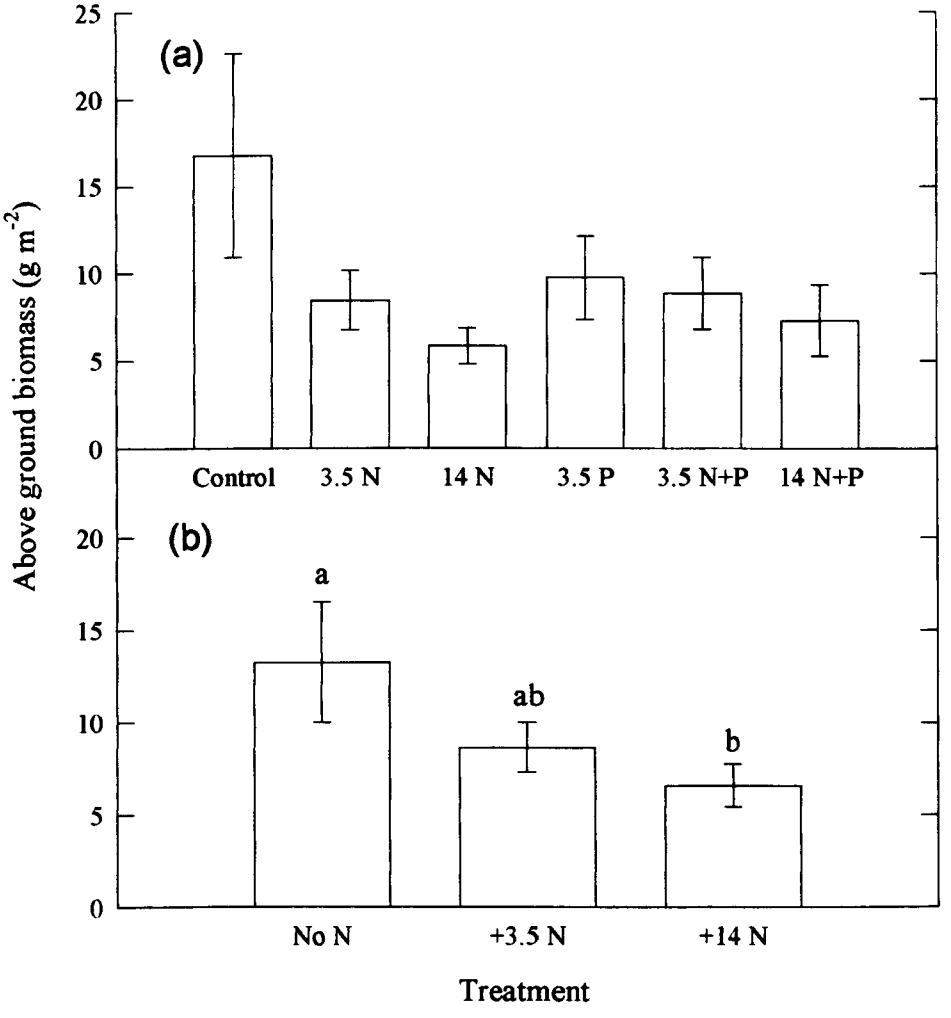


Fig. 3.8. Above ground biomass of moss in calcareous grassland plots that have received (a)18 months N and 12 months P additions (Bars are not significantly different (P>0.05); treatment codes as for Fig. 3.3.), and (b) the overall effect of 18 months N additions (0, 3.5 or 14 g m^{-2} y^{-1}; ± SEM). Bars sharing a letter are not significantly different (P>0.005).

ii) Acid grassland

Despite the lack of grazing exclosures, above ground biomass was 46 % greater in control plots of the acid grassland as compared to the calcareous grassland (Figs. 3.9a and 3.3). Similarly, the vegetation in the acid grassland site was not significantly affected by factorial additions of N and P (Fig. 3.9a) but instead was strongly influenced by inputs of N, regardless of whether P was applied in combination. Additions of 3.5 g N m^{-2} increased total live above ground biomass by 27 % from 162 to 205 g m^{-2} and significantly increased monocotyledon above ground biomass by 58 % from 65 to 104 g m^{-2} (P<0.05; Fig. 3.9b). However, application of N at 14 g m^{-2} reduced total above ground biomass by 16 % to 136 g m^{-2} but increased monocotyledon biomass by 21 % to 79 g m^{-2}. Dicotyledon productivity did not change significantly (P>0.05) in response to any of the treatments, although N supplied at 3.5 g m^{-2} y^{-1} (+3.5 N) reduced above ground biomass by 21 % from 23 to 18 g m^{-2}, and by 50 % to 11 g m^{-2} when supplied at 14 g m^{-2} y^{-1} (+14 N; Fig. 3.9b). The contrasting responses of dicotyledons and monocotyledons to inputs of N is illustrated in the dicotyledon:monocotyledon ratio which was significantly lower in both the +3.5 N and +14 N treatment combinations (P<0.05; Fig. 3.9b). *Anemone nemorosa* and *Galium saxatile* were the main contributors to the total above ground dicotyledonous biomass in the control plots. Despite there being no significant overall treatment effect on the dicotyledon group, significant effects were observed on these key species. The above ground biomass of *A. nemorosa* was not affected by factorial combinations of N and P (Fig. 3.10a) but was strongly influenced by addition of N where it decreased by 52 % from 14.5 to 6.9 g m^{-2} in the +3.5 N treatment and by 97 % (P<0.05) to 0.5 g m^{-2} in the +14 N treatment (Fig. 3.10c). Similarly, above ground biomass of *G. saxatile* was not affected by N and P treatments (Fig. 3.10b) in combination but was instead reduced in plots that had received P where it decreased by 65 % from 4.5 to 1.6 g m^{-2} (Fig. 3.10c).

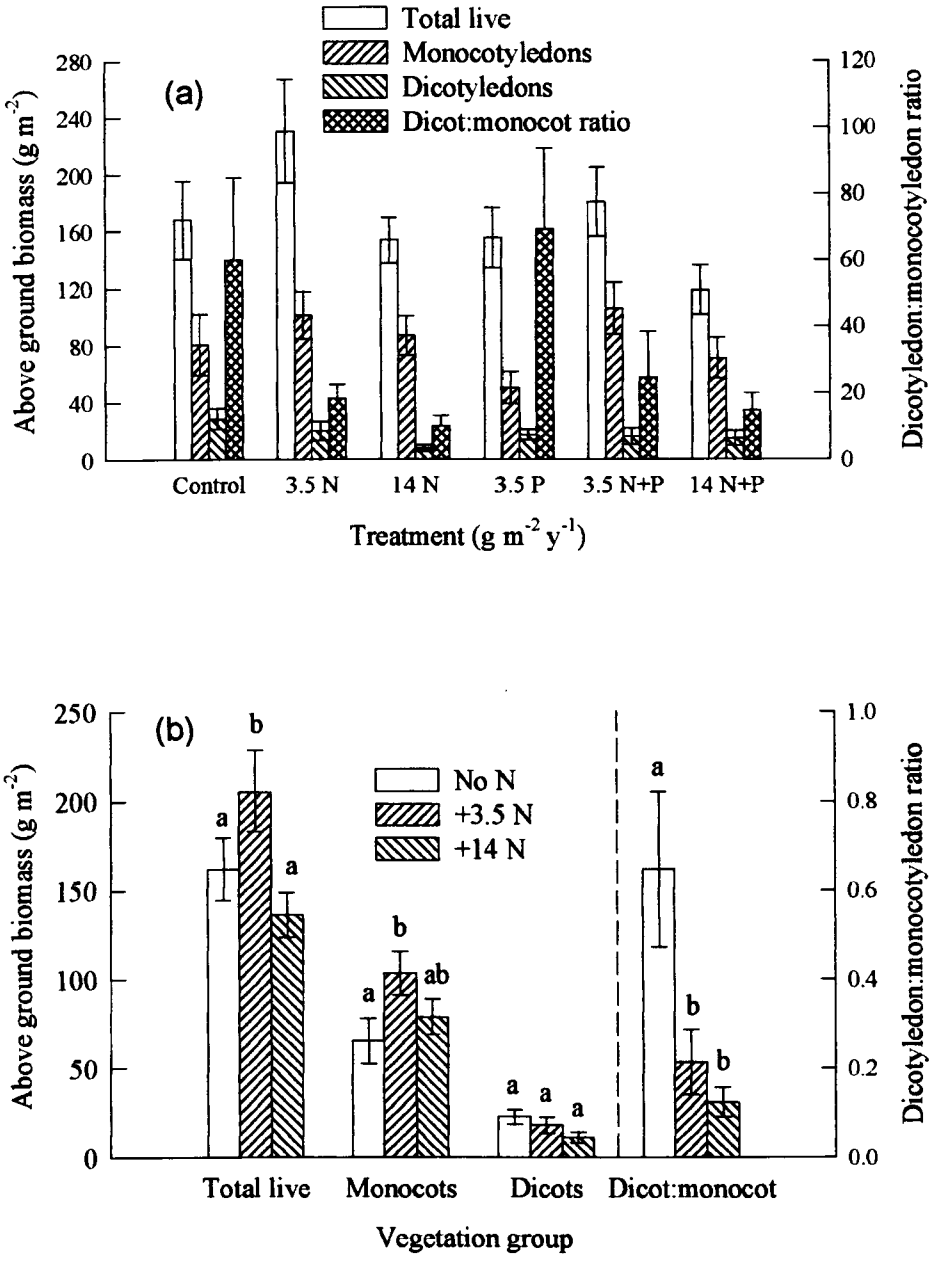


Fig. 3.9. Total live, monocotyledon and dicotyledon above ground biomass and the dicotyledon:monocotyledon ratio (g g^{-1}) in acid grassland plots that have received (a) 12 months of P and 18 months N (Treatment codes as for Fig. 3.3.; Bars are not significantly different; $P>0.05$), and (b) the overall effect of 18 months N additions (± SEM). Bars sharing a letter are not significantly different ($P>0.05$). Each species group is analysed independently.

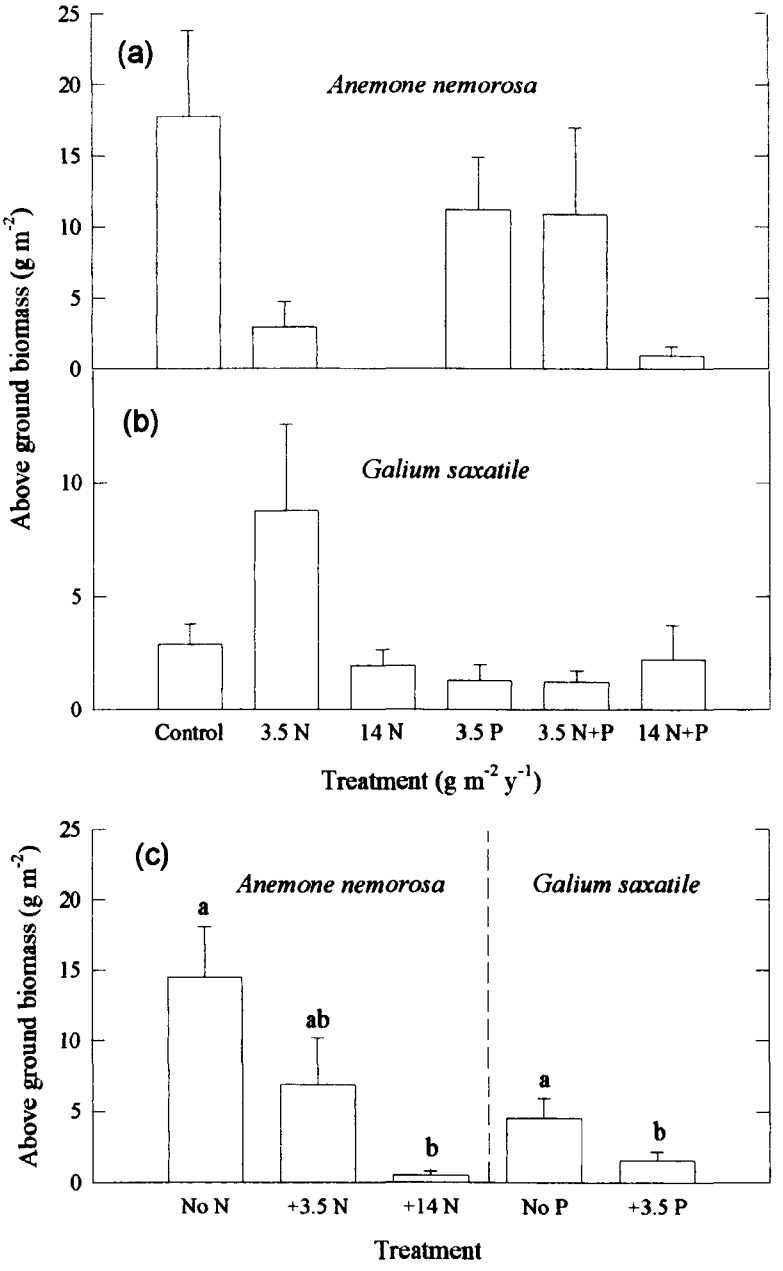


Fig. 3.10. Above ground biomass of (a) *Anemone nemorosa* and (b) *Galium saxatile* in acid grassland plots that have received 18 months N and 12 months P treatments (Treatment codes as for Fig. 3.3; Bars not significantly different; $P>0.05$), and (c) overall effects of 18 months N additions (0, 3.5 and 14 g N m^{-2} y^{-1}) on *A. nemorosa* and 12 months P additions (0 and 3.5 g N m^{-2} y^{-1}) on *G. saxatile* (+ SEM). Bars sharing a letter are not significantly different ($P>0.05$).

3.3.2.2 *Carex flacca*

Shoot density, standing biomass and shoot biomass of *C. flacca* were not affected by factorial combinations of N and P aditions (Figs. 3.11a-c). Significant differences ($P<0.05$) were only measured in plots that had received additions of P (Fig. 3.12). Shoot density was reduced by 60 % from approximately 500 to 200 shoots m^{-2}, standing biomass by 70 % from 28 to 9 g m^{-2} and shoot biomass by 28 % from 55 to 45 mg $shoot^{-1}$. Shoot N and P concentrations were measured only in shoots removed from the control, 3.5 P, 14 N and 14 N+P plots. The greatest increases in N concentration were seen in the 14 N and 14 N+P treatments while shoot P concentrations appeared to be influenced most by addition of P (Fig. 3.13). Shoot N concentrations ranged from 20.2 mg g^{-1} in the control to 26.5 mg g^{-1} in the 14 N treatment and shoot P from 1.3 mg g^{-1} in the control to 1.9 mg g^{-1} in the 14 N+P treatment (Fig. 3.13). Changes in shoot N and P concentrations were reflected in the N:P ratio which decreased from 16.5 in the control and 14 N treatments to 11 in the 3.5 P and 13 in the 14 N+P treatments (Fig. 3.14a). The overall effect of P treatments was to significantly ($P<0.05$) reduce the ratio by 25 % from 33.1 to 24.8 (Fig. 3.14b).

3.3.2.3 *Gentianella amarella*

The number of plants varied greatly across the treatments, ranging from 9.4 shoots m^{-2} in the control to 0.2 shoots m^{-2} in the 14 N+P treatment (Fig. 3.15). Significant reductions were observed regardless of the treatment combination ($P<0.01$) where even the lowest N addition (3.5 g m^{-2}) caused a 75 % decrease. Although not statistically significant, the addition of 3.5 g P m^{-2} further reduced the number of shoots by 56 % in the 3.5 N treatment and by 90 % in the 14 N treatment (Fig. 3.15). Linear regression revealed a significant (r^2 = -0.31, $P<0.012$) and negative correlation between the density of *G. amarella* plants and total live above ground biomass (Section 3.3.1.3; Fig. 3.16).

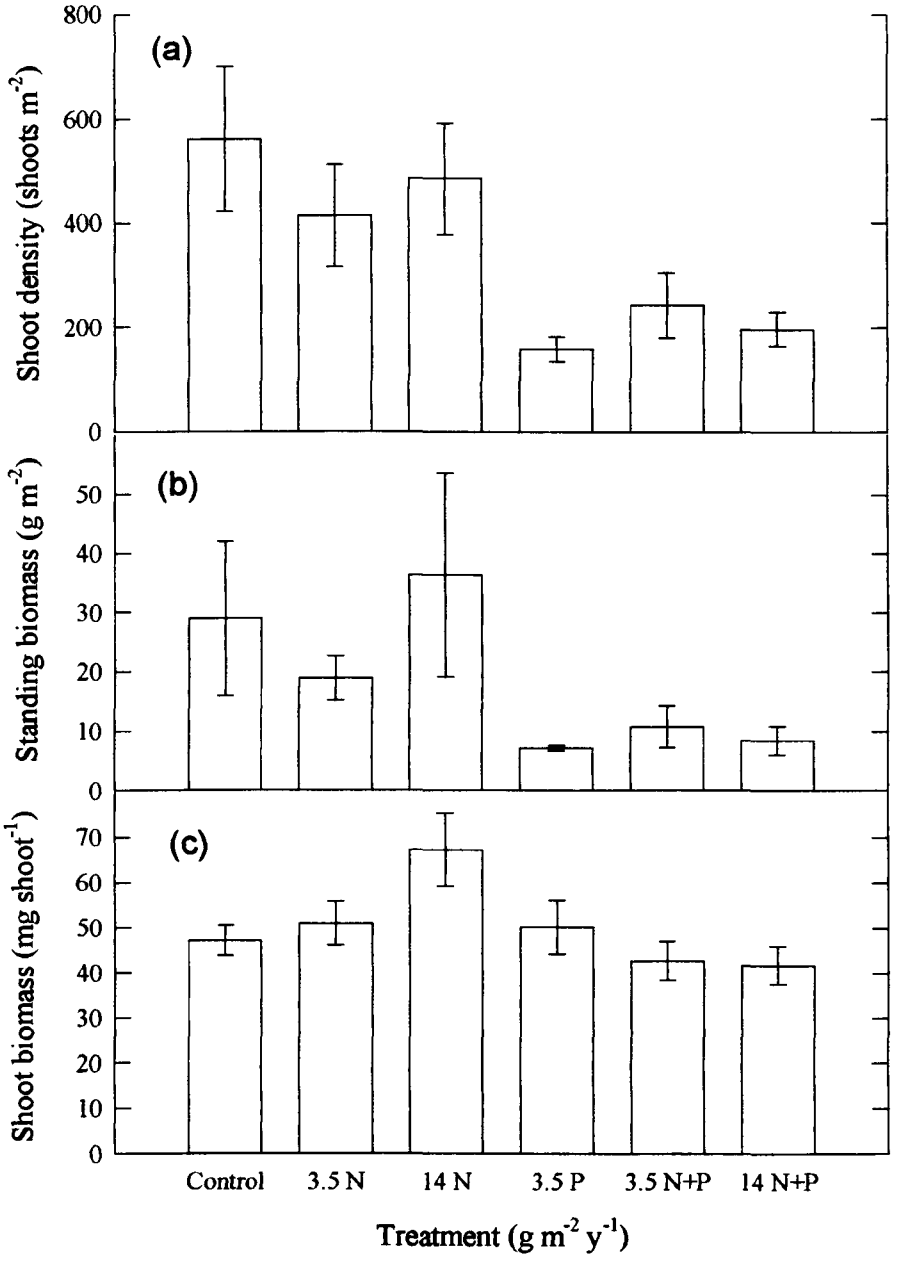


Fig. 3.11. Effect of 18 months N and 12 months P additions on (a) shoot density, (b) standing biomass, and (c) shoot biomass of *Carex flacca* in calcareous grassland plots (± SEM). Treatment codes as for Fig. 3.3. Bars are not significantly different (P>0.05).

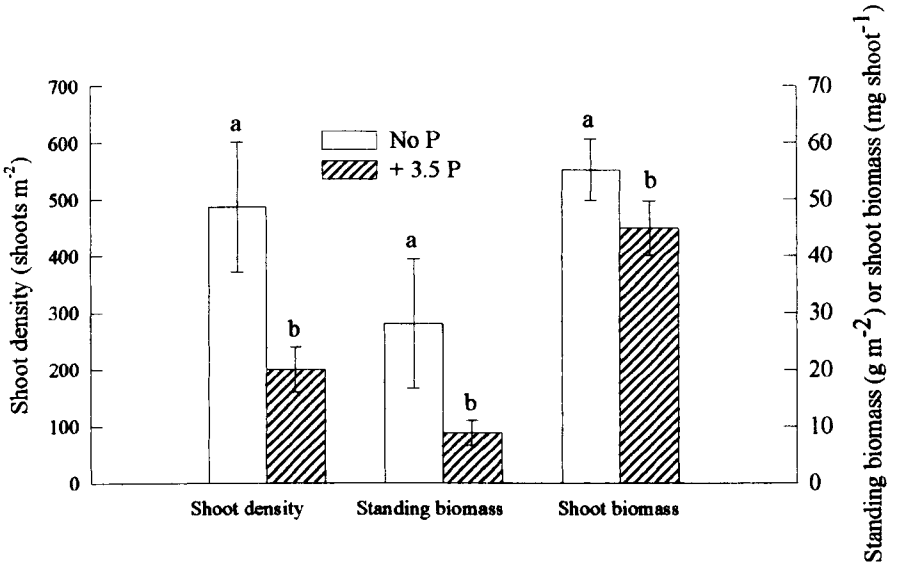


Fig. 3.12. Changes in shoot density, standing biomass and shoot biomass of *Carex flacca* resulting from 12 months P treatments (0 or 3.5 g m^{-2} y^{-1}) in calcareous grassland plots that have received factorial combinations of N and P (± SEM). Bars sharing a letter are not significantly different (P>0.01).

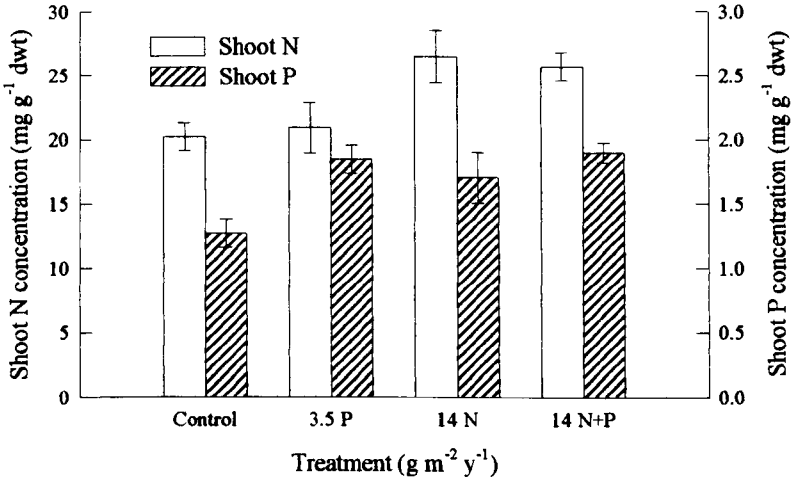


Fig. 3.13. N and P concentrations of *Carex flacca* shoots in calcareous grassland plots that have received 12 months N and 18 months P treatments (± SEM). Treatment codes as for Fig. 3.3. Bars are not significantly different (P>0.05).

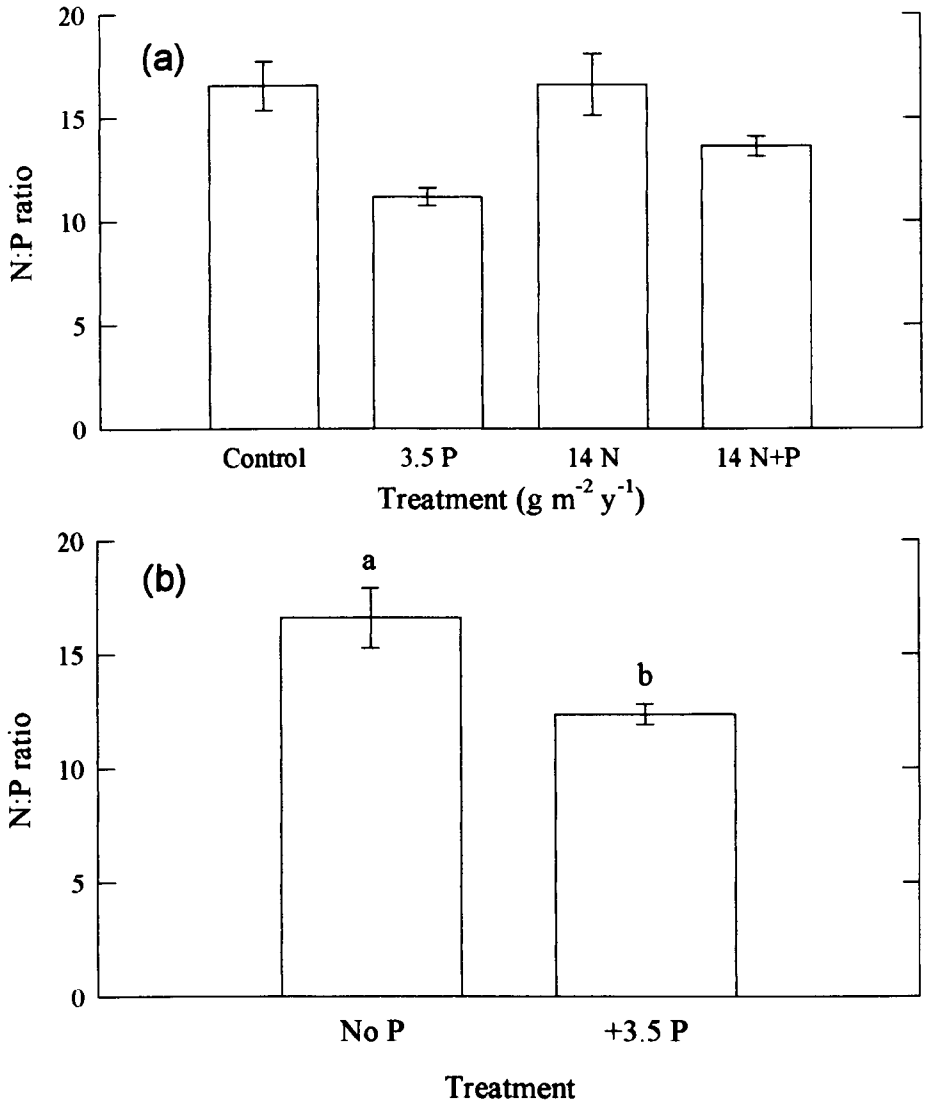


Fig. 3.14. N:P ratio (mg mg^{-1} dwt) of *Carex flacca* shoots in calcareous grassland plots that have received (a) 18 months N and 12 months P additions (Treatment codes as for Fig. 3.3; Bars are not significantly different; $P>0.05$), and (b) overall effect of 12 months P additions (0 or 3.5 g m^{-2} y^{-1}; ± SEM). Bars sharing a letter are not significantly different ($P>0.001$).

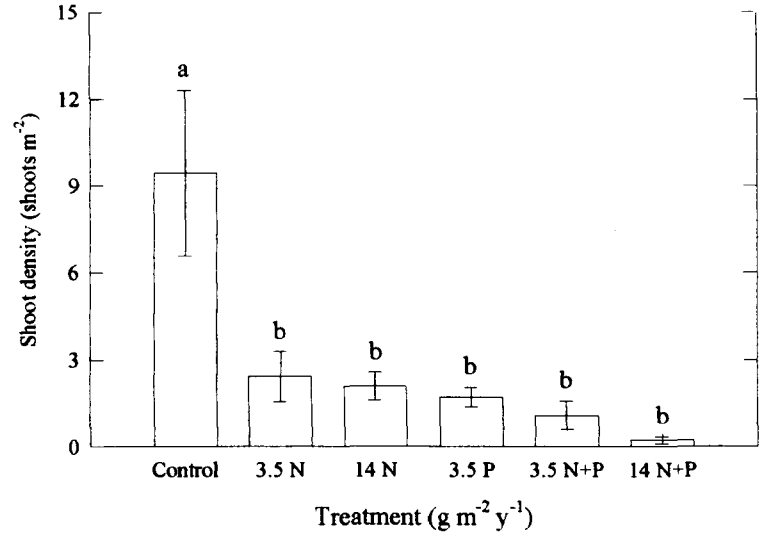


Fig. 3.15. Number of rooted *G. amarella* shoots in calcareous grassland plots that have received 12 months of P and 18 months of N treatments (± SEM). Treatment codes as for Fig. 3.3. Bars sharing a letter are not significantly different (P>0.01).

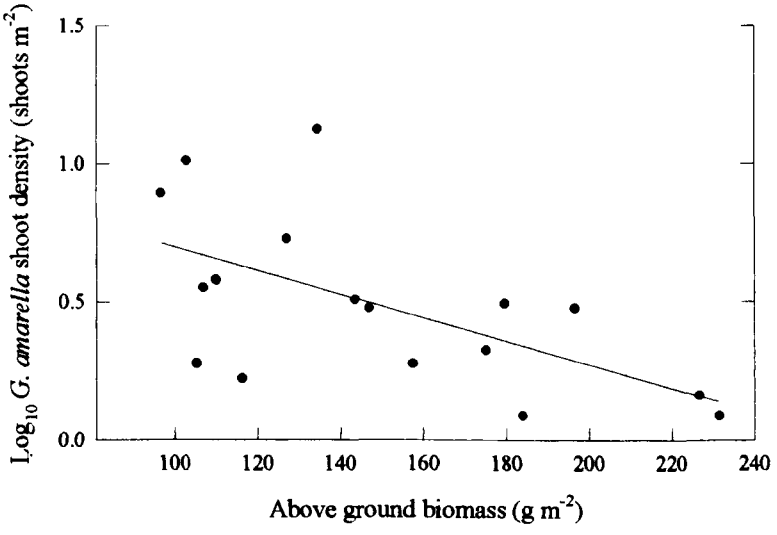


Fig. 3.16. Relationship between number of rooted *G. amarella* shoots and total live above ground biomass in calcareous grassland plots that have received 18 months N and 12 months P additions (r^2 = -0.31, P<0.012).

3.4 DISCUSSION

Significant changes in plant productivity and abundance occurred at both the calcareous and acid grasslands in response to the nutrient enrichment experiments. The vegetation at the calcareous grassland was, in general, more responsive to P rather than N inputs, although additions of both N and P significantly reduced the dicotyledon:monocotyledon ratio. In contrast, the vegetation of the acid grassland was more responsive to inputs of N.

3.4.1 Calcareous grassland

3.4.1.1 Effect of P treatments

Short-term (18 months) factorial combinations of N and P to the calcareous grassland have resulted in substantial changes to the vegetation community. The most striking effects occurred in the plots that had received applications of P, whether in combination with N or not, which is supported by both qualitative and quantitative evidence. Significant increases in plant productivity in the 3.5 P treated plots together with the less marked increases in the 3.5 N+P and 14 N+P treated plots provides strong evidence that the calcareous grassland is co-limited by N and P. This confirms the observations of Morecroft *et al.* (1994) who observed a significant increase in sward height following addition of 5 g P m^{-2} either with or without 10 g N m^{-2} to the same grassland. The total live above ground biomass increased significantly when P was applied ($P<0.001$; Fig. 3.4a, Plate 3.2). Further increases were observed in the plots receiving combinations of N and P, although these were not significantly different ($P>0.05$). These effects were most apparent on the grasses which clearly dominated the vegetation. The substantial increase in the number of flowering grasses, particularly in the 14 N+P treatment, indicates strong reproductive capabilities by these species, despite the cessation of P applications after just one year. The negative response of *Carex flacca* to raised P supply mirrored the observations by Willis (1963) of the same species at the Braunton Burrows dune system. However, the slight decrease in the abundance of *C. flacca* in response by to the 3.5 and 14 N treatments in this study was unexpected since Willis (1963) reported significant increases in growth by this species following additions of N. The author suggested that this species has a low P requirement and is preferentially favoured by additions of N. This was supported by shoot P concentrations which were 0.7 mg g^{-1} in the control plots, 0.5 mg g^{-1} in the -P plots and 2.2

mg g^{-1} in the N+P plots. The equivalent shoot P concentrations in the present study were 1.3, 1.7 and 1.9 mg g^{-1} indicating that this system is initially less deficient in P. It may be expected, therefore, that any stimulatory effect of N on the growth of *C. flacca* may only occur when the plants have adapted to highly P deficient soils or when they are initially very strongly N limited. Similar responses have been observed for *Carex lasiocarpa* Ehrh. growing in a P limited mesotrophic fen system in The Netherlands (Aerts *et al.*, 1995). In parallel with the present study using *C. flacca*, the productivity did not increase following regular fertilisation with 10 g N m^{-2} y^{-1} during the growing season, although significant increases in N uptake and shoot N:P ratio were reported. These parameters were not significantly affected in the *C. flacca* plants analysed in this study in response to the N treatments. This may in part be explained by the greater dominance of *C. lasiocarpa* in the Dutch system (>70 %) minimising competition for the added N.

The only dicotyledons which significantly increased productivity in response to the P treatments were the occasional species *Plantago lanceolata, Scabiosa columbaria* and *Hieracium pilosella*. The contribution from this group to total dicotyledon above ground biomass increased from 6 to 13 %. However, this represents an increase of just 2.83 g m^{-2} and has therefore marginal ecological significance in relation to the response of the entire community to the P treatments. It would be expected that the substantial increases in growth of grasses in these plots would competitively exclude the lower growing forbs, primarily by restricting light influx. This was, however, only observed in the biennial *Gentianella amarella* where the number of rooted shoots decreased significantly in all treatments. The spread of the grass *Brachypodium pinnatum* at the expense of phanerogamic species in Dutch calcareous grasslands has been attributed to changes in the vertical structure of the vegetation reducing the quantity and quality of light (Bobbink *et al.*, 1988; Bobbink, 1991). Willis (1963) also reported increased dominance of *Festuca rubra* and *Poa pratensis* ssp. *subcaerulea* (L.) at the expense of low growing broad leafed plants following application of P to a dune system. The lack of any loss of dicotyledon diversity and productivity, other than *G. amarella*, following applications of P is therefore unexpected. Willis (1963) noted initial stimulatory effects of P treatments on the productivity of some forbs, notably *Arenaria serpyllifolia* L. and *Sedum acre* L., although this was restricted to the initial (within 12 months) stages of the experiment and to the plots

in which grasses were initially a minor component. In the present study, the increased nutrient status of the treated plots, coupled with vigorous growth of monocotyledons, may have caused light to become the major resource limiting dicotyledon productivity rather than the availability of P. This may also explain the lack of any significant change in the productivity of forbs in other nutrient manipulation experiments in similar P limited soils (e.g. Jeffrey and Piggot, 1973) where grasses have become the dominant vegetation type.

3.4.1.2 The loss of *Gentianella amarella*

The number of rooted *G. amarella* plants decreased by between 75 and 98 % in the treated plots. A significant negative relationship ($r^2 = -0.31$, $P<0.012$) was found between total live above ground biomass and the abundance of *G. amarella* (Fig. 3.16). Similar relationships with plant productivity (Verkaar and Schenkeveld, 1984; Kelly, 1989a; Fischer *et al.*, 1997), litter accumulation (Lennartsson and Svensson, 1996) and temperature (Kelly, 1989b) have been described for other members of the Gentianaceae. Fischer *et al.* (1997) reported that the survival of *G. germanica* (Willd.) Boerner seedlings was negatively correlated to total plot above ground biomass ($r^2 = -0.67$, $P<0.05$). It is likely that other factors may be influencing the occurrence of *G. amarella* in the present study since total above ground biomass only accounted for 31 % of the variation and significant losses of *G. amarella* occurred even in those plots where nutrient additions had only caused marginal increases in above ground biomass (i.e. 3.5 N and 14 N). Keizer *et al.* (1985) found that seedling emergence of the short-lived forbs *Carlina vulgaris* L., *Euphrasia officinalis* L. and *Linum catharticum* L. was negatively correlated with bryophyte cover, although no relationship was found for *G. germanica* and *Scabiosa columbaria*. Similarly, no relationship between *G. amarella* and bryophyte above ground biomass was found in the present study ($r^2 = 0.031$, $P<0.233$). An important factor that may affect the occurrence of *G. amarella* in plots receiving elevated nutrient inputs is the degree of mycorrhizal colonisation. Most members of the Gentianaceae are known to be colonised by VA mycorrhizal fungi (Harley and Harley, 1987) that have an important role in nutrient, and particularly P, acquisition (Smith and Read, 1997). It would be of interest to quantifiy any changes in the degree of infection that may be occurring in plants growing in the treated plots. The loss of *G. amarella*, even in the plots receiving low inputs of N, after just 18 months of treatments has particular significance given its high conservation value. Similar

losses have occurred in Sweden where both *G. amarella* and *G. campestris* (L.) Boerner have disappeared from between 88 and 98 % of localities (Lennartsson and Svensson, 1996). The apparent sensitivity of species of the Gentianaceae to increased productivity, and perhaps also other factors such as mycorrhizal colonisation, underpin the importance of elucidating the mechanisms by which increased N deposition may be affecting these processes.

3.4.1.3 Effects of N treatments

Visual observations of the plots indicated that the vegetation was not affected by simulated N deposition, especially in comparison to the dramatic responses arising from the P treatments. The data obtained from the exclosures show that there have been no significant losses of species diversity, as has been reported for other calcareous grasslands (Bobbink, 1991). In the study by Bobbink (1991), three years of N treatments led to *Brachypodium pinnatum* becoming the dominant grass coupled with a loss of species diversity. This was caused largely by a decrease in the abundance of lower growing forbs whose growth became limited by lower light levels as a consequence of the dense cover of *B. pinnatum*. In the grassland used in the present study, *B. pinnatum* and other rhizomatous nitrophiles are not present. Although no individual monocotyledonous species has become dominant, the N treatments decreased dicotyledon above ground biomass by approximately 20 % while the 3.5 N treatment increased monocotyledon above ground biomass by 55 %. These changes are reflected by a significant decrease in the dicotyledon:monocotyledon ratio in the 3.5 N treatment (Fig. 3.7). Further supporting evidence of the deleterious effects caused by N treatments arises from the decreases in both moss and *G. amarella* cover in response to the N amendments, although the latter was also affected by the P treatments. Changes in the productivity and abundance of bryophytes are often regarded as indicative of pollution damage to vegetation communities as a result of their sensitivity to solute supply (Lee *et al.*, 1993). The greater impact of the 3.5 g N m^{-2} treatment give these results particular significance since this level of application falls closer to projected N pollution loads for the UK. In addition, since these effects have only occurred in the short-term (18 months), further detailed monitoring needs to be undertaken in order to assess the potential impacts of these treatments in the longer-term. The increase in the dicotyledon:monocotyledon ratio and the decreases in moss productivity were not reported by Morecroft *et al.* (1994)

who applied similar levels of N, including 14 g N m^{-2} y^{-1} as ammonium sulphate, to the same grassland for three years. It is likely that methodological differences account for most of these disparities. Morecroft *et al.* (1994) employed a limited number of point quadrats which, although necessary for gaining long-term trends in plots of limited area, would almost certainly be insensitive to changes in moss productivity. Furthermore, their plots had no protection from grazing cattle and sheep and may have been subject to selective grazing.

3.4.2 Acid grassland

In contrast to the calcareous grassland, the application of P had virtually no effect on the vegetation of the acid grassland. The only species which was affected by the P treatments was *Galium saxatile*, which had significantly lower above ground biomass in these plots (Fig. 3.10c). This response may have been the result of selective grazing since visual observations of unharvested vegetation within grazing exclosures at this site suggested substantially greater numbers of *G. saxatile* flowers in comparison to the unprotected areas of the plots. The most substantial vegetation changes were seen in the plots that had received applications of N, or combinations of N and P, where both the total live and monocotyledon above ground biomass were greater following application of N. No overall significant changes in dicotyledon above ground biomass were seen although there was evidence of a trend for decreasing productivity with increasing increments of N. In the long-term acid grassland plots, the shoot biomass of *A. nemorosa* increased slightly at the lowest rate of N addition (3.5 g N m^{-2} y^{-1}) prior to decreasing in response to subsequent applications. Analysis of the nutrient status of the shoots revealed progressively higher N concentrations and progressively lower P concentrations in response to N applications resulting in significant increases in the shoot N:P ratio at all treatment levels. This provides strong evidence that *A. nemorosa* is limited by P in the long-term plots particularly when interpreted together with the reduced biomass and purple coloration of shoots found in the 14 N and 14 N AS treatments. In the short-term acid grassland plots, the productivity of *A. nemorosa* had a strong influence on the overall dicotyledon response. Above ground biomass decreased by 52 % in the +3.5 N treatments and significantly by 97 % in the +14 N treatments (Fig. 3.10c). No visual signs of nutrient deficiency (e.g. leaf discoloration and reduced leaf size) were seen that would indicate plant growth was limited by P, although

growth was stimulated (but not significantly) in the 3.5 P and 3.5 N+P treatments (Fig. 3.10a). This may indicate an initial shift from N to P as the main nutrient limiting *A. nemorosa*, which certainly was apparent after seven years of treatments, and provides further evidence of the important interactions occurring below ground between the deposition of N and the cycling of P. Regular monitoring of the N and P status of *A. nemorosa* in the short-term plots is needed in order to establish the timing of this shift in nutrient limitation.

The observations by Morecroft *et al.* (1994) of reduced occurrence of the moss *Rhytidiadelphus squarrosus* in the 14 N AS treatment were supported by the more detailed survey undertaken in this study. The shoot density of this species decreased significantly in all except the lowest N application (Fig. 3.2a) while shoot N concentrations increased significantly in all treatments (Fig. 3.2b). A similar effect was seen for the less dominant moss *Pleurozium schreberi* for which shoot biomass decreased significantly even in the lowest N treatment (Fig. 3.2a). *Sphagnum* spp. have been shown to absorb large quantities of atmospherically deposited inorganic N which is subsequently released as organic forms (Silcock and Williams, 1995; Baxter *et al.*, 1992). The result of the loss of a mat of bryophytes in the grassland systems used in the present study may be to alter both the form and amount of N reaching the soil surface and further accelerate any eutrophication processes.

3.4.3 Importance of plot size in studies of plant ecology

The larger plots used in the present study (compared to the plots in the long-term investigation) have enabled more detailed investigations of the vegetation responses to be undertaken through the use of destructive harvests and grazing exclosures. However, the size of experimental plots is of fundamental importance, particularly when investigating communities that are species rich or which contain a number of rare (and therefore dispersed) species. For example, species-area curves published for Cressbrookdale (a species rich calcareous grassland in Derbyshire similar to the calcareous grassland used in this study) by Al-Mufti *et al.* (1977) show that a 1 m^2 quadrat, as used by Morecroft *et al.* (1994), would contain approximately 30 species while the 9 m^2 quadrats used in the present

study would contain approximately 47. The effects of the use of the larger plots in identifying loss of species as a result of the nutrient manipulations is reflected in the *G. amarella* data. This species was not recorded in any previous long-term monitoring of this site (J. Carroll pers. comm.), yet the number of rooted shoots was found to decrease by 75 % in the 3.5 N treatment in the short-term calcareous grassland plots.

3.4.4 N/P interactions

The productivity of both the dominant and minor components of the vegetation community has been shown to be strongly influenced by the availability of P. These results demonstrate the importance of the role of P in the maintenance of species rich vegetation communities, such as the calcareous grassland used in this study. The effects of N additions on the vegetation community were also significant, particularly at relatively low application rates (3.5 g N m^{-2} y^{-1}). Since P availability has such importance in these systems, it is vital that we identify and understand potential changes in the cycling of P by both plants and microorganisms in response to elevated levels of N enabling the development of meaningful pollution control strategies, such as critical loads, for other similar grassland ecosystems.

CHAPTER 4

BELOW GROUND VEGETATION RESPONSES TO LONG-TERM INPUTS OF N AND SHORT-TERM INPUTS OF N AND P

4.1 INTRODUCTION

The short-term plots receiving factorial combinations of N and P have provided strong evidence of interactions between increased N deposition and P cycling. The importance of P supply in the short-term calcareous grassland plots was highlighted by substantial increases in the total live above ground biomass in response to P additions (Chapter 3), which demonstrated that plant productivity is limited or co-limited by P. Although monocotyledonous species were particularly responsive to the treatments, the abundance and biomass of *Carex flacca* was reduced following addition of P. In the short-term acid grassland plots, the above ground biomass increased in response to N treatments while the addition of P had little effect, indicating an N rather than P limited system. Evidence for significantly altered nutrient limitation only occurred after 7-years of treatments, as reflected by increases in the shoot N:P ratio and decreases in leaf biomass of *Anenome nemorosa* plants in the N treated plots. In the calcareous grassland, it would be expected that enhanced aerial deposition of N may cause P limitation to become even more critical as increased N supply leads to greater plant demand for P. In the acid grassland, the availability of P may only become important when N limitation is reduced in response to long-term N additions. The observed changes in above ground biomass do not, however, give any indication of the processes controlling the mineralisation and supply of P to plants which may by affected by increased N supply.

Plants primarily utilise P in the inorganic form, although there is increasing evidence that organic forms may be accessed directly, particularly in ericaceous plants where roots are colonised by ericoid mycorrhizas (e.g. Leake and Miles, 1996). Inorganic P can be supplied to plants from the solubilisation of P containing minerals such as apatite by organic acid excretion from plants or carbonic acid production by microbial respiration (Paul and Clark, 1996). More importantly, P is mineralised from organic sources, predominantly as a result of microbial activity, or to a lesser extent, by the direct action of plant roots and taken up directly from the soil solution. The mineralisation of P sources in the soil is catalysed by the phosphatase group of enzymes, which includes phosphomonoesterases (PME), phosphodiesterases (PDE) and phosphotriesterases (Florkin and Stotz, 1964; see Section 1.3.2). Phosphatases may be of microbial (bacterial or fungal) or plant origin and are found in soil solution, bound onto colloids and clay minerals and on the surface of roots. Phosphatase enzymes associated with the root surface of plants may originate from

mycorrhizal or saprotrophic fungi, bacteria or root exudates. The importance of root surface phosphatases in maintaining an adequate supply of P for the plant is considerable. Inorganic P produced by this mechanism has been calculated to contribute 65 % of the annual P demand of *Eriophorum vaginatum* subsp. *spissum* (L.) plants growing in an arctic tundra system (Kroehler and Linkens, 1988). Measurement of root surface phosphatase activities has largely been restricted to agricultural and horticultural crops including wheat, barley, oats, onion and papaya (Ridge and Rovira, 1971; Dodd *et al.*, 1987; Lee, 1988; Tarafdar and Claassen, 1988; Mohandas, 1992) where, in most cases, increased plant growth was coupled with greater phosphatase activity. Lee (1988) found a significant correlation between PME activity and the influx of ^{32}P labelled orthophosphate, indicating the enzyme may also have a function in phosphate transport.

Investigations of the effects of root surface phosphomonoesterase activities in response to nutrient manipulations have almost exclusively focused on effects of orthophosphate availability since this is a potent repressor of the enzyme and an inhibitor of its activity (e.g. Silberbush *et al.*, 1981; Furlani *et al.*, 1984; Caradus and Snaydon, 1987; Kroehler and Linkins, 1988; Lee, 1988; Speir and Cowling, 1991). In a pot experiment, Silberbush *et al.* (1981) found that root surface PME activity of *Aegilops peregrina* L. was stimulated when grown in a P-poor soil, as compared to the activity of those grown in a P-rich soil. However, very few studies have measured root surface PME activity in plants growing in natural systems that have received factorial additions of P.

No information is available regarding the effects of increased N supply, either as chronic inputs simulating N pollution or acute fertiliser inputs, on root surface phosphatase activity in nutrient limited semi-natural grasslands where the vegetation is typically regarded as being sensitive to increased pollution loads. However, parallels may be drawn with studies investigating the effects of increased N supply on the root surface phosphatase activity of coniferous trees, since semi-natural forest ecosystems are also dependent on belowground nutrient cycling processes and where enhanced N deposition is thought to affect tree health (Aber *et al.*, 1989). Kieliszewska-Rokicka (1992) demonstrated that root surface PME activity of *Pinus sylvestris* L. seedlings was stimulated when grown in soil receiving high (18.9 mM) levels of N, and was even more active when seedlings were inoculated with the ectomycorrhizal fungus, *Paxillus involutus* (Fr.) Fr. This stimulatory effect on PME

activity may be expected since addition of N is likely to lead to increased P demand in soils (or growth media) with a low P status, as seen in the forest N enrichment experiments at Aber, North Wales (Emmett, 1995). In addition to phosphatase production by plant roots colonised by ectomycorrhizas, there is increasing evidence that the vesicular-arbuscular (VA) mycorrhizal fungi, which are universally found in long-term grasslands, may contribute considerably to soil phosphatse activity and play a direct role in mobilising P from organic sources (Jayachandran *et al.*, 1992; Joner and Jakobsen, 1995). Dodd *et al.* (1987) reported significantly higher acid phosphatase activity in wheat (*Triticum aestivum* L.) and onion (*Allium cepa* L.) when inoculated with the VA-mycorrhizal fungi *Glomus mosseae* (Nicol & Gerd.) Gerd & Trappe and *G. geosporum* (Nicol & Gerd.) Gerd & Trappe.

However, some grassland plant species are unlikely to be dependent on mycorrhizal associations for P nutrition. The Cyperaceae are not normally considered to form mycorrhizas (Harley and Harley, 1987), although 'dark septate' fungi have been observed within the roots of several sedges, including *Carex flacca*, from alpine ecosystems (Read and Haselwandter, 1981; Treu *et al.*, 1996), that have been shown to increase both growth and shoot P content (Haselwandter and Read, 1982). Instead their roots are often characterised by distinctive carrot-shaped swellings, to which the name 'dauciform' is given (Lamont, 1974). These unique structures, first described by Davies *et al.* (1973) on *C. flacca* plants from dune slacks at Newborough National Nature Reserve, North Wales, occur primarily on the root laterals in organic matter enriched upper soil horizons. Their morphology has been likened to the proteoid roots found in certain members of the Proteaceae in that the swellings are often encompassed by dense clusters of root hairs that adhere strongly to soil organic matter (Lamont, 1974). Close observations of their development has led to the identification of distinct stages in growth (Davies *et al.*, 1973), which can be further simplified into four basic stages (Plate 4.1). Little is known concerning either the function or the factors controlling the formation of dauciform roots. The presence of the roots in the surface soil horizons together with the substantially increased surface area afforded by the hairy clusters suggests that the structures may have a role in nutrient uptake. An insight into the function of dauciform roots may be obtained from investigations into the function of proteoid cluster roots, since both structures have a similar morphologly. Proteoid roots are known to have an important role in P uptake. In a

pot experiment, Lamont *et al.* (1984) reported that P uptake by *Leucadendron laureolum* (Lam.) Fourcade was related to the number of proteoid roots. Lamont (1982) demonstrated between 1 and 13 times greater uptake of ^{32}P by proteoid roots as compared to non-proteoid roots in a liquid culture experiment. The author suggested that greater accessibility to P alone could not account for the increases in uptake and that in the field, solubilisation processes may also occur. Greater surface PME activity has also been reported on root clusters of *Lupinus albus* L. as compared to non-cluster roots (Adams and Pate, 1992). The increase in enzyme activity may be a result of different functional groups of microorganisms associated with the root surface. For example, Wenzel *et al.* (1994) isolated functionally different groups of bacteria that were capable of solubilising calcium phosphates from the proteoid cluster roots of waratah (*Telopea speciosissima* (Sm.) R.Br.).

An understanding of the factors controlling the distribution and development of dauciform roots is crucial given the likely role of the swellings in nutrient acquisition. Lamont (1973) reported that the number of dauciform swellings present on clones of *Cyathochaete avenacea* L. grown in dune sand was on average five times that in the absence of added N and also following addition of 0.62 mM N. This study suggests that enhanced N supply may have an important role in controlling the abundance of dauciform roots and therefore maintaining above ground productivity of *C. avenacea*. No further information is available regarding the role of other nutrients, such as P, on this species or the effect of N and P additions in combination on the abundance of dauciform swellings of other members of the Cyperaceae, such as *Carex flacca*. However, the extent of proteoid cluster root formation has been found to be negatively correlated with the amount of extractable soil P and soil organic matter in established roots of *Myrica gale* L. (Crocker and Schwintzer, 1994). The significant decreases in shoot density and biomass of *C. flacca* in response to raised P supply (Chapter 3) may be mirrored by similar changes in the abundance of dauciform swellings. In contrast, if dauciform swellings are actively involved in P acquisition, additions of N may lead to greater production of proteoid roots as the demand for P increases.

4.1.1 Aims

The principal aims of this chapter were to investigate the effect of simulated pollutant N deposition alone and in combination with P additions on root surface PME activity and the abundance of dauciform swellings in *Carex flacca*. Measurement of root surface phosphatase activity in species rich turfs is confounded by a number of difficulties, including i) the separation of the roots of individual species from other roots and organic matter ii) root systems must be kept intact since cell leakage would result in overestimation of root surface enzyme activity, and iii) the age of roots must be consistent between samples since younger fine roots are likely to be more active producers of enzymes. A more satisfactory approach is the use of the bioassay concept first described by (Harrison, 1975) in which the author transplanted seedlings of a known age in order to measure P uptake rates. Using a similar approach, Birch (1988) demonstrated that *Plantago lanceolata* seedlings were colonised by VA-mycorrhizal fungi after just 5 days growth in a calcareous grassland. In the acid grassland, preliminary studies revealed that the field bioassay approach was less practicable as a result of the taller sward and the deep (1-3 cm) F horizon. This layer comprises primarily dead or decaying roots and litter and is very freely draining which led to transplanted seedlings of *Agrostis capillaris*, the dominant grass at this site, to be prone to droughting. Instead, seedlings were grown in a controlled environment facility within tubes containing soil removed from the Ah horizon.

Initial experiments aimed to optimise the assay of root surface PME (Sections 4.2.1.i and 4.2.1.ii). The second series of experiments aimed to assess the effect of long-term N and short-term N and P additions on root surface PME activity of *Plantago lanceolata* in the calcareous grassland and *Agrostis capillaris* in the acid grassland (Section 4.2.1.iii and 4.2.1.iv). The third group of experiments (Section 4.2.2) comprises quantification of the abundance and developmental stages of dauciform swellings in *C. flacca* root systems removed from the short-term calcareous grassland plots (3 m × 3 m).

4.2 MATERIALS AND METHODS

4.2.1 Assay of root surface enzyme activity

i) pH optimisation

The optimum pH for assay of root surface phosphomonoesterase (PME) activity was determined using TRIS/maleate buffer in the range pH 5-7 in half unit increments. PME

activity was determined using the artificial substrate *p*-nitrophenyl phosphate (*p*-NPP) from which the release of *p*-nitrophenol (*p*-NP) is measured (Leake and Miles, 1996; Tabatabai and Bremner, 1969). Whole root systems were excised from *Plantago lanceolata* seedlings that had grown for two weeks in silica sand moistened with de-ionised water in a controlled environment room (20 °C day, 15 °C night; 80 μmol $m^{-2} s^{-1}$ PFD, 16 h day). The roots were cleaned in distilled water and assayed in 10 ml plastic centrifuge tubes containing 0.5 M *p*-NPP dissolved in 4 ml buffer in a shaking water bath (37 °C). Up to 500 μl were removed and added to plastic cuvettes containing 2 ml 2 M NaOH. Optical density was measured immediately at 410 nm using a Cecil CE1020 spectrophotometer. The procedure was repeated three times every 30 min in order to check the enzyme reaction was linear. Standards were prepared in the range 1-10 mmol *p*-NP using stock solutions dissolved in TRIS/maleate buffer adjusted to each pH. PME activity was expressed as nmol *p*-NP g^{-1} fwt s^{-1}. Assays of root surface PME of *A. capillaris* seedlings were buffered at soil pH (4.5) and *P. lanceolata* at pH 5.

ii) Choice of buffer

An assessment of the effectiveness of four buffers in assay of root surface phosphomonoesterase activity was undertaken. Root surface PME activity of *P. lanceolata* seedlings was measured as described in Section 4.2.1.i except the roots were incubated in either Na acetate, Na citrate, K citrate or TRIS/maleate buffers (0.5 M in each case).

iii) Assay of root surface PME activity in microcosms

P. lanceolata seeds were germinated and grown on moistened builders sand for fourteen days prior to being transplanted into microcosms for a further 30 days (growth room conditions as for Section 4.2.1.i). Microcosms were constructed using 5 ml plastic pipette tips and contained soil removed (Ah horizon) from calcareous grassland plots that had received six years of N treatments (Fig. 4.1a). Whole root systems were cleaned and assayed for root surface PME activity as described in Section 4.2.1.i. The experiment was repeated using *Agrostis capillaris* seedlings grown for 56 days in soil removed (Ah horizon) from acid grassland plots that had received seven years of N treatments, and *A. capillaris* seedlings grown for 36 days in soil removed from plots that had received 12 months of P and 18 months of N additions. N and P concentrations in *A. capillaris* shoots

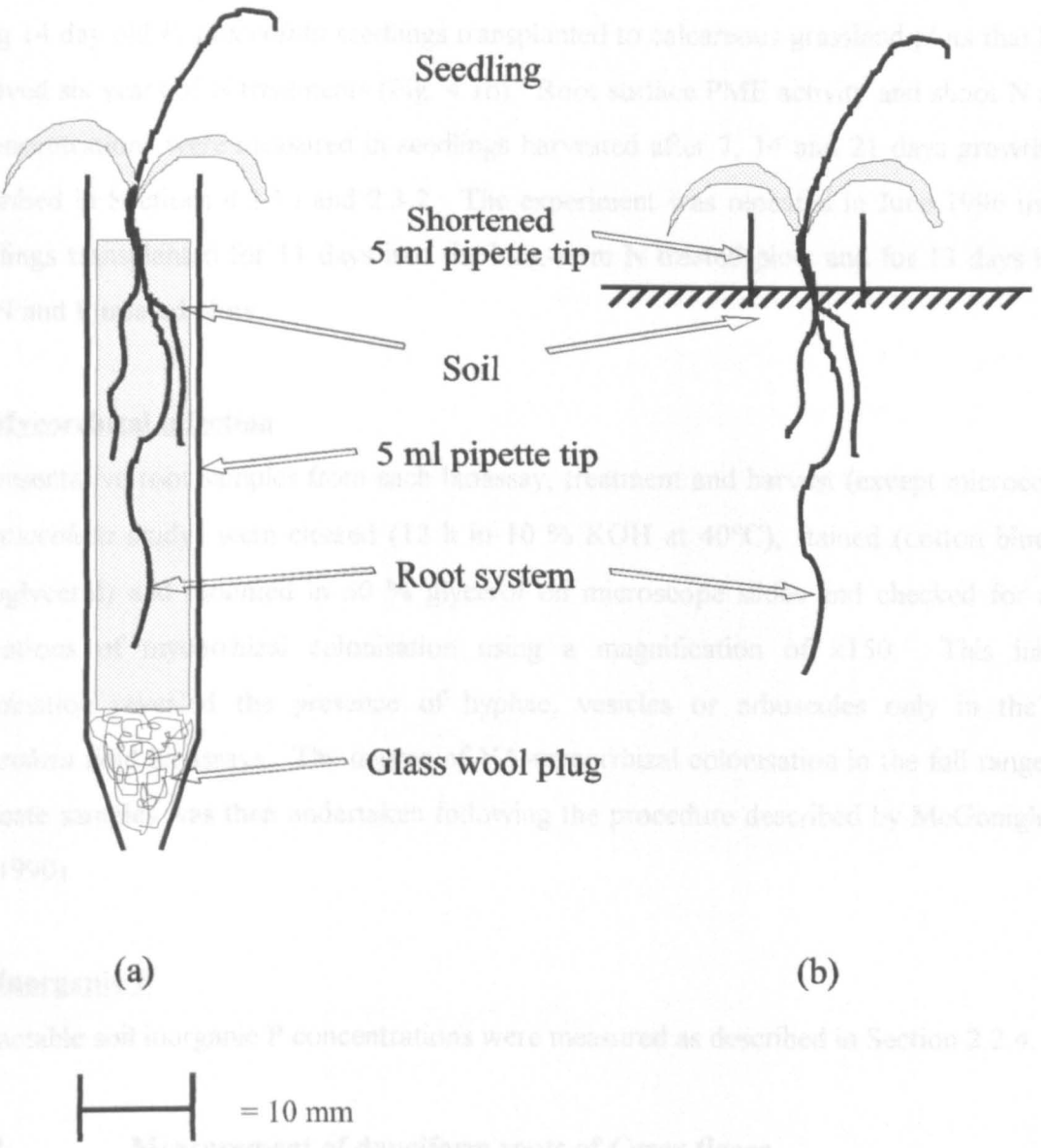


Fig. 4.1. Experimental arrangements for assay of root surface phosphomonoesterase activity (a) in the controlled temperature rooms and (b) in the field.

were determined (Section 2.2.2). Assays of root surface PME of *A. capillaris* seedlings were buffered at soil pH (pH 4.5).

iv) Field bioassay of root surface PME activity

A field-based bioassay of root surface PME activity was undertaken in November 1995 using 14 day old *P. lanceolata* seedlings transplanted to calcareous grassland plots that had received six years of N treatments (Fig. 4.1b). Root surface PME activity and shoot N and P concentrations were measured in seedlings harvested after 7, 14 and 21 days growth as described in Sections 4.2.1.i and 2.3.2. The experiment was repeated in June 1996 using seedlings transplanted for 11 days into the long-term N treated plots and for 13 days into the N and P treated plots.

v) Mycorrhizal infection

Representative root samples from each bioassay, treatment and harvest (except microcosm *P. lanceolata* study) were cleared (12 h in 10 % KOH at 40°C), stained (cotton blue in lactoglycerol) and mounted in 50 % glycerol on microscope slides and checked for any indications of mycorrhizal colonisation using a magnification of ×150. This initial examination revealed the presence of hyphae, vesicles or arbuscules only in the *P. lanceolata* field bioassays. The degree of VA-mycorrhizal colonisation in the full range of replicate samples was then undertaken following the procedure described by McGonigle *et al.* (1990).

vi) Inorganic P

Extractable soil inorganic P concentrations were measured as described in Section 2.2.4.

4.2.2 Measurement of dauciform roots of *Carex flacca*

C. flacca plants were removed from the short-term calcareous grassland plots during November 1996 using a 4 cm diameter soil corer to a maximum depth of 10 cm from which the root systems were excised and cleaned. Root length (cm) per volume of soil was measured using the modified line intersect method (Tennant, 1975; Newman, 1966) using a 5 mm grid and a conversion factor of ×0.3928. Dauciform swellings, which were classified according to their age and morphology (young smooth, young hairy, old smooth and old

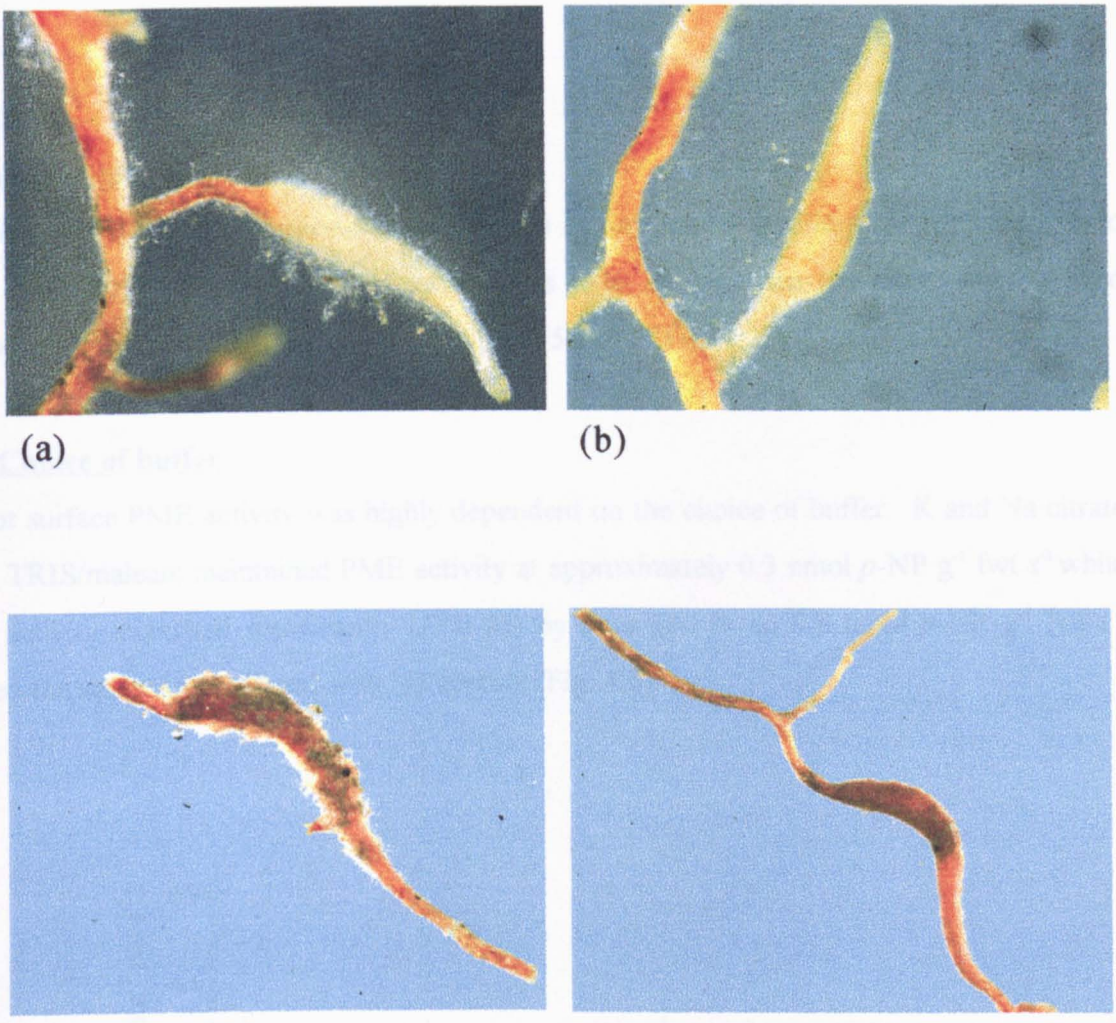


Plate 4.1. The four groups of dauciform swellings characteristic of the roots of *Carex flacca* from the short-term calcareous grassland plots. (a) young hairy, (b) young smooth, (c) old hairy, and (d) old smooth.

hairy; Plate 4.1), were also scored when they intersected a grid line from which the percentage of root comprising the different type of swellings was calculated.

4.3 RESULTS

4.3.1 Root surface PME

i) pH optimisation

Root surface phosphomonoesterase activity in *P. lanceolata* seedlings ranged from 0.3 to 0.1 nmol *p*-NP g^{-1} fwt s^{-1}. The enzyme was most active when the assay was buffered between pH 5-6 but least active between pH 6.5 and 7 (Fig. 4.2).

ii) Choice of buffer

Root surface PME activity was highly dependent on the choice of buffer. K and Na citrate and TRIS/maleate maintained PME activity at approximately 0.3 nmol *p*-NP g^{-1} fwt s^{-1} while the activity increased significantly ($P<0.05$) by over 100 % to 0.8 nmol *p*-NP g^{-1} fwt s^{-1} when the assay was buffered with Na acetate (Fig. 4.3).

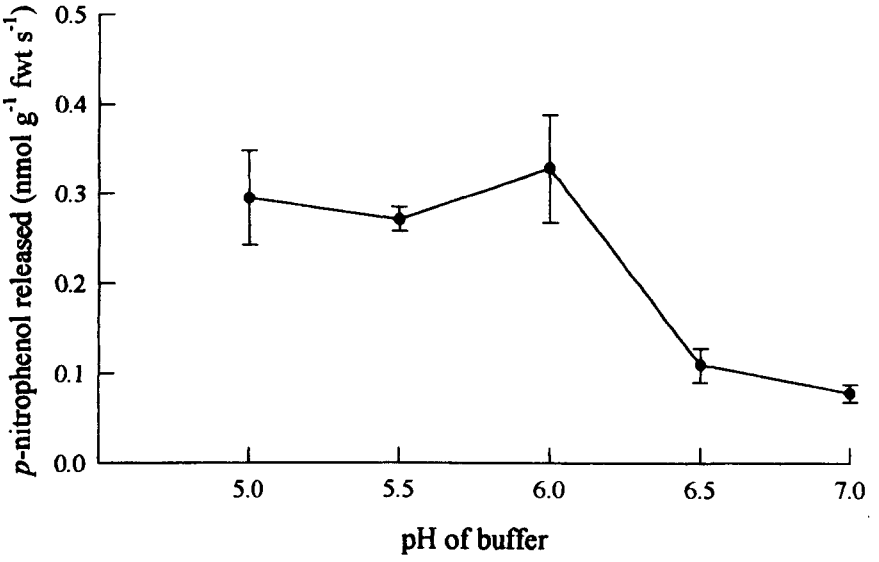


Fig. 4.2. pH optima (TRIS/maleate buffer) for assay of root surface phosphomonoesterase activity in *Plantago lanceolata* seedlings (± SEM).

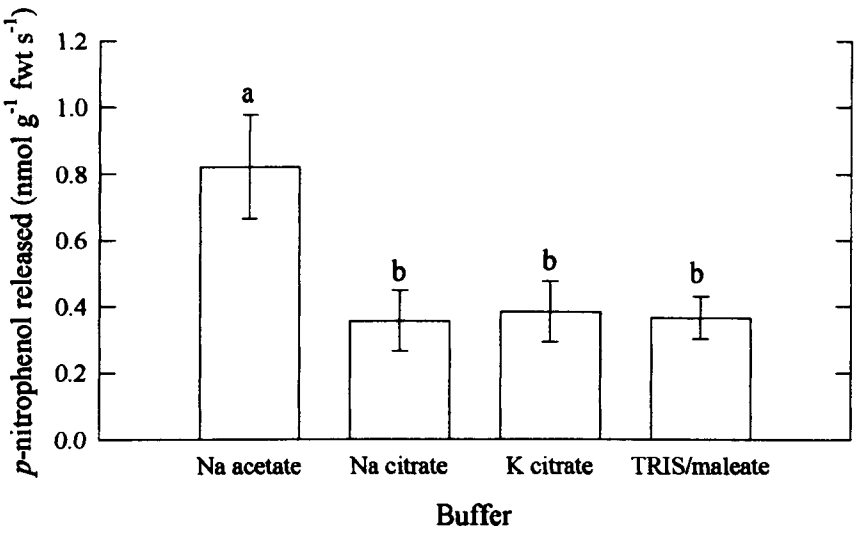


Fig. 4.3. Root surface phosphomonoesterase activity in *Plantago lanceolata* seedlings assayed in different buffers at pH 5.5 (± SEM). Bars sharing a letter are not significantly different (P>0.05).

iii) Assay of root surface PME activity in microcosms

PME activity in *P. lanceolata* seedlings grown for 30 days in microcosms increased from 21 nmol *p*-NP g^{-1} fwt s^{-1} in the control to 30 nmol *p*-NP g^{-1} fwt s^{-1} in the 3.5 N and 14 N treatments and 36 nmol *p*-NP g^{-1} fwt s^{-1} in the 14 N AS treatment (Fig. 4.4), but these differences were not significant (P>0.05).

A similar response was observed for *A. capillaris* seedlings which had grown for 56 days in microcosms containing soil from the acid grassland plots that had received N for seven years. In contrast to *P. lanceolata* seedlings, not only did all of the N treatments significantly increase root surface PME activity (P<0.01), but the activity of the enzyme was an order of magnitude greater. The activity increased from 150 nmol *p*-NP g^{-1} fwt s^{-1} in the control to between 275 nmol *p*-NP g^{-1} fwt s^{-1} in the 3.5 N treatment and 350 nmol *p*-NP g^{-1} fwt s^{-1} in the 7 N treatment (Fig. 4.5). A highly significant (P<0.001) quadratic relationship was seen between KCl extractable soil ammonium concentrations and root

surface PME activity of *A. capillaris* in the long-term acid grassland plots (Fig. 4.6). The ammonium concentrations accounted for 86 % of the variation in the root surface PME activity. An almost identical relationship was seen when total extractable inorganic N (ammonium + nitrate) was correlated with root surface PME activity ($r^2 = 0.84$, $P<0.001$). N additions had no effect on root surface PME activity of *A. capillaris* seedlings when grown for 36 days in microcosms containing soil from the short-term N and P treated plots (Fig. 4.7a). However, the application of P, either with or without N, significantly reduced ($P<0.05$) PME activity by 20 % from 400 to 325 nmol *p*-NP g^{-1} fwt s^{-1} (Fig. 4.7b).

In the microcosms containing soils removed from the long-term acid grassland plots, the N treatments did not significantly affect ($P>0.05$) shoot N concentration which ranged from 40 to 47 mg g^{-1} dwt. (Fig. 4.8a). However, shoot P concentration was reduced from 0.55 mg g^{-1} dwt in the control to 0.4 mg g^{-1} dwt. in the 3.5 N treatment while subsequent N treatments progressively raised the P concentration to 0.8 mg g^{-1} dwt. (Fig. 4.8a). This initial decrease in shoot P concentration was reflected by a significant increase ($P<0.05$) in N:P ratio from 100 in the control to 145 in the 3.5 N treatment before falling to 60 in the 14 N AS treatment ($P>0.05$; Fig. 4.8b). The shoot N concentrations of seedlings grown in microcosms containing soil from the short-term N and P treated acid grassland plots were not affected by any of the nutrient treatments and ranged from 55 to 65 mg g^{-1} dwt. (Fig. 4.9a), a level similar to those measured in seedlings grown in soil from the long-term plots (Fig. 4.8a). Shoot P concentration and shoot N:P ratio of plants grown in short-term microcosms were also unaffected by any of the nutrient treatments, although shoot P concentration decreased to 2.4 mg g^{-1} dwt. (Fig. 4.9a) and shoot N:P ratio increased to 27 in the 14 N+P treatment (Fig. 4.9b). However, shoot P concentrations were considerably greater and shoot N:P ratio considerably lower in plants grown in microcosms containing soil from the short-term acid grassland plots compared to those grown in microcosms containing control soils from the long-term plots. For example, the shoot N:P ratio was approximately 20 in the former microcosms (Fig. 4.9b) compared to a value of 80 in the latter (Fig. 4.8b). Root surface PME activity in *A. capillaris* seedlings grown in microcosms containing soils from the short-term plots was not correlated with shoot N, P or N:P ratio or soil extractable inorganic N and P concentrations ($P>0.05$).

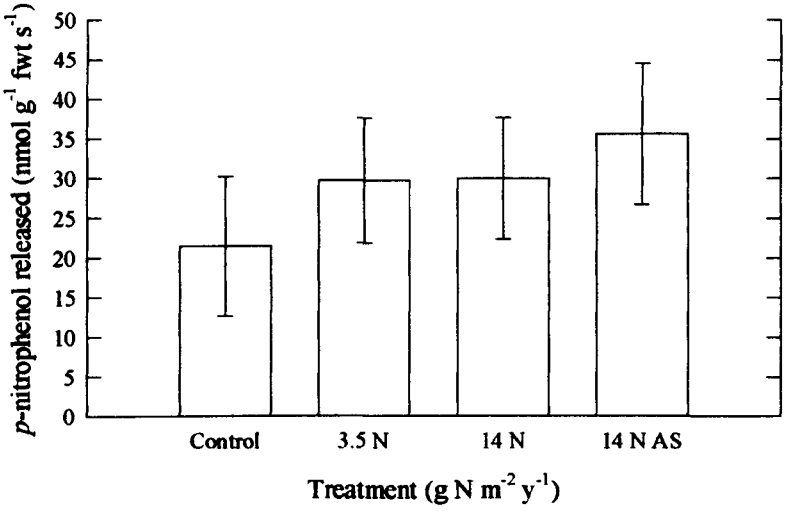


Fig. 4.4. Root surface phosphomonoesterase activity in *Plantago lanceolata* seedlings harvested after 30 days growth in microcosms containing soil removed from calcareous grassland plots that have received six years of N treatments (± SEM). Bars are not significantly different (P>0.05).

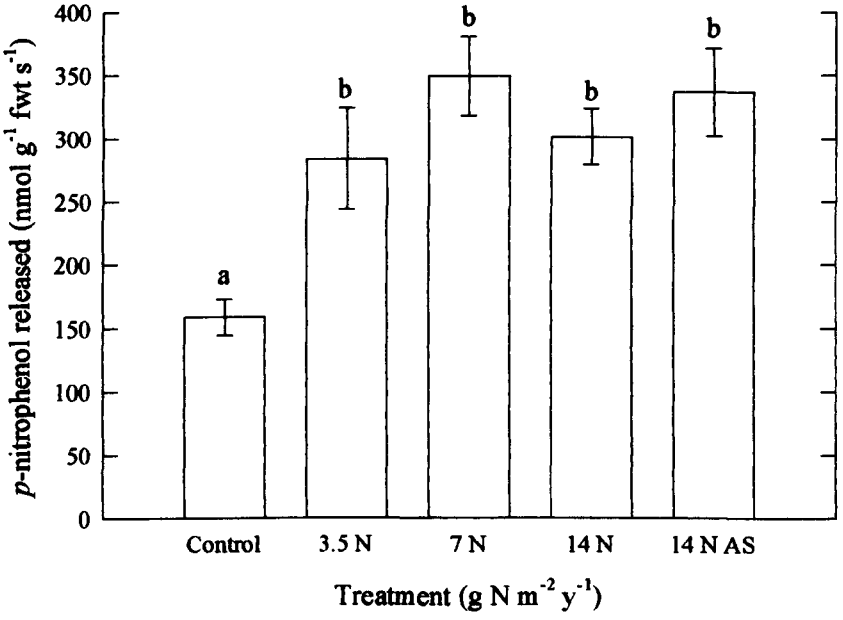


Fig. 4.5. Root surface phosphomonoesterase activity in *Agrostis capillaris* seedlings harvested after 56 days growth in microcosms containing soil removed from acid grassland plots that have received seven years of N treatments (± SEM). Bars sharing a letter are not significantly different (P>0.01).

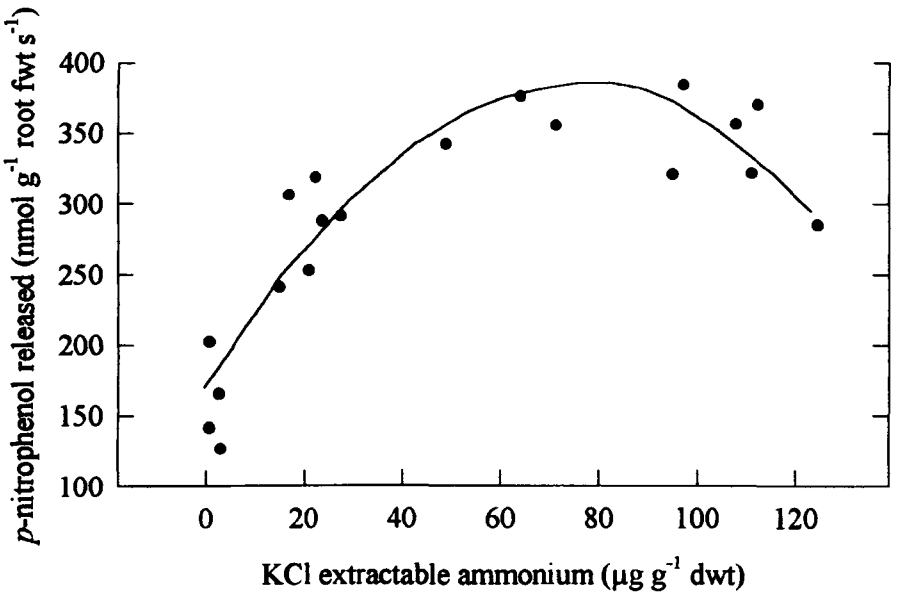


Fig. 4.6. Quadratic relationship between extractable ammonium concentrations and root surface phosphomonoesterase activity of *Agrostis capillaris* seedlings grown for 56 days in microcosms containing soil removed from acid grassland plots that have received seven years of N treatments ($r^2 = 0.859$, $P<0.001$).

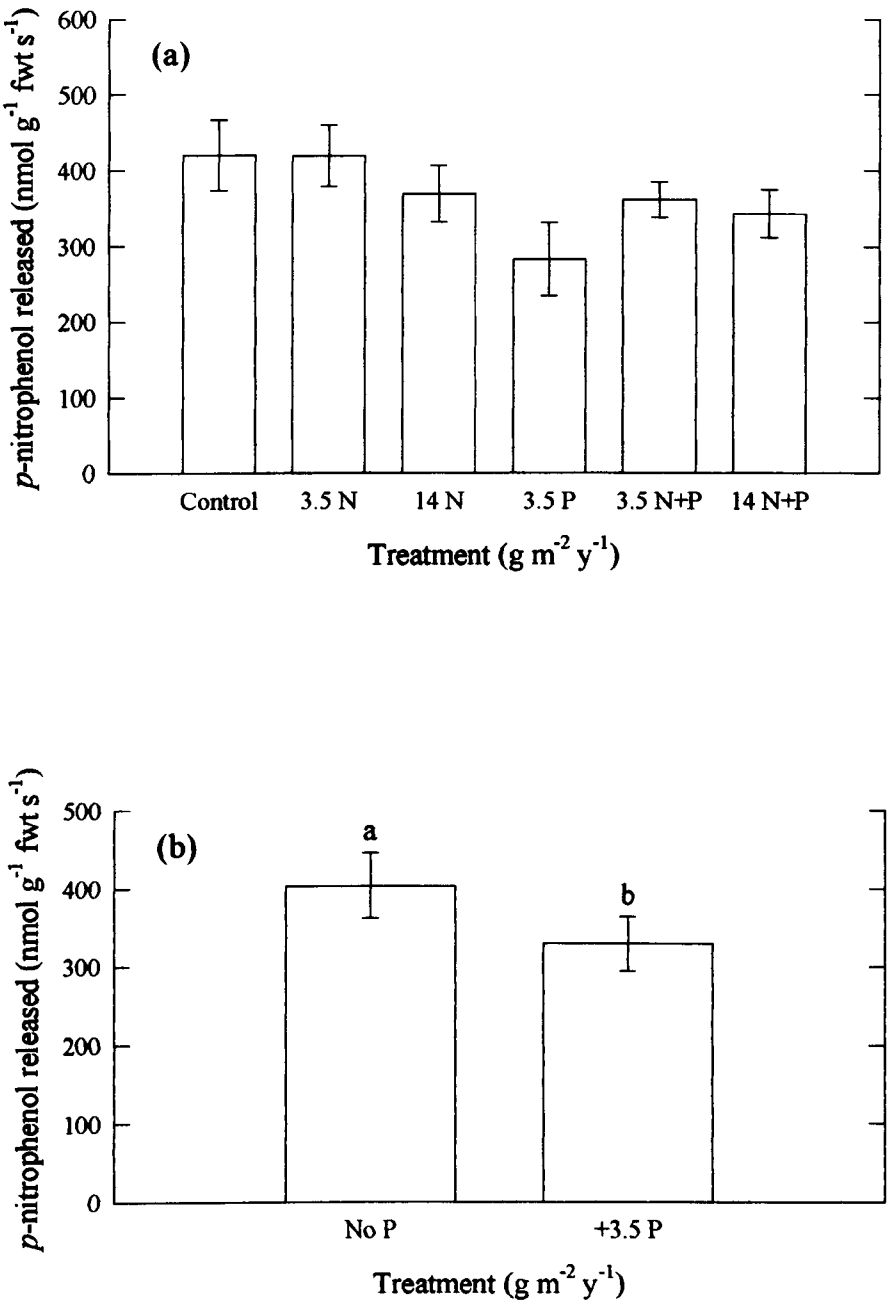


Fig. 4.7. Root surface phosphomonoesterase activity in *Agrostis capillaris* seedlings harvested after 36 days growth in microcosms containing soil removed from acid grassland plots that have received 18 months N and 12 months P additions (± SEM). (a) Effect of N and P interactions (no significant differences), and (b) overall effect of P (bars are significantly different; $P<0.05$).

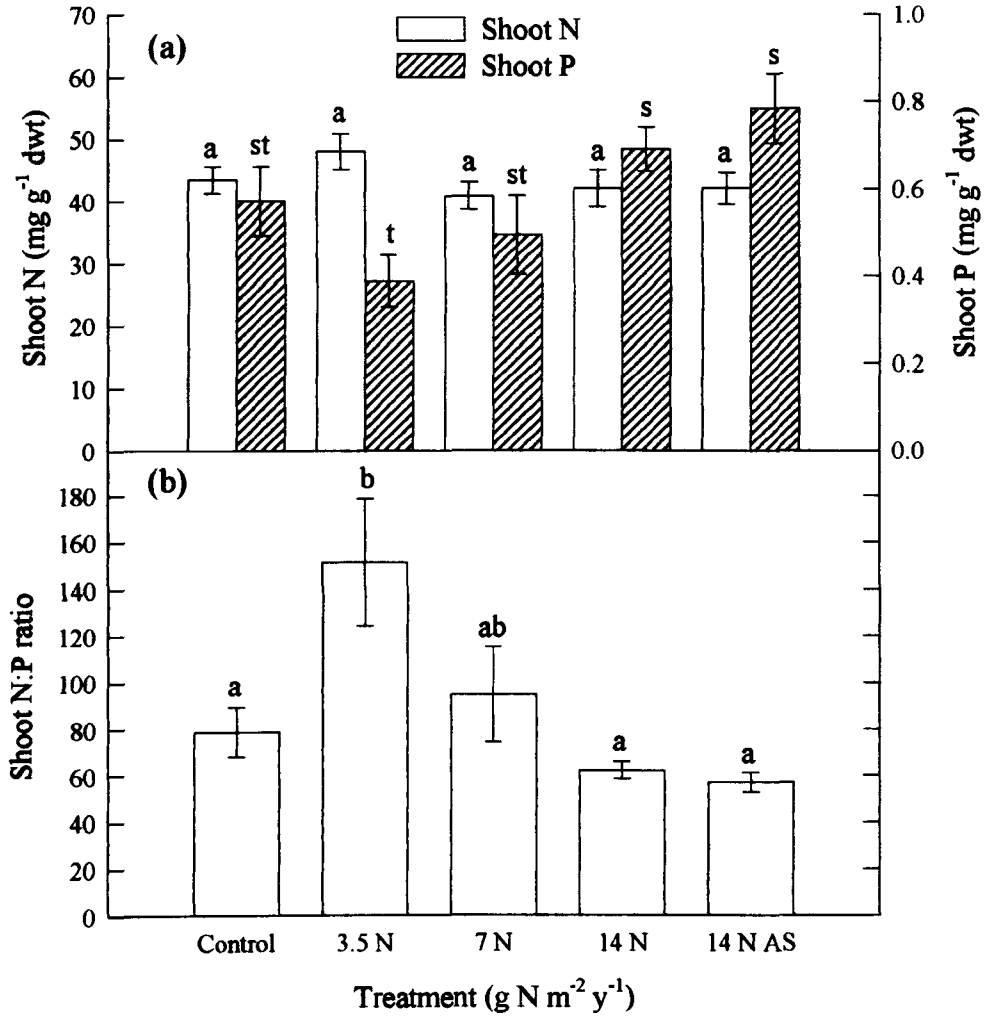


Fig. 4.8. Changes in (a) shoot N and P concentrations, and (b) shoot N:P ratio of *Agrostis capillaris* seedlings harvested after 56 days growth in microcosms containing soil removed from acid grassland plots that have received seven years of N additions (± SEM). Bars sharing a letter are not significantly different (P>0.05). Shoot N and P concentrations analysed independently.

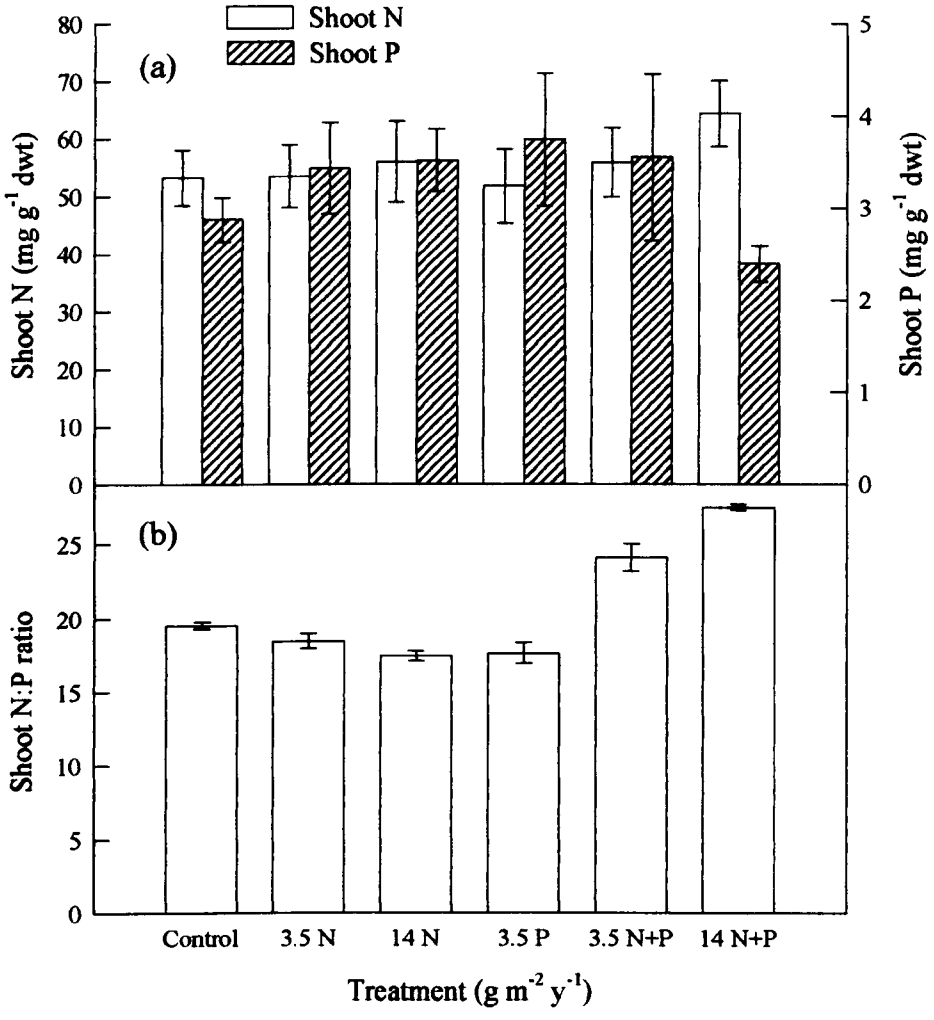


Fig. 4.9. Changes in (a) shoot N and P concentrations, and (b) shoot N:P ratio of *Agrostis capillaris* seedlings harvested after 36 days growth in microcosms containing soil removed from acid grassland plots that have received 12 months of P and 18 months of N additions (± SEM). Bars are not significantly different ($P>0.05$).

iv) Field bioassay of root surface PME activity

Root surface PME activity of *P. lanceolata* seedlings transplanted to the calcareous grassland in November 1995 increased significantly after 7 and 21 days growth in both the 14 N and 14 N AS treated plots and after 14 days growth in the 14 N treatment (Fig. 4.10). In the seven day harvest, the activity increased from 1.25 nmol *p*-NP g^{-1} fwt s^{-1} in the control to 1.75 and 1.85 nmol *p*-NP g^{-1} fwt s^{-1} in the 14 N and 14 N AS treatments respectively. After 14 days, the activity in seedlings removed from the control plots increased by 48 % to 1.85 nmol *p*-NP g^{-1} fwt s^{-1} while the seedlings removed from the 14 N treatment increased by 26 % to 2.2 nmol *p*-NP g^{-1} fwt s^{-1} and from the 14 N AS treated plots by only 16 % to 2.15 nmol *p*-NP g^{-1} fwt s^{-1}. Following 21 days growth, root surface PME activity in seedlings from the control plots decreased considerably by 38 % to a level observed in the 7 day harvest, while the enzyme activity of seedlings removed from the 14 N treated plots decreased by only 11 % to 1.95 nmol *p*-NP g^{-1} fwt s^{-1} and those removed from the 14 N AS plots increased by 12 % to 2.4 nmol *p*-NP g^{-1} fwt s^{-1}.

A similar effect of N treatments on root surface PME activity was observed when *P. lanceolata* seedlings were transplanted for 11 days in June 1996. PME activity increased from 6.5 nmol *p*-NP g^{-1} fwt s^{-1} in the control to 10 nmol *p*-NP g^{-1} fwt s^{-1} in the 3.5 N treatment and significantly increased ($P<0.05$) to approximately 20 nmol *p*-NP g^{-1} fwt s^{-1} in the 14 N and 14 N AS treated plots (Fig. 4.11). Root surface PME activity of *P. lanceolata* seedlings transplanted to the calcareous grassland for 13 days was stimulated following 18 months N additions, where it increased from 8 nmol *p*-NP g^{-1} fwt s^{-1} in the control to 17 nmol *p*-NP g^{-1} fwt s^{-1} in the 14 N treatment (Fig. 4.12). However, both the lowest N application rate (3.5 g N m^{-2} y^{-1}) and additions of P did not increase PME activity, even when P was applied in combination with the 14 N treatment. Indeed, enzyme activity significantly decreased ($P<0.05$) from 8 nmol *p*-NP g^{-1} fwt s^{-1} in the 3.5 N treatment to 6.5 nmol *p*-NP g^{-1} fwt s^{-1} in the 3.5 N+P treatment (Fig. 4.12).

Shoot P concentrations of *P. lanceolata* seedlings transplanted for 11 days to the long-term N treated calcareous grassland plots ranged from 21 to 26 mg g^{-1} dwt. and were not affected by the N treatments (Fig. 4.13). In contrast, shoot N concentrations increased significantly ($P<0.05$) from 20 mg g^{-1} dwt. in the control to 25 mg g^{-1} dwt. in the 3.5 and 14 N treatments and 30 mg g^{-1} dwt. in the 14 N AS treatments (Fig. 4.13). A significant

positive decelerating quadratic relationship was observed between root surface PME activity and shoot N concentration (r^2=0.197; P<0.038; Fig. 4.14a). Root surface PME was also strongly correlated to extractable soil inorganic P concentration (r^2=0.356; P<0.014; Fig. 4.14b). The shoot N and P concentrations of *P. lanceolata* seedlings transplanted for 13 days to the short-term calcareous grassland plots were not significantly affected by the N and P treatments (Fig. 4.15a). Shoot N concentrations ranged from 29 to 35 mg g^{-1} dwt. while shoot P concentrations ranged from 2.8 to 4.8 mg g^{-1} dwt. The shoot N:P ratio increased, although not significantly (P>0.05), with each increment of added N from 13 in the control to 15 in the 3.5 N and 17 in the 14 N treatment (Fig. 4.15b). However, the trend was reversed when P was applied in combination with N where the N:P ratio decreased to 10 in the 3.5 N+P treatment and decreased significantly (P<0.01) to 7 in the 14 N+P treatment.

VA-mycorrhizal colonisation of field grown *P. lanceolata* seedlings was almost zero. However, many of the root samples were colonised by unidentified septate extra-radical hyphae. Regression analysis revealed no correlation between the degree of hyphal colonisation and root surface phosphatase activity (P>0.05).

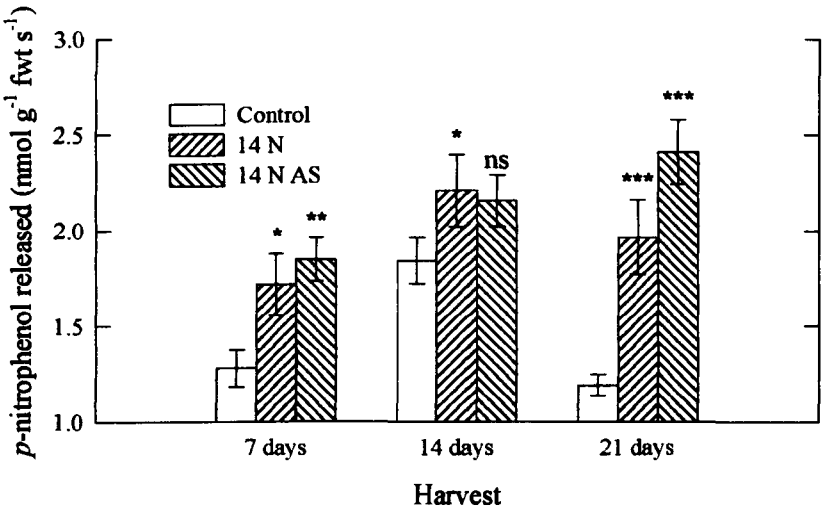


Fig. 4.10. Bioassay of root surface phosphomonoesterase activity in *Plantago lanceolata* seedlings introduced for 7, 14 and 21 days in November 1995 into calcareous grassland plots that have received six years of N treatments (± SEM). Asterisk indicates significant differences from the controls: * $P<0.05$, ** $P<0.01$, *** $P<0.001$, ns = not significantly different from control.

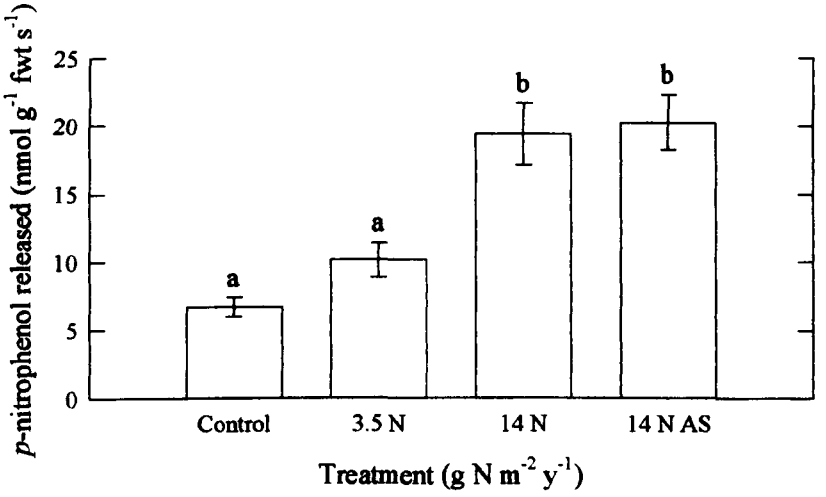


Fig. 4.11. Bioassay of root surface phosphomonoesterase activity in *Plantago lanceolata* seedlings introduced for 11 days during June 1996 into calcareous grassland plots that have received nearly seven years of N treatments (± SEM). Bars sharing a letter are not significantly different ($P>0.05$).

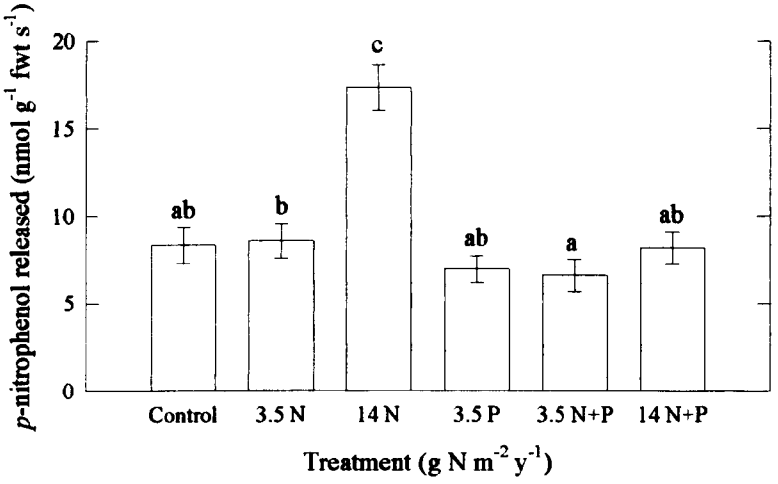


Fig. 4.12. Bioassay of root surface phosphomonoesterase activity in *Plantago lanceolata* seedlings introduced for 13 days during June 1996 into calcareous grassland plots that have received 12 months of N and P treatments (± SEM). Bars sharing a letter are not significantly different (P>0.05).

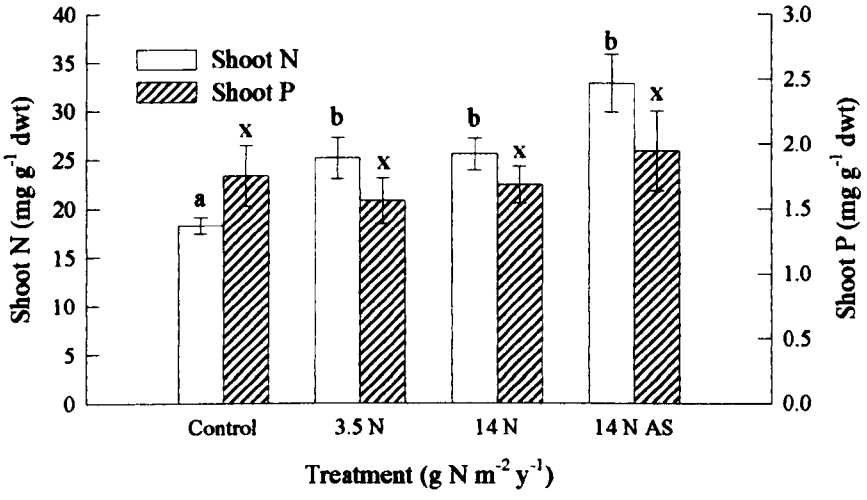


Fig. 4.13. Shoot N and P concentrations in *Plantago lanceolata* seedlings following an 11 day bioassay during June 1996 of root surface phosphomonoesterase activity in calcareous grassland plots (± SEM). Bars sharing a letter are not significantly different (P>0.05). Shoot N and P concentrations analysed independently.

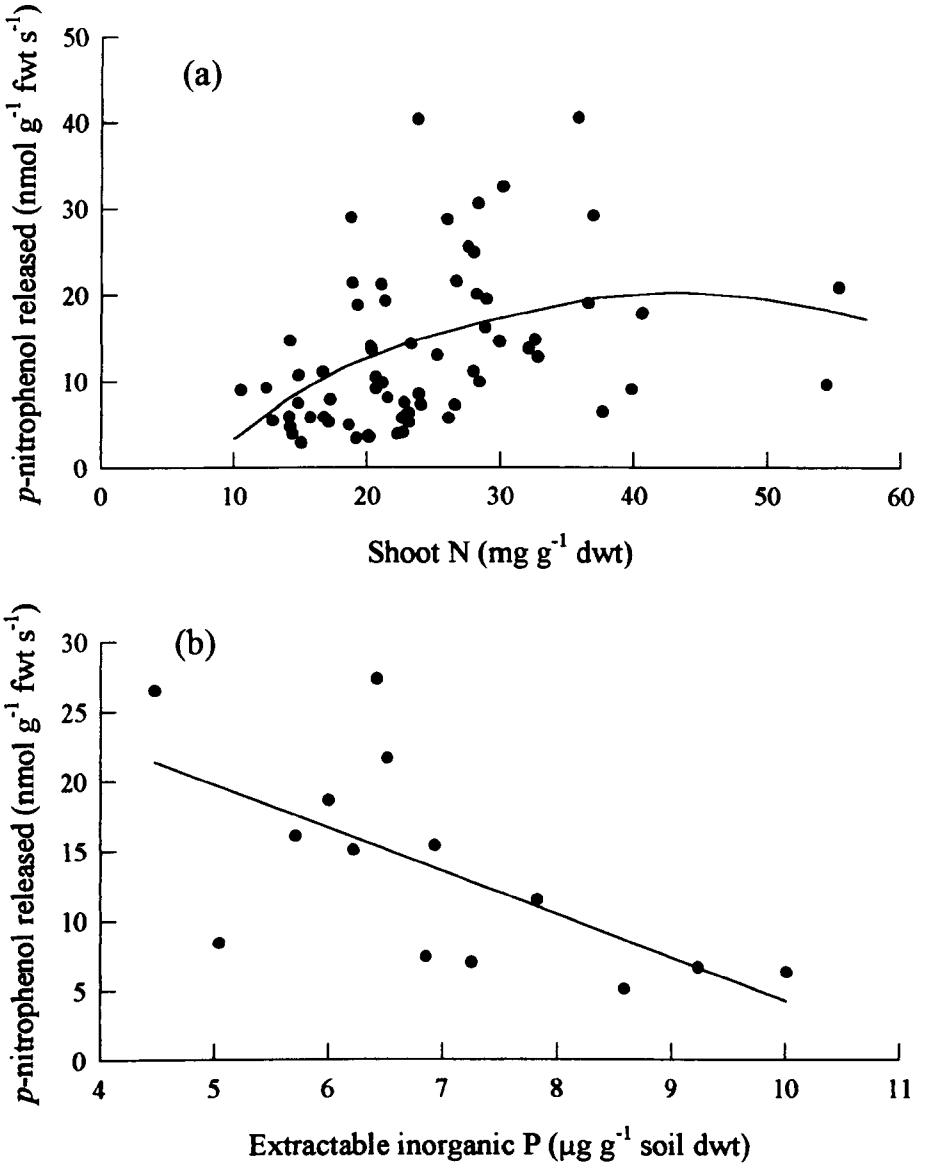


Fig. 4.14. Factors affecting root surface phosphomonoesterase activity in *Plantago lanceolata* seedlings introduced for 11 days during June 1996 into calcareous grassland plots that have received seven years of N treatments. (a) Positive decelerating relationship with shoot N concentration (r^2 = 0.197; linear: P<0.005; quadratic: P<0.038), and (b) relationship with extractable soil inorganic P concentration (r^2 = 0.356, P<0.014).

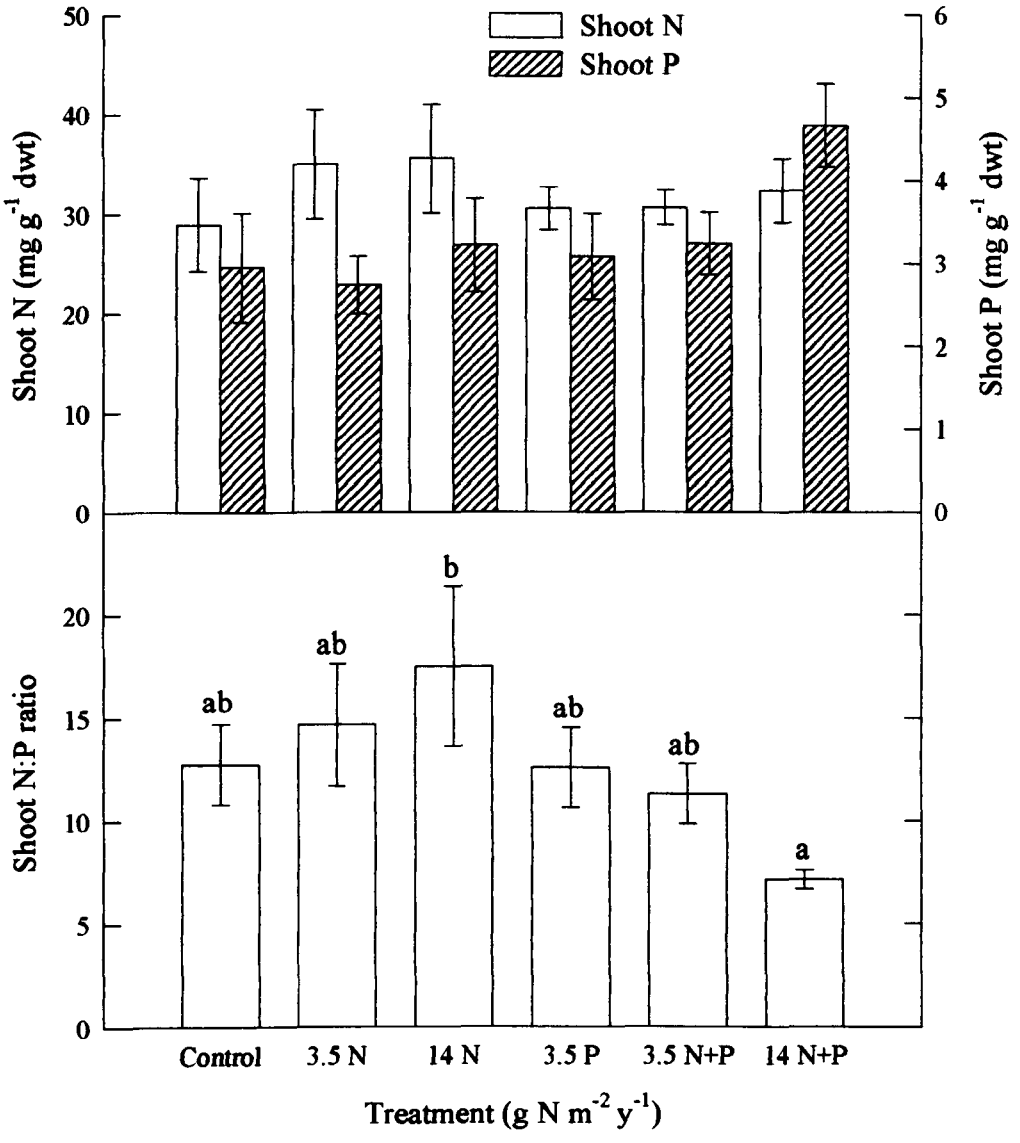


Fig. 4.15. Changes in (a) shoot N and P concentrations (no significant differences), and (b) N:P ratio of *Plantago lanceolata* seedlings (Bars sharing a letter are not significantly different; $P>0.01$) following a 13 day bioassay in June 1996 of root surface phosphomonoesterase activity in calcareous grassland plots (± SEM).

4.3.2 Effect of N treatments on the root system of *Carex flacca*

Root length was not significantly affected by additions of either N or P and ranged from 86 cm 100 cm^{-3} in the 3.5 P treatment to 113 cm 100 cm^{-3} in the 14 N treatment (Fig. 4.16). In contrast, substantial changes occurred in the percentage of root containing dauciform swellings. The combined effect of N and P had no effect on the abundance of all types of dauciform swellings although the percentage root containing young smooth swellings ranged from 0 % in the 3.5 P to 2.5 % in the 14 N+P treatment (Fig. 4.17a). Significant differences ($P<0.05$) in the percentage of dauciform swellings per length of root only occurred in plants removed from plots receiving P, either with or without N (Fig. 4.17b). Here the percentage root length containing all categories of dauciform swellings decreased from 22 to 12 % in response to added P, while the young hairy category decreased from 2 to 0.2 % and the old hairy category decreased from 7 to 2.5 %. No effects were observed on either the young smooth and old smooth categories. In all plots, the old smooth and old hairy categories were the dominant type of dauciform swelling.

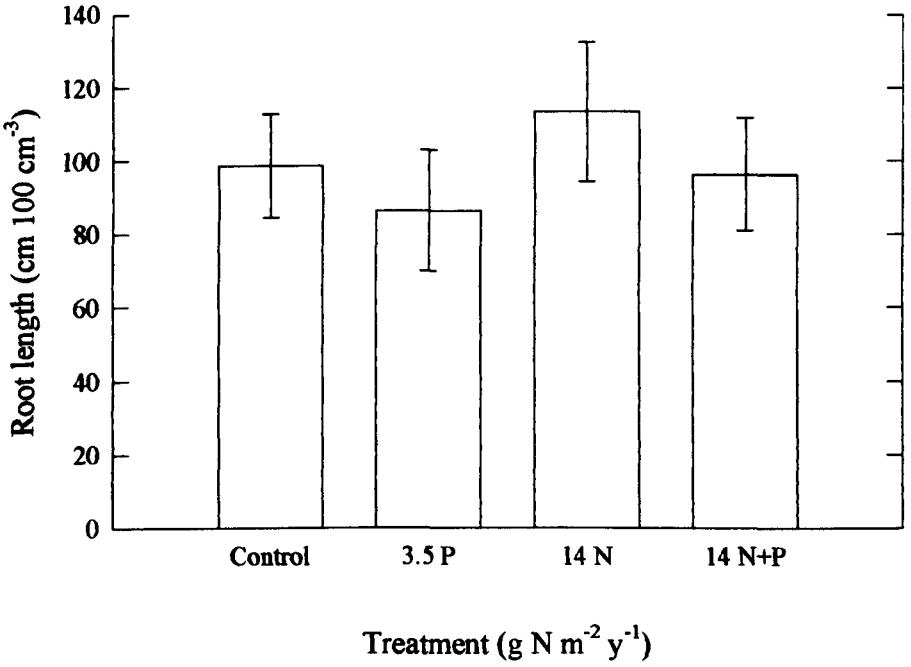


Fig. 4.16. Root length of *Carex flacca* removed from calcareous grassland plots that have received 18 months N and 12 months P additions (± SEM). Bars are not significantly different ($P>0.05$).

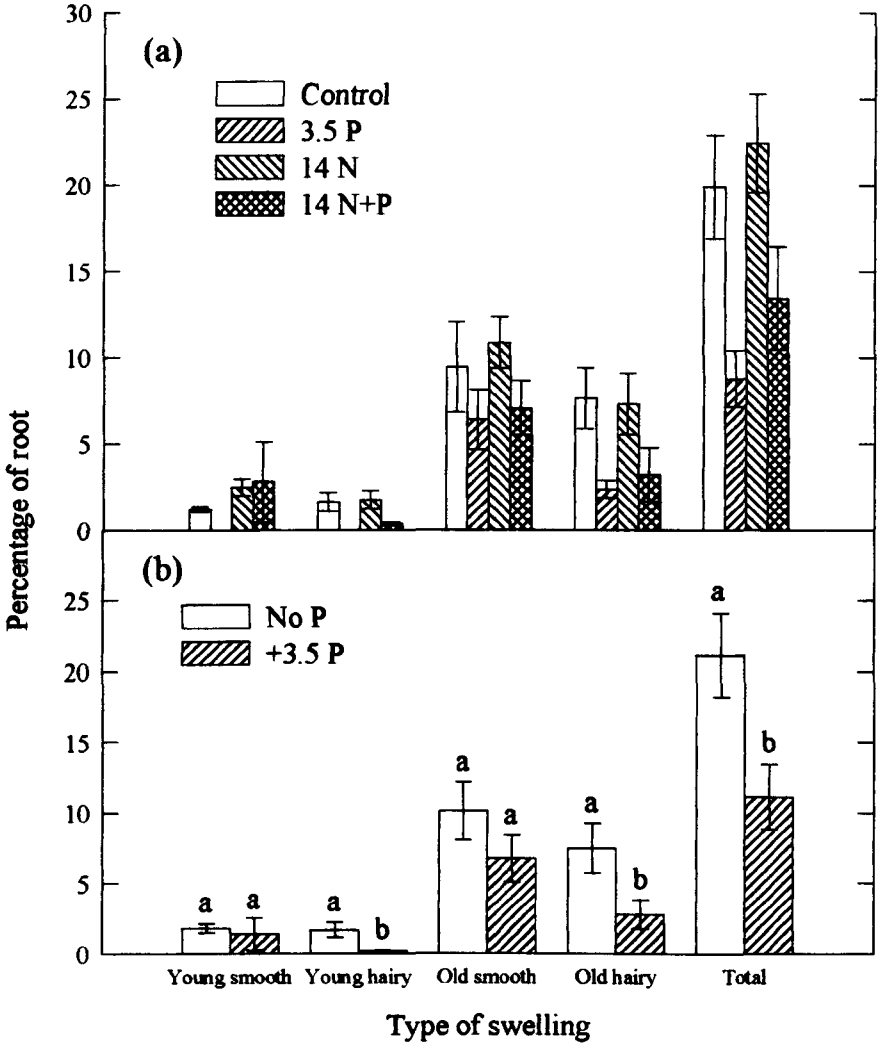


Fig. 4.17. Percentage of *Carex flacca* roots (± SEM) comprising different morphological types of dauciform swellings in plants removed from calcareous grassland plots that have received (a) 18 months N and 12 months P (no significant differences), and (b) the overall effect of 12 months P additions (bars sharing a letter are not significantly different; $P>0.05$). Type of swellings analysed independently.

4.4 DISCUSSION

The optimum pH for assay of root surface phosphomonoesterase (PME) activity of *Plantago lanceolata* seedlings was between pH 5 and 6 (Fig. 4.2). The close similarity between the optimum pH for root surface PME activity and the pH of soil (pH 6.5) on which *P. lanceolata* is found in nature is expected. The optimal pH for assay of root surface PME of *Eriophorum vaginatum* seedlings has been found to be between 3.5 and 4, while the mean soil solution pH of the tussocks from which the plants were removed was 3.7 (Kroehler and Linkins, 1988). Similar results have also been reported for bulk soil PME activity (which originates from either microbial biomass, mycorrhizas or root exudates), whereby acid PME is predominant in acidic soils and alkaline PME is predominant in alkaline soils (Eivazi and Tabatabai, 1977). Root surface PME activity was consistent when the assay was buffered using Na and K citrate and TRIS/maleate. The substantial increase in activity when the assay was buffered using Na acetate almost certainly indicates leakage of the enzyme, probably from epidermal cells of apical root zones, since PME and other enzymes are typically concentrated in this area (Marschner, 1995). Acetic acid, and other monocarboxylic acids, have been shown to cause excessive membrane leakiness as reflected by leakage of potassium and nitrate ions from root tissue (Lee, 1977).

Root surface PME activity of *Plantago lanceolata* seedlings were an order of magnitude lower than for *Agrostis capillaris* seedlings, regardless of whether they were grown in the field or microcosms. PME activities of *P. lanceolata* seedlings grown in microcosms were, however, greater than field grown plants. Root surface PME activity of plants grown in control soils were 21 nmol g^{-1} fwt s^{-1} for the microcosm study and between 1.2 and 8 nmol g^{-1} fwt s^{-1} for the field bioassay. The inter species differences can probably be explained by the finer root systems typical of grasses as compared to forbs. Fine roots are known to have a more active role in nutrient acquisition than coarser roots (Marschner, 1995). Differences in mycorrhizal colonisation was not a factor since neither of the root systems had any significant level of infection. The intra-species differences observed between field and microcosm grown *P. lanceolata* seedlings is likely to result from the more favourable environmental conditions experienced by the seedlings grown in the microcosms, therefore resulting in more active young root biomass. Comparison of the root surface enzyme activities reported in this study with published data from other investigations is confounded

by the use of a number of different units and assay procedures. Many other workers have used acetate buffer which is known to cause leakage of enzyme and overestimate true activities as confirmed in the present study (Fig. 4.3) and by Lee (1977). The approach taken here whereby results were expressed as nmol fresh weight^{-1} was found to be more reproducible (i.e. lower coefficient of variation) than using dry weights or root length (data not shown). The root surface PME activities of *P. lanceolata* seedlings fall within the range of published values measured using a number of different species, while the activities of *A. capillaris* roots were approximately an order of magnitude greater (Table 4.1).

When grown in microcosms, the long-term N amendments significantly increased root surface PME activity of *Agrostis capillaris* seedlings on the acid soil, while a parallel effect was seen in *Plantago lanceolata* seedlings grown in the calcareous soil, but this was not significant (P>0.05). The increased PME activities in *A. capillaris* seedlings in response to long-term N additions suggests greater phosphate limitation in plants following soil N enrichment. This was clearly supported by the relationship between root surface PME activity and KCl extractable soil ammonium concentrations, which accounted for over 85 % of the variation in enzyme activity. The relationship indicated a progressive increase in PME activity with increasing soil ammonium concentrations, reaching a maximum when the ammonium concentration was approximately 75 μg N g^{-1} dwt. Above this concentration, PME activity progressively declined which may indicate a toxic effect of ammonium. Toxicitiy symptoms have been reported for *Sinapsis alba* L. seedlings subjected to 1 mM ammonia (Mehrer and Mohr, 1989), although no visible signs (e.g. stunted root growth, shoot discoloration) were apparent in the present study. The importance of ammonium over nitrate in determining root surface PME activity of *A. capillaris* reflects their concentrations in the soil; ammonium concentrations extended over a 180-fold variation between 0.67 and 124 μg g^{-1} soil dwt which contrasts markedly to those for nitrate which ranged from only 2.9 to 4.3 μg g^{-1} soil dwt (Chapter 2, Table 2.4). No relationship was found between root surface PME activity and shoot N and P concentrations or shoot N:P ratio. It would be expected that plants with low shoot P concentrations, reflecting increased demand for P, would have higher root surface phosphatase activities. In the *A. capillaris* seedlings, a decrease in shoot P concentration was observed in plants grown in the 3.5 N treatment, which was reflected by a significant increase in shoot N:P ratio. It may be possible that the differences in shoot N and P concentrations are a result of a gradient

Table 4.1. Comparison of root surface PME activities of plants measured during this study with published values.

Species	Reported activity	Activity (nmol g^{-1} fwt s^{-1})	Comments	Reference
Plantago lanceolata		1.2-35	pH 5	This study
Agrostis capillaris		155-415	pH 4.5	This study
Sitka spruce	0.14-0.27 nmol g^{-1} fwt min^{-1}	2.33-4.5	pH 4.6	Alexander and Hardy (1981)
4 ecotypes of *Aegilops peregrina*	1.38-3.95 nmol mm^{-2} h^{-1}	0.00038-0.00109†	pH 4.8, 6.4 and 7.3	Silberbush *et al.* (1981)
Deschampsia flexuosa *Festuca ovina* *Juncus squarrosus* L. *Nardus stricta*	1.39-36.7 nmol g^{-1} fwt s^{-1}	1.39-36.7	pH 4.5 Root tips	Atkinson (1983)
Rape, wheat and onion	1.94-11.38 µmol 100 mg^{-1} fwt h^{-1}	5.38-31.6	M and NM (wheat and onion) pH 5.2 Acetate buffer	Dodd *et al.* (1987)
Eriophorum vaginatum	0.2-9 nmol mm^{-2} h^{-1}	0.000055-0.0025†	pH 4 1-48°C	Kroehler and Linkins (1988)
Barley	199-2511 nmol g^{-1} fwt min^{-1}	3.33-41.85	pH 6	Lee (1988)
Clover	0.094-0.378 EU × 10^{-3}	0.00157-0.0063	pH 5.4 Acetate buffer	Tarafdar and Claassen (1988)
22 vascular epiphytes from tropical rain forest	14.9-112.8 µmol g^{-1} dwt h^{-1}	4.14-31.33‡	pH 5 21-24°C	Antibus and Lesica (1990)
Phaseolus vulgaris L.	2.7-14.5 µmol $plant^{-1}$ h^{-1}	0.084-0.428	pH 4, 5, 6	Helal (1990)
Lupinus albus *L. augustifolius* L.	5.9-34.7 mg *p*-NP g^{-1} fwt h^{-1}	0.0118-0.0693	pH 4.8 ± cluster roots	Adams and Pate (1992)

† Values expressed per mm^{-2}. A tentative conversion factor to fresh weight of ×7000 has been suggested by Alexander and Hardy (1981).

‡ Values expressed on a dry weight basis.

NM=non mycorrhizal, M=mycorrhizal

between the N treatments in the availability of phosphatase hydrolysable organic P sources (Tarafdar and Claassen, 1988). Root surface PME activity of a number of species has been found to be strongly influenced by the P status of the growth medium, since inorganic P is a potential inhibitor of the enzyme (Alexander and Hardy, 1981; Kroehler and Linkins, 1988). It would therefore be expected that the P status of the soils from the treated plots is related to root surface PME activity. Despite concentrations of extractable inorganic P being 18 % lower in the 3.5 N treated plots as compared to the control (Table 2.7), no correlation with the root surface PME activity of *A. capillaris* was found (r^2 = 0, P>0.596). However, Atkinson (1983) reported a similar effect and found that the root surface PME activity of 3 grassland species, including *Festuca ovina*, *Juncus squarrosus* and *Nardus stricta*, did not change significantly when the plants were supplied with either 0.05 or 1.06 mol P m^{-3} to their growth medium. In contrast, root surface PME activity of *Deschampsia flexuosa* was reduced by more than 50 % in the high P treatment, possibly indicating inter-species differences in the demand for P. It must also be remembered that the soil P concentrations measured in the present study represent the bulk soil and that root surface PME activity is more likely to be affected by the nutrient status of the rhizosphere. 18 months of N additions had no effect on root surface PME activity of *A. capillaris* seedlings but PME activity was significantly lower in plots that had received applications of P. Shoot N and P concentrations were not correlated with root surface PME activity. As with the long-term treatments, no correlation between PME activity and extractable soil inorganic P concentration was found (r^2 = 0, P>0.892) despite a 70 % increase in extractable P concentrations in plots that had received additions of P (Table 2.7). The lack of any short-term N treatment effect on root surface PME activity of *A. capillaris*, but a significant increase following long-term treatments supports the conclusions from Chapter 3 that in the long-term, N deposition leads to a shift from N to P as the main nutrient limiting plant growth.

The field-bioassays of root surface PME activity in the calcareous grassland were highly sensitive to long-term N treatments. Root enzyme activity of *P. lanceolata* seedlings significantly increased after just 7 days growth in November 1995 and after 11 days growth in June 1996. In contrast to the acid grassland, root surface PME activity was also stimulated in the short-term 14 N treatment. The rapidity of this adaptation when transplanted to N amended soils clearly illustrates increased P limitation to plant growth. In

addition, shoot N concentration was positively correlated (P<0.038) with root surface PME, whereas extractable inorganic soil P concentrations were negatively correlated (P<0.014) in seedlings transplanted into the long-term plots in June 1996 (Fig. 4.14a, b). This further strengthens the interrelationship occurring between enhanced N deposition and P cycling. Few studies have focused on the interactions between N and P cycling. Interrelationships between PO_4-P and NH_4-N supply and nitrification have been reported for forest soils (Pastor *et al.*, 1984) and savanna grasslands (Purchase, 1974), while Van Oorschot *et al.* (1997) found that N and P availability in a low productivity riverine system had different controlling variables. The results obtained from the bioassay provide clear evidence of plant adaptation to a system that is increasing in P limitation as a result of (simulated) enhanced aerial N deposition.

Unexpectedly, the N treatments had no effect on either root length or the number of dauciform swellings of *Carex flacca* plants despite the likely increase in P limitation. However, removal of this limitation following additions of P greatly reduced the abundance of swellings. In particular, P treatments led to significant decreases in the percentage of root comprising of young hairy and old hairy types of swelling. Although the older types of swelling were the most dominant types, it is likely that the most important functional types are those surrounded by hairs. The hairs were found to adhere strongly with soil particles and probably have a role in nutrient acquisition, as reported for proteoid cluster roots (e.g. Lamont *et al.*, 1984). The significant effects of P on these clusters indicates they may specifically be involved in P mobilisation although the exact mechanisms can only be speculated upon. Preliminary measurements undertaken in this study (data not shown) revealed no differences in root surface PME activity in dauciform and non-dauciform roots when expressed on a fresh weight basis. However, the PME activity per volume of soil in contact with the roots would be substantially greater for the hairy dauciform swellings. Other mechanisms may involve solubilisation of calcium phosphates through the secretion of organic acids, as suggested by Lamont (1974), or colonisation by P-solubilising bacteria, as reported for proteoid root clusters in waratah (*Telopea speciosissima* (Sm.) R.Br.; Wenzel *et al.*, 1994).

In the future, it would be desirable to extend the bioassay to include species representative of a range of genotypes. This would be particularly important in the calcareous grassland

which is host to a very rich flora and where N treatments have resulted in significant changes in species abundance and biomass. Furthermore, the role of VA-mycorrhizal infection in the production of root surface PME needs to be addressed since these fungi have been found to stimulate root surface and rhizosphere PME activity of wheat and onion (Dodd *et al.*, 1987). Although other studies have shown that *P. lanceolata* can be colonised very rapidly (Birch, 1988), the seedlings used in the present bioassay had virtually no mycorrhizal infection (data not shown).

CHAPTER 5

CHANGES IN MICROBIAL BIOMASS C, N AND P IN RESPONSE TO LONG-TERM INPUTS OF N AND SHORT-TERM INPUTS OF N AND P

5.1 INTRODUCTION

Soil microbial biomass comprises a diverse range of microorganisms including bacteria, fungi (saprotrophic and mycorrhizal), actinomycetes and protozoa. In terms of numbers, bacteria are generally regarded as being most abundant, with up to several billions g^{-1} soil (Paul and Clarke, 1996), while fungi typically account for the greatest biomass. The microbial biomass has a fundamental role in the decomposition of organic residues and subsequent cycling of C, N and P. One of the most important nutrient cycling processes undertaken by the microbial biomass is the breakdown of organic N and P compounds, since the productivity of most semi-natural plant communities is limited by one or both of these nutrients (Ellenberg, 1988). The net result can be either mineralisation, resulting in the release of plant available inorganic nutrients, or immobilization, whereby nutrients are held within microbial tissues. Often both processes are undertaken simultaneously by different groups of organisms in soil microsites. Microbially-driven nutrient cycling processes are of fundamental importance for the maintenance of plant productivity, particularly in semi-natural ecosystems, since unlike most agricultural systems they do not receive any fertiliser inputs of nutrients. These processes include ammonification, nitrification, denitrification and N fixation (see Section 1.3.1). A number of studies have shown that the microbial biomass is considerable in semi-natural systems with typical values of 0.5-9 mg C g^{-1} dwt for semi-natural grassland and forest soils (Anderson and Domsch, 1978; Sparling and Williams, 1986; Ross *et al.*, 1996; Anderson and Joergensen, 1997) as compared to only 0.1-1 mg C g^{-1} dwt for agricultural soils (Anderson and Domsch, 1978; Sparling *et al.*, 1981; Kaiser and Heinemeyer, 1993). This highlights the importance of microbial biomass as a sink for C. In addition, microbial biomass has further importance as a sink for N and P. Biomass P has been found to account for approximately 4 % of total organic P in arable soils and up to 24 % in long-term grasslands (Brookes *et al.*, 1984), while contributions to $NaHCO_3$ extractable P of between 4 and 76 % have been reported for New Zealand pasture soils (Sparling *et al.*, 1985a). The authors suggested that microbial P may be particularly important in organic matter enriched soils. This indicates that the amount of biomass P in heathlands and other semi-natural ecosystems, where organic P can account for 90 % or more of the total P, is also likely to be considerable. In addition, the microbial biomass is considered to be a relatively labile soil component that is susceptible to management practices and fluctuations in soil water content (Sparling *et al.*, 1985a). Phosphodiesters in particular, which are the most abundant sources of P in

microbial biomass (Kapoor and Haider, 1982) may, therefore, have an important role as a P source for plants, as demonstrated for ericaceous plants and their associated mycorrhizas (Leake and Miles, 1996; Myers and Leake, 1996).

Very little work has focused on changes to the microbial biomass resulting from chronic atmospheric inputs of N, although other anthropogenic inputs including potentially toxic elements (Bååth, 1989), sewage sludge (Chander and Brookes, 1991), large scale fertiliser additions of N (Bååth *et al.*, 1981) and atmospheric deposition of SO_2 (Wainwright, 1980) have been shown to affect the biomass. This is surprising considering the pivotal role of microorganisms in nutrient cycling and maintaining ecosystem functioning, particularly in the low productivity ecosystems that are considered to be most sensitive to enhanced N inputs. Fertiliser inputs of N often reduce microbial biomass C (C_{mic}) and soil respiration rates as reported for Swedish spruce forests (Bååth *et al.*, 1981; Söderström *et al.*, 1983), long-term pastures (Lovell *et al.*, 1995) and agricultural clay loams and sands (Kowalenko *et al.*, 1978). Such a large ionic influx may result in toxicity due to an increase in the osmotic potential of the soil solution (Broadbent, 1965) which is to be expected given the magnitude of many fertiliser applications. For example, Bååth *et al.* (1981) applied between 150 and 600 kg N ha^{-1} y^{-1} (15-60 g m^{-2} y^{-1}) of ammonium nitrate to a podsolic soil in single applications. The treatments in the present study are more comparable to those of Lovell *et al.* (1995) who applied 200 kg N ha^{-1} y^{-1} in nine equal applications during the grazing season for 10 years to a long-term grassland. They found that microbial biomass C and N both decreased in response to the treatments. Although this response was consistent throughout the year, there was an increase in microbial biomass during Spring in all treated plots.

It is likely that any changes in plant productivity would be mirrored by changes in C_{mic}, since litter and root biomass are both important sources of C for microbial biomass. Above ground biomass of upland *Calluna* heaths can be as high as 2000 g m^{-2} y^{-1} (Barclay-Estrup, 1970), with an annual litter production in the range 179-485 g m^{-2} y^{-1} (Robertson and Davies, 1965), depending on the age of the stand. Fine root biomass of dry *Calluna* heaths has been found to be in the range 303-612 g m^{-2} (Tinhout and Werger, 1988). In the acid and calcareous grasslands used in the present study, above ground biomass was approximately 200 g m^{-2} (Chapter 3). The greatest interception of the treatments is therefore likely to occur in the heathland where the vegetation canopy is the greatest. At

the heathland site, plant cover has increased in response to the treatments, while in the acid grassland cover has decreased and in the calcareous grassland no change has occurred (J. Carroll pers. comm.). It is hypothesised that changes in C_{mic} may reflect plant productivity in the grasslands.

The results from Chapters 3 and 4 provide strong evidence that the vegetation in the calcareous grassland continues to be strongly limited by P after 7 years of N treatments. In contrast the acid grassland was initially N limited, but may have moved closer towards P limitation following the N additions. Increased microbial demand for P mirrored by parallel changes in immobilisation rates as a consequence of enhanced N deposition may lead to even greater plant demand for P as the supply of mineralised orthophosphate decreases. Although there is no information addressing the effects of N deposition on microbial biomass P, fertiliser additions of N to a permanent pasture have been found to lead to commensurate increases in both biomass C and P. In contrast, additions of P substantially increased the biomass C:P ratio (Brookes *et al.*, 1984). An understanding of any changes in the proportions of N and P held in the microbial biomass is critical in determining interactions between increased N deposition and P and N cycling.

A number of techniques are available which enable the quantification of C_{mic} including fumigation incubation (FI), fumigation extraction (FE) and substrate induced respiration (SIR). Other methods give relative measurements of biomass by determining key metabolic activities such as dehydrogenase, or by extraction of compounds representative of the microbial biomass including nucleic acids, ergosterol and adenosine triphosphate (ATP). The FI method is generally regarded as a benchmark in soil biomass determinations against which many of the other methods are calibrated. Briefly, it involves the measurement of a temporary flush of respired CO_2 and extractable NH_4 in a soil sample previously fumigated with $CHCl_3$. The increase in CO_2 evolution during a 10 day incubation period is used to estimate C_{mic} (Jenkinson and Powlson, 1976a, b) and extractable NH_4 to estimate biomass N (Jenkinson, 1988; Voroney and Paul, 1984).

The SIR method involves the addition of glucose to a soil sample from which the initial (1-4 h) respiratory response is measured (Anderson and Domsch, 1978). C_{mic} determinations are based on the significant relationship obtained between maximum initial SIR rates and C_{mic} as

measured by FI. The relationship was obtained using 50 soil samples with varying chemical properties. Subsequent studies have derived similar equations by extending the range of soils or adjusting the assay conditions (e.g. temperature) and procedures (West and Sparling, 1986; Cheng and Coleman, 1989; Heinemeyer *et al.*, 1989; Kaiser *et al.*, 1992).

The FE method uses the difference in the quantity of extractable microbial constituents from fumigated and non-fumigated soils as an index of C_{mic} (Vance *et al.*, 1987), N (Brookes *et al.*, 1985) and P (Brookes *et al.*, 1987). All of the methods outlined above have potential advantages and disadvantages. The SIR method is particularly advantageous since it allows for the simultaneous determination of basal respiration rates (Sparling *et al.*, 1981), C_{mic} (Anderson and Domsch, 1978), metabolic quotient (Pirt, 1975) and glucose induced respiration rates. The metabolic quotient (the ratio of basal respiration:C_{mic}) has been shown to be a sensitive indicator of environmental stress on soil microbial communities (Anderson and Domsch, 1993). In addition, SIR-biomass is derived from actual measurements of microbial activity. In contrast, the FE method does not measure microbial activity but is dependent on extraction of microbial constituents. This can present problems in that only a proportion of the extracted C, N or P is actually of microbial origin. The proportion of non-microbial C, N or P is accounted for by the application of k_x values, which have been derived by a number of workers. However, the application of both the FI and FE methods to soils in which organic compounds contribute markedly to the total N and P concentrations can also result in erroneous values, particularly for estimates of biomass N and P, due to breakdown of non-microbial material following fumigation (Couteaux *et al.*, 1990). In the present study, this may be especially problematic for the heathland soil which has a high organic matter content (Table 2.1) but is also likely to contain large concentrations of lipidic compounds which may be particularly sensitive to the chloroform treatments. However, no other method exists which attempts to quantify microbial N and P. Given the importance of this measurement, the FE has been applied to the three field sites, but the results are interpreted with a great degree of caution. In particular, no emphasis is placed on making comparisons with other studies. Despite these potential problems, intra-site comparisons are likely to be more valid. The development of reliable methods for determination microbial N and P in highly organic soils certainly warrents further research.

A pragmatic approach was therefore taken in this study whereby the SIR method was applied to all the soils for measurements of C_{mic}. Biomass C, N and P were measured using FE. Although the FE data are described as microbial biomass, it is recognised that they may include additional non-microbial constituents.

5.1.1 Aims

This chapter aims to investigate the effects of long-term N treatments on microbial biomass C, N and P. Measurements of C_{mic} were made by SIR in the F and H horizons of the heathland, the F and Ah horizons of the acid grassland and the Ah horizon of the calcareous grassland. Biomass C, N and P were also measured by FE in the F horizon of the heathland and Ah horizons of the long-term acid and calcareous grassland plots. Only FE-biomass C and P were measured in the short-term acid grassland plots.

5.2 MATERIALS AND METHODS

5.2.1 Determination of respiration rates

i) Basal respiration

Basal respiration is defined as the CO_2-C that is normally evolved from soils which have had no substrate amendment (Sparling *et al.*, 1981). Measurements of basal respiration were undertaken in August 1995 using soil removed from the F and H horizons of the heathland, the F and Ah horizons of the acid grassland and the Ah horizon of the calcareous grassland, and in August 1997 using soil removed from the F horizon of the heathland and the Ah horizons of the acid and calcareous grasslands. All samples were stored and prepared as described in Section 2.2.1 and had their moisture contents adjusted to 60 % by adding up to 1 ml of de-ionised water to 1 g soil (dwt). Respiration (oxygen consumption) was measured at 22°C in 15 minute intervals for up to 5 h with an automated Merit 20 manometric electrolytic respirometer (E. R. Addington Engineers Ltd.). Oxygen consumption ($\mu l\ h^{-1}$) was converted to CO_2-C evolution ($\mu g\ CO_2$-C g^{-1} soil dwt h^{-1}) using the following equation:

$$\mu g\ CO_2\text{-}C = \mu l\ O_2 \times 16.5 \qquad \text{(Equation 5.1)}$$

ii) Substrate induced respiration (SIR) for determination of biomass C

Determinations of C_{mic} by SIR were undertaken in parallel with the basal respiration measurements. The optimal rate of glucose amendment, determined following addition of between 1 and 50 mg glucose g^{-1} soil dwt, was found to be 5 mg glucose g^{-1} soil dwt. for all of the soils, a value similar to that obtained by a number of other studies (e.g. Sparling *et al.*, 1981). One of the main problems with the original SIR method (Anderson and Domsch, 1978) was ensuring that the glucose was fully dispersed within the soil sample. In the present study, this was overcome by adding glucose in solution. The solution was added so each soil sample had an equal moisture content (60 %) sufficient to remove any potential water limitation (West and Sparling, 1986). Substrate induced respiration was measured as described in Section 5.2.1i but the oxygen consumption was converted to CO_2 evolution rather than CO_2-C. Basal respiration was subtracted from these values in order to differentiate between the CO_2 normally respired by the soil and that evolved as a consequence of glucose metabolism. C_{mic} was then calculated using the equation:

$$x = 40.04y + 0.37$$

(only valid when the incubation temperature is 22 ± 0.5 °C)

where:

x = mg C_{mic} g^{-1} soil

y = ml CO_2 g^{-1} soil h^{-1}

iii) Metabolic quotient

The metabolic quotient (qCO_2 or specific respiration rate) was determined using the following equation:

$$qCO_2\ (\mu g\ CO_2\text{-}C\ g^{-1}\ C_{mic}\ h^{-1}) = \frac{\text{basal respiration } (\mu g\ CO_2\text{-}C\ g^{-1}\ \text{soil})}{C_{mic}\ (\text{mg C}\ g^{-1}\ \text{soil})}$$

iv) Glucose induced respiration rates

The maximum respiration rates in response to glucose amendments during incubations of 5 h duration were calculated as described in equation 5.1.

5.2.2 Fumigation extraction (FE)

All FE experiments were undertaken using soil samples removed during May 1997 from the F horizon of the heathland and the Ah horizon of the acid grassland and during June from the Ah horizon of the calcareous grassland.

i) Biomass C

C_{mic} is determined by the difference in the amount of extractable total organic carbon (TOC) in an unfumigated and a fumigated soil sample multiplied by a calibration factor, K_{ec} (Vance *et al.*, 1987). Fumigation is achieved by boiling chloroform with one set of the soil samples in a vacuum desiccator. In the original method, approximately 50 ml chloroform was added to a separate beaker which was placed within the desiccator. However, preliminary studies showed that addition of chloroform directly into the vials aided fumigation efficiency. Duplicate 2 g (dwt) soil samples (prepared as described in Section 2.2.1) were placed in 20 ml universal vials. To each vial of the fumigated soil was added 0.5 ml ethanol-free $CHCl_3$, which had previously been washed 6 times with distilled water to remove any residual C. The desiccator was lined with moist paper towels (to maintain humidity), evacuated until the $CHCl_3$ had boiled for 5 minutes, covered with a black plastic sack and incubated for 24 h. The duplicate set of vials were also kept in darkness for 24 h. After fumigation, the desiccator was evacuated 6 times and left open in a fume cupboard for 20 mins to ensure removal of any residual chloroform. Each vial was shaken (side-to-side) for 45 min with 20 ml 0.5 M K_2SO_4, the soil suspensions were then vacuum filtered (Whatman No 1.) and frozen (-20 °C) prior to analysis of TOC by sodium persulfate digestion/UV absorption (LabTOC, Pollution and Process Monitoring Ltd.). A set of blank filtrates were run for both fumigated and unfumigated samples. The FE method has been found to be unreliable indicator of C_{mic} in organic matter enriched soils (40 % organic carbon) that are very wet (75 % water content; Couteaux *et al.*, 1990). The authors suggested the high water content of the soil protected the microbial cells owing to the low solubility of chloroform and so in the present study, the moisture content of all the soils was not higher than 45 %.

The concentration of TOC in the soil extracts was converted to C_{mic} using the following equation:

$$C_{mic} = (C_f - C_{uf})/K_{ec}$$

where

C_f = C in the fumigated extract

C_{uf} = C in the unfumigated extract

K_{ec} = is the proportion of the microbial C that is extracted from the soil (0.38; Vance *et al*, 1987).

ii) Biomass N

Biomass N was determined in the same extracts as for C_{mic} (Brookes *et al.*, 1985), but was not measured in the short-term acid grassland plots. The extracts were digested as described in Section 2.2.2 and NH_4^+ measured colorimetrically (Cecil 2010; Scheiner, 1976). Biomass N was determined using the following equation:

$$\text{Biomass N} = (N_f - N_{uf})/K_{en}$$

where

N_f = N in the fumigated extract

N_{uf} = N in the unfumigated extract

K_{en} = is the proportion of the microbial N that is extracted from the soil (0.54; Brookes *et al*, 1985).

Biomass N was also expressed as a percentage of C_{mic}:

$$\% \text{ biomass N} = \text{biomass N} \times \frac{C_{mic}}{100} \qquad \text{(equation 5.2)}$$

iii) Biomass P

The determination of biomass P uses the same fumigation procedure as for biomass C and N. Inorganic P (P_i) is extracted with 0.5 M $NaHCO_3$ adjusted to pH 8.5. Correction for absorption of P_i onto soil organic matter was accounted for by addition of 0.25 ml 80 μg P_i ml^{-1} to a duplicate set of samples prior to extraction (Brookes *et al.*, 1982). P_i in the extracts was determined colorimetrically by autoanalysis (Techator FIAStar 5010 auto-analyser). Biomass P (μg P_i g^{-1} soil dwt) was calculated using the following equation:

$$\text{Biomass P} = \frac{20(P_f - P_{uf})}{K_{ep}(P_{ufp} - P_{uf})}$$

where

$P_f = P_i$ (μg P_i g^{-1} soil dwt) in the fumigated extract

$P_{uf} = P_i$ (μg P_i g^{-1} soil dwt) in the unfumigated extract

$P_{ufp} = P_i$ (μg P_i g^{-1} soil dwt) in the unfumigated extracted spike with 20 μg P_i g^{-1}soil dwt

K_{ep} = is the proportion of the microbial P that is extracted from the soil (0.4; Brookes *et al.*, 1982).

Biomass P was also expressed as a percentage of C_{mic} in accordance with equation 5.2.

5.3 RESULTS

5.3.1 Heathland

During August 1995, SIR-C_{mic} ranged from 13.4 to 18.7 mg C g^{-1} soil dwt in the F horizon and from 7.5 to 11.1 mg C g^{-1} soil dwt in the H horizon (Fig. 5.1a). Repeated measurements of C_{mic} in the F horizon during August 1997 gave constantly lower values. SIR-biomass C ranged from just 1.8 to 2.7 mg C g^{-1} soil dwt while FE-biomass C ranged from 2.7 to 3.6 mg C g^{-1} soil dwt (Fig. 5.1b). However, patterns of response in C_{mic} to the N treatments was similar for both years. During 1995, SIR-biomass C increased in all of the treated plots, although this was only significant ($P<0.05$) in response to the 8 N and 12 N treatments in the F horizon (Fig. 5.1a) where C_{mic} increased by 40 % and 34 % respectively. The same pattern was observed in 1997 where SIR-biomass C increased significantly by 47 % in the F horizon of the 8 N treated plots (12 N not analysed). Although not significant ($P>0.05$), FE-biomass C increased in response to all levels of N addition (Fig. 5.1b). The greatest change occurred in the 4 N treatment where C_{mic} increased by 33 % as compared to the control.

Basal respiration measured in 1995 ranged from 787 to 922 μg CO_2-C g^{-1} soil in the F horizon and from 426 to 695 μg CO_2-C g^{-1} soil in the H horizon (Table 5.1). Basal respiration was not affected by the N treatments and did not show any parallels between the 1995 SIR-biomass C values in either the F or the H horizons. As with the C_{mic} values, basal

respiration rates were considerably lower in 1997 than in 1995 and ranged from 12 to 92 μg CO_2-C g^{-1} soil h^{-1} (Table 5.1). In contrast to 1997 SIR and FE-C_{mic}, basal respiration decreased in response to the N treatments. This was most apparent in the 8 N treatment where the respiration rates were 87 % lower. The response of the microbial biomass to additions of glucose closely mirrored C_{mic} in both the F and H horizons and in each year (Table 5.1). The 1995 glucose induced respiration rates ranged from 4.8 to 6.4 mg CO_2-C g^{-1} soil h^{-1} in the F horizon and from 2.8 to 3.7 mg CO_2-C g^{-1} soil h^{-1} in the H horizon while in 1997 they were an order of magnitude lower and ranged from 0.47 to 0.68 mg CO_2-C g^{-1} h^{-1} (Table 5.1). Significant increases (P<0.05) were only observed in the F horizon in response to the 8 and 12 N treatments.

The metabolic quotient (qCO_2-C) was significantly (P<0.05) lower in the 8 and 12 N treatments as compared to the control during both 1995 and 1997 (Table 5.1). The most striking effect occurred in 1997 when qCO_2-C fell by 92 % in response to the 8 N treatment.

Microbial biomass N ranged from approximately 600 to 1070 μg g^{-1} soil in the F horizon during 1997 (Fig. 5.2a). Significant increases were observed at all treatment levels (P<0.05) where biomass N increased progressively from the control by 39, 52 and 76 % with each added increment of N (Fig. 5.2a). Biomass N expressed as a percentage of C_{mic} ranged between 15 and 20 % but was not significantly (P>0.05) affected by any of the treatments (Fig. 5.2b). The quantity of biomass P was similar to biomass N and ranged from 760 to 920 μg g^{-1} soil (Fig. 5.2a), while biomass P as a percentage of C_{mic} ranged between 27 and 32 %. Neither the quantity or the proportion P within C_{mic} were affected by any of the N treatments (P>0.05).

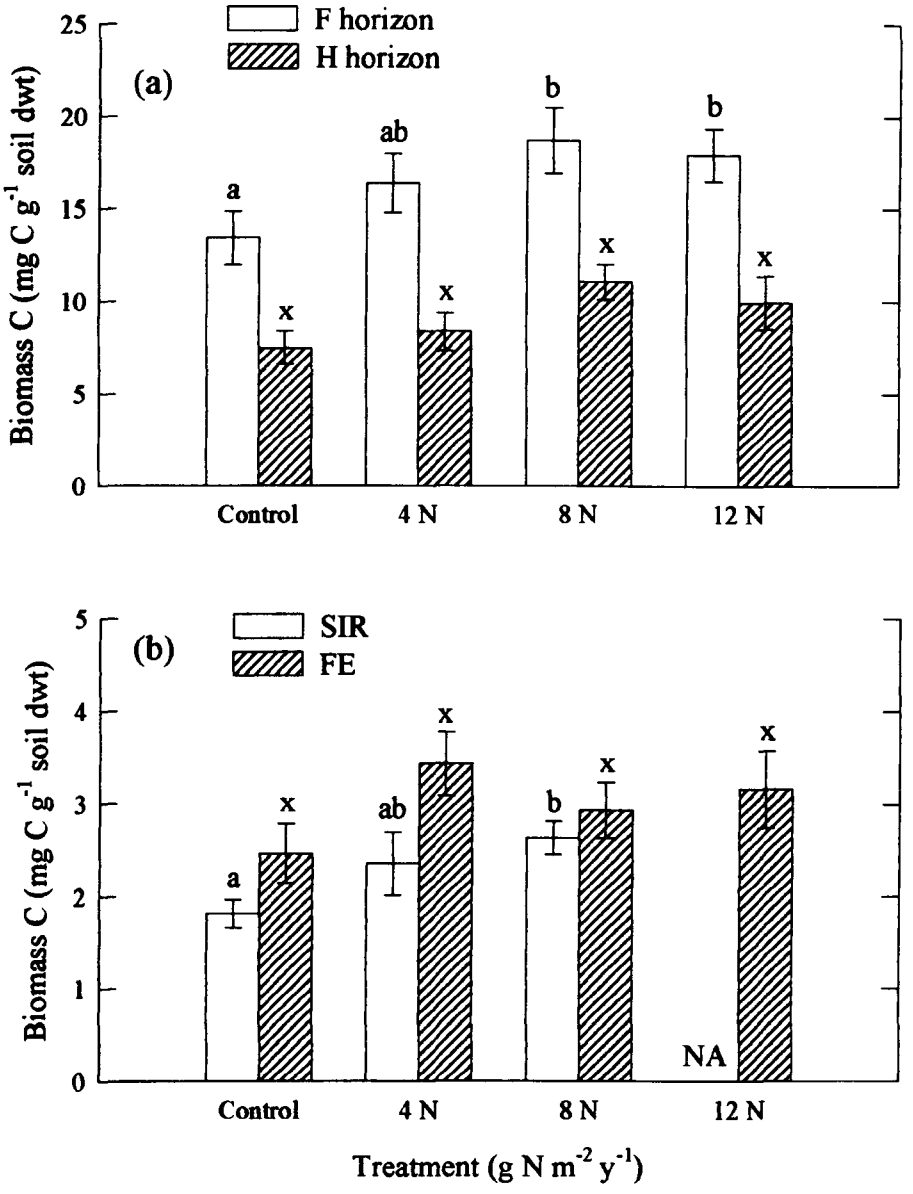


Fig. 5.1. Microbial biomass C (C_{mic}) in a heathland soil measured in (a) the F and H horizons during August 1995 by SIR following 6 years of N treatments and (b) the F horizon during August 1997 by SIR and FE following 8 years of N treatments (± SEM). Bars sharing a letter are not significantly different ($P>0.05$). NA = Not analysed. Treatment codes: Control = water only, 4, 8 and 12 N = 4, 8 or 12 g N m^{-2} y^{-1} (as ammonium nitrate).

Table 5.1. Glucose induced and basal respiration rates and metabolic quotient (qCO_2-C) in the F and H horizons of a heathland soil that has received up to 8 years N treatments (± SEM). NA = Not analysed. Values sharing a letter are not significantly different (P>0.05).

	F horizon					
	Basal respiration ($\mu g\ CO_2\text{-}C\ g^{-1}\ dwt\ h^{-1}$)		Glucose induced respiration ($mg\ CO_2\text{-}C\ g^{-1}\ dwt\ h^{-1}$)		qCO_2-C ($mg\ CO_2\text{-}C\ g^{-1}\ C_{mic}\ h^{-1}$)	
Treatment	1995	1997	1995	1997	1995	1997
Control	786.6^a (± 90.6)	92.40^a (± 28.89)	4.81^a (± 0.49)	0.472^a (± 0.053)	61.83^a (± 6.74)	41.89^a (± 15.71)
4 N	921.7^a (± 133.4)	50.60^a (± 18.35)	5.84^{ab} (± 0.59)	0.629^a (± 0.104)	55.77^{ab} (± 5.45)	21.78^a (± 6.00)
8 N	758.7^a (± 106.2)	12.10^b (± 9.34)	6.36^b (± 0.55)	0.680^a (± 0.057)	44.35^b (± 6.12)	3.74^b (±2.66)
12 N	821.7^a (± 156.8)	NA	6.20^b (± 0.49)	NA	47.42^b (± 7.60)	NA
	H horizon					
Control	516.2^a (± 70.2)	NA	2.75^a (± 0.32)	NA	69.67^a (± 6.90)	NA
4 N	509.0^a (± 72.6)	NA	3.01^a (± 0.34)	NA	66.21^a (± 11.54)	NA
8 N	426.1^a (± 71.0)	NA	3.75^a (± 0.31)	NA	40.77^b (± 5.83)	NA
12 N	695.1^a (± 151.3)	NA	3.67^a (± 0.54)	NA	75.61^a (± 16.49)	NA

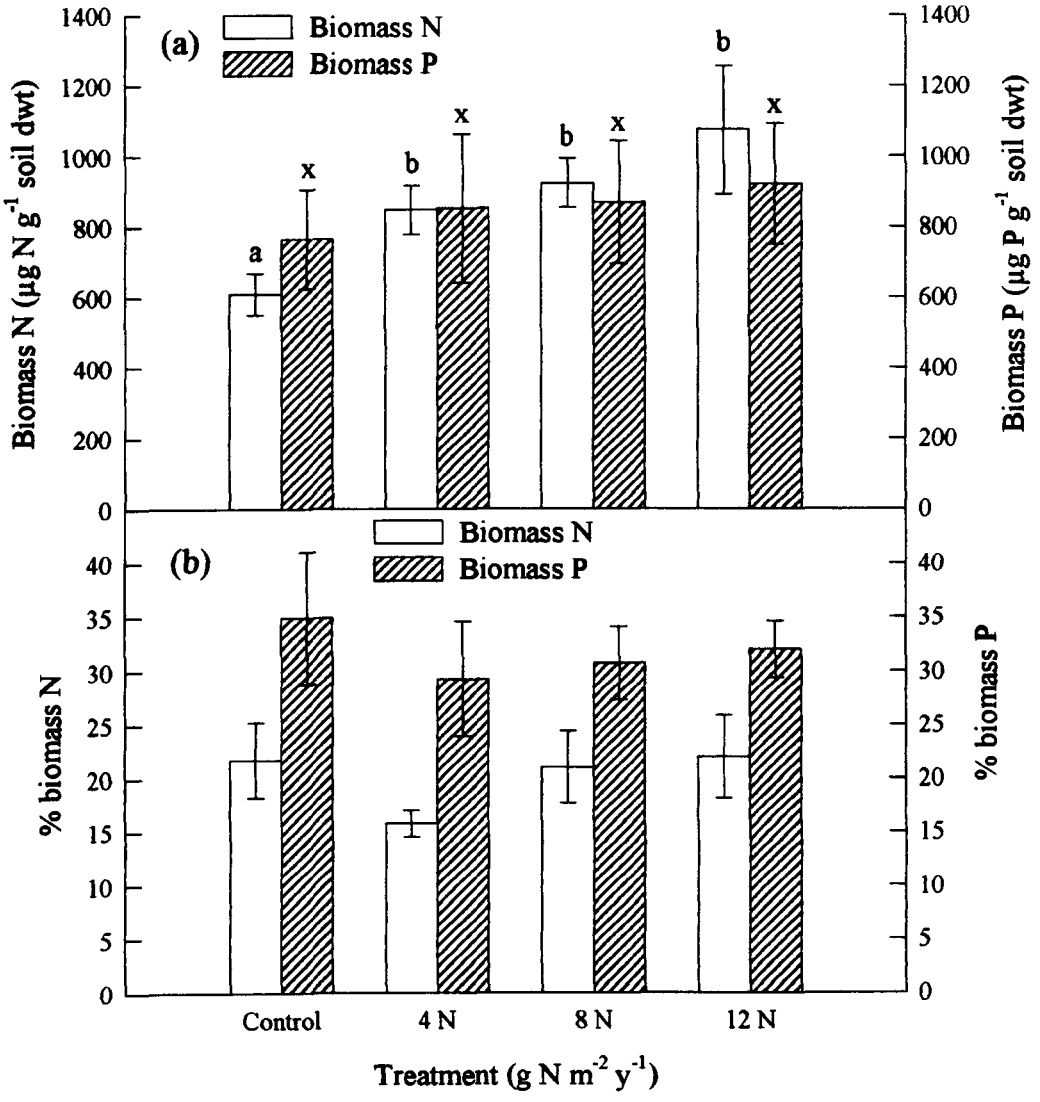


Fig. 5.2. Changes in (a) microbial biomass N and P (bars sharing a letter are not significantly different; $P>0.05$) and (b) the percentage N and P held in the microbial biomass in a heathland soil (bars are not significantly different; $P>0.05$) that has received 8 years of nitrogen treatments (± SEM). Treatment codes as for Fig. 5.1. % biomass N or P calculated according to equation 5.2.

5.3.2 Acid grassland

i) Effect of 7 years N treatments

During August 1995, SIR-C_{mic} in the F horizon of the long-term acid grassland plots ranged from 22 to 29 mg C g^{-1} soil but was not affected by any of the N treatments (Fig. 5.3a). In the Ah horizon, C_{mic} ranged from 8.2 to 15.7 mg C g^{-1} (Fig. 5.3a) and was consistently reduced by approximately 40 % in the N treated plots. This effect was significant ($P<0.05$) only in the 14 N AS treatment where C_{mic} was 48 % lower than in the control. A similar response to the N additions was seen in FE-C_{mic} during May 1997 where it was significantly ($P<0.05$) reduced by between 39 and 47 % in the 3.5, 7 and 14 N treatments and by 63 % in the 14 N AS treatment (Fig. 5.3b). However, the quantities of C_{mic} were approximately an order of magnitude lower than the 1995 SIR-C_{mic} values. Basal respiration ranged from 1076 to 1274 μg CO_2-C g^{-1} soil h^{-1} in the F horizon and from 372 to 528 μg CO_2-C g^{-1} soil h^{-1} in the Ah horizon (Table 5.2) but was not affected by the N treatments. Similarly, no significant N treatment effects were seen on the glucose induced respiration rates although the responses to the treatments mirrored those observed for C_{mic}. The metabolic quotients were similar for both the F and Ah horizons ranging from 2.5 to 59.4 mg CO_2-C g^{-1} C_{mic} h^{-1} in the former to 44.2 to 66.8 mg CO_2-C g^{-1} C_{mic} h^{-1} in the latter (Table 5.2). Significant treatment effects ($P<0.05$) only occurred in the 7 N treatment in the Ah horizon where qCO_2-C was 61 % higher.

Biomass N as a percentage C_{mic} was approximately half that of the heathland soil, and ranged from 170 to 260 μg g^{-1} soil (Fig. 5.4a). No significant treatment effects were observed with respect to the control, although biomass N increased significantly ($P<0.05$) by over 50 % in the 14 N treatment compared with the 7 N treatment. The percentage N in the microbial biomass in control soil was 23 %, a value also similar to the heathland (Fig. 5.4b). This increased significantly ($P<0.05$) to 43 % in the 14 N and 45 % in the 14 N AS treatments. Microbial biomass P tended to mirror the response of C_{mic} to the N treatments (Fig. 5.3a, b) ranging from 155 μg g^{-1} soil in the control to 80 μg g^{-1} soil in the 14 N AS treatment (Fig. 5.4a). All of the treatments consistently reduced biomass P by approximately 45 %, although these reductions were only significant ($P<0.05$) in the 14 N and 14 N AS treatments which both resulted in a decrease of 48 %. Biomass P as a percentage C_{mic} was somewhat lower than the heathland and remained at a consistent level

of approximately 16 % in all treatments, although there was a suggestion of a stimulatory effect in the 14 N AS treatment where it increased to 23 % (Fig. 5.4b).

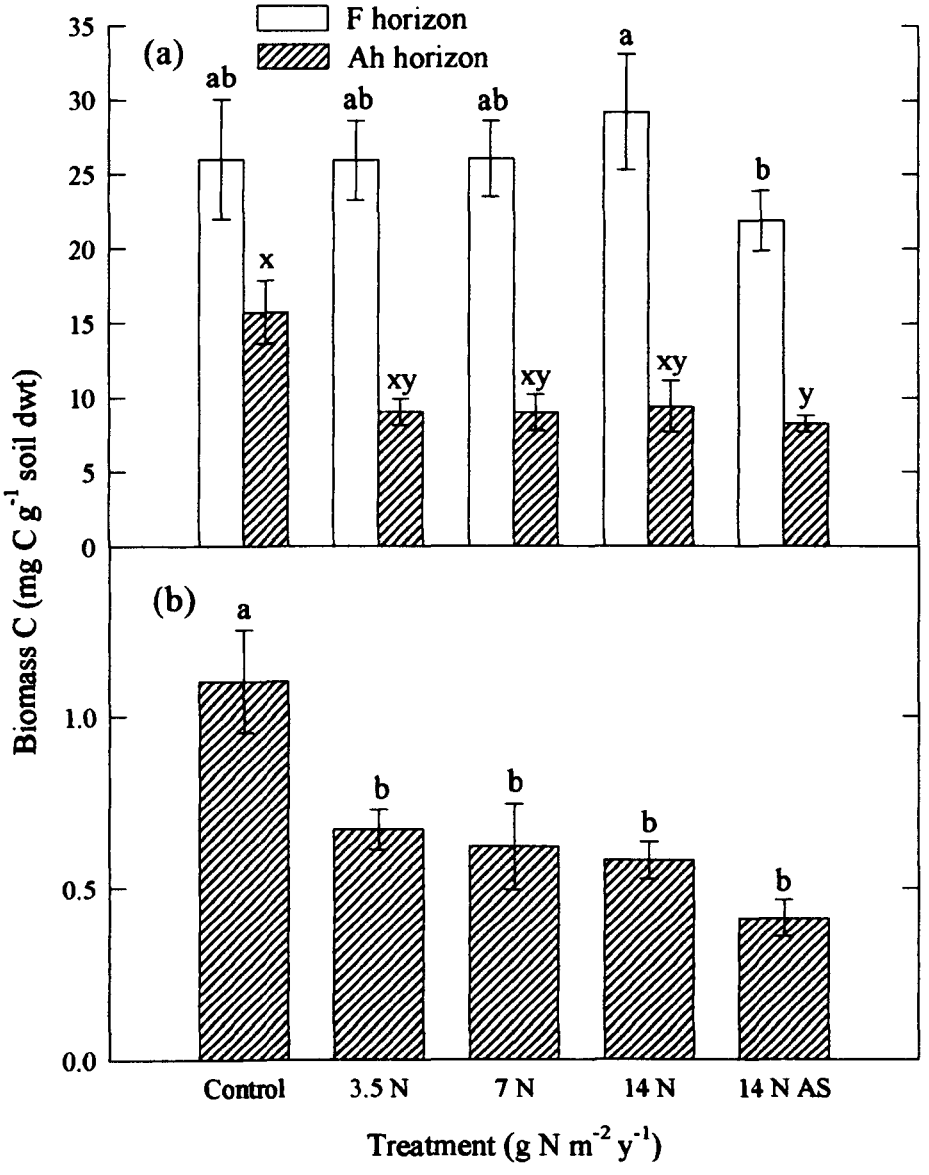


Fig. 5.3. Microbial biomass C (C_{mic}) in an acid grassland soil measured in (a) the F and Ah horizons during August 1995 by SIR following 5 years of N treatments and (b) the Ah horizon during May 1997 by FE following 7 years of N treatments (± SEM). Bars sharing a letter are not significantly different (P>0.05). Treatment codes: Control = water only, 3.5, 7 and 14 N = 3.5, 7 or 14 g N m^{-2} y^{-1} (as ammonium nitrate) and 14 N AS =14 g N m^{-2} y^{-1} (as ammonium sulphate).

Table 5.2. Glucose induced and basal respiration rates and metabolic quotient (qCO_2-C) in the Ah and F horizons of an acid grassland soil that has received 5 years N treatments (± SEM). Values sharing a letter are not significantly different (P>0.05).

	F horizon		
Treatment	Basal respiration ($\mu g\ CO_2\text{-}C\ g^{-1}\ dwt\ h^{-1}$)	Glucose induced respiration ($mg\ CO_2\text{-}C\ g^{-1}\ dwt\ h^{-1}$)	qCO_2-C ($mg\ CO_2\text{-}C\ g^{-1}\ C_{mic}\ h^{-1}$)
Control	1224^{a} (± 270)	9.01^{a} (± 1.41)	46.02^{a} (± 5.37)
3.5 N	1076^{a} (± 132)	8.85^{a} (± 0.89)	43.53^{a} (± 4.12)
7 N	1108^{a} (± 79)	8.91^{a} (± 8.27)	44.43^{a} (± 2.59)
14 N	1274^{a} (± 153)	10.01^{a} (± 1.28)	42.54^{a} (± 3.71)
14 N AS	1180^{a} (± 92)	7.73^{a} (± 0.60)	59.38^{a} (± 3.37)
	Ah horizon		
Control	498.0^{a} (± 85.7)	4.57^{a} (± 0.73)	41.62^{a} (± 5.10)
3.5 N	425.6^{a} (± 58.8)	3.12^{a} (± 0.31)	48.65^{ab} (± 6.59)
7 N	528.1^{a} (± 53.6)	3.22^{a} (± 0.39)	66.78^{b} (± 7.40)
14 N	442.3^{a} (± 70.1)	3.18^{a} (± 0.54)	60.30^{ab} (± 9.86)
14 N AS	372.4^{a} (± 57.8)	2.84^{a} (± 0.20)	44.20^{a} (± 6.20)

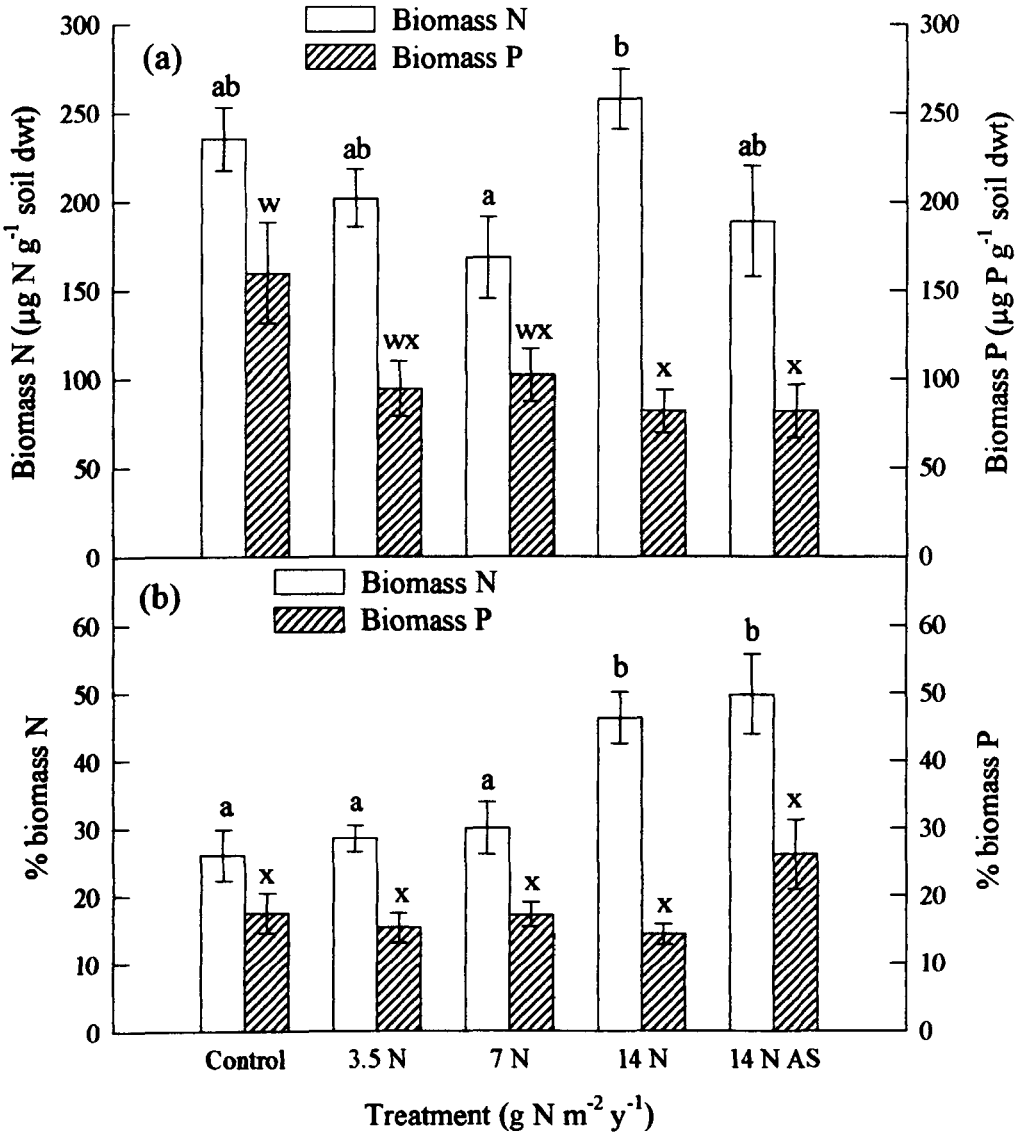


Fig. 5.4. Changes in (a) microbial biomass N and P and (b) the percentage N and P held in the microbial biomass in the Ah horizon of an acid grassland soil that has received 7 years of nitrogen treatments (± SEM). Bars sharing a letter are not significantly different ($P>0.05$). Treatment codes as for Fig. 5.3. % biomass N or P calculated according to equation 5.2.

ii) Effect of 18 months N and P treatments

In the 18 month acid grassland plots, C_{mic} was approximately 3 fold that of the long-term plots and ranged from 5.0 to 6.6 mg C g^{-1} soil (Fig. 5.5a). The effect of N additions alone appeared to be stimulatory. In the 3.5 N treatment C_{mic} increased by 12 % while the 14 N treatment significantly increased ($P<0.05$) C_{mic} by 33 %. However, when applied in combination with 3.5 g P m^{-2} y^{-1}, C_{mic} decreased significantly ($P<0.05$) compared to the 3.5 N and 14 N treatments to levels approximately 30 % lower than the control (Fig. 5.5a). The quantity of biomass P was similar to the values obtained in the long-term plots ranging from 128 to 269 μg g^{-1} soil (Fig. 5.5a). Likewise, the response to the N and P treatments was similar to that of C_{mic}. The greatest effect was observed in the 3.5 P treatment where biomass P increased significantly ($P<0.05$) by 100 %. This response was removed when N was applied in combination with P causing biomass P to fall to control levels. Biomass P increased slightly by 24 % in response to the 14 N treatment, although this was not significant. Biomass P as a percentage C_{mic} was considerably lower than in the long-term plots and ranged from 2.5 to 4.5 % (Fig. 5.5b). The overall effect of P additions was to significantly increase the percentage P in the microbial biomass, while additions of N had no effect (Fig. 5.5c).

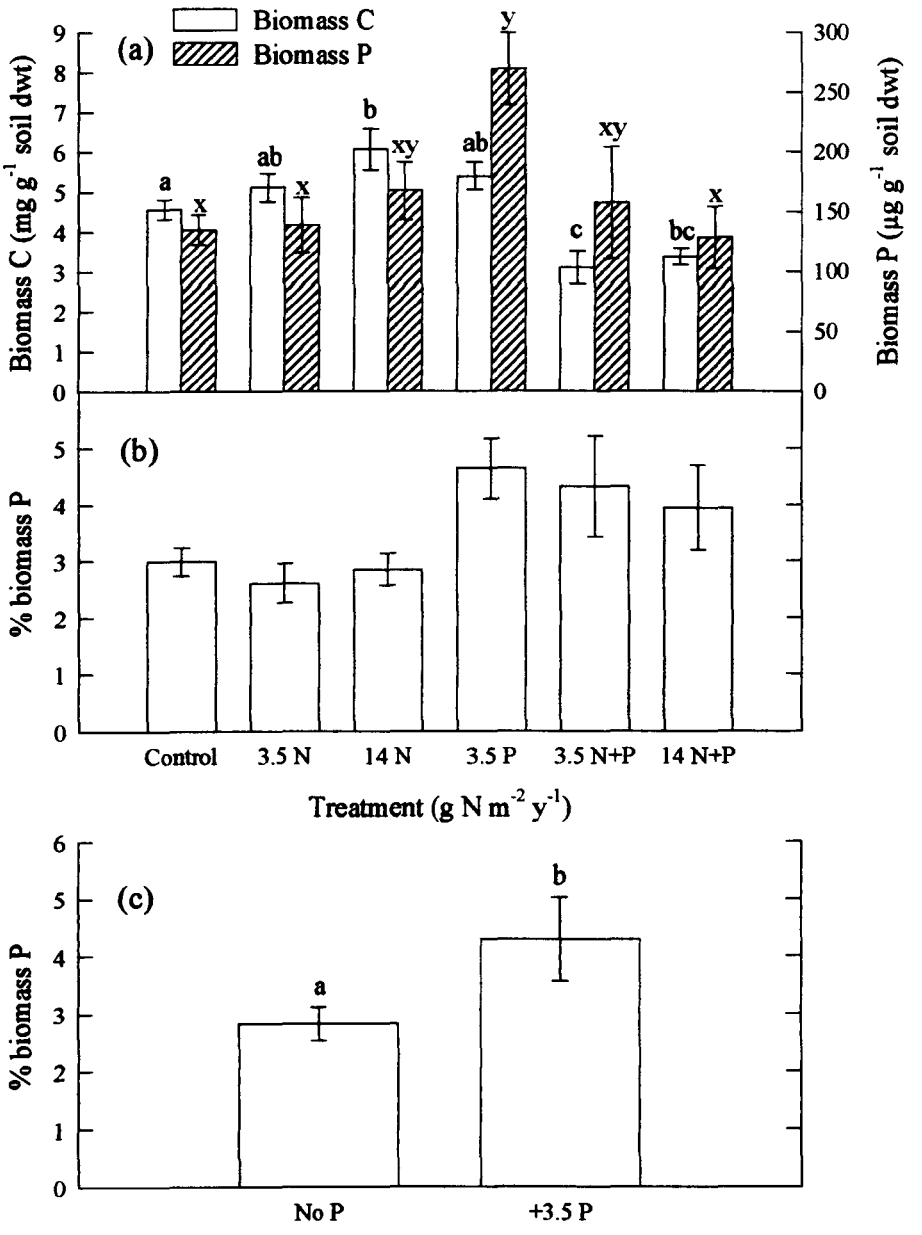


Fig. 5.5. Changes in (a) microbial biomass C (C_{mic}) and P, (b) the percentage P in the microbial biomass in response to 18 months N and P treatments (not significantly different; $P>0.05$) and (c) the overall effect of the P treatments (0 or 3.5 g P m^{-2} y^{-1}) on the percentage P in the microbial biomass in an acid grassland soil in May 1997 (± SEM). Bars sharing a letter are not significantly different ($P>0.05$). Treatment codes (m^{-2} y^{-1}): Control = 0 g N, 3.5 N = 3.5 g N, 14 N = 14 g N, 3.5 P = 3.5 g P, 3.5 N+P = 3.5 g N + 3.5 g P, 14 g N+P = 14 g N + 3.5 g P. % biomass P calculated according to equation 5.2.

5.3.3 Calcareous grassland

SIR-C_{mic} ranged from 4.9 to 6.7 mg C g^{-1} soil during August 1995 and from 1.4 to 2.1 mg C g^{-1} soil during August 1997 (Fig. 5.6a), while FE-C_{mic} ranged from 3.4 to 4.4 mg C g^{-1} (Fig. 5.6b). The N treatments had similar effects on SIR-C_{mic} irrespective of the year (Fig. 5.6a). The 14 N treatment decreased C_{mic} by 26 % in 1995 and by 29 % in 1997, although the decrease was only significant ($P<0.05$) in the latter year, while the 14 N AS treatment had no effect. In contrast, FE-C_{mic} was not affected by additions of ammonium nitrate but instead increased significantly ($P<0.05$) in the 14 N AS treatments (Fig. 5.6b). Basal respiration was considerably lower in the calcareous grassland as compared to the acid grassland and ranged from 103 to 177 μg CO_2-C g^{-1} soil h^{-1} in 1995 and from 138 to 158 μg CO_2-C g^{-1} soil h^{-1} in 1997 (Table 5.3). Basal respiration was not affected by any of the N treatments. The effects of the N treatments on glucose induced respiration rates were mirrored by the SIR-C_{mic} responses. However, in contrast to the 1995 SIR-C_{mic}, the glucose induced respiration rate was significantly lower ($P<0.05$) in the 14 N treated plots (Table 5.3). The metabolic quotient was considerably lower in 1995 than in 1997 and ranged from 21 to 31 mg CO_2-C g^{-1} C_{mic} h^{-1} in the former and from 80 to 125 mg CO_2-C g^{-1} C_{mic} h^{-1} in the latter (Table 5.3). Significant ($P<0.05$) stimulatory effects were only observed in response to the 14 N treatment which increased qCO_2-C by 55 %. Biomass N ranged from 323 to 349 μg g^{-1} soil and was not affected by any of the treatments (Fig. 5.7a). Similarly, no changes were seen in the percentage N held in the microbial biomass, which consistently accounted for approximately 10 % of C_{mic} in each treatment (Fig. 5.7b). Biomass P ranged from 90 to 260 μg g^{-1} soil but, in contrast, was significantly ($P<0.01$) lower with each added increment of N (Fig. 5.7a). This effect was also reflected in the percentage P held in the microbial biomass which decreased significantly ($P<0.01$) from 8 % in the control to 2 % in the 14 N AS treatment (Fig. 5.7b).

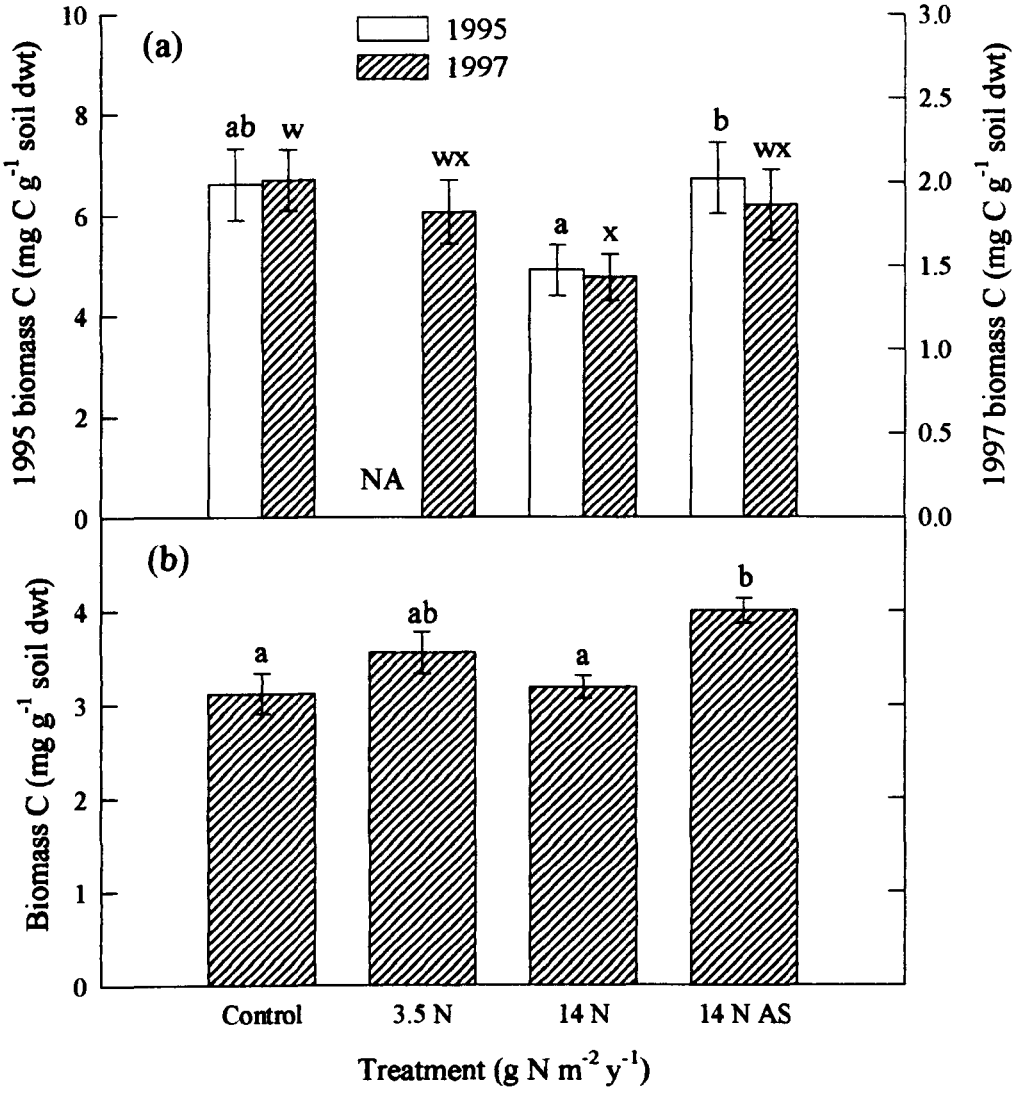


Fig. 5.6. Microbial biomass C (C_{mic}) in a calcareous grassland soil measured (a) by SIR in the Ah horizon during August 1995 and 1997 following 5 and 7 years of N treatments respectively, and (b) by FE in the Ah horizon during June 1997 (± SEM). Bars sharing a letter are not significantly different (P>0.05). Treatment codes as for Fig. 5.3. NA = not analysed.

Table 5.3. Glucose induced and basal respiration rates and metabolic quotient (qCO_2-C) in the Ah horizon of a calcareous grassland soil that has received up to 7 years N treatments (± SEM). NA = Not analysed. Values sharing a letter are not significantly different (P>0.05).

Treatment	Basal respiration ($\mu g\ CO_2\text{-}C\ g^{-1}\ dwt\ h^{-1}$)		Glucose induced respiration ($mg\ CO_2\text{-}C\ g^{-1}\ dwt\ h^{-1}$)		qCO_2-C ($mg\ CO_2\text{-}C\ g^{-1}\ C_{mic}\ h^{-1}$)	
	1995	1997	1995	1997	1995	1997
Control	126.6^{a} (± 25.5)	151.3^{a} (± 12.7)	2.11^{a} (± 0.21)	0.614^{a} (± 0.043)	21.14^{a} (± 4.42)	79.88^{a} (± 10.14)
3.5 N	NA	137.5^{a} (± 10.3)	NA	0.543^{ab} (± 0.059)	NA	80.29^{a} (± 8.58)
14 N	103.4^{a} (± 40.7)	157.9^{a} (± 10.1)	1.57^{b} (± 0.15)	0.441^{a} (± 0.033)	23.21^{a} (± 9.76)	124.6^{b} (± 22.4)
14 N AS	177.0^{a} (± 36.5)	158.4^{a} (± 7.9)	2.20^{a} (± 0.20)	0.555^{ab} (± 0.065)	31.11^{a} (± 7.95)	92.06^{ab} (± 11.58)

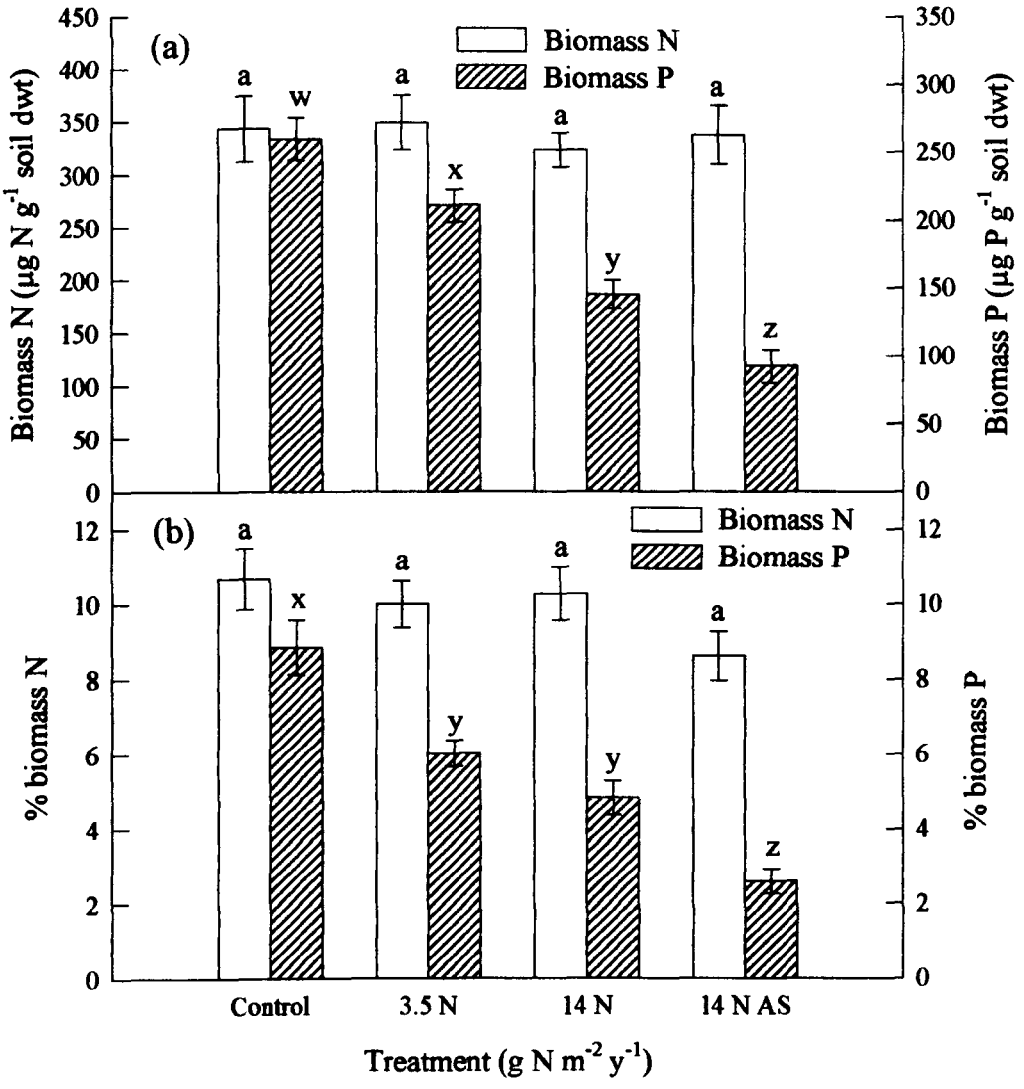


Fig. 5.7. Changes in (a) microbial biomass N and P and (b) the percentage N and P held in the microbial biomass in a calcareous grassland soil that has received 7 years of nitrogen treatments (± SEM). Bars sharing a letter are not significantly different (P>0.01). Treatment codes as for Fig. 5.3. % biomass N or P calculated according to equation 5.2.

5.4 DISCUSSION

5.4.1 Methodologies

Determinations of microbial biomass C, N and P provide information fundamental to the understanding of nutrient cycling in soil:plant systems. However, the measurement of these parameters is often not straight forward. There is a wealth of information addressing the effectiveness of a number of different methodologies for measurements of microbial biomass (e.g. Beck *et al.*, 1997). The SIR and fumigation techniques are likely to give different values since they measure contrasting groups of organisms, i.e. glucose responsive for SIR and chloroform susceptible for FE (Wardle and Ghani, 1995). In the present study, only in the heathland were SIR and FE-C_{mic} significantly correlated ($r^2 = 0.32$, $P<0.001$). The relationship between the two methods is often variable with some studies reporting strong correlations (Anderson and Joergensen, 1997; Beck *et al.*, 1997) and others very weak or no correlations (Ross *et al.*, 1984).

Of fundamental importance to the FE methods are the k factors (the proportion of microbial C, N or P released upon fumigation). The k factors used in the present study are based on those derived by Vance *et al.* (1987) and Brookes *et al.* (1982, 1985). Ingham *et al.* (1991) highlighted 3 critical assumptions of the k factor: "1) all organisms release the same proportions of C, N or P upon fumigation, 2) all microorganisms are killed by the fumigation process, and 3) the substrates respired after fumigation come only from the killed microbes, or from the same pool of non-microbial organic matter." In many studies, these assumptions have been violated (Couteaux *et al.*, 1989; Ingham and Horton, 1987), which can thus lead to large variations in the k factor for different soils. For example, Beck *et al.* (1997) reported k_{ec} to fluctuate from 0.35 to 0.45 depending on the soil type. It is therefore quite possible that k factors could be different for the 3 soils used in the present study. Between site comparisons must therefore be undertaken with caution. However, the response of the microbial biomass to the treatments in each site is possible since the k factors are unlikely to fluctuate markedly within each soil type. Determination of reliable k factors for soils with high organic matter content is an area of research that needs to be addressed in the future. Previous determinations of k factors are based on release of C, N or P from cultured microorganisms. This will always lead to inaccuracies, even if the organisms are isolated from representative soil samples, due to the highly selective nature of culture media. Of greater concern is the chloroform fumigation procedure which is likely to

break down a wide range of non-microbial constituents, particularly in organic soils (Couteaux *et al.*, 1990). Consequently, it is recognised that the biomass N and P values quoted in this thesis, and many other publications, may actually represent microbial biomass + chloroform soluble N and P.

5.4.2 Biomass C

C_{mic} was considerably greater in 1995 than in 1997 in all three field sites. Whilst minor differences are to be expected when comparing different methodologies (see section 5.4.1), the considerable changes in SIR-C_{mic} in the heathland and calcareous grassland and between SIR-C_{mic} and FE-C_{mic} in the acid grassland in 1995 and 1997 are surprising. C_{mic} was approximately 5 times greater in 1995 in the heathland, 3.5 times greater in the calcareous grassland and 13 times greater in the acid grassland. Seasonal variations in C_{mic} have been reported in the literature. Lovell *et al.* (1995) reported up to 2 times more C_{mic} in Spring than in Winter in an agricultural soil. It may be possible that seasonal or climatic effects could result in the large discrepancies observed between years in the present study. However, soil moisture contents were consistent between the two sample dates at all of the sites. Other factors such as operator error or differences in sample preparation/storage are unlikely to be a cause of the variation since the experimental procedures were identical in each year. In light of the high values, it is unfortunate that C_{mic} was not measured in the 1995 samples using another method, such as FE. A tentative comparison of the 1995 C_{mic} measured in the present study with published data also indicates unusually high values. Sparling *et al.* (1986) found C_{mic} ranged from 0.26 to 1.26 mg C g^{-1} soil dwt in 20 permanent pastures. However, soils with high organic matter content tend to have high C_{mic} values (Wardle, 1992). In addition, high C_{mic} values are to be expected in this study since soil was sampled from the top 5 cm of soil. In agricultural soils, the more biologically active surface horizons are typically mixed to form a much less active plough layer (Kaiser and Heinemeyer, 1993). The upper horizons of forest soils have been shown to have considerable quantities of C_{mic}. Al Fassi (1985) measured 6.9 mg C_{mic} g^{-1} soil dwt in the L horizon of a spruce forest and Ross *et al.* (1996) reported 7.7 mg C_{mic} g^{-1} soil dwt in the FH horizons of beech forest. Soil with high fungal biomass such as ectomycorrhizal mycellial mats have C_{mic} values of up to 370 mg C g^{-1} dwt (Ingham *et al.* 1991). Vance *et al.* (1987) reported of 3.2 mg C_{mic} g^{-1} soil dwt in a grassland soil with 10 % organic matter (considerably less than the heathland and acid grassland soils used in this study).

The long-term application of N (as ammonium nitrate) increased SIR-C in the heathland but had no effect or decreased it in the acid and calcareous grasslands (Figs. 5.1a,b, 5.3a, 5.6a). These responses are parallel to the effects on plant cover which, in response to increasing N deposition systematically increased in the heathland, but progressively decreased in the two grasslands (Table 5.4).

Table 5.4. The decrease (-) in vegetation cover of an acidic and calcareous grassland and increase in cover (+) of a heathland as a percentage of the cover in control plots following seven years N treatments (Caporn *et al.*, 1998; J. Carroll, pers. comm.). Treatments in parentheses refer to the heathland. NA = Not applicable. Values sharing a letter are not significantly different within sites ($P>0.05$).

Treatment†	Acid grassland	Calcareous grassland	Heathland
Control	0[a]	0[a]	0[a]
3.5 N (4 N)	-4[b]	-4[b]	+11[b]
7 N (8 N)	-8[c]	-5[b]	+15[c]
14 N (12 N)	-14[d]	-13[c]	+29[d]
14 N AS	-26[e]	-34[d]	NA

† Treatments (g N m^{-2} y^{-1}) applied as ammonium nitrate (N) or ammonium sulphate (N AS).

Although plant productivity has not been measured it is likely to correlate strongly with the cover determinations, which thus provide an index of C input into the soil via litter. These results support the hypothesis that changes in plant productivity are likely to mirror C_{mic}. Whilst direct effects of chronic long-term N enrichment upon soil microorganisms cannot be excluded, it is likely that one of the main determinants of the microbial response to pollutant N is the productivity of the plant communities since this determines a high proportion of the C input into soils. The microbial community may also be affected by changes in the composition of the litter entering the decomposer subsystem. Incubation of up to eight species of plant litter held in nylon bags has shown strong effects of litter type on the C_{mic} of

the litter (Wardle *et al.*, 1997). The soil (myco-) rhizosphere is host to a diverse and very active microbial community and so other factors of importance may include changes in the quantity and quality of root exudation products and root/mycorrhizal turnover. For example, Ennik *et al.* (1980) reported that N fertilisation reduced sward root mass and root C input. Bacteria are the most abundant free-living organisms in the rhizosphere and so are most likely to be affected by changes in root C flow (Clarholm, 1985). In the calcareous grassland, no parallels were seen between plant cover and SIR-C_{mic} in the 14 N AS treatment. In this treatment, soil pH was markedly lower than control soils (Table 2.4) and is likely to have an important role in controlling the activity and size of the microbial biomass (Wardle, 1992). However, decreasing soil pH often reduces C_{mic}, although microbial activity may not be significantly affected until soil pH is severely lowered (Killham *et al.*, 1983), or has no effect (e.g. Bitton *et al.*, 1985) while raising the pH of acid soils often leads to increases in C_{mic} (e.g. Adams and Adams, 1983). In the present study, the substantial decreases in plant cover, possibly as a consequence of the significant drop in pH, could be coupled with large amounts of senescing root material. This could form an effective and readily available C substrate for saprotrophic fungi and bacteria thus counteracting reductions in C input from litter fall and explaining the lack of any effect of the treatment on SIR-C_{mic} (Fig. 5.6a) and the increase in FE-C_{mic} (Fig. 5.6b).

The response of the soil microbial biomass to 18 months N and P treatments in the acid grassland contrasted markedly to the changes in the long-term plots. A stimulatory effect was seen in the 14 N treatment which was suppressed when applied in combination with P (Fig. 5.5a). Changes in C inputs from plant litter as a consequence of alterations in species composition were unlikely to be determining factors since both above ground biomass and the dicotyledon:monocotyledon ratio were suppressed in response to N when supplied either with or without P (Fig. 3.9). It is possible that the plant growth responses to the treatments could affect the quantitiy of inorganic N reaching the soil, which may then affect the microbial biomass directly. If the vegetation is naturally strongly P limited, the N only treatments may simply wash off the vegetation surface onto the soil. However, when P is applied in combination, more N could be utilised as plant demand increases thus limiting the quantity of inorganic N reaching the soil. Furthermore, enhanced foliar uptake of N could also occur as a consequence of the increased surface area of the vegetation canopy in the P treated plots.

The metabolic quotient (qCO_2-C) has been shown to be a sensitive indicator of environmental stress on soil microbial communities (Anderson and Domsch, 1993). Microorganisms that are stressed tend to have high qCO_2-C values which indicates a greater amount of energy being partitioned from biosynthesis into cell maintenance (Odum, 1985). High qCO_2-C values have been reported for *Fagus* and *Fagus-Quercus* stands where soil pH is low (Anderson and Domsch, 1993) and as a consequence of increased salt stress and heavy metal loading (Killham, 1985). The sensitivity of qCO_2-C as an indicator of environmental stress in preference to other measures, such as respiration rate, is highlighted in the values obtained for the heathland. Here, qCO_2-C was significantly lower during both 1995 and 1997 in the F and H horizons of the 8 N treated plots, while significant changes in the respiration rate only occurred during 1995 in the F horizon of the 8 N treated plots. In contrast, qCO_2-C in the acid grassland remained constant in the F horizon, but was significantly higher in the 7 N treated plots in the Ah horizon. In the calcareous grassland, qCO_2-C was significantly greater in the Ah horizon of the 14 N treated plots only in 1997. These data suggest that the N treatments (as ammonium nitrate) are reducing the stress on soil microorganisms in the heathland, but increasing microbial stress in the Ah horizons of the acid and calcareous grasslands. This can be explained by the changes in C input discussed earlier: in the heathland, C inputs have significantly increased while the reverse was seen in the acid and calcareous grasslands. Care must be taken when interpreting qCO_2-C values in soils where pH change has occurred since pH has been shown to be an important determinant of environmental stress (Anderson and Domsch, 1993). In the calcareous grassland, large pH shifts were measured in the 7 and 14 N and highly significant changes in the 14 N AS plots treated. Consequently, qCO_2-C may be affected by more than one variable, each with the potential to operate in different directions thus limiting the sensitivity of qCO_2-C.

5.4.3 Biomass N and P

The microbial biomass is a comparatively labile fraction of the soil organic matter (Jenkinson and Ladd, 1981) and is thus a potential source of nutrients for plants. Changes in the absolute amounts of N and P held in the microbial biomass as a consequence of nutrient manipulations are of interest since the relative importance of this pool as a nutrient source can be assessed. Of further importance, in terms of ecosystem functioning, are changes in the percentage of N and P in the microbial biomass. This may reflect alterations

in the functional diversity of the microbial biomass either as a result of modifications to the functional capacity of existing microorganisms or changes in the species composition of the community.

The absolute values for biomass N and P must be interpreted with caution since they may represent microbial + non-microbial N and P. This may be particularly true for the heathland soil which probably contains the greatest amount of chloroform soluble lipidic compounds. In the heathland, biomass N and P ranged from 600-1100 μg g^{-1} soil dwt. which is considerably greater than values reported for agricultural soils. Lovell *et al.* (1995) reported biomass N in the range 159 and 275 μg g^{-1} soil dwt in a long-term grassland soil while Brookes *et al.* (1984) measured between 5.3 and 106 μg biomass P g^{-1} soil dwt in a study of 15 arable and grassland soils. Similarly, the percentage N and P in the microbial biomass was also higher than published values. Brookes *et al.* (1984) reported between 2.8 and 9.4 % P in the 'wet' microbial biomass (i.e. making no correction for fwt:dwt ratio of the biomass) in a range of grassland soil. Clearly, considerably more biomass N and P is found in non-agricultural soils. The surface horizons of beech forests and tussock grassland can comprise up to 10 mg g^{-1} soil dwt as microbial N (Ross *et al.* 1996). Biomass N and P as a percentage of C_{mic} were exceptionally high in the heathland and acid grasslands. Biomass N ranged from 15-50 % and biomass P from 15-35 % for both sites. This strongly suggests solubilisation of non-microbial N and P compounds by the chloroform fumigation, indicating the need to reevaluate the method for soils of high organic matter. However, it is interesting to note that large within site fluctuations of biomass N (as a percentage of C_{mic}) are occurring in the acid grassland (discussed below; Fig. 5.4b).

Biomass N responded positively to the nutrient amendments in the heathland, negatively or neutrally in the acid grassland, and remained constant in the calcareous grassland. The heathland was particularly responsive to the treatments where significant increases resulted from even the lowest (4 g N m^{-1} y^{-1}) application rate. In the acid grassland, biomass N was not significantly affected by the N treatments as compared to the control. However, both the 3.5 and 7 N treatments led to progressive decreases in biomass N, while biomass N was significantly greater in the 14 N treatment as compared to the 7 N treatment. In the heathland biomass N as a percentage C_{mic} did not increase. This suggests that the functional

capability of the microbial community to acquire N has not changed. In contrast, biomass N as a percentage C_{mic} was significantly greater in the 14 N and 14 N AS treatments in the acid grassland, suggesting either i) an increased capacity for microbial N immobilisation, ii) an increase in the quantity of chloroform soluble N compounds, or iii) a combination of these. The lack of any progressive increase in the 14 N AS treated plots indicates that it is N *per se*, rather than the form of N, that affects the biomass N as a percentage C_{mic}. In the calcareous grassland, the N treatments had no effect on biomass C or N. Instead, both the absolute amounts and the proportion of P in the biomass were substantially reduced. This may indicate a change in the ability of the microbial community to immobilise P, which could occur through either a change in the species composition of the community, or a change in the functional capabilities of the existing microbial biomass. The progressive decreases in pH observed at this site may be resulting in a gradual shift towards a community dominated by fungi, which tend to have a higher tolerance for acid conditions than bacteria (Killham, 1994). Other factors that could influence the biomass P measurements include changes in root growth, biomass and morphology and mycorrhizal infection, all of which can be affected by N fertilisation (Marschner, 1995). However, in the present study, interference from these factors is unlikely to be of significance since only biomass P was affected. Changes in the abundance of fine roots is known to have equivalent effects on biomass C, N and P (Sparling *et al.* 1985b).

In the long-term acid grassland, the percentage P in the biomass did not change significantly despite the absolute amounts of biomass P decreasing consistently in all of the treated plots. The increase in the percentage of N in the biomass combined with no change in the percentage P, suggests that either the microbial community is N rather than P limited or that the microbial biomass does not have the ability to immobilise additional quantities of P. It is unlikely the former argument is correct given the response of the vegetation at this site. Both shoot P and shoot biomass of *Anenome nemorosa* progressively decreased while root surface PME activity of *Agrostis capillaris* progressively increased, providing strong evidence the plants were P rather than N limited. Short-term (18 months) applications of N had similar effects on biomass P, while the percentage P in the biomass increased significantly when additional P was supplied. If the microbial biomass was N rather than P limited, it is unlikely this increase would have occurred. Consequently, the lack of any

increase in the percentage P in the biomass in response to the long-term treatments suggests that the microbial community is operating at its maximum potential for P immobilsation.

The differing inter- and intra-site responses of microbial biomass C, N and P to long-term additions of N and short-term additions of N and P is not surprising considering the varied effects of nutrient amendments reported in the literature. Although up to one third of studies have measured stimulatory effects of mineral N addition the remainder have reported either inconsistent or neutral effects (Wardle, 1992). The results presented in this chapter illustrate that both long-term and short-term additions of N can have significant effects on microbial biomass C, N and P. Furthermore, changes in the biomass N and P as a percentage C_{mic} in the acid and calcareous grasslands indicate possible alterations in the functional capabilities of the microbial communities in these soils, the subject of Chapters 6 and 7. The results also indicate that present methodologies for measuring absolute quantities of microbial biomass N and P may be of only limited value in organic matter enriched soils.

CHAPTER 6

SOIL PHOSPHOMONOESTERASE ACTIVITY IN RESPONSE TO LONG-TERM INPUTS OF N AND SHORT-TERM INPUTS OF N AND P

6.1 INTRODUCTION

In plant communities that have approached or have reached N saturation, the availability and rates of recycling of nutrients other than N is of increasing importance. These nutrients may subsequently exert the main control on plant productivity. In ombrotrophic bogs (Aerts *et al.*, 1992), fens (Verhoeven and Schmitz, 1991) and heathlands (Aerts and Berendse, 1988), N enrichment has led to P being the main nutrient limiting plant productivity. There is also increasing evidence of strong interrelationships between the cycling of N and P in oligotrophic ecosystems with low rates of productivity (Pastor *et al.*, 1984; Van Oorschot *et al.*, 1997). This is also reflected by results from the previous chapters of this study which highlight increases and decreases in the shoot N:P ratios for *A. nemorosa* and *C. flacca* respectively, increases in root surface phosphomonoesterase (PME) activities of *P. lanceolata* and *A. capillaris* and decreases in microbial biomass P concentrations at the acid and calcareous grasslands. It is hypothesised that the change from N to P as the main nutrient limiting plant productivity in some ecosystems, is likely to lead to increasing P turnover and therefore more rapid rates of organic P mineralisation by the soil biota.

P mineralisation occurs through the production of organic P degrading enzymes, such as phosphatases, by both soil microorganisms and plants, which catalyse the breakdown of organic P compounds. The most important phosphatase enzyme present in the soil is thought to be PME which is responsible for the breakdown of phosphomonoesters such as inositol P. At present, there are no data quantifying changes in soil PME activity in response to chronic additions of N. However, large single fertiliser applications to forest soils have been found sometimes to increase PME activity on mycorrhizal tree roots (Kieliszewska-Rokicka, 1992; O'Connell and Grove, 1985) but in other studies had a strong inhibitory effect on PME activity in soil (Bååth *et al.*, 1981; Pang and Kolenko, 1986). A similar inhibitory effect was also reported by Lovell *et al.* (1995) who applied N fertiliser to a long-term grassland for 10 years. In systems such as the calcareous grassland that are naturally strongly P limited, it is possible that soil PME activity will be unresponsive to the N treatments since enzyme activity may already be at a maximum. However, the significant increases in root surface PME activity measured at this site (Chapter 4) could suggest the contrary, since this activity comprises enzymes produced by the plant and by soil microorganisms adhering to the root.

Lovell *et al.* (1995) also found that PME activity was greatest following recent applications of N. Changes in PME activity in response to N additions may therefore be highly dependent on the duration of the treatments. It may therefore be crucial to consider the effects of N treatments following both long-term and short-term applications of N treatments. Based on this evidence, it is hypothesised that PME activity will be more responsive, at least in the grasslands, to short-term applications of N.

In forest soils, PME activity is influenced by a number of factors, including soil depth, organic matter, moisture, clay and silt, total N, extractable magnesium, exchangeable P and pH (Harrison, 1983). In addition, since a large proportion of PME found in soils is produced by the microbial biomass, the overall soil PME activity is likely to be influenced by the amount of active biomass. Consequently, the response of soil PME activity to the N treatments may mirror the changes in C_{mic} reported in Chapter 5. The soil P status has been shown to have considerable importance particularly in the growing season where it can account for 70 % of the variation in the PME activity of forest soils (Harrison, 1983). Since PME is an inducible enzyme, its activity is likely to be greatest when organisms are P limited (Reid and Bieleski, 1970). P fertilisation would therefore be expected to reduce PME activity. Indeed, Speirs and McGill (1979) reported that fertilisation at 2.7 and 5.4 g P m^{-2} y^{-1} for 5 years decreased soil PME activity in a 'high' organic matter soil (6 % organic C) with high initial PME activity. However, the treatments were found to increase PME activity in a 'low' organic matter soil (1.3 % organic C) with low initial PME activity. This indicates that the response of PME to increased P availability may be dependent upon the soil properties. The grassland soils used in the present study have high but contrasting organic matter contents (42 % in the acid grassland; 30 % in the calcareous grassland; Table 2.1). The low extractable soil P concentrations indicates that the calcareous grassland may be initially more P limited that the acid grassland. It is hypothesised that addition of P to both grasslands will suppress PME activity, although this may be more marked in the calcareous grassland owing to its greater degree of initial P limitation.

6.1.1 Aims

This chapter aims to investigate the effects of long-term N treatments on soil PME activity in the heathland, acid grassland and calcareous grassland. The following hypotheses will be tested:

1. Long-term N deposition will lead to increases in soil PME activity.
2. PME activity in the calcareous grassland, which is already strongly P limited may be less responsive to the N treatments.
3. PME activity is more responsive to short-term N treatments (grasslands only).
4. Addition of P to the grasslands will supress PME activity, particularly in the calcareous system which is naturally strongly P limited.

6.2 MATERIALS AND METHODS

6.2.1 Soil phosphomonoesterase activity

Soil suspensions from the F and H horizons of the heathland and the Ah horizons of the acid and calcareous grasslands were assayed for PME activity using the artificial substrate *p*-nitrophenyl phosphate (*p*-NPP). The enzyme is quantified by measuring the release of *p*-nitrophenol (*p*-NP; Tabatabai and Bremner, 1969). Glass universal vials containing 1 ml of soil suspension (1 g soil:14 ml water) were incubated with 4 ml 13.6 mM *p*-NPP in a shaking water bath (37 °C) for up to 45 minutes. The use of toluene is often recommended as an inhibitor of microbial growth during the assay (Tabatabai and Bremner, 1969; Speirs and McGill, 1979). However, preliminary studies (Appendix A) revealed that it had no effect on PME activities, possibly due to the short duration of the assays, and it was therefore omitted from the procedure. A sub-sample (100 to 500 µl) was added to 2 ml 2 M NaOH and the optical density measured immediately using a Cecil CE1020 spectrophotometer at a wavelength of 410 nm. The assays were undertaken without buffer in order to allow the enzyme to operate at the pH of the soil. PME activity was expressed as nmol *p*-NP released g^{-1} soil dwt s^{-1} after correction for background colour of each soil and any binding of *p*-NP onto the soil suspension (Vuorinen, 1993).

In the heathland and acid grassland, the N treatments have not significantly affected the pH of the soil. In contrast, there has been significant acidification of soil in the calcareous grassland (Table 2.4). For this soil, the PME assays were repeated using the substrate dissolved in 0.5 M Na citrate buffer in the pH range 4.5-6.5 to distinguish pH effects from N treatment effects. The buffered assays were conducted on soil from the control, 14 N and 14 N AS treatments.

6.2.2 Extractable N and P

Nitrate (NO_3^-), ammonium (NH_4^+) and phosphorus (PO_4) were determined as described in Section 2.2.4.

6.3 RESULTS

6.3.1 Effects of long-term treatments

Long-term applications of N affected soil PME activity at all of the field sites. PME activity was particularly responsive to the treatments in the heathland soil and increased significantly at all treatment levels (Fig. 6.1). The greatest increase in PME per g of added N occurred in the plots receiving 4 g N m^{-2} y^{-1}. Here the enzyme activity increased significantly ($P<0.001$) from 19.5 to 41.6 nmol g^{-1} soil s^{-1} in the F horizon and from 10.8 to 24.1 nmol g^{-1} soil s^{-1} in the H horizon ($P<0.01$). The 8 and 12 N treatments further increased PME activity but these increases were progressively smaller with each increment of N, particularly in the F horizon (Fig. 6.1).

PME activity ranged from 34.1 to 46.2 nmol g^{-1} soil s^{-1} in the long-term acid grassland plots (Fig. 6.2), and from 13.3 to 22.5 nmol g^{-1} soil s^{-1} in the long-term calcareous grassland plots (Fig. 6.3). In the control plots, the enzyme activity was considerably higher in the acid grassland than in the heathland, but in the calcareous grassland it was lower than in the heathland. In the acid grassland, PME activity was only affected by the 14 N treatment where the enzyme activity increased significantly ($P<0.01$) by 35 % (Fig. 6.2). In the calcareous grassland, excepting the lowest N treatment in which there was a slight reduction in PME activity relative to the control, there was a trend for increased enzyme activity with increasing N application. This effect was only significant ($P<0.001$) in the 14 N AS treatment where the enzyme activity increased by 80 % to 23 nmol g^{-1} soil s^{-1} in June 1996 and by 110 % to 15.7 nmol g^{-1} soil s^{-1} in January 1997 (Fig. 6.3). This marked response to ammonium sulphate in the calcareous grassland contrasts with the lack of effect of the treatment in the acid grassland. At the calcareous site the N additions caused highly significant soil acidification (Table 2.4), an effect which was most marked in the plots receiving 14 g N as ammonium sulphate. In contrast there were no significant ($P>0.05$) change in soil pH at the other two sites. In the calcareous soil, the PME activity could have simply been a reflection of the differences in soil pH since the assays were unbuffered. However, when the samples from this site were assayed in buffer in the pH range 4.5-6.5 it

was apparent that the ammonium sulphate treatment significantly (P<0.001) increased PME activity regardless of the pH of the buffer (Fig. 6.4). The highest activity was found in the assays buffered at pH 6.5 and this was unaffected by the N treatments. This indicates that in the unbuffered assays soil acidification will tend to underestimate rather than overestimate the increased activity in the 14 N AS treatment.

Paired soil samples from the calcareous grassland in which total KCl extractable inorganic N and PME activity were determined revealed a highly significant (P<0.001) linear relationship between these variables (Fig. 6.5). This relationship extended over a forty-fold variation (3.5-135 μg N g^{-1} soil dwt) in the concentration of extractable N in the soil, and accounted for over 70 % of the variation in soil PME activity. The relationship between PME activity and extractable ammonium was considerably stronger (r^2 = 0.57) than the relationship with extractable nitrate (r^2=0.27), and the slope of the regression was steeper than with nitrate. This suggests that the ammonium concentration exerts the main controlling effect on PME production, but the strength of the relationship with total extractable N indicates that nitrate also has some effect. The concentration of extractable N in the soil was exceptionally high, which is consistent with the soil system being 'N saturated'.

Soil PME activity and SIR biomass C data were combined to indicate the PME activity per mg Cmic (Fig. 6.6). This expression provides a useful measure of phosphate demand by soil organisms in sites with very different microbial biomass. The values ranged from 1.5-5.79 nmol p-NP mg C_{mic} s^{-1}. These are similar to those which can be calculated from the study by Sparling et al. (1986) of microbial biomass and PME activity in 20 permanent pastures where it ranged from 3.1-8.97 nmol p-NP mg C_{mic} s^{-1}. The results for all three sites are more comparable than for PME per g soil which will be strongly influenced by the quantity of active microbial biomass, one of the main producers of the enzyme. The results show a consistent pattern of response to increased N deposition, with PME activity per mg C_{mic} always increasing in response to N enrichment. This effect was highly significant (P<0.001) over the three sites.

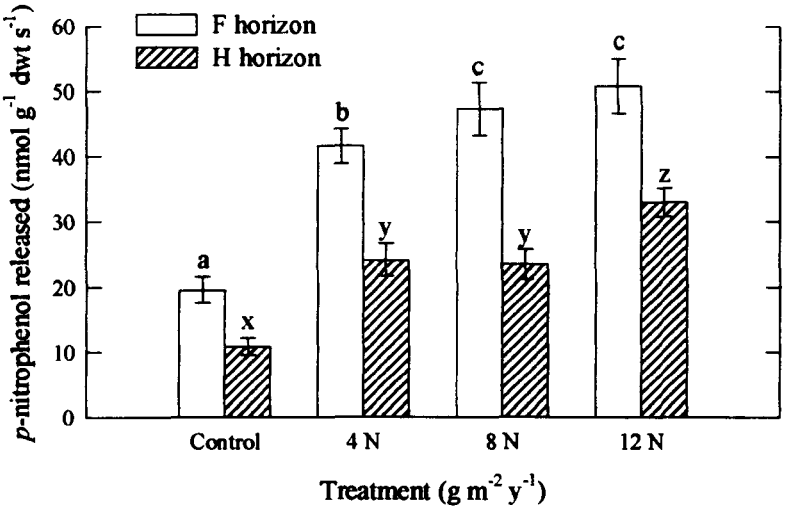


Fig. 6.1. Soil phosphomonoesterase activity in the F and H horizons of a heathland measured during August 1996 in response to seven years of N treatments (± SEM). Bars sharing a letter are not significantly different (P>0.01). Treatment codes: Control = water only, 4, 8 and 12 N = 4, 8 or 12 g N m^{-2} y^{-1} (as ammonium nitrate).

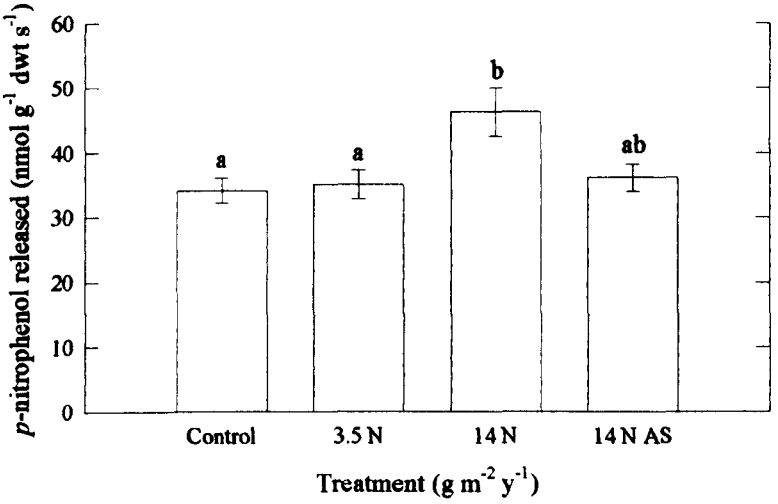


Fig. 6.2. Soil phosphomonoesterase activity in the Ah horizon of an acid grassland measured during November 1996 in response to seven years of N treatments (± SEM). Bars sharing a letter are not significantly different (P>0.01). Treatment codes: Control = water only, 3.5 and 14 N = 3.5 and 14 g N m^{-2} y^{-1} (as ammonium nitrate), 14 N AS = 14 g N m^{-2} y^{-1} (as ammonium sulphate).

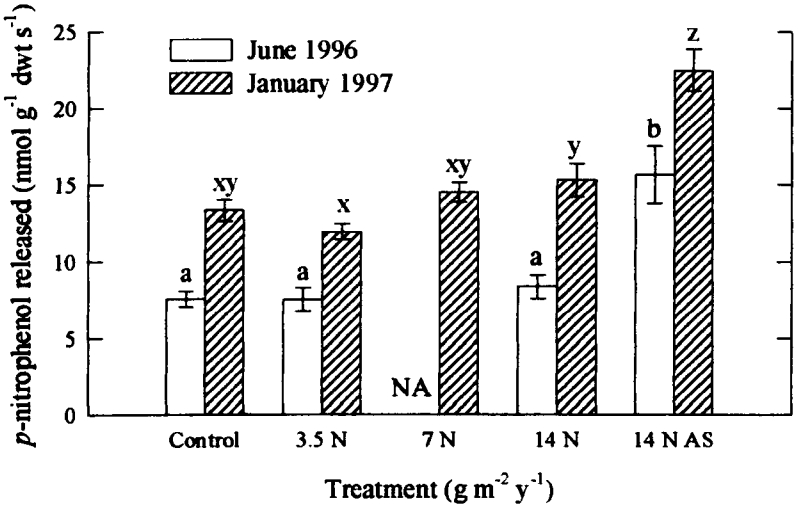


Fig. 6.3. Phosphomonoesterase activity in the Ah horizon of a calcareous grassland soil measured during June 1996 and January 1997 that has received up to seven years of N treatments (± SEM). Bars sharing a letter are not significantly different (P>0.05). NA = not analysed. Treatment codes as for Fig. 6.2.

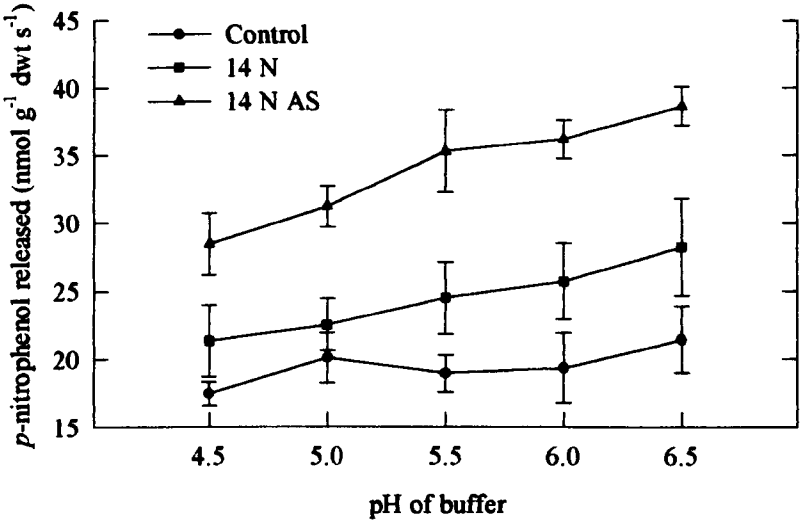


Fig. 6.4. Effect of assay pH on phosphomonoesterase activity in the Ah horizon of a calcareous grassland soil measured during January 1997 that has received seven years of N treatments (± SEM).

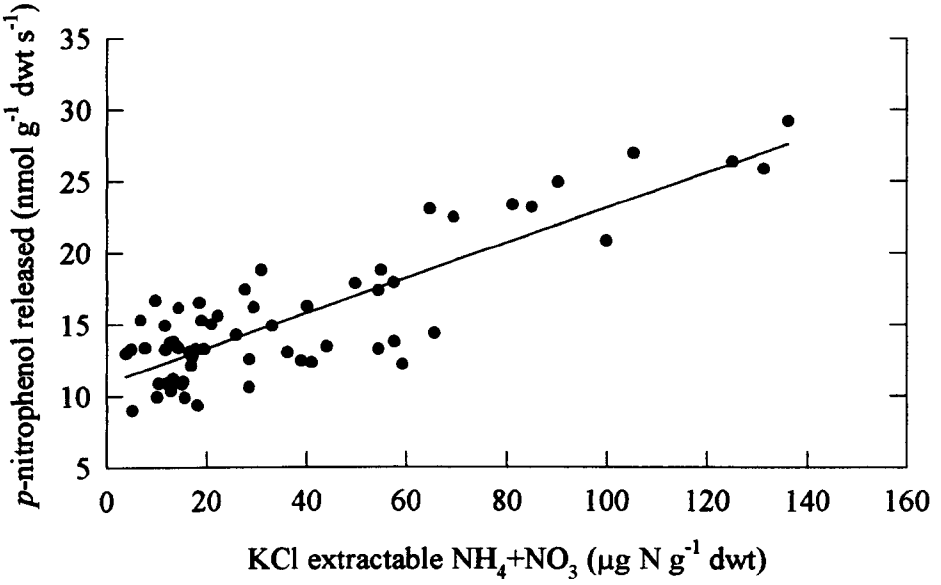


Fig. 6.5. Relationship between KCl extractable N (NH_4^+ and NO_3^-) and soil phosphomonoesterase activity in calcareous grassland plots that have received seven years of N treatments ($r^2 = 0.71$, $P<0.001$).

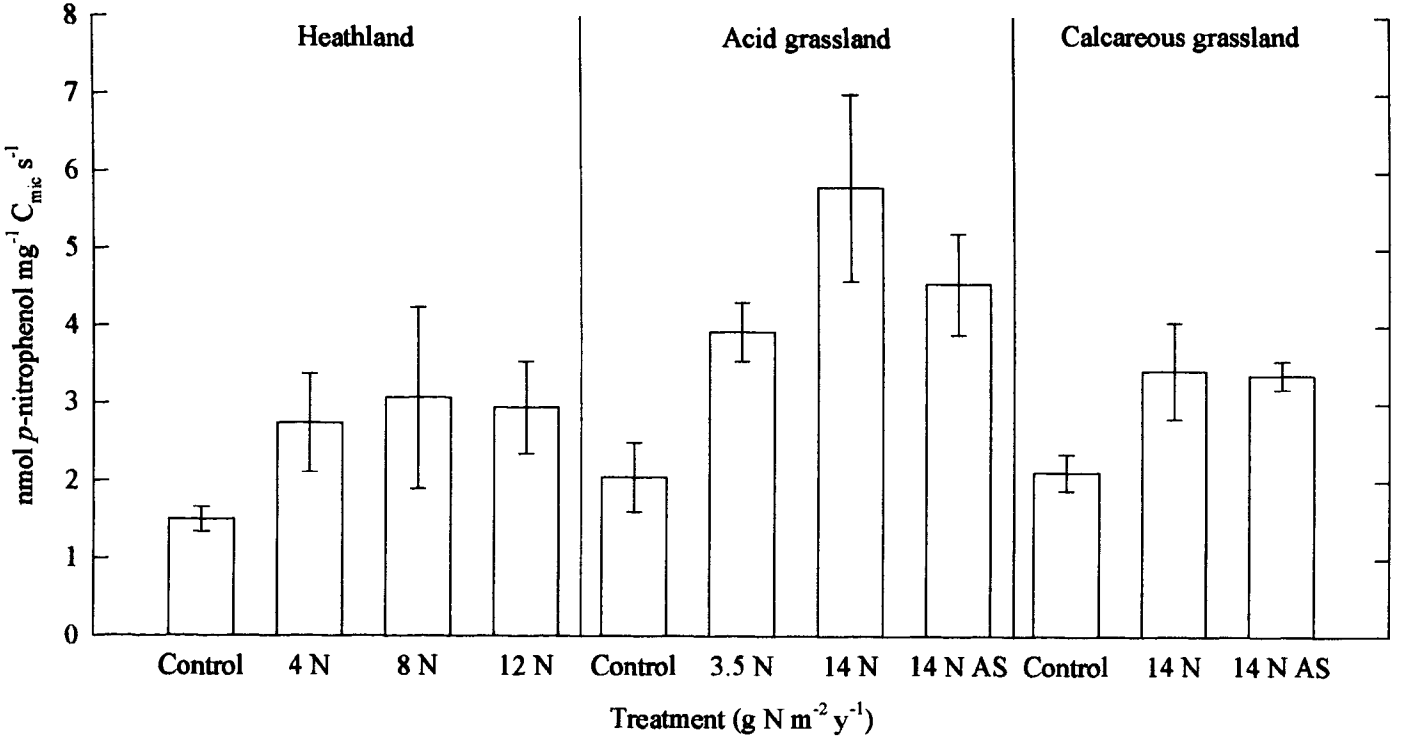


Fig. 6.6. Phosphomonoesterase activity per mg SIR C_{mic} in heathland, acid grassland and calcareous grassland soils that have received up to seven years N treatments (± SEM). Bars are not significantly different. Treatment codes as for Fig 6.1 and 6.2.

6.3.2 Effects of short-term treatments

Soil PME activities in the short-term acid grassland plots ranged from approximately 22 to 40 nmol *p*-NP g^{-1} s^{-1} (Fig. 6.7a). These contrasted to the long-term plots where they ranged from 33 to 45 nmol *p*-NP g^{-1} s^{-1} (Fig. 6.2). Short-term (18 months) applications of N and P had no significant effect on PME activity when they were applied in combination (Fig. 6.7a). However, the application of 14 g N, either with or without P, significantly ($P<0.05$) increased PME activity by 33 % from 27 to 36 nmol *p*-NP g^{-1} s^{-1} (Fig. 6.7b). This effect was comparable to that seen in the long-term plots. In the calcareous grassland, PME activity was higher in the long-term plots than in the short-term plots, where it ranged from 17 to 23 nmol *p*-NP g^{-1} s^{-1} (Fig. 6.8). In the short-term plots, only the 3.5 N treatment had a significant ($P<0.05$) stimulatory effect on PME activity (Fig. 6.8). Here, the activity increased by 35 % from 17 to 23 nmol *p*-NP g^{-1} s^{-1}. However, this effect was reversed when P was applied in combination with the 3.5 N treatment which significantly ($P<0.05$) lowered PME activity by 10 %. The addition of P alone did not affect PME activity.

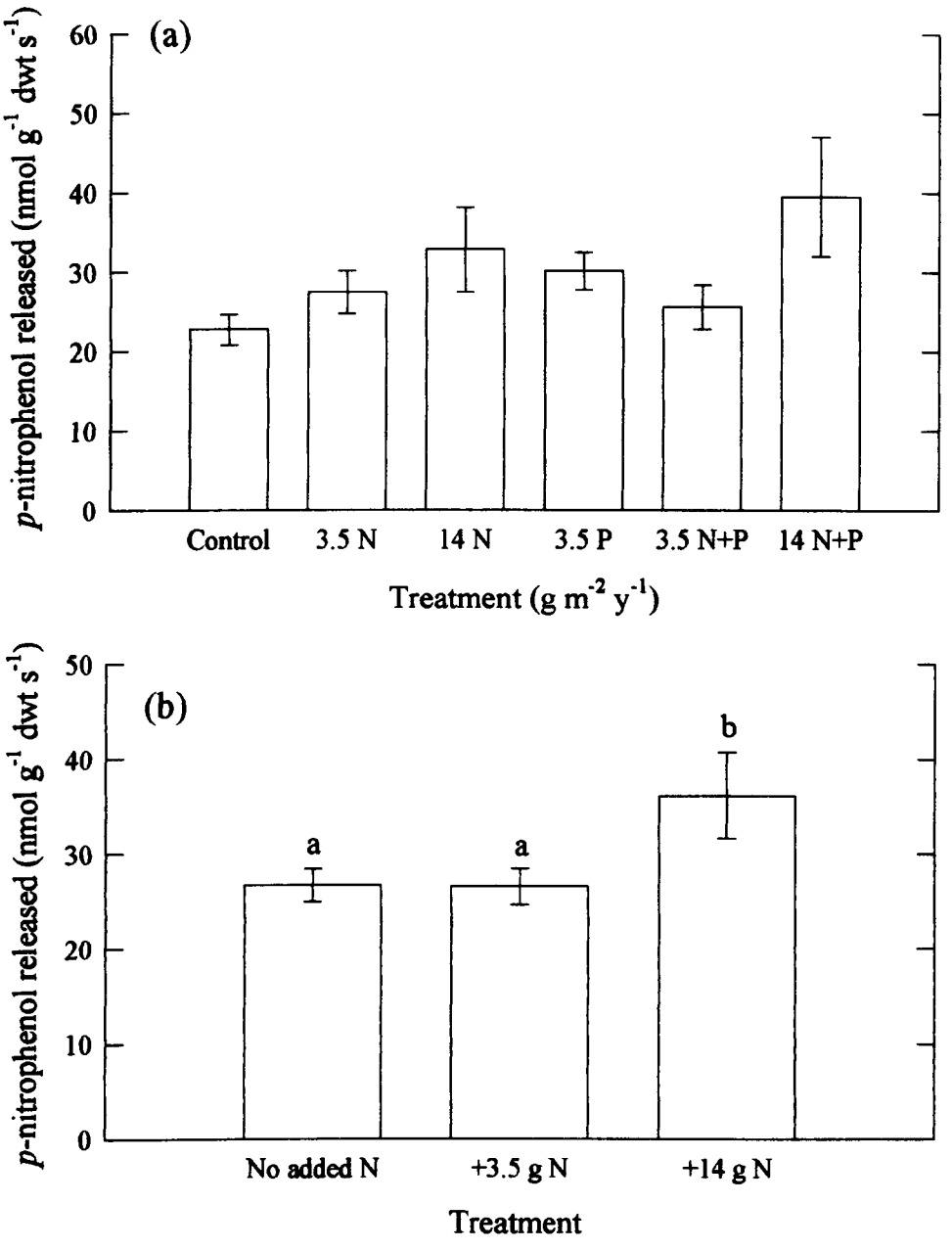


Fig. 6.7. Changes in soil phosphomonoesterase activity in the Ah horizon of an acid grassland measured during October 1996 in response to (a) 18 months of N and P treatments (no significant differences) and (b) the overall effect of 18 months N additions (0, 3.5 and 14 g N m^{-2} y^{-1}; ± SEM). Bars sharing a letter are not significantly different ($P>0.05$). Treatment codes (m^{-2} y^{-1}): Control = 0 g N, 3.5 N = 3.5 g N, 14 N = 14 g N, 3.5 P = 3.5 g P, 3.5 N+P = 3.5 g N + 3.5 g P, 14 g N+P = 14 g N + 3.5 g P.

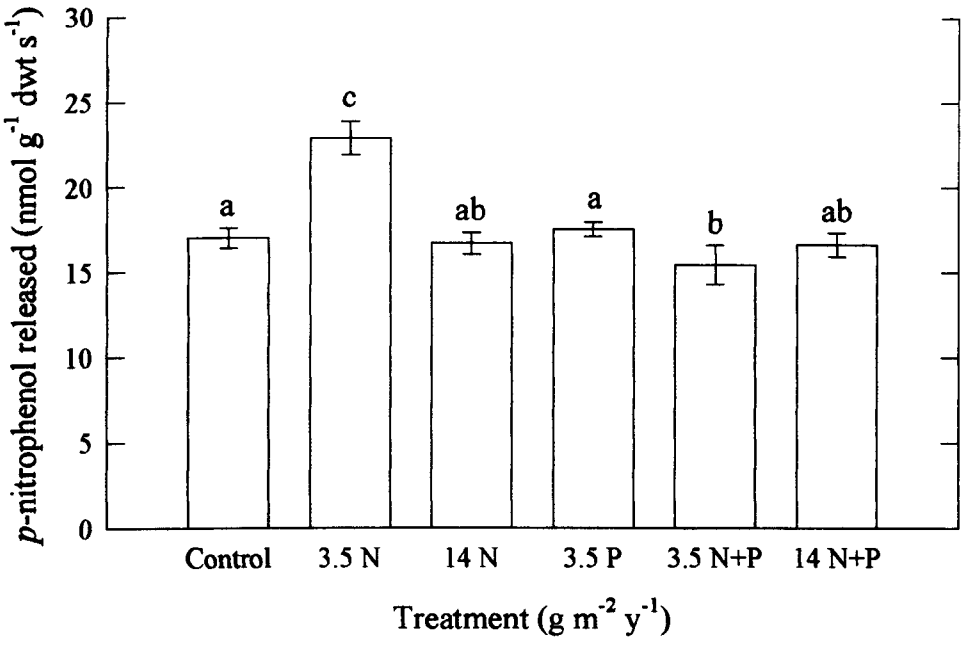


Fig. 6.8. Soil phosphomonoesterase activity in the Ah horizon of a calcareous grassland in response to 18 months N and 12 months P treatments measured during June 1996 (± SEM). Bars sharing a letter are not significantly different (P>0.05). Treatment codes as for Fig. 6.7a.

6.4 DISCUSSION

Previous field and laboratory studies of the effects of N additions on PME activity in soils have indicated both increases (Bååth *et al.*, 1981; Khan, 1970; Speirs and McGill, 1979) and decreases (Bååth *et al.*, 1981; Pang and Kolenko, 1981; Lovell *et al.*, 1995) in activity due to N. These contrasting responses may arise from different rates and sources of N being applied and differences in the N demand and 'N saturation' of the sites being investigated. Bååth *et al.* (1981) added up to 60 g N m^{-2} y^{-1} to a Scots pine forest and found that PME activity was influenced by the form of the applied N; ammonium nitrate decreased it but urea increased it. However, short-term treatments with high N doses of this kind are unlikely to provide a reliable guide to the long-term effects of chronic inputs of pollutant N. In the present study, short-term (18 months) applications of up to 14 g N m^{-2} y^{-1} provided a reliable indicator of the long-term treatment effects (7 years) in the acid grassland but not in the calcareous grassland. Whilst short-term N treatments resulted in significant increases in PME activity in the grasslands, the magnitude of the responses were similar for the long-term treatments. This disagrees with the hypothesis that PME activity would be more sensitive to short-term N treatments than long-term treatments.

It is clear from this chapter that soil PME activity can be very sensitive to prolonged inputs of N at the current rate of deposition of the pollutant in the UK: in the heathland, even the lowest N treatment (4 g N m^{-2} y^{-1}) applied for seven years led to a significant twofold increase in soil PME activity. In the heathland and acid grassland, PME activity per mg C_{mic} doubled in the 4 N and 3.5 N treatments respectively. Soil microbial biomass decreased in the acid grassland with increasing N deposition (Fig. 5.3), a feature which may explain the smaller and less consistent increase in PME activity per g soil than seen at the other sites (Fig. 6.1-6.3). However, PME activity per mg C_{mic} in the Ah horizon of the acid grassland site was highly responsive to the N treatments (Fig. 6.6) where it increased more than twofold in the highest treatments. This suggests that despite the decreasing microbial biomass, P limitation becomes more severe with increasing simulated N deposition as was seen in the calcareous grassland and the heathland site. The sensitivity of PME to increased N deposition at the calcareous grassland is unexpected where the strength of initial P limitation would suggest that the enzyme systems are already operating at their maximum. These results do not support the hypothesis that PME activity at this site is any less sensitive than the acid grassland and heathland. The response of PME to N deposition

indicates that this enzyme may have the potential to be used as a sensitive tool for calculating critical loads of N. The results support the hypothesis that long-term N treatments lead to increases in soil PME activities.

The substantial increases in PME activity (g^{-1} soil) suggest that either the functional characteristics of the microbial communities or that the availability of P at the three sites may have changed in response to the long-term N treatments, or a combination of the two. Increased PME activity is most likely to arise from a population change, whereby microorganisms with high organic P degrading capabilities are being selected for, but could also occur through a general increase in microbial activity. However, the former appears most likely given the progressive increase in PME activity mg^{-1} C_{mic}, which takes into account any change in microbial biomass. SIR microbial biomass is probably closely related to microbial activity, since the SIR technique actually measures the respiratory activity of glucose responsive organisms.

The adverse effects of N addition to the grasslands on plant cover and microbial biomass C are almost certainly a response to 'N saturation' in these ecosystems which are mainly P rather than N limited. In the acid grassland soil, PME activity was very high, even in the control plots, and the simulated pollution further increased it only in the highest ammonium nitrate treatment (Figs. 6.2 and 6.7b). In contrast, the results from the two other sites provided evidence of increased microbial P demand in response to N enrichment. In both the heathland and calcareous grassland sites, PME activity increased in proportion to the amount of N added (Figs. 6.1 and 6.3). In the calcareous grassland, the increased microbial demand for P was also reflected by significant reductions in microbial biomass P (Chapter 5, Figs. 5.7a, b). Furthermore, the KCl extractable N (ammonium and nitrate) in the calcareous grassland soil accounted for more than 70 % of the variation in its PME activity (Fig. 6.5) indicating a strong link between N saturation and PME activity. When correlated separately, ammonium accounted for 58 % of the variation and nitrate 28 %. The much greater increase in PME activity in the 14 N AS treatment than the equivalent ammonium nitrate treatment (Fig. 4c), is therefore probably due to the stronger effect of extractable ammonium than nitrate on PME activity. This may be as a consequence of the greater mobility of nitrate in soil, the concentrations of which are likely to be more variable than for ammonium.

P additions did not affect soil PME activity in either the calcareous or acid grassland. However, in the calcareous grassland, PME activity was significantly lower ($P<0.05$) in the 3.5 N+P treatment. The significant effect of the P additions when applied in combination with 3.5 g N to the calcareous grassland is expected given the strength of initial P limitation at this site. These results therefore only partially support the hypothesis that P treatments will lead to decreases in soil PME activity. A similar effect to the present study was observed by Speirs and McGill (1979) who applied P fertiliser to a soil with initially high PME activity and organic matter content. A similar inhibitory effect would be expected in the acid grassland given the uniform increase in extractable P concentrations in the plots receiving additions of P (Table 2.7). However, extractable soil P was not correlated with PME activity ($r^2 = 0.1$, $P<0.916$). It may be possible that the added P is rendered less available than expected at this site by being tightly bound to soil organic matter or preciptating to form insoluble hydrous oxides of Al or Fe. Extraction with a strong alkaline solution such as 0.5 M $NaHCO_3$ may thus give an over estimation of plant and microbial available P. However, it may also be possible that considerable foliar absorption and subsequent uptake of P is occurring, given the method of application in which the treatments are applied as a very fine mist. The contrasting effects of P in controlling soil PME activity has been reported by a number of workers (e.g. Speirs and McGill, 1979; Tarafdar and Claassen, 1988). Adams (1992) suggested the unpredictable role of P may reflect the diverse origin, specificity and stability of soil phosphatases.

The PME activities measured in the present study ranged from 11-51 nmol g^{-1} s^{-1} in the heathland, 23-46 nmol g^{-1} s^{-1} in the acid grassland and 12-39 nmol g^{-1} s^{-1} in the calcareous grassland (Table 6.1). These activities tend to be greater than most published values which typically ranged from 0.1-12.5 nmol g^{-1} s^{-1}, although they are most comparable to studies that have used organic soils (e.g. Bååth *et al.*, 1981; Table 6.1). However, as for root surface enzyme activities, many studies use different assay conditions and express results in different units. Malcolm (1983) reported that the amounts of *p*-NPP g^{-1} soil used in 12 studies of PME activity ranged from 2.5 µmol to 125 µmol, despite the original method of Tabatabai and Bremner (1969) stating a concentration of 115 µmol g^{-1} soil. In the present study, the amount of *p*-NPP was typically 500 µmol g^{-1} soil. Most investigations have used assay systems buffered at a pH where enzyme activity is at a maximum. This enables a measure of the maximum potential enzyme activity but not the potential enzyme activity in

Table 6.1. Comparison of soil PME activities measured during this study with published values.

Soil types	Activity (nmol g^{-1} dwt s^{-1})	Assay pH and temperature	Reference
Heathland	11-51	Unbuffered (pH 3.7) 37°C	This study
Acid grassland	23-46	Unbuffered (pH 4.8) 37°C	This study
Calcareous grassland	12-23	Unbuffered (pH 6.8) 37°C	This study
Calcareous grassland	17-39	pH 4.5-6.5; 37°C	This study
8 soils of low organic matter	0.42-1.65	pH 6.5; 37°C	Tabatabai and Bremner (1969)
Surface horizon of 5 agricultural soils	0.10-0.41	pH 6.5; 37°C	Eivazi and Tabatabai (1977)
Agricultural soil	36.4	pH 6.5; 37°C	Mathur and Rayment (1977)
Ap horizon of 8 chernozems	0.339-2.53	pH 6.5; 37°C	Speirs and McGill (1979)
Spruce podsol	8.88-12.5	pH 6 20°C	Bååth *et al.* (1981)
Pahokee muck, fallow	1.1-2.0	Buffered at soil pH; 37°C	Duxbury and Tate (1981)
10 soils	0.10-11.99	pH 6; 37°C	Sarathchandra and Perrott (1981)
Deciduous podsol	0.064-5.31	Buffered at soil pH; 37°C	Harrison (1983)
1 organic, 4 mineral soils	5.3×10^{-7}-2.2×10^{-6}	pH 6; 37°C	Bitton and Boylan (1985)†
2 Brunosolic forest soils	0.23-9.86	pH 7; 37°C	Pang and Kolenko (1986)
17 soils	1.54-10.4	pH 6; 37°C	Sparling *et al.* (1986)
Eucalyptus soil	0.18-2.61	pH 6; 37°C	Adams (1992)
Sand	0.47-1.01	pH 6; 37°C	Adams and Pate (1992)
Podsol	0.98-5.39	pH 6; 37°C	Fox and Cromford (1992)
Long-term grassland	3.59-6.47	pH 6; 37°C	Lovell *et al.* (1995)

† Unlikely to be an accurate measure of PME activity.

the natural environment (Malcolm, 1983). Furthermore, treatment effects on PME activity may only be apparent when the system is operating at its natural pH. Most workers have assayed PME activity at blood temperature (37 °C; Table 6.1), probably due to the origins

of these investigations in the medical industry. This temperature was used in the present study simply to enable closer comparisons with other studies, since temperature can have substantial effects on PME activity (e.g. Kroehler and Linkins, 1988). However, it would be of further interest to repeat the assays at temperatures closer to those observed in nature (Ca 0-10 °C).

The higher values reported in the present study may be due to the samples being derived from the upper 5 cm of soil in which free living and rhizosphere microorganisms are typically most active (Kaiser and Heinemeyer, 1993) and in which most organic P is found (Harrison, 1983). In addition, the relatively nutrient rich surface soil horizons tend to have the greatest concentration of active plant roots (Tinhout and Werger, 1988; Mamolos *et al.*, 1995). In dry *Calluna* heathlands, up to 55 % of the total root mass can be located in the upper 5 cm of soil (Tinhout and Werger, 1988). The PME activity of soil in the (myco-) rhizosphere is typically significantly greater than in the bulk soil (Tarafdar and Jungk, 1987; Tarafdar and Claassen, 1988). At the heathland site, there is an abundance of heavily mycorrhizal hair roots in the surface soil (Caporn *et al.*, 1995) which produce active extracellular and cell wall bound acid phosphatase (Pearson and Read, 1975; Straker and Mitchell, 1986; Leake and Miles, 1996). At present, it is not possible to accurately partition soil PME activity into its component parts (i.e. microbial, mycorrhizal, root exudation, residual). However, a tentative estimate was made by Speir *et al.* (1986) using microwave radiation to selectively inactivate intra- and extracellular PME. The authors attributed 17 % of the total soil PME activity to microbial sources. Clearly, further work is needed in this area, particularly using (myco-) rhizosphere soil since it is within this zone that plants access the majority of their nutrients. A number of studies have shown PME activity to decrease with increasing depth. For example, in deciduous and coniferous forest soils, PME activity can be between 4 and 18 times greater in the surface 5 cm than at 15 cm depth (Harrison, 1983; Pang and Kolenko, 1986). Of further importance is the P limitation of the sites. The calcareous grassland is strongly P limited, even prior to N treatments, and the apparent increase in P limitation with N enrichment at all three sites which may ensure particularly high rates of PME production. The extremely high PME activity in the acid grassland and heathland plots receiving N applications reflects the relatively large microbial biomass and organic matter content of these soils in comparison to the calcareous site.

It is clear that N treatments can have significant effects on PME activity. This was reflected most strongly in the heathland, where PME activity increased significantly even at the lowest level of N application, and in the calcareous grassland where PME activity was significantly correlated with KCl extractable ammonium and nitrate. It is important now to investigate any actual changes in the utilisation of organic P compounds, rather than the potential changes that are reflected by measurements of PME activity.

CHAPTER 7

UTILISATION OF N AND P SOURCES BY SOIL MICROORGANISMS IN RESPONSE TO LONG-TERM INPUTS OF N

7.1 INTRODUCTION

It is becoming clear that long-term N treatments are increasing the P limitation and therefore the P demand in the three systems under investigation in this study. This is reflected by shoot N and P concentrations, soil and root surface PME activities and microbial biomass N and P concentrations. Significant increases in soil PME activity in response to the N treatments are observed at all of the sites (Chapter 6), although the effect is most apparent in the heathland and calcareous grassland, while substantial increases in root surface PME activity are seen in the grasslands (Chapter 4). This indicates that the ability of both plants and microorganisms to mineralise organic P for their nutritional requirements may be of increasing importance in systems where N saturation has led to enhanced P limitation. For most plants, organic P mineralisation by the soil microbial community remains the most important mechanism for P uptake (Tate, 1984), although there is increasing evidence that organic P compounds can be utilised through the action of mycorrhizal fungi and root surface phosphatases (Jayachandran *et al.*, 1992). For example, the ericoid mycorrhizal fungus *Hymenoscyphus ericae* has been shown to produce phosphodiesterase and to utilise DNA as a P source (Leake and Miles, 1996). The situation is similar for N, where mineralisation by the soil microbial biomass remains fundamental in maintaining an adequate supply of N for many plant species, despite the ability of both ericoid and ectomycorrhizas to produce active extracellular proteinases and chitinases (Leake and Read, 1989; Leake and Read, 1990a, b) and increasing evidence of direct uptake of organic N sources in some plant communities (e.g. Kielland, 1994).

The importance of mineralisation/immobilisation reactions for plant nutrition has led to a considerable wealth of information concerning net N and P mineralisation rates in a number of soils including heathlands (Berendse *et al.*, 1989) and acid and calcareous grasslands (e.g. Morecroft *et al.*, 1994). Both acute and chronic inputs of N have often resulted in contrasting changes in net N mineralisation rates (Hall *et al.*, 1994; Morecroft *et al.*, 1994). Morecroft *et al.* (1994) demonstrated a significant linear relationship between N mineralisation and N deposition rates after 3 years of treatments at the present study site. However, less information is available regarding the utilisation of specific substrates by soil microorganisms. Changes in respiration rates following amendment of soils with organic substrates can be used as an indicator of microbial substrate utilisation (Sparling *et al.*, 1981). This approach was adopted by Barak *et al.* (1990) and Hopkins *et al.* (1994, 1997)

who reported increased microbial activity following amendment of soils with a number of different amino acids. Hopkins *et al.* (1994) reported significant differences in metabolism of D- and L-isomers of alanine, glutamine and glutamic acid. The D-amino acid induced respiration rate was apparently sensitive to streptomycin which implicated bacteria in D-amino acid metabolism. More recently, Hopkins *et al.* (1997) showed that long-term (97 years) fertiliser additions of ammonium sulphate at 3.6 g N m^{-2} y^{-1} significantly reduced metabolism of both the D- and L-isomers of alanine, glutamine and glutamic acid during a 6 h incubation. These results indicate that bacteria may have an important role in amino acid mineralisation, and that amino acid mineralisation by the whole soil microbial community can be affected by long-term N treatments.

The potential for P mineralisation by the soil microbial biomass is often assessed by measuring soil PME activity. This enzyme has been measured in a number of soils in response to a range of perturbations and nutrient enrichments (e.g. Bååth *et al.*, 1981) or in soils with contrasting physical, chemical and biological properties (e.g. Harrison, 1983). Strong relationships between total organic P and soil PME activity have been reported (Dalal, 1977), but there is little information relating PME activity with the utilisation of specific P compounds. This is primarily because few studies have quantified microbial utilisation of organic P sources in whole samples, despite it being well known that many fungal and bacterial isolates can produce active extracellular phosphatases and utilise a range of P sources (Cosgrove, 1967). Nevertheless, the ability of the microbial biomass of three alkaline soils to utilise a number of organic P sources, including glycerophosphate and nucleic acid, has been investigated (El-Shinnawi *et al.*, 1991; Waly *et al.*, 1991). The authors found that addition of 500 ppm N affected the mineralisation of these compounds and the availability of the inorganic P subsequently released. The form of applied N also had a strong effect; ammonium sulphate resulted in the highest amount of P released followed by ammonium nitrate and calcium nitrate respectively. Nordgren (1992) used a similar approach to determine the principal limiting nutrient in a forest soil by measuring respiration rates following amendment of soil samples with glucose in combination with either N or P. The greatest respiratory response occurred in response to the glucose + N addition indicating that the microbial population was more limited by N than by P. These approaches may be of considerable value in the present study where significant increases in

soil PME activity have been reported, and where changes from N to P as the principal limiting nutrient are thought to be occurring.

The rate of utilisation of specific groups of C sources is increasingly being used for genetic, metabolic and taxonomic studies of microorganisms (Bochner and Savageau, 1977; Garland and Mills, 1991; Colores *et al.*, 1996). This has led to the development of commercially available sole C source utilisation plates ('BIOLOG'). The plates comprise 96 wells of which 95 contain a C source and a tetrazolium based redox dye which forms the insoluble purple formazan following reduction of NAD to NADH during microbial respiration. Inoculation of the wells with soil suspensions has enabled the characterisation of the functional diversity of bacteria from soil supporting different plant communities based on the metabolic discrimination between the C sources (Garland and Mills, 1991; Zak *et al.*, 1994; Garland, 1996). This approach has been taken further by adding more ecologically relevant user defined substrates to BIOLOG plates that contain only the redox dye. Campbell *et al.* (1997) combined standard and customised plates in an investigation of grassland rhizosphere microbial communities. These results indicate that the use of smaller plates containing ecologically relevant user defined C sources provide a more meaningful and economical alternative to the standard plates. This approach has not been applied to soils receiving chronically enhanced atmospheric N deposition. The technique may be particularly useful since the addition of different P sources to the plates may provide evidence for changes in the rates of P turnover in these soils, further strengthening the view that these sites are becoming principally P limited. The information gained from BIOLOG plates relates only to a sub-sample of the whole microbial community (i.e. culturable bacteria). However, by combining the technique with other measurements of the activity of soil microorganisms (PME activity; respiration), a more accurate picture of the functional capabilities of the entire soil microbial community can be attained. Although saprotrophic and, especially, mycorrhizal fungi are the dominant microorganisms in acid soils (in terms of biomass) and are therefore probably the most important in nutrient cycling processes, there is little information regarding the functional capabilities of bacteria in these soils, particularly in their response to enhanced N deposition.

7.1.1 Aims

The aims of this chapter is to investigate the effects of long-term N additions on the utilisation of C, N and P sources by soil microorganisms. The potential rates of C, N and P source utilisation by soil bacteria will be examined using standard BIOLOG microtitre plates. In addition, a series of customised BIOLOG plates will be developed which contain a range of inorganic and organic N and P sources. The effect of N treatments on the utilisation of C, N and P sources by microorganisms within whole soil samples will be investigated by monitoring the respiratory response of fresh soil samples to nutrient amendments.

7.2 MATERIALS AND METHODS

7.2.1 BIOLOG microtitre plates

i) Standard plates

Standard BIOLOG GN micro plates were used for the heathland and acid grassland soils, and GP plates for the calcareous grassland. The GN and GP plates differ only in the range of substrates, although there is considerable overlap (Appendix B, Tables B1 and B2). Both plates include a range of carbohydrates, carboxylic acids, amino acids and phenolic acids, the remainder being a miscellaneous mixture of polymers, alcohols and heterocyclic N compounds. In the GN plates, this included three P containing compounds, glucose-1-phosphate, glucose-6-phosphate and D, L-*α*-glycerol phosphate. The GP plates included 4 additional P sources, adenosine-5'-monophosphate, thymidine-5'-monophosphate, uridine-5'-monophosphate and fructose-6-phosphate. The preformed plates comprise 96 wells of 200 µl capacity, of which 95 contain 5 mg dry weight of an individual C source while the remaining C free well acts as a control.

ii) Custom plates

BIOLOG MT plates, which are identical to the GN and GP types except they do not contain any C sources, were used as the basis for investigations into the utilisation of a number of user defined C sources. One set of plates[1], constructed at the Macaulay Land Use Research Institute ('MT1 plates'), contained 30 ecologically relevant C sources which were primarily phenolic acids (Johnson, 1994; Campbell *et al.*, 1997). The second set of plates ('MT2 plates'), constructed in Sheffield, included both organic and inorganic N and P

[1] The MT1 plates were constructed by Dr. C. D. Campbell whose help is gratefully acknowledged.

sources (Table 7.1). Unlike the standard BIOLOG plates, the substrates in this set were added on a dry weight C, N or P basis. The amount of C added to each well was 1.2 mg. This was calculated to be the same as the wells of the standard plates containing glucose as a sole C source. Glucose was added with substrates that did not contain any C, i.e. inorganic N and P sources. Substrates containing either C and N or C and P were added using C as the weight determining factor. N was used as the weight determining factor for $NH_4H_2PO_4$ which contains both N and P (C added separately as glucose).

The substrates were made as solutions (100 ml for glucose; 20 ml for all others) with deionised water and sterilised either by autoclaving for 20 minutes (all inorganic N and P sources) or by filtration (0.45 μm). Any contamination was accounted for by using appropriate blank plates (see section 7.2.1.iii below). Sterile glucose solution was added to the inorganic substrates after each had been sterilised. 200 μl of each substrate combination were added aseptically to a well of a BIOLOG MT plate in a laminar flow cabinet so that each plate contained 6 complete sets of substrate. The plates were left to dry in the cabinet with their lids removed for up to 48 hours.

iii) Preparation of soil suspensions and inoculation of plates

Initial experiments with soil from the heathland and acid grassland used GN and MT1 plates in combination, while the GP plate was used for the calcareous grassland. Subsequent investigations on the utilisation of inorganic and organic N and P sources by soil bacteria in the acid and calcareous grassland used the customised MT2 plates. The plates were inoculated with soil suspensions from the H horizon in the heathland and Ah horizon in the acid and calcareous grasslands. A 10 g sample of field moist soil was added to 100 ml sterile deionised water and shaken on a wrist action shaker for 10 minutes. Ten fold serial dilutions were made from which the 400× dilution for the heathland and the 1000× for the acid and calcareous grasslands were identified as being the lowest dilutions with minimal background colour. In the initial study using standard GN, GP and MT1 plates, the diluted samples were centrifuged at 750 *g* for 10 minutes. The standard and MT1 plates had 150 μl of soil suspension inoculated into each well while the MT2 type received 200 μl. All plates were incubated at 20 °C for up to 5 days. Potential contamination of the substrates in the customised plates was accounted for by inoculating a plate with sterile (by filtration through

a 0.45 μm filter) deionised water, and subtracting the optical density values from those obtained with soil suspensions. The plates were shaken on an automated shaker-plate reader for 20 s and the colour development of the redox dye in each well was measured at 590 nm approximately every 24 h.

Absolute rates of colour development were compared in wells containing all C sources, organic P compounds and amino acids in response to the N treatments. The mean average well colour development (AWCD) of each substrate group during the initial 96 h of incubation were compared using two-way ANOVA and Tukey multiple comparison test. Absorbance values are expressed as optical density (OD) units.

Table 7.1. Substrate composition (mg C, N or P well^{-1}) and treatment codes of customised BIOLOG MT plates.

Substrate	Formula	Treatment code	mg C	mg N	mg P
Glucose	$C_6H_{12}O_6$	G	1.20	0	0
Calcium nitrate + glucose	$Ca(NO_3)_2$-$4H_2O$	CNG	1.20	0.24	0
Ammonium sulphate + glucose	$(NH_4)_2SO_4$	ASG	1.20	0.24	0
Ammonium nitrate + glucose	NH_4NO_3	ANG	1.20	0.24	0
Ammonium orthophosphate + glucose	$NH_4H_2PO_4$	APG	1.20	0.24	0.53
Sodium dihydrogen orthophosphate + glucose	NaH_2PO_4-H_2O	SPG	1.20	0	0.24
Glycerophosphate	$C_3H_7O_6PNa_2.5H_2O$	GP	1.20	0	1.03
a-naphthyl phosphate	$C_{10}H_7O_4PNa$	NP	1.20	0	0.31
Histidine	$C_6H_9N_3O_2H_2O$	Hi	1.20	0.70	0
Glutamic acid	$C_5H_{10}O_3N_2$	Gl	1.20	0.56	0
Asparagine	$NH_2COCH_2(NH_2)COOH.H_2O$	As	1.20	0.733	0

7.2.2 Soil respiration

Changes in soil respiration rates were used as indicators of microbial utilisation of inorganic and organic N and P sources added to whole soil samples from the heathland and calcareous grassland. Soils were obtained from the H horizon of the heathland and Ah horizon of the calcareous grassland and prepared as described in section 2.2.1. The substrates and their concentrations are shown in Table 7.2. Substrates were added as a solution with deionised water to enable even and rapid distribution throughout the soil samples with a minimum of disturbance and to remove any potential water limitation (West and Sparling, 1986). Glucose was added in combination (5 mg g^{-1} soil dwt) in order that substrates were utilised as N or P sources, rather than as C sources, and also to ensure that the microbial population was in its most active state. The respiration rate was measured in a series of glucose controls in which parallel samples of soil had received only additions of glucose. The substrate solutions were added to fresh soil samples (1 g dry weight equivalent) and the respiration rates measured every 15 minutes for 5 h at 22 °C with a 20 channel automated manometric electrolytic respirometer (Merit 20, E. R. Addington (Engineers) Ltd.).

Table 7.2. Substrates and their concentrations used in combination with glucose (5 mg g^{-1} soil dwt) that were added to the heathland and calcareous grassland soils.

Substrate	Concentration (g^{-1} soil dwt)
DNA	2 mg
Glycerophosphate	2 mg
Histidine	2 mg
Sodium orthophosphate	0.2 mg P
Ammonium nitrate	0.2 mg N

7.3 RESULTS

7.3.1 Utilisation of C, N and P sources in BIOLOG plates

i) Standard and MT1 plates

The 96 h average well colour development (AWCD) measured in optical density units (OD) ranged from 0.011 to 0.25 OD in the heathland, 0.23 to 0.48 OD in the acid grassland and from 0.25 to 0.95 OD in the calcareous grassland (Table 7.3). There were significant effects of N treatments on the utilisation of C sources and the combined C-P and C-N sources in BIOLOG plates. The AWCD for all 125 C sources inoculated with the heathland soil increased significantly from 0.06 OD in the control to 0.20 OD in the N treated plots ($P<0.05$), there being no further increase from the 8 to the 12 g N m^{-2} y^{-1} treatment (Table 7.3). In the wells containing amino acids as sole C sources there was a similar N treatment effect, AWCD increasing significantly ($P<0.05$) by a factor of approximately 3 from 0.075 OD in the control to 0.25 OD in the 8 N and 0.22 OD in the12 N treatments (Table 7.3), again there being no difference between the two levels of N treatment. In contrast, the AWCD for the organic P compounds not only increased by an order of magnitude from 0.01 OD in the control to 0.1 OD in the 8 N treatment ($P<0.01$), but increased further to 0.19 OD in the 12 N treatment ($P<0.05$).

Unlike the heathland site, C source utilisation in the acid grassland soil was consistently reduced by the N treatments, the effects being similar for all C sources (Table 7.3). The AWCD of all three groups of C sources was significantly ($P<0.05$) lower in the 14 N AS treatment when compared with the control while the 14 N treatment significantly reduced only the utilisation of amino acids and organic P compounds ($P<0.05$).

A similar pattern was seen in the calcareous grassland, where utilisation of C sources in the plates containing soil suspensions from the N treated plots was lower than in the controls. However, significant differences were only seen in the 14 N and 14 N AS treatments. The AWCD for all 95 C sources decreased significantly ($P<0.05$) by 53 % from 0.68 OD to 0.32 OD in the 14 N treatment and by 43 % to 0.39 OD ($P>0.05$) in the 14 N AS treatment (Table 7.3). As with the heathland site, the N treatment effects were not of a consistent magnitude for each group of substrates. The AWCD for the amino acid group was only significantly lower in the 14 N treatment, where it decreased by 39 % from 0.95 OD to 0.58

OD. For the organic P groups however, AWCD decreased significantly by over 70 % from 0.86 OD to 0.25 OD in the 14 N treatment, and by over 60 % to 0.33 OD in the 14 N AS treatment (Table 7.3).

Table 7.3. Average well colour development of BIOLOG plates inoculated with soil suspensions from heathland, acid grassland and calcareous grassland plots that have received seven years of N treatments (± SEM). Values sharing a letter are not significantly different (P>0.05). Sites and substrate groups analysed independently.

	Heathland		
Treatment (m^{-2} y^{-1})	Total (all C sources)	Amino acids	Organic P
Control (water only)	0.06^{a} (± 0.02)	0.08^{a} (± 0.03)	0.011^{a} (± 0.007)
8 N (8 g NH_4NO_3-N)	0.20^{b} (± 0.06)	0.25^{b} (± 0.07)	0.10^{b} (± 0.03)
12 N (12 g NH_4NO_3-N)	0.20^{b} (± 0.05)	0.22^{b} (± 0.06)	0.19^{c} (± 0.06)
Treatment (m^{-2} y^{-1})	Acid grassland		
Control (water only)	0.39^{a} (± 0.09)	0.48^{a} (± 0.10)	0.34^{a} (± 0.10)
14 N (14 g NH_4NO_3-N)	0.33^{ab} (± 0.08)	0.40^{b} (± 0.10)	0.27^{b} (± 0.07)
14 N AS (14 g $(NH_4)_2SO_4$-N)	0.30^{b} (± 0.07)	0.36^{b} (± 0.10)	0.23^{b} (± 0.08)
Treatment (m^{-2} y^{-1})	Calcareous grassland		
Control (water only)	0.68^{a} (± 0.11)	0.95^{a} (± 0.05)	0.86^{a} (± 0.17)
3.5 N (3.5 g NH_4NO_3-N)	0.53^{ab} (± 0.08)	0.80^{a} (± 0.06)	0.52^{ab} (± 0.12)
14 N (14 g NH_4NO_3-N)	0.32^{b} (± 0.04)	0.58^{b} (± 0.02)	0.25^{b} (± 0.03)
14 N AS (14 g $(NH_4)_2SO_4$-N)	0.39^{b} (± 0.03)	0.77^{ab} (± 0.04)	0.33^{b} (± 0.09)

ii) MT2 plates

The AWCD of customised MT2 plates containing glucose with and without added inorganic N and P sources and inoculated with soil suspensions from the acid grassland ranged from 0.04 OD to 1.25 OD (Fig. 7.1a). The AWCD for glucose added in combination with ammonium sulphate (ASG), ammonium nitrate (ANG), ammonium orthophosphate (APG) and sodium orthophosphate (SPG) was higher than for glucose alone. The utilisation of glucose and glucose + calcium nitrate (CNG) was not affected by the N treatments (Fig. 7.1a), although the overall AWCD was considerably lower in wells supplied with CNG than glucose alone. However, significant N treatment effects were seen for ASG, ANG, APG and SPG. This was most marked for ASG where the AWCD significantly ($P<0.05$) decreased from 1.25 OD in the control to 0.75 OD in the 14 N treatment and further decreased to 0.5 in the 14 N AS treatment (Fig. 7.1a). Similar effects were also seen for ANG, APG and SPG where AWCD was lower in the 3.5 N treatment and significantly ($P<0.05$) lower in the 14 N treatment. However, the 14 N AS treatment had no effect on AWCD of wells containing ANG. For APG and SPG, AWCD was significantly ($P<0.05$) lower in the 14 N AS treatment, but only to the same degree as the 14 N treatment.

The AWCD of customised MT2 plates containing organic N and P sources and inoculated with soil suspensions from the acid grassland ranged from 0.02 OD to 1.53 OD (Fig. 7.1b). The lowest values were seen in wells containing glycerophosphate (GP) and histidine (Hi), and the highest in wells containing glutamic acid (Gl) and asparagine (As). No absorbance readings could be obtained for α-naphthyl phosphate for this soil. Significant ($P<0.05$) N treatment effects were seen only for GP and Hi (Fig. 7.1b). For GP, AWCD decreased from 0.2 OD in the control to 0.06 OD in the 14 N treatment and 0.02 OD in the 14 N AS treatments. For Hi, AWCD increased significantly ($P<0.05$) from 0.3 OD in the control to 0.46 OD in the 3.5 N treatment but subsequently decreased to 0.11 OD in the 14 N AS treatment, while the 14 N treatment had no effect. Although no significant N treatment effects were seen in the wells containing Gl and As, there was an indication of a less active microbial population in the 14 N treatment where AWCD was reduced by 25 % for Gl and 31 % for As (Fig. 7.1b).

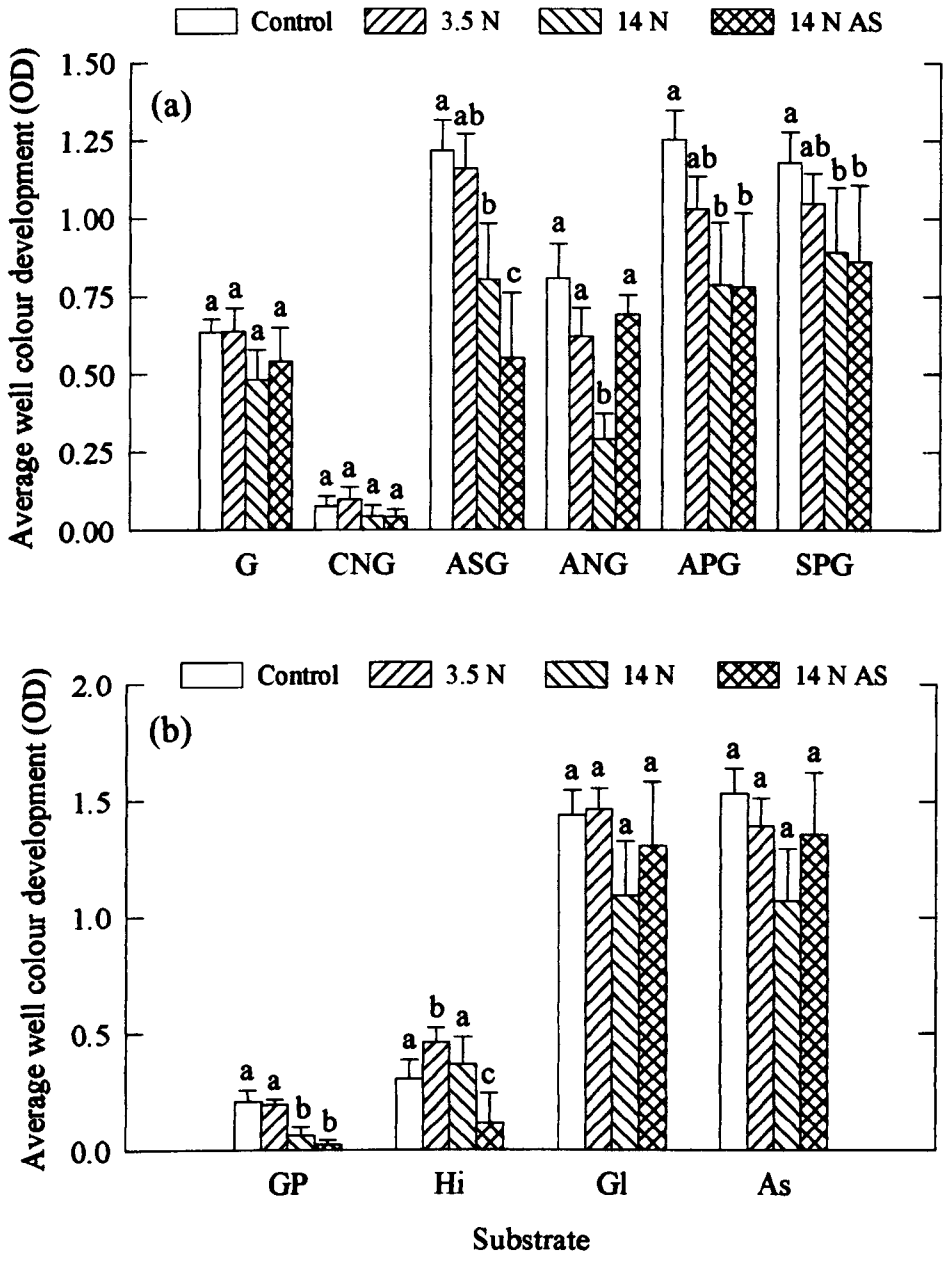


Fig. 7.1. Utilisation of (a) glucose and inorganic N and P sources and (b) organic N and P compounds in customised BIOLOG MT2 plates containing soil suspensions from the Ah horizon of acid grassland plots that have received 0 (Control), 3.5 (3.5 N) and 14 (14 N) g N m^{-2} y^{-1} as ammonium nitrate and 14 (14 N AS) g N m^{-2} y^{-1} as ammonium sulphate for seven years (+SEM). Values sharing a letter are not significantly different (P>0.05). Substrate codes as for Table 7.1.

The AWCD of customised MT2 plates containing glucose with and without added inorganic N and P sources and inoculated with soil suspensions from the calcareous grassland ranged from 0.06 OD to 1.43 OD (Fig. 7.2). The AWCD in wells containing glucose only (G) was not significantly affected by the N treatments. However, in contrast to the acid grassland, there was a strong trend for increased AWCD in response to the 14 N and 14 N AS treatments. In the 14 N treatment, the AWCD increased by 55 % from the control and significantly by 65 % from the 3.5 N treatment (Fig. 7.2). This trend was seen in only the ammonium nitrate + glucose combination (ANG) where AWCD increased significantly in both the 14 N and 14 N AS treatments. AWCD was not affected by the N treatments in any of the remaining substrate combinations (Fig. 7.2). The stimulatory effect of the 14 N and to a lesser extent, the 14 N AS treatments on the AWCD of wells containing glucose alone could disguise the effect of the inorganic N and P sources on bacterial activity in the wells. When the glucose only response was subtracted from the combined substrate responses, the N treatments were found to have a stronger effect on utilisation of the inorganic N and P sources (Table 7.4). AWCD in the ammonium nitrate and sodium orthophosphate wells was significantly ($P<0.05$) lower in response to the 14 N treatment. Similar but non-significant decreases were also seen in wells containing ammonium sulphate and ammonium orthophosphate (Table 7.4).

The AWCD of MT2 plates containing organic N and P sources and inoculated with soil suspensions from the calcareous grassland ranged from 0.01 OD to 1.5 OD (Fig. 7.3). The AWCD in glutamic acid (Gl) and asparagine (As) wells was considerably greater than for the other substrates and ranged from 1.1 OD to 1.5 OD. The AWCD of wells containing glycerophosphate (GP) was significantly ($P<0.05$) lower in the 14 N treatment while both the 3.5 N and 14 N treatments significantly reduced the AWCD of wells containing histidine (Hi). No N treatment effects were seen for *a*-napthyl phosphate (NP), Gl or As.

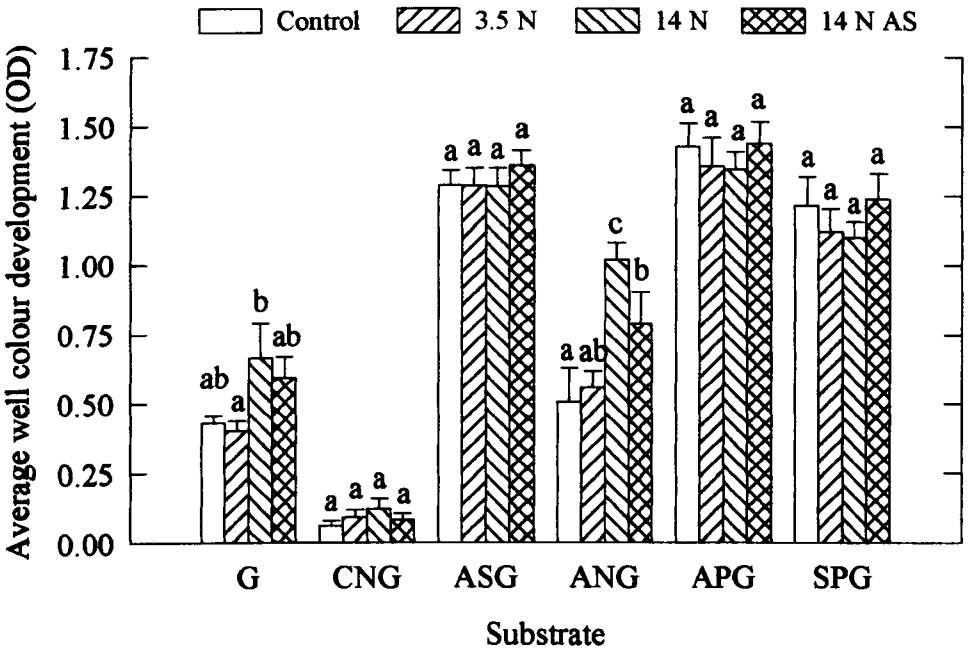


Fig. 7.2. Utilisation of glucose and glucose + inorganic N and P sources in customised BIOLOG MT2 plates containing soil suspensions from calcareous grassland plots that have received 0 (Control), 3.5 (3.5 N) and 14 (14 N) g N m^{-2} y^{-1} as ammonium nitrate and 14 (14 N AS) g N m^{-2} y^{-1} as ammonium sulphate for seven years (+ SEM). Values sharing a letter are not significantly different (P>0.05). Substrate codes as for Table 7.1.

Table 7.4. The increase (+) or decrease (-) in AWCD of customised BIOLOG MT2 plates containing inorganic N and P sources inoculated with soil suspensions from a calcareous grassland soil that has received 0 (control), 3.5 (3.5 N) or 14 (14 N) g N m^{-2} y^{-1} as ammonium nitrate or 14 (14 N AS) g N m^{-2} y^{-1} as ammonium sulphate for seven years in relation to the AWCD of plates containing glucose alone (± SEM). Values sharing a letter are not significantly different (P>0.05).

Treatment	Calcium nitrate	Ammonium sulphate	Ammonium nitrate	Ammonium orthophosphate	Sodium orthophosphate
Control	-0.37^{ab} (± 0.03)	+0.86^{a} (± 0.05)	+0.07^{a} (± 0.11)	+0.99^{a} (± 0.08)	+0.78^{a} (± 0.10)
3.5 N	-0.31^{a} (± 0.04)	+0.88^{a} (± 0.06)	+0.15^{a} (± 0.06)	+0.95^{a} (± 0.11)	+0.72^{ab} (± 0.09)
14 N	-0.55^{b} (± 0.14)	+0.72^{a} (± 0.06)	+0.46^{b} (± 0.08)	+0.79^{a} (± 0.07)	+0.53^{b} (± 0.06)
14 N AS	-0.51^{b} (± 0.07)	+0.77^{a} (± 0.06)	+0.20^{a} (± 0.09)	+0.84^{a} (± 0.10)	+0.65^{ab} (± 0.12)

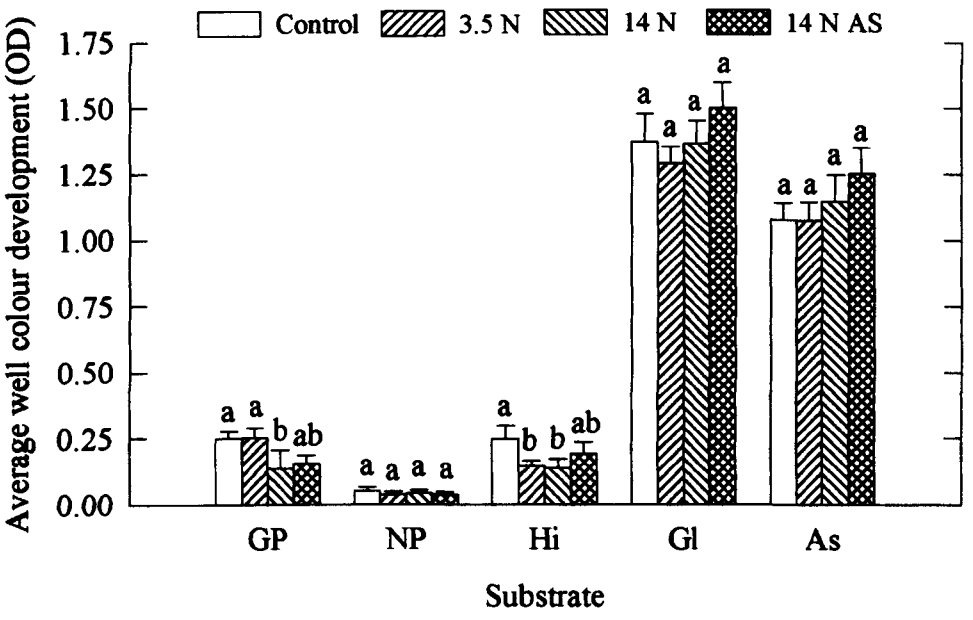


Fig. 7.3. Utilisation of organic N and P sources in customised BIOLOG MT2 plates containing soil suspensions from calcareous grassland plots that have received 0 (Control), 3.5 (3.5 N) and 14 (14 N) g N m^{-2} y^{-1} as ammonium nitrate and 14 (14 N AS) g N m^{-2} y^{-1} as ammonium sulphate for seven years (+ SEM). Values sharing a letter are not significantly different (P>0.05). Substrate codes as for Table 7.1.

7.3.2 Soil respiratory response following addition of C, N and P sources

Soil respiration rates ranged from approximately 1200 to 2300 μg CO_2 g^{-1} soil dwt h^{-1} in the heathland and from 1200 to 2000 μg CO_2 g^{-1} soil dwt h^{-1} in the calcareous grassland (Fig. 7.4a, b). In the heathland, the 4 N and 8 N treatments resulted in a progressive increase in respiration rates in response to glucose (G), DNA + glucose (DNA) and sodium orthophosphate + glucose (SP). However, this effect was only significant (P<0.05) in response to the 8 N treatment in the soils receiving SP where the respiration rate increased from 1200 μg CO_2 g^{-1} soil dwt h^{-1} in the control to 2000 μg CO_2 g^{-1} soil dwt h^{-1} (Fig. 7.4a). Both the 8 N and 4 N treatments had a stimulatory effect the respiration rate when glucose

was added in combination with glycerophosphate (GP), histidine (Hi) and Ammonium nitrate (AN). The slightly stronger effect of the 8 N treatment resulted in significant ($P<0.05$) increases in the respiration rate following addition of the GP and Hi substrate combinations (Fig. 7.4a).

In contrast to the heathland, there were no significant changes to the respiration rate of the calcareous grassland soil in response to the N treatments, regardless of the type of substrate added. There was, however, a trend for a progressive decrease in respiration rate with increasing N deposition (as ammonium nitrate) following addition of all substrates except SP (Fig. 7.4b). For example, the respiration rate in the glucose only amended soil decreased by 11 % in the 3.5 N treatment and by 30 % in the 14 N treatment. The respiration rates remained relatively constant in response to the 14 N AS treatment for all substrate combinations.

The effects of the N treatments on the respiration rate of the heathland soil for the entire duration of the incubations (300 minutes) were less apparent when the glucose only responses were removed from the glucose + N or P responses (Table 7.4). Significant ($P<0.05$) increases were only seen following addition of sodium orthophosphate, where the respiration rate increased from 7 $\mu g\ CO_2\ g^{-1}$ soil dwt h^{-1} in the control to 298 $\mu g\ CO_2\ g^{-1}$ soil dwt h^{-1} in the 8 N treatment. Ammonium nitrate was the only substrate that consistently reduced the respiration rate in response to the N treatments. Here the respiration rate was reduced by 300 % in the 4 N and by 490 % in the 8 N treatments, although these decreases were not significant ($P>0.05$). The addition of histidine resulted in a stimulatory effect in response to the 4 N treatment, while a slight inhibitory effect was seen in the 8 N treatment. The N treatments appeared to have a greater impact on the respiration rates during the initial 60 minutes of the incubations. The treatments significantly increased the respiration rate following addition of DNA, glycerophosphate, histidine, and sodium orthophosphate (Table 7.4).

The removal of the glucose only response in the calcareous grassland soil reversed the trend that was previously seen. The N treatments progressively increased the respiration rates following addition of DNA, glycerophosphate and histidine, although these differences were not significant ($P>0.05$). However, when the responses for all of the substrates were

analysed in combination, the respiration rate increased significantly ($P<0.05$) from 36 μg CO_2 g^{-1} soil dwt h^{-1} in the control to 323 μg CO_2 g^{-1} soil dwt h^{-1} in the 14 N AS treatment (Table 7.5). In contrast to the heathland, the respiratory responses were consistent throughout the incubation period.

Soil PME activities in the heathland and calcareous grassland plots were plotted against the respiration rates following amendment with glycerophosphate (glucose only respiration subtracted). Significant linear relationships were seen for both sites, although this was most marked at the heathland where soil PME activity accounted for 47 % of the variation of the respiratory response (Fig. 7.5a, b). A stronger relationship ($r^2 = 0.77$; $P<0.001$) was found for the AWCD of BIOLOG GN plates containing organic P compounds inoculated with soil suspensions from the heathland plots (Fig. 7.5c). No relationship was found for BIOLOG GP plates inoculated with soil from the calcareous grassland plots ($r^2 = 0.13$; $P<0.17$).

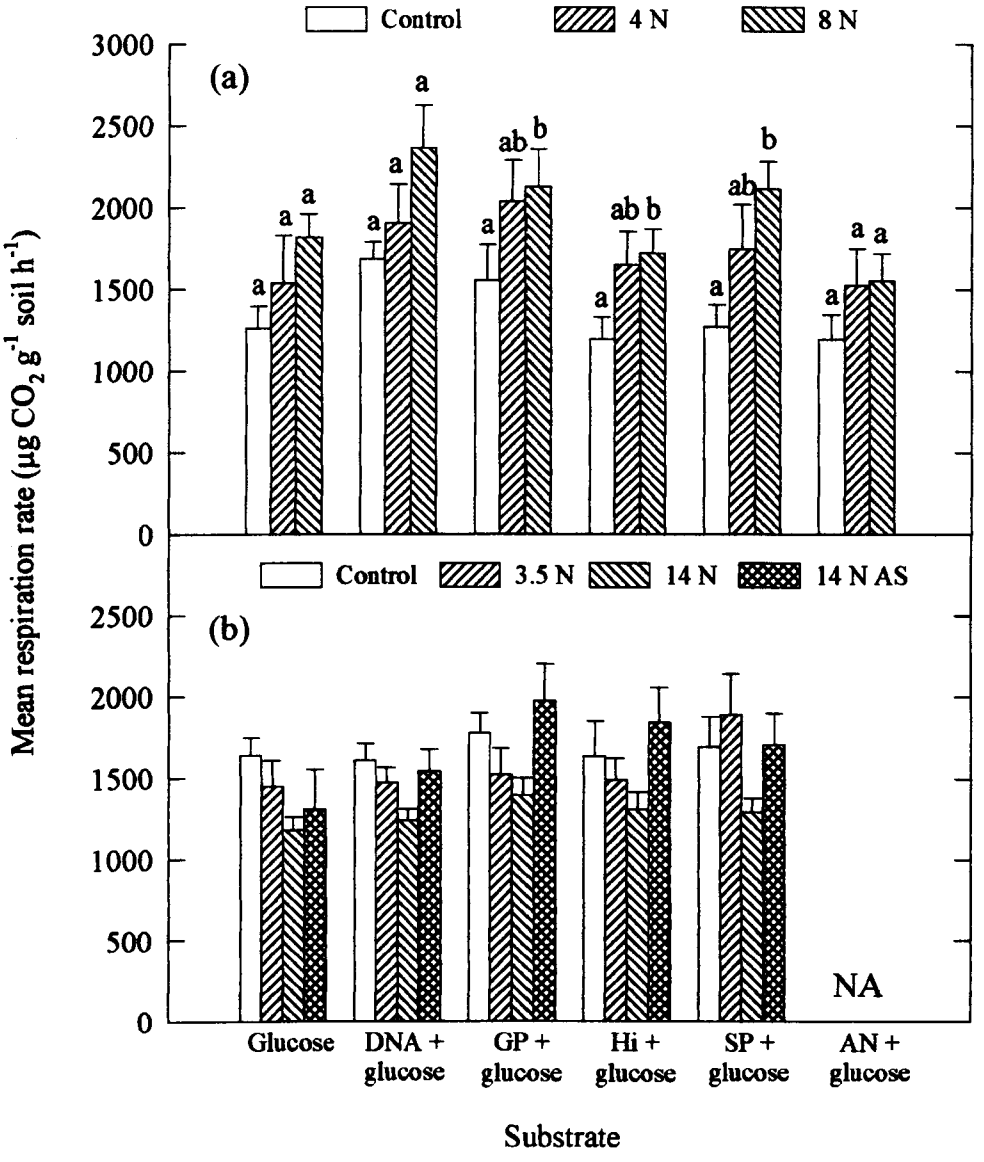


Fig. 7.4. Mean respiration rates of (a) a heathland soil that has received 0 (Control), 4 (4 N) and 8 (8 N) g N m^{-2} y^{-1} as ammonium nitrate for eight years and (b) a calcareous grassland soil (no significant differences; $P>0.05$) that has received 0 (Control), 3.5 (3.5 N) and 14 (14 N) g N m^{-2} y^{-1} as ammonium nitrate and 14 (14 N AS) g N m^{-2} y^{-1} as ammonium sulphate for seven years following amendment with C, N and P sources during a 5 hour incubation (+ SEM). Values sharing a letter are not significantly different ($P>0.05$). NA = not analysed. Substrate codes as for Table 7.1.

Table 7.5. The increase (+) or decrease (-) in the glucose induced respiration rate of a heathland soil that has received 8 years of N treatments following amendment with C, N and P sources (μg CO_2 g^{-1} soil dwt h^{-1}). Values sharing a letter are not significantly different (P>0.05).

Treatment	0-60 minutes				
	DNA	Glycerophosphate	Histidine	Sodium orthophosphate	Ammonium nitrate
Control	-167^{a}	+84^{a}	-337^{a}	-455^{a}	-414^{a}
4 N	-4^{a}	+645^{b}	+66^{b}	+216^{b}	-389^{a}
8 N	+1063^{b}	+554^{ab}	-117^{ab}	+183^{b}	-513^{a}
	0-300 minutes				
Control	+313^{a}	+237^{a}	-67^{ab}	+7^{a}	-67^{a}
4 N	+488^{a}	+501^{a}	+111^{a}	+208^{ab}	-268^{a}
8 N	+668^{a}	+312^{a}	-95^{b}	+298^{b}	-395^{a}

Table 7.6. The increase (+) or decrease (-) in the glucose induced respiration rate of a calcareous grassland soil that has received 7 years of N treatments following amendment with C, N and P sources (μg CO_2 g^{-1} soil dwt h^{-1}). Values sharing a letter are not significantly different (P>0.05).

Treatment	0-300 minutes				
	DNA	Glycerophosphate	Histidine	Sodium orthophosphate	All substrates
Control	-26^{a}	+123^{a}	-10^{a}	+57^{a}	+36^{a}
3.5 N	+36^{a}	+77^{a}	+40^{a}	+429^{a}	+145^{a}
14 N	+62^{a}	+196^{a}	+121^{a}	+100^{a}	+120^{a}
14 N AS	+212^{a}	+617^{a}	+502^{a}	-41^{a}	+323^{b}

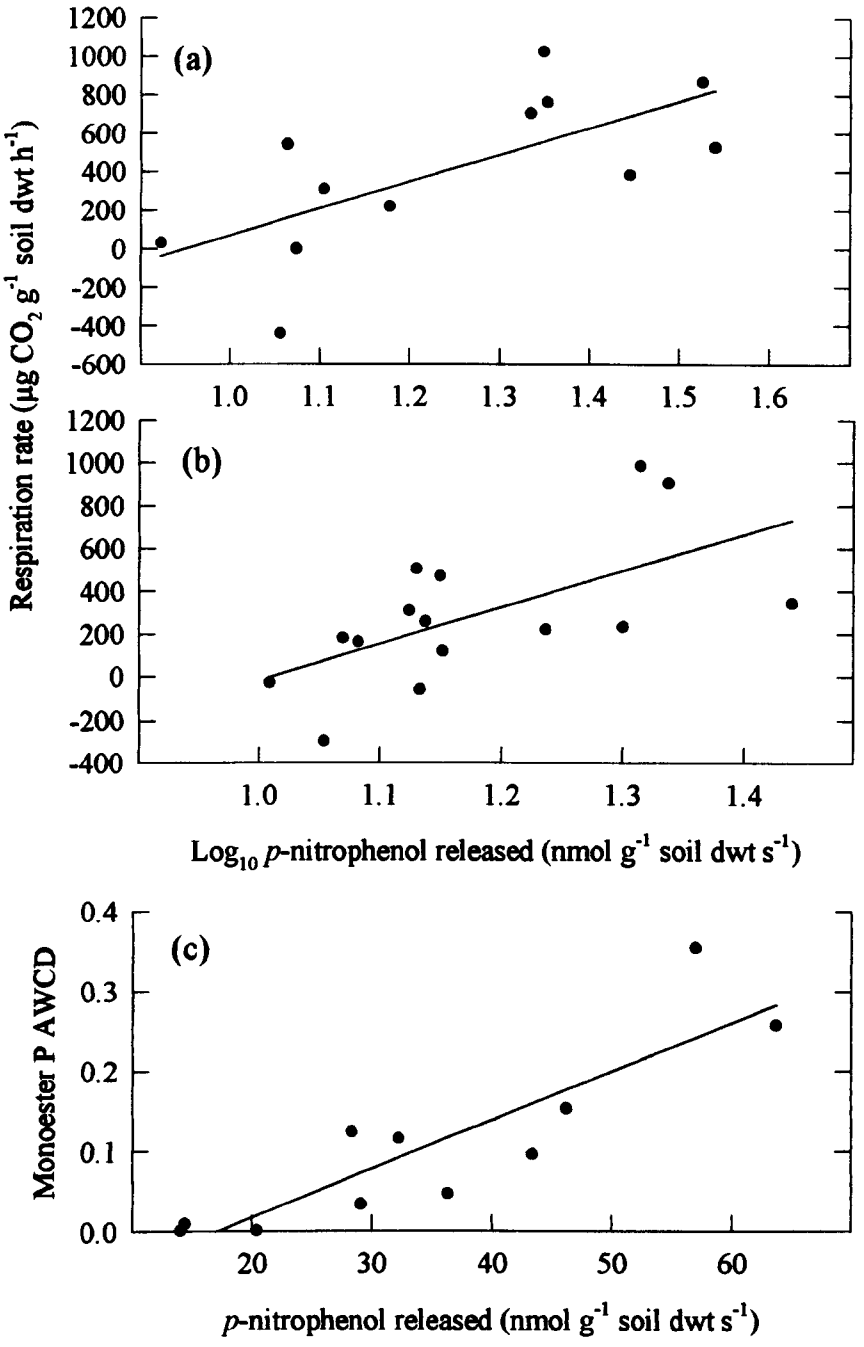


Fig. 7.5. Relationship between soil phosphomonoesterase activity and glycerophosphate induced soil respiration rate in (a) a heathland (r^2 = 0.47; P<0.014) and (b) a calcareous grassland (r^2 = 0.38; P<0.038) and soil phosphomonoesterase activity and (c) AWCD of BIOLOG GN plates containing organic P sources inoculated with soil suspensions from heathland plots (r^2 = 0.77; P<0.001).

7.4 DISCUSSION

The BIOLOG profiles revealed close parallels between the changes in C_{mic} and the average rate of C source utilisation and provide independent verification of the effects of N treatments on C_{mic} and potential catabolic activities. At the heathland site, not only was the average rate of C source utilisation in the standard and MT1 plates increased by N additions (Table 7.3), but there was also specific enhancement of rates of utilisation phosphomonoesters which increased 10-fold in the 8 N and 18-fold in the 12 N treatments. This is most likely to have arisen from a change in the bacterial population in this soil, with the N treatments selecting for bacteria with high rates of phosphomonoester utilisation. In the control plots of the heathland site, the rate of utilisation of phosphomonoester C sources was only one sixth of the average for all C sources in the BIOLOG plates. However, phosphomonoester use rose sharply with each increment of added N, and in the 12 N treatment was equal to the average for all C sources, reflecting the shift from N towards P as the principal limiting mineral nutrient. This effect was mirrored by the respiration data which increased following addition of DNA and glycerophosphate (Table 7.5). The respiratory response was most apparent during the initial 60 minutes of the incubations which may indicate that N saturation has resulted in microbial populations that are adapted for more efficient utilisation of organic P compounds. The decrease in utilisation of these compounds after 60 minutes may thus indicate either substrate limitation or an increase in the amount of energy diverted for biosysnthesis and cell maintenance rather than respiration, as reported for salt stressed streptomycetes (Killham and Firestone, 1984).

Significant increases were seen in respiration rates following amendment of soils from the heathland with histidine (Table 7.5). Histidine is thought to be an important amino acid in heathland soils where its concentration can fluctuate markedly depending on the time of year. In an analysis of 13 amino acids, Abuarghub (1987) reported that histidine and glutamic acid were found in the highest concentrations in the surface horizons of a *Calluna* heathland where they reached a peak of 28 $\mu g\ g^{-1}$ soil dwt. Plants growing in soils which have become N saturated might be expected to produce more organic N exudates and have litter with lower C:N ratios (Williams and Silcock, 1997), so that the change in potential amino acid utilisation would be consistent with adaptation of a microbial community to the increased availability of organic N substrates.

In the BIOLOG plates inoculated with soil from the acid grassland, the rate of phosphomonoester utilisation in the control was almost the same as the average rate of utilisation of all of the C sources (Table 7.3). This suggests the presence of bacterial populations potentially capable of efficient organic P utilisation at this site even prior to N treatments. N treatments here reduced C_{mic} and the average rate of utilisation of the 125 C sources, with no evidence that bacterial activity against organic P sources was any less effected. Furthermore, there was a consistent reduction in AWCD of MT2 plates containing a range of inorganic and organic N and P sources (Fig. 7.1a, b). It is therefore likely that the differences in C source utilisation between the treatments are indicating a shift in the activity of the bacterial community as a whole, rather than a functional change towards increased utilisation of specific groups of organic nutrient sources, as was seen in the heathland soil. At first sight this appears to be in conflict with the data on soil PME per mg C_{mic} (Fig. 6.6) which showed that the greatest increase in response to the N additions occurred in the acid grassland. However, the source of the soil PME has not been determined, and may include significant contributions from plant roots and fungi.

The patterns of C source utilisation in the BIOLOG GP plates inoculated with soil from the long-term calcareous grassland were similar to the acid grassland, where N treatments caused a decrease in utilisation of all C sources, amino acids and organic P compounds. This pattern was also seen to a certain extent in the MT2 plates where there was a decrease in the utilisation of glycerophosphate and histidine in response to the N treatments. There was a more pronounced decrease in utilisation of organic P compounds in the GP plates compared to the entire range of C sources and amino acids (Table 7.3). This appears to be in contrast with the MT2 plates where there was an absence of any change in the utilisation of *a*-naphthyl phosphate (NP) and may indicate that soil bacteria at this site are able to utilise a limited range of organic P sources (Fig. 7.3). This may be partially explained by the C:P ratio of NP (3.9) which is considerably higher than for glycerophosphate (1.2), indicating that it is a lower quality P source. Alternatively, bacteria may not be able to utilise NP as a C source, or NP, at least in the concentrations used in the MT2 plates, may be toxic. Furthermore, in the heathland, where there was clear evidence of selective P utilisation, the AWCD in wells containing monoester P sources in the 12 N treatment was 6× greater than for all of the C sources, while it was only 1.5× greater for the 14 N treatment in the calcareous grassland. The overall decrease in C source utilisation in the N

treated calcareous grassland plots is in agreement with the 1997 SIR C_{mic} data (Chapter 5) where similar decreases were measured. However, the results are not in agreement with the measurements of root surface and soil PME activities at this site which both increased in response to the N treatments. This indicates that long-term N treatments may have contrasting effects on different components of the soil biota; soil bacteria may have a lower demand for P when the system has become N saturated than other organisms. There are many studies that report solubilisation of phosphates by bacteria through secretion of organic acids such as oxalic, malic or citric acid (e.g. Cosgrove, 1977; Marschner *et al.*, 1987), rather than through metabolic pathways. The overall decrease in C source utilisation but increase in PME activity suggests that bacteria may have only a marginal role in P mineralisation at the calcareous grassland site, although further work is clearly necessary to elucidate the source of the PME activity. For example, VA-mycorrhizal fungi which are the most abundant mycorrhizas in most grasslands, are known to contribute significantly to the P nutrition of their host plants which is at least in part due to the production of phosphatase enzymes (Joner and Jakobsen, 1995). The absence of any raised demand for P by soil bacteria extracted from the calcareous grassland in response to the N treatments is also reflected by the AWCD in wells containing inorganic P. Here, the AWCD decreased significantly in the N treated plots, but the effect was alleviated to a certain extent in wells containing inorganic N and P and was completely reversed in the presence of ammonium nitrate-N alone where the AWCD increased significantly (Table 7.4). The soil respiration data relate to a much wider group of microorganisms than the BIOLOG plates, including both bacteria and fungi. In the calcareous grassland, there was a strong trend which indicated increased utilisation of organic N and P sources (Table 7.6) which provides further evidence for potential contrasts in the response of different components of the soil microbial biomass to increased N deposition. The increased respiratory response following amendment with histidine to the N treated calcareous grassland plots is in agreement with Morecroft *et al.* (1995) who observed increased N mineralisation at this site in response to N additions.

Significant relationships were found between soil PME activity and utilisation of monoester P compounds in both the heathland and calcareous grassland (Fig. 7.5a-c). This suggests that measurements of PME activity using the artificial substrate *p*-NPP can indicate actual changes in the utilisation of important organic P sources found in soil. In the heathland,

significant relationships were found between PME activity and both glycerophosphate induced respiration and AWCD of BIOLOG plates containing monoester P sources (Fig. 7.5a, c). This may suggest that bacteria are contributing to the bulk soil PME activity, and may thus indicate that the N treatments can stimulate P mineralisation by this group of organism. However, the significance of any contribution from soil bacteria to P turnover at the heathland site is not known. The acidity of the soil and abundance of ericoid mycorrhizal fungi (Caporn *et al.*, 1995) would suggest that they have a marginal role. Alternatively, the relationship between PME activity and monester P utilisation in BIOLOG plates may be reflecting parallel responses between bacteria and other soil microorganisms, such as fungi. Although some pot experiments have shown no apparent effect of VA-mycorrhizal fungal mycelium on numbers and activities of bacteria (e.g. Olsson *et al.*, 1996), others have demonstrated differences between bacterial populations in non-mycorrhizal and mycorrhizal plants (e.g. Secilia and Bagyaraj, 1987).

The results presented here provide further evidence of increased P demand by soil microorganisms in response to enhanced atmospheric N deposition, particularly in the heathland and calcareous grassland. In general, the results from BIOLOG plates closely mirror other microbial parameters, such as soil PME activity and microbial biomass, despite the limited group of organisms (soil bacteria) present in the plates. It is suggested that customised BIOLOG plates, such as the MT2 plates, can provide a more economical and ecologically relevant measure of microbial activity than the standard GN or GP plates.

CHAPTER 8

GENERAL DISCUSSION

8.1 GENERAL DISCUSSION

The work presented in this thesis provides an in-depth analysis of the effects of long-term (eight years) N additions to a heathland, an acid grassland and a calcareous grassland. The results provide evidence that long-term chronic inputs of N can lead to significant alterations in the nutrition of vegetation and the size and activity of the soil microbial biomass in these semi-natural ecosystems. The importance of below ground processes and the lack of data regarding impacts of chronic N inputs in semi-natural ecosystems gives these results added significance. Whilst this work has filled some of the gaps in our knowledge on the effects of N deposition on above and below ground processes in semi-natural ecosystems, the data clearly highlight the need for research into new areas, particularly concerning N and P interactions. For example, changes in root surface PME activities in the grasslands following short-term (18 months) applications of N and P provided evidence of strong interrelationships between enhanced N deposition and P demand.

The main effects of the long-term N treatments are summarised in Table 8.1. It is clear that all three sites may have become 'N saturated', although the degree of saturation appears to be most apparent in the heathland and calcareous grassland, where both extractable inorganic soil N concentrations and shoot N concentrations increased and extractable inorganic soil P decreased. N saturation in some semi-natural ecosystems has been shown to lead to considerable changes in the composition of vegetation, including widespread conversion of Dutch heathlands into grasslands and the spread of the grass *Brachypodium pinnatum* in calcareous grasslands (Bobbink, 1991; Pearson and Stewart, 1993). UK heathlands have yet to be similarly affected by increased N deposition. In the present study, *Calluna vulgaris* has in fact shown marked growth increases in response to eight years simulated N deposition (see Table 5.4). The lack of any dramatic changes in UK heathland vegetation is probably due to a number of factors. In Dutch heaths, grasses out-compete *Calluna* by colonising gaps in the canopy (Van der Eerden *et al.*, 1991). Although this process normally takes several decades, the transition can be dramatically stimulated by other factors such as drought and heather beetle attack. The heather beetle thrives on N enriched shoots and causes defoliation and reduced plant health (Heil and Diemont, 1983), but in many UK upland heaths, the unfavorable climate could prevent heather beetle population explosions. Furthermore, in the UK, heather moors are managed by regular

Table 8.1. Summary of the main effects of long-term N additions to a heathland, an acid grassland and a calcareous grassland. Figures in parentheses refer to the relevant chapter(s). ND = not determined.

Parameter	Field site		
	Heathland	Acid grassland	Calcareous grassland
Soil pH (2)	No change	No change	Reduction
Soil inorganic N (2)	Increase	Increase	Increase
Soil inorganic P (2)	Decrease	No change	Decrease
Shoot N (3,4)	Increase†	Increase	Increase
Shoot P (3,4)	ND	Decrease	No change
Root surface PME (4)	ND	Increase	Increase
Microbial C (5)	Increase	Decrease	No change
Microbial N (5)	No change	No change	No change
Microbial P (5)	No change	No change	Decrease
Soil PME (6)	Increase	Increase	Increase
Organic P utilisation (7)	Increase	No change	Increase

† Caporn *et al.* (1998)

burning which can release considerable quantities of accumulated N (Allen *et al.*, 1969). Consequently, N can only be allowed to accumulate between the periods of burning (approximately 15 years). In the present study, the increased productivity of *Calluna* will have resulted in greater uptake of P, which was largely reflected by significant reductions in soil solution orthophosphate concentrations. However, soil inorganic P concentrations were slightly higher in the 12 N treatment when compared to the 8 N treatment (Table 2.4) but plant productivity (as reflected by cover) increased significantly between these treatments (Table 5.4). This indicates that organic forms of P may have been utilised in order to maintain plant productivity. In nutrient poor mixed upland grassland communities, organic forms of P are thought to have an important nutritional role, nearly half of the organic and residual components of soluble P are in forms suitable for rapid mineralisation or direct plant uptake (Macklon *et al.*, 1994). The very fine root system of *Calluna*, which

is typically concentrated in the organic matter enriched surface horizons, and the ability of the ericoid mycorrhizal endophyte, *Hymenoscyphus ericae* to degrade a wide range of mono and diester P compounds (Mitchell and Read, 1981; Straker and Mitchell, 1986; Leake and Miles, 1996) certainly suggests that *Calluna* is suitably adapted for organic P uptake. Further work is clearly necessary to quantify the nutritional importance of organic P for *Calluna*, particularly when subjected to enhanced pollutant N deposition.

The data presented in Chapter 3 indicate that the structure of the grassland vegetation communities may be more sensitive to N inputs than the heathland. Mosses appear to be particularly sensitive to the N treatments. In the calcareous grassland, the above ground biomass of moss was reduced following just 18 months of N treatments while in the acid grassland, the shoot density of *R. squarrosus* and *P. schreberi* decreased with increasing N supply following seven years of treatments. Numerous other workers have also reported substantial decreases in moss abundance in response to atmospheric pollution (Baddeley *et al.*, 1994; Pitcairn *et al.*, 1995). Morecroft *et al.* (1994) reported a decrease in abundance of *R. squarrosus* after three years of N treatments to the acid grassland site used in the present study, but only in the ammonium sulphate treatment (14 g N m^{-2} y^{-1}). In addition, the authors did not report any decrease in moss cover at the calcareous grassland. However, the methods used by Morecroft *et al.* (1994) are likely to be less reliable than those used in the present study for the calcareous grassland, where the total above ground biomass was harvested and sorted. Decreases in moss cover in response to N additions have been reported for numerous other systems (e.g. Baddeley *et al.*, 1994). Mosses have been shown to readily absorb N from atmospheric deposition (Silcock and Williams, 1995; Baxter *et al.*, 1992). The disappearance of the bryophyte mat in the acid grassland may lead to enhanced levels of inorganic N deposition directly onto the soil surface which could stimulate soil eutrophication.

No changes in the cover of higher plant species were observed by Morecroft *et al.* (1994) after three years of N treatments. Subsequent annual surveys using the same methodology have continued which indicate reduced cover of a number species (J. Carroll, pers. comm.). The most affected species in the calcareous grassland include *Helianthemum nummularium*, *Thymus praecox* and *Carex flacca* while in the acid grassland, only the cover of *Agrostis capillaris* was significantly reduced. However, the present study revealed that the

long-term N treatments have also reduced the above ground biomass of *Anemone nemorosa* in which significant increases in the shoot N:P ratio were seen. Similar decreases in cover and biomass of *A. nemorosa* have been reported following addition of up to 18 g N m^{-2} y^{-1} to a Swedish beech stand (Falkengren-Grerup, 1993). The root system of *A. nemorosa* is characterised by a rhizome, approximately 5 mm in diameter (Shirreffs, 1985), which is thought to have an important role for starch storage (Meyer and Hellwig, 1997). Although the plants in the N treated plots may have become P limited, the reduced above ground biomass may indicate reduced C allocation from the rhizome. The rhizomes of *A. nemorosa* are often colonised by fungi of which little is known about their function. Ernst (1983) reported significant alterations in the mineral nutrition of plants which were infected with *Ochropsora ariae*, *Sclerotinia tuberosa* and *Tranzschelia anemones* and so it is likely that they could contribute to the nutrition of the plants. In the present study, the rhizomes were not removed and so no data are available regarding the degree of fungal colonisation in these plants. Further work is clearly necessary to elucidate both the function of the colonising fungi and their response when host plants are grown in soils subjected to chronically enhanced inputs of N.

It is clear from the results presented in Chapter 3 that the number and type of species at both sites, but particularly the calcareous grassland, are strongly dependent on the supply of nutrients (i.e. N or P). Grime (1973a) proposed a 'hump-backed' model describing the control of species density in herbaceous vegetation. The model suggests that the result of moderate intensities of stress is to increase species density by reducing the vigour of potential dominants, thus allowing coexistence of secondary species, while at high intensities of stress, species diversity falls to produce the characteristic 'hump-back'. The model has been shown to adequately describe changes in species richness in response to a number of environmental stresses (e.g. Grime, 1973b; Muotka and Virtanen, 1995) and to increasing plant biomass (Al-Mufti *et al.*, 1977). However, the data obtained in this study from the short-term calcareous grassland grazing exclosure experiment do not agree with this pattern. Negative linear correlations between species richness (number species m^{-2}) and total extractable inorganic N (nitrate and ammonium) and the soil inorganic N:P ratio were observed (Table 8.2) which indicates that as N deposition increases, species richness decreases. A positive linear relationship was seen between species richness and extractable soil P (Table 8.2). This contrasts to a number of other studies where addition of fertiliser to

Table 8.2. Correlation coefficients (r^2) between species richness and above ground biomass and soil N and P concentrations and above ground biomass and soil N and P concentrations in short-term calcareous grassland plots (ns = not significant ($P>0.05$); * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$; NA = not applicable).

	Above ground biomass	N	P	N:P
Species richness	-0.03^{ns}	-0.37**	0.57***	-0.49***
Above ground biomass	NA	0.11^{ns}	-0.15^{ns}	0.07^{ns}

nutrient poor environments has been shown to have an inverse effect on species diversity (e.g. Willis, 1963). In the present study, this may be explained by the importance of monocotyledons, even prior to any experimental additions of N and P. The dominant species at the calcareous grassland were *Festuca ovina* and *Carex caryophyllea* which had mean percent cover values of 80.5 and 44.2 % respectively (Table 2.2). This contrasts to the studies of Willis (1963), working on a low productivity dune system, where grasses were initially a minor component of the vegetation. Thus, in the present study, species that may otherwise be out-competed following substantial increases in growth of grasses (mainly low growing forbs such as *Thymus* sp.), as observed by Willis (1963), may have already adapted or be absent in the already grass dominated community. Consequently, at least in the short-term (18 months), these species may also be able to survive in plots where monocotyledon above ground biomass has shown only moderate increases (as compared to Willis, 1963) in response to the P treatments.

No relationship was found between species richness and above ground biomass + litter (Table 8.2). This may in part be explained by the relatively narrow range of biomass + litter values (162 to 374 g m^{-2}), which is in marked contrast to the study of Al-Mufti *et al.* (1977) who sampled within the range 175 to 2600 g m^{-2}. The optimum above ground biomass for the maximum number of species was 510 g m^{-2}. Similarly, Vermeer and Berendse (1983) reported significant negative linear relationships between these two parameters in grassland communities where the above ground biomass ranged from 400 to 1150 g m^{-2}, and the optimum biomass was between 400 to 500 g m^{-2}. These figures are considerably higher than the maximum biomass measured in the present study. It is quite conceivable,

therefore, that the vegetation response to the P additions simply is not great enough to influence the species density of the whole plant community. Clearly, other factors are much better discriminants of species richness (i.e. soil inorganic N and P concentrations and N:P ratio). Whilst species richness of the calcareous grassland community may not be related to above ground biomass, at least after 18 months nutrient additions, it is clear that individual species can be dramatically affected. For example, the virtual loss of the biennial *Gentianella amarella* (not included in the above ground biomass determinations) in both the N and P treated plots was significantly correlated to above ground biomass (Fig. 3.16). It is surprising that no relationship was found between total above ground biomass and extractable soil N and P concentrations or the soil extractable N:P ratio (Table 8.2). However, when taken together with the changes in shoot N and P concentrations, the results imply substantial foliar uptake of the applied nutrients. In many plant communities that receive elevated levels of N deposition foliar uptake of nutrients is of increasing importance (Pearson and Stewart, 1993). The observations in the present study confirm the effectiveness of the mode of treatment application as a method for simulating N deposition.

Despite the dramatic loss of some plant communities, such as Dutch lowland heaths, virtually no work has focused on the impact of chronic N inputs on the activity of soil microorganisms and nutrient cycling processes. This is surprising considering the importance of the soil microbial biomass in nutrient turnover and providing plants with an adequate supply of N and P. Furthermore, since litter-fall is a major mechanism for C input into the soil environment, any changes in plant productivity are likely to have considerable impact on the soil microbial biomass. This was seen in the present study where the loss of plant cover at the grassland sites and the increase in productivity at the heathland (Table 5.4) closely mirrored the changes in soil microbial biomass C (C_{mic}). The microbial biomass may also be affected by changes in the composition of the litter entering the soil (Wardle *et al.*, 1997). Other factors of importance may include changes in the quantity and quality of root exudation products and root/mycorrhizal turnover. For example, Ennik *et al.* (1980) reported that N fertilisation reduced sward root mass and root C input. It would therefore be of further interest to investigate possible changes in root biomass and root exudation products at the three sites.

Perhaps not surprisingly, in view of the long duration of the experimental treatments, and the fact that the highest treatments are considerably above the current rate of pollutant N deposition in the UK, the simulated aerial deposition of N has been shown to lead to significant alterations in the size and functioning of the soil microbial biomass. However, the effects in the highest N treatments were also reflected in the lower treatments (although they were not always significant). Some effects, particularly in the heathland were significant even in the lowest treatment (4 g N m^{-2} y^{-1}), which is well within the current UK rates of N deposition (UKRGAR, 1997). These included increased demand for P as reflected by soil PME activity, PME activity per mg C_{mic} and the utilisation of organic P sources, as reflected in BIOLOG plates and substrate induced respiration rates.

The consistent effects of N deposition on PME activity per mg C_{mic} at all three sites demonstrates a crucial link between the supply of N and the cycling of P in these low productivity systems. In addition, the study confirms that sites like the heathland which are initially N limited are most vulnerable to pollutant N deposition. Since this increases plant growth, microbial biomass and nutrient cycling activity, the fertility and the potential for floristic changes through competitive invasion may also increase as has occurred in Dutch lowland heaths (Van der Eerden *et al.*, 1991). Alternatively, sites such as the grasslands used in the present study in which P is the main nutrient limiting plant growth may show reduced C_{mic} and reduced plant growth. The increasing P limitation of these sites with increasing N deposition may prevent major floristic changes by reducing plant growth and preventing dominance by any one species. In the calcareous grassland site, there were no differences in higher-plant species composition after three years of N applications (Morecroft *et al.*, 1994), although more subtle changes in the proportions of species have occurred in the subsequent four years (J. Carroll, pers. comm.). This contrasts with the study of Bobbink (1991) who reported substantial increases in the productivity of the grass *Brachypodium pinnatum* following similar N additions to two chalk grasslands in Holland. However, the Dutch sites differed from the grasslands used in the present study since most species were not P limited (Bobbink, 1991). In the present study, the P limitation at the calcareous grassland was dramatically illustrated by substantial increases in above ground biomass, particularly of monocotyledonous species, following only 12 months P additions (3.5 g P m^{-2} y^{-1}). More recently, ten Harkel and van der Meulen (1996) found no effect on species composition of five years N additions to two coastal dune grasslands, and suggested

the absence of treatment effects was due to the vegetation being P rather than N limited. These studies support the view that in order for us to understand and predict effects of pollutant N on ecosystems we need to consider the availabilities of other plant growth-limiting nutrients such as P where, as in the case of the heathland and calcareous grassland sites the addition of N may increase the rates of organic P utilisation and PME activities.

A schematic model has been developed which illustrates some of the effects of increased N supply in the systems used in the present study (Fig. 8.1). The model illustrates the strong interrelationships between N deposition, N supply and P limitation. In both the grasslands, sustained elevated N deposition has resulted in higher rates of N mineralisation (Morecroft *et al.*, 1994; J. Carroll, pers. comm.) which has led to elevated concentrations of available N (i.e. KCl extractable nitrate and ammonium). Inorganic N concentrations in the heathland also increased. Although N mineralisation rates have not been measured, it is likely that this process results in only very low amounts of inorganic N at this site due to the inactivity of the microbial biomass. In the calcareous grassland and heathland, available P (i.e. $NaHCO_3$ extractable P) concentrations decreased with increasing N deposition. The decrease in soil extractable P concentrations in plots that have reduced plant cover, and therefore reduced plant biomass, may arise from slower rates of P mineralisation. Furthermore, in the calcareous grassland, it is possible that the considerable soil acidification has resulted in higher solution Ca concentrations as a consequence of dissolution of carbonates (Faurie and Fardeau, 1990), which may then increase the potential for the formation of insoluble Ca phosphates. The changes in soil inorganic N and P status were largely mirrored by shoot N and P concentrations. The subsequent alterations in plant cover and productivity, and, therefore C inputs to the soil via litter-fall are likely to be the main causes of the changes in C_{mic}. The reduced inorganic P concentrations and increased P limitation was reflected by considerable increases in soil PME activity. In the grasslands, the root surface PME activity of *Plantago lanceolata* and *Agrostis capillaris* was also stimulated by the N additions. The increased P limitation was also reflected by increased utilisation of organic P compounds and a reduction in microbial biomass P, although the exact values reported for the latter must be viewed with caution due to the methodological limitations.

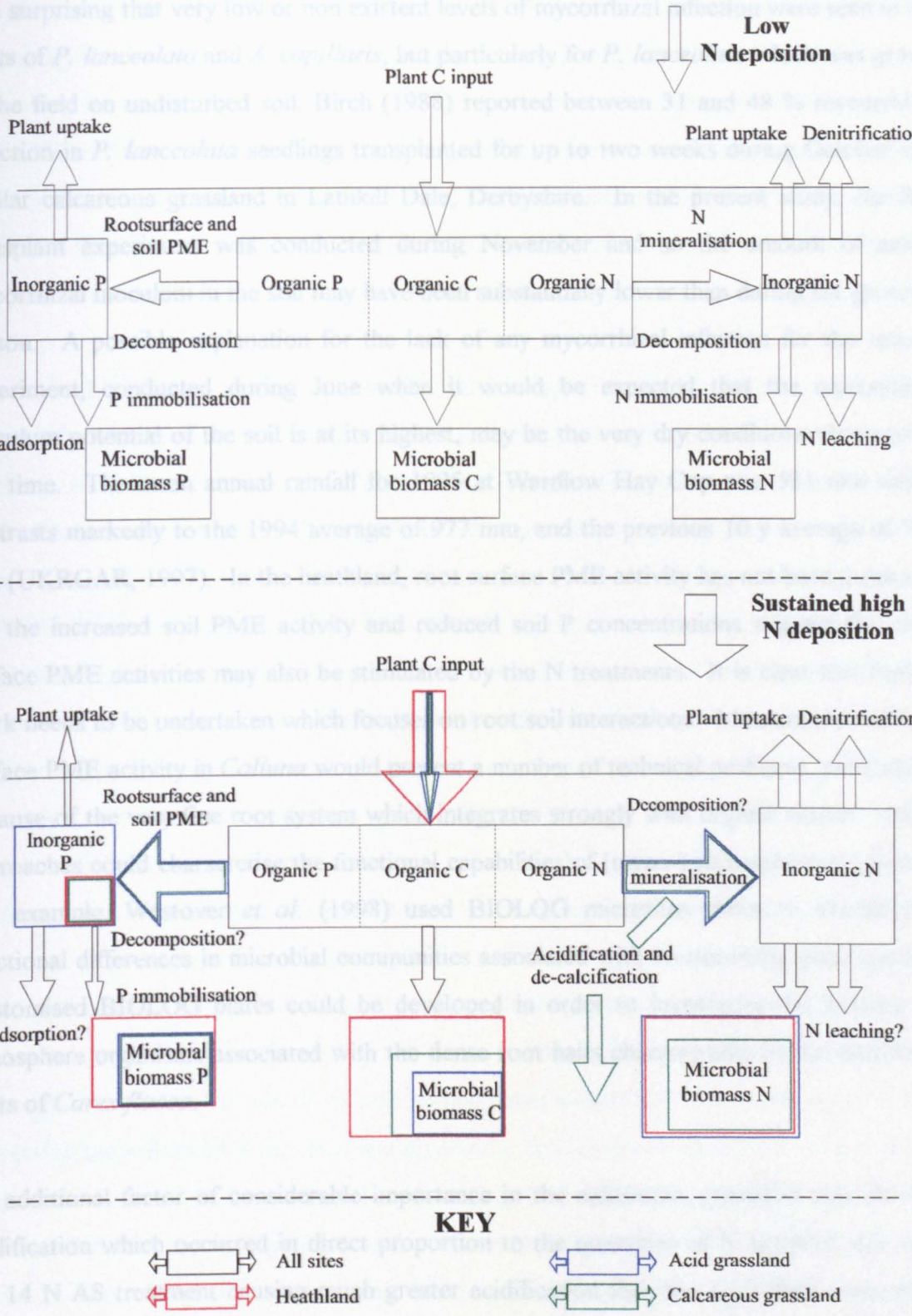


Fig. 8.1. Schematic model of the interactions between ambient and enhanced N deposition in the heathland, acid grassland and calcareous grassland used in the present study. Changes in the size/width of boxes/arrows indicate overall increases or decreases in these parameters. Question marks indicate present areas of greatest uncertainty.

It is surprising that very low or non existent levels of mycorrhizal infection were seen in the roots of *P. lanceolata* and *A. capillaris*, but particularly for *P. lanceolata* which was grown in the field on undisturbed soil. Birch (1988) reported between 31 and 48 % mycorrhizal infection in *P. lanceolata* seedlings transplanted for up to two weeks during October in a similar calcareous grassland in Lathkill Dale, Derbyshire. In the present study, the first transplant experiment was conducted during November and so the amount of active mycorrhizal inoculum in the soil may have been substantially lower than during the growing season. A possible explanation for the lack of any mycorrhizal infection for the second experiment, conducted during June when it would be expected that the mycorrhizal inoculum potential of the soil is at its highest, may be the very dry conditions observed at this time. The mean annual rainfall for 1995 at Wardlow Hay Cop was 581 mm which contrasts markedly to the 1994 average of 977 mm, and the previous 10 y average of 795 mm (UKRGAR, 1997). In the heathland, root surface PME activity has not been measured but the increased soil PME activity and reduced soil P concentrations suggest that root surface PME activities may also be stimulated by the N treatments. It is clear that further work needs to be undertaken which focuses on root:soil interactions. Measurement of root surface PME activity in *Calluna* would present a number of technical problems, particularly because of the very fine root system which integrates strongly with organic matter. Other approaches could characterise the functional capabilities of (myco-) rhizosphere organisms. For example, Westover *et al.* (1998) used BIOLOG microtitre plates to characterise functional differences in microbial communities associated with co-occurring plant species. Customised BIOLOG plates could be developed in order to investigate the function of rhizosphere organisms associated with the dense root hairs characteristic of the dauciform roots of *Carex flacca*.

An additional factor of considerable importance in the calcareous grassland was the soil acidification which occurred in direct proportion to the quantities of N supplied, but with the 14 N AS treatment causing much greater acidification than the equivalent ammonium nitrate treatment. In view of the extent of soil acidification, it is surprising that both the microbial biomass and PME activities showed only modest changes in response to the N additions at this site. The main source of acidification in the calcareous grassland is likely to be nitrification as reported for other N enriched ecosystems (Makarov and Kiseleva, 1995; Nambu *et al.*, 1994; Wilson and Skeffington, 1994). Morecroft *et al.* (1994) showed

that during the first three years of N treatment at this site nitrification increased with increasing N deposition, reaching a maximum of over 20 mg N m^{-2} day^{-1} in the 14 N AS treatment, in which the greatest drop in pH was seen. In contrast, nitrification was negligible at the acid grassland site for the same time period and is likely to be similarly unimportant in the heathland due to the low soil pH which inhibits the process (Adams, 1986; De-Boer *et al.*, 1989). This may explain the lack of any significant acidification in response to the N additions to the acid grassland and heathland sites.

The model highlights a number of areas (marked as question marks) which have not been thoroughly addressed in this study, notably denitrification rates, litter decomposition, N leaching and P adsorption, but which could form the basis of future research. Denitrification is an important but little studied microbially mediated process by which N escapes from the soils and is released into the atmosphere as nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen (N_2). Aerts (1997) reported that natural Dutch peat soils, which receive large amounts of atmospheric N, have a high potential for denitrification. In the present study, preliminary data suggest that the heathland plots that have received long-term inputs of N may also have an increased denitrification potential (Appendix C), although a more rigorous evaluation needs to be undertaken. The lack of any increase in nitrate concentrations in the acid grassland indicates that either denitrification or nitrate leaching may be occurring at this site. Nitrate leaching has important consequences since it can cause an increase in base cation depletion, and therefore reduce the buffering capacity of the soil, and also pollute standing and running waters (DoE, 1994).

Although a considerable wealth of information, summarised by Fog (1988), is available addressing the effects of N on litter decomposition, few studies have focused on the effects of chronic N additions. The standard methodology for studying litter decomposition in the field involves measuring weight and nutrient losses from litter incubated in mesh bags. This approach is confounded by a somewhat arbitrary choice of mesh sizes: meshes too small prevent both root penetration, which therefore inhibits decomposition by rhizosphere microorganisms, and access by larger organisms such as Enchytraeid worms, which have an important role in litter comminution (Yesmin *et al.*, 1995), while meshes too large can result in litter loss or weight and nutrient gain as a consequence of excessive root

in-growth. Nevertheless, litter decomposition rates provide important information which can often be used to infer microbial activities and nutrient mineralisation rates.

8.2 CRITICAL LOADS OF N

The differing definitions of a critical load result in considerable problems when attempting to evaluate a critical load for a particular ecosystem. For example, the critical load of a pollutant element or compound can be defined as the input "below which empirical detectable changes in ecosystem structure and function do not occur according to present knowledge" (Grennfelt and Thornelof, 1992) or alternatively, "the quantitative estimate of an exposure to one or more pollutants below which significant harmful effects on specified sensitive elements of the environment do not occur according to present knowledge" (Nilsson and Grennfelt, 1988). Although the latter definition has been used by a number of expert groups, such as the UNECE (United Nations Economic Commission for Europe), the phrase "harmful effects" introduces a high degree of subjectivity. Changes in the vegetation structure may, for example, be considered to be a negative or a positive effect depending on the preferred use of the system. Nevertheless, critical loads of N have been estimated for 14 types of semi-natural and natural ecosystems. The value derived for acid grassland is <2 g N m^{-2} y^{-1} and for species rich calcareous grassland is 1.4-2.5 g N m^{-2} y^{-1} (Grennfelt and Thornelof, 1992). Although no estimates are available for upland heaths, a comparison can be made with lowland wet heathland which was given a critical load of 1.7-2.2 g N m^{-2} y^{-1}. It must be remembered that these figures are based entirely upon vegetation effects, and do not take into account changes in key below ground nutrient cycling processes. This study has highlighted a number of below ground parameters that are sensitive to enhanced N deposition, including soil and root surface PME activity and PME activity per mg C_{mic}, that may be useful for developing critical loads. It is clear that these substantial changes in PME activity represent "detectable...changes in ecosystem structure and function" (Grennfelt and Thornelof, 1992), but it is not known if they constitute "harmful effects". In general, PME activities were stimulated by all levels of N addition. Further monitoring is necessary to see if these changes are eventually mirrored by above ground vegetation effects. Should this prove to be the case, the sensitivity of soil and root surface PME activities to enhanced pollutant N deposition may suggest that the critical load of N for the three ecosystems used in this study may be less than 3.5-4 g N m^{-2} y^{-1}.

The work presented in this thesis suggests that a knowledge of the availability of nutrients other than N, particularly P, is necessary before reliable calculations of critical loads of N can be calculated for semi-natural soils in the UK.

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APPENDIX A

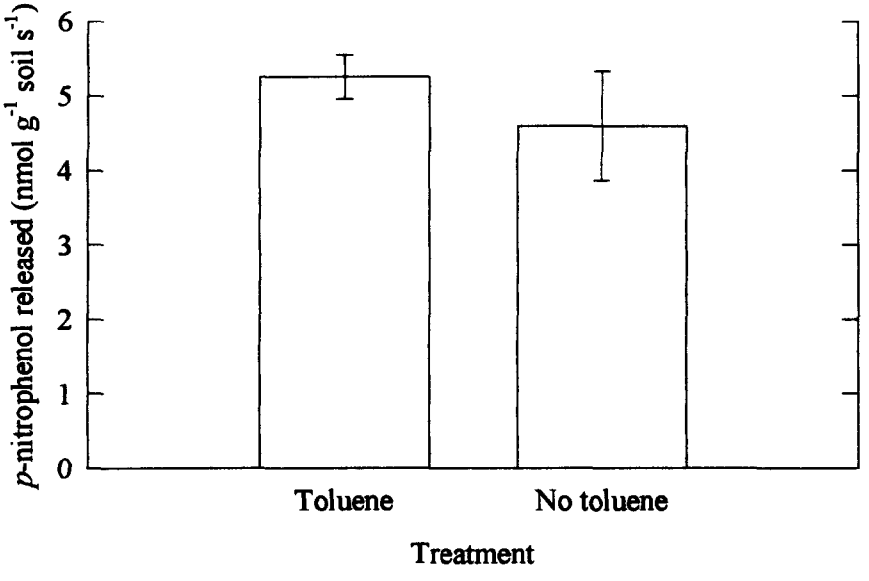

Fig. A1. Effect of toluene on phosphomonoesterase activity of a calcareous grassland soil (± SEM). Bars are not significantly different (P>0.05).

APPENDIX B

Table B1. Carbon sources used in BIOLOG GN microtitre plates

Carbon source	Carbon source	Carbon source
Alpha cyclodextrin	Xylitol	L-alanine
Dextrin	Methyl pyruvate	L-alanyl-glycine
Glycogen	Mono-methyl succinate	L-asparagine
Tween 40	Acetic acid	L-aspartic acid
Tween 80	Cis-aconitic acid	L-glutamic acid
N-acetyl-D-galactosamine	Citric acid	Glycyl-L-aspartic acid
N-acetyl-D-glucosamine	Formic acid	Glycyl-L-glutamic acid
Adonitol	D-galactonic acid lactone	L-histidine
L-arabinose	D-galacturonic acid	Hydroxy L-proline
D-arabitol	D-gluconic acid	L-leucine
Cellobiose	D-glucosamine	L-ornithine
I-ethritol	D-glucoronic	L-phenylalanine
D-fructose	Alpha-hydroxy butyric acid	L-proline
L-fructose	Beta-hydroxy butyric acid	L-pyroglutamic acid
D-galactose	Gamma-hydroxy butyric acid	D-serine
Gentibiose	P-hydroxy phenylacetic acid	L-serine
Alpha-D-glucose	Itaconic acid	L-threonine
M-inositol	Alpha-keto butyric acid	D, L-carnitine
Alpha-D-lactose	Alpha-keto glutaric acid	Gamma-amino butyric acid
Lactulose	Alpha-keto valeric acid	Urocanic acid
Maltose	D, L-lactic acid	Inosine
D-mannitol	Malonic acid	Uridine
D-mannose	Propionic acid	Thymidine
D-melibiose	Quinic acid	Phenyl ethylamine
Beta-methyl D-glucosidise	D-saccharic acid	Putrescine
D-psicose	Sebacic acid	2-amino ethanol
D-raffinose	Succinic acid	2, 3-butanediol
L-thamnose	Bromo succinic acid	Glycerol
D-sorbitol	Succinamic acid	D, L-alpha-glycerol phosphate
Sucrose	Glucoronamide	Glucose-1-phosphate
D-trehalose	Alaninamide	Glucose-6-phosphate
Turanose	D-alanine	

Table B2. Carbon sources used in BIOLOG GP microtitre plates

Carbon source	Carbon source	Carbon source
Alpha cyclodextrin	Beta-methyl D-galactoside	Methyl pyruvate
Beta cyclodextrin	3-methyl glucose	Mono-methyl succinate
Dextrin	Alpha-methyl D-glucoside	Propionic acid
Glycogen	Beta-methyl D-glucoside	Pyruvic acid
Inulin	Alpha-methyl D-mannoside	Succinamic acid
Mannan	Palailnose	Succinic acid
Tween 40	D-psicose	N-acetyl L-glutamic acid
Tween 80	D-raffinose	Alaninamide
N-acetyl-D-galactosamine	L-mammose	D-alanine
N-acetyl-D-mannosamine	D-ribose	L-alanine
Amygdalin	Salicin	L-alanyl-glycine
L-arabinose	Sedoheptulosan	L-asparagine
D-arabitol	D-sorbitol	L-glutamic acid
Arbutin	Stachylose	Glycyl-L-glutamic acid
Cellobiose	Sucrose	L-pyroglutamic acid
D-fructose	D-lagatose	L-serine
L-fructose	D-trehalose	Putrescine
D-galactose	Turanose	2, 3-butanediol
D-galacturonic acid	Xylitol	Glycerol
Gentibiose	D-xylose	Adenosine
D-gluconic acid	Acetic acid	2-deoxy adenosine
Alpha-D-glucose	Alpha-hydroxy butyric acid	Inosine
M-inositol	Beta-hydroxy butyric acid	Thymidine
Alpha-D-lactose	Gamma-hydroxy butyric acid	Uridine
Lactulose	P-hydroxy phenylacetic acid	Adenosine-5'-monophosphate
Maltose	Alpha-keto glutaric acid	Thymidine-5'-monophosphate
Mallotriose	Alpha-keto valeric acid	Uridine-5'-monophosphate
D-mannitol	Lactamide	Fructose-6-phosphate
D-mannose	D-lactic acid methyl ester	Glucose-1-phosphate
D-melezitose	L-lactic acid	Glucose-6-phosphate
D-melibiose	D-malic acid	D, L-alpha-glycerol phosphate
Alpha-methyl D-galactoside	L-malic acid	

APPENDIX C

Table C1. Potential denitrification rate in a heathland soil that has received seven years of N treatments (± SEM). Values are not significantly different.

Treatment	Potential denitrification ($\mu g\ N\ g^{-1}$ soil dwt h^{-1})
Control (0 g N m^{-2} y^{-1})	4.67 (± 1.26)
4 N (4 g N m^{-2} y^{-1})	5.18 (± 0.52)
8 N (8 g N m^{-2} y^{-1})	5.79 (± 0.72)
12 N (12 g N m^{-2} y^{-1})	6.60 (± 0.61)