NITROGEN-PHOSPHORUS RELATIONSHIPS IN LICHENS

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Abstract

Nitrogen enrichment promotes phosphomonoesterase (PME) activity in the common heathland lichen *Cladonia portentosa*. This is associated with a marked increase in thallus N:P mass ratio and significant up-regulation of inorganic phosphate uptake capacity, evidencing a shift from N-limited to P-limited growth. Phosphomonoesterase activity in *C. portentosa* responds rapidly to change in N deposition load, with a significant increase in activity recorded within 6 months of transplantation from a low-N to high-N site. The location of PME activity in *C. portentosa* was revealed using a fluorescent marker and was found to be concentrated on both the outer and inner surfaces of the hollow 'tube-like' thallus branches. Activity appeared to be associated exclusively with the mycobiont and was located within the hyphal-lumina, consistent with a membrane bound ecto-enzyme. High PME activity in axenic mycobiont cultures of *C. portentosa* provided further evidence of a fungal location and confirmed that rates of activity in this lichen are amongst the highest reported for any other plant/fungal system in the literature. Different classes of phosphatases were assayed in a range of N_2-fixing and non-N_2-fixing lichens in an oligotrophic subarctic environment. No relationship was found between the capacities for nitrogenase and PME activities. Maximum rates of PME activity were recorded in fruticose mat-forming lichens which capture nutrients predominantly from atmospheric deposits. 5´ nucleotide phosphodiesterase activity was readily detected in N_2-fixing lichens and was particularly high in rhizine-rich regions of foliose terricolous lichen thalli, consistent with the utilisation of organic phosphates from soil and litter sources.

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Abbreviations (excluding standard SI units)

5′ ND	5′ nucleotide phosphodiesterase
asl	above sea level (m)
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bis-*p*NPP	bis (*para*)-nitrophenyl phosphate
C→C	'native' Caithness back to Caithness transplanted control thalli
C→D	Caithness to Derbyshire transplanted thalli
D→C	Derbyshire to Caithness transplanted thalli
D→D	'native' Derbyshire to Derbyshire transplanted control thalli
DMG	3, 3 dimethylglutaric acid
EH	Eadie-Hofstee
ELF	Enzyme Labelled Fluorescence
ELF 97 phosphate	(2-(5′-chloro-2′-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone)
HW	Hanes-Woolf
K_m	Michaelis constant (substrate concentration at half V_{max})
LB	Lineweaver-Burke
LSD	least significant difference
MM	Michaelis-Menten
MUP	4-methylumbelliferyl phosphate
$\mathbf{N}$	wet inorganic N deposition (kg N ha^{-1} y^{-1})
$\mathbf{N}_m$	measured wet inorganic N deposition (kg N ha^{-1} y^{-1})
$\mathbf{N}_s$	modelled wet inorganic N deposition (kg N ha^{-1} y^{-1})
$[N]_{apex}$	N concentration in the apical region of the thallus (mg g^{-1})
$[N]_{base}$	N concentration in the basal region of the thallus (mg g^{-1})
$[N]_{lichen}$	thallus N concentration (mg g^{-1})
$[N]_m$	measured volume-weighted concentration of inorganic N in rainfall
$[N]_{medium}$	N concentration in growth medium
$[N]_{myc}$	N concentration in fungal mycelium (mg g^{-1})
$[N]_{ppt}$	volume-weighted concentration of inorganic N in precipitation
$[N]_s$	modelled volume-weighted concentration of inorganic N in rainfall
$([N]/[P])_{apex}$	N:P mass ratio in the apical region of the thallus
$([N]/[P])_{base}$	N:P mass ratio in the basal region of the thallus
$([N]/[P])_{medium}$	N:P mass ratio in growth medium
$([N]/[P])_{myc}$	N:P mass ratio in fungal mycelium
PDE	phosphodiesterase
PME	phosphomonoesterase
PTE	phosphotriesterase
*p*NP	*para*-nitrophenyl
*p*NPP	*para*-nitrophenyl phosphate
*p*NPPP	*para*-nitrophenyl phenylphosphate
PPFR	photosynthetic photon fluence rate
$[P]_{apex}$	P concentration in the apical region of the thallus (mg g^{-1})
$[P]_{base}$	P concentration in the basal region of the thallus (mg g^{-1})
$[P]_{lichen}$	thallus P concentration (mg g^{-1})

$[P]_{medium}$	P concentration in growth medium
$[P]_{myc}$	P concentration in fungal mycelium
$[P]_{ppt}$	total phosphorus concentration in precipitation
RG	relative growth (mg g^{-1})
RGR	relative growth rate (mg g^{-1} y^{-1})
[S]	substrate concentration
SI	system I
SIa	system Ia
SIb	system Ib
SII	system II
UE	uptake efficiency (V_{max}/K_m)
V_{max}	maximum velocity (rate) of a reaction

CHAPTER 1. General Introduction

1.1 Lichen Biology

Lichens are symbiotic systems, formed from an association between a mycobiont and a photobiont. In most lichens (*c*. 98 %), the mycobiont is an ascomycete fungus, but the photobiont can be either a unicellular green alga, most commonly of the genera *Trebouxia* and *Trentepohlia* (90 % of all lichens), or a cyanobacterium such as *Nostoc* or *Stigonema spp* (10 % of all lichens). The two symbionts grow together forming a lichen 'thallus' and are able to survive and compete under harsh environmental conditions which could not be tolerated by either symbiont growing in the free-living state (Purvis 2000). Most commonly, lichen associations are referred to as individual organisms or 'species' which, although not biologically accurate, for simplicity is how they are referred to throughout this thesis.

1.2 Nutrient acquisition in lichens

Lichens have evolved to inhabit nutrient poor environments, and are frequently among the primary colonisers of bare soil and rock. As they do not possess roots and lack a vascular system, terrestrial lichens have evolved highly efficient mechanisms to capture nutrients directly from trace quantities present in atmospheric deposits (Crittenden 1989; Nash and Gries 1995; Crittenden 1996; Hyvärinen and Crittenden 1998a; 1998b). In the British Isles, the concentration of

inorganic nitrogen in precipitation ($[N]_{ppt}$) ranges between 0.1 and 1.5 mg l^{-1} and phosphorus concentrations ($[P]_{ppt}$) are recorded between 10 and 100 µg l^{-1} (Gore 1968; Newman 1995; Hayman *et al.* 2005, C. Jordan, Department of Agriculture for Northern Ireland, via AEA Technology, Pers. Comm.). Both wet and dry deposition processes contribute to the nutrient supply of lichens (Crittenden 1996), with dry-impacted nutrients absorbed across the thallus surface during periods of hydration. Uptake processes involve passive ion exchange, particulate entrapment (extracellular) and both passive and active transport across the cell membrane (intracellular) (Nieboer *et al.* 1978).

Several lines of evidence lend support to the suggestion that the mycobiont is the principal partner involved in nutrient capture by the thallus. First, the mycobiont partner constitutes the largest proportion of the thallus by both mass and volume (*c*. 90 %, Smith 1975) and forms the cortex, the outermost tissue that is the interface for nutrient exchange with the environment (Dahlman *et al.* 2004). Second, Pavlova and Maslov (2008) demonstrated that the freshly isolated photobiont of *Parmelia sulcata* (*Trebouxia sp*.) was unable to take up nitrate (NO_3^-), whilst mycobiont cultures and thallus fragments containing both symbionts took up 50 % and 90 %, respectively, of the NO_3^- supplied. The authors showed that in the intact thallus, NO_3^- was first absorbed by the mycobiont before being translocated to different regions of the thallus (Pavlova and Maslov 2008). Third, evidence of N and P internal recycling in the mat-forming lichen *Cladonia portentosa* (Hyvärinen and Crittenden 2000; Ellis *et al.* 2005) is

consistent with the findings of Pavlova and Maslov (2008), and most likely represents nutrient transport in the mycobiont. Fourth, Honegger (1996) reports no cytological differences between lichenized and non-lichenized fungi, and as such, it seems reasonable to assume that the lichen mycobiont has similar nutrient uptake properties.

1.2.1 Nitrogen and phosphorus acquisition in lichens

Lichen habitats are generally considered to be N-deficient (Crittenden 1991; Crittenden *et al.* 1994; Crittenden 1996), and there is evidence that N supply can limit lichen growth (Verhoeven *et al.* 1996; Palmqvist and Dahlman 2006; Makkonen *et al.* 2007; Nash 2008). Efficient capture of N is therefore likely to be advantageous for lichen growth and metabolism and, indeed, several mechanisms operate to maximise N uptake by the thallus. Both passive and active uptake systems have been identified for the trans-membrane uptake of nitrogenous compounds, including inorganic (NO_3^- and NH_4^+) and organic forms (e.g. amino acids) (Lang *et al.* 1976; Rai 1988; Crittenden 1996; Crittenden 1998; Kinoshita *et al.* 2001; Bhattacharya *et al.* 2002; Dahlman *et al.* 2004; Ellis *et al.* 2005). There is evidence of preferential uptake of NH_4^+ by lichens when supplied with multiple forms of N in equally high concentrations (Dahlman *et al.* 2004; Ellis *et al.* 2005). However, during natural rainfall episodes which deliver trace quantities of NH_4^+ and NO_3^-, lichens can capture both ions with similar efficiency (Crittenden 1998). This capacity to acquire multiple forms of N is no doubt beneficial to lichens in their natural habitat where the form and availability of N varies greatly. In lichens

which contain a cyanobacterial photobiont, the problem of N deficiency is solved due to the capacity to fix atmospheric N_2.

As P is present in only trace concentrations in atmospheric deposits, lichens have evolved highly efficient uptake pathways to ensure maximum phosphate capture (Smith 1960; Farrar 1976) and to minimise P-deprivation (Crittenden *et al.* 1994). Farrar (1967) and Hyvärinen and Crittenden (1998c) confirmed that the lichens *Hypogymnia physodes* and *Cladonia portentosa*, respectively, are highly efficient at capturing phosphate ions from dilute solutions. Farrar (1967) demonstrated that PO_4^{3-} uptake rate in *H. physodes* was highly dependent on the concentration of phosphate supplied and followed Michaelis-Menten kinetics, consistent with active, energy driven uptake processes. He suggested that the P-requirement of this lichen could be met by just 1 h rainfall per week (Farrar, 1967). For the purposes of simplicity, all forms of inorganic phosphate available for lichen uptake (e.g. H_3PO_4, $H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}) are represented by 'PO_4^{3-}' in this thesis.

Phosphorus exists in multiple forms in atmospheric deposits, including inorganic phosphates and organic molecules. Although no data yet exists to suggest that phosphorylated organic molecules are directly transported across the cell membrane, indirect evidence points to the possibility that lichens can access this source of 'biologically unavailable' P by the activity of phosphatase enzymes (Lane and Puckett 1979; LeSueur and Puckett 1980; Stevenson 1994).

1.3 Atmospheric nitrogen and phosphorus

1.3.1 Natural sources of N and P

Biologically available N and P are emitted into the atmosphere by several processes. Ammonia (NH_3) is emitted from decomposing animal and plant matter, and rapidly converted to NH_4^+ in solution. Nitrogen oxides (NO_x) including N_2O (nitrous oxide), NO (nitric oxide) and NO_2 (nitrogen dioxide) are emitted by denitrification and during electrical processes (including lightning and corona discharges) (Sprent 1987; NEGTAP 2001). Phosphorus occurs in the atmosphere principally as dust derived from soil erosion and the weathering of rocks. Other natural sources of atmospheric P include long-range wind-blown dusts, pollen, spores, seeds, sand and oceanic foam, and considerable quantities of N and P are emitted into the atmosphere during volcanic activity and natural fires (Niemi and Eklund 1986; Sprent 1987; Newman 1995).

1.3.2 Pollution sources of N and P

During the past 100 years, anthropogenic activity has increased the rate at which N compounds are discharged into the atmosphere (Brimbelcombe and Stedman 1982; Rodhe and Rood 1986; Pitcairn *et al.* 1995; Fowler *et al.* 2004). The majority of pollutant N emitted across the British Isles is in the form of NH_3 (Pitcairn *et al.* 1998), and is attributed to intensive agricultural practices (Fowler *et al.* 1998a; 1998b). For example, a large proportion of NH_3 is released during the

decomposition of urea in animal wastes and uric acid in poultry excreta, generated in large quantities in densely stocked animal houses (Misselbrook *et al.* 2000; NEGTAP 2001). Combustion processes associated with transport and industry along with the disposal of sewage sludge contribute to the emission of NO_x (NEGTAP 2001).

There is a dearth of information regarding pollution sources of P. Nonetheless, anthropogenic practices are recognised as significant contributors, with pollution sources including industry (e.g. phosphate manufacture and processing), and emissions from the combustion of coal, timber and agricultural waste (Graham and Duce 1979). Particles of soil containing P may also be dispersed into the atmosphere during construction and agricultural activity which, if enriched with agricultural fertilizers, can contribute significantly to atmospheric P loads.

1.3.3 Deposition of N and P in the British Isles

Both wet and dry deposition processes deliver nutrients to lichen thalli (Nash and Gries 1995). Wet deposition occurs during precipitation events and during periods of occult precipitation (dew and fog) (Nash 2008). Dry deposition occurs by sedimentation and impaction of particulates and by adsorption of gases (Nash 2008). In the British Isles there is large geographical variation in the relative importance of these different processes in the delivery of nutrients to lichens. For example, in upland ecosystems, nutrient inputs are dominated by wet deposition processes (Leith *et al.* 1999). Here, due to seeder-feeder enrichment of rainfall

over high ground, upland vegetation (which is frequently rich in lichens) receives disproportionately large quantities of N compared with lowland habitats (Fowler *et al.* 1988; NEGTAP 2001; Britton and Fisher 2007a). Leith *et al.* (1999) estimate that *c*. 65 % of British uplands (> 300 m asl) receive > 10 kg N ha^{-1} y^{-1}, with *c*. 5 % receiving > 30 kg N ha^{-1} y^{-1}. In lowland ecosystems, N inputs are predominantly supplied in dry deposits (Sutton *et al.* 1993).

In the British Isles, there are few reliable estimates of natural 'background' rates of N and P deposition. The available historical data suggests that background N deposition during the early 1900s ranged from 1 – 3 kg N ha^{-1} y^{-1} (Bobbink *et al.* 2002) and by the mid 1900s was in the range 0.74 – 9.25 kg N ha^{-1} y^{-1} (Eriksson 1952; Stevenson 1968). Today, measured N deposition ($\mathbf{N}_m$) ranges between 2.7 – 22.4 kg N ha^{-1} y^{-1} (total wet inorganic N) (Lawrence *et al.* 2007) and values of modelled N deposition ($\mathbf{N}_s$) are given in the range 5 – 35 kg ha^{-1} y^{-1} (R. I. Smith, CEH, Edinburgh, Pers. Comm.). In contrast, the availability of P in atmospheric deposits appears to be less influenced by anthropogenic activity. Historical estimates for the deposition of P in the British Isles lie in the range 0.17 – 0.43 kg P ha^{-1} y^{-1} (Carlisle *et al.* 1966; Gore 1968); this can be compared with deposition values of 0.27 – 0.55 kg P ha^{-1} y^{-1} (C. Jordan, Department of Agriculture for Northern Ireland, via AEA Technology, Pers. Comm) and 0.1 kg P ha^{-1} y^{-1} (Tomassen *et al.* 2004) measured in Ireland in 1993 and 2000, respectively, and the estimate given by Newman (1995) for the British Isles of < 1 kg P ha^{-1} y^{-1}.

1.3.4 Estimating N and P deposition in the British Isles

Measurement of N and P deposition is complicated by the wide range of compounds which exist in both the gaseous (e.g. NH_3, NO_x) and aqueous phase (e.g. NO_3^-, NH_4^+, PO_4^{3-}) (Pitcairn *et al.* 2003). Currently, the UK Acid Deposition Monitoring Network (AEA Energy & Environment) measures the concentrations of major inorganic cations and anions in bulk (wet and dry) deposits at 2 weekly intervals, at 38 sites in England, Wales, Scotland and Northern Ireland (Lawrence *et al.* 2007). Concentrations of NO_x and NH_3 are also measured at some sites permitting co-location of sampling with other national monitoring programmes (e.g. the National Ammonia Monitoring Network) (Lawrence *et al.* 2008). Measurements have been made since 1986, permitting the identification of large-scale temporal and spatial changes in the chemical composition of precipitation (Lawrence *et al.* 2007). However, this system has several shortcomings. First, collection stations are frequently positioned at easy access, low altitude sites, which can under-estimate the chemical composition of precipitation on higher ground. Second, bulk collectors are unable to discriminate between wet and dry processes. Third, wind-blown precipitation is collected at a low efficiency. Fourth, rainwater samples are highly susceptible to contamination from bird faeces, leaf litter and ash (Gore 1968), and to the loss of nutrients to collection-funnel adhesion and microbial assimilation.

Some of the above measurement errors have been corrected for in modelled data sets. Here, measured data are combined with a 5 x 5 km gridded simulated rainfall

field (generated by the UK Meteorological Office), values in which have been adjusted to correct for the low collection efficiencies of rain gauges. Measured values of ion concentrations are also adjusted to take into account the effects of seeder-feeder processes. Deposition of NO_x and NH_3 is also modelled using ecosystem-specific deposition velocities and taking into account proximity to sources of N (Leith *et al.* 1999). Since this modelling corrects for largely underestimations, it results in a range of deposition values up to 3 times greater than are currently measured (see Chapter 4). These modelled data sets and resultant interpolative deposition maps (Fig. 1.1) remain to be critically evaluated.

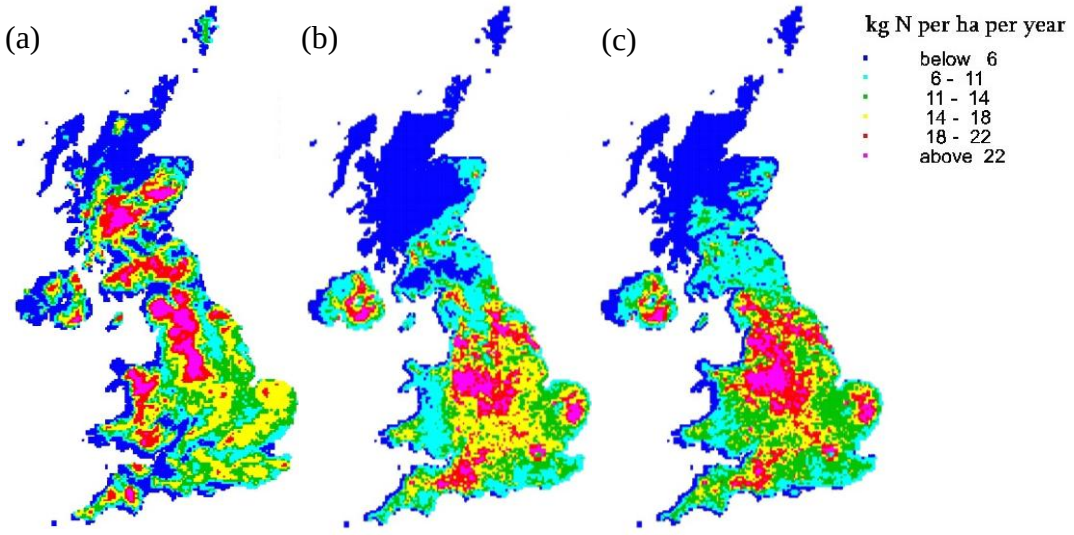


Figure 1.1 Simulated rates of wet (a), dry (b) and total (c) N deposition in the British Isles. Data modelled at a resolution of 5 x 5 km^2 for moorland terrain (2001 - 2003 models, R. I. Smith, CEH Edinburgh, Pers. Comm.).

1.4 Impact of N enrichment in the British Isles

The majority of natural plant communities in the British Isles have evolved under conditions of low N input (Leith *et al.* 1999; Grime 2001; Britton and Fisher 2007b), resulting in floristically diverse communities (including calcareous grasslands, lowland heaths and upland moors). In todays landscape of intensive farmland and urban and industrial development, many of these ecosystems have become fragmented and species composition has been altered by the introduction of non-native species (Aerts and Heil 1993). As such, several semi-natural ecosystems have been identified as susceptible to the impacts associated with N enrichment (Verhoeven *et al.* 1996; Fowler *et al.* 1998a; Carroll *et al.* 1999; Leith *et al.* 2001; Britton and Fisher 2007a). Heathlands with their rich fruticose lichen communities, in particular are increasingly recognised as threatened by N pollution (Aerts and Heil 1993; Pitcairn *et al.* 1995; Carroll *et al.* 1999; Britton and Fisher 2007b).

Nitrogen enrichment alters the natural competitive balance between individual species as there is a high degree of variation in the capacity of plants to utilise excess N (Prins *et al.* 1991; Pearce and van der Wal 2002; Pitcairn *et al.* 2003). In oligotrophic heathland habitats, the more competitive, faster-growing graminoids (including *Deschampsia flexuosa*, *Festuca ovina* and *Molinia caerulea*) respond more positively to increased N supply than ericaceous shrubs and lichens (Falkengren-Grerup 1998; van der Wal *et al.* 2003). Graminoid species produce more tillers in response to N enrichment (Leith *et al.* 1999), thereby increasing

their ground-cover and light interception (Pearce and van der Wal 2002; van der Wal *et al.* 2003). This occurs at the expense of species of ericaceous shrubs (e.g. *Calluna vulgaris*, *Erica tetralix*) (Heil and Diemont 1983; Aerts and Heil 1993), and commonly results in a marked decline in the abundance and diversity of slow growing cryptogam species (e.g. lichens, mosses, and liverworts) (Heil and Diemont 1983; Gordon *et al.* 2001; Leith *et al.* 2001; Pearce and van der Wal 2002; Britton and Fisher 2007a). Such shifts in species composition can also act as a catalyst for further ecosystem change as the decomposition of vegetation with high tissue N further enriches the soil, increasing the supply of N to new plant growth (Pearce and van der Wal 2002; Nordbakken *et al.* 2003). However, recent observations suggest that disturbance events (e.g. mowing, grazing or fire) frequently counteract the detrimental effects of N enrichment by altering canopy structure and increasing light and nutrient availability to slow growing vegetation, in particular bryophytes and lichens (van der Wal *et al.* 2003; Britton and Fisher 2007b).

1.4.1 Impact of N enrichment on lichens

The majority of studies into the impact of N pollution in the British Isles have focussed on vascular plants and bryophytes, in particular those of nutrient-poor habitats (e.g. Baddeley *et al.* 1994; Pitcairn *et al.* 1995; Lee and Caporn 1998; Carroll *et al.* 1999; Kirkham 2001; Bragazza *et al.* 2005; Pilkington *et al.* 2005). These impacts not only include shifts in plant community structure (Pitcairn *et al.* 1995; Carroll *et al.* 1999; Britton and Fisher 2007b), but are also associated with

increased tissue N concentration (Baddeley *et al.* 1994; Morecroft *et al.* 1994; Pitcairn *et al.* 1995; 1998; Carroll *et al.* 1999; Leith *et al.* 1999; Kirkham 2001; Carroll *et al.* 2003; Pitcairn *et al.* 2003; Mitchell *et al.* 2004; Skinner *et al.* 2006), increased sensitivity to drought (Fangmeier *et al.* 1994; Lee and Caporn 1998; Gordon *et al.* 1999), increased susceptibility to heather beetle outbreaks (Brunsting and Heil 1985; Power *et al.* 1998), reduced cell membrane integrity (Woolgrove and Woodin 1996; van der Wal *et al.* 2003), decreased rates of N assimilation (Morecroft *et al.* 1994; Pearce and van der Wal 2002; van der Wal *et al.* 2003) and reduced growth (Bell *et al.* 1994; van der Wal *et al.* 2003). Nonetheless, there are instances where the impact of N supply on lichen chemistry and physiology have been investigated (Bruteig 1993; Søchting 1995; Hyvärinen and Crittenden 1998a; 1998b; Walker *et al.* 2003; Sheppard *et al.* 2004b). Lichens are highly efficient at capturing trace concentrations of N in atmospheric deposits (Crittenden 1983; 1989; Hyvärinen & Crittenden 1998a; 1998b; 1998c). As such, under conditions of N enrichment, several lichen species, in particular mat-forming terricolous lichens of the genera *Cladonia* and *Flavocetraria* have been shown to accumulate significant concentrations of N (Søchting 1995; Hyvärinen and Crittenden 1998a; Walker *et al.* 2003; Skinner *et al.* 2006). For example, Hyvärinen and Crittenden (1998a) demonstrated that *c*. 60 % of variation in the thallus N content ($[N]_{lichen}$) of *C. portentosa* could be explained by differences in wet inorganic N deposition at 31 sites in the British Isles. Søchting (1995) reported values of $[N]_{lichen}$ in *C. portentosa* to range between 3.3 and 10.3 mg g^{-1} at different locations in Western Europe, related to variation in N deposition.

More recently, Walker *et al.* (2003) recorded a decrease in tissue N concentration in *Cladonia stellaris* from 8 to 6 mg g^{-1} along a putative gradient of decreasing N deposition in northern Russia. In none of these studies did the authors report evidence of toxic effects of N enrichment. However, other authors have documented some deleterious effects. For example Tomassen *et al.* (2004) reported that application of 20 kg N ha^{-1} y^{-1} (as ammonium and nitrate) to vegetation-free plots in an ombrotrophic bog in Ireland resulted in a 68 % suppression of the re-growth of *C. portentosa* when compared with plots receiving natural 'background' deposition of 4.6 kg N ha^{-1} y^{-1}. Vagts and Kinder (1999) also report a 2 fold decrease in the thallus growth rate of *C. portentosa* in response to the addition of 150 kg N ha^{-1} y^{-1} (NH_4^+ and NO_3^-), and Søchting (1990) recorded evidence of widespread injury to thalli of *C. portentosa* in Denmark, which he attributed to N enrichment. Recently, Sheppard *et al.* (2004b) reported that application of 42.7 µg m^{-3} NH_3 to an ombrotrophic bog in Scotland over 8 months resulted in a 50 % reduction in the photosynthetic efficiency (measured as F_v/F_m) of *C. portentosa*, and was associated with severe thallus bleaching.

There are several reasons why lichens might be more susceptible than vascular plants to the harmful effects of excess N. First, as lichens do not posses stomata or a cuticle, atmospheric deposits are received and absorbed over the entire thallus surface (Pitcairn *et al.* 1995). Second, dry deposited aerosols are rapidly absorbed upon re-hydration of the thallus (Nash and Gries 1995). Third, some vascular plants can avoid toxicity by shedding toxic-laden leaves, a strategy which appears

unavailable to lichens (Nash and Gries 1995). Nitrogen enrichment has also been associated with a decline in lichen diversity and abundance in habitats such as grasslands and low-alpine and upland heathlands (Lee and Caporn 1998; Britton and Fisher 2007b; Pilkington *et al.* 2007). For example, Britton and Fisher (2007b) reported a significant loss of lichen diversity in upland heathlands following the addition of 10 or 50 kg $N\text{-}NH_4NO_3$ ha^{-1} y^{-1} and Pilkington *et al.* (2007) recorded almost complete loss of lichens from an upland moor site treated with 10 or 20 kg $N\text{-}NH_4NO_3$ ha^{-1} y^{-1}. A reduction in the abundance and species diversity of lichens has been attributed to competitive exclusion by higher plants, which increase ground cover in response to increased N supply, reducing light and nutrient interception by lichens.

1.5 Lichens as bio-indicators of N enrichment

Lichens have been used widely as bio-indicators of atmospheric chemistry (Ferry *et al.* 1973; Ferry and Coppins 1979; Seaward 1992; Bruteig 1993; Halonen *et al.* 1993; Søchting 1995; Loppi *et al.* 1997; Conti and Cecchetti 2001; Nimis *et al.* 2002; Lambley and Wolseley 2004; Nash 2008), with two major classes of effect recognised. First, there are the well documented shifts in lichen community structure and composition (e.g. Hawksworth and Rose 1970; de Bakker 1989; Benfield 1994; van Dobben and de Bakker 1996; van Dobben and ter Braak 1998; van Herk 1999) and second, changes in the chemical composition of thalli occur as lichens accumulate contaminants (Bruteig 1993; Halonen *et al.* 1993; Søchting 1995; Hyvärinen and Crittenden 1996; 1998a; Walker *et al.* 2003; Skinner *et al.*

2006). Probably the best example of change in lichen community composition as a result of atmospheric pollution is the simplification of epiphytic lichen communities in response to sulphur dioxide (Hawksworth and Rose 1970). However, N pollution has also been shown to significantly modify lichen communities. In particular, increased N deposition has been associated with a shift in epiphytic lichen species, with invasion and population expansion of N-tolerant 'nitrophytic' species (e.g. *Physicia spp*., *Xanthoria parietina*, *X. polycarpa*) at the expense of N-sensitive 'acidophyte' species (e.g *Cladonia spp*., *Evernia prunastri, H. physodes, Parmelia saxatilis, Plasmatia glauca, Usnea spp.*) (de Bakker 1989; Benfield 1994; van Dobben and ter Braak 1998; van Herk 1999; Wolseley *et al.* 2004; 2006; Sparrius 2007; Sutton *et al.* 2009), which cannot tolerate a decrease in bark pH associated with N pollution (Wolseley *et al.* 2004; 2006).

The chemistry of lichens also changes in a predictable way in response to N pollution. Thallus N concentration and N:P mass ratio in epiphytic (e.g. *H. physodes*) and fruticose terricolous mat-forming lichens (e.g. *Cladonia spp*., *Flavocetraria spp*.) have repeatedly been found to correlate strongly with N enrichment (Bruteig 1993; Halonen *et al.* 1993; Hyvärinen and Crittenden 1998a; Walker *et al.* 2003; Skinner *et al.* 2006), as such providing chemical biomarkers for atmospheric N. According to Hyvärinen and Crittenden (1998a), coupling between thallus chemistry of mat-forming terricolous lichens and that of atmospheric deposits is particularly close for three reasons. First, lichen mats are

highly efficient at capturing trace concentrations of N present in atmospheric deposits (Crittenden 1983, 1989). For example, Crittenden (1989) revealed that 80 % of inorganic N (NH_4^+ and NO_3^-) delivered to mats of *C. stellaris* in rainfall was retained, whilst for *Stereocaulon paschale*, inorganic N capture efficiency was 90.5 %. Second, lichen mats generally develop in open areas, permitting direct interception of atmospheric deposits, minimising confounding effects due to the modified chemistry of canopy through-fall (cf. Farmer *et al.* 1991; Søchting 1995). Third, development of a deep layer of dead and/or decaying thallus material at the base of well-developed lichen mats isolates the lichen from the underlying substratum and hence soil chemical influences (Crittenden 1991; Ellis *et al.* 2003; 2004). However, there is marked vertical variation in the chemical composition of mat-forming lichen thalli, with maximum element concentrations in the apices with values decreasing towards the base (Pulliainen 1971; Pakarinen 1981; Crittenden 1991; Hyvärinen and Crittenden 1998a), and the relationship between lichen and atmospheric chemistries can depend on the stratum within a lichen mat that is analysed. For example, Hyvärinen and Crittenden (1998a) showed that N concentration in the thallus apices of *C. portentosa* (0 – 5 mm measured from the apices downwards, $[N]_{apex}$) varied by *c*. 50 %, while that in a deeper stratum (40 – 50 mm, $[N]_{base}$) varied by 70 %, and that this variation was strongly related to wet inorganic N deposition. The authors suggested that increased N capture in the apices decreased the rate of internal N recycling, thereby increasing retention and concentration of N in the thallus bases. In contrast, Walker *et al.* (2003) observed changes in $[N]_{apex}$, but not $[N]_{base}$, in *C.*

stellaris, along a putative N deposition gradient in northern Russia, and Pakarinen (1981) demonstrated significant increases in $[N]_{apex}$ in *C. stellaris* and *C. arbuscula* in response to increased N supply in Finnish ombrotrophic bogs. Thus it is possible that the strength of thallus N concentration as a biomarker for N deposition changes from $[N]_{base}$ to $[N]_{apex}$ in mat-forming lichens as the rate of N income decreases. It is therefore essential that a standardised region of the thallus is selected should mat-forming lichens be adopted as biomarkers for N enrichment.

In the study by Hyvärinen and Crittenden (1998a), co-variation between $[N]_{lichen}$ and thallus P concentration ($[P]_{lichen}$) in *C. portentosa* was recorded in both apices and bases. The authors attempted to explain variation in $[N]_{lichen}$ as a reflection of different rates of N deposition in the British Isles. However, they could not attribute the 4-fold variation in $[P]_{lichen}$ values to concomitant variation in P deposition, as available evidence suggests that this remains uniformly low across all regions (< 1 kg P ha^{-1} y^{-1}) (Newman 1995; Tomassen *et al.* 2004, C. Jordan, Pers. Comm.). They also speculated that reduced lichen growth rates due to phytotoxic agents might result in reduced growth-led dilution of P and increased $[P]_{lichen}$ values in polluted regions. It was this observed co-variation between $[N]_{lichen}$ and $[P]_{lichen}$ which partly prompted the current investigation, as it was of interest to explain mechanisms underlying variation in $[P]_{lichen}$. Furthermore, nitrogen-fixing lichens generally contain higher concentrations of both N and P compared to co-occurring non-N_2-fixing lichens (Crittenden *et al.* 1994). An

explanation for the co-variation in $[N]_{lichen}$ and $[P]_{lichen}$ in N enriched lichens not hitherto explored is that up-regulation of phosphatase activity might promote P capture. Lane and Puckett (1979) and LeSueur and Puckett (1980) together with a more recent unpublished study by Stevenson (1994) provide evidence of surface-bound phosphatase activity in a range of lichens, including several mat-forming species. In addition, evidence of PO_4^{3-} enrichment of snow melt-water that had percolated though a canopy of the Antarctic lichen *Usnea sphacelata* (Crittenden 1998) and the loss of ^{15}N from ^{15}N-labelled thalli of *C. portentosa* (Ellis *et al.* 2005) have also been attributed to the possible activity and loss, respectively, of surface-bound phosphatase enzymes.

1.6 Phosphatase enzymes

Phosphatases are enzymes which catalyse the hydrolysis of organic phosphorus compounds by cleaving a C-O-P ester linkage to release an organic moiety and orthophosphate (Cembella *et al.* 1984; Jansson *et al.* 1988). There are several distinct classes of phosphatase enzymes, including phosphomonoesterases (PME) phosphodiesterases (PDE) and phosphotriesterases (PTE) (Fig. 1.2).

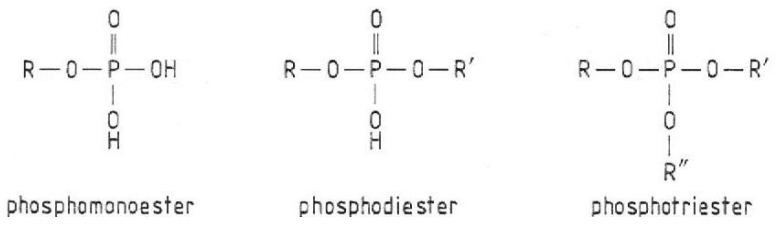
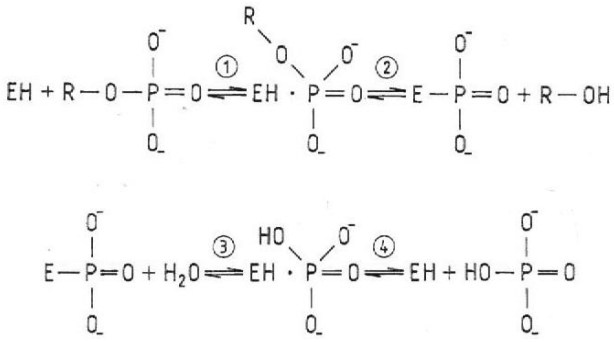


Figure 1.2 General formula for different types of phosphate esters. '*R'* represents the organic part of the molecule. Adapted from Jansson *et al.* (1988).

Non-specific PME enzymes hydrolyse only phosphomonoester substrates, which include glucose-6-phosphate, ß-glycerophosphate, trehalose-6-phosphate and phosphoenolpyruvate (Bartlett and Lewis 1973; Ezawa *et al.* 1999; Turner *et al.* 2004). Alternatively, phosphodiesterases (including 5′ nucleotide phosphodiesterase (5′ ND)) catalyse hydrolysis of phosphodiester substrates, including phospholipids and nucleic acids such as DNA and RNA (Leake and Miles 1996; Turner 2008). The reaction mechanism for all phosphatases is identical and can be divided into four steps, outlined in Fig. 1.3 (McComb *et al.* 1979; Jansson *et al.* 1988).



1. Non-covalent binding of the organic P substrate to the phosphatase enzyme.
2. Alcohol release from the complex and covalent binding of orthophosphate to the enzyme forming a phosphoryl-enzyme compound.
3. Conversion of the phosphoryl-enzyme compound, through uptake of water, to a non-covalent complex.
4. Release of orthophosphate and regeneration of free enzyme.

Figure 1.3 Reaction-scheme for the enzyme catalysed hydrolysis of phosphate esters as described by McComb *et al.* (1979), and illustrated by Jansson *et al.* (1988).

1.6.1 Phosphatase activity in lichens, plant/root systems and soils

Phosphatases are ubiquitous enzymes, present in multiple forms within a single organism. Intracellular phosphatases are typically involved in metabolic pathways, whilst extracellular phosphatases (present in the cell membrane and/or cell wall) are recognised as a means of enhancing P capture (McComb *et al.* 1979). Phosphatase activity is reported for algae (Cembella *et al.* 1984; Hernández *et al.* 2002), bacteria (Banerjee *et al.* 2000; Kretschmer 2007), and unicellular and filamentous fungi, including those in symbiotic associations (e.g. mycorrhizas and lichens) (Lane and Puckett 1979; Beever and Burns 1980; LeSueur and Puckett 1980; Dighton 1983; Antibus *et al.* 1986; Egger *et al.* 1994; Stevenson 1994; Leake and Miles 1996; Yadav and Tarafdar 2003), as well as bryophytes and vascular plants (Press and Lee 1983; Duff *et al.* 1994; Turner *et al.* 2001; Ellwood *et al.* 2002). In addition, phosphatase activity is reported in river systems, lakes and soils as a result of enzymes secreted into the environment, typically from bacteria, algae and fungi (Eivazi and Tabatabai 1977; Pettersson 1980; Jansson *et al.* 1988; Siuda and Güde 1994; Christmas and Whitton 1998a; 1998b; Turner *et al.* 2002; Turner and Haygarth 2005) (see also Appendix 1).

Most of the studies cited above concentrate on the role of extracellular phosphatases in the P nutrition of a particular organism or as a ‘bio-marker’ to assess the nutrient status of a particular environment. For example, up-regulation of PME activity is interpreted as an indication of P-limitation (Press and Lee 1983; Christmas and Whitton 1998a), and has been recorded in several plant/fungal

systems in response to N enrichment (Johnson *et al.* 1999; Treseder and Vitousek 2001; Turner *et al.* 2001; Phoenix *et al.* 2003). For example Phuyal *et al.* (2008) reported that phosphatase activity in the green tissue of *Hypnum jutlandicum* increased by *c.* 40 % in line with increasing wet inorganic N deposition from 8 to 64 kg N ha^{-1} y^{-1}.

1.7 Research objectives

The principal objective of this research project was to investigate relationships between nitrogen enrichment, and phosphatase activity and N/P stoichiometry in lichens. The heathland lichen *Cladonia portentosa* provided a model system to investigate the response of PME activity to N enrichment under field and experimental conditions. Both modelled and measured data for N deposition were used to indicate differences between sites in N enrichment providing an opportunity to assess the reliability of modelled interpolative N deposition maps. It is suggested that the close coupling between atmospheric chemistry and thallus physiology and chemistry in *C. portentosa* might provide the basis for a sensitive 'bio-indicator' of N deposition in British heathlands. The effect of N enrichment on N and P uptake kinetics was also examined in *C. portentosa*, whilst interspecific variation in phosphatase activity was investigated in N_2-fixing lichens.

The specific objectives of this research project were:

(i) To establish the characteristics and optimum conditions for PME activity in *C. portentosa*.

(ii) To determine the location of PME activity in *C. portentosa* thalli.

(iii) To investigate the response of PME activity in *C. portentosa* to variation in wet inorganic N deposition under field and experimental conditions.

(iv) To compare changes in PME activity in thalli of *C. portentosa* transplanted between N-polluted and non-polluted sites.

(v) To investigate the rate of PME activity in axenic cultures of *C. portentosa* in response to N and P availability.

(vi) To examine N and PO_4^{3-} uptake kinetics in *C. portentosa* exposed to contrasting conditions of N supply.

(vii) To investigate the relationship between N_2-fixing capacity and PME activity in N_2-fixing lichens.

CHAPTER 2. Preliminary investigations on phosphatase activity in *Cladonia portentosa*

2.1 Introduction

Lichens typically colonise nutrient poor habitats, and derive key nutrients directly from atmospheric sources (Crittenden *et al.*, 1994). As the availability of N and P in atmospheric deposits is generally low, lichens have evolved mechanisms to maximise nutrient capture and conservation and to grow slowly so as not to outstrip these resources. It has been shown that lichens readily absorb and assimilate multiple forms of N, including inorganic NO_3^- and NH_4^+, and organic molecules such as amino acids (Dahlman *et al.*, 2002, 2004; Ellis *et al.*, 2005; Palmqvist and Dahlman, 2006). This strategy is likely to prove beneficial to lichens where the form and quantity of N available in atmospheric deposits is spatially and temporally variable. Phosphorus acquisition by lichens has received less attention, however, uptake of inorganic phosphates (PO_4^{3-}) is known to be rapid and highly efficient (Farrar, 1976; Hyvärinen and Crittenden, 1998c). As with N, P exists in a number of different forms, including both inorganic and organic molecules. A capacity for lichens to utilise organic P has been indirectly implied by research into the action of phosphatase enzymes (Lane and Puckett, 1979; LeSueur and Puckett, 1980; Stevenson, 1994), which catalyse the hydrolysis of organic P molecules releasing biologically available orthophosphate (Jansson *et al.*, 1988).

In an early study of phosphatase activity in lichens, Lane and Puckett (1979), examined phosphomonoesterase (PME) activity in *Cladonia rangiferina* and reported its response to several environmental factors including pH, temperature, and metal toxicity. The authors also reported rates of phosphatase activity in other lichen species in the genera *Cetraria*, *Lobaria*, *Peltigera*, *Platismatia* and *Umbilicaria* (Lane and Puckett, 1979). This study was followed by an investigation into the effects of vanadium on PME activity in 5 lichen species (LeSueur and Puckett, 1980), and more recently by a yet unpublished investigation of surface phosphatase activity in species of *Peltigera* and *Cladonia* (Stevenson, 1994).

In the aforementioned reports, lichen phosphatase activity was quantified using the *para*-nitrophenyl phosphate (*p*NPP) and bis *para*-nitrophenyl phosphate (bis-*p*NPP) methods. These methods are among the most widely cited in the phosphatase literature, and have been applied to a broad range of different organisms and biological materials (Whitton *et al.*, 2005; Turner *et al.*, 2001; Phuyal *et al.*, 2008; Phoenix *et al.*, 2003; McElhinney and Mitchell, 1993; Press and Lee, 1983; e.g. Lane and Puckett, 1979). Enzymatic hydrolysis of the artificial substrate *p*NPP by PME enzymes releases inorganic phosphate and the organic moiety para-nitrophenol (*p*NP) in the ratio 1:1, whilst bis-*p*NPP is broken down by phosphodiesterase (PDE) enzymes into a phosphomonoester and *p*NP; thereby requiring PME activity to release orthophosphate (Turner and Haygarth, 2005). Both enzyme activities can be determined colourimetrically by measuring the quantity of *p*NP released spectrophotometrically at 405 nm.

These methods offer several advantages compared to other approaches to the determination of phosphatase activity, namely that both *p*NPP and bis-*p*NPP are soluble over the entire pH range, and the assay itself is simple, rapid and the substrates are relatively cheap (Tibbett, 2002, 2002b; Whitton *et al.*, 2002a). It is also considered useful as a first step in identifying non-specific phosphatase activity in previously un-tested samples. However, it is noteworthy that Attias and Bonnet (1972) discovered a phosphatase enzyme expressed by *Saccharomyces cerevisiae* which proved entirely specific to *p*NPP and showed little or no activity when exposed to other common phosphate monoesters. In addition, the *p*NPP assay can only provide a measure of total monoesterase activity, and cannot distinguish between several enzymes with different substrate specificities (Cembella *et al.*, 1984), and when supplied at very high concentrations, subsequent high *p*NP release may be toxic to some organisms (Whitton *et al.*, 2002b). Thus although there are no standard assay conditions, it is recommended that samples are incubated for less than 1 h at substrate concentrations intermediate between substrate saturating and that typically found in nature (Whitton *et al.*, 2002b, 2005).

Phosphomonoesterase activity can also be determined fluorometrically using a fluorogenic substrate, of which 4-methylumbelliferyl phosphate (MUP) is the most widely used (Pettersson and Jansson, 1978; Whitton *et al.*, 2002b). Although the general response of phosphatase activity is considered to be similar between colourimetric and fluorometric methods (Stevenson, 1994; Christmas and Whitton, 1998b; Ellwood *et al.*, 2002), quantitative differences are known to arise,

frequently with higher recordings made using the *p*NPP method (Pettersson and Jansson, 1978; Banerjee *et al.*, 2000). This has been shown in lichens where Stevenson (1994) compared hydrolysis of MUP and *p*NPP, confirming that *p*NPP provided uniformly higher estimates of PME activity in *Peltigera canina* and *Cladonia arbuscula* across the pH range 3-11. However, it is important to note that as fluorometric methods are considered to be up to 100 times more sensitive than colourimetric assays (Pettersson and Jansson, 1978), they have the advantage that they can be performed on much smaller quantities of biological material, for shorter time periods and at substrate concentrations most closely resembling those found in nature (Whitton *et al.*, 2002b).

Phosphatase activity has most recently been detected using the technique of enzyme-labelled fluorescence (ELF, Invitrogen, Molecular Probes). This procedure involves treating samples with a soluble, weakly fluorescent substrate such as ELF-97 phosphate (2-(5′-chloro-2′-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone), which once enzymatically cleaved forms a fluorescent precipitate at the site of enzyme activity. This can be quantified using fluorescent microscopy techniques; it has the advantage that the fluorescent precipitate has high photostability and is readily detected above any background fluorescence (Nedoma *et al.*, 2003). Other advantages of this method are that both acid and alkaline phosphatases are readily measurable, and it is highly sensitive to the addition of inhibitors, in particular orthophosphate. However, drawbacks associated with this method include low sensitivity, inability to detect PDE activity, and a non-linearity of precipitate formation with time (Huang *et al.*, 1992;

Nedoma *et al.*, 2003). The latter problem might result from the development of fluorescent precipitates within the immediate enzyme environment, slowing down the rate at which enzyme-substrate complexes can form. Since ELF-97 phosphate has become commercially available only very recently, few studies have compared the reliability of this technique for measuring PME activity against other more established methods. Nedoma *et al.* (2003) report that values of the PME kinetic constants K_m (Michaelis constant) and V_{max} (maximum rate of reaction) for several algal genera (including *Chlamydomonas*, *Chlorogonium* and *Gymnodium*) were two to four times higher when determined using ELF 97 phosphate as opposed to MUP; and van Aarle *et al.* (2001) confirm that both acid and alkaline phosphatases were detected in *Aspergillus fumigatus* with ELF-97 phosphate, however, only acid phosphatase activity was observed using *p*NPP. More recently, research by Alvarez *et al.* (2006), confirmed no significant differences in PME activity measured with either *p*NPP or ELF-97 phosphate substrates in the ectomycorrhizal fungi *Cencoccum geophilium* and *Paxillus involutus*.

Regarding all analytical techniques, the largest source of error in accurately determining phosphatase activity arises from a lack of distinction between different sample fractions. The vast majority of phosphatase studies report cell-wall associated and extracellular PME activity, in which inaccuracies might arise if cells are damaged and the activity of intracellular metabolic-associated phosphatases is recorded (Tibbett *et al.*, 2000). This has been avoided in several studies by the physical separation of cell fractions into those likely to contain phosphatases. Straker and Mitchell (1986) and McElhinney and Mitchell (1993)

divided mycorrhizal fungal phosphatase activity into that associated with extracellular, wall-bound and cytoplasmic fractions. It is also important to consider that the most meaningful comparative studies on phosphatase activity might be those in which consideration is given to the surface area to which the substrate is exposed and to the likely non-random distribution of enzymes throughout the organism (Tibbett *et al.*, 2000).

Although there is a large literature reporting PME and PDE activities in different organisms, it is often difficult for direct comparisons to be made between studies due to large inconsistencies in assay conditions and methods followed (Pettersson and Jansson, 1978; Tibbett, 2002). It is also worth considering whether phosphatases show the same affinity for artificial substrates as they do for natural ones (Jansson *et al.*, 1988). However despite the drawbacks associated with the different methods discussed above, phosphatase assays can provide an invaluable insight into the ability of an organism to utilise organic P fractions within their immediate environment.

This chapter therefore reports investigations to characterise phosphatase activity in *C. portentosa*, using the *p*NPP and bis-*p*NPP methods. Assay conditions were optimised for pH, incubation temperature and time, and substrate concentration. In addition, a preliminary investigation was undertaken to determine the response of PME activity to variation in simulated wet inorganic N deposition ($\mathbf{N}_s$).

2.2 Materials and Methods

2.2.1 Study sites

Cladonia portentosa is a common terricolous lichen found in *Calluna vulgaris*-dominated lowland heathland and upland moorland throughout the British Isles. Study sites supporting *C. portentosa* from which samples could be removed without seriously depleting local populations were sought and collections were made on various dates between 25th October 2005 and 4th June 2007 (Table 2.1).

Table 2.1 Details of study sites from which samples of *Cladonia portentosa* were collected for preliminary investigations on lichen phosphatase activity. Values of $\mathbf{N}_s$ are provided by R. I. Smith (Pers. Comm., CEH Edinburgh), for full details see Chapter 4, section 4.2.1.

Name of site	Geographical location	Grid reference	$\mathbf{N}_s$ (kg N ha^{-1} y^{-1})	Date of collection	Collected by:*
River Etherow	Derbyshire	SK 125 986	20.3	07.02.06	EJH, PDC, GM
Ridgewalk Moor	Derbyshire	SK 147 952	19.3	04.06.07	EJH, PDC, ZM
North Lees Estate	Derbyshire	SK 231 835	19.2	09.12.05	EJH, PDC
The Rock, (Stiperstones)	Shropshire	SO 359 970	8.8	19.11.05 21.03.06	PDC EJH, PDC, GM
The Halsary	Caithness	ND 195 493	4.2	25.10.05 - 27.10.05 08.12.06	EJH, PDC EJH, PDC, GM

* Names abbreviated as follows; EJH, E. J. Hogan; PDC, P. D Crittenden; ZM, Z. Mtshali; GM, G. Minnullina

2.2.2 Collection and pre-treatment of *Cladonia portentosa*

At each site 5 - 10 replicate samples of *C. portentosa* were collected, each one typically a clump of podetia 50 - 150 mm in diameter. The samples were collected from open areas to minimise tree canopy effects and from locations > 10 m apart to minimise the chances of collecting genetically identical material. Samples were transported to the laboratory either in seed trays or polythene bags, and air-dried for 24 – 48 h at room temperature. Material was then either stored in seed trays in a growth room at 10 ^{o}C under a 12 h light (photosynthetic photon fluence rate (PPFR) of 50 - 200 µmol m^{-2} s^{-1} over the waveband 400 - 700 nm) / 12 h dark cycle, or sealed in polythene bags and stored at -15 ^{o}C (unless stated otherwise, see section 2.2.2.1). Powder-free latex gloves were worn at all times when handling lichens both in the field and in the laboratory to minimize contamination.

Optimal conditions for sample storage were confirmed by performing phosphatase assays on lichens at the following times and subject to the following pre-treatments after collection: within 8 h and samples not allowed to dry (i.e. naturally hydrated); after 24 h and 7 days stored dry in a growth room at 10 ^{o}C under a 12 h light (PPFR 50 - 200 µmol m^{-2} s^{-1}) / 12 h dark cycle; after 7 days and 8 weeks stored dry in sealed polythene bags at -15 ^{o}C.

2.2.3 Sample preparation

Thalli were rehydrated overnight in water-saturated air (over water in a desiccator) at 10 ^{o}C, then saturated by spraying lightly with deionised water and cleaned of extraneous debris. Samples selected from different lichen cushions (to avoid pseudo-replication), were then dissected into horizontal strata by cutting with a razor blade at some or all of the following distances downwards from the apices: 5, 10, 15, 25, 35, 40, 50, 65, 80 mm.

2.2.4 Phosphatase assay

Phosphomonoesterase (PME) and phosphodiesterase (PDE) activities were determined using *p*-nitrophenyl phosphate (*p*NPP) and bis *p*-nitrophenyl phosphate substrates respectively, as described by Turner *et al*. (2001). Samples of *C. portentosa* were added to 2.9 ml 0.02 M citric acid-trisodium citrate buffered assay medium (pH 2.5 unless otherwise stated) made up in simulated rainfall containing major ions representative of precipitation chemistry in the British Isles (20 mmol l^{-1} $MgSO_4 \cdot 7H_2O$, 8 mmol l^{-1} $CaCl_2 \cdot 2H_2O$, 150 mmol l^{-1} NaCl, 15 mmol l^{-1} NH_4NO_3, 5 mmol l^{-1} KNO_3) (Hayman *et al.*, 2004b). Assays were initiated by the addition of 0.1 ml analogous substrate, to yield a final concentration of either 0.5 mM, 3 mM or other values where stated. Samples were then placed in a shaking water-bath at 15 ^{o}C for 20 min in the dark (unless otherwise stated) after which the reaction was terminated by transferring 2.5 ml assay medium into 0.25 ml terminator solution (1.1 M NaOH, 27.5 mM EDTA, 0.55 M K_2HPO_4) and the absorbance measured at 405 nm using a NanoDrop ND-1000 spectrophotometer

(Labtech International Ltd, Ringmer, East Sussex). Thalli were then blotted dry, oven dried for 24 h at 80 ^{o}C and weighed. Enzyme activity is expressed as mmol substrate hydrolyzed g^{-1} dry mass h^{-1} using *p*-nitrophenyl to calibrate the assay. No-lichen controls were included in each set of assays and used to correct for interference due to absorbance by *p*NPP at 405 nm and any non-enzymatic substrate hydrolysis (Fig. 2.1).

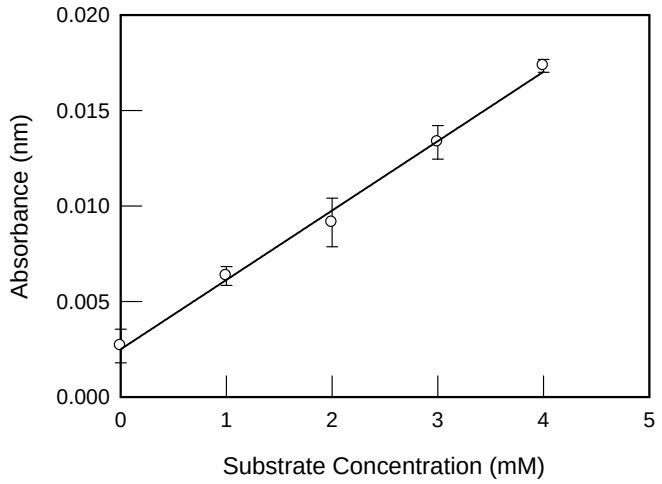


Figure 2.1 Background absorbance of *p*NPP (no lichen controls) at substrate concentrations between 0 – 4 mM *p*NPP (r = 0.999, P < 0.001). Plotted values are means (n = 3) ± 1 SEM.

2.2.4.1 Influence of assay medium pH

The citric acid tri-sodium citrate PME assay medium was adjusted to pH values of 2.5, 2.8 (0.02 M) 3.2, 3.9, 4.3, 4.6, 5.3, 5.9 and 6.6 (0.01 M) (Table 2.2) following slight alterations to the 0.1 M citric acid-trisodium citrate buffer solutions recipe described by Dawson *et al*. (1986).

2.2.4.2 Selection of buffer

A comparative study was designed to test the influence of buffer type on PME activity. 0.1 M DMG (3, 3-dimethylglutaric acid) was adjusted with 0.2 M NaOH to obtain pH values of 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 and 5.5 following Dawson *et al*. (1986). Citric acid tri-sodium citrate buffer was made up to the pH values outlined in Table 2.2.

Table 2.2 Citric acid tri-sodium citrate buffer solutions, pH 2.5 - 6.6. *x* ml 0.1 M citric acid and *y* ml 0.1 M trisodium citrate are mixed and made up to 1 l, following Dawson *et al*. (1986).

pH	*x* ml 0.1 M citric acid	*y* ml 0.1 M trisodium citrate
2.5	19.5	0.5
2.8	18.2	1.8
3.2	8.2	1.8
3.9	6.85	3.15
4.3	5.9	4.1
4.6	4.95	5.05
5.3	3.5	6.5
5.9	2.1	7.9
6.6	0.8	9.2

2.2.4.3 Influence of light

Phosphomonoesterase assays were conducted at 10 ^{o}C either in the dark (incubation vials double wrapped in aluminium foil) or beneath a light bank providing a PPFR of 65.6 µmol m^{-2} s^{-1} over the waveband 400 – 700 nm.

2.2.4.4 Influence of incubation temperature

Phosphomonoesterase activity was determined with incubation temperatures of 5, 10, 15, 20, 30, 40 and 50 ^{o}C.

2.2.4.5 Influence of incubation time

The duration of assays for PME activity was varied from between 5 and 60 min.

2.2.4.6 Influence of substrate concentration

The response of PME activity to increasing substrate concentration was determined for samples of *C. portentosa* collected from sites with contrasting rates of $\mathbf{N}_s$; The Halsary, Caithness and Ridgewalk Moor, Derbyshire (for values of $\mathbf{N}_s$ see Table 2.1). In order to determine enzyme kinetics, PME assays were conducted with final *p*NPP concentrations varying across the range 0.5 – 10 mM.

If graphical presentations of data plotted as initial velocity (v) against substrate concentration ([S]) reveals a rectangular hyperbolic curve, it is indicative of first order Michaelis-Menten enzyme kinetics. The rate of reaction increases with increasing substrate concentration up to a certain value whereby the reaction proceeds at maximum velocity (known by the kinetic parameter V_{max}). The Michaelis constant (K_m), can also be visualised on the hyperbolic curve as it represents the substrate concentration at which the rate of reaction proceeds at half V_{max}, and provides an indication of the affinity of an enzyme for its substrate

(Cornish-Bowden, 1995). The ratio V_{max}/K_m estimates the efficiency of an enzyme, which is ultimately determined by the rate at which substrate comes into contact with the enzyme. Values for K_m and V_{max} were estimated by fitting data to the Michaelis-Menten equation (Eqn. 2.1) using regression analysis (hyperbola, single rectangular, two parameters) in SigmaPlot 8.0 (SPSS, Inc., Chicago, IL). However, this method is not considered to provide the most accurate determinations of K_m and V_{max}. Therefore, linear regression analysis following linear transformation of the Michaelis-Menten equation was also used. The three linear transformations of the data which were applied in order to derive accurate values for K_m and V_{max} were Lineweaver-Burke, Eadie-Hofstee and Hanes-Wolf (Equations 2.2 -2.4).

Michaelis-Menten equation:

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad \textbf{Equation 2.1}$$

Lineweaver-Burke re-arrangement of the Michaelis-Menten equation:

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \text{ x } \frac{1}{[S]} \qquad \textbf{Equation 2.2}$$

Eadie-Hofstee re-arrangement of the Michaelis-Menten equation:

$$v = V_{max} - K_m \text{ x } \frac{v}{[S]} \qquad \textbf{Equation 2.3}$$

Hanes-Woolf re-arrangement of the Michaelis-Menten equation:

$$\frac{[S]}{v} = \frac{K_m}{V_{max}} + \frac{[S]}{V_{max}} \qquad \textbf{Equation 2.4}$$

where, v is the velocity of the reaction at any given substrate concentration ([S]), K_m is the Michaelis-Menten constant (substrate concentration at which the reaction is proceeding at half the maximum uptake rate) and V_{max} is the maximum uptake rate achieved at substrate saturation (Wanek and Pörtl, 2008).

The three transformations give different values for the kinetic parameters as they each apply different weighting to various sources of error (Cornish-Bowden, 1995; Turner *et al.*, 2001; Wanek and Pörtl, 2008). The Lineweaver-Burk transformation is a double reciprocal plot of 1/[S] against 1/v giving a straight line with K_m/V_{max} as the gradient, $1/V_{max}$ at the y-intercept and $-1/K_m$ at the x-intercept (Cornish-Bowden, 1995). This plot is considered to over-emphasise the error associated with values at low substrate concentrations; conditions in which slow uptake rates occur, often leading to inaccurate readings (Wanek and Pörtl, 2008). The Eadie-Hofstee plot gives a gradient value equating to $-K_m$, y axis intercept as V_{max} and the x axis intercept as V_{max}/K_m. However, in determining values for the x and y axes (v/[S] and v, respectively), there is no independence of the variables and any sources of experimental error will therefore be apparent on both axes (Wanek and Pörtl, 2008). The Hanes-Woolf transformation is often considered to give the most reliable values for K_m and V_{max} (Cornish-Bowden, 1995; Wanek and

Pörtl, 2008) whereby the plot of [S] against [S]/*v* provides a gradient value equating to 1/*V*max, *x*-intercept of K_m and *y*-intercept of K_m/V_{max}.

2.2.5 Influence of wet N deposition

The influence of wet inorganic N deposition on PME activity in *C. portentosa* was investigated by recording activity in lichen samples collected from sites receiving 4.2, 8.8, 19.2 and 20.3 kg N ha^{-1} y^{-1}. Sites were selected from a database of $\mathbf{N}_s$ values at 5 x 5 km grid square resolution across Great Britain (R. I. Smith, CEH Edinburgh, Pers. Comm.). For full details see Chapter 4, section 4.2.1.

2.2.6 Thallus surface pH

Dry thalli were hydrated as described above and then pressed against the bottom of a Petri dish using two microscope slides positioned 15 mm apart. 0.025 M KCl was added to the exposed thallus between the slides (to enhance cation exchange) until a visible water film was formed on the surface of the thallus. A flat tip pH electrode (BDH Gelplas double junction flat tip electrode) was placed firmly against the wet thallus and the pH was noted at 15 s intervals for 1 min.

2.2.7 Sequence of assays

Assays were performed in batches where thalli selected from each batch were exposed to the full range of experimental variables. Replication (n = 5 - 10) was generated by analysis of further batches.

2.2.8 Statistical analyses

The statistical software package SPSS version 12.0.1 (SPSS, Inc., Chicago, IL) was used to perform standard statistical analyses. All data were initially subjected to tests for normality and homogeneity of variances. If these assumptions were not violated, data was subjected to linear regression analysis, *t*-tests or one or two-way analysis of variance (ANOVA). Where ANOVA produced significant results, Fishers LSD (least significant difference) post-hoc tests were also performed. Where data did not fit a normal distribution, $\log_{10}$ or square root transformations were performed. If data remained discontinuous, differences between different groups of data were analysed by non-parametric Mann Whitney U tests.

2.3 Results

2.3.1 Response to environmental variables

Cladonia portentosa had readily measurable PME and PDE activities. Both were detected across the range of pH intervals tested, with PME activity consistently significantly higher than PDE activity by a factor of *c.* 4 (Fig. 2.2). For both enzyme systems there was a marked trend for increasing activity with decreasing pH, with maximum values recorded at pH 2.5. Rates of activity were approximately 3 and 5 times greater at pH 2.5 than pH 6.6 for PME and PDE, respectively (Fig. 2.2). This apparent pH optimum was consistent with the

measured thallus pH of 3.09 recorded after 1 min in 0.025 KCl. At each pH interval investigated there was no significant difference between the effects of either DMG or citric acid tri-sodium citrate buffers (P = 0.213) on PME activity (Fig. 2.3). There was no effect of final citric acid tri-sodium buffer concentration at either 0.01 or 0.02 M on PME activity (P = 0.818).

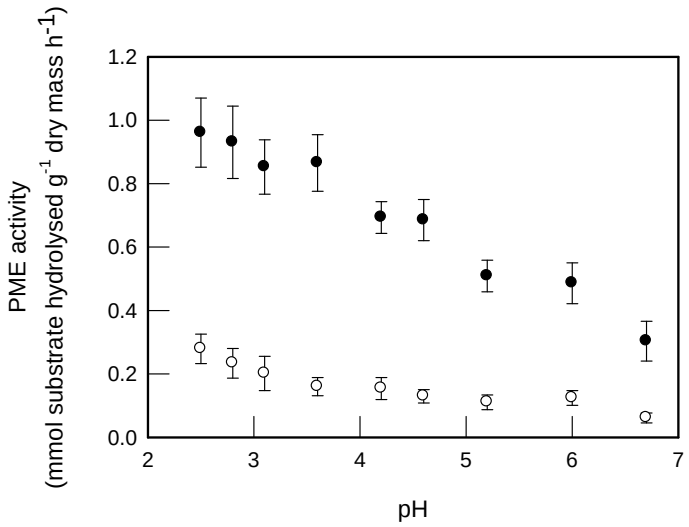


Figure 2.2 The effect of pH on PME (●; r^2 = 0.971, P < 0.001) and PDE (○; r^2 = 0.931, P < 0.001) activity in 10 mm apical segments of *Cladonia portentosa*. Assays were performed with 0.5 mM *p*NPP or bis-*p*NPP for 20 min at 15 ^{o}C in the dark. Material collected from the North Lees Estate, Derbyshire. Plotted values are means (n = 6) ± 1 SEM.

In *C. portentosa*, ANOVA revealed that PME activity significantly decreased (P = 0.001) with increasing distance from the apices (Fig. 2.4b). Although PME activity was readily measurable to a depth of 80 mm below the apices, the lowermost stratum assayed, it was highest in the apices and decreased by *c.* 40 % at depths > 40 mm from the apices (Fig. 2.4b).

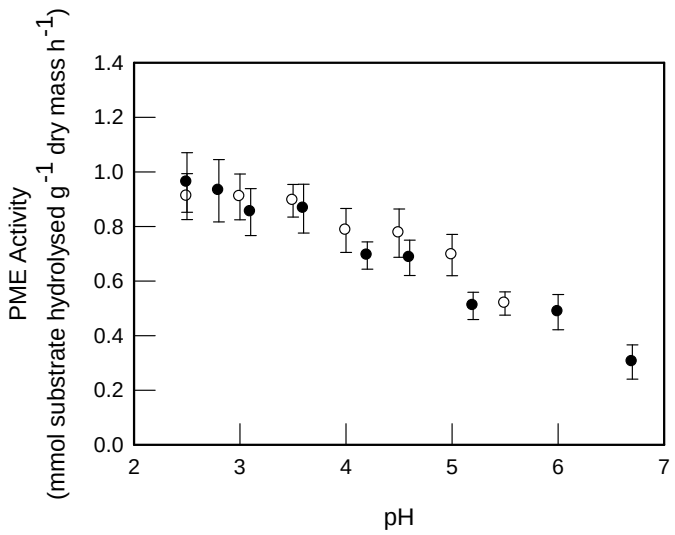


Figure 2.3 The effect of pH buffered by citric acid tri-sodium citrate (○) and DMG (●) on PME activity in 10 mm apical segments of *Cladonia portentosa*. Assays were performed on material collected from the North Lees Estate, Derbyshire with 0.5 mM *p*NPP for 20 min at 15 ^{o}C in the dark. Plotted values are means (n = 6) ± 1 SEM.

A Fishers *t* test suggested that PME activity in *C. portentosa* was independent of light (Fig. 2.5). The rate of reaction of the PME assay responded linearly to increasing temperature between 5 and 50 ^{o}C (Fig. 2.6a), with an ecologically relevant incubation temperature of 15 ^{o}C selected for subsequent assays. Generation of product (*p*NP) was linear with respect to time up to 60 min, at a rate of *c.* 0.4 mmol *p*NP g^{-1} dry mass h^{-1} (Fig. 2.6b). Results of PME activity measured subsequently using a 20 min assay could therefore be accurately extrapolated to represent mmol substrate hydrolysed g^{-1} dry mass h^{-1}. Although collections of *C. portentosa* were made on several different occasions (Table 2.1), there was no evidence of seasonal variation in PME activity.

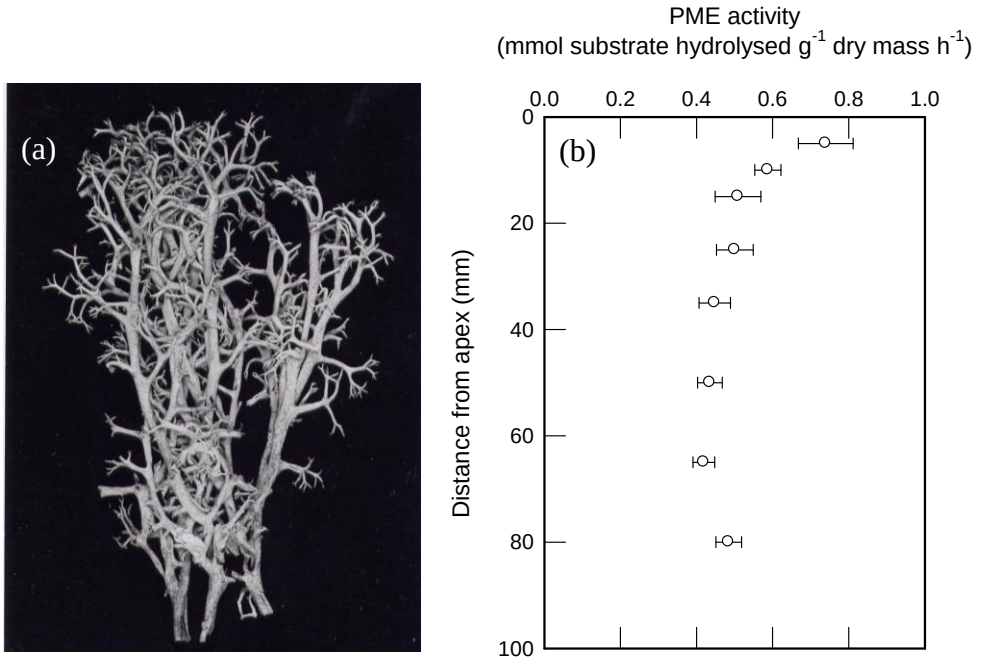


Figure 2.4 Thallus of *Cladonia portentosa* (50 mm tall) (a) and vertical distribution of PME activity in podetia collected from The Halsary, Caithness (b). Assays were performed with 0.5 mM *p*NPP for 20 min at 15 ^{o}C in the dark. Plotted values are means (n = 6) ± 1 SEM. Each mean value is plotted at the lower extremity of the depth interval that they represent (e.g. 0 – 5 mm plotted at 5 mm from apices).

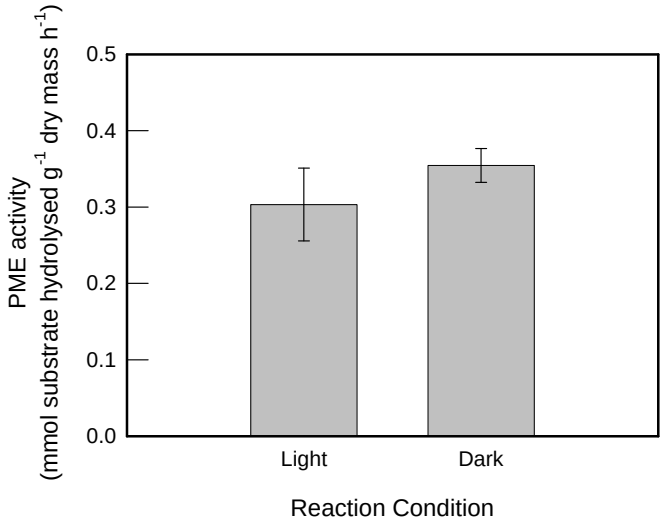


Figure 2.5 The effect of light on PME activity in 10 mm apical segments of *Cladonia portentosa* (P = 0.161). Assays were performed on material collected from The Halsary, Caithness with 0.5 mM *p*NPP for 20 min at 15 ^{o}C, light conditions (65.5 µmol m^{-2} s^{-1}). Plotted values are means (n = 5) ± 1 SEM.

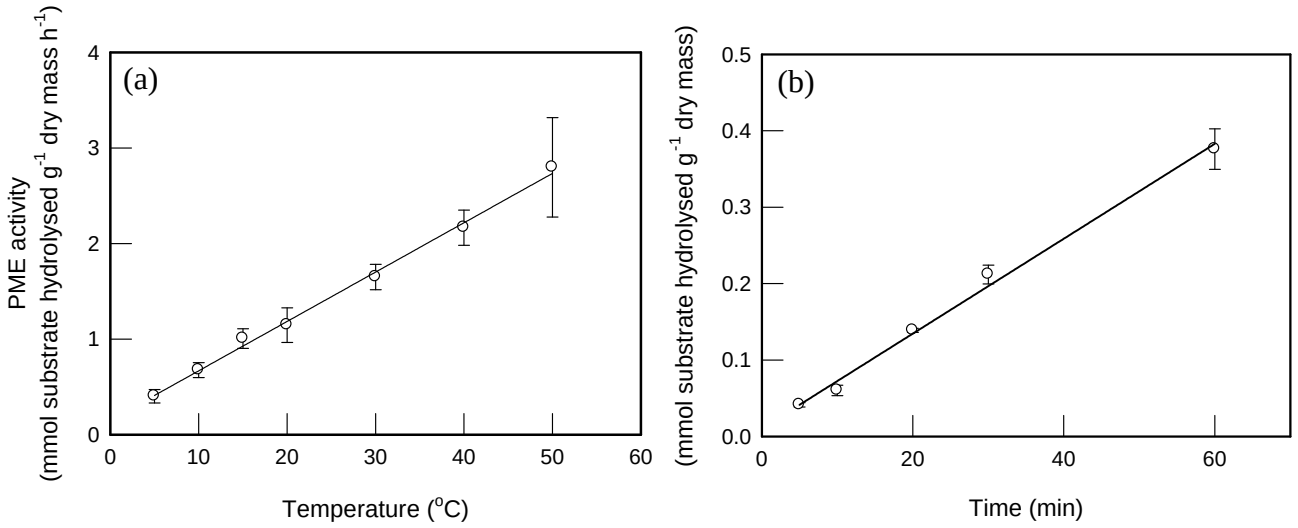


Figure 2.6 The effect of incubation temperature (a, r^2 = 0.960, P < 0.001) and duration (b, r^2 = 0.994, P < 0.001) on PME activity in 10 mm apical segments of *Cladonia portentosa*. Assays were performed on material collected from The Rock, Shropshire (a) and The Halsary, Caithness (b) using 0.5 mM *p*NPP for 20 min (a) or 15 ^{o}C (b) in the dark. Plotted values are means (n = 5 - 6) ± 1 SEM.

2.3.2 Response to substrate concentration and N availability

Preliminary investigations were undertaken using a substrate concentration of 0.5 mM following Turner *et al.* (2001). In response to increasing substrate concentration, PME activity in *C. portentosa* followed Michaelis-Menten kinetics (Fig. 2.7a). Substrate response curves and subsequent linear transformations (Fig. 2.7b) were performed for material collected from two study sites, which differ in the rate at which N is naturally deposited. The three separate linear transformations of the Michaelis-Menten equation gave different values for the parameters K_m and V_{max} (Table 2.3).

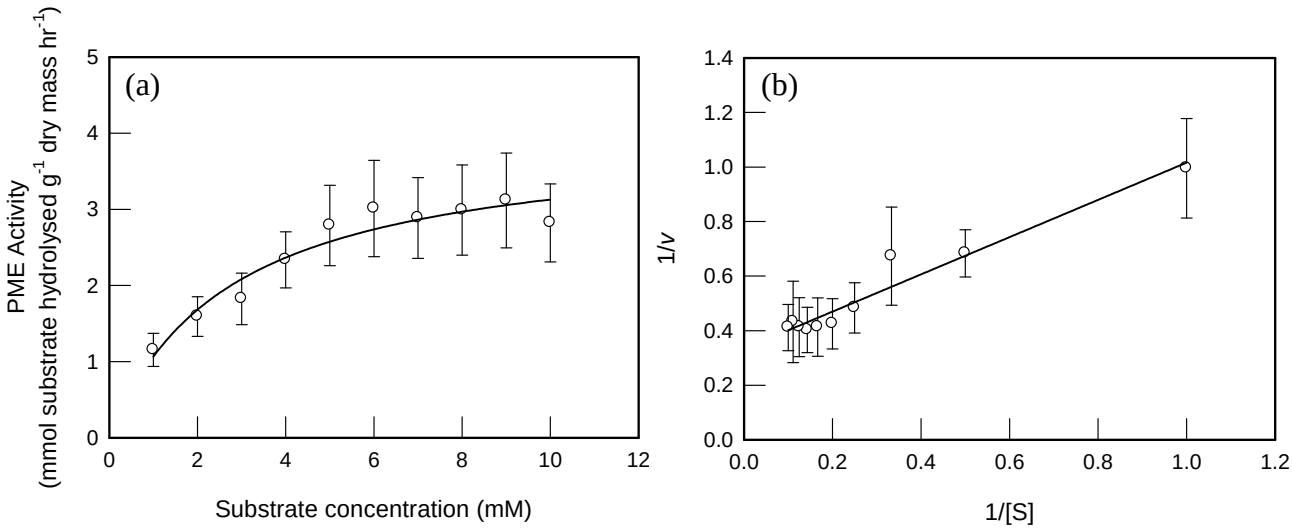


Figure 2.7 Relationship between PME activity and substrate concentration in 10 mm apical segments of *Cladonia portentosa* (a) Michaelis-Menten plot (b) Lineweaver-Burke linear transformation of data set where the *x* axis (1/[S]) is the reciprocal of substrate concentration and the *y* axis (1/*v*) is the reciprocal velocity of the reaction (r^2 = 0.947, $P < 0.001$). Assays performed on material collected from Ridgewalk Moor, Derbyshire at 15 ^{o}C for 20 min in the dark. Plotted values are means (n = 6) ± 1 SEM.

Mean K_m and V_{max} values were 0.974 mM and 1.219 mmol substrate hydrolysed g^{-1} dry mass h^{-1}, respectively for material collected at The Halsary, a site receiving low N inputs (4.2 kg N ha^{-1} y^{-1}). Values of K_m and V_{max} were significantly higher (P = 0.05) by factors of 2.5 and 3, respectively, in samples from Ridgewalk Moor, Derbyshire which receive high N inputs (19.3 kg N ha^{-1} y^{-1}) and enzyme efficiency (V_{max}/K_m) in lichen from this site was significantly higher (P = 0.013) by *c*. 17 %. For all subsequent assays, a concentration of 3 mM *p*NPP was selected to represent a concentration intermediate between saturation and that found in nature.

Table 2.3 Kinetic parameters of PME activity in *Cladonia portentosa* collected from sites with contrasting rates of $\mathbf{N}_s$ as determined following three linear transformations of the Michaelis-Menten equation. The units of K_m are mM and V_{max} are mmol substrate hydrolysed g^{-1} dry mass h^{-1}; r^2 values are provided to demonstrate the goodness of fit of the straight line following linear transformations. Values of $\mathbf{N}_s$ are provided by R. I. Smith (CEH Edinburgh, Pers. Comm.), for full details see Chapter 4, section 4.2.1.

	Study Site and $\mathbf{N}_s$ values					
	The Halsary $\mathbf{N}_s$ 4.2 kg N ha^{-1} y^{-1}			Ridgewalk Moor $\mathbf{N}_s$ 19.3 kg N ha^{-1} y^{-1}		
Equation	K_m	V_{max}	r^2	K_m	V_{max}	r^2
Michaelis-Menten	0.674	0.998	0.847	2.737	3.984	0.295
Lineweaver-Burk	1.499	1.667	0.995	2.277	3.333	0.947
Eadie-Hofstee	0.843	1.101	0.654	2.441	3.824	0.820
Hanes-Woolf	0.878	1.111	0.849	2.221	3.333	0.967
Mean	0.974	1.219		2.419	3.619	

Phosphomonoesterase activity was highly positively related to $\mathbf{N}_s$ (Fig. 2.8), increasing by *c.* 70 % between sites receiving 5.3 and 19.3 kg N h^{-1} y^{-1}.

2.3.3 Response of PME activity to pre-treatment

Results for the preliminary investigations above were obtained using freshly collected lichen material stored air dry in a growth room (see Methods section 2.2.2). However, the initial enzyme activity at the time of collection could be maintained by storage at -15 ^oC in the air dry state (Fig. 2.9). Accordingly, this storage method was used for lichen collections used in subsequent investigations.

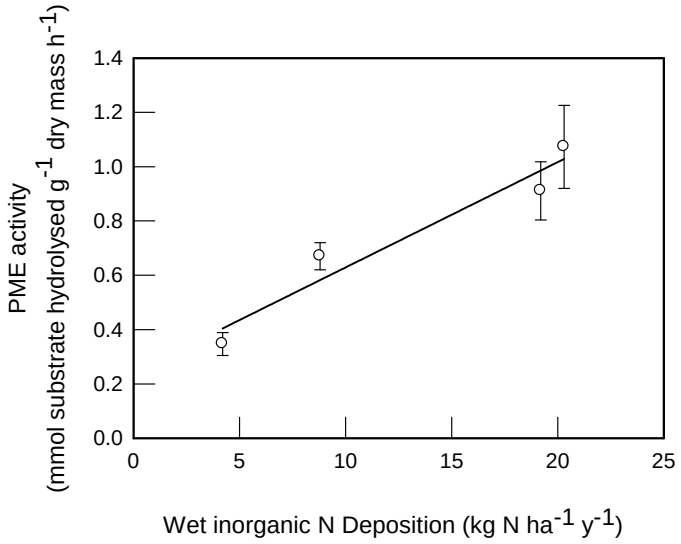


Figure 2.8 The relationship between $\mathbf{N}_s$ and PME activity in 10 mm apical segments of *Cladonia portentosa* (r^2 = 0.928, P = 0.036). Assays were performed on material collected from The Halsary (Caithness), The Rock (Shropshire), the North Lees Estate and River Etherow (Derbyshire) using 0.5 mM *p*NPP for 20 min at 15 ^{o}C in the dark. Plotted values are means (n = 10) ± 1 SEM.

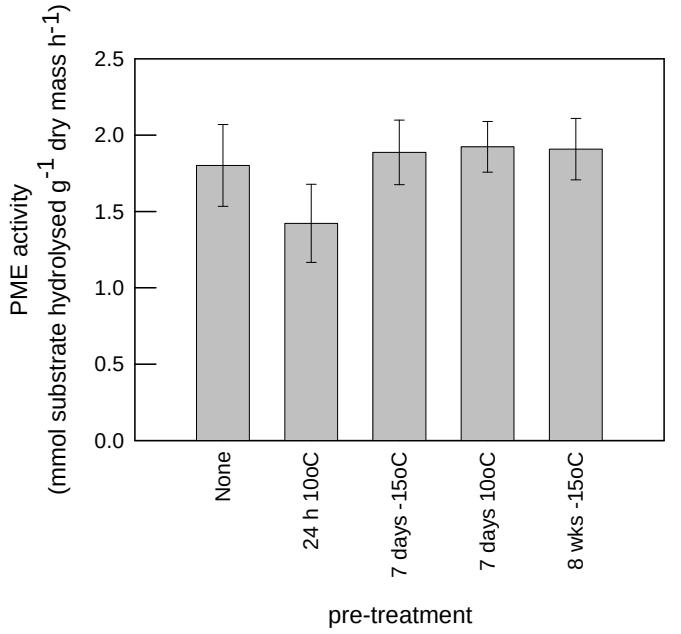


Figure 2.9 The effect of pre-treatment on PME activity in 10 mm apical segments of *Cladonia portentosa*. The effect of storage time was not significant (ANOVA, P = 0.482). Assays were performed on material collected at The Rock (Shropshire) with 0.5 mM *p*NPP for 20 min at 15 ^{o}C in the dark. Plotted values are means (n = 10) ± 1 SEM.

2.4 Discussion

The characteristics of phosphatase activity in *C. portentosa* are comparable to those reported for other plant, microbial and fungal systems (McElhinney and Mitchell, 1993; Joner *et al.*, 2000; Turner *et al.*, 2001; Whitton *et al.*, 2005). Activity was detected across a broad range of pH values (pH 2.5 – 6.6), consistent with the data of Lane and Puckett (1979) for *C. rangiferina* and *Lobaria pulmonaria*, both of which were shown to have acid phosphatases and apparent pH optima of *c.* 2.2. Since *C. portentosa* produces a range of secondary metabolites, including usnic and perlatolic acids (Ahti, 1961; Thomson, 1967; Houvinen and Ahti, 1986; Osyczka, 2006), it is likely that phosphatase enzymes are located in a low pH environment. However, not all lichens produce acidic secondary compounds, and the pH optimum for PME activity in some lichen species (e.g. *Peltigera spp., Xanthoria spp.)* is in the range 5 - 6 (see Chapter 9 & Stevenson 1994), consistent with a neutral to alkaline class of phosphatase (Jansson *et al.*, 1988; Nedoma *et al.*, 2003). In agreement with the findings of Turner *et al.* (2001) for several species of moss, PME activity in *C. portentosa* was significantly higher than PDE activity. A similar ratio has also been recorded in species of *Trebouxia* and *Nostoc* (Whitton *et al.*, 1990; Banerjee *et al.*, 2000), which commonly form lichenized associations, and might reflect the availability of different phosphate esters to which *C. portentosa* is exposed in heathland habitats. There is also a greater physiological cost involved in the release of PO_4^{3-} from a phosphodiester molecule (as this involves both PDE and PME activities). Molecules of bis-*p*NPP are also considerably larger than *p*NPP, and may diffuse

less readily through the cell wall (Turner *et al.*, 2001). Nonetheless, hydrolysis of phosphodiesters provides a source of both the major growth-limiting nutrients, N and P (Leake and Miles 1996).

Although it is suggested that the results of lab based phosphatase assays can be influenced by the buffer selected (Whitton *et al.*, 2005), in the present work there was a strong agreement between the rates of activity recorded using DMG and citric acid tri-sodium citrate buffers. A concentration of 0.01 or 0.02 M citric acid tri-sodium citrate buffer was selected for use in all subsequent assays since it was felt this system would minimise potential interference, whilst at the same time maintain a stable pH throughout the duration of the assay.

The rate of product generation was linear with time up to 60 min, consistent with previous findings for *C. rangiferina* (Lane and Puckett, 1979) and *Peltigera canina* (Stevenson, 1994). This linear accumulation with time of *p*NP in the bathing medium provides indirect evidence that hydrolysis of *p*NPP might result from the action of an extracellular phosphatase, as there was no delay in *p*NP release which might be expected if hydrolysis occurs inside the cell (Banerjee *et al.*, 2000). In addition, this response indicated that there was no evidence of substrate depletion or potential inhibition of activity by un-absorbed orthophosphate accumulating in the bathing solution.

Maximum PME activity occurred in the apices, decreasing significantly towards the bases, consistent with the distribution of physiological activity (Plakunova and

Plakunova, 1984; Sveinbjörnsson, 1987; Crittenden, 1991) and element distribution (N, P and K) (Pulliainen, 1971; Pakarinen, 1981; Crittenden, 1991; Hyvärinen and Crittenden, 1998a) in thalli of *C. portentosa*. Within large cushions of *C. portentosa*, maximum PME activity in the apices is likely to increase the efficiency of substrate utilisation, since apical regions will be exposed to the largest quantities of atmospheric deposits and are likely to have the greatest nutrient demand.

It was essential to examine the influence of light, temperature and substrate availability on PME activity, as these represent key abiotic factors which are most likely to influence enzyme activity in the lichen habitat. Phosphomonoesterase activity in *C. portentosa* was independent of light, consistent with the findings of Lane and Puckett (1979) on *C. rangiferina*, suggesting P hydrolysis can function during the complete diurnal cycle. However, it is important to note that both light availability and light quality have been demonstrated to significantly influence PME activity in cyanobacteria (Banerjee *et al.*, 2000), algae (Hernández *et al.*, 1996; Banerjee *et al.*, 2000) and phytoplankton (Wynne and Rhee, 1988). A reduction in PME activity in the light might result from increased susceptibility of extracellular enzymes to high irradiance (Whitton *et al.*, 2005). Alternatively, increased PME activity in the light might be due to a positive influence of light on PO_4^{3-} uptake (Whitton *et al.*, 2005), reducing the concentration of this competitive inhibitor in the immediate environment.

In agreement with the work of Lane and Puckett (1979) on *C. rangiferina*, PME activity in *C. portentosa* increased linearly with temperature up to 50 ^{o}C. Activity was measurable down to 5 ^{o}C, inferring a broad temperature tolerance, covering the typical range to which *C. portentosa* is exposed. The temperature required to denature PME in *C. portentosa* was not achieved, however this process typically occurs at temperatures considerably higher than those experienced by organisms in their natural habitat (Banerjee *et al.*, 2000; Whitton *et al.*, 2005). In Antarctic endolithic microbial communities containing *Trebouxia* (which infrequently experience temperatures > 10 ^{o}C), Banerjee *et al.* (2000) measured PME activity up to temperatures of 15 ^{o}C. Lichens are usually desiccated when exposed to high temperatures as a means of reducing thermal stress (Kappen, 1973). Moderate heat resistance has been reported in hydrated lichen thalli (< 46 ^{o}C in members of the subgenus *Cladina*), whilst desiccated thalli have been shown to tolerate temperatures of 80 ^{o}C for 0.5 h (Lange, 1953). In their natural habitat, lichens are exposed to broad temperature fluctuations, and intermittent periods of desiccation and rehydration. Tolerance to extremely high temperatures might therefore reflect a 'flexibility' of the enzyme (Banerjee *et al.*, 2000) ensuring physiological machinery survives periods of desiccation and can regain full functionality upon onset of rehydration. Whitton *et al*. (1990) report no detectable lag in PME and PDE activity following rehydration of desiccated samples of the cyanobacterium *N. commune*.

Hyperbolic substrate-saturation curves indicative of Michaelis-Menten kinetics were generated in response to increasing substrate concentration up to 10 mM.

Values of K_m and V_{max} calculated from different models were in general agreement, and ranged between 0.97 – 2.42 mM and 1.22 and 3.62 mmol substrate hydrolysed g^{-1} dry mass h^{-1}, respectively. These are comparable with published K_m and V_{max} values for fungal extracellular PME activity (K_m, 0.07 - 17.5 mM) (Antibus *et al.*, 1986; McElhinney and Mitchell, 1993), (V_{max}, 1.7 nmol to 4.8 mmol substrate hydrolysed g^{-1} dry mass h^{-1}) (Antibus *et al.*, 1986; McElhinney and Mitchell, 1993; Gibson and Mitchell, 2005). Using a similar range of substrate concentrations (0.76 – 13.7 mM) to those used in the current investigation, Lane and Puckett (1979) derived a K_m value of 8.9 mM for PME activity in *C. rangiferina.* However, no values for lichen PME V_{max} have previously been published. Calculated values of K_m for lichen PME activity are several orders of magnitude greater than typical environmental substrate concentrations. This implies that rapid substrate hydrolysis can occur should *C. portentosa* become exposed to large concentrations of organic P (Kroehler *et al.*, 1988), e.g. in leaf litter or animal excreta (Tomassen *et al.*, 2005).

Values of K_m were significantly lower in *C. portentosa* collected from a low N deposition environment compared to thalli from a site with a high N input. This variation in the affinity of PME for organic P substrates might imply that different PME enzymes (or systems) dominate P hydrolysis in environments with differing nutrient availabilities. As such, it seems reasonable to suggest that PME activity in *C. portentosa* might result from the activity of several enzymes. A significantly higher V_{max} value calculated for PME activity in *C. portentosa* from a N-rich environment is consistent with the findings of Turner et *al.* (2001) who reported

the highest V_{max} values for PME in *Racomitrium langinosum* collected from upland sites in Great Britain (high N) when compared to samples taken from Swedish Lapland (low N). The authors suggest that increased PME activity might indicate greater competition for P, implying that phosphatase enzyme kinetics could be influenced by variation in nutrient availability, a suggestion also made for ectomycorrhizal fungi (Antibus *et al.*, 1986) and algae (Pettersson, 1980). An increased capacity for P hydrolysis (high V_{max}) under conditions likely to impose P limitation is likely to reflect an increase in PME enzymes.

Phosphomonoesterase activity was strongly positively related to $\mathbf{N}_s$, in line with data for several plant-fungal systems (Johnson *et al.*, 1999; Treseder and Vitousek, 2001; Phoenix *et al.*, 2003). Since *C. portentosa* typically inhabits nutrient poor environments, it is likely that enhanced PME activity is induced by a shift in nutrient status from N to P-limitation (Aerts *et al.*, 1992). It is widely accepted that an inverse relationship exists between PME activity and phosphate availability (either in the surrounding environment or within the cell) (McComb *et al.*, 1979; Whitton *et al.*, 1990; Phuyal *et al.*, 2008). However, it should be noted that some authors have reported the opposite relationship (Straker and Mitchell, 1986; McElhinney and Mitchell, 1993) or no correlation at all (Dighton, 1983).

A capacity to access an alternative source of P might help meet the increased demand for P generated by N enrichment. The up-regulation of PME activity in response to N enrichment might result from increased investment of cellular N in

the synthesis of new enzymes (Treseder and Vitousek, 2001) or by altering the kinetic properties of the enzyme (Alvarez *et al.,* 2003). This preliminary finding has led to the studies presented in Chapters 4 and 5, in which PME activity and cellular nutrient concentrations in *C. portentosa* are investigated in relation to natural and artificial variation in wet inorganic N deposition patterns.

Although the effect of increasing substrate concentration to enzyme saturating values has been investigated, most of the preliminary studies reported in this chapter were performed at a substrate concentration intermediate between saturation and that found in nature (mean annual soluble organic P in precipitation 4 – 23 µg l^{-1}, C. Jordan, Department of Agriculture for Northern Ireland, Pers. Comm., via AEA Technology). The advantages of this selection of substrate concentrations include minimising potential toxicity from excessively high substrate concentrations whilst also allowing sufficient product release for accurate measurement (Whitton *et al.,* 2005). Several more detailed investigations utilizing a large range of substrate concentrations have identified the presence of dual affinity phosphatase systems (Pasqualini *et al.,* 1992; Turner *et al.,* 2001; Whitton *et al.*, 2005). For species of algae, fungi and moss, it has been shown that in response to low organic P availability (similar to concentrations available in nature), a high affinity system operates. This can rapidly switch to a low affinity system as substrate concentration increases (Turner *et al.*, 2001). It has been suggested that the promotion of low affinity hydrolysis by high substrate concentrations might be a mechanism to minimise enzyme damage and cellular saturation of phosphate (Whitton *et al.*, 2005). Although this system was not

evidenced in *C. portentosa*, it cannot be ruled out as the presence of a high affinity system is likely to be detected only at very low substrate concentrations (e.g. µM range), for which enzyme kinetics were not investigated.

During this suite of investigations, PME activity in the apical 10 mm of *C. portentosa* ranged between *c*. 0.3 and 3 mmol substrate hydrolysed g^{-1} dry mass h^{-1}. These measured rates can be compared favourably with the maximum reported activities documented for vascular plant roots and fungal mycelia, which range from 30 nmol to 7.5 mmol g^{-1} dry mass h^{-1} (Pilkington *et al.*, 2005; Press and Lee, 1983; Phuyal *et al.*, 2008), and with bryophytes ranging from 0.02 – 3.9 mmol substrate hydrolyzed g^{-1} dry mass h^{-1} (Phuyal *et al.*, 2008; Press and Lee, 1983) (see Appendix 1). Furthermore, since there is evidence from Lane and Puckett (1979) and LeSueur and Puckett (and more recently from unpublished data submitted in this thesis, see Chapter 9) that rates of PME activity in other lichens species (e.g. *Flavocetraria cucullata, Stereocaulon alpinum*, *Usnea sphacelata*) are similar to those in *C. portentosa*, it seems probable that lichen-forming fungi as a group have a highly developed capacity for PME activity.

CHAPTER 3. Localisation of PME activity in *Cladonia portentosa*

3.1 Introduction

Cladonia portentosa has a remarkable capacity for phosphatase activity, with preliminary studies in this thesis suggesting rates of activity amongst the highest reported in the literature for any plant-microbial system (Appendix 1). While the occurrence of phosphatase activity in lichens is long established (Kuziel, 1972; Lane and Puckett, 1979; LeSueur and Puckett, 1980; Stevenson, 1994), there have been no detailed reports on the location of activity in the thallus or on the relative contribution to PME activity made by the fungal or algal symbionts.

Stevenson (1994) suggested that lichen phosphatase activity is dominated by the mycobiont. This is in line with the fungal partner constituting the largest proportion of both the mass and volume of the thallus (Smith, 1975; Lawrey, 1984; Purvis, 2000), and the likely importance of the fungus in nutrient capture from atmospheric deposits (Crittenden, 1989). In an early investigation, Kuziel (1972) was unable to find significant differences in phosphatase activity between different locations within a lichen thallus. However, Stevenson (1994) later applied chromogenic staining techniques to reveal distinct locations of phosphatase activity in *Peltigera canina*. Activity was predominantly associated within the rhizines and veins on the underside of the thallus, whereas comparatively low activity was recorded in the medulla and upper cortex. Staining of rhizines revealed that phosphatase activity was greatest between

hyphal branches, termed ‘hyphal junctions’, and was suggested by the author to result from both cell-wall bound and cytoplasmic phosphatase activity.

Multiple locations of phosphatase activity have been reported in fungal hyphae (a situation similar to that in plant cells), including extracellular sites on the cell membrane and cell wall (McElhinney and Mitchell 1993; van Aarle *et al.* 2001), and intracellular sites in the cytosol and on membranes of phosphate-accumulating vacuoles (tonoplast) (Gianinazzi *et al.*, 1979; Jacquelinet-Jeanmougin *et al.*, 1987; Tisserant *et al.*, 1993). It is reported that phosphatases located in different regions of the cell possess different enzyme characteristics (e.g. in their response to pH and substrate availability) (McElhinney and Mitchell, 1993). Although preliminary investigations on the characteristics of PME in *C. portentosa* frequently showed smooth response curves to variation in pH, temperature, time and substrate concentration (Chapter 2, section 2.3), the marked differences in enzyme kinetics at sites differing in N input suggest that PME activity in this lichen might represent several enzymes (or systems), perhaps located within an enzyme complex.

Despite a wealth of information on fungal phosphatases, the role of the photobiont in lichen phosphatase activity cannot be ignored. There is a vast literature reporting phosphatase activity associated with green-algae (Roberts and Rozenkrantz, 1967; Cembella *et al.*, 1984; Whitton *et al.*, 2005) and cyanobacteria (Livingstone *et al.*, 1983; Wood *et al.*, 1986; Grainger *et al.*, 1989; Whitton *et al.*, 1990), and in addition, several authors have examined phosphatase activity in

isolated lichen photobionts (Roberts and Rozenkrantz, 1967; Boissière, 1973). Boissière (1973) revealed phosphatase activity on the cell membrane of the photobiont of *P. canina* (*Nostoc*) by staining thallus tissue and observing lead precipitate formation, while Roberts and Rosenkrantz (1967) recorded several substrate specific phosphatases associated with the isolated photobiont of *Lasallia papulosa*. In addition, free-living species of cyanobacteria and green-algae from genera including *Nostoc* and *Trebouxia* (species of which commonly form lichen associations) have measurable rates of phosphatase activity (Whitton *et al.*, 1990; Banerjee *et al.*, 2000; Whitton *et al.*, 2005). Banerjee *et al.* (2000) measured PME activity in endolithic microbial communities dominated by *Trebouxia* at rates between 0.15 and 0.8 µmol substrate hydrolysed g^{-1} dry mass h^{-1} and as *c.* 0.1 µmol substrate hydrolysed g^{-1} dry mass h^{-1} for PDE activity. For *Nostoc*, extracellular PME activity has been measured at 1.25 mmol substrate hydrolysed g^{-1} dry mass h^{-1}, whilst a rate of 800 µmol substrate hydrolysed g^{-1} dry mass h^{-1} has been recorded for PDE activity (Whitton *et al.*, 1990).

The results of previous studies on phosphatase activity in lichens indicate that the hydrolysis of organic P molecules most likely results from the action of fungal enzymes, possibly located within the cell wall (Lane and Puckett, 1979; LeSueur and Puckett, 1980; Stevenson, 1994); the most conclusive evidence coming from the study by Stevenson (1994). However, indirect evidence can also be drawn from the results of studies presented previously in this thesis (Chapter 2) which utilise the colorimetric *p*NPP assay. In this assay, the artificial substrate (*p*NPP) is considered not to be absorbed, thus any measured activity must result from

hydrolysis external to the cell membrane (Whitton *et al.*, 2005). In addition, *p*NP (the product of hydrolysis) accumulates linearly with time in the bathing solution during enzyme assays, consistent with catalysis being effected by exo-enzymes located within the cell wall or outer surface of the cell membrane.

Several chromogenic stains and fluorescent markers coupled with microscopy have previously been employed to identify the location of phosphatase enzymes in a range of organisms (Gianinazzi *et al.*, 1979; Livingstone *et al.*, 1983; Grainger *et al.*, 1989; Turner *et al.*, 2001). Stevenson (1994) revealed the location of phosphatase enzymes in *P. canina* by staining thalli with three dyes: 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 3-hydroxy-2-naphtholic acid 2, 4-dimethylanilide phosphate sodium salt (naphthol AS-MX phosphate), and ß-naphthyl phenylphosphonate. BCIP is an azo-dye which forms a blue/purple colour following hydrolysis (Coston and Holt, 1958). Staining with BCIP has been applied to reveal distinct locations of phosphatase activity in several species of moss (Turner *et al.*, 2001) and in the cyanobacterium *Nostoc commune* (Whitton *et al.*, 1990). However, results obtained with this method must be interpreted with caution, since in order to obtain clearly visible staining, high substrate concentrations are required which have been shown to cause damage to cell membranes (Whitton *et al.*, 2005). Furthermore, staining with BCIP is a two-step process in which the second step requires oxygen (Coston and Holt, 1958; Whitton *et al.*, 1990; Stevenson, 1994), and using the cyanobacteria *Calothrix parietina*, Grainger *et al.* (1989) showed that absence of O_2 can lead to erroneous results. The chromogenic stain involving naphthol AS-MX phosphate results in

the formation of a violet precipitate when coupled with diazotized 4-benzylamino-2, 5-dimethoxyanilide zinc chloride (Fast Blue RR diazonium salt). Staining by this method has been favoured in locating fungal and cyanobacterial phosphatases (Whitton *et al.* 1990; Tisserant *et al.* 1993; van Aarle *et al.* 2001). Staining with ß-naphthyl phenylphosphonate produces a violet insoluble dye at the site of phosphatase activity when coupled with diazotized 2-methyl-4-[(2-methylphenyl)azo] benzenediazonium sulphate salt (Fast Garnet GBC sulphate salt). Stevenson (1994) found this technique to produce the most intense staining in *P. canina*, and is of particular interest because in contrast to the other dyes it specifically identifies the location of PDE enzymes. In revealing the location of PME enzymes in blue-green algae, Livingstone and Whitton (1983) used a staining technique based on the deposition of lead phosphate (formed in response to inorganic phosphate release), which the authors reported to give more accurate results than could be achieved with azo-dye staining.

A draw-back associated with staining methods is that negative results following staining (i.e. no activity detected) must be treated with caution and ideally followed up with confirmatory tests for phosphatase activity (i.e. colorimetric or fluorometric assays, see Chapter 2) (Whitton *et al.*, 2005). More recently, the advent of Enzyme Labelled Fluorescence (ELF, Invitrogen, Molecular Probes) has made possible the visualisation of enzyme location in tissues (van Aarle *et al.* 2001; Alvarez *et al.* 2003; Nedoma *et al.* 2003). Enzyme Labelled Fluorescence is considered to be a highly sensitive and accurate technique in locating PME activity. When comparing the use of ELF 97 phosphate against Fast Blue RR

chromogenic staining, van Aarle *et al.* (2001) reported that both techniques produced precipitations at the same locations within arbuscular mycorrhizal fungi, but that phosphatase activity visualised with ELF 97 phosphate was much clearer and apparently more abundant. This high degree of sensitivity can be attributed to the fact that the artificial substrate 2-(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone) is only weakly fluorescent, yet upon enzymatic removal of the phosphate group is able to form a bright green fluorescent precipitate at the site of activity (van Aarle *et al.* 2001). The emission λ_{max} (540 nm) is also readily distinguishable from the maximum excitation wavelength (360 nm) (ELF 97 endogenous phosphatase detection kit (E6601) product information sheet, Molecular Probes, Invitrogen). Such properties suggest that this technique might be highly advantageous in the visualisation of sites of lichen phosphatase activity, as many lichen species synthesise secondary metabolites, some of which cause thalli to auto-fluoresce.

In this chapter I present the results of a study using ELF 97 phosphate to locate sites of PME activity in *C. portentosa*. Using this technique I was able to test the following hypotheses: i) phosphatase activity is distributed throughout the entire lichen thallus, ii) phosphatase activity is dominated by the action of exo-enzymes, and iii) extracellular phosphatase activity in thalli is the property of the fungal partner.

3.2 Materials and Methods

3.2.1 Study site

Cladonia portentosa was collected from Ridgewalk Moor, Derbyshire (SK 147 952) on 4th July 2007 by E. J. Hogan, P. D Crittenden and Z. Mtshali.

3.2.2 Collection and pre-treatment of *Cladonia portentosa*

Ten replicate samples of *C. portentosa* each consisting of an aggregate of podetia approximately 50 mm in diameter were removed from separate cushions growing at distances > 10 m apart. Samples were transported back to the laboratory in polythene bags and maintained in a hydrated state overnight in a desiccator over water at 10 ^{o}C in the dark. Powder-free latex gloves were worn at all times when handling lichen samples.

3.2.3 Enzyme labelling and preparation of material for fluorescence microscopy

Thalli were cleaned of extraneous debris and sprayed lightly with deionised water. The apical parts of thalli were selected by cutting with a razor blade at a distance of 5 mm downwards from the apices. These apical branches were shaken gently in 2 ml ELF 97 phosphate substrate solution for 20 min at 15 ^{o}C, the solution being prepared by adding 100 µl ELF 97 stock solution (ELF 97 endogenous phosphatase detection kit (E6601), Invitrogen, Molecular Probes, Europe) to 1.9

ml 0.02 M citric acid tri-sodium citrate buffer (pH 2.5). The samples were then washed twice in deionised water for 5 min and once in 0.1 M sodium phosphate buffer (pH 7) for 10 min with hand cut sections prepared at this stage. Material was processed for thin sectioning following Davey *et al.* (1993) by fixing thalli overnight at 4 ^{o}C in 2 % (v/v) EM grade glutaraldehyde (made in 0.1 M sodium phosphate, pH 7). Briefly, samples were dehydrated in a series of ethanol washes (25, 50 and 80 % ethanol in water) each lasting 1 h at 4 ^{o}C before a final incubation in 100 % ethanol at 4 ^{o}C for 2 h. Samples were then added to a well mixed 50:50 solution of LR White hard grade acrylic resin (Agar Scientific) in 100 % ethanol and left overnight in the dark at 4 ^{o}C. Thalli, now visibly bleached, were sequentially incubated in two solutions of 100 % LR White hard grade acrylic resin at 4 ^{o}C; initially for 4 h and then overnight. The following day samples were removed from the resin and fine branches were dissected and placed in gelatin capsules containing LR White hard grade acrylic resin. Capsules were incubated at 60 ^{o}C for 3 days. Blocks were mounted on a Sorvall Porter-Blum MT-2 Ultra Microtome and sections cut (*c.* 1 µm). Microtome sections of *C. portentosa* were counterstained with Toluidine Blue and mounted in either Citifluor CFPVOH or Euparal media (Agar Scientific). Control thalli, not exposed to the fluorescent substrate, were fixed, embedded and sectioned similarly. Hand-cut sections were used in preliminary investigations.

3.2.4 Bright field and fluorescence microscopy

Sections were observed first on a Nikon Microphot-SA light microscope (10x, 40x and 100x objectives, using bright field and phase-contrast) in order to check integrity of the sample. Fluorescence was observed using a Zeiss Axioskop 50 fluorescence microscope fitted with a 48 79 02 long pass filter set for UV-G 365 nm excitation at 10x, 40x and 100x magnifications. Digital images were taken with a black and white Zeiss Axiocam camera.

3.2.5 Confocal laser scanning microscopy

Sections of *C. portentosa* were observed by confocal laser scanning microscopy (LSM) using one of two systems. The first, a Leica SP2 Confocal Laser Scanning Microscope with an argon ion laser set to excitation at 350 nm and an emission filter at LP505 nm. The second system was a Zeiss LSM uv META Kombi confocal on a Zeiss Axiovert 100M microscope using an argon laser set to excitation at 363 nm and an LP475 emission filter with a 10x/0.3 Ph1 Plan-Neofluar objective.

Cladonia portentosa thalli incubated with ELF 97 phosphate showed maximum enzymatically-activated fluorescence at *c.* 500 nm. This could be distinguished from auto-fluorescence of secondary metabolites in control lichen tissue which fluoresced with a much lower level of intensity and λ_{max} of around 450 nm (Fig. 3.1). Captured images of thalli treated with ELF 97 phosphate and observed using the Zeiss LSMuv META Combi confocal on a Zeiss Axiovert 100 microscope

fitted with an Argon laser were automatically corrected to remove the small amount of auto-fluorescence observed from control samples.

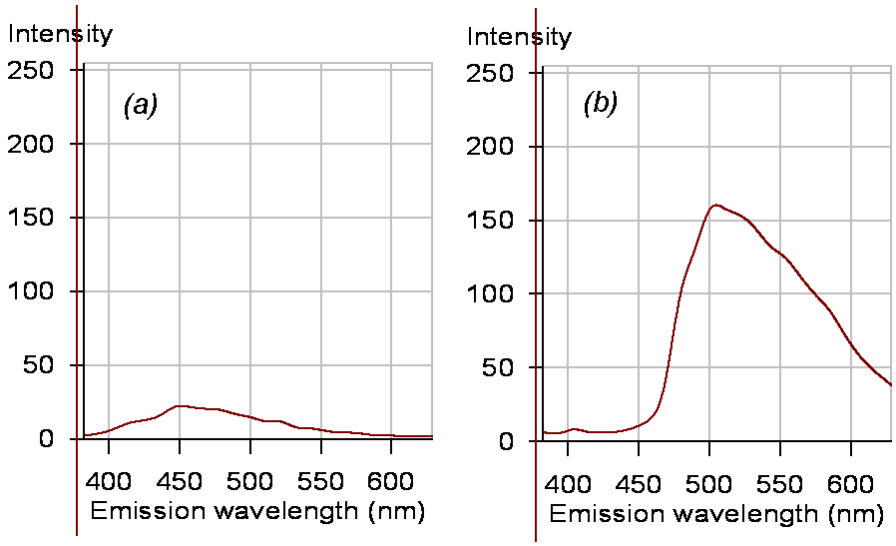


Figure 3.1 Emission spectra for *Cladonia portentosa*: untreated control thalli (a) and thalli treated with ELF 97 phosphate (b). Spectra obtained with a Zeiss LSMuv META Combi confocal on a Zeiss Axiovert 100 microscope with an argon laser set to excitation at 363 nm and an LP475 emission filter.

3.3 Results

Fluorescent precipitates of the ELF 97 phosphate reaction product were visualised in all treated samples of *C. portentosa*. Maximum PME activity was concentrated in the outer and inner surfaces of the hollow tube-like branches of *C. portentosa* thalli (Fig. 3.2). This pattern of enzyme distribution was most clearly observed when thalli were cut in transverse section, however, fluorescent precipitates were

also visualised running continuously along both internal and external surfaces of thalli sectioned longitudinally (Fig. 3.2b).

Scanning electron micrographs (SEM) of *C. portentosa* (Fig 3.3) provided by Sabine Freitag (Environmental Research Institute, Thurso, Scotland, Pers. Comm) show that the outer surface of the thallus is composed of a comparatively loose network of fungal hyphae. The surface contours have a faintly verrucose appearance probably due to clusters of algal cells beneath the raised areas (Fig. 3.3). These clusters of photobiont cells are evident in high powered light micrographs of longitudinal sections (Fig. 3.4). The inner surface of the thallus is composed of dense mycelium of the cartilaginous layer of the medulla from which several short protrusions are visible (Fig. 3.3). Since both the inner and outer surfaces of the thallus are composed of the mycobiont, and since PME is predominantly located on the surface of the thallus (Fig. 3.2), it seems reasonable to conclude that the high rate of PME activity in this lichen is a property of the fungus.

More detailed observations on thin sections under high magnification suggested that PME activity was exclusively associated with the fungal symbiont (Fig. 3.4). The use of ELF 97 phosphate did not reveal any PME activity associated with photobiont cells.

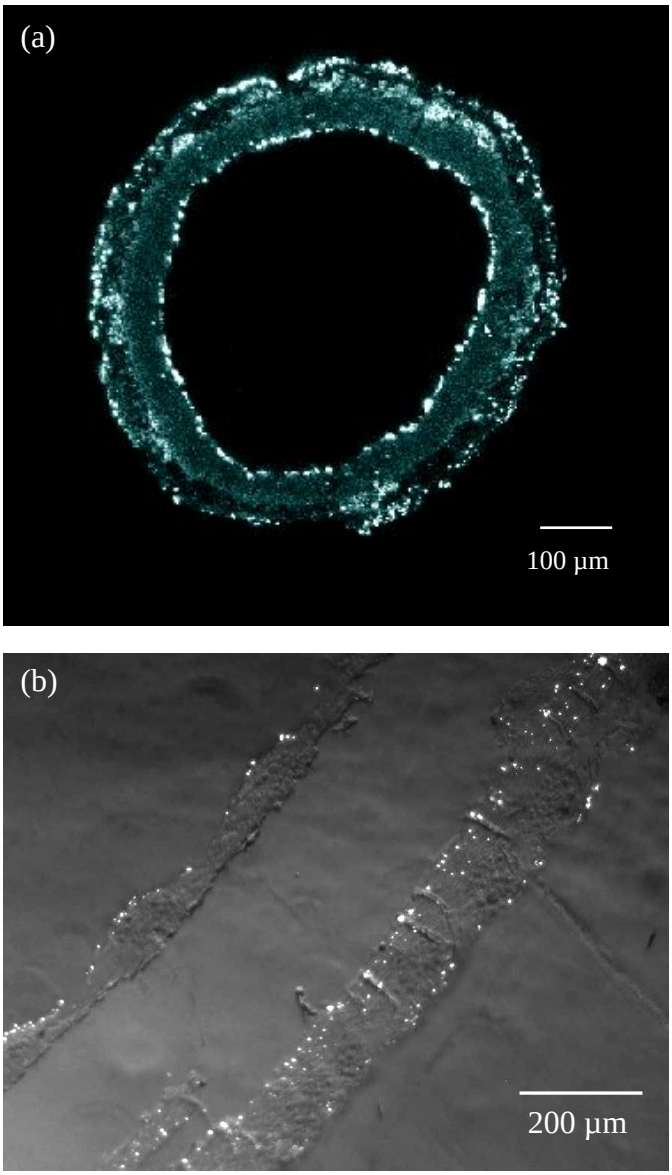


Figure 3.2 High power fluorescence images of transverse (a) and longitudinal (b) sections of thalli of *Cladonia portentosa* treated with ELF 97 phosphate. Sites of PME activity are visualised as areas of maximum fluorescence, illustrated in white, and are seen to be associated maximally with both the inner and outer surfaces of the hollow tube-like podetium. Assays were performed for 20 min at 15 ^{o}C in the dark using the fluorescent ELF 97 phosphatase substrate and images were obtained through a Zeiss Axioskop 50 fluorescence microscope as outlined in section 3.2.4.

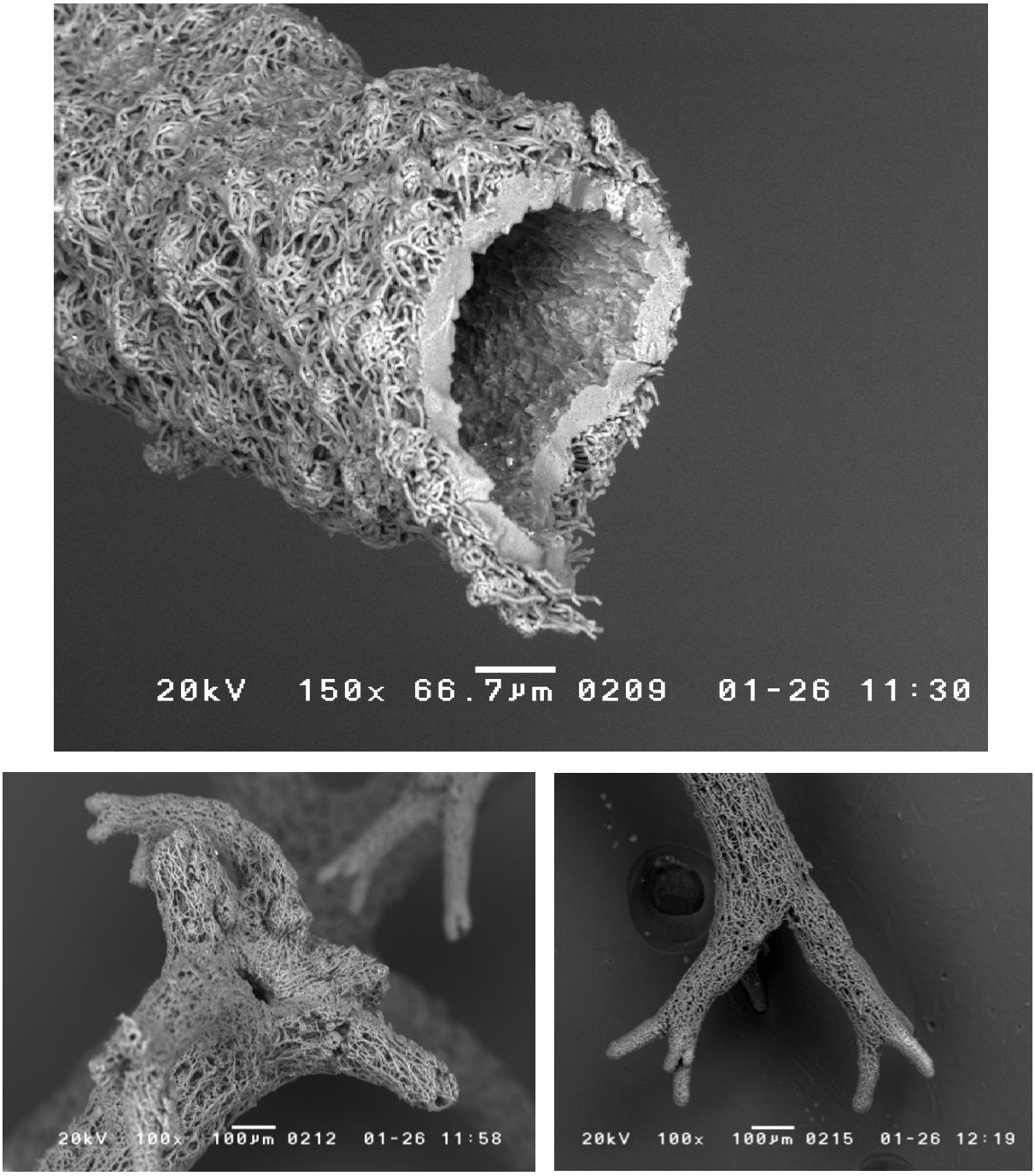


Figure 3.3 Scanning electron micrographs of thalli of *Cladonia portentosa* illustrating the hollow tube like thallus with a network of fungal hyphae forming the surface. The verrucose appearance of the outer thallus surface is consistent with clusters of algal cells located beneath the surface. Images courtesy of Sabine Freitag, Environmental Research Institute, Thurso.

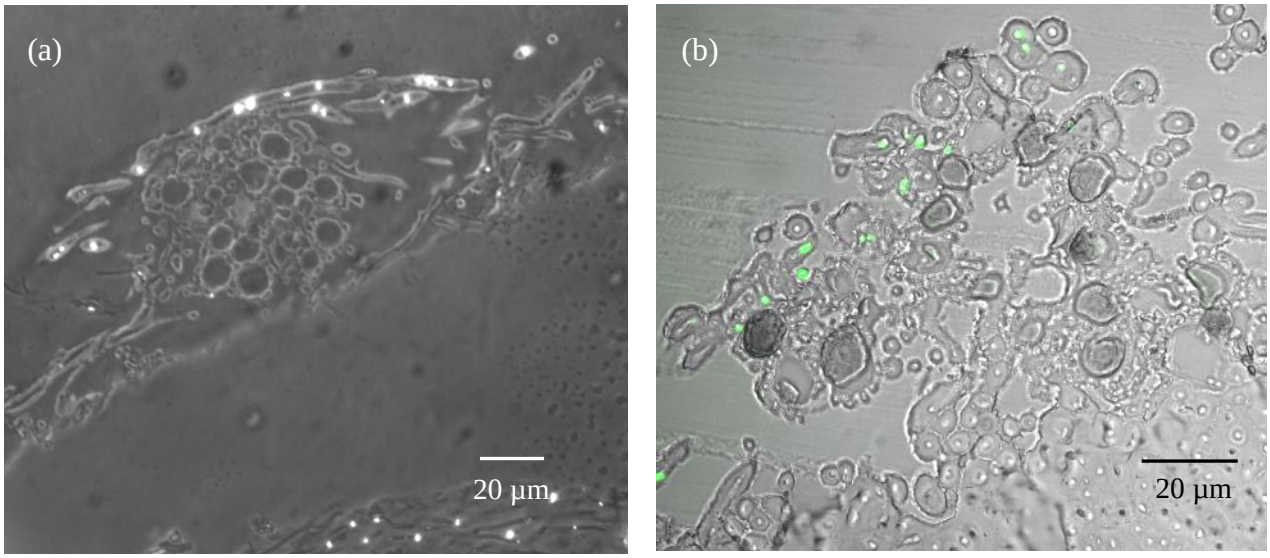


Figure 3.4 High power fluorescence images of longitudinal (a) and transverse (b) sections of thalli of *Cladonia portentosa* treated with ELF 97 phosphate. Sites of enzyme activity are revealed by areas of maximum fluorescence, illustrated in white (a) or green (b) and are associated exclusively with the fungal symbiont. Initial counter staining of sections with Toluidine Blue resulted in the algal cells taking up the stain and thus appearing dark and spherical in contrast to the unstained fungal hyphae. Assays were performed for 20 min at 15 ^{o}C in the dark using the fluorescent ELF 97 phosphate substrate. Images obtained using a Zeiss Axioskop 50 fluorescence microscope (a) or a Zeiss LSM uv META Kombi confocal on a Zeiss Axiovert 100M microscope (b) as outlined in sections 3.2.4 – 3.2.5.

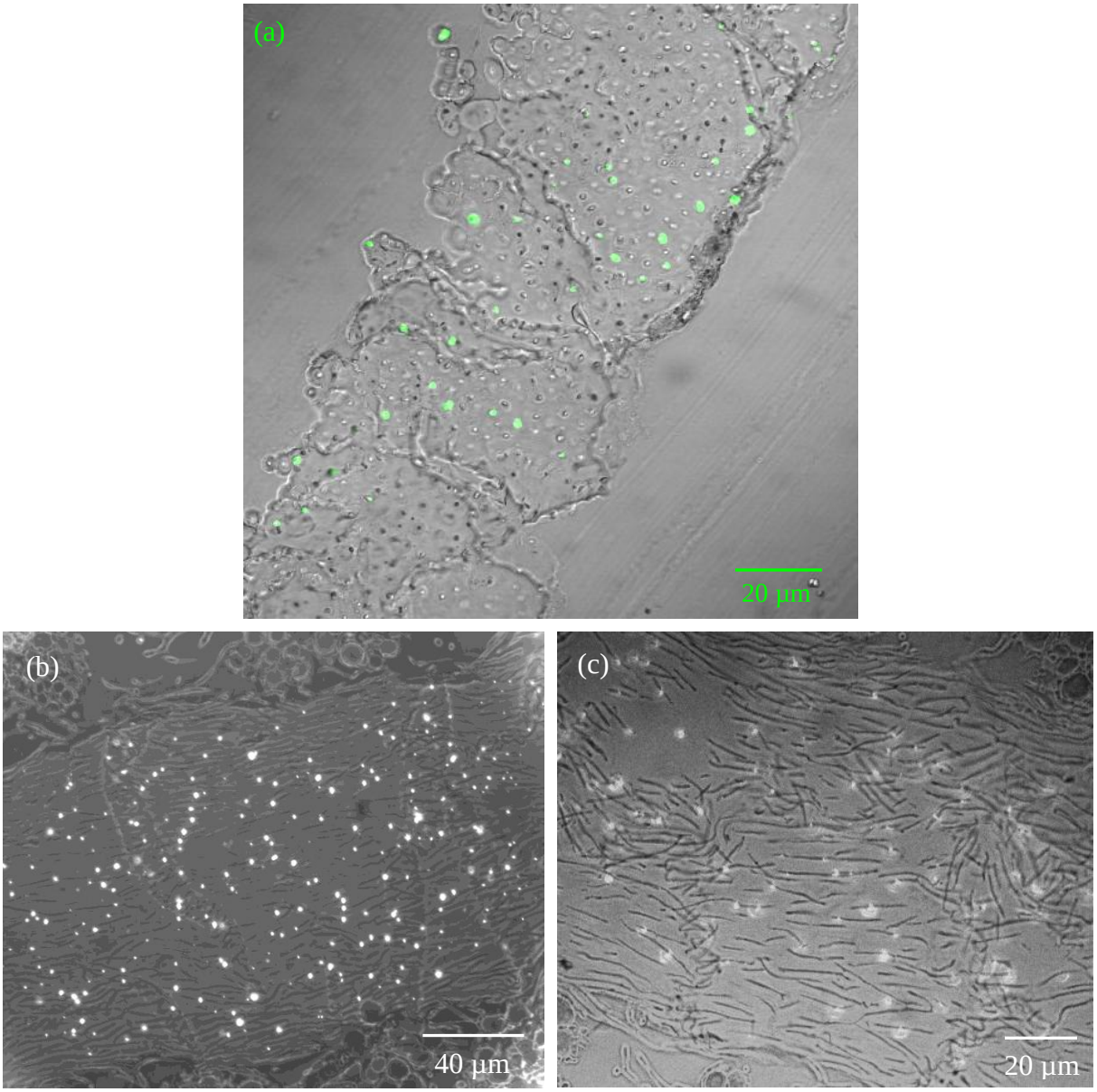


Figure 3.5 High power fluorescence images of transverse (a) and longitudinal (b and c) sections of the cartilaginous medullary region of *Cladonia portentosa* treated with ELF 97 phosphate. This region is composed of prosoplectenchyma tissue, characterised by the arrangement of periclinal hyphae with thick conglutinated cell walls. Sites of enzyme activity are revealed by areas of maximum fluorescence, illustrated in green (a) or white (b and c) and are associated with the hyphal lumen. Assays were performed for 20 min at 15 ^{o}C in the dark using the fluorescent ELF 97 phosphate substrate and visualised through a Zeiss LSM uv META Kombi confocal on a Zeiss Axiovert 100M microscope as outlined in section 3.2.5.

Although PME activity is concentrated in the inner and outer surfaces of podetia (Fig. 3.2), it is not exclusively restricted to these locations. Isolated patches of fluorescent precipitate were observed throughout the cartilaginous medullary region of the thallus (Fig. 3.5). Hyphae in this tissue are arranged periclinally and have thick and heavily conglutinated cell wells (prosoplectenchyma) which produce a much different appearance when viewed in cross section compared to longitudinal sections (Fig. 3.5) (cf. Lawrey, 1984). Here, fluorescence is seen to be consistently associated with the hyphal lumen and not the cell wall. Fluorescent precipitates were also observed in the centre of hyphae located on the inner surface of podetia.

3.4 Discussion

Phosphomonoesterase activity in *C. portentosa* results chiefly from enzyme activity in the mycobiont. This is consistent with the findings of Stevenson (1994), obtained using chromogenic stains, which showed that PME and PDE enzymes were located in the rhizines and veins of *P. canina*, and that maximum activity was concentrated at 'hyphal junctions', i.e. regions of branching (Stevenson, 1994).

The dominant role of the mycobiont to the total PME activity in the thallus of *C. portentosa* is also consistent with the contribution of this symbiont to other physiological processes. For example, the mycobiont constitutes the greatest

proportion of a lichen thallus (> 80 %) (Smith, 1975; Lawrey, 1984; Purvis, 2000) and is generally accepted to play a key role in nutrient capture (Crittenden, 1989). However, it is important to note that the absence of fluorescent precipitates observed in algal cells cannot be taken as conclusive evidence for an absence of PME activity in the photobiont. For example, it is worth considering that ELF 97 phosphate will be hydrolysed only by PME enzymes, and the possibility exists that algal cells produce other classes of phosphatases. These might include highly substrate specific monoesterases or, alternatively, PDE, PTE, or phytase enzymes. Phosphomonoesterase activity has been measured in axenic cultures of *Trebouxia* (the photobiont partner in *C. portentosa*) at rates between 2.3 and 3.5 nmol *p*NP g^{-1} dry mass h^{-1} (Roberts and Rozenkrantz, 1967). Yet, in comparison, rates of PME activity in axenic mycobiont cultures of *C. portentosa* are *c*. 2 orders of magnitude higher those measured by Roberts and Rozenkrantz (1967) in *Trebouxia* (see Chapter 8), and it therefore seems plausible to suggest any PME activity associated with the photobiont of *C. portentosa* might be too low to detect using the technique of ELF.

Cladonia portentosa produces high levels of acid PME activity, which has been detected throughout the lichen thallus from the growing apices to decaying bases (Chapter 2, section 2.3.1). As is characteristic of the subgenus *Cladina*, the thallus of *C. portentosa* is dichotomously to polytomously branched (Ahti, 1961; Thomson, 1967). The branches are of unequal length (anisotomic) and are hollow, generating a 'tube-like' structure. When treated with ELF 97 phosphate, sites of PME activity were visualised in both the outer and inner surfaces of these hollow

branches (Fig 3.2). It was hypothesised that enzyme activity might be concentrated in the outer-most lichen surface, since *C. portentosa* utilises nutrients deposited onto this surface via atmospheric processes. A concentration of PME enzymes in the outer thallus surface might be expected to maximise the probability of enzyme-substrate complexes forming and hydrolysis of organic P molecules occurring. Accordingly, high PME activity in the inner surface of thalli was a surprising discovery. This distribution might promote additional substrate capture should nutrient deposits penetrate to the interior of the thallus tubes; this could occur via perforated axils. In addition, Cardinale *et al*. (2006, 2008) have shown that microbial communities are present on the inner surface of several *Cladonia* species, which could provide a source of organic P. Nonetheless, it seems probable that the contribution to total thallus P capture made by PME enzymes located on the inside of thallus branches is relatively small. An alternative explanation for the presence of PME activity on both the inner and outer surfaces might be that these enzymes play a role in P conservation, by re-capturing organic P leaked from the symplast (J. Leake, Pers. Comm.). This might be of particular importance if solutes are leaked from the thallus during re-hydration following a period of desiccation (Lang *et al.*, 1976; Smith, 1980).

Leakage of P compounds from the lichen thallus was illustrated by Farrar and Smith (1976) who showed an appreciable quantity of PO_4^{3-} and phosphate esters (including hexose-phosphate) leaked from air-dry thalli of *Hypogymnia physodes* upon sudden rehydration. The release of solutes most commonly results from damage to the cell membrane during either desiccation or subsequent rehydration

(Farrar and Smith, 1976). Yet, re-establishment of membrane integrity in lichens is extremely rapid; Farrar and Smith (1976) report active PO_4^{3-} uptake occurring within just 10 – 20 s of thallus rehydration. Furthermore, solute efflux is most commonly recorded following submersion of desiccated thalli into a bathing solution (Farrar and Smith, 1976; Lang *et al.*, 1976), and Crittenden (1983, 1998) has argued that there is little evidence of this process occurring in nature. Nonetheless, Coxon (1991) has shown that 0.2 kg P ha^{-1} y^{-1} can be lost from epiphytic bryophyte mats upon the rewetting of desiccated tissue by natural rainfall episodes.

Phosphomonoesterase activity in *C. portentosa* appears to be associated with the hyphal lumen (Fig. 3.5). Evidence for this location was most clearly observed in thin sections through prosoplectenchyma in the cartilaginous medullary region (e.g. Fig 3.5a). Here, enzyme activity was visualised by fluorescent precipitates distributed in a non-random manner throughout the tissue, appearing to form at the centre of fungal hyphae. Kretschmer (2007) reported homogeneous distribution of phosphatases in the hyphal lumen of *Streptomyces noursei*, and ELF has revealed the presence of extracellular PME enzymes in several species of fungi (including *Cenococcum geophilum, Paxillus involutus* and *Pisolithus tinctorius*) (Alvarez *et al.*, 2006). Intracellular phosphatase activity might reflect a role in the storage of P and/or in metabolic and physiological processes, whilst extracellular PME activity in the cell membrane/inner region of the cell wall might enhance P capture efficiency since PO_4^{3-} will be released in close proximity to phosphate transporters (Bartlett and Lewis, 1973), resulting in rapid intake of the anion in the possible

face of competition from a surface microbial community (Cardinale *et al.*, 2006, 2008). In addition, a surface-bound location might facilitate recovery of PME enzymes via endocytosis (Stenberg, 2007), a mechanism which might serve to maximise nutrient conservation in oligotrophic habitats. Although it is reported that the hydrophobic nature of the plasma membrane acts as an effective barrier to ELF 97 phosphate (which is highly hydrophilic) (Hernández *et al.*, 1996; Nedoma *et al.*, 2003; Alvarez *et al.*, 2006), evidence from the current study suggests that intracellular phosphatases can be located using ELF 97 phosphate.

The thallus of *C. portentosa* has a large surface area-to-volume ratio. This, in combination with a concentration of PME activity on both inner and outer surfaces of thallus branches, might partially explain the high rates of activity in this species. On the basis of work presented later in this thesis (Chapter 9) and in the literature (Lane and Puckett, 1979; LeSueur and Puckett, 1980; P. D. Crittenden, Pers. Comm.), there is some evidence to suggest that foliose lichens with lower surface area-to-volume ratios have low rates of PME activity.

This study represents the first time the ELF 97 phosphate substrate has been used to detect and locate PME activity in a lichen. It proved a sensitive method and can be recommended for future investigations (e.g. to locate of PME activity in different lichen thalli and/or isolated symbionts). Although colourimetric assays reveal high rates of PME activity in *C. portentosa*, further research is necessary to confirm whether this measured activity results exclusively from the action of enzymes located in this chapter.

CHAPTER 4. Phosphomonoesterase activity in *Cladonia portentosa* at field sites subject to different N deposition characteristics

4.1 Introduction

Nitrogen enrichment is believed to be one of the principal potential drivers of ecosystem change at mid to high latitudes (Sala *et al.* 2000; Köchy and Wilson 2001; Magnani *et al.* 2007) and is predicted to increase in importance in the future (Sanderson *et al.* 2006; Civerelo *et al.* 2008). Nitrogen pollution is relevant to the biology of lichens for two principal reasons. First, lichens respond to increased nitrogen supply in a coherent and predictable manner both in terms of changes in community structure (Armstrong 1984; van Dobben and ter Braak 1998; van Herk 1999) and changes in chemical composition (Bruteig 1993; Søchting 1995; Hyvärinen and Crittenden 1998a; Walker *et al.* 2003). Thus lichens have considerable potential as indicators of N deposition, particularly across large areas of remote northern terrain in which they are often abundant and for which measurements of N load are generally sparse. Second, since most lichen habitats are highly depleted in N and P, an analysis of lichen physiological response to elevated N supply might provide an understanding of how lichens are adapted for growth in ultra-oligotrophic environments.

Mat-forming terricolous lichens in such genera as *Cladonia* (subgenus *Cladina*) and *Flavocetraria* have been shown to provide coherent biomarkers for N enrichment (Hyvärinen and Crittenden 1998a; Walker *et al.* 2006); these species

are widespread and locally abundant on well-drained terrain in subarctic heathlands and boreal forests as well as in temperate heathlands and uplands. Hyvärinen and Crittenden (1998a) demonstrated that *c.* 60 % of variation in tissue N concentration in *C. portentosa* at different sites across Great Britain could be explained by variation in inorganic N deposition, and Walker *et al.* (2003) found that N concentration in *Cladonia stellaris* decreased northwards along a 240 km transect across the taiga-tundra ecotone in north European Russia which, according to Ryaboshapko *et al.* (1998), lies along a gradient of decreasing N deposition. Coupling between the chemical composition of mat-forming lichens and that of atmospheric deposits might be particularly close because (i) lichen mats typically develop in open situations where rainfall is intercepted directly, and hence relationships between lichen chemistry and atmospheric inputs are not confounded by nutrient exchanges between rainfall (or snow meltwater) and overlying vascular plant canopies (cf. Farmer *et al.* 1991; Søchting 1995), (ii) accumulation, below well-developed lichen mats, of copious quantities of structurally intact dead thallus or necromass partially isolates living thalli from the chemical influence of underlying soil (Ellis *et al.* 2003; 2004), and (iii) lichen mats are efficient scavengers of key ions in precipitation such as NO_3^-, NH_4^+ and PO_4^{3-} (Crittenden 1983; 1989; Hyvärinen and Crittenden 1996; 1998c).

In their studies on *C. portentosa*, Hyvärinen and Crittenden (1998a) found that concentrations of thallus N and P ($[N]_{lichen}$ and $[P]_{lichen}$, respectively) co-varied and that both were positively correlated with at least some components of N deposition. The reason for such co-variation between $[N]_{lichen}$ and $[P]_{lichen}$ is not

known but two suggestions were advanced. First, N and P deposition might co-vary spatially due to man-made P contamination of the atmosphere deriving from, e.g. mechanized agriculture. Unfortunately, data on P deposition are very scarce and thus it is difficult to evaluate this possibility. Second, lower lichen growth rates in N-polluted regions might result in lower growth dilution of tissue P concentrations, but evidence of lower growth rates in *C. portentosa* at N polluted sites was not found (Hyvärinen & Crittenden, 1998b). A further possibility is that N enrichment might promote P capture possibly involving up-regulation of phosphatase enzymes catalyzing the release of orthophosphate from organic P sources. This suggestion is partially consistent with studies on plant root and mycorrhizal systems in which N enrichment is known to promote phosphomonoesterase (PME) activity (Johnson *et al.* 1999; Treseder and Vitousek 2001; Phoenix *et al.* 2003; Pilkington *et al.* 2005). However, such up-regulation of PME activity in plant root systems is usually associated with reduction in plant tissue P concentration and an increase in N:P mass ratio values.

In this chapter PME activity and thallus N:P mass ratios in *C. portentosa* are measured at sites in Great Britain with contrasting N deposition characteristics. Relationships are then examined between lichen chemistry and physiology and N deposition rate, N concentration in precipitation, and rainfall depth. Sites at which the lichen was collected were selected using both modelled and measured N deposition data facilitating evaluation of the accuracy of modelled deposition values.

4.2 Materials and methods

4.2.1 Site selection

Cladonia portentosa was collected from sites in Great Britain subject to widely differing N deposition rates and mean N concentrations in precipitation. Site selection utilized two data sets: modelled data and measured data. Modelled data were supplied by R. I. Smith (Pers. Comm., CEH, Edinburgh) and were derived using an atmospheric deposition model parameterized for moorland terrain (Smith and Fowler 2001). The model utilizes a simulated rainfall field for Great Britain generated by the UK Meteorological Office and interpolated maps derived using measurements from the UK Acid Deposition Monitoring Network operated by the National Environmental Technology Centre (NETCEN, AEA Technology). Modelled data were provided as values of annual mean total wet deposited inorganic N (NO_3^- + NH_4^+) ($\mathbf{N}_s$) and annual mean volume-weighted concentration of dissolved inorganic N ($[N]_s$) in rainfall as 5 x 5 km gridded data sets for Great Britain. Each grid square was assigned a site code and, using a plot of $\mathbf{N}_s$ against $[N]_s$ (Fig. 4.1a), individual grid squares were then selected to represent two gradients: one in $\mathbf{N}_s$ but in which grid squares had broadly similar $[N]_s$ values and the other in $[N]_s$ but in which grid squares had similar $\mathbf{N}_s$ values (Fig. 4.1b, Table 4.1).

Measured data used the site measurements from the UK Acid Deposition Monitoring Network, which comprises 38 rainfall collection stations distributed in

rural areas of Great Britain (Hayman *et al.* 2005). Values of total annual rainfall and annual mean volume-weighted concentration of inorganic N ($[NO_3^-]_m$ + $[NH_4^+]_m$) ($[N]_m$) were available for these sites from which annual mean total wet inorganic N deposition ($\mathbf{N}_m$) could be calculated (Table 4.2). This procedure is likely to underestimate N deposition as this type of collector does not catch all rainfall, and the catch efficiency will vary between sites, but unlike the modelled data it does not rely on model assumptions. Using 3 yearly mean values for 2002-2004, subsets of sites were selected representing gradients in $\mathbf{N}_m$ and $[N]_m$ (Fig. 4.2, Tables 4.1 and 4.2) as described above for modelled data. Heathlands and moorlands supporting *C. portentosa* were sought within each of the 5 x 5 km grid squares selected from the modelled data set (13 sites), and as close as possible to each of the selected rainfall chemistry monitoring stations (9 sites) (Figs. 4.1b & d and 4.2, Table 4.1). Relationships between lichen chemistry and $\mathbf{N}_m$ or $[N]_m$ were analysed using 4 yearly mean values (2002 - 2005) (Table 4.1) as data on rainfall chemistry for 2005 became available (Hayman *et al.* 2007).

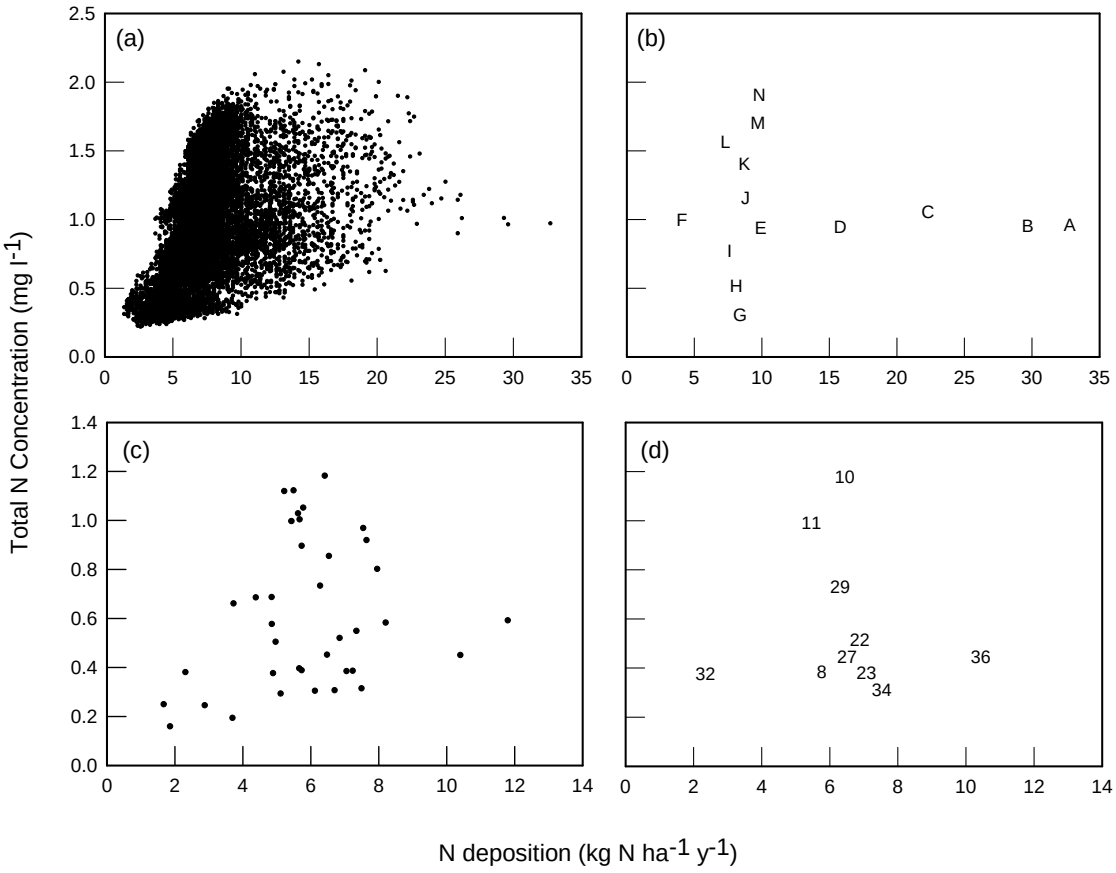


Fig. 4.1 Relationship between annual total wet inorganic N (NO_3^- + NH_4^+) deposition and volume-weighted annual mean inorganic N concentration in precipitation for sites in Great Britain: modelled values for all 5 x 5 km grid squares (a); modelled values for selected 5 x 5 km grid squares representing distinct gradients in N deposition and N concentration in precipitation (b); measured values for each of the rainfall collection stations in the UK Acid Deposition Monitoring Network (2002 – 2004) (c); measured values for selected rainfall collection stations in the UK Acid Deposition Monitoring Network (2002 - 2004) representing distinct gradients in wet inorganic N deposition and inorganic N concentration in precipitation (d). Individual grid squares are represented by either dots (all sites) or by a unique site code (selected study sites). Measured data calculated from Hayman *et al.* 2004a, 2004b, 2005.

Table 4.1 Details of sites at which *Cladonia portentosa* was collected (sites selected from Figure 1a, c) including values of modelled* and measured** annual mean total inorganic N deposition (**N**), volume-weighted inorganic N concentration in precipitation ($[N]_{ppt}$), and precipitation for each study site (ss). Discrepancies in altitude between study sites and rain gauges (rg) are provided for measured data (ss deviation from rg), along with collection dates for all sites. Sites varying in measured inorganic N deposition and N concentration were selected using 3 years' average data (2002 - 2004)†, whilst relationships reported in the text between lichen chemistry and $\mathbf{N}_m$ or $[N]_m$ are based on 4 years' average data (2002 - 2005)‡.

Site code	Name of site	National Grid Reference	(**N**) Wet N deposition (kg N ha^{-1} y^{-1})	$[N]_{ppt}$ (mg l^{-1})	Annual precipitation (mm y^{-1})	Discrepancy in altitudes between ss and rg (m)	Date of lichen collection
Modelled data*							
A	Wythburn Fells	NY 315125	32.8	0.96	3279	-	24 Apr 2006
B	Tarn at Leaves Res.	NY 258123	29.7	0.96	3000	-	24 Apr 2006
C	Honister Pass	NY 227133	22.3	1.05	2014	-	05 May 2006
D	Alwen Reservoir	SH 923514	15.8	0.94	1591	-	24 Apr 2006
E	Glasnant	SO 170512	9.9	0.94	912	-	21 Mar 2006
F	Strabauchlinn	NT 496855	4.1	1.00	399	-	08 May 2006
G	Five Sisters	NG 952167	8.4	0.31	2625	-	11 May 2006
H	Nant-y-moch Res.	SN 757861	8.1	0.51	1371	-	03 Apr 2006
I	Dalswinton Wood	NX 958873	7.6	0.77	952	-	13 May 2006
J	Stiperstones	SO 359970	8.8	1.16	655	-	21 Mar 2006
K	Winterton Dunes	TG 492202	8.7	1.40	605	-	24 Mar 2006
L	Moira Tip	SK 318149	7.3	1.56	450	-	25 July 2006
M	The Brand	SK 535131	9.7	1.70	547	-	27 Mar 2006
N	Wooley Mine	SE 310117	9.8	1.90	504	-	28 Mar 2006

* 2004 data for moorland ecosystems from R. I. Smith (CEH Edinburgh, Pers. Comm.)

Table 4.1 continued

Site code	Name of site	National Grid Reference		(**N**) Wet N deposition (kg N ha^{-1} y^{-1})		$[N]_{ppt}$ (mg l^{-1})		Annual Precipitation (mm y^{-1})		Discrepancy in altitudes between ss and rg (m)	Date of lichen collection
Measured data**				2002-2004†	2002-2005‡	2002-2004†	2002-2005‡	2002-2004†	2002-2005‡		
8	Llyn Brianne Res.	SN	815503	5.77	5.16	0.38	0.45	1526	1275	-40	03 Apr 2006
10	Wangford Warren	TL	757840	6.44	6.77	1.18	1.32	557	529	-1	24 Apr 2006
10	Stoke Ferry	TF	722012	6.44	6.77	1.18	1.32	557	529	-8	24 Apr2006
11	Whixall Moss	SJ	492357	5.46	5.61	0.99	1.06	549	533	25	21 Apr 2006
11	Stiperstones	SO	359970	5.46	5.61	0.99	1.06	549	533	320	21 Apr 2006
11	The Cliff	SJ	393207	5.46	5.61	0.99	1.06	549	533	80	08 Jun 2006
22	Cow Green	NY	818298	6.88	5.85	0.52	0.46	1357	1267	3	05 May 2006
23	Loch Dee	NX	480794	7.08	7.32	0.38	0.44	1897	1749	-10	13 May 2006
27	Strathyre	NN	556160	6.51	6.48	0.45	0.46	1529	1481	170	12 May 2006
29	Glen Dye	NO	649846	6.31	5.88	0.73	0.71	905	859	45	09 May 2006
32	The Halsary	ND	195493	2.34	2.32	0.38	0.36	633	649	-8	10 May 2006
34	Llyn Llagi	SH	647481	7.53	7.85	0.31	0.35	2426	2276	5	04 Apr 2006
36	Scoat Tarn	NY	227133	10.44	9.26	0.45	0.49	2355	1991	-145	05 May 2006

** Calculated from 3 years (2002-2004)† or 4 years (2002-2005)‡ average data with wet inorganic N deposition calculated from annual mean volume-weighted $[N]_{ppt}$ multiplied by rainfall volume. Data taken from Hayman *et al.* (2004a; 2004b; 2005; 2007)

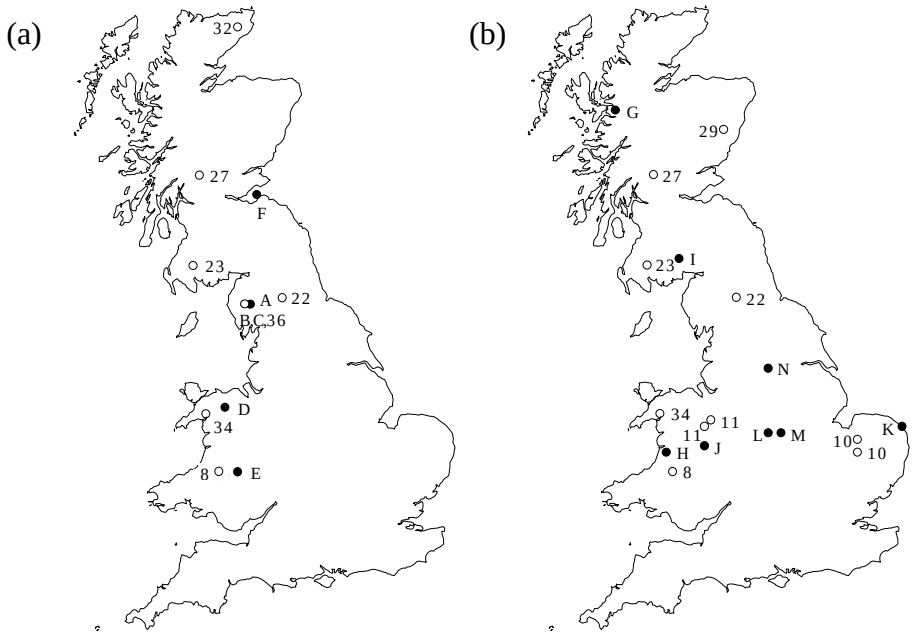


Figure 4.2 The location of lichen collection sites in Great Britain identified in Fig. 1 b & d. Sites representing gradients in wet N deposition (a), and N concentration in rainfall (b); sites selected on the basis on modelled N values (●), sites selected on the basis of measured data (○, 2002 - 2004). Site distribution maps plotted using Dr. A. Morton's DMAP program (R. Morton, Windsor, UK) inputting site specific UK grid references.

Table 4.2 Annual mean volume-weighted concentrations and wet depositions of NH_4^+ and NO_3^- and total inorganic N at rain gauge sites selected from the UK Acid Deposition Monitoring Network during 2002 - 2004.

Site	N concentration (mg l^{-1})*			Deposition (kg N ha^{-1} y^{-1})**			Annual rainfall
no.	$[NO_3^-]$	$[NH_4^+]$	Total N	NO_3^-	NH_4^+	Total N	(mm)*
8	0.20	0.19	0.38	2.91	2.86	5.77	1526
10	0.54	0.63	1.18	2.98	3.46	6.44	558
11	0.31	0.69	0.99	1.69	3.77	5.46	549
22	0.25	0.26	0.52	3.36	3.53	6.88	1357
23	0.17	0.21	0.38	3.15	3.93	7.08	1897
27	0.21	0.24	0.45	3.10	3.41	6.51	1529
29	0.41	0.32	0.73	3.60	2.71	6.31	905
32	0.20	0.18	0.38	1.27	1.07	2.34	632
34	0.16	0.15	0.31	3.90	3.63	7.53	2426
36	0.20	0.24	0.45	4.75	5.69	10.44	2355

* Calculated from 3 years average data (2002-2004) taken from Hayman *et. al.* (2004a, 2004b, 2005)
** Calculated from 3 years average data (2002-2004) with wet inorganic N deposition calculated from annual mean volume-weighted $[N]_{ppt}$ multiplied by rainfall. Data taken from Hayman *et. al.* (2004a, 2004b, 2005)

Sites were selected using data sets for wet inorganic N deposition. This was for two reasons. First, the concentrations of NO_x and NH_3 are measured directly at only a limited number of sites in Great Britain (Lawrence *et al.* 2008). As a result, highly derived modelled data sets are produced for dry N deposition, resulting in large uncertainties (Magnani *et al.* 2007). Second, Hyvärinen and Crittenden (1998a) investigated multiple regression analyses between lichen chemistry and wet inorganic N deposition and reported that these regressions could not be improved by incorporating modelled data for NH_3 and NO_x.

4.2.2 Collection and pre-treatment of *Cladonia portentosa*

Samples were collected between 21.03.06 and 13.05.06 (Table 4.1) with 10 replicate samples of *C. portentosa* collected at each site. Each sample was typically a clump of podetia 50 - 150 mm in diameter, collected from a cushion > 10 m apart from other replicates. The samples were collected and transported back to the laboratory as outlined in section 2.2.2. Air dry samples were sealed in polyethylene bags and stored at either 4 ^{o}C for chemical analysis or -15 ^{o}C for phosphatase assays. Samples stored at -15 ^{o}C for phosphatase assays were analysed within 8 weeks in line with the pre-treatment conditions reported in Chapter 2 (section 2.3.3, Fig. 2.9). Samples collected from The Cliff (SJ 393 207) and Moira Tip (SK 318 419) were maintained in a hydrated state overnight and assayed for phosphatase activity within 24 h. Powder-free latex gloves were worn at all times when handling lichens to minimise contamination.

4.2.3 Sample preparation

Thalli were prepared for chemical analysis and phosphatase assays following the method outlined in section 2.2.3. Samples from each replicate collection were then dissected into the following horizontal strata by cutting with a razor blade: 0 - 5 and 40 - 50 mm (for chemical analysis) and 0 - 10 mm (for phosphatase assays) (distances measured downward from the apices in each case, Fig. 4.3). The strata selected for chemical analyses were chosen in line with the study by Hyvärinen and Crittenden (1998a) in order to facilitate comparison of the two data sets.

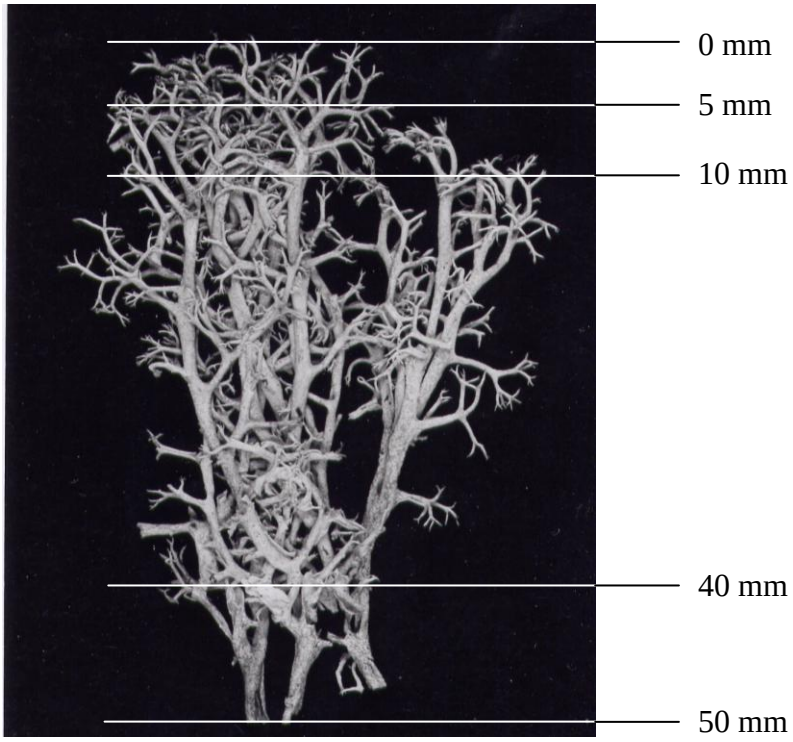


Figure 4.3 Thallus of *C. portentosa* illustrating selected distances measured downwards from the apices used to dissect samples into the following strata: 0 – 5 mm, 0 – 10 mm and 40 – 50 mm.

4.2.4 Phosphatase assay

Phosphomonoesterase activity was determined using the *p*-nitrophenyl phosphate (*p*NPP) method, following Turner *et al.* (2001) as outlined in section 2.2.4. Assay conditions included 0.02 M citric acid-trisodium citrate buffered assay medium (pH 2.5), a final *p*NPP concentration of 3 mM, and incubation for 20 min at 15 ^{o}C in the dark.

4.2.5 Determination of total N and P concentration

Samples of the apical 5 mm and the 40 - 50 mm strata were oven dried for 24 h at 80 ^{o}C, weighed and digested in 1 ml 1:1.2 (v/v) mixture of H_2O_2 and H_2SO_4 at 375 ^{o}C for 1 h following Allen (1989). All digests were diluted to 50 ml with deionised water before analysis. Blank control digests were performed without lichen material. Ammonium-N in the digest was determined using the fluorometric method of Holmes (1999). A 0.25 ml sample digest was diluted 1:1 with control digest and added to 10 ml borate buffer (Fl_{BB}, 40 g sodium tetraborate in 1 l deionised water) or 10 ml working reagent (Fl_{WR}, 1 l borate buffer, 5 ml 1 mM sodium sulfite and 50 ml 0.75 mM orthophthaldialdehyde (OPA) in ethanol). The reaction mixtures were incubated in the dark for 2.5 h following which fluorescence was determined using a Wallac 1420 Victor^2 multilabel counter and samples adjusted for background fluorescence (i.e. $Fl_{NH_4} = Fl_{WR} - Fl_{BB}$). Total N was determined using a standard curve over the range 0 – 4 µg ml^{-1}. Phosphorus was assayed by the malachite green variant of the methylene blue method after Van Veldhoven and Mannaerts (1987). 1.5 ml digest was diluted 1:1 with control

digest following which 0.6 ml of Van Veldhoven and Mannaerts (1987) reagent A (1.75 % (w/v) $(NH_4)_6Mo_7O_{24}.4H_2O$ in 4.25 N H_2SO_4) and 0.6 ml reagent B (0.035 % (w/v) malachite green, 0.35 % (w/v) polyvinyl alcohol (PVA) in 1 l dH_2O) were added at 10 min intervals. Samples were left for 30 min and analysed at 610 nm using a Pye Unicam SP6-350 visible spectrophotometer. Total P was determined using a standard curve over the range 0 – 0.6 µg ml^{-1}. Determination of total N and P in *C. portentosa* for this investigation was undertaken by G. Minnullina.

4.2.6 Statistical analyses

The statistical software package SPSS was used to perform standard statistical analyses. All data were subject to checks for normality and homogeneity of variances. Where these assumptions were met, the relationships between lichen chemistry and variables describing atmospheric N inputs (measured data 2002-2005) were subjected to correlation analyses and linear regression. Discontinuous data were subject to Spearman's rank correlation analyses.

4.3 Results

Phosphomonoesterase activity assayed in the apical 10 mm of *C. portentosa* was strongly positively related to wet N deposition (Table 4.3; Fig. 4.4a & c). Activity varied roughly by a factor of 2 between sites with the lowest and highest $\mathbf{N}_s$ values. Note that the goodness of fit of the regression line in Fig. 4.4a can be

improved by using ln $\mathbf{N}_s$ values (r^2 = 0.93). Phosphomonoesterase activity was also weakly negatively related to $[N]_{ppt}$ (Fig. 4.4b & d). However, the selected gradients in increasing $\mathbf{N}$ and decreasing $[N]_{ppt}$ are both also gradients in increasing rainfall and there are positive relationships between PME and precipitation among the sites selected to represent gradients in $\mathbf{N}_s$, $\mathbf{N}_m$ and $[N]_m$ (Table 4.3).

Thallus N concentration in both the apical 5 mm ($[N]_{apex}$) and the basal stratum 40-50 mm downwards from the apices ($[N]_{base}$) were strongly positively correlated to both $\mathbf{N}_s$ and $\mathbf{N}_m$, and also precipitation, (Table 4.3, Fig. 4.5a & c) but of the two lichen measurements $[N]_{apex}$ was the better correlate. Relationships between $[N]_{ppt}$ and $[N]_{lichen}$ were generally weaker: neither $[N]_{apex}$ nor $[N]_{base}$ were significantly related to $[N]_m$ (Fig. 4.4d) while only $[N]_{base}$ was significantly related to $[N]_s$ (Fig. 4.5b, Table 4.3).

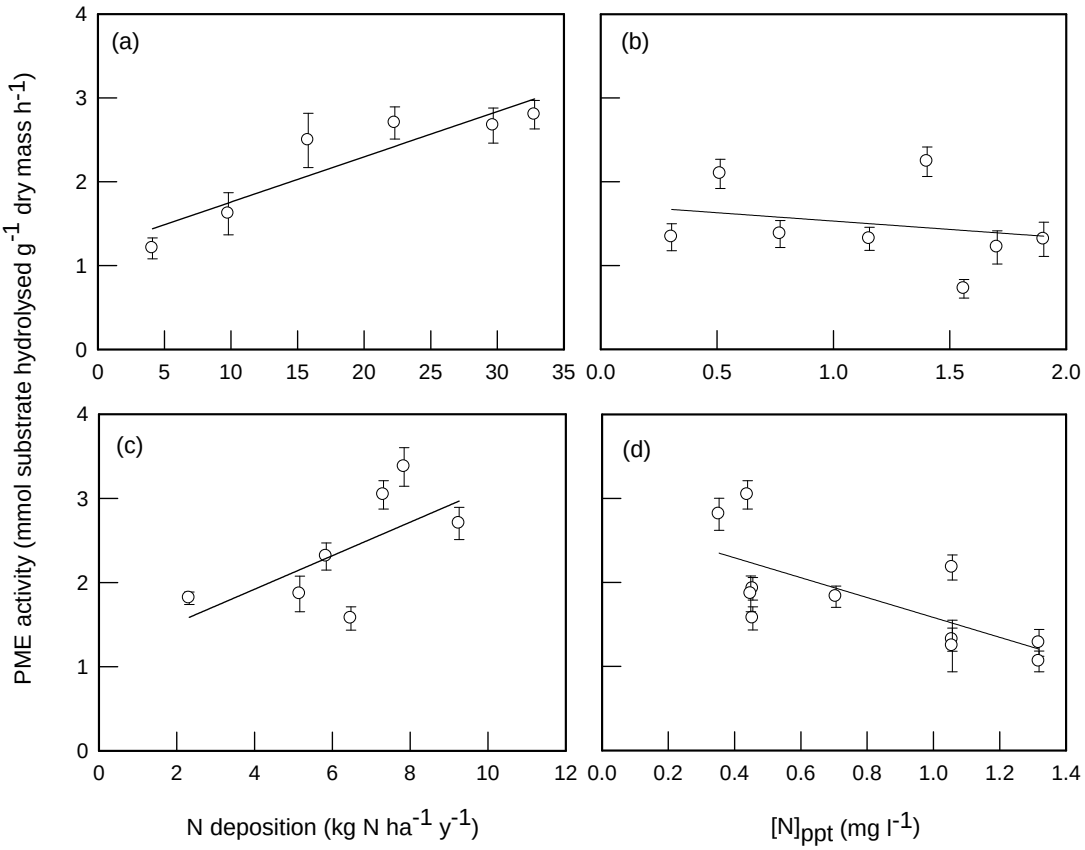


Figure 4.4 Relationships between PME activity in the apical 10 mm segment of *Cladonia portentosa* and modelled total wet inorganic N deposition (a, $r^2 = 0.827$, $P < 0.05$); modelled mean inorganic N concentration in precipitation (b, $r^2 = 0.101$, $P = 0.443$); measured total wet inorganic N deposition (c, $r^2 = 0.424$, $P = 0.113$); measured mean inorganic N concentration in precipitation (d, $r^2 = 0.376$, $P < 0.05$). Assays were performed with 3 mM *p*NPP for 20 minutes at 15 ^{o}C in the dark. Plotted values are means ($n = 10$) ± 1 SEM.

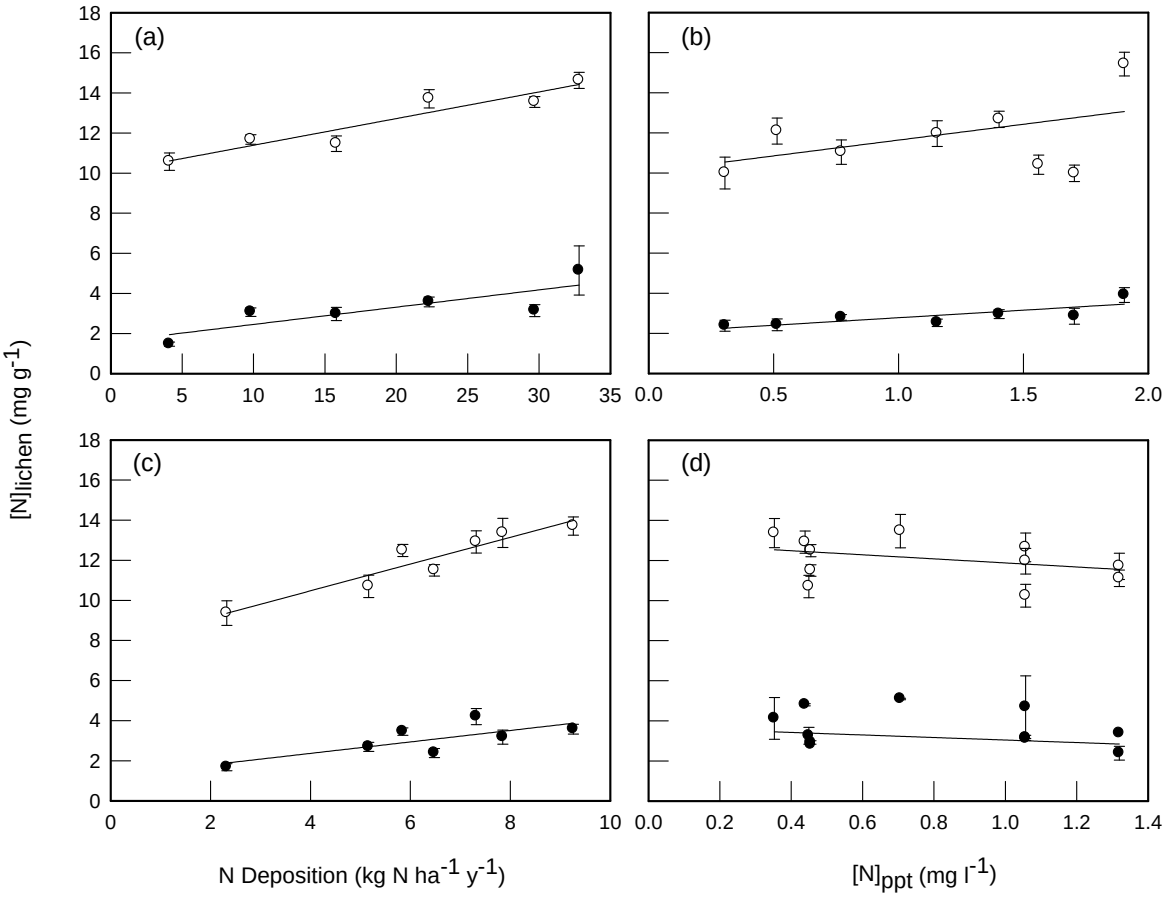


Figure 4.5 Relationships between the concentration of total N in apical (○; 0 – 5 mm) and basal (●; 40 – 50 mm) strata in *Cladonia portentosa* and modelled annual wet inorganic N deposition (a, ○, r^2 = 0.895, P <0.01; ●, r^2 = 0.690, P <0.05); modelled mean inorganic N concentration in precipitation (b, ○, r^2 = 0.245, P = 0.212; ●, r^2 = 0.697, P <0.05); measured total wet inorganic N deposition (c, ○, r^2 = 0.895, P <0.01; ●, r^2 = 0.578, P <0.05); measured mean inorganic N concentration in precipitation (d, ○, r^2 = 0.142, P = 0.253; ●, r^2 = 0.068, P = 0.439). Plotted values are means (n = 10) ± 1 SEM.

Table 4.3 Bivariate Pearson correlation coefficients (*r*) between PME activity, $[N]_{lichen}$ and $[P]_{lichen}$ in *Cladonia portentosa* and both modelled ($_s$) and measured (2002-2005) ($_m$) values of mean inorganic total wet N deposition ($\mathbf{N}_s$, $\mathbf{N}_m$), volume-weighted inorganic N concentration in rainfall ($[N]_s$, $[N]_m$) and annual mean precipitation.

					Annual precipitation at collection sites (ppt)			
	$\mathbf{N}_s$	**$[N]_s$**	**$\mathbf{N}_m$**	**$[N]_m$**	**$\mathbf{N}_s$ sites**	**$[N]_s$ sites**	**$\mathbf{N}_m$ sites**	**$[N]_m$ sites**
PME	0.909*	-0.318	0.651	-0.613*	0.893*	0.162	0.787*	0.719*
$[N]_{apex}$	0.886*†	0.495	0.946**	-0.436†	0.886*†	-0.426	0.546	0.427†
$[N]_{base}$	0.886*†	0.835*	0.679†	-0.327†	0.886*†	-0.542	0.536†	0.227†
$[N]_{apex}/[N]_{base}$	-0.600†	-0.571†	-0.393†	0.136†	-0.600†	0.571†	-0.214†	-0.027†
$[P]_{apex}$	-0.919**	0.500†	0.012	0.418†	-0.898*	-0.521†	-0.014	-0.327†
$[P]_{base}$	-0.222	0.881**†	-0.040	0.699*†	-0.217	-0.810*†	-0.063	-0.721*†
$[P]_{apex}/[P]_{base}$	-0.657†	-0.821*	-0.286†	-0.358†	-0.657†	0.821*†	-0.286†	0.491†
$([N]/[P])_{apex}$	0.954**	-0.048†	0.714†	-0.664*	0.931**	0.000†	0.571†	0.627*†
$([N]/[P])_{base}$	0.895*	-0.312	0.607†	-0.588†	0.864*	0.112	0.393†	0.576†

* -correlation is significant at the $P \leq 0.05$ level
** - correlation is significant at the $P \leq 0.01$ level
† - Spearman's correlation coefficient (nonparametric equivalent of the Pearson's correlation)

Relationships between $[P]_{lichen}$ and N enrichment were less clear than those for $[N]_{lichen}$. There were no obvious trends between $[P]_{lichen}$ and $\mathbf{N}_m$ (Fig. 4.6c) but both $[P]_{apex}$ and $[P]_{base}$ were negatively related to $\mathbf{N}_s$ (Fig. 4.6a). Despite the lack of consistent relationships between $[P]_{lichen}$ and N deposition, the N:P mass ratio in both lichen apices and bases was positively correlated with $\mathbf{N}_s$ and $\mathbf{N}_m$ (Fig. 4.7, Table 4.3), with $([N]:[P])_{apex}$ increasing by a factor of 1.9 over the range of modelled depositions. In turn, PME activity was broadly positively related to N:P mass ratio across all sites investigated (Fig. 4.8). Activity was also significantly negatively related to $[P]_{apex}$ (Fig. 4.9). Values of $[N]_{apex}$ ranged between 9.4 and 15.4 mg g^{-1} and $[P]_{apex}$ between 0.5 and 0.9 mg g^{-1} (Fig. 4.9), with the concentration of both elements approximately 2.5 - 8 times greater in the apices than in the bases (Fig. 4.10). There was weak co-variation between $[N]_{lichen}$ and $[P]_{lichen}$ in both the apices and basal strata.

Correlations between PME, $[N]_{lichen}$ and $[P]_{lichen}$ and N deposition parameters were frequently stronger for the modelled data sets than for measured data. In five of the ten regressions that utilize measured data, the relationships improved when they were recalculated using the modelled values pertaining to the lichen collection site improved the correlations. However, this procedure also frequently changed the order of sites on the wet inorganic N deposition ($\mathbf{N}$) and $[N]_{ppt}$ gradients such that some of the sites with measured rainfall chemistry might not have been selected if the original choice of sites had been on the basis of modelled data. Modelled values spanned a larger range ($\mathbf{N}_s$, 4.1 – 32.8 kg N ha^{-1}

y^{-1}; $[N]_s$, 0.31 – 1.9 mg l^{-1}) than measured values (2002 – 2005, $\mathbf{N}_m$, 2.3 – 9.3 kg N ha^{-1} y^{-1}; $[N]_m$, 0.35 – 1.32 mg l^{-1}, respectively).

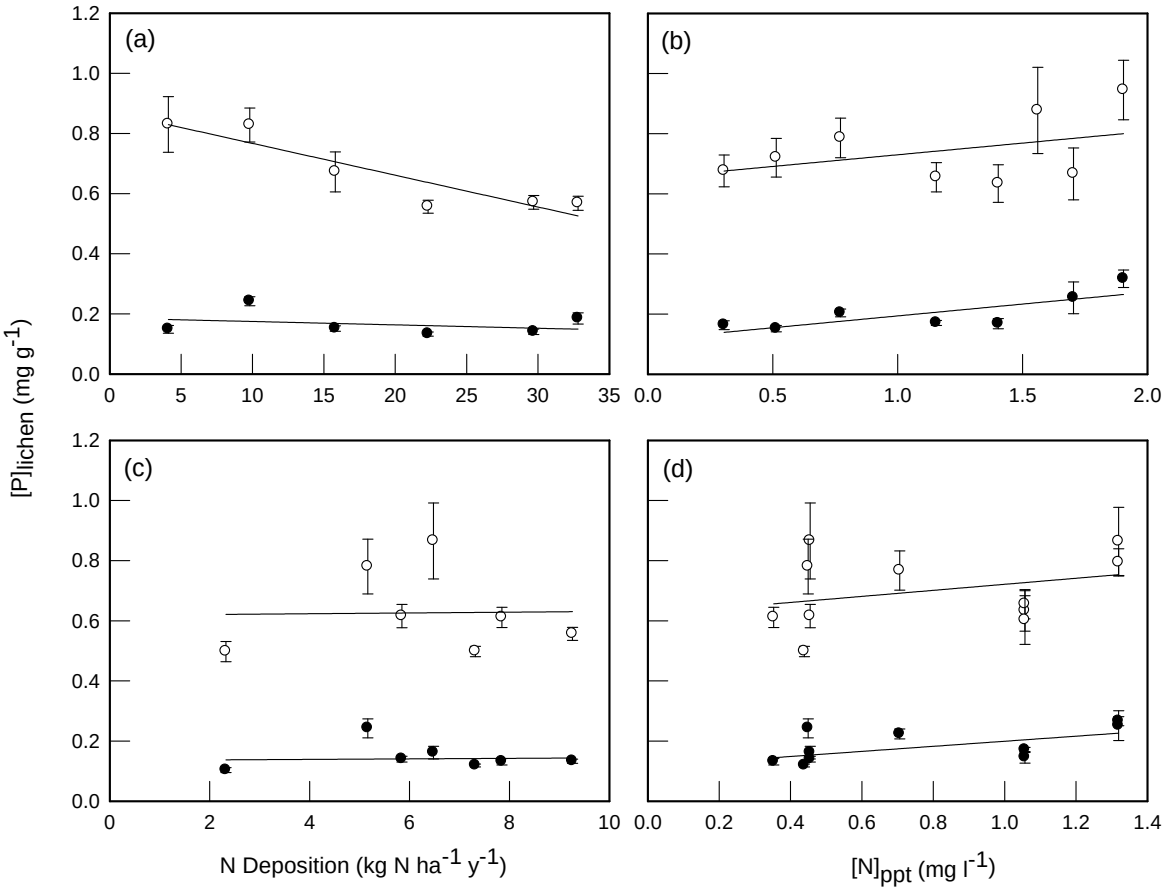


Figure 4.6 Relationships between the concentration of total P in apical (○; 0 – 5 mm) and basal (●; 40 – 50 mm) strata in *Cladonia portentosa* and modelled annual wet inorganic N deposition (a, ○, r^2 = 0.845, P <0.05; ●, r^2 = 0.049, P = 0.673); modelled mean inorganic N concentration in precipitation (b, ○, r^2 = 0.272, P = 0.185; ●, r^2 = 0.610, P <0.05); measured total wet inorganic N deposition (c, ○, r^2 = 0.000, P = 0.979; ●, r^2 = 0.002, P = 0.933); measured mean N concentration in precipitation (d, ○, r^2 = 0.081, P = 0.397; ●, r^2 = 0.331, P = 0.082). Plotted values are means (n = 10) ± 1 SEM.

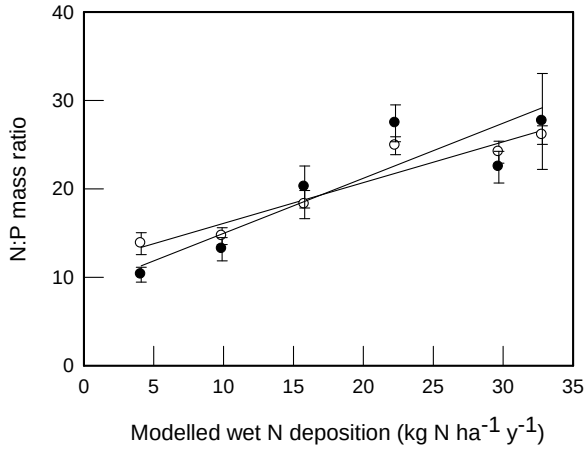


Figure 4.7 Relationship between modelled annual total wet inorganic N deposition and N:P mass ratio in apical (○, 0 – 5 mm) and basal (●, 40 – 50 mm) strata in *Cladonia portentosa* (○, r^2 = 0.909, P <0.01; ●, r^2 = 0.800, P <0.01). Plotted values are means (n = 5 - 10) ± 1 SEM.

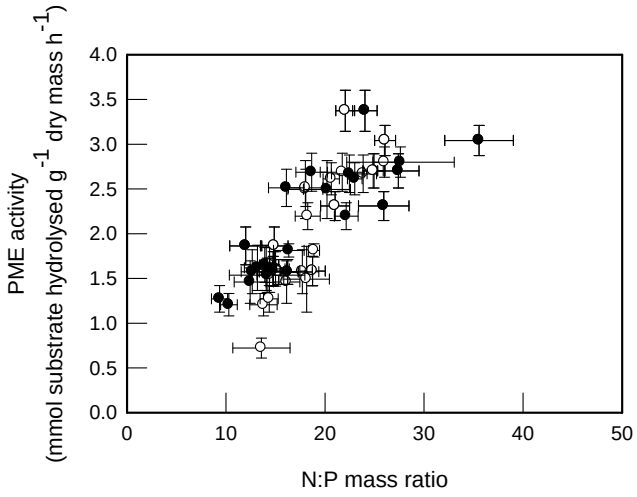


Figure 4.8 Relationship between N:P mass ratio in apical (○, 0 – 5 mm) and basal (●, 40 – 50 mm) strata in *Cladonia portentosa* and PME activity in the 10 mm apical segment. (○, r = 0.845, P < 0.001; ●, r = 0.835, P < 0.001). PME assays were performed with 3 mM *p*NPP for 20 minutes at 15 ^{o}C in the dark. Plotted values are means (n = 5 - 10) ± 1 SEM.

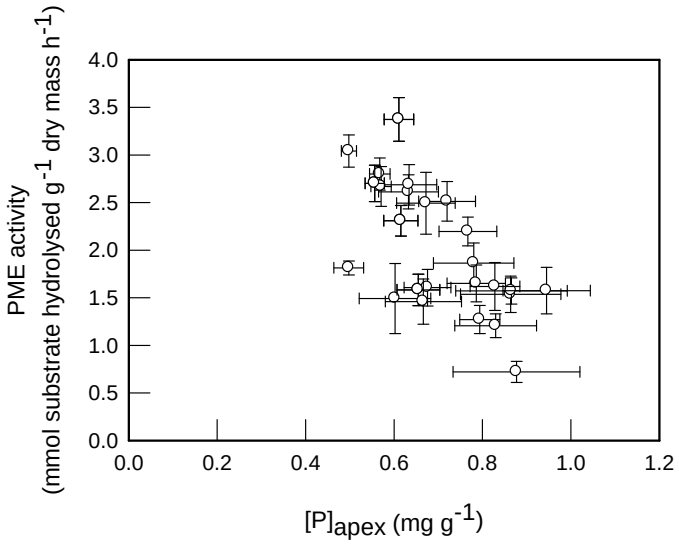


Figure 4.9 Relationship between $[P]_{apex}$ and PME activity in *Cladonia portentosa* (r = -0.660; $P < 0.001$). PME assays were performed on the apical 10 mm of thalli using 3 mM *p*NPP for 20 minutes at 15 ^{o}C in the dark. Plotted values are means (n = 5 -10) ± 1 SEM.

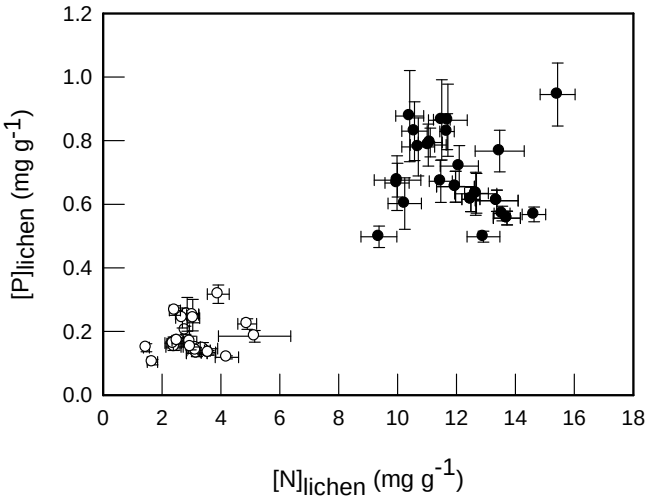


Figure 4.10 Relationship between $[N]_{lichen}$ and $[P]_{lichen}$ in apical (●, 0 - 5 mm, r = -0.146, P = 0.449) and basal (○, 40 – 50 mm, r = 0.126, P = 0.532) segments of *Cladonia portentosa*. Plotted values are means (n = 5 -10) ± 1 SEM.

4.4 Discussion

Phosphomonoesterase activity in *C. portentosa* was positively associated with N deposition. Values increased by a factor of *c.* 2 between sites with the lowest and highest N deposition rates, and in the case of both measured and predicted N deposition rates. In the following chapter (Chapter 5), it is demonstrated that this relationship is causal since experimental addition of N as NH_4^+ and NO_3^- to peatland plant communities containing *C. portentosa* resulted in up-regulation of PME activity in this lichen species. The present results for *C. portentosa* are in line with data for plant root associated PME activity in rainforests (Treseder and Vitousek 2001), acid and calcareous grasslands (Johnson *et al.* 1999; Phoenix *et al.* 2003), and heathlands (Pilkington *et al.* 2005) where N fertilization typically increased PME activity by factors of 2 - 3. Since the habitats for *C. portentosa*, a species usually associated with *Calluna vulgaris*, are typically N-limited, the probable explanation for this response is that increased N availability and capture from atmospheric deposits shifts deficiency from N to P, an element that frequently becomes the limiting factor when N-limited tundra-like ecosystems become N-enriched (Aerts and Berendse 1988). This explanation is consistent with the positive coupling between PME and thallus N:P mass ratio. Values of N:P mass ratio in *C. portentosa* generally ranged between 10 - 26 (Fig. 4.8). According to Güsewell (2004), N:P mass ratios in vascular plants > 20 are indicative of P limitation, a situation that obtained in *C. portentosa* at sites with $\mathbf{N}_s$ values > 18.5 kg ha^{-1} y^{-1}. Up-regulation of PME activity under conditions of N enrichment might result from new enzyme synthesis supported by excess cellular

[N] (Cembella *et al.* 1984; Treseder and Vitousek 2001), or by the alteration of the kinetic properties of PME to increase the rate of P hydrolysis (as evidenced by a significant increase in K_m and V_{max}, Chapter 2, Table 2.3), or perhaps via a combination of these two processes (Pettersson 1980).

Phosphomonoesterase capacity was not promoted by higher concentrations of N in rainfall alone when this was not associated with increased N deposition. The trend for negative relationships between PME and $[N]_{ppt}$ among sites with similar N deposition values (Fig. 4.4b & d) might be explained by the underlying rainfall differences between the sites potentially resulting in higher annual lichen growth rates, and hence greater growth-led demand for nutrients at high precipitation sites with low $[N]_{ppt}$ values (but with N deposition values equivalent to those at sites with high $[N]_{ppt}$). There is abundant evidence that lichen growth rates are higher at sites, or in years, with higher rainfall (e.g. Kärenlampi 1971; Crittenden *et al.* 1994), although it should be noted that Hyvärinen and Crittenden (1998b) found that growth in *C. portentosa* at physiographically distinct sites in Great Britain was not correlated with annual rainfall. There is no evidence of seasonal variation in PME activity in *C. portentosa* (see Chapter 2). There are a few published reports of seasonal changes in soil/root systems (Turner *et al.* 2002) and in mosses (Turner *et al.* 2003) but in lichens one would expect nutrient capture to be maximised throughout the year and such seasonal variation to be small.

It is instructive to compare the results of the present work with those of Hyvärinen and Crittenden (1998a) for *C. portentosa* collected between 1993 - 1994 as close as possible to 31 rainfall collections stations in the UK Acid Deposition Monitoring Network. The current range of values for $[N]_{lichen}$ and $[P]_{lichen}$ both in the apices and bases were highly similar to data obtained by Hyvärinen and Crittenden (1998a), and comparable with other species of *Cladina* (Solberg 1967; Pulliainen 1971; Scotter 1972; Pakarinen 1981). In the present study, the performance of $[N]_{apex}$ and $[N]_{base}$ as predictors of N deposition were largely indistinguishable, whereas Hyvärinen and Crittenden (1998a) found $[N]_{base}$ to be the stronger correlate. Their explanation for the accentuation of N enrichment in lichen bases was that increased N capture in the apices interferes with internal N cycling thereby reducing relocation of N from basal regions of the thallus to the apices. The anomaly between the results of Hyvärinen and Crittenden's (1998a) survey and the present findings might reflect changes in the pollution climate during the 12 year period between the surveys which include decreasing SO_2 and N emissions (NEGTAP 2001; Fowler *et al.* 2004). However, there was no significant difference in $\mathbf{N}_m$ values between 1994 and 2006 at 8 sites common to both studies. It is noteworthy that Walker *et al.* (2003) found variation in $[N]_{apex}$, but not that in $[N]_{base}$, in *C. stellaris* to reflect a putative gradient in N deposition along a 240 north-south transect in northern Russia, and Pakarinen (1981) reported a significant positive correlation between N enrichment and $[N]_{lichen}$ in the apical 2 cm of *Cladonia arbuscula* and *C. stellaris* along a N deposition gradient in

Finland. Thus it is possible that the usefulness of $[N]_{lichen}$ as a biomarker for N deposition shifts from $[N]_{base}$ to $[N]_{apex}$ as N deposition decreases.

Also in agreement with the present study, Hyvärinen and Crittenden (1998a) found that $[P]_{apex}$ and $[P]_{base}$ were negatively correlated, and $([N]/[P])_{apex}$ and $([N]/[P])_{base}$ positively correlated, with rainfall depth. However, in the earlier study $[N]_{apex}$ and $[N]_{base}$ were not related to rainfall depth in contrast to the present data (see Table 4.4), and there was co-variation between $[N]_{lichen}$ and $[P]_{lichen}$ in both thallus apices and bases (only weakly shown in the present study), an observation that partially prompted the current study. In both surveys, the range of values of $[N]_{apex}$, $[N]_{base}$, $[P]_{apex}$ and $[P]_{base}$ are broadly similar but in the present survey there were fewer sites yielding high values of both $[N]_{apex}$ and $[P]_{apex}$. This might reflect differences between surveys in site locations and/or the different criteria used in each study to select collection sites. For example, high **N** sites with high $[N]_{ppt}$ values (i.e. $\mathbf{N}_s$ *c.* 10 – 26 kg N ha^{-1} y^{-1} and $[N]_{ppt}$ 1 – 2 µg l^{-1}) were omitted from the present work.

In making comparisons between the efficacy of modelled and measured N deposition data in explaining variation in lichen eco-physiological variables, it should be remembered that the two series of analyses utilized *C. portentosa* collections from different sets of locations (Fig. 4.1). Notwithstanding this caveat, outcomes of the regression analyses using modelled and measured data were in good general agreement, although modelled values sometimes proved superior explanatory variables. This might partially be due to the very much larger range

of $\mathbf{N}_s$ compared to $\mathbf{N}_m$ values. Two factors underlie the generally large values of $\mathbf{N}_s$. First, the moorland deposition model, the output of which was used in the present study, compensates for variable collection efficiencies of rain gauges by taking into account wind-driven rain, occult precipitation, and orographic seeder-feeder effects which enhance $[N]_{ppt}$ and $\mathbf{N}$ values at high altitude. Second, among the *c*. 11,200 5 x 5 km grid squares for which $\mathbf{N}_s$ values are available, there is inevitably a greater range of values available than among the 38 measurement sites (Fig. 4.1); i.e., it was possible to collect *C. portentosa* from sites representing almost the full range of modelled deposition values (4.1 – 32.8 kg N ha^{-1} y^{-1}) which might have contributed to the strong relationships observed with modelled data. Thus, for a combination of these two reasons, values of $\mathbf{N}_s$ always exceeded $\mathbf{N}_m$.

CHAPTER 5. Response of PME activity in *Cladonia portentosa* to N and P enrichment in a field manipulation experiment – Whim Moss

5.1 Introduction

Anthropogenic nitrogen pollution in the British Isles results in marked spatial variation in N deposition rates. This variation in N availability is associated with co-variation in metabolic functioning and chemical composition in the common heathland lichen *C. portentosa*. For example, in the previous chapter, it was shown how variation in modelled wet inorganic N deposition ($\mathbf{N}_s$) is associated with significant increases in PME activity, N:P mass ratio and N concentration in the apical 5mm ($[N]_{apex}$) and basal 40 – 50 mm strata ($[N]_{base}$) of *C. portentosa* thalli. In addition, research described later in this thesis shows that nutrient uptake kinetics (for PO_4^{3-}, NO_3^-, NH_4^+ and glycine) are significantly modified by N enrichment (see Chapter 7).

A rise in $\mathbf{N}_s$ from 4.1 to 32.8 kg N ha^{-1} y^{-1} is associated with an increase in thallus N:P mass ratio from *c.* 14 to 26, and in PME activity from 1.2 to 2.8 mmol substrate hydrolysed g^{-1} dry mass h^{-1} (Chapter 4). It was of interest to discover by means of controlled experimentation whether these relationships were causal. Use of the Whim Moss experimental site provided an opportunity to assess this causality.

At Whim Moss, an unmanaged ombrotrophic bog located approximately 30 km south of Edinburgh, natural peatland communities (in which *C. portentosa* is abundant) occur in a region of low atmospheric N deposition (8 kg N ha^{-1} y^{-1}). Since 2002, simulated rainfall containing elevated N concentrations has been applied to the natural vegetation growing at Whim Moss in order to help understand the impacts of N enrichment on N-sensitive ecosystems (Leith *et al.* 2001; Sheppard *et al.* 2004a; 2004b). The N treatments range from 8 – 64 kg N ha^{-1} y^{-1} (including ambient deposition), the highest N treatment being equivalent to approximately twice the maximum $\mathbf{N}_s$ value in Great Britain (32.8 kg N ha^{-1} y^{-1}, R. I. Smith, CEH Edinburgh, Pers. Comm.). In addition, the experiment has been designed to identify dose-dependent effects, to distinguish between the effects of reduced (NH_4^+) and oxidised (NO_3^-) forms of N and also to investigate the effects of phosphorus enrichment.

In this chapter I report the response of *C. portentosa* to factorial additions of wet N deposition and the influence of both N form and PK enrichment on lichen physiology and chemistry.

5.2 Materials and Methods

5.2.1 Experimental site

The Whim Moss experimental site is located approximately 30 km south of Edinburgh (NT 204 532) (Sheppard *et al.* 2004a) and represents a transition between a lowland raised bog and a blanket bog (National Vegetation Classification M19a) (Rodwell 1991). The site has been unmanaged for approximately 60 years, although there is a small degree of grazing by rabbits. Mean annual rainfall is *c.* 900 mm, with very low levels of pollutants (4 kg S ha^{-1} y^{-1} and 8 kg N ha^{-1} y^{-1}) (Sheppard *et al.* 2004a). The vegetation has been described by Sheppard *et al*. (2004a) and is dominated by heathers (including *Calluna vulgaris* and *Erica tetralix*), *Empetrum nigrum*, *Eriophorum vaginatum*, and bryophytes (including *Pleurozium schreberi*, *Sphagnum capillifolium* and *S. papillosum*). Lichen abundance is high and includes several *Cladonia* species, of which *C. portentosa* is estimated to have a percentage cover of up to 30 % (Sheppard *et al.* 2004a).

The experimental treatment plots cover an area of 0.5 ha (Sheppard *et al.* 2004a) and are accessed by a network of boardwalks (Fig. 5.1) to minimise trampling effects on the vegetation. Eleven wet N treatments have been applied since June 2002 to a total of 44 circular plots (Fig. 5.1), each are 12.5 m^2 in area and separated by a distance of 3 m (Sheppard *et al.* 2004b). Four control plots receive only natural rainfall (8 kg N ha^{-1} y^{-1}), with experimental N treatments applied at

doses of 8, 24 and 56 kg N ha^{-1} y^{-1} mixed with rainwater collected on site. Treatment plots therefore receive a total of 16, 32 or 64 kg N ha^{-1} y^{-1} as both oxidised ($NaNO_3$) and reduced (NH_4Cl) N forms. Additional treatments at 16 and 64 kg N ha^{-1} y^{-1} are supplemented with P and K by adding K_2HPO_4 at a 1:14 PN ratio to give 16 kg N + 0.57 kg P ha^{-1} y^{-1} and 64 kg N + 4 kg P ha^{-1} y^{-1} (Sheppard *et al.* 2004b).

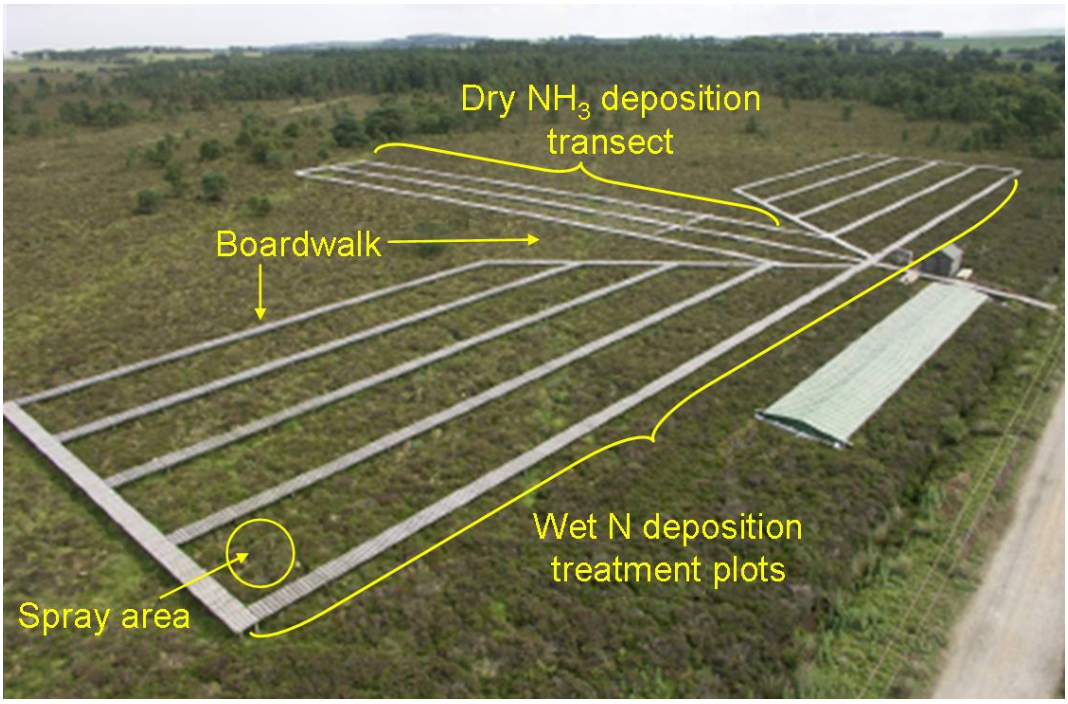


Figure 5.1 Aerial view of Whim Moss experimental site

The main experimental site is divided into 4 separate blocks which each contain all 11 wet N treatments with oxidised and reduced N treatments side-by-side (helping to maximise any distinguishing effects of N form) (Fig. 5.2). An automated system, designed to simulate natural deposition patterns applies treatments only

when strict meteorological conditions are met. Thus treatments are only sprayed onto plots during rainfall events coupled with windspeeds < 5 m s^{-1}.

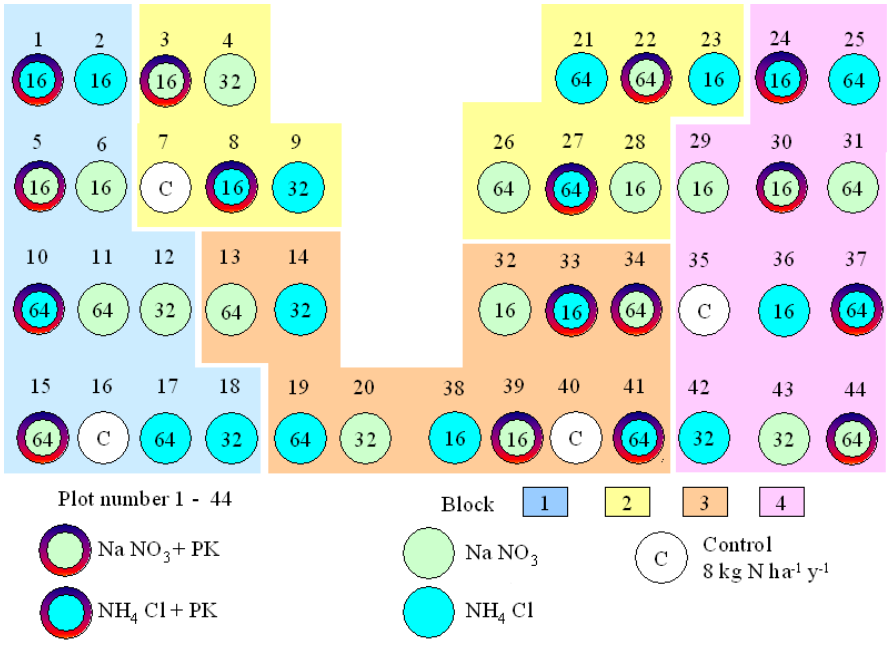


Figure 5.2 Map of nitrogen treatment plots at Whim Moss. Four separate blocks each contain 11 wet N treatments (each individual treatment is illustrated as a circle with the representative N deposition value (kg N ha^{-1} y^{-1}). Plots receiving $N\text{-}NO_3^-$ are shown in green, $N\text{-}NH_4^+$ in blue and those fertilised with PK by a red halo.

5.2.2 Collection and pre-treatment of *Cladonia portentosa*

A sample of *C. portentosa* (typically a clump of podetia 20 – 40 mm in diameter) was collected from 34 of the 44 treatment plots on 8 February 2006 providing a replication of n = 2 - 3 for each treatment. The samples were rinsed briefly in deionised water, air dried and stored in plastic vials at either 4 ^oC for 7 days (for

phosphatase analysis) or at -15 ^{o}C for 12 weeks (for chemical analysis). Powder-free latex gloves were worn at all times when handling lichens to minimise contamination.

5.2.3 Sample preparation

Thalli were rehydrated overnight in water-saturated air (over water in a desiccator) at 10 ^{o}C, then saturated by spraying lightly with deionised water and cleaned of extraneous debris. The apical parts of thalli were selected for analysis by cutting each replicate with a razor blade at either 5 mm (for chemical analysis) or 10 mm (for phosphatase assays) downwards from the apices.

5.2.4 Phosphatase assay

Phosphomonoesterase activity was determined using the *p*-nitrophenyl phosphate (*p*NPP) method as described by Turner *et al*. (2001) and outlined in section 2.2.4. Assay conditions included 0.02 M citric acid-trisodium citrate buffered assay medium (pH 2.5), a final *p*NPP concentration of 3 mM, with incubation conditions of 15 ^{o}C, for 20 min in the dark.

5.2.5 Determination of total N and P concentration

Samples were weighed and digested following the sulphuric acid-hydrogen peroxide procedure of Allen (1989), outlined in section 4.2.5. Ammonium-N in the digest was determined using the fluorometric method of Holmes (1999), and

phosphorus was assayed by the malachite green variant of the methylene blue method after Van Veldhoven and Mannaerts (1987). For full details of both methods see section 4.2.5. Determination of total N and P in *C. portentosa* for this investigation was undertaken by G. Minnullina.

5.2.6 Statistical analyses

SPSS was used to perform standard statistical analyses. All data were initially checked for normality and statistical tests performed accordingly. The effects of N deposition amount and N form on enzyme activity and lichen chemistry were tested by ANCOVA. As there is not a complete set of treatments receiving additional PK, ANOVA was performed on data for the 16 and 64 kg N ha^{-1} y^{-1} treatments to test the effects of N deposition amount, form and addition of PK on PME activity and lichen chemistry. Pearson's correlation coefficients were derived for plots of thallus N or P concentration or N:P mass ratio against PME activity.

5.3 Results

Phosphomonoesterase activity in the apical 10 mm of *C. portentosa* was strongly positively related to the quantity of N applied, the response being independent of the N form (Table 5.1, Fig. 5.3). Activity increased by *c.* 70 % when N deposition increased from 8 (control plots) to 64 kg ha^{-1} y^{-1}. Combining data for both NH_4^+ and NO_3^- enhanced the significance of the N effect (ANOVA calculated for PME

values at each N load with form omitted, $P < 0.001$, Fig. 5.3c). Up-regulation of PME activity in response to N enrichment was not evident in plots fertilised with PK: in *C. portentosa* receiving 16 kg N + 0.57 kg P ha^{-1} y^{-1}, PME activity was similar to that in control plots while in lichens receiving 64 kg N + 4 kg PK ha^{-1} y^{-1}, PME activity was depressed by *c*. 57 % compared to control plots. Phosphomonoesterase activity in PK treated material was highly significantly reduced ($P < 0.001$) compared to treatment plots receiving similar N loads but not fertilised with PK, and there was a significant interaction between the quantity of N applied (16 or 64 kg N ha^{-1} y^{-1}) and the addition of PK (Table 5.2, Fig. 5.3c).

Increasing wet N deposition from 8 to 64 kg N ha^{-1} y^{-1} also significantly increased $[N]_{apex}$ by a factor of 1.4 with no modification under regimes fertilised with PK (Table 5.1, Fig. 5.4a, c, e). The concentration of P in thalli was unaffected by N enrichment but was markedly elevated in plots receiving P additions (by 60 % and 165 % in the 16 kg N + 0.57 kg P ha^{-1} y^{-1} and 64 kg N + 4 kg P ha^{-1} y^{-1} plots, respectively) (Table 5.2, Fig. 5.4b, d, f). Phosphomonoesterase activity was strongly negatively related to $[P]_{apex}$ and only weakly positively related to $[N]_{apex}$ (Fig. 5.5) and there was no relationship between $[N]_{apex}$ and $[P]_{apex}$ ($r = -0.371$, $P = 0.468$).

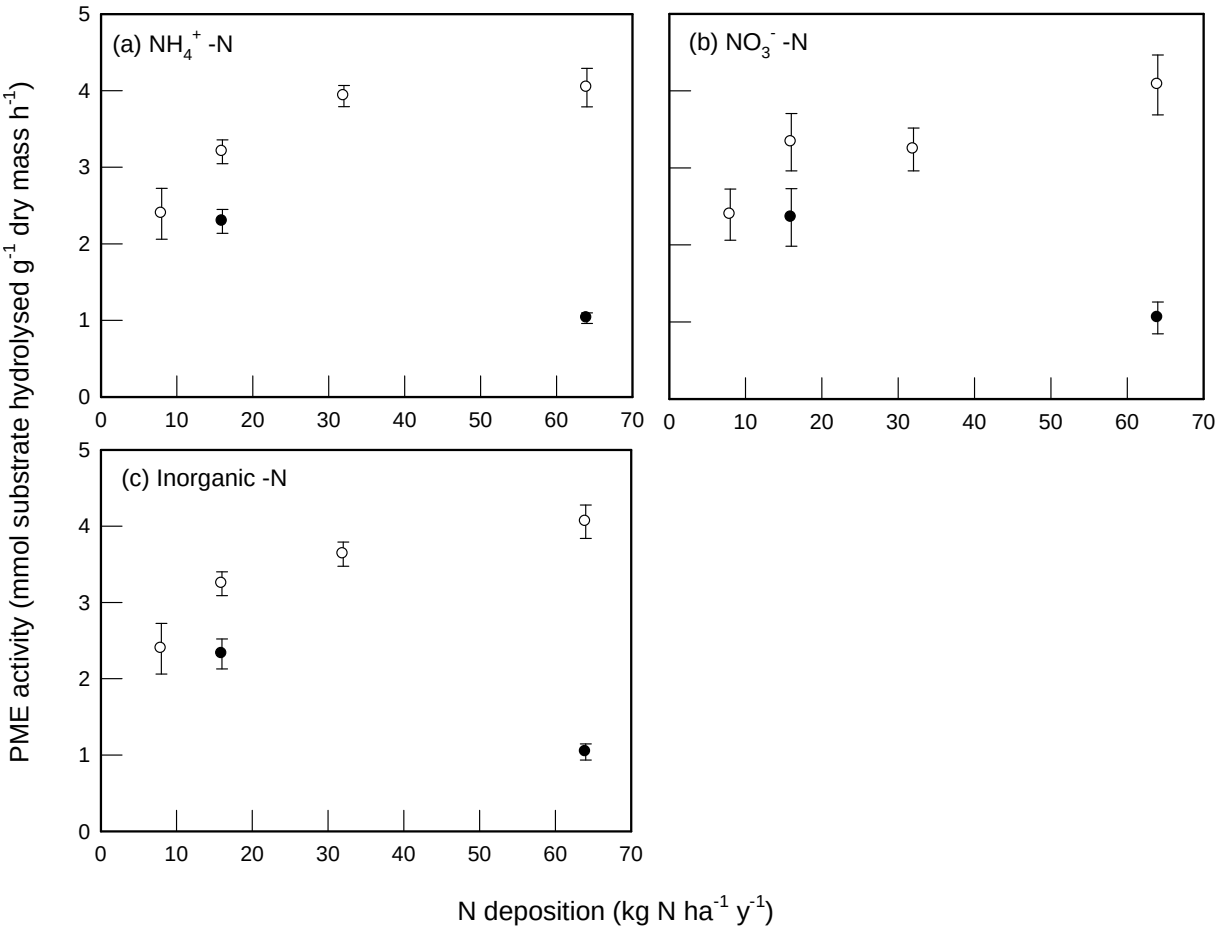


Figure 5.3 Relationships between PME activity in the apical 10 mm of *Cladonia portentosa* and addition of NH_4^+-N (a), NO_3^--N (b), or inorganic N (c) (○, nitrogen only treatments; ●, nitrogen + PK). Assays were performed with 3 mM *p*NPP for 20 minutes at 15 ^oC in the dark. Plotted values are means (n = 2 - 6) ± 1 SEM.

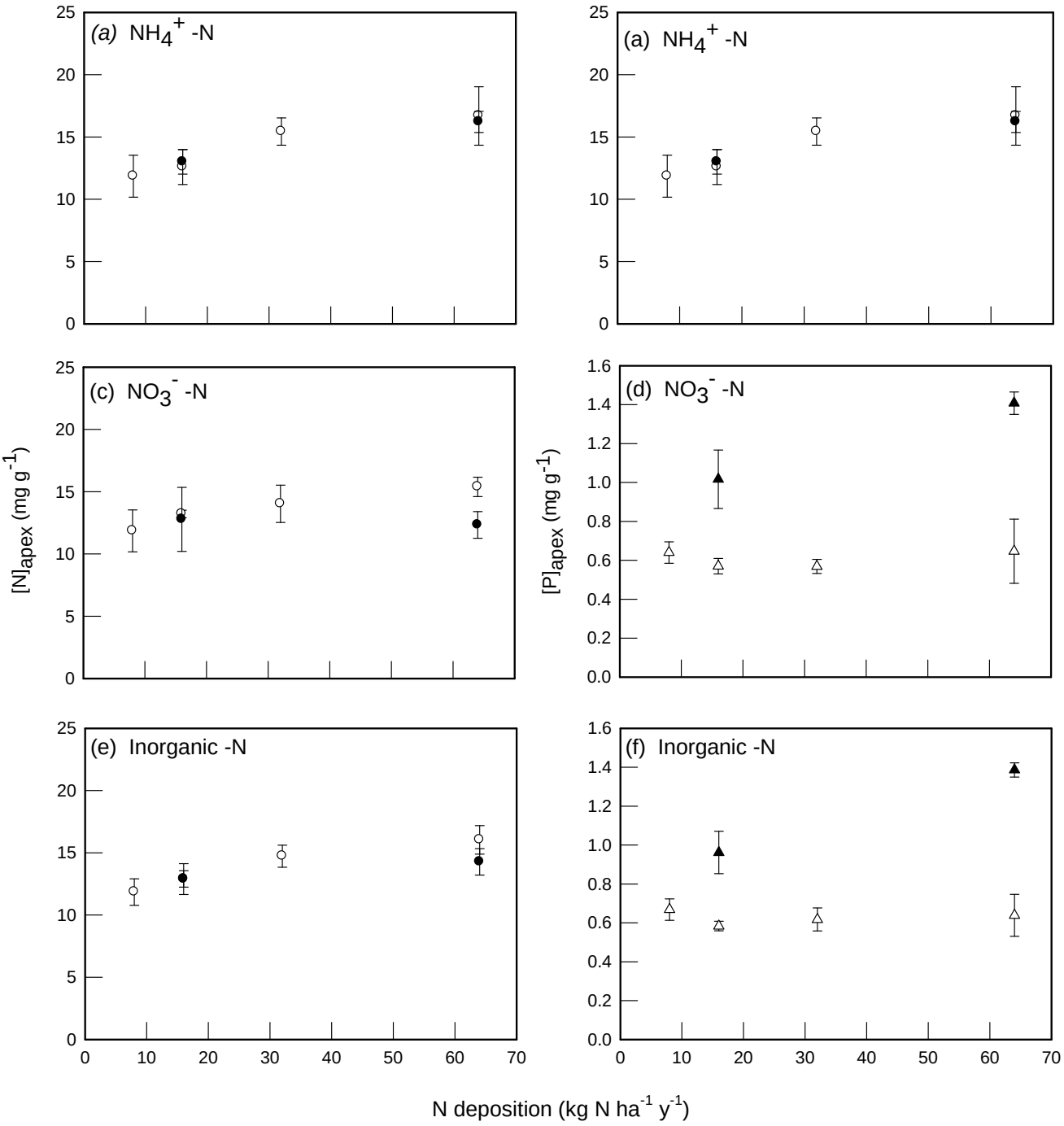


Figure 5.4 Relationships between the N concentration (○) or P concentration (Δ) in the apical 5 mm of *Cladonia portentosa* and addition of NH_4^+-N (a & b), NO_3^--N (c & d), or inorganic N (e & f). Additional points (●, ▲) are for treatments receiving PK in addition to N. Plotted values are means (n = 3) ± 1 SEM.

Table 5.1 Results of two-way ANOVA comparing PME activity, $[N]_{apex}$, $[P]_{apex}$ or thallus N:P mass ratio in *Cladonia portentosa* between N deposition treatments (8 – 64 kg N ha^{-1} y^{-1}) and between different N forms (NH_4^+ or NO_3^-)

	df				*F*-ratio				*P*-value			
Source of variation	PME	[N]	[P]	[N]/[P]	PME	[N]	[P]	[N]/[P]	PME	[N]	[P]	[N]/[P]
Between N deposition treatments	3	3	3	3	12.982	3.705	0.106	4.524	<0.001	0.019	0.956	0.008
Between N forms	1	1	1	1	0.448	0.000	0.072	0.102	0.506	0.995	0.790	0.751
N deposition load x N form	3	3	3	3	1.022	0.358	0.311	0.261	0.389	0.784	0.817	0.853
Error	66	43	43	43								
Total	74	51	51	51								

PME (r^2 = 0.398); [N] (r^2 = 0.234); [P] (r^2 = 0.033); [N]/[P] (r^2 = 0.256)

Table 5.2 Results of a three-way ANOVA comparing PME activity, $[N]_{apex}$, $[P]_{apex}$ or thallus N:P mass ratio in *Cladonia portentosa* between N deposition treatments (16 or 64 kg N ha^{-1} y^{-1}), between different N forms (NH_4^+ or NO_3^-) and between treatments with/without the addition of P.

	df				*F*-ratio				*P*-value			
Source of variation	PME	[N]	[P]	[N]/[P]	PME	[N]	[P]	[N]/[P]	PME	[N]	[P]	[N]/[P]
Between N deposition treatments	1	1	1	1	1.859	4.643	7.428	0.028	0.178	0.047	0.015	0.869
Between N forms	1	1	1	1	0.124	1.299	0.166	0.006	0.726	0.271	0.689	0.937
With/without addition of PK	1	1	1	1	119.639	0.721	40.976	40.065	<0.001	0.408	<0.001	<0.001
N deposition treatment x N form	1	1	1	1	0.035	1.770	0.004	0.031	0.853	0.202	0.948	0.863
N form x addition of PK	1	1	1	1	0.013	0.668	0.216	0.277	0.911	0.426	0.649	0.606
N deposition treatment x addition of PK	1	1	1	1	32.814	0.699	4.410	5.322	<0.001	0.415	0.052	0.036
N deposition x N form x addition of PK	1	1	1	1	0.006	0.171	0.098	0.342	0.939	0.685	0.759	0.567
Error	63	16	16	16								
Total	71	24	24	24								

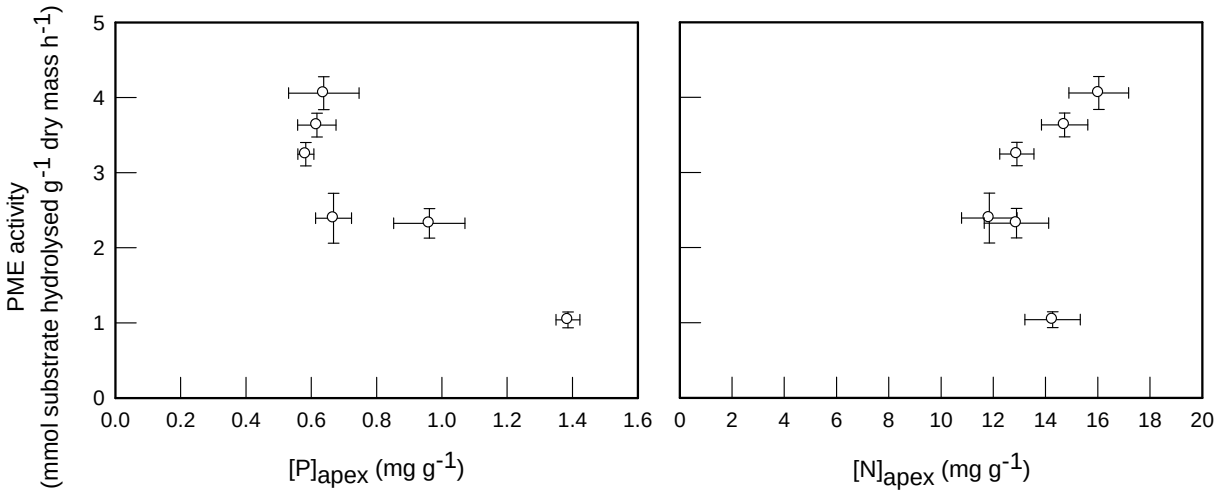


Figure 5.5 Relationships between PME in the apical 10 mm of *Cladonia portentosa* and phosphorus concentration (a, r = -0.878, P = 0.021) or nitrogen concentration (b, r = 0.419, P = 0.409) in the apical 5 mm. PME assays were performed with 3 mM *p*NPP for 20 minutes at 15 ^{o}C in the dark. Plotted values are means (n = 10) ± 1 SEM.

Lichen N:P mass ratio was significantly positively related to both wet N deposition, (increasing by a factor of 1.5 between 8 and 64 kg N ha^{-1} y^{-1} treatments) (Fig. 5.6) and PME activity (increasing from 8.7 to 27) (Fig. 5.7). The addition of PK resulted in a significant decrease in lichen N:P mass ratio ($P < 0.001$, Fig. 5.6) and was demonstrated by ANOVA to interact significantly with the quantity of N, P and K added (Table 5.2). Under all concentration regimes, the form of N applied had no significant effect on phosphatase activity or lichen chemistry (Tables 5.1 & 5.2, Figs. 5.3 & 5.4) with the strength of any significant findings increased by combining data obtained for both NH_4^+ and NO_3^- treatments.

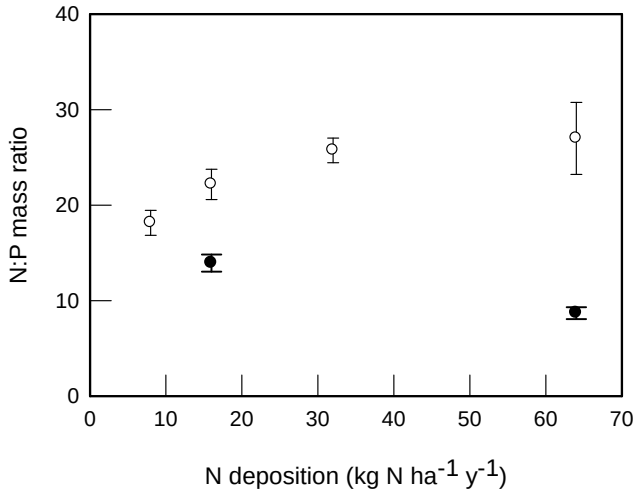


Figure 5.6 Relationship between wet inorganic N deposition (○) or wet N deposition + PK (●) and N:P mass ratios in the apical 5 mm of *Cladonia portentosa*. Plotted values are means (n = 5 – 6) ± 1 SEM.

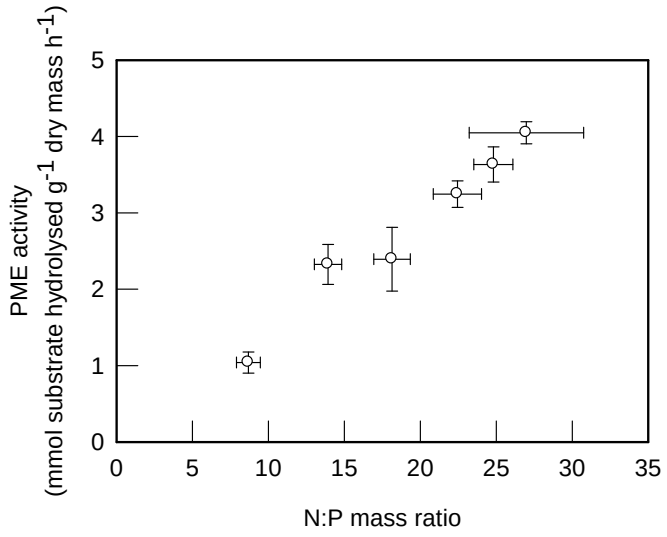


Figure 5.7 Relationship between N:P mass ratio in the apical 5 mm of *Cladonia portentosa* and PME activity in the apical 10 mm (r = 0.981, P = 0.001). Plotted values are means (n = 2 – 6) ± 1 SEM.

A comparison between the data collected from the survey of different lichen populations across Great Britain (reported in Chapter 4) and data from the study at Whim Moss revealed similar relationships in both cases between wet N deposition and PME activity, $[N]_{lichen}$, $[P]_{lichen}$ and thallus N:P mass ratio (Figs. 4.8 – 4.10). However, rates of PME activity were significantly higher in *C. portentosa* from the Whim Moss experiment at all N deposition values investigated (Fig. 5.8, Table 5.3). For example, at 16 and 32 kg N ha^{-1} y^{-1}, activity was *c*. 23 % higher at Whim Moss at rates of 3.2 and 3.6 mmol substrate hydrolysed g^{-1} dry mass h^{-1}, respectively, when compared with PME activity in lichens collected from sites across Great Britain. Phosphomonoesterase activity at the highest N deposition rate investigated at Whim Moss (64 kg N ha^{-1} y^{-1}) was *c*. 1.5 times higher than the maximum activity recorded in the field at 32.8 kg N ha^{-1} y^{-1}.

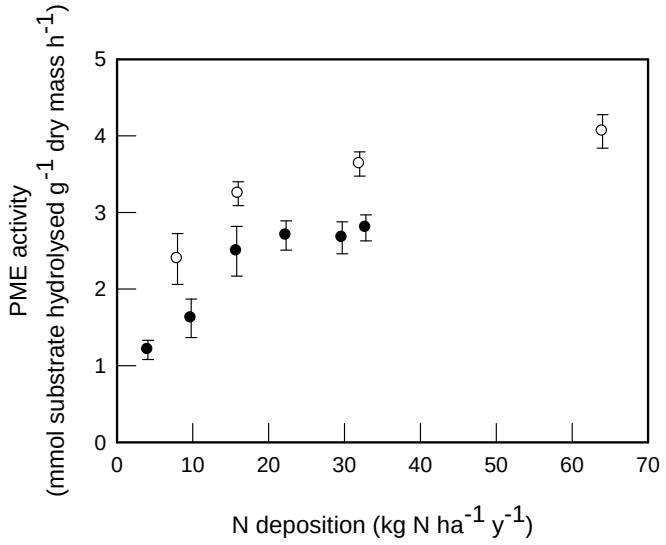


Figure 5.8 Relationship between wet inorganic N deposition and PME activity in the apical 10 mm of *Cladonia portentosa* collected from Whim Moss (○) or sites across Great Britain (●) (for site details see Chapter 4). Assays were performed with 3 mM *p*NPP for 20 minutes at 15 ^{o}C in the dark. Plotted values are means (n = 6 – 10) ± 1 SEM.

Values of $[N]_{apex}$, $[P]_{apex}$ and thallus N:P mass ratio did not differ significantly between the two investigations (Figs. 5.9 & 5.10, Table 5.3).

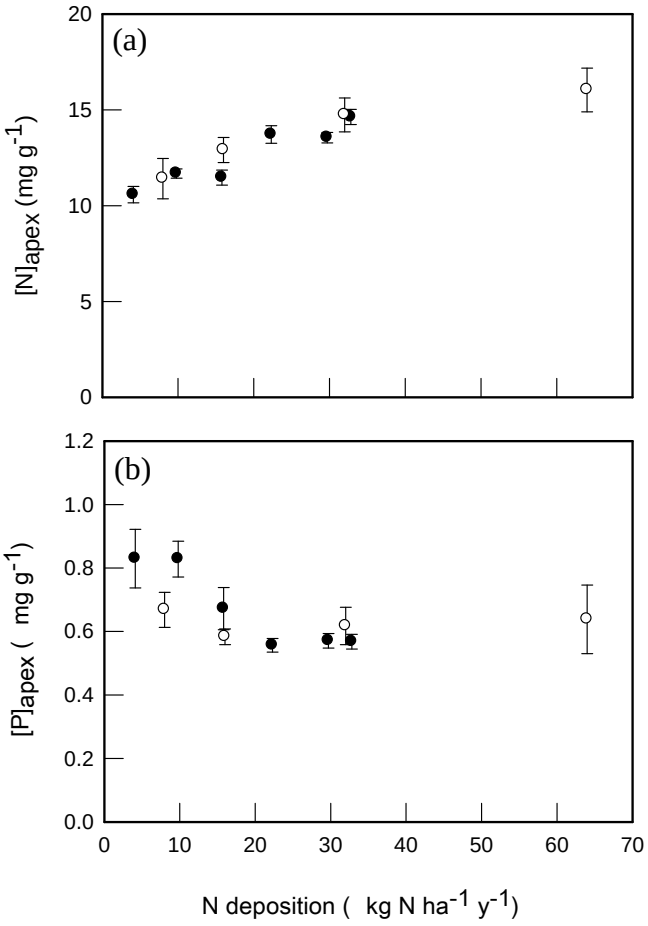


Figure 5.9 Relationship between wet inorganic N deposition and the nitrogen concentration (a) or phosphorus concentration (b) in the apical 5 mm of *Cladonia portentosa* collected from Whim Moss (○) or sites across Great Britain (●) (for site details see Chapter 4). Plotted values are means (n = 6 – 10) ± 1 SEM.

Table 5.3 Results of one-way ANOVA comparing PME activity, $[N]_{apex}$, $[P]_{apex}$ or thallus N:P mass ratio in *Cladonia portentosa* between two different investigations into the effects of wet N deposition (Whim Moss and sites across Great Britain varying in N deposition load (Chapter 4)). For comparative purposes, values of lichen chemistry and PME activity measured at 64 kg N ha^{-1} y^{-1} at Whim Moss were excluded from these analyses.

	df				*F*-ratio				*P*-value			
Source of variation	PME	[N]	[P]	[N]/[P]	PME	[N]	[P]	[N]/[P]	PME	[N]	[P]	[N]/[P]
Between N deposition treatments	1	1	1	1	34.075	0.597	0.909	1.200	< 0.001	0.442	0.343	0.277
Total	107	77	77	77								

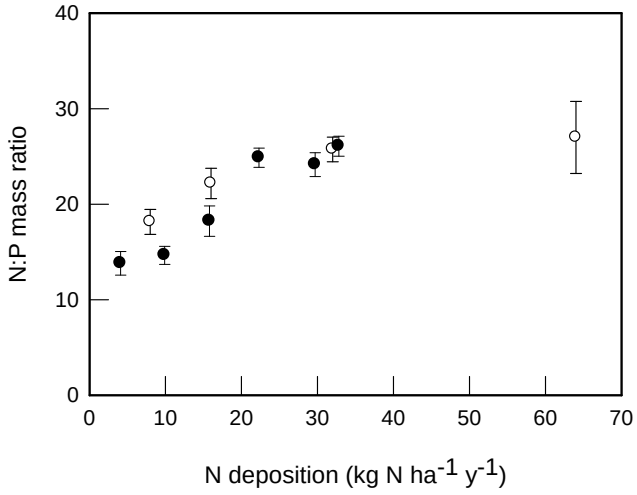


Figure 5.10 Relationship between wet inorganic N deposition and thallus N:P mass ratio in the apical 5 mm of *Cladonia portentosa* collected from Whim Moss (○) or sites across Great Britain (●) (for site details see Chapter 4). Plotted values are means (n = 6 – 10) ± 1 SEM.

5.4 Discussion

Phosphomonoesterase activity in *C. portentosa* is strongly up-regulated in response to increased N deposition. The magnitude and variation in activity measured in controlled experimental N treatments at Whim Moss are consistent with the observations made on natural populations of *C. portentosa* at a range of sites across Great Britain (see Chapter 4), and establishes causality between N enrichment and increased PME activity in this lichen. However, at similar rates of N deposition, PME activity measured in lichen from Whim Moss was significantly higher than that recorded in populations from un-manipulated field sites elsewhere in Britain. Similar values of $[N]_{apex}$ and $[P]_{apex}$ recorded in *C. portentosa* from

both studies suggest that discrepancies in PME activity cannot be accounted for by differences in nutrient availability and/or uptake. However, it is possible that higher PME activities at Whim Moss might reflect a non-steady state situation in which initial response to sudden N enrichment is greater than the long-term steady state response. Evidence to support this suggestion is presented in the next chapter where I show that PME activity in *C. portentosa* transplanted from a low- to high-N site is rapidly up-regulated to a level above that in lichen 'native' to the high-N site. There are also precedents for such a response in vascular plants. Carroll *et al.* (1999) reported a significant increase in the annual shoot extension in *Calluna vulgaris* over a four year period in response to N fertilization (0 - 120 kg N ha^{-1} y^{-1}), but during the subsequent 3 years this stimulus to annual shoot extension was progressively lost until after 8 years the rate had returned to original pre-treatment values.

The present findings for *C. portentosa* compare well with data for other plant/soil systems in which N enrichment has been shown to promote PME activity. These include calcareous and acid grasslands (Johnson *et al.* 1999; Phoenix *et al.* 2003), heathlands (Pilkington *et al.* 2005), and peat bogs (Bragazza *et al.* 2006). Similarly under the same experimental N regimes at Whim Moss, Phuyal *et al.* (2008) report a significant increase in PME activity in the mosses *Sphagnum capillifolium* and *Hypnum jutlandicum*, from *c.* 1 to 2.7 mmol substrate hydrolysed g^{-1} dry mass h^{-1} and 1.5 to 2.1 mmol substrate hydrolysed g^{-1} dry mass h^{-1}, respectively. It is noted that the rates of PME activity recorded in *C. portentosa* at Whim Moss are amongst the highest reported in the literature for a plant/fungal

system (see Appendix 1) and comparable to those measured by Phuyal *et al.* (2008) at the same site. The high rates of PME activity in a lichen that is largely dependent on atmospheric deposits for supplies of N and P (Hyvärinen and Crittenden 1998a; Ellis *et al.* 2003; 2004) might suggest that intermittent but spatially uniform inputs of trace quantities of organic P in wet and dry deposits are significant in the P-economy of this lichen. Alternatively, *C. portentosa* might source organic phosphates from larger depositions such as animal excreta and leaf litter that are spatially and temporally infrequent and erratic (Tomassen *et al.* 2005).

Heathlands and ombrotrophic bog habitats which commonly support populations of *C. portentosa* are generally considered to be oligotrophic and predominantly N-limited (Aerts and Heil 1993; Britton and Fisher 2007a). Increased N input to such habitats results in a change in the relative availability of essential plant nutrients with P frequently becoming growth limiting (Aerts and Berendse 1988; Aerts *et al.* 1992). This is evident in *C. portentosa* at Whim Moss in which N:P mass ratio rises from *c.* 18 to 27 in response to an increase in N deposition rate from 8 to 64 kg N ha^{-1} y^{-1}. This level of N enrichment elevates values of $[N]_{apex}$ from *c.* 12 to 16 mg g^{-1} but has an insignificant effect on values of $[P]_{apex}$. Thus thallus N:P mass ratio responds strongly to N enrichment, supporting observations previously reported for naturally occurring populations of *C. portentosa* across Great Britain (see Chapter 4). Nonetheless, an increase in wet inorganic N deposition > *c.* 23 kg N ha^{-1} y^{-1} was not associated with a further increase in thallus N:P mass ratio which remained relatively constant at a value of *c.* 26 in *C.*

portentosa collected from both Whim Moss and several sites in Great Britain (Fig. 5.9). Thus, the possibility exists that a value of *c*. 26 represents the maximum achievable thallus N:P mass ratio in the apical 5 mm of *C. portentosa*. This compares favourably with a maximum thallus N:P mass ratio of 23.4 reported by Hyvärinen and Crittenden (1998a) in an earlier study on the same lichen. However, Tomassen *et al.* (2004) reported a value of 58 for thallus N:P mass ratio in *C. portentosa*, but the authors do not state the region of the thallus investigated and values for $[N]_{lichen}$ and $[P]_{lichen}$ are between 1.5 - 2.5 times smaller than values recorded in this thesis.

In a review of variation and functional significance of N:P ratios in terrestrial plants (including mosses and lichens), Güsewell (2004) suggests that N:P mass ratios of < 10 and > 20 correspond to N-limited and P-limited biomass production, respectively. Following Güsewell (2004), the N:P mass ratios obtained for *C. portentosa* growing at Whim Moss suggest that this lichen experiences P-limitation at N deposition rates > 16 kg N ha^{-1} y^{-1}. This is highly comparable with the findings on natural populations of *C. portentosa* which were identified as experiencing P-limitation at heathland sites in Great Britain receiving > 18.5 kg N ha^{-1} y^{-1}. This strong positive correlation between thallus N:P mass ratio and PME activity in *C. portentosa* provides evidence that the up-regulation of phosphatase activity in this species is induced by a shift to a P-limited state. Furthermore, N:P mass ratio and PME activity in *C. portentosa* vary regionally in Great Britain and are both positively correlated with N deposition (see Chapter 4). However there are currently insufficient data to confirm whether the increase in PME activity

observed in *C. portentosa* at Whim Moss result from either differences in enzyme kinetics, simply an increase in the abundance of the enzyme, or a combination of these two factors (Pettersson 1980).

Addition of P to plots receiving both 16 and 64 kg N ha^{-1} y^{-1} significantly increased $[P]_{apex}$ and was associated with a marked decrease in PME activity and N:P mass ratio. A negative relationship between PME activity and tissue P concentration has been recorded in several different plant and fungal systems, including grasses (Johnson *et al.* 1999; Phoenix *et al.* 2003), bryophytes (Press and Lee 1983; Phuyal *et al.* 2008), mycorrhizas (Beever and Burns 1980; Kroehler *et al.* 1988; Joner *et al.* 2000) and in field populations of *C. portentosa* (as reported in Chapter 4). For example, Press and Lee (1983) recorded an inverse linear relationship between acid phosphatase activity and tissue P concentration in 11 species of *Sphagnum*, with activity ranging from 12 to 108 µmol substrate hydrolysed g^{-1} dry mass h^{-1} in line with a decrease in tissue P concentration from 2.4 to 0.3 mg g^{-1} dry mass. Phoenix *et al.* (2003) reported that a decrease in shoot P concentration from 4.1 to 3.2 mg g^{-1} dry mass in *Koeleria macrantha* seedlings was associated with a 40 % increase in root-surface PME activity, and that application of 0.35 kg P ha^{-1} y^{-1} significantly reduced activity by a factor of 1.6. Thus, it is not surprising that P fertilization reduced P-limitation in *C. portentosa*, as indicated by the decreased N:P mass ratio and significant down-regulation of PME activity.

Under experimentally controlled N treatments, the physiological responses of *C. portentosa* to additions of NH_4^+ and NO_3^- were indistinguishable. These findings are in line with Phuyal *et al.* (2008) who reported no significant effect of N form on PME activity in *H. jutlandicum* growing on the same treatment plots at Whim Moss. Although there is evidence to suggest that lichens preferentially absorb NH_4^+ compared with NO_3^- or amino-N (Dahlman *et al.* 2002; 2004; Ellis *et al.* 2005; Palmqvist and Dahlman 2006; Chapter 7), it is apparent that *C. portentosa* is efficient in the uptake of both NH_4^+ and NO_3^- when supplied together at trace concentrations in natural rainfall (Crittenden 1989), a strategy which is likely to prove beneficial under unpolluted natural conditions where N supply might limit growth (Crittenden 1989; Crittenden *et al.* 1994).

CHAPTER 6. A reciprocal transplant experiment to investigate the response of PME activity in *Cladonia portentosa* to change in N deposition load

6.1 Introduction

Phosphomonoesterase activity in *Cladonia portentosa* is significantly positively related to wet inorganic N deposition. Across Great Britain, rates of PME activity in this lichen vary between 1.2 ± 0.1 and 2.8 ± 0.2 mmol substrate hydrolysed g^{-1} dry mass h^{-1} (see Chapter 4, section 4.3). A broadly comparable increase in activity was recorded at Whim Moss where PME activity was up-regulated from 2.4 ± 0.3 to 4.1 ± 2.2 mmol substrate hydrolysed g^{-1} dry mass h^{-1} in response to N additions between 8 and 64 kg N ha^{-1} y^{-1} over a period of *c*. 3.5 years (Chapter 5). The consistency between up-regulated PME activity measured in *C. portentosa* under field and controlled experimental conditions provided evidence of causality between N enrichment and increased PME activity in this lichen. The objective of work reported in this chapter was to provide further information on potential rates of adjustment of PME activity in *C. portentosa* to change in N supply rate.

Studies on PME activity in *C. portentosa* reported in previous chapters were confined to the apical 10 mm of podetia. However, it is of interest to know whether or not N-induced change in PME activity occurs throughout a podetium or whether it is restricted to new growth that occurs post-exposure to a modified N deposition regime. Therefore, a reciprocal transplant experiment was undertaken in which *C. portentosa* was transplanted between sites receiving high or low

atmospheric N inputs. Phosphomonoesterase activity was measured in transplanted lichen following 6 and 12 months growth to investigate the rate at which enzyme activity could respond to change in the rate of N supply. In addition, the vertical profile of PME activity was measured to investigate which regions of the lichen are most responsive to changes in N availability.

6.2 Materials and Methods

6.2.1 Study sites

Field work was conducted at two heathland sites. These were selected due to contrasting rates of modelled wet inorganic N deposition ($\mathbf{N}_s$), and are relatively protected from disturbance by livestock and the general public. The site selected as representing 'background' N deposition was The Halsary, Caithness (ND 195 493) where annual N deposition (2002-2005) is measured at 2.32 kg N ha^{-1} y^{-1} (AEA Technology) (Hayman *et al.* 2004a; 2004b; 2005; 2007) and $\mathbf{N}_s$ is 4.2 kg N ha^{-1} y^{-1} (R. I. Smith, CEH Edinburgh, Pers. Comm). Vegetation at The Halsary is classified as a *Calluna vulgaris-Scirpus cespitosus* blanket mire (Rodwell 1991; Ellis *et al.* 2005) of which *C. portentosa* forms an abundant component. The N polluted site is located on the North Lees Estate, Derbyshire (SK 231 835) where $\mathbf{N}_s$ is 14.6 kg N ha^{-1} y^{-1} (R. I. Smith, CEH Edinburgh, Pers. Comm.). Here a well-developed but isolated population of *C. portentosa* exists amongst heathland vegetation dominated by large bushes of *C. vulgaris*.

6.2.2 Collection of *Cladonia portentosa*

In the field, single podetia (*n* = 30) were removed from naturally hydrated cushions of *C. portentosa* and cut to a uniform length of 50 mm (measured downwards from the apex). Each sample was collected from a cushion > 10 m apart from other replicates. Samples were transported in sealed plastic 100 ml vials, cleaned of any extraneous debris and left to dry and equilibrate with unheated laboratory air for 24 h at the Environmental Research Institute, Thurso, (material from The Halsary) or The University of Nottingham (North Lees Estate material). Powder-free latex gloves were worn at all times when handling lichen tissue.

6.2.3 Growth experiments

Air-dry thalli were weighed, and then tagged with a small acetate label attached by polyester thread. Tagged samples were returned to the field during the period 08 - 10.11.06. Samples were transported in sealed plastic 100 ml vials and placed in protective, cylindrical stainless steel mesh cages (*c.* 35 mm diameter x 85 mm tall; 0.4 mm gauge wire with 16 holes cm^{-2}) which were embedded vertically into undisturbed cushions of *C. portentosa* (Fig. 6.1). Approximately 3 - 6 cages were inserted into each lichen cushion, containing both transplanted and control podetia. The cages were open at the top and plugged at the bottom with *c.* 30 mm compacted lichen litter (collected from surrounding cushions), to provide a stable substratum permeable to rainwater and supporting experimental thalli with the

apices at the same height as those in the surrounding lichen cushion to minimise shading effects (Fig 6.1). Samples were transplanted from Caithness to Derbyshire (C→D) and Derbyshire to Caithness (D→C). For control purposes, thalli were also transplanted back into their native field site at both Caithness (C→C) and Derbyshire (D→D).

6.2.4 Harvests and measurements

A subsample of the C→D and D→D transplanted thalli (n = 3) were retrieved from the field site in Derbyshire after *c.* 6 months' growth (167 days on 24.04.07). The remaining thalli were collected after approximately 12 months' growth (349 days growth on 26.11.07); all samples in Caithness were collected on 22.11.07 (351 days growth). Recovery of samples was high with the loss of only one replicate from the C→D transplant. Integrity of samples was also high with evidence of livestock trampling over 2 sample cages and a further 2 instances of fallen leaves covering experimental thalli at the Derbyshire field site. At the Caithness site, an increase in the height of surrounding lichen cushions with respect to the experimental cages might have imposed a degree of shading on 5 of the samples.

Figure 6.1 Experimental thalli of *C. portentosa* cut to a uniform length of 50 mm and inserted into metal cages (a) with several cages positioned in an intact lichen cushion (b). Experimental thalli sit on a bed of lichen necromass, the depth of which is adjusted so that the apices are at the same height as those in the surrounding lichen cushion.

In addition to retrieving transplanted thalli, previously undisturbed samples of *C. portentosa* (1 or 2 interconnected podetia cut to a distance of 50 mm from the apices) were selected from intact lichen cushions at both field sites at the time of the final harvest.

At each harvest, the length of each podetium in its naturally hydrated state was measured in the field. Then, harvested lichen thalli were transported back to the laboratory in sealed 100 ml plastic vials, cleaned of extraneous debris and left to equilibrate with laboratory air for 24 h. Tags were carefully removed and air dry lichen mass recorded. Oven dry mass was estimated for each sample using the air dry: oven dry (80 ^{o}C, 24 h) mass ratio obtained for additional 'dummy' thalli (collected at the same time and cut to 50 mm in the field alongside experimental thalli). Air dry samples were stored in sealed vials at -15 ^{o}C until PME assays (< 4 weeks).

6.2.5 Phosphatase assay

Thalli were rehydrated overnight in water-saturated air (over water in a desiccator) at 10 ^{o}C, then sprayed lightly with deionised water and cleaned of any remaining debris. They were then dissected into horizontal strata by cutting with a razor blade at some or all of the following distances downwards from the apices: 5, 10, 15, 25, 35, 40, 50, 65, 70, 75, 80, 85, 90, 95 mm. Phosphomonoesterase analysis followed the *p*NPP method adapted from Turner (2001), as described in section 2.2.4.

6.2.6 Calculation of growth rate

Relative growth (RG, mg g^{-1}) and relative growth rate (RGR, mg g^{-1} y^{-1}) were calculated following Hunt (1982) (Eqns. 6.1 & 6.2).

$$RG = 1000\left(\frac{m_{tx+1} - m_{tx}}{m_{tx}}\right) \qquad \textbf{Equation 6.1}$$

$$RGR = 1000\left(\frac{\ln m_{tx+1} - \ln m_{tx}}{t_{x+1} - t_x}\right) \qquad \textbf{Equation 6.2}$$

where m is mass (g) and t is time (y)

6.2.7 Statistical analyses

All data were checked for normality, and where necessary homogeneity of variances. In cases where data were not normally distributed, a $\log_{10}$ transformation of the data was applied. If data met both of these assumptions, a *t*-test or a two-way ANOVA with LSD post-hoc tests was performed to identify significant differences between mean values. If data still remained discontinuous, a Scheirer-Ray-Hare (SRH) test was performed, the non-parametric equivalent of a two-way ANOVA. However, because it makes fewer assumptions about the data than the parametric ANOVA, the statistical power of this test is considered weaker and results must be interpreted with caution.

6.3 Results

6.3.1 Growth of reciprocal transplants

After 6 months growth in the field, *C. portentosa* transplanted from a low to high N environment (Caithness to Derbyshire) achieved a mean increase in thallus length of 4.33 ± 0.33 mm. This was significantly lower than the length increment of 10 mm (P = 0.003) for native D control podetia (Table 6.1). This increase in length was associated with an increase in mass of corresponding magnitude (Table 6.1).

Data collected at the 12 month harvest suggested that C→C and C→D transplants grew at similar rates according to all measurements made (length increment, % mass increment, RG and RGR; Table 6.2). D and D→C transplants also grew at similar rates (Table 6.2) and thus there appeared to be no effect of site on the growth rate of *C. portentosa*. However, consistent with the measurements made at 6 months, lichen from Derbyshire grew significantly faster than samples from Caithness, the difference ranging from 45 to 57 % depending on the measurement applied (Fig. 6.2, Table 6.2).

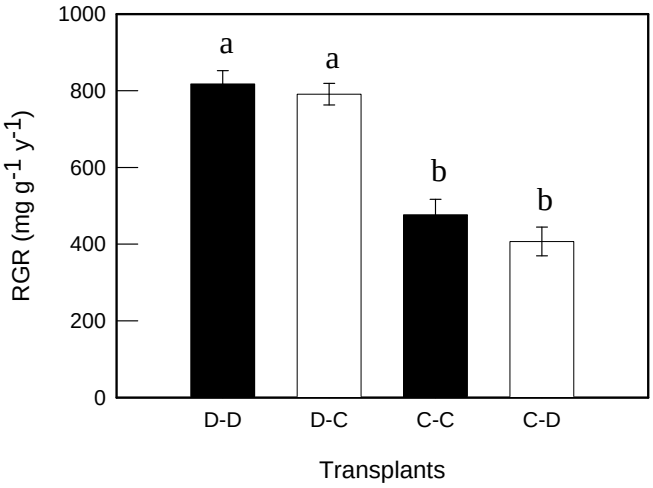


Figure 6.2 Relative growth rates of *C. portentosa* podetia: □, thalli transplanted between sites with contrasting wet inorganic N deposition (Derbyshire (D), 4.2 kg N ha^{-1} y^{-1} and Caithness (C), 14.6 kg N ha^{-1} y^{-1}; D→C and C→D); ■, native transplants, D→D and C→C. Plotted values are means (n = 11 - 15) ± 1 SEM. Columns illustrated by the same letter are not significantly different at the $P \leq 0.05$ level.

6.3.2 PME activity in reciprocal transplants

Phosphomonoesterase activity in *C. portentosa* was strongly affected by change in $\mathbf{N}_s$. At 6 months, PME activity was significantly higher in thalli transplanted from C→D compared with native D→D material (Fig. 6.4, Table 6.3). This difference was most apparent in the top 25 mm of thalli investigated (Fig. 6.4)

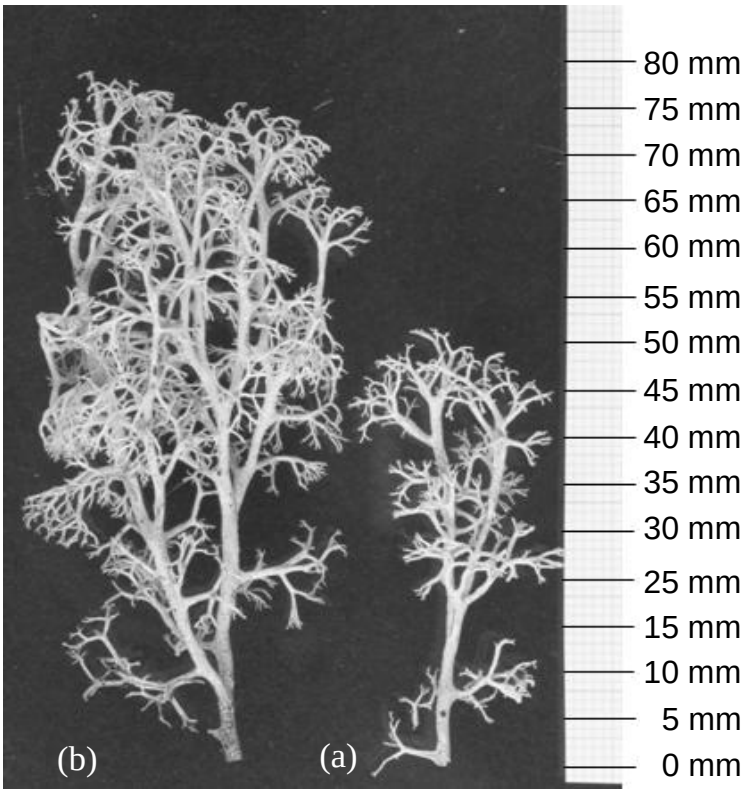


Figure 6.3 Podetia of *C. portentosa* selected from North Lee Estate, Derbyshire cut to a distance of 50 mm from the apices in its naturally hydrated state (a) and transplanted between D→D after 12 months growth in the field (b). (*nb*. Images were taken on different air dry thalli in the laboratory thus length is less than when naturally hydrated).

Table 6.1 Measurements made on transplanted podetia of *Cladonia portentosa* (initially cut to 50 mm) after 6 months growth in the field between Derbyshire to Derbyshire (D→D) and Caithness to Derbyshire (C→D). Data are given as means (n = 3) ± 1 SEM.

Transplant	Length increase (mm)	Start mass (mg)	End mass (mg)	Percentage mass increase (%)	RG (mg g^{-1})	RGR (mg g^{-1} y^{-1})
D→D	10.00 ± 0.00	84.43 ± 3.02	110.89 ± 5.27	31.26 ± 2.92	312.62 ± 29.23	543.05 ± 44.64
C→D	4.33 ± 0.33	93.90 ± 20.09	106.12 ± 19.37	14.63 ± 3.78	146.29 ± 37.84	270.87 ± 66.27

Table 6.2 Measurements made on transplanted podetia of *C. portentosa* (initially cut to 50 mm) after 12 months growth in the field between Derbyshire to Derbyshire (D→D), Caithness to Derbyshire (C→D), Caithness to Caithness (C→C) and Derbyshire to Caithness (D→C). Data are given as means (n = 11-15) ± 1 SEM.

Transplant	Length increase (mm)	Start mass (mg)	End mass (mg)	Percentage mass increase (%)	RG (mg g^{-1})	RGR (mg g^{-1} y^{-1})
D→D	26.33 ± 2.49	88.62 ± 6.14	202.31 ± 15.62	128.08 ± 8.11	1280.79 ± 81.13	817.83 ± 34.64
C→D	14.00 ± 1.95	92.15 ± 5.11	137.80 ± 6.75	51.24 ± 5.50	512.42 ± 54.98	406.87 ± 37.36
C→C	20.47 ± 2.18	90.15 ± 5.29	146.58 ± 9.74	62.88 ± 6.33	628.84 ± 63.35	476.85 ± 40.12
D→C	27.73 ± 2.35	109.75 ± 10.89	243.57 ± 24.74	121.79 ± 6.39	1217.94 ± 63.90	791.01 ± 27.93

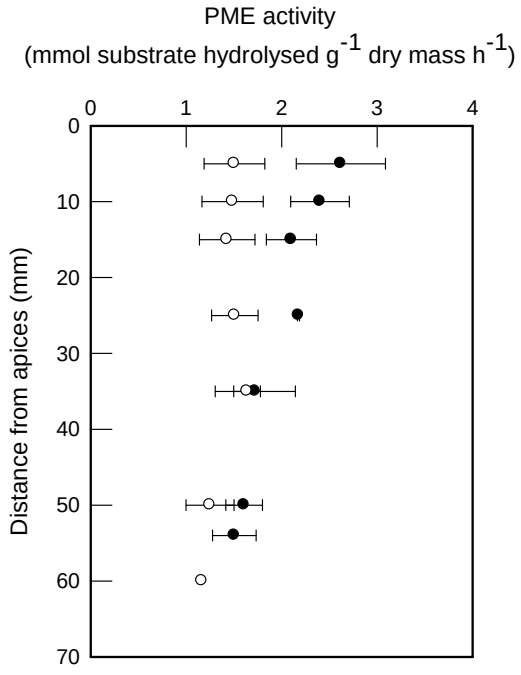


Figure 6.4 Vertical distribution of PME activity in *Cladonia portentosa* at 6 months after transplantation between C→D (●) and D→D (○). Assays were performed with 3 mM *p*NPP for 20 min at 15 ^{o}C in the dark. Plotted values are means (n = 3) ± 1 SEM. Each mean value is plotted at the lower extremity of the depth interval that they represent (e.g. 0 – 5 mm plotted at 5 mm from apices).

Table 6.3 Results of a two-way ANOVA comparing PME activity at different depths in *C. portentosa* and between the transplants C→D and D→D following 6 months growth in the field.

Source of variation	df	*F*-ratio	*P*-value
Between transplants	1	12.535	0.002
Between depths	6	1.315	0.287
Transplant x depth	6	0.725	0.633
Error	25		
Total	38		

At 12 months, PME activity was highly significantly influenced by transplantation (Fig. 6.5, Table 6.4). The vertical variation in PME activity in all treatments was broadly similar to that observed in previous investigations (e.g. Chapter 2, section 2.3), with maximum activity occurring in the top 5 – 10 mm and decreasing towards the base (Fig. 6.5). In thalli transplanted from D→C, PME activity decreased in all horizontal strata compared with D→D thalli (Fig. 6.5a, Table 6.4a). In contrast, in thalli transplanted from a low to a high $\mathbf{N}_s$ site (C→D), PME activity increased significantly in all strata between the apices and 50 mm depth compared to C→C controls (Fig 6.5b, Table 6.4b).

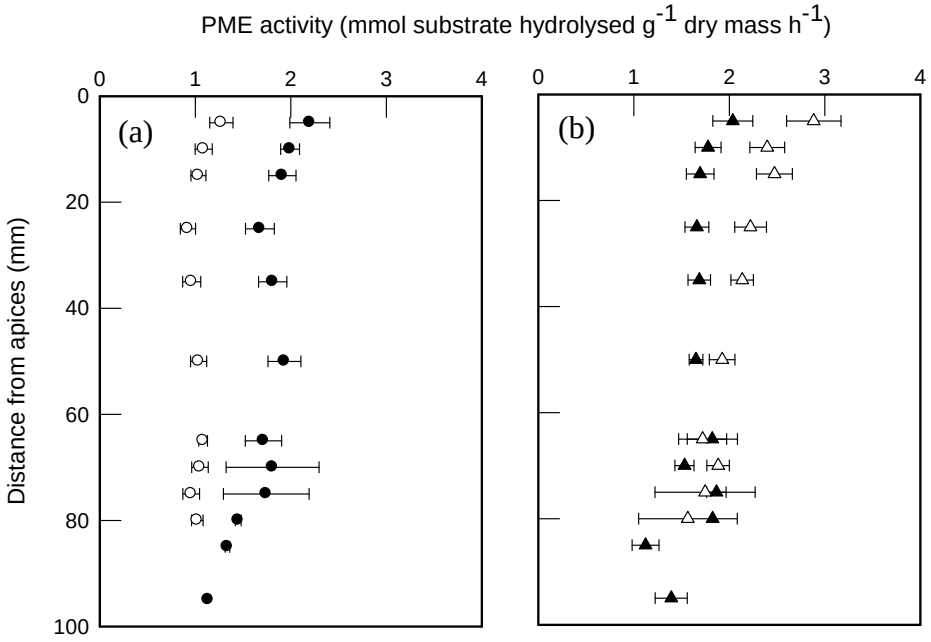


Figure 6.5 Vertical distribution of PME activity in *Cladonia portentosa* 12 months after transplantation between D→D (●) and D→C (○) (a), and C→C (▲) and C→D (Δ) (b). Assays were performed with 3 mM *p*NPP for 20 min at 15 ^{o}C in the dark. Plotted values are means (n = 11 - 15) ± 1 SEM. Each mean value is plotted at the lower extremity of the depth interval that they represent (e.g. 0 – 5 mm plotted at 5 mm from apices).

Table 6.4 Results of two-way ANOVA comparing the effect of transplantation between sites of contrasting N input on the rate and vertical variation in PME activity in *C. portentosa*. Transplants were undertaken between Derbyshire (D, $\mathbf{N}_s$ 14.6 kg N ha^{-1} y^{-1}) and Caithness (C, $\mathbf{N}_s$ 4.2 kg N ha^{-1} y^{-1}), with ANOVA comparing transplants between D→D and D→C (a) and C→C and C→D (b).

(a)

Source of variation	df	*F*-ratio	*P*-value
Between transplants	1	105.122	< 0.001
Between depths	12	1.435	0.153
Transplant x depth	8	0.521	0.839
Error	190		
Total	212		

(b)

Source of variation	df	*F*-ratio	*P*-value
Between transplants	1	6.481	0.012
Between depths	11	1.856	0.049
Transplant x depth	9	0.963	0.472
Error	165		
Total	187		

The lichen culture technique used had a detectable effect on the vertical distribution of PME activity in *C. portentosa* (Fig. 6.6a & b, Table 6.5). However, these differences in activity were small compared to the effects of a change in $\mathbf{N}_s$ brought about by transplantation (Fig. 6.5).

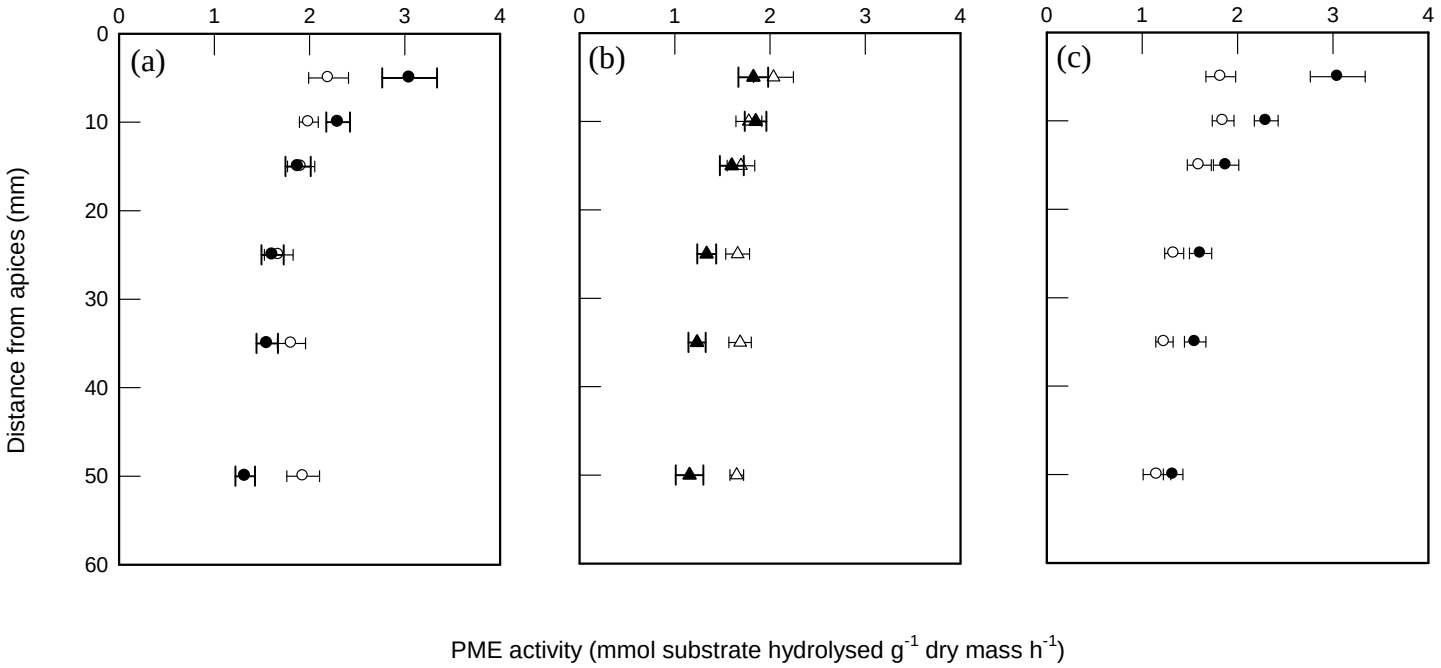


Figure 6.6 Vertical distribution of PME activity in undisturbed *C. portentosa* podetia in Derbyshire (●) and D→D transplants after 12 months growth (○, cf. Fig. 6.5a) (a); undisturbed lichen podetia in Caithness (▲) and C→C transplants after 12 months growth (Δ, cf. Fig. 6.5b) (b); undisturbed lichen podetia in Derbyshire (●) and Caithness (○) (c). Assays were performed with 3 mM *p*NPP for 20 min at 15 ^{o}C in the dark. Plotted values are means (n = 11 - 15) ± 1 SEM. Each mean value is plotted at the lower extremity of the depth interval that they represent (e.g. 0 – 5 mm plotted at 5 mm from apices).

Table 6.5 Results of a Scheirer-Ray-Hare test comparing PME activity at different depths in *Cladonia portentosa* and between PME activity in undisturbed lichen podetia in Derbyshire and D→D native transplants (a) and undisturbed lichen podetia in Caithness and C→C native transplants (b).

(a)

Source of variation	df	*F*-ratio	*P*-value
Between transplants	1	510.588	< 0.001
Between depths	11	5.091	0.990
Transplant x depth	5	1.660	0.990
Error	155		
Total	173		

(b)

Source of variation	df	*F*-ratio	*P*-value
Between transplants	1	347.037	< 0.001
Between depths	11	2.317	1.000
Transplant x depth	5	0.516	1.000
Error	152		
Total	170		

Rates of PME activity in undisturbed *C. portentosa* cushions were significantly higher in Derbyshire than in Caithness (Fig. 6.6c, Table 6.6). In thalli from Derbyshire, activity was markedly higher in the apices and progressively decreased towards the base, with rates approximately 2.3 times greater in the apices when compared to the 35 - 50 mm segment (Fig. 6.6c). Phosphomonoesterase activity in each segment of Caithness podetia was distinctly lower than that measured at the same depth in Derbyshire lichen.

Table 6.6 Results of a Scheirer-Ray-Hare test comparing PME activity at different depths in undisturbed podetia of *Cladonia portentosa* collected from two sites contrasting in $\mathbf{N}_{s,}$ North Lees Estate, Derbyshire (14.6 kg N ha^{-1} y^{-1}) and The Halsary, Caithness (4.2 kg N ha^{-1} y^{-1}).

Source of variation	df	*F*-ratio	*P*-value
Between locations	1	510.588	< 0.001
Between depths	11	5.091	0.990
Transplant x depth	5	1.660	0.990
Error	155		
Total	173		

6.4 Discussion

6.4.1 Growth of reciprocal transplants

Lichens are considered to have a relatively slow rate of growth, on average achieving a few mm new growth per year, dependent upon species type (Purvis 2000) and nutrient input regimes (Crittenden *et al.* 1994). Data presented in this chapter suggests that *C. portentosa* is a relatively productive lichen, with RGR ranging between 406.9 ± 37.4 and 817.8 ± 34.6 mg g^{-1} y^{-1}. These values compare favourably with RGRs in the range 260 - 431 mg g^{-1} y^{-1} measured in *C. portentosa* at various sites in Great Britain using a similar method (Hyvärinen and Crittenden 1998b; Ellis *et al.* 2005). In addition, the values are consistent with reported RGRs of other species of *Cladonia* (including *C. stellaris*, *C. rangiferina* and *C. mitis*) which range from 650 to 840 mg g^{-1} y^{-1} (Kärenlampi 1971; Crittenden 1983; Kytövita 1993). High rates of growth in mat-forming *Cladonia spp.* has been

attributed to a specialised mode of growth (Crittenden 1989; 1991; Ellis *et al.* 2005). *Cladonia portentosa* grows acropetally (from the apices vertically upwards), with older, basal material decaying to form a layer of necromass isolating the lichen from the underlying substratum (Crittenden 1991). Recently, it has been shown that the development of a deep layer of basal necromass provides a source of nutrients for *C. portentosa*, with N and P translocated vertically upwards to the growing apices (Hyvärinen and Crittenden 2000; Ellis *et al.* 2005). It is therefore possible that high rates of apical growth observed in *C. portentosa* are promoted by the remobilisation of nutrients from decaying basal tissue. This might be more prevalent under conditions of low nutrient inputs (e.g. Caithness, low $\mathbf{N}_s$), and could enable *C. portentosa* to sustain growth at a rate greater than that which could be supported solely by the income of atmospheric deposits (Ellis *et al.* 2005).

Cladonia portentosa originating from Caithness and Derbyshire had significantly different rates of growth. The relative growth rate of *C. portentosa* from Derbyshire was approximately twice that of material from Caithness and this difference was consistent between both control transplants (C→C, D→D) and between-site transplants (C→D, D→C). This suggests that differences in growth rates might be due to different growth capacities of the respective lichen populations and not due to the immediate operating environment at either Caithness or Derbyshire. There are two possible explanations for this difference. First, there is a strong positive correlation between $\mathbf{N}_s$ and thallus N concentration in the apices and base of *C. portentosa* (see Chapters 4 and 5, Hyvärinen and

Crittenden, 1998a). Therefore, it is possible that prior to transplantation, lichen growing at the North Lees Estate, Derbyshire contained a higher N content than lichen at Caithness. If so, this might support a high growth rate in Derbyshire lichen over a short (12 month) period of time (Ellis *et al.* 2005). Second, genetic variation might explain the observed differences in RGR, as the two populations are considerably spatially separated. Although at present there is insufficient data to confirm whether phenotypic or genotypic influences explain the observed differences in growth rate, further investigation could confirm the relative contribution of these processes. For example, measuring growth rates of mycobiont cultures isolated from different populations of *C. portentosa* might reveal genetic variation. However, the relevance of such data to the growth of intact lichen thalli under natural field conditions is uncertain. Alternatively, a similar reciprocal transplant experiment maintained over a longer time period (e.g. to measure annual growth) might reveal phenotypic differences since over a longer time interval growth rate modified by N deposition might be expected to change as $[N]_{lichen}$ increases or decreases.

6.4.2 PME activity in reciprocal transplants

Phosphomonoesterase activity in *C. portentosa* is highly responsive to changes in N supply. Six months exposure to an increased N load (C→D, 4.2 - 14.8 kg N ha^{-1} y^{-1}) was sufficient to significantly increase PME activity. After 12 months, the change in PME activity was even more pronounced, with activity up to 20 % higher in the majority of strata investigated. This contrasted markedly with *C.*

portentosa exposed to a reduced N load (D→C), where PME activity was significantly down-regulated throughout the entire thallus. The rapid response of PME activity to change in nutrient regime compares favourably with reports that N enrichment significantly influences PME activity in vascular plants over a very short time periods (Johnson *et al.* 1999; Phoenix *et al.* 2003). For example, Johnson *et al.* (1999) report a significant increase in the root-surface PME activity of *Plantago lanceolata* and *Agrostis capillaris* within 7 days growth of plants transplanted to N enriched soils (treated with either short term (18 months) or long term (7 years) additions of 14 g N m^{-2} y^{-1}). Furthermore, Phoenix *et al.* (2003) measured a significant increase in PME activity in the grasses *Carex flacca* and *Koeleria macrantha* within 14 days exposure to N enriched soil (supplied with 3.5 g N m^{-2} y^{-1} for 7 years).

In the current investigation, rapid up-regulation of PME activity in lichen transplanted from a low to high N environment might be explained simply by increased investment of cellular N into the synthesis of phosphatase enzymes (Treseder and Vitousek 2001). Hyvärinen and Crittenden (1998b) showed that thalli of *C. portentosa* treated in a similar experimental manner significantly increased $[N]_{lichen}$ within 1 year of transplantation from a low to high N environment. It has also been shown that thallus N:P mass ratio is significantly positively associated with N deposition (Chapters 4 & 5). Therefore it is possible that in extreme cases, *C. portentosa* experiences rapid P-limitation in response to a sudden increase in N availability. In response to P-deficiency, it has been shown previously (e.g. Chapters 4 & 5) that *C. portentosa* up-regulates PME activity as a

means of maximising P capture and redressing cellular N:P stoichiometry. *Cladonia portentosa* experiencing a decrease in N availability is likely to have a reduced P demand. Thus it might be expected that PME activity is down-regulated as *C. portentosa* re-directs resources into maximising N uptake. It is a further possibility that the faster rate of growth observed in samples transplanted from Derbyshire to Caithness might be sustained not just by a high $[N]_{lichen}$, but by the breakdown of redundant nutrient-rich surface-bound PME enzymes (Treseder and Vitousek 2001). Data from the current investigation suggests that the capacity to down-regulate PME activity was stronger than the capacity to increase activity in *C. portentosa* (Fig. 6.5, Table 6.4).

It was initially hypothesised that change in PME activity might be restricted to regions of new apical growth. However, results from the current investigation confirm that activity is modified throughout all regions of the thallus and that such change is relatively rapid, consistent with the proposal that PME activity in *C. portentosa* is dynamic. The apoplastic location for PME activity in this lichen has previously been discussed (see Chapter 3), and is considered to be the cell membrane and/or inner region of the cell wall. Turn-over and recycling of extracellular enzymes via endocytosis (Stenberg 2007) might be a mechanism by which rapid down-regulation of PME activity occurs. Data presented later in this thesis reveals a comparatively fast turn-over time for PME enzymes in the lichens *Lobaria linita, Stereocaulon alpinum* and *Peltigera aphthosa* (see Chapter 9). Storage of these lichens at -15 ^{o}C for 10 months resulted in a significant decrease in enzyme activity (e.g. from 720 to 370 mmol substrate hydrolysed g^{-1} dry mass

h^{-1} in L. *linita*). It is possible that a fast natural turn-over rate of PME enzymes might explain the rapid change in activity measured throughout all regions of fully differentiated *C. portentosa* thalli. Also consistent with this suggestion is the earlier hypothesis of Ellis *et al.* (2005) that the turnover of surface-bound enzymes (including phosphatases) might explain the loss of ^{15}N from ^{15}N-labelled thalli of *C. portentosa* over a 2 year period of growth in the field.

The present study demonstrates that a reciprocal transplant experiment can conveniently detect comparatively rapid lichen responses to variation in atmospheric chemistry. Nonetheless, it is worth considering the influences of potential confounding factors. These might include differences in site climatic conditions, the microclimate experienced by the lichen in mesh cages (Ellis *et al.* 2005) and the physical effect of transplantation (Ferry and Coppins 1979). The principal climatic differences between the two transplant sites which might potentially influence lichen physiology are probably temperature and rainfall (Mitchell *et al.* 2004; 2005). In 2007, mean annual temperature was 7.9 ± 1.2 ^{o}C in Caithness and 10.2 ± 1.3 ^{o}C in Derbyshire with corresponding rainfall depths of 600.4 mm and 903.2 mm, respectively (Lawrence *et al.* 2008; www.metoffice.gov.uk/climate/uk/2007/annual/averages1.html). However, despite previous reports of a correlation between rainfall and lichen growth rate (Kärenlampi 1971; Crittenden *et al.* 1994), results from the current investigation suggest that rainfall differences at the host site did not affect growth; instead this was most strongly influenced by the source population site.

This study represents the first reciprocal transplant investigating the physiological responses of growth and PME activity to variations in atmospheric N supply in a fruticose lichen species. The results show that a period of between 6 to 12 months is sufficient to detect changes in the PME response of *C. portentosa* to altered N supply, and that the change in enzyme activity is detectable at all depths of the thallus investigated. Thus it is suggested that PME activity in *C. portentosa* either native to a site or transplanted might serve as a sensitive, reliable and cheap bio-monitor for $\mathbf{N}_s$. However, further research is required in order to fully understand the role of phenotype and genotype expression on observed physiological responses to N pollution.

In the current investigation non-fertile thalli of *C. portentosa* were selected for analysis of growth responses. This is consistent with previous studies investigating the growth and physiology of this lichen (Hyvärinen and Crittenden 1996; 1998a; 1998b; 1998c; Ellis *et al.* 2003; 2005), which have not included fertile thalli. This is due to the uncertainty as to whether fertile and non-fertile thalli respond in a similar manner to experimental treatments applied. However, undertaking the current investigation provided an opportunity to examine whether fertile and non-fertile podetia of *C. portentosa* have similar growth rates. The results of this investigation can be found in Appendix 2.

CHAPTER 7. The effect of N enrichment on phosphate and nitrogen uptake efficiencies in *Cladonia portentosa*

7.1 Introduction

Under conditions of elevated wet N deposition, *C. portentosa* is associated with increased thallus N concentration, N:P mass ratio and PME activity (see Chapters 4 - 6). This combination of physiological changes suggests that *C. portentosa* experiences P-deficiency as a consequence of increased N supply. A strong inverse relationship between thallus P concentration ($[P]_{lichen}$) and PME activity has previously been discussed (Chapters 4 - 5), consistent with observations on vascular plants, bryophytes, fungi and algae (Dighton 1983; Press and Lee 1983; Antibus *et al.* 1986; Kroehler *et al.* 1988; Johnson *et al.* 1999; Turner *et al.* 2001; Hernández *et al.* 2002), and which can be attributed to repression of enzyme synthesis by elevated tissue P concentrations (McComb *et al.* 1979; Phoenix *et al.* 2003; Whitton *et al.* 2005).

A primary role of surface-bound phosphatase enzymes is the hydrolysis of organic P molecules; providing a means of releasing phosphate from otherwise unavailable sources (McComb *et al.* 1979; Jansson *et al.* 1988). This system for nutrient capture appears to be of great significance to *C. portentosa* in low-nutrient environments, as evidenced by rates of activity amongst the highest reported for any other plant/fungal or soil system in the literature (e.g. Appendix 1). The action of membrane- or wall-bound phosphatases results in release of

orthophosphate in the apoplast. Although there is evidence for fungi that enzymatically released PO_4^{3-} is absorbed via phosphate uptake transporters (Beever and Burns 1980), there is little evidence to suggest direct coupling between PME activity and phosphate uptake capacity (Kroehler and Linkins 1991; Muchhal *et al.* 1996; Phuyal *et al.* 2008).

It is therefore of interest to examine whether up-regulation of PME activity in *C. portentosa* under conditions of N enrichment (hence P-limitation) is also associated with increased PO_4^{3-} uptake capacity. It is also of interest to discover whether N uptake processes are additionally altered. Hence, the results presented in this chapter aim to address the following two hypotheses concerning nutrient uptake capacity in *C. portentosa*. First, N enrichment is associated with an increase in PO_4^{3-} uptake capacity. Second, N enrichment is associated with a decrease in N-NO_3^-, N-NH_4^+ and N-glycine uptake capacities. In addition, details regarding the uptake mechanisms employed by *C. portentosa* for the capture of PO_4^{3-} (representing all forms of inorganic P available for uptake) and N-NO_3^-, N-NH_4^+, and N-glycine are also reported.

7.2 Materials and Methods

7.2.1 Study sites

Cladonia portentosa was collected from four sites; The Migneint, north Wales; Wythburn Fells, Lake District; Alwen Reservoir, north Wales and Strabauchlinn Knowe; nr Edinburgh. The sites represent a gradient in wet inorganic N deposition with values of $\mathbf{N}_s$ ranging from 4.1 kg ha^{-1} y^{-1} (Strabauchlinn Knowe), to 15.8 kg ha^{-1} y^{-1} (Alwen Reservoir) and 32.8 kg ha^{-1} y^{-1} (Wythburn Fells), and were previously used as sample sites in an earlier survey (see Chapter 4). Material collected from The Migneint was used in preliminary assays. Details of site location, N deposition and concentration characteristics and collection dates are given in Table 7.1.

Table 7.1 Details of *Cladonia portentosa* collection sites for studies on N and phosphate uptake kinetics. Data for $\mathbf{N}_s$ and $[N]_{ppt}$ were provided by R. I. Smith (CEH, Edinburgh, Pers. Comm.)

Name of site	Grid reference	$\mathbf{N}_s$ (kg N ha^{-1} y^{-1})	$[N]_{ppt}$ (mg l^{-1})	Date of collection	Collectors:[†]
Migneint	SH 748 438	9.2	0.57	14 Feb. 2008	EJH, PDC
Wythburn Fells	NY 315 125	32.8	0.96	09 Mar. 2008	EJH, JW
Alwen Reservoir	SH 923 514	15.8	0.94	06 Mar. 2008	EJH, PDC
Strabauchlinn	NT 496 855	4.1	1.00	08 Mar. 2008	EJH, JW

[†] Names abbreviated as follows; EJH, E.J. Hogan; PDC, P.D Crittenden; JW, J. Whiteford

7.2.2 Collection and pre-treatment of *Cladonia portentosa*

At each site 5 - 10 replicate samples of *C. portentosa* were collected, each typically a clump of podetia 50 - 150 mm in diameter. Samples were collected and stored in seed-trays at 10 ^{o}C under a 12 h light (PPFR over the waveband 400 - 700 nm of 50 - 200 µmols m^{-2} s^{-1}) / 12 h dark cycle following the method outlined in section 2.2.2.

7.2.3 Sample preparation

Preparation of samples for phosphate uptake experiments followed section 2.2.3. For N uptake investigations, thalli were sprayed with deionised water and left to rehydrate at *c*. 18 ^{o}C for 24 h in a glass cabinet. The apical 10 mm of thalli were removed (by cutting with a razor blade) for phosphate uptake investigations, and to determine N ($[N]_{apex}$) and P ($[P]_{apex}$) content. The apical 15 mm were removed for N uptake experiments.

7.2.4 Phosphate uptake assays

7.2.4.1 Effect of pH on phosphate uptake

Replicate thalli were each added to a vial containing 19 ml assay medium comprising simulated rainwater and either 0.01 or 0.02 M citric acid, tri-sodium citrate buffer adjusted to one of the following pH values; 2.7, 2.9 (0.02 M), 3.3, 3.9, 4.3, 4.6, 5.2, 5.9 and 6.4 (0.01 M) following the method outlined in section 2.2.4.1. The vials were placed on ice and assays were initiated by the addition of 1

ml 100 µM KH_2PO_4 containing 0.074 MBq ^{33}P-H_3PO_4 and placing the vial in a shaking water-bath at 15 ^oC in the dark. After 20 min, assays were terminated by removing thalli from the solution, blotting with absorbent paper (to remove any remaining surface solution) and washing for 1 min in excess 100 µM KH_2PO_4. Thalli were transferred to 2 further solutions of 100 µM KH_2PO_4 where they were washed for 1 min each before being blotted dry, oven dried for 24 h at 80 ^oC and weighed. Samples were then digested in 1 ml H_2O_2 and H_2SO_4 (1:1.2 v/v) solution at 375 ^oC for 1 h following Allen (1989). The digest was diluted to 50 ml with deionised water and then 0.5 ml transferred to 5 ml Hionic Fluor liquid scintillation cocktail (Perkin Elmer, USA). Samples were analysed for ^{33}P content by liquid scintillation counting (TRI-CARB 2100TR Liquid Scintillation Analyzer, Packard Bioscience, USA). Rates of phosphate uptake from the assay medium were calculated using decay adjusted values for percentage uptake of ^{33}P.

7.2.4.2 Phosphate uptake kinetics

Each replicate lichen sample was added to 9.5 ml 0.02 M citric acid tri-sodium citrate buffered assay medium at pH 2.8 and stored on ice. Assays were initiated by the addition of 5 ml KH_2PO_4 to give final phosphate concentrations of 0.002, 0.004, 0.01, 0.02, 0.04, 0.5, 1.5, 2.5, 7.5 and 10 mM. The concentration range was chosen to cover the natural range available to *C. portentosa* in the lichen habitat and also to ensure saturation of the uptake system. The phosphate solutions contained ^{33}P-orthophosphate, the quantity of which was varied taking into

account the degree of interference with the final phosphate concentration, likely percentage uptake rates and scintillation counting detection limits (Table 7.2).

Samples were incubated in a shaking water-bath at 15 ^{o}C in the dark for 10 min following which thalli were analysed for phosphate uptake as described above (see section 7.2.4.1). An incubation period of 10 min was selected in order to measure rates of phosphate influx, to minimise the chance of nutrient efflux occurring, and was comparable with phosphate uptake work on other plant/fungal systems (Burns and Beever 1977; Pettersson and Jansson 1978; Straker and Mitchell 1987).

Table 7.2 Phosphate concentrations and ^{33}P content in assay media used in studies on phosphate uptake kinetics in *C. portentosa*.

Phosphate concentration (mM)	KBq ^{33}P
0.002	24.2
0.004	24.2
0.01	24.2
0.02	24.2
0.04	24.2
0.5	121.1
1.5	242.2
2.5	605.5
7.5	1210.9
10	1210.9

7.2.5 Nitrogen uptake kinetics

Nitrogen uptake investigations were carried out by Dr. W. Wanek at the University of Vienna, Austria. Short-term influx studies were undertaken by adding *c.* 100

mg (fresh weight) lichen sample to 20 ml ^{15}N labelled un-buffered solution of either ^{15}N-KNO_3, ^{15}N-$(NH_4)_2SO_4$ or ^{15}N-glycine (all 98 % ^{15}N). All 3 N solutions were dissolved in deionised water and amended with 0.1 mM $CaCl_2$ (giving an approximate pH of 4.5 - 5.5). Nitrogen uptake was investigated over the range of ^{15}N concentrations: 0.001, 0.004, 0.02, 0.05, 0.1, 0.25 and 0.5 mM, again selected to bracket the physiological range experienced by *C. portentosa* for each substrate in nature. Samples were incubated at a PPFR of 100 µmol m^{-2} s^{-1} for 10 min at 20 ± 1 ^{o}C following which termination was achieved by removing samples and rinsing once in tap water followed by three washes in 10 mM $CaSO_4$ for 1 min each. Samples were blotted dry and allowed to air dry following which they were weighed and homogenised in a ball mill (Retsch MM2, Haan, Germany); 1.5 mg was transferred to a tin capsule for ^{15}N analysis by isotope radio mass spectrometry (IRMS) (DeltaPLUS, Finnigan MAT, Bremen, Germany).

Net ^{15}N uptake was also measured by incubating lichen samples in 0.02 and 0.25 mM substrate for 60 min, during which time ^{15}N solutions were exchanged after each 15 min to hinder depletion of N in the tracer solution (Wanek and Pörtl 2008). Data presented in this chapter are those from influx studies and data on net uptake (accounting for efflux) are not shown.

7.2.6 Analysis of kinetic parameters

The Michaelis-Menten equation (Eq. 2.1) characterises steady-state enzyme catalysed reactions (Cornish-Bowden 1995) and is represented graphically by a

rectangular hyperbolic curve. The equation consists of two kinetic constants, K_m (the Michaelis-Menten constant, which defines the substrate concentration at half the maximum rate of reaction) and V_{max}, the maximum rate of reaction at substrate saturation (Wanek and Pörtl 2008). Although the hyperbolic curve is described by the Michaelis-Menten equation, estimating the values of K_m and V_{max} from such a plot is considered inaccurate (Wanek and Pörtl 2008). Accordingly, re-arrangement of the Michaelis-Menten equation to formulate straight-line graphs is deemed the most accurate way of determining values for the kinetic constants. Three linear transformations of the Michaelis-Menten equation are most commonly applied: Lineweaver-Burke (Eq. 2.2), Eadie-Hofstee (Eq. 2.3) and Hanes-Woolf (Eq. 2.4). As outlined in Chapter 2, (section 2.2.4.6), each of the three linear transformations applies a different weighting to various sources of experimental error and as such give different estimates for K_m and V_{max}. The Hanes-Woolf transformation is considered to give the most accurate values for K_m and V_{max} because a plot of [S] against [S]/V avoids influencing the independent y-axis data ([S]) by any errors introduced in measuring uptake rates (Wanek and Pörtl 2008). The ratio between values of the two kinetic constants (UE; V_{max}/K_m) provides a measure of uptake efficiency.

7.2.7 Determination of total N and P concentration

Approximately 30 mg of oven dried (80 ^{o}C) lichen sample was digested in sulphuric acid-hydrogen peroxide following Allen (1989) (for details see section 4.2.5). Phosphorus was then assayed using the malachite green variant of the

methylene blue method after Van Veldhoven and Mannaerts (1987) using a Pye Unicam SP6-350 visible spectrophotometer (see section 4.2.5). For N analysis, samples were oven dried at 80 ^{o}C for 24 h and homogenised in a ball mill (Retsch MM2, Haan, Germany). Aliquots of 1 – 2 mg were weighed into tin capsules and analysed by continuous flow isotope ratio mass spectroscopy (IRMS) (DeltaPLUS, Finnigan MAT, Bremen, Germany) attached to an elemental analyser (Flash EA 1112, CE Instruments, Milan, Italy) by a ConFlo III interface (Finnigan MAT).

7.2.8 Statistical analyses

All data were subject to normality tests and checks to determine homogeneity of variances. Where data violated one or both of these assumptions, $\log_{10}$ or square root transformations were performed and when data met both assumptions, they were subjected to either one-way or two-way ANOVA, linear regression analysis, correlation or paired sample comparisons. Where ANOVA revealed significant differences, post-hoc tests were carried out using Fishers LSD tests. If data could not be transformed to fit a normal distribution, non-parametric Kruskal-Wallis tests were performed. All statistical analyses were undertaken using SPSS version 12.0.1 (SPSS, Inc., Chicago, IL). Deriving the kinetic constants K_m and V_{max} by fitting uptake data to the Michaelis-Menten equation was achieved with regression analysis (hyperbola, single rectangular, two parameters) using SigmaPlot 8.0 (SPSS, Inc., Chicago, IL).

7.3 Results

7.3.1 Relationships between $\mathbf{N}_s$ and lichen chemistry

Thallus N concentration in the apical 10 mm of *C. portentosa* was weakly positively related to $\mathbf{N}_s$ (Fig. 7.1a). In contrast, $[P]_{apex}$ was highly negatively related to $\mathbf{N}_s$, decreasing from 0.58 to 0.42 mg g^{-1} at sites receiving 4.1 and 32.8 kg N ha^{-1} y^{-1}, respectively (Fig. 7.1b). Thallus N:P mass ratio was positively related to $\mathbf{N}_s$, increasing by a factor of *c*. 2 between sites (Fig. 7.1c).

7.3.2 Effect of pH on phosphate uptake

Phosphate uptake in the apical 10 mm of *C. portentosa* increased significantly in response to decreasing pH ($P < 0.001$), with maximum uptake rates occurring at pH 2.9 (Fig. 7.2). Reducing the pH from 6.4 to 2.9 increased phosphate uptake by a factor of *c*. 4.

7.3.2 Relationships between $\mathbf{N}_s$ and phosphate uptake rate

Phosphate uptake by *C. portentosa* followed Michaelis-Menten kinetics as evidenced by hyperbolic substrate saturation curves (Fig. 7.3). ANOVA performed on the complete data set revealed that phosphate uptake was significantly influenced by both $\mathbf{N}_s$ and thallus N:P mass ratio (Table 7.3). The highest rates of PO_4^{3-} uptake were recorded in thalli exposed to the greatest N income and corresponding high N:P mass ratio (Fig. 7.3).

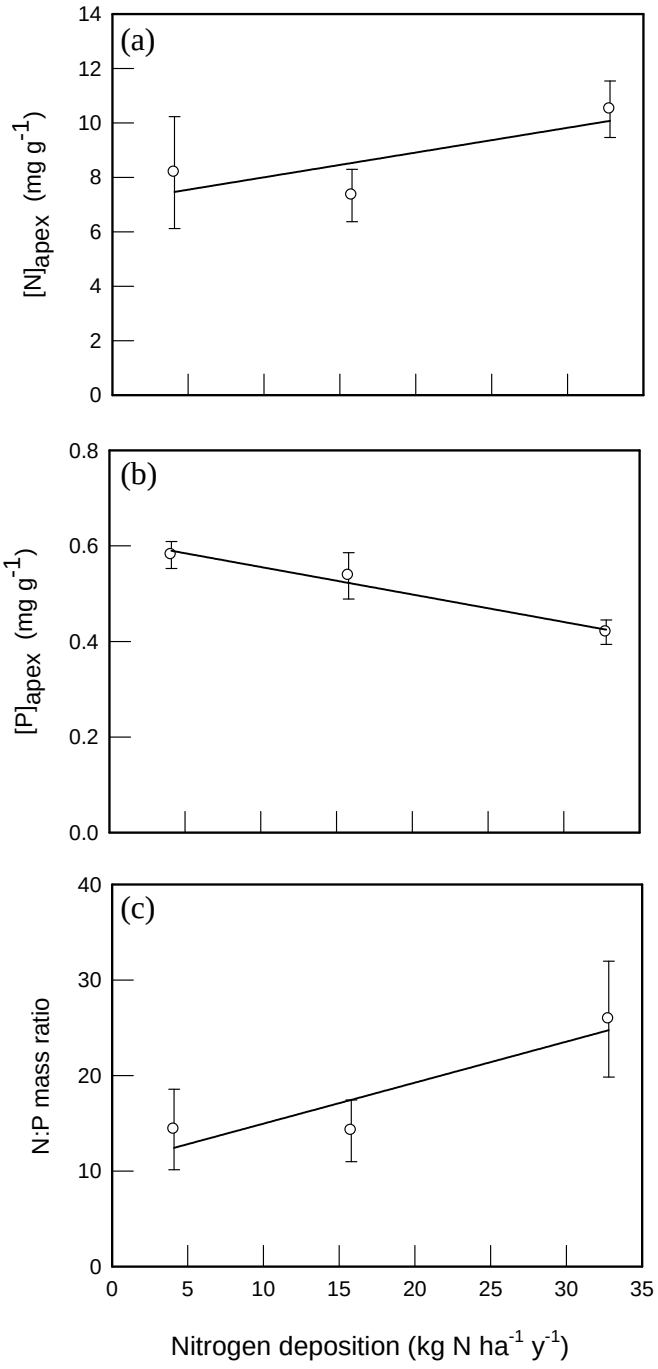


Figure 7.1 Relationship between wet inorganic N deposition and $[N]_{apex}$ (a, r^2 = 0.228, P = 0.683); $[P]_{apex}$ (b, r^2 = 0.977, P = 0.098) and thallus N:P mass ratio (c, r^2 = 0.742, P = 0.340) in *Cladonia portentosa*. Plotted values are means (n = 7 - 8) ± 1 SEM.

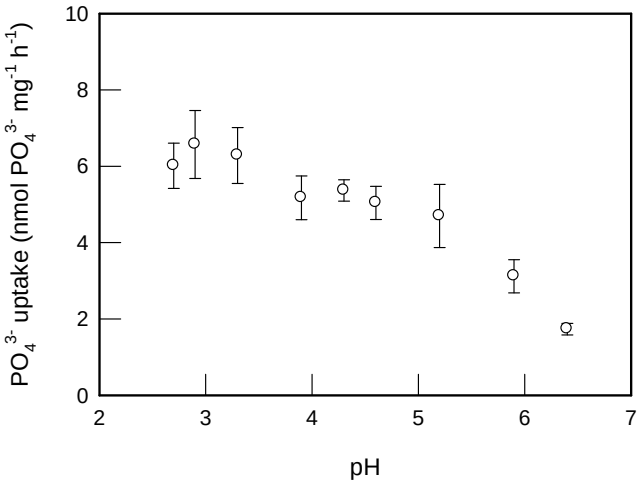


Figure 7.2 The effect of pH on phosphate uptake in 10 mm apical segments of *Cladonia portentosa*. Assays were performed with 74 kBq ^{33}P-H_3PO_4 in 20 ml 100 µmol l^{-1} KH_2PO_4 for 20 min at 15 ^{o}C in the dark. Material collected from the Migneint, North Wales. Plotted values are means (n = 5) ± 1 SEM.

Table 7.3 Results of two-way ANOVA to compare phosphate uptake rate in *Cladonia portentosa* between sites with different $\mathbf{N}_s$ (4.1 – 32.8 kg ha^{-1} y^{-1} and corresponding thallus N:P mass ratios, 14.34 – 25.89), and between different PO_4^{3-} concentrations (0.002 – 10 mM)

Source of variation	df	*F*-ratio	*P*-value	LSD Post Hoc $\mathbf{N}_s$ (kg N ha^{-1} y^{-1})	*P*-value
Between PO_4^{3-} concentrations	9	673.830	<0.001		
Between sites (or N:P mass ratio)	2	6.867	0.002	4.1 – 15.8	0.968
				4.1 – 32.8	0.002
				15.8 – 32.8	0.002
PO_4^{3-} concentration x $\mathbf{N}_s$	18	0.665	0.839		
Error	120				
Total	150				

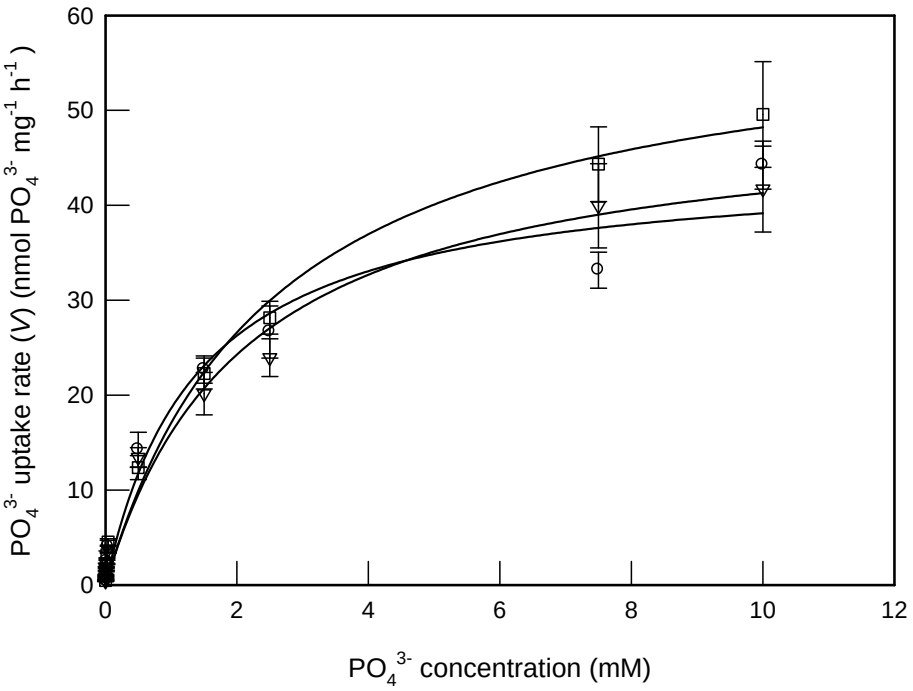


Figure 7.3 Effect of phosphate concentration on PO_4^{3-} uptake rate (*V*) in *Cladonia portentosa* collected from sites differing in $\mathbf{N}_s$; 4.1 kg ha^{-1} y^{-1} (○); 15.8 kg ha^{-1} y^{-1} (▽); 32.8 kg ha^{-1} y^{-1} (□). Assays performed for 10 min at 15 ^oC in the dark using 24.2 – 1211 kBq ^{33}P in 20 ml assay medium containing PO_4^{3-} concentrations in the range 0.002 – 10 mM KH_2PO_4 (See Table 7.2). Plotted values are means (*n* = 5) ± 1 SEM.

Linear transformation of data fitted to the Michaelis-Menten equation revealed the presence of two distinct PO_4^{3-} uptake systems at low (0.002 - 0.04 mM, System I, SI) and high (0.5 - 10 mM, System II, SII) substrate concentrations (Fig. 7.4). This was evidenced by a point of flexion in each linear transformation graph highlighting a change in the rate of PO_4^{3-} uptake (Figs. 7.4 & 7.6). By separating the data into uptake by either system, separate Michaelis-Menten saturation curves were plotted (Fig. 7.5), with ANOVA revealing the positive relationship between $\mathbf{N}_s$ (and corresponding thallus N:P mass ratio) and phosphate uptake was due to significant differences in System I uptake (0.002 – 0.04 mM [PO_4^{3-}]) (Table 7.4).

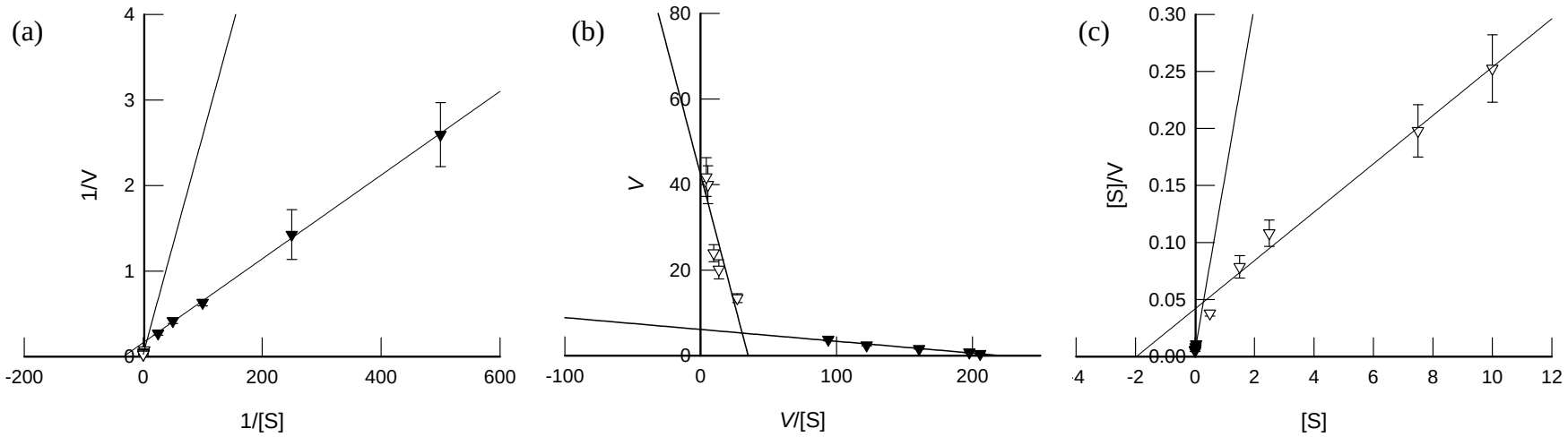


Figure 7.4 Linear transformations of the Michaelis-Menten equation representing phosphate uptake in *Cladonia portentosa*: Lineweaver Burke (a); Eadie-Hofstee (b); Hanes-Woolf (c), evidencing two systems for PO_4^{3-} uptake over low (0.002 – 0.04 mM, System I, closed symbols) and high (0.5 – 10 mM, System II, open symbols) substrate concentration ranges. *Cladonia portentosa* was collected from Alwen Reservoir, $\mathbf{N}_s$ 15.8 kg N ha^{-1} y^{-1}. Assays performed at 15 ^oC for 10 min in the dark. Plotted values are means (n = 5) ± 1 SEM.

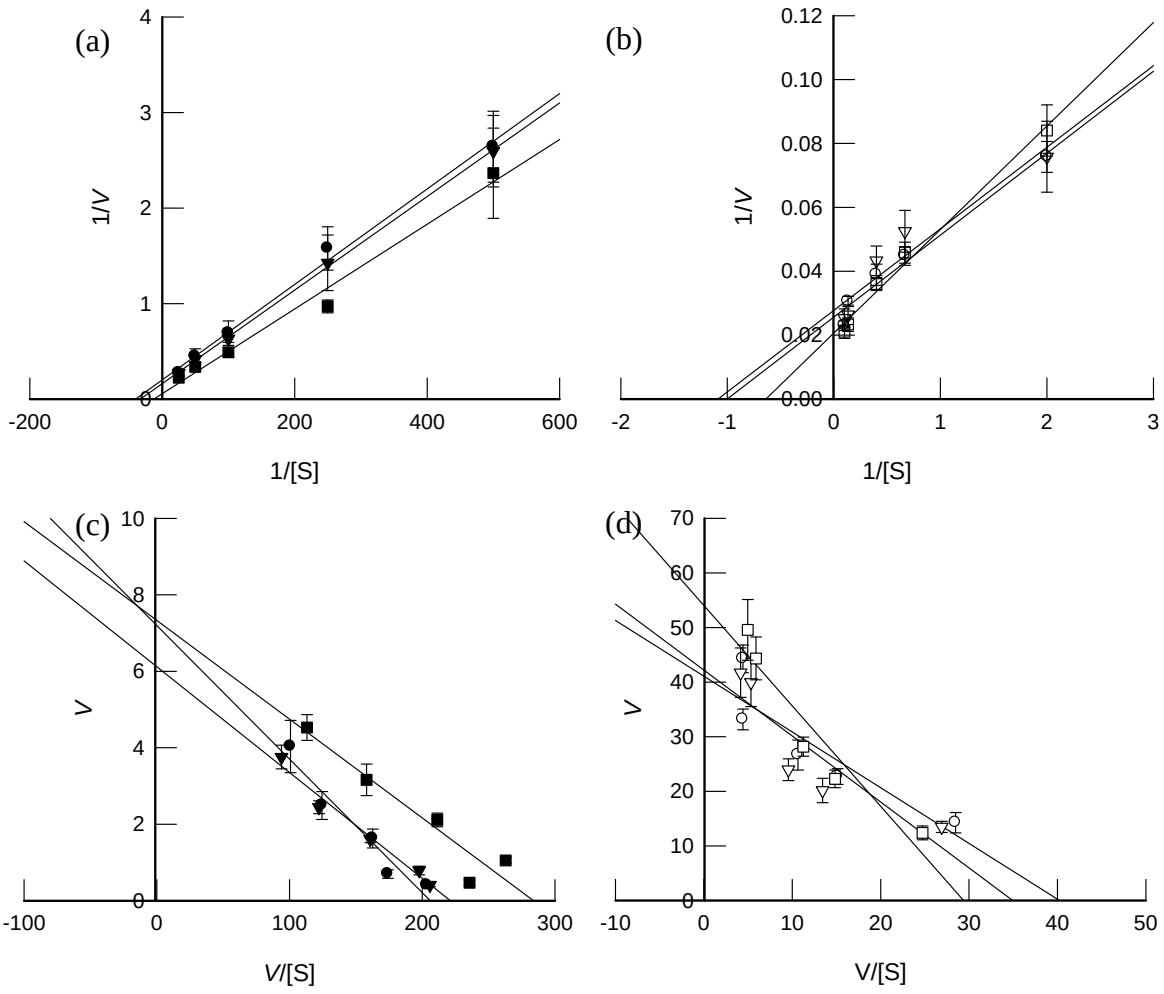


Figure 7.5 Linear transformations of the Michaelis-Menten equation representing phosphate uptake in *Cladonia portentosa*: Lineweaver-Burke (a & b); Eadie-Hofstee (c & d); Hanes-Woolf (e & f). Data illustrate System I (substrate concentration range 0.002 – 0.04 mM; closed symbols) and System II (substrate concentration range 0.5 – 10 mM; open symbols) PO_4^{3-} uptake systems in *C. portentosa* collected from sites receiving 4.1 kg N ha^{-1} y^{-1} (circles), 15.8 kg N ha^{-1} y^{-1} (triangles) and 32.8 kg N ha^{-1} y^{-1} (squares). Assays performed at 15 ^{o}C for 10 min in the dark. Plotted values are means (n = 5) ± 1 SEM.

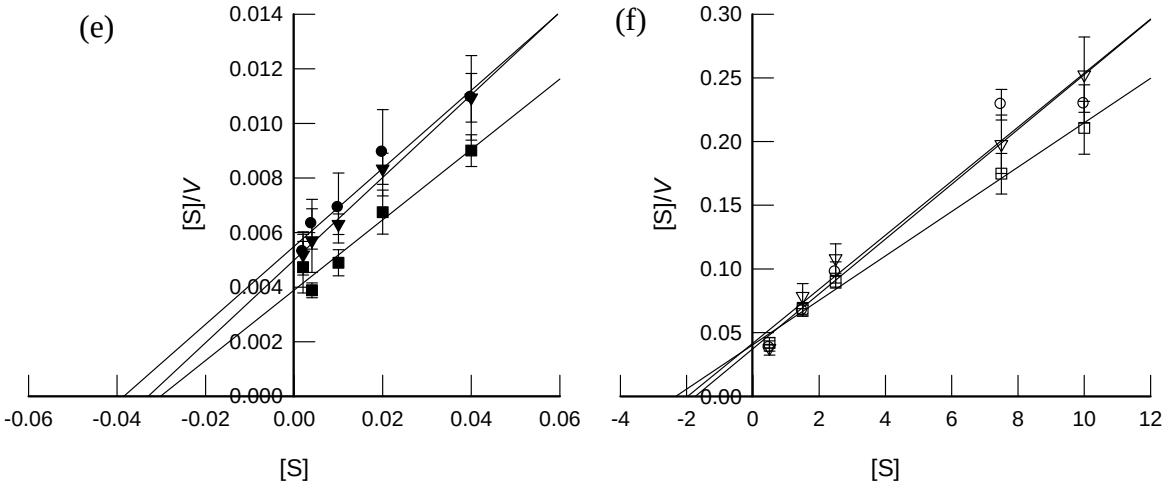


Figure 7.5 continued.

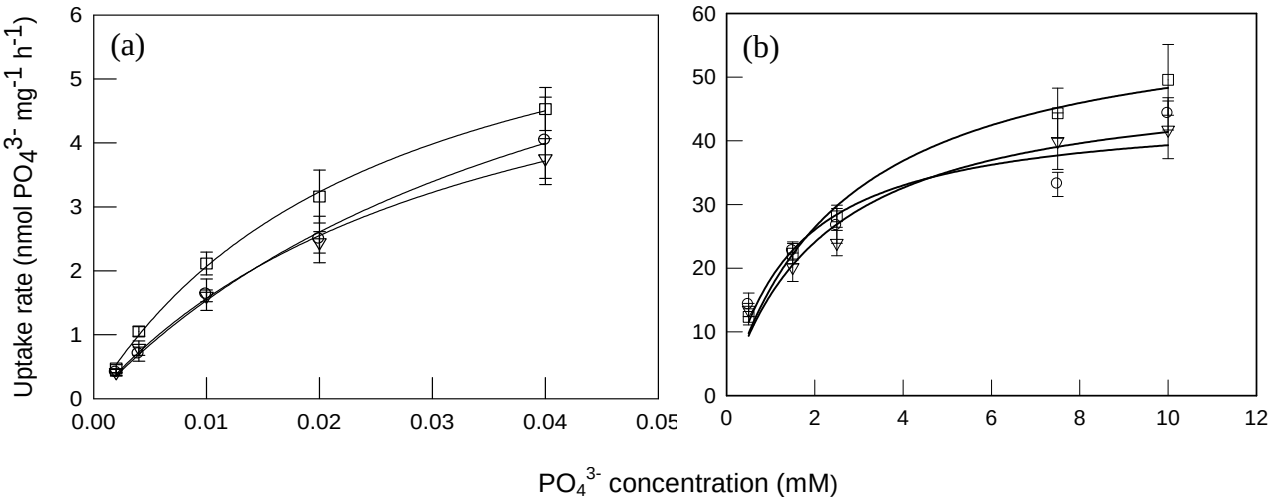


Figure 7.6 Effect of phosphate concentration on the rate of PO_4^{3-} uptake in *Cladonia portentosa* collected from sites differing in $\mathbf{N}_s$: 4.1 kg ha^{-1} y^{-1} (○); 15.8 kg ha^{-1} y^{-1} (▽); 32.8 kg ha^{-1} y^{-1} (□), over the range of concentrations 0.002 – 0.04 mM PO_4^{3-} (a) and 0.5 – 10 mM PO_4^{3-} (b). Significance levels are given in table 7.4. Assays performed for 10 min at 15 ^{o}C in the dark using 24.2 – 1211 kBq ^{33}P in 20 ml assay medium containing PO_4^{3-} concentrations in the range 0.002 – 10 mM KH_2PO_4 (See Table 7.2). Plotted values are means (n = 5) ± 1 SEM.

Table 7.4 Results of two-way ANOVA to compare phosphate uptake rates in *Cladonia portentosa* between sites with different $\mathbf{N}_s$: (4.1, 15.8 and 32.8 kg ha^{-1} y^{-1} and corresponding thallus N:P mass ratios) and between different PO_4^{3-} concentrations: 0.002 – 0.04 mM PO_4^{3-} (a, System I uptake); 0.5 – 10 mM PO_4^{3-} (b, System II uptake).

(a) System I				LSD Post Hoc	
Source of variation	df	*F*-ratio	*P*-value	$\mathbf{N}_s$ (kg N ha^{-1} y^{-1})	*P*-value
Between PO_4^{3-} concentrations	4	152.065	< 0.001		
Between sites (or N:P mass ratios)	2	6.103	0.004	4.1 – 15.8	0.691
				4.1 – 32.8	0.002
				15.8 – 32.8	0.007
PO_4^{3-} concentration x $\mathbf{N}_s$	8	0.256	0.977		
Error	60				
Total	75				

(b) System II				LSD Post Hoc	
Source of variation	df	*F*-ratio	*P*-value	$\mathbf{N}_s$ (kg N ha^{-1} y^{-1})	*P*-value
Between PO_4^{3-} concentrations	4	81.224	< 0.001		
Between sites (or N:P mass ratios)	2	1.363	0.264	4.1 – 15.8	0.540
				4.1 – 32.8	0.313
				15.8 – 32.8	0.107
PO_4^{3-} concentration x $\mathbf{N}_s$	8	0.937	0.493		
Error	60				
Total	75				

The kinetic constants K_m and V_{max} were calculated for *C. portentosa* collected from each of the three sites and for both uptake systems by curvilinear fitting of uptake data to the Michaelis-Menten equation and Lineweaver-Burke, Hanes-Woolf and Eadie-Hofstee transformations (Table 7.5).

Table 7.5 Values of the kinetic parameters K_m and V_{max} derived from high affinity (System I) and low affinity (System II) PO_4^{3-} transport systems in *Cladonia portentosa*. *Cladonia portentosa* was collected from sites differing in $\mathbf{N}_s$ (4.1, 15.8 and 32.8 kg ha^{-1} y^{-1}). Kinetic parameters were determined following fitting of data to the hyperbolic Michaelis-Menten equation or after Lineweaver-Burke, Eadie-Hofstee and Hanes-Woolf transformations. The units of K_m and V_{max} are mM and nmol PO_4^{3-} mg^{-1} dry mass h^{-1}, respectively. r^2 values are provided to demonstrate the goodness of fit of regression models. Values are means (n = 4) ± 1 SEM

		Michaelis-Menten			Lineweaver-Burk			Eadie-Hofstee			Hanes-Woolf			Mean ± 1 SEM	
Study site and $\mathbf{N}_s$ (kg N ha^{-1} y^{-1})	**System** and $[PO_4^{3-}]$ conc. range (mM)	K_m	V_{max}	r^2	K_m	V_{max}	r^2	K_m	V_{max}	r^2	K_m	V_{max}	r^2	K_m	V_{max}
Strabauchlinn	**SI** 0.002 – 0.04	0.05	8.55	0.76	0.03	5.97	0.99	0.04	7.20	0.94	0.04	7.00	0.97	0.04 ± 0.004	7.18 ± 0.53
Knowe (4.1)	**SII** 0.5 – 10	1.46	45.01	0.75	1.00	46.73	0.97	1.02	41.08	0.81	1.74	46.51	0.95	1.31 ± 0.18	44.83 ± 1.31
Alwen Reservoir	**SI** 0.002 – 0.04	0.03	6.85	0.92	0.03	7.38	0.99	0.03	6.12	0.98	0.03	6.61	0.99	0.03 ± 0.001	6.74 ± 0.26
(15.8)	**SII** 0.5 – 10	2.18	50.42	0.72	0.92	43.10	0.92	1.20	42.19	0.79	1.97	47.17	0.99	1.57 ± 0.30	45.72 ± 1.90
Wythburn Fells	**SI** 0.002 – 0.04	0.03	7.41	0.89	0.08	20.96	0.98	0.03	7.33	0.91	0.03	7.76	0.95	0.04 ± 0.01	10.87 ± 3.37
(32.8)	**SII** 0.5 – 10	2.62	60.96	0.51	1.57	58.14	0.99	1.84	54.05	0.91	2.33	57.47	0.99	2.09 ± 0.24	57.65 ± 1.42

Mean values of K_m for System I PO_4^{3-} uptake were up to 50 times lower than values of K_m for System II uptake (Table 7.5), evidencing higher affinity uptake. There was a weak positive relationship between $\mathbf{N}_s$ and values of System I K_m, whilst the relationship with System II K_m values was much stronger (Fig. 7.7a). Values of System I V_{max} were *c*. 5 times smaller than for System II (Table 7.5), with positive relationships between V_{max} and $\mathbf{N}_s$ observed for both uptake systems (Fig. 7.7b)

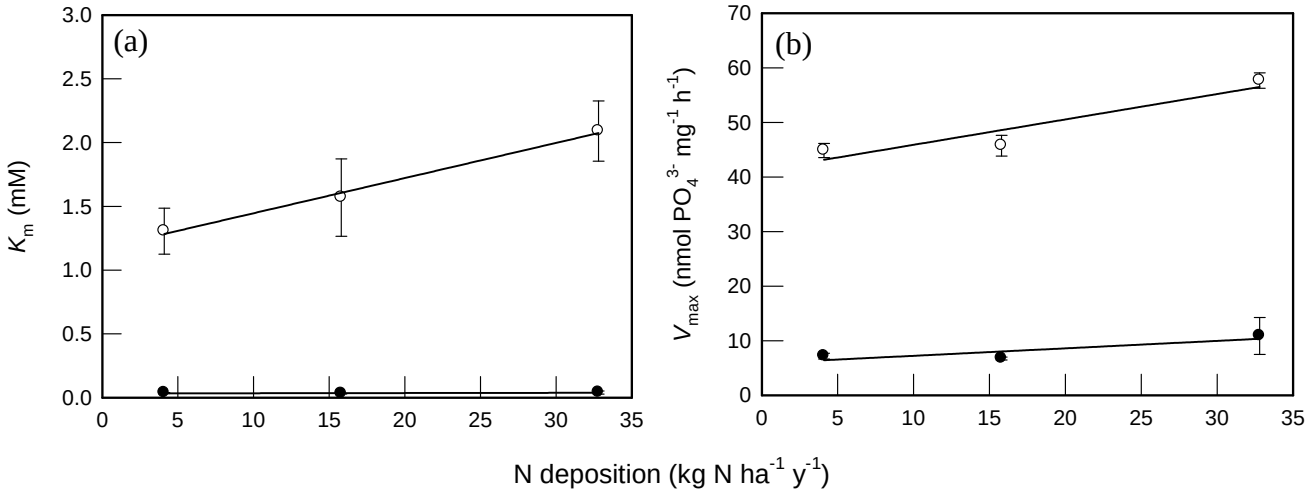


Figure 7.7 Relationship between wet inorganic N deposition and values of K_m (a) or V_{max} (b) for high affinity (●, System I) and low affinity (○, System II) PO_4^{3-} uptake in *Cladonia portentosa*. (a) ●, r^2 = 0.271, P = 0.651; ○, r^2 = 0.993, P = 0.052 (b) ●, r^2 = 0.758, P = 0.328; ○, r^2 = 0.879, P = 0.226. Mean values are calculated from four models with $\mathbf{N}_s$ data provided from an interpolative model (R. I. Smith, CEH Edinburgh, Pers. Comm.). Plotted values are means (n = 4) ± 1 SEM.

Phosphate uptake efficiency (UE; V_{max}/K_m) for System I was significantly higher than UE for System II at all 3 sites investigated ($P < 0.001$) (Fig. 7.8a). There was a positive relationship between high affinity System I UE and $\mathbf{N}_s$, whilst for low affinity UE, this relationship was weakly negative (Fig. 7.8a, Table 7.6). The ratio between UE_{SI} : UE_{SII} was positively related to $\mathbf{N}_s$, increasing by *c*. 40 % in line with increasing $\mathbf{N}_s$ from 4.1 to 32.8 kg N ha^{-1} y^{-1} (Fig. 7.8b). A plot of V_{max} against K_m (values calculated from both uptake systems) revealed strong positive co-variation between the two parameters, and provided further evidence of increased UE for System I high affinity uptake (i.e. steeper gradient) (Fig. 7.9).

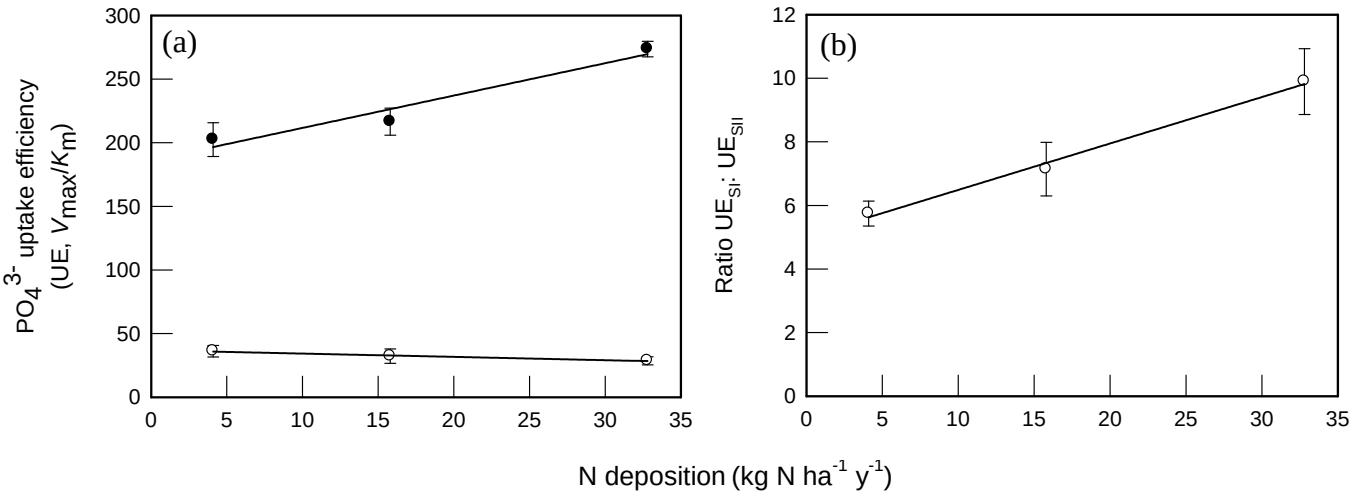


Figure 7.8 Relationship between wet inorganic N deposition and PO_4^{3-} uptake efficiency (UE, V_{max}/K_m) for high affinity (●, System I, $r^2 = 0.948$, $P = 0.146$) and low affinity (System II,○, $r^2 = 0.986$, $P = 0.077$) PO_4^{3-} transport systems in *Cladonia portentosa* (a), and the ratio between UE for both systems (UE_{SI} : UE_{SII}, $r^2 = 0.994$, $P = 0.051$) (b). Uptake efficiencies are calculated from mean K_m and V_{max} values derived from four models ($n = 4$) with inorganic N deposition data provided from an interpolative model (R. I. Smith, Pers. Comm.). Plotted values are means ($n = 3$ - 4) ± 1 SEM.

Table 7.6 Phosphate uptake efficiencies (UE, V_{max}/K_m) calculated for high affinity (System I) and low affinity (System II) PO_4^{3-} transporter systems in *Cladonia portentosa*. *Cladonia portentosa* was collected from sites with contrasting $\mathbf{N}_s$ (4.1, 15.8 and 32.8 kg ha^{-1} y^{-1}). Parameters were determined following fitting of data to the hyperbolic Michaelis-Menten equation or following Lineweaver-Burke, Eadie-Hofstee and Hanes-Woolf transformations Mean values are given (n = 4) ± 1 SEM.

Study site $\mathbf{N}_s$ (kg N ha^{-1} y^{-1})	**System**, $[PO_4^{3-}]$ (mM)	Michaelis-Menten	Lineweaver-Burk	Eadie-Hofstee	Hanes-Woolf	Mean ± 1 SEM
Strabauchlinn	**SI** 0.002 – 0.04	185.80	238.68	205.73	179.56	202.44 ± 13.31
(4.1)	**SII** 0.5 – 10	30.92	46.73	40.23	26.67	36.14 ± 4.53
Alwen Reservoir	**SI** 0.002 – 0.04	201.50	246.00	218.54	200.15	216.55 ± 10.67
(15.8)	**SII** 0.5 – 10	23.08	46.95	35.13	23.92	32.27 ± 5.61
Wythburn Fells	**SI** 0.002 – 0.04	285.00	268.77	282.00	258.60	273.59 ± 6.12
(32.8)	**SII** 0.5 – 10	23.30	37.03	29.39	24.63	28.59 ± 3.10

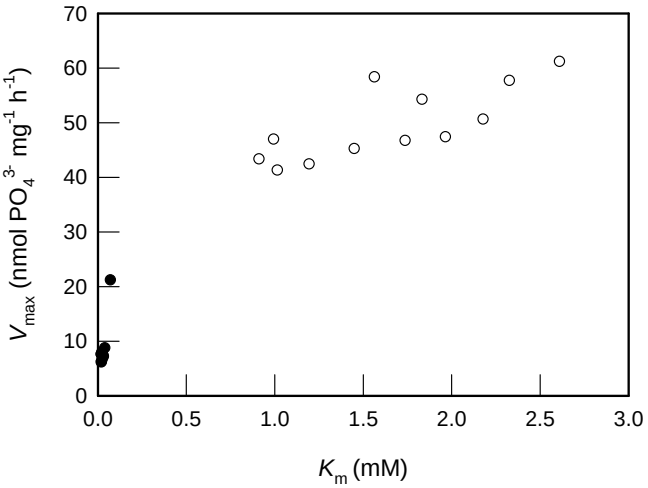


Figure 7.9 Relationship between maximum PO_4^{3-} uptake rates (V_{max}) and corresponding K_m values (substrate concentration at half V_{max}) for high affinity (●, System I, r = 0.442, P = 0.150) and low affinity (○, System II, r = 0.776, P = 0.003) PO_4^{3-} transporter systems in *Cladonia portentosa*. Kinetic parameters were determined following fitting of data to the hyperbolic Michaelis-Menten equation and after Lineweaver-Burke, Eadie-Hofstee and Hanes-Woolf transformations.

7.3.3 The relationships between $\mathbf{N}_s$ and nitrogen uptake rate

Inorganic (NO_3^- and NH_4^+) and organic (glycine) nitrogen uptake by *C. portentosa* followed Michaelis-Menten kinetics, evidenced by hyperbolic substrate saturation curves (Fig. 7.10). Nitrogen uptake capacity was significantly negatively related to $\mathbf{N}_s$ (and corresponding N:P mass ratios) for each form investigated (Fig. 7.10, Table 7.7). At all sites, rates of NO_3^- uptake were significantly lower than for the other forms of N ($P < 0.008$, Fig. 7.10). In line with literature on N uptake in bryophytes and vascular plants (Wanek and Pörtl 2008), the range of substrate

concentrations selected for analysis of N uptake capacity in *C. portentosa* (0.002 – 0.5 mM) was unable to reveal low affinity uptake. This is despite the fact that Michaelis-Menten uptake curves suggest a linear component indicating substrate saturation. The three linear transformations applied to N uptake data each independently revealed a point of inflexion at which the rate of change in uptake velocity (*V*) altered (Fig. 7.11). This was attributed to the presence of two distinct high affinity systems (System Ia (SIa) and System Ib (SIb)). The substrate concentration at which the point of inflexion occurred varied amongst the three N forms. For NO_3^- and glycine this value was 0.05 mM and for NH_4^+ the value was 0.02 mM (Fig. 7.11).

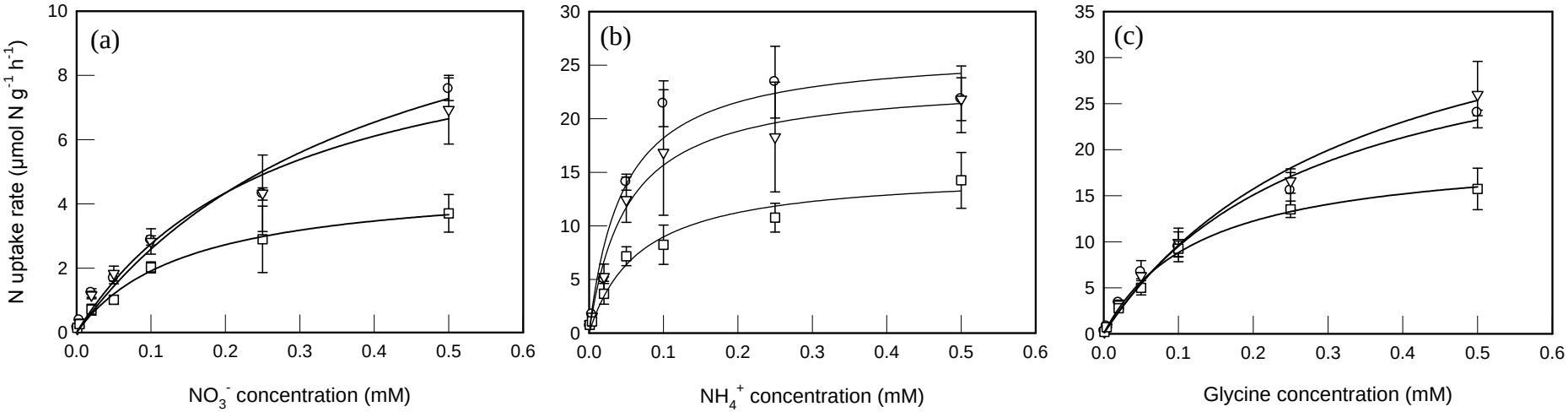


Figure 7.10 Effect of varying nitrate (a), ammonium (b) and glycine (c) concentrations on N uptake rates in *Cladonia portentosa* collected from sites differing in $\mathbf{N}_s$; 4.1 kg ha^{-1} y^{-1} (○), 15.8 kg ha^{-1} y^{-1} (▽) and 32.8 kg ha^{-1} y^{-1} (□). Assays were performed for 10 mins at a PPFR of 100 µmol m^{-2} s^{-1} and 20 ^{o}C in 20 ml ^{15}N-KNO_3, $^{15}N(NH_4)_2SO_4$ or ^{15}N-glycine at final ^{15}N concentrations of 0.001, 0.004, 0.02, 0.05, 0.1, 0.25 and 0.5 mM. Plotted values are means (n = 3) ± 1 SEM.

Table 7.7 Results of two-way ANOVA to compare nitrate (a), ammonium (b) and glycine (c) uptake rates in *Cladonia portentosa* between sites with different $\mathbf{N}_s$ values (4.1, 15.8 and 32.8 kg ha^{-1} y^{-1} and corresponding N:P mass ratios) and between different substrate concentrations (0.001 - 0.5 mM).

(a)				LSD Post Hoc	
Source of variation	df	*F*-ratio	*P*-value	$\mathbf{N}_s$ (kg N ha^{-1} y^{-1})	*P*-value
Between NO_3^- concentrations	6	105.401	< 0.001		
Between sites (and N:P mass ratios)	2	13.523	< 0.001	4.1 – 15.8	0.627
				4.1 – 32.8	< 0.001
				15.8 – 32.8	< 0.001
NO_3^- concentration x $\mathbf{N}_s$	12	1.416	0.197		
Error	42				
Total	63				

(b)				LSD Post Hoc	
Source of variation	df	*F*-ratio	*P*-value	$\mathbf{N}_s$ (kg N ha^{-1} y^{-1})	*P*-value
Between NH_4^+ concentrations	6	70.784	< 0.001		
Between sites (and N:P mass ratios)	2	15.873	< 0.001	4.1 – 15.8	0.193
				4.1 – 32.8	< 0.001
				15.8 – 32.8	< 0.001
NH_4^+ concentration x $\mathbf{N}_s$	12	1.426	0.193		
Error	42				
Total	63				

(c)				LSD Post Hoc	
Source of variation	df	*F*-ratio	*P*-value	$\mathbf{N}_s$ (kg N ha^{-1} y^{-1})	*P*-value
Between glycine concentrations	6	558.993	< 0.001		
Between sites (and N:P mass ratios)	2	4.920	0.012	4.1 – 15.8	0.804
				4.1 – 32.8	0.007
				15.8 – 32.8	0.013
Glycine concentration x $\mathbf{N}_s$	12	0.640	0.796		
Error	42				
Total	63				

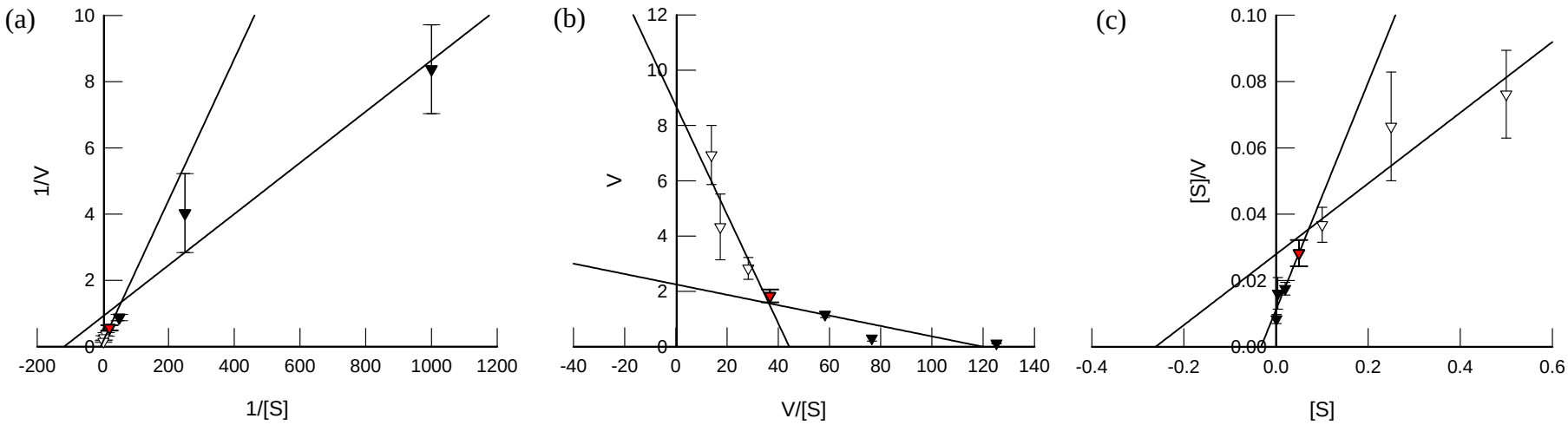


Figure 7.11a Linear transformations of the Michaelis-Menten equation evidencing two high affinity nitrate uptake systems in *Cladonia portentosa*: Lineweaver Burke (a); Eadie-Hofstee (b); Hanes-Woolf (c). System Ia (▼) operates at 0.001 – 0.05 mM NO_3^- and System Ib (▽) at 0.05 – 0.5 mM NO_3^-. Data presented are for *C. portentosa* collected from Alwen Reservoir, $\mathbf{N}_s$ 15.8 kg N ha^{-1} y^{-1}. Values illustrated in red represent possible uptake by either system. Assays were performed for 10 min at a PPFR of 100 µmol m^{-2} s^{-1} and 20 ^oC in 20 ml ^{15}N-KNO_3. Plotted values are means (n = 3) ± 1 SEM.

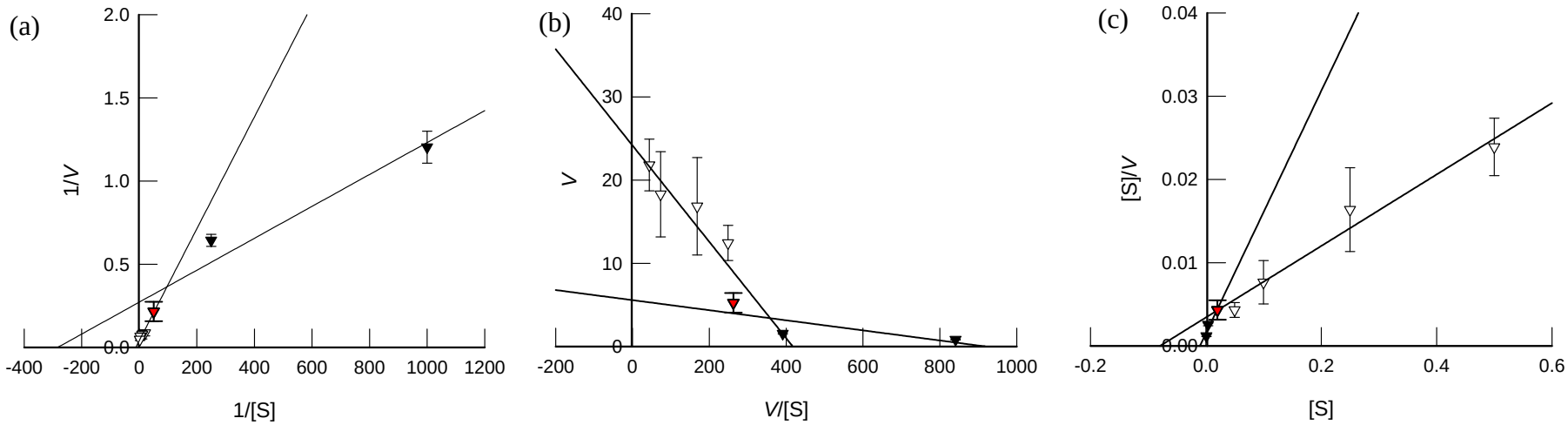


Figure 7.11b Linear transformations of the Michaelis-Menten equation evidencing two high affinity ammonium uptake systems in *Cladonia portentosa*: Lineweaver Burke (a); Eadie-Hofstee (b); Hanes-Woolf (c). System Ia (▼) operates at 0.001 – 0.02 mM NH_4^+ and System Ib (▽) at 0.02 – 0.5 mM NH_4^+. Data presented are for *C. portentosa* collected from Alwen Reservoir, $\mathbf{N}_s$ 15.8 kg N ha^{-1} y^{-1}. Values illustrated in red represent possible uptake by either system. Assays were performed for 10 min at a PPFR of 100 µmol m^{-2} s^{-1} and 20 ^oC in 20 ml ^{15}N-$(NH_4)_2SO_4$. Plotted values are means (n = 3) ± 1 SEM.

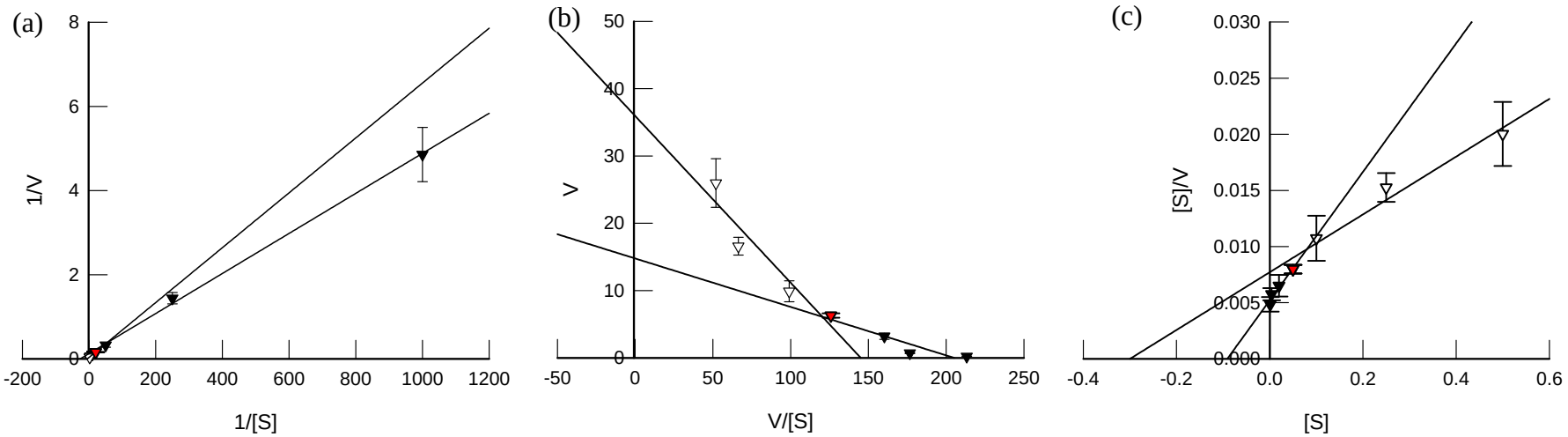


Figure 7.11c Linear transformations of the Michaelis-Menten equation evidencing two high affinity glycine uptake systems in *Cladonia portentosa*: Lineweaver Burke (a); Eadie-Hofstee (b); Hanes-Woolf (c). System Ia (▼) operates at 0.001 – 0.05 mM glycine and System Ib (▽) at 0.05 – 0.5 mM glycine. Data presented are for *C. portentosa* collected from Alwen Reservoir, $\mathbf{N}_s$ 15.8 kg N ha^{-1} y^{-1}. Values illustrated in red represent possible uptake by either system. Assays were performed for 10 min at a PPFR of 100 µmol m^{-2} s^{-1} and 20 ^{o}C in 20 ml ^{15}N-glycine. Plotted values are means (n = 3) ± 1 SEM.

Table 7.8 Values of the kinetic parameters K_m and V_{max} derived from high affinity nitrate (a), ammonium (b) and glycine (c) uptake rates for System Ia (substrate concentration range 0.001 – 0.05 mM NO_3^- and glycine, 0.001 – 0.02 mM NH_4^+) and System Ib (substrate concentration range, 0.05 – 0.5 mM NO_3^- and glycine, 0.02 – 0.5 mM NH_4^+) high affinity N transporter systems in *Cladonia portentosa*. *Cladonia portentosa* was collected from sites differing in $\mathbf{N}_s$ (4.1, 15.8 and 32.8 kg ha^{-1} y^{-1}). Kinetic parameters were determined following fitting of data to the hyperbolic Michaelis-Menten equation or after Lineweaver-Burke, Eadie-Hofstee and Hanes-Woolf transformations. The units of K_m are mM and V_{max} are µmol substrate g^{-1} dry mass h^{-1}. r^2 values are provided to demonstrate the goodness of fit of regression models. Values are means (n = 4) ± 1 SEM.

(a) NITRATE		Michaelis-Menten			Lineweaver-Burke			Eadie-Hofstee			Hanes-Woolf			Mean ± 1 SEM	
Study site $\mathbf{N}_s$ (kg N ha^{-1} y^{-1})	**System**, [NO_3^-] (mM)	K_m	V_{max}	r^2	K_m	V_{max}	r^2	K_m	V_{max}	r^2	K_m	V_{max}	r^2	K_m	V_{max}
Strabauchlinn Knowe	**SIa** 0.001 – 0.05	0.02	2.30	0.95	0.01	1.72	0.99	0.02	2.21	0.96	0.02	2.24	0.99	0.02	2.12
(4.1)	**SIb** 0.05 – 0.5	0.49	14.51	0.95	0.24	9.63	0.99	0.26	10.24	0.81	0.36	12.27	0.89	0.34	11.66
Alwen Reservoir	**SIa** 0.001 – 0.05	0.03	3.04	0.92	0.01	1.10	0.95	0.02	2.25	0.78	0.03	2.92	0.89	0.02	2.33
(15.8)	**SIb** 0.05 – 0.5	0.31	10.91	0.71	0.15	6.87	0.97	0.20	8.72	0.86	0.26	9.37	0.88	0.23	8.97
Wythburn Fells	**SIa** 0.001 – 0.05	0.02	1.34	0.80	0.002	0.36	0.45	0.06	0.95	0.73	0.03	1.51	0.69	0.03	1.04
(32.8)	**SIb** 0.05 – 0.5	0.16	4.88	0.57	0.17	4.42	0.93	0.17	4.90	0.88	0.19	4.58	0.90	0.17	4.70

Table 7.8 continued.

(b) AMMONIUM		Michaelis-Menten			Lineweaver-Burke			Eadie-Hofstee			Hanes-Woolf			Mean ± 1 SEM	
Study site $\mathbf{N}_s$ (kg N ha^{-1} y^{-1})	**System**, [NH_4^+] (mM)	K_m	V_{max}	r^2	K_m	V_{max}	r^2	K_m	V_{max}	r^2	K_m	V_{max}	r^2	K_m	V_{max}
Strabauchlinn Knowe	**SIa** 0.001 – 0.02	0.02	9.29	0.99	0.01	5.86	0.99	0.01	7.37	0.90	0.01	8.42	0.98	0.01	7.74
(4.1)	**SIb** 0.02 – 0.5	0.05	26.36	0.76	0.13	40.16	0.95	0.05	26.32	0.49	0.04	23.81	0.98	0.07	28.41
Alwen Reservoir	**SIa** 0.001 – 0.02	0.02	10.93	0.79	0.004	3.68	0.94	0.01	5.57	0.60	0.01	6.87	0.91	0.01	6.76
(15.8)	**SIb** 0.02 – 0.5	0.05	23.58	0.50	0.08	24.63	0.96	0.06	24.16	0.82	0.08	23.31	0.98	0.07	23.92
Wythburn Fells	**SIa** 0.001 – 0.02	0.02	7.14	0.68	0.001	1.60	0.94	0.004	3.24	0.47	0.01	4.43	0.70	0.01	4.10
(32.8)	**SIb** 0.02 – 0.5	0.07	15.12	0.66	0.08	14.79	0.96	0.06	14.29	0.90	0.09	15.22	0.99	0.07	14.86

(c) GLYCINE		Michaelis-Menten			Lineweaver-Burk			Eadie-Hofstee			Hanes-Woolf			Mean ± 1 SEM	
Study site $\mathbf{N}_s$ (kg N ha^{-1} y^{-1})	**System,** [glycine] (mM)	K_m	V_{max}	r^2	K_m	V_{max}	r^2	K_m	V_{max}	r^2	K_m	V_{max}	r^2	K_m	V_{max}
Strabauchlinn Knowe	**SIa** 0.001 – 0.05	0.10	11.95	0.88	0.03	7.38	0.99	0.06	13.89	0.87	0.06	13.74	0.97	0.06	11.74
(4.1)	**SIb** 0.05 – 0.5	0.31	37.58	0.89	0.18	26.88	0.98	0.19	29.57	0.84	0.31	37.18	0.94	0.24	32.80
Alwen Reservoir	**SIa** 0.001 – 0.05	0.09	11.79	0.97	0.04	8.01	0.99	0.07	14.77	0.88	0.09	17.48	0.94	0.07	13.01
(15.8)	**SIb** 0.05 – 0.5	0.37	44.08	0.85	0.20	29.94	0.98	0.25	35.95	0.90	0.30	38.91	0.97	0.28	37.22
Wythburn Fells	**SIa** 0.001 – 0.05	0.06	11.07	0.92	0.04	8.00	1.00	0.05	9.85	0.99	0.05	9.05	0.99	0.05	9.49
(32.8)	**Sib** 0.05 – 0.5	0.13	20.05	0.82	0.19	23.70	0.98	0.14	20.67	0.92	0.13	19.12	0.99	0.15	20.88

Values of K_m differed significantly between the form of N supplied and the system dominating uptake (Table 7.9a). Values of K_m for NH_4^+ uptake were significantly lower than for NO_3^- or glycine (Fig. 7.12a), and among all N forms, values of K_m were significantly lower for System Ia (< 0.05 mM), suggesting higher affinity uptake (Fig. 7.12a, Table 7.9). Maximum uptake rates (V_{max}) for all 3 N forms were significantly higher for System Ib reflecting increased substrate availability (Fig. 7.12b, Table 7.9b). Values of V_{max} also differed significantly between N form, increasing in the series: nitrate < ammonium < glycine (for both uptake systems) (Fig. 7.12b).

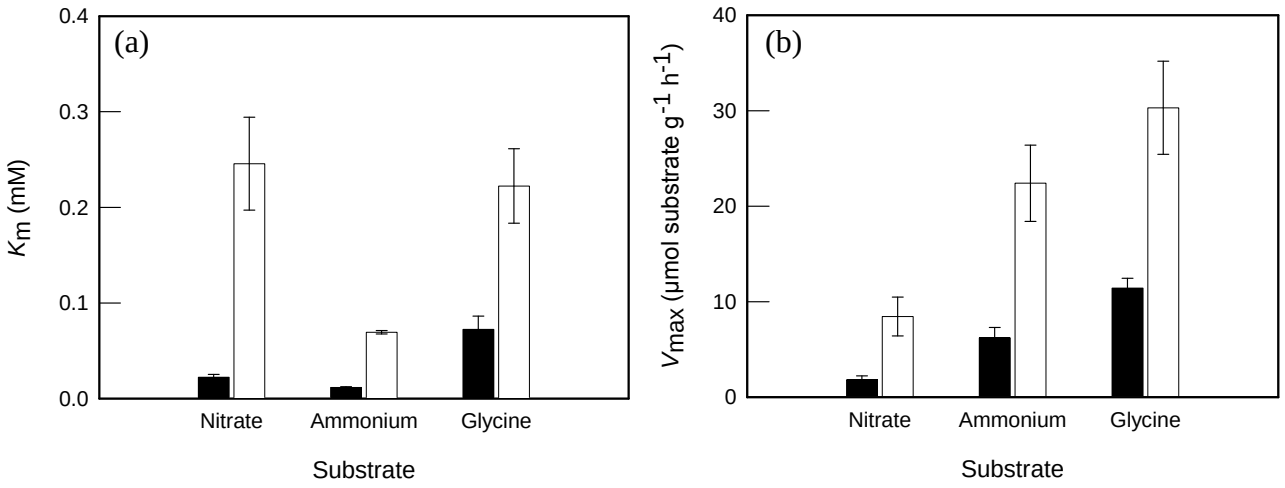


Figure 7.12 Values of the kinetic constants K_m (a) and V_{max} (b) calculated for high affinity NO_3^-, NH_4^+ and glycine transporter systems in *Cladonia portentosa* (□, System Ia; ■, System Ib). Values were calculated by fitting data to the Michaelis-Menten equation and Lineweaver-Burke, Eadie-Hofstee and Hanes-Woolf transformations. Plotted values are means (n = 4) ± 1 SEM.

Table 7.9 Results of two-way ANOVA to compare values of K_m (a) and V_{max} (b) calculated for high affinity N uptake in *Cladonia portentosa* between the different substrates NO_3^-, NH_4^+ and glycine and between the two high uptake systems, System Ia (substrate concentration range, 0.001 – 0.05 mM NO_3^- and glycine; 0.001 – 0.02 mM NH_4^+) and System Ib (substrate concentration range, 0.05 – 0.5 mM NO_3^- and glycine; 0.02 – 0.5 mM NH_4^+).

(a) K_m				LSD	
Source of variation	df	*F*-ratio	*P*-value	Post Hoc	*P*-value
Between substrates	2	9.954	0.003	NO_3^- - NH_4^+	0.004
				NO_3^- - Glycine	0.619
				NH_4^+ - Glycine	0.001
Between uptake systems	1	45.470	< 0.001		
Substrate x uptake system	2	5.061	0.025		
Error	12				
Total	18				

(b) V_{max}				LSD	
Source of variation	df	*F*-ratio	*P*-value	Post Hoc	*P*-value
Between substrates	2	16.194	< 0.001	NO_3^- - NH_4^+	0.006
				NO_3^- - Glycine	< 0.001
				NH_4^+ - Glycine	0.036
Between uptake systems	1	37.630	< 0.001		
Substrate x uptake system	2	2.704	0.107		
Error	12				
Total	18				

There was no clear pattern to the relationships between $\mathbf{N}_s$ and K_m for all 3 N forms investigated (Fig 7.13). Values of K_m for high affinity System Ib NO_3^- and glycine uptake were negatively related to $\mathbf{N}_s$ whilst for NH_4^+, this relationship was weakly positively (Fig. 7.13). Relationships were in general weaker when K_m values for System Ia were investigated. In contrast, there was a more uniform response between values of V_{max} and $\mathbf{N}_s$, for which there was a negative

relationship for all three N forms investigated and both high affinity uptake systems (Fig. 7.14). Nonetheless, relationships were consistently stronger for values of System Ib V_{max}.

At all sites, the uptake efficiency calculated for all three forms of N was markedly higher for System Ia than System Ib (Fig. 7.15, Table 7.10). There was no overriding pattern for the relationship between UE and $\mathbf{N}_s$ for each N form. Whilst the UE for glycine changed little with increasing $\mathbf{N}_{s,}$ values of UE for NO_3^- (System Ia and Ib) and NH_4^+ (System Ib) decreased (Fig. 7.15). There was a strong positive relationship between $\mathbf{N}_s$ and the ratio of UE_{SIa} : UE_{SIb} for NH_4^+ (Fig. 7.16b). All three forms of N showed strong positive co-variation between values of K_m and V_{max} for both uptake systems (Fig. 7.17), and despite stronger co-variation between the two kinetic parameters calculated for System Ib uptake, a steeper gradient between values of V_{max} and K_m for System Ia N uptake provided further evidence of greater uptake efficiency (Fig. 7.17).

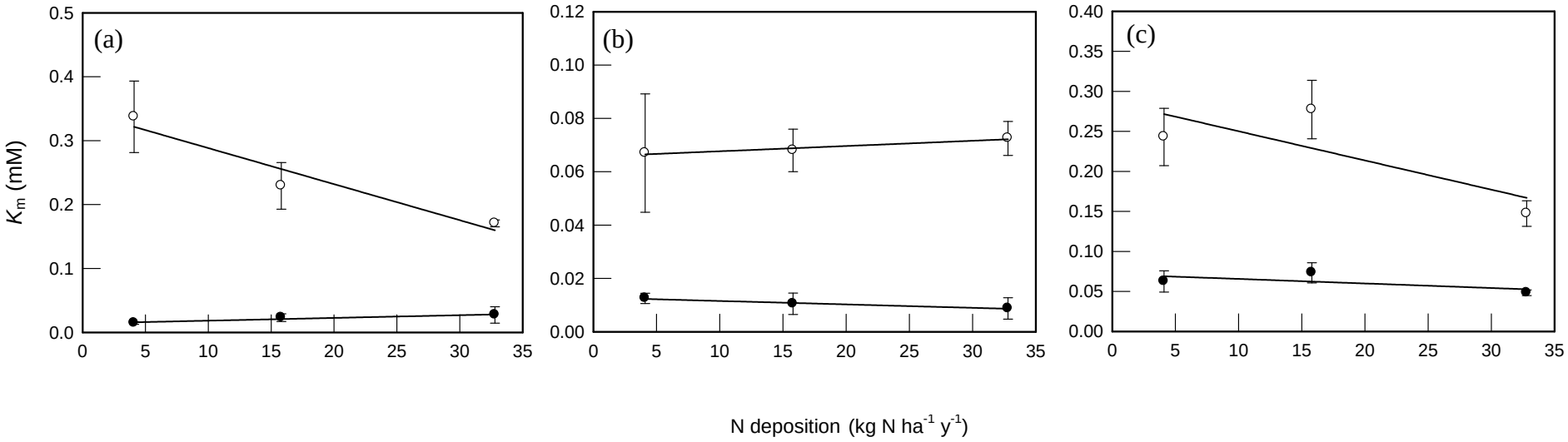


Figure 7.13 The relationship between wet inorganic N deposition ($\mathbf{N}_s$) and values of K_m for high affinity System Ia (●) and System Ib (○) N uptake in *Cladonia portentosa*: NO_3^- (a, ○, r^2 = 0.924; P = 0.177; ●, r^2 = 0.952, P = 0.141); NH_4^+ (b, ○, r^2 = 0.933, P = 0.166; ●, r^2 = 0.653, P = 0.401); glycine (c, ○, r^2 = 0.612, P = 0.428; ●, r^2 = 0.459, P = 0.526). Plotted values are means (n = 4) ± 1 SEM.

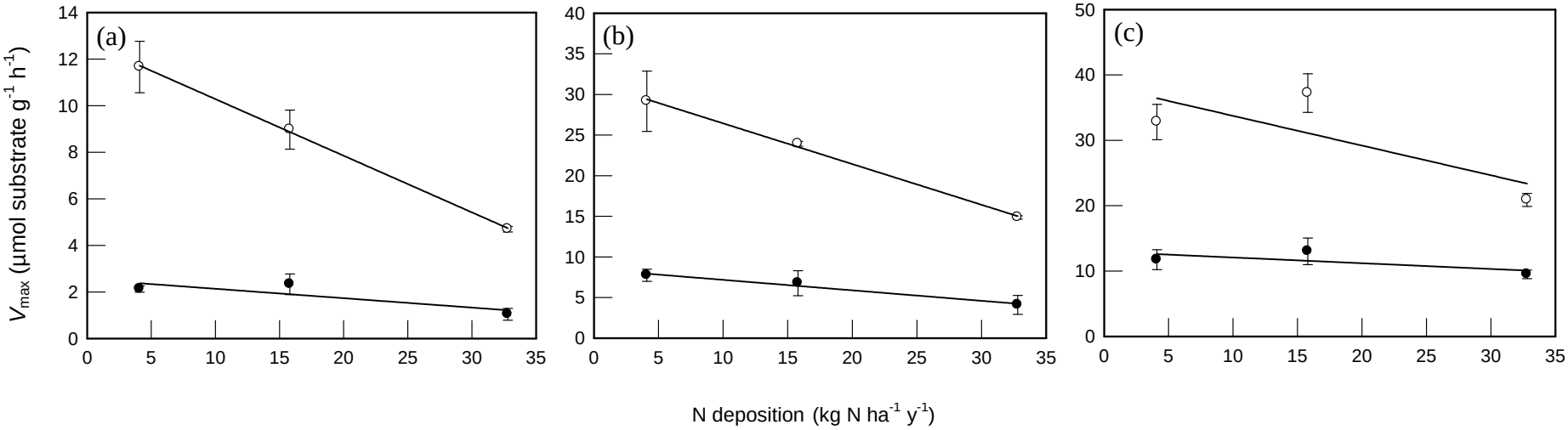


Fig. 7.14 The relationship between wet inorganic N deposition ($\mathbf{N}_s$) and values of V_{max} for high affinity System Ia (●) and System Ib (○) N uptake in *Cladonia portentosa*: NO_3^- (a, ○, r^2 = 0.999, P = 0.015; ●, r^2 = 0.712, P = 0.361); NH_4^+ (b, ○, r^2 = 0.993, P = 0.055; ●, r^2 = 0.975, P = 0.101); glycine (c, ○, r^2 = 0.603, P = 0.434; ●; r^2 = 0.503, P = 0.498). Plotted values are means (n = 4) ± 1 SEM

Table 7.10 Nitrogen uptake efficiencies (UE, ratio of V_{max}/K_m) calculated for dual high affinity (System Ia and System Ib) NO_3^- (a), NH_4^+ (b) and glycine (c) N transporter systems in *Cladonia portentosa. Cladonia portentosa* was collected from sites with contrasting $\mathbf{N}_s$ (4.1, 15.8 and 32.8 kg ha^{-1} y^{-1}). Parameters were determined following fitting of data to the hyperbolic Michaelis-Menten equation or following Lineweaver-Burke, Eadie-Hofstee and Hanes-Woolf transformations Mean values are given (n = 4) ± 1 SEM.

(a) Study site $\mathbf{N}_s$ (kg N ha^{-1} y^{-1})	**System**, $[NO_3^-]$ (mM)	Michaelis-Menten	Lineweaver-Burk	Eadie-Hofstee	Hanes-Woolf	Mean ± 1 SEM
Strabauchlinn	**SIa** 0.001 – 0.05	127.94	132.39	130.53	124.56	128.85 ± 1.70
(4.1)	**SIb** 0.05 – 0.5	29.78	39.45	39.38	34.27	35.72 ± 2.32
Alwen Reservoir	**SIa** 0.001 – 0.05	92.00	137.13	118.58	91.19	109.72 ± 11.13
(15.8)	**SIb** 0.05 – 0.5	34.86	47.04	44.26	35.91	40.52 ± 3.03
Wythburn Fells	**SIa** 0.001 – 0.05	79.06	181.50	15.27	53.86	82.42 ± 35.54
(32.8)	**SIb** 0.05 – 0.5	30.32	26.30	29.36	24.60	27.65 ± 1.33

Table 7.10 continued

(b) Study site $\mathbf{N}_s$ (kg N ha^{-1} y^{-1})	**System** $[NH_4^+]$ (mM)	Michaelis-Menten	Lineweaver-Burk	Eadie-Hofstee	Hanes-Woolf	Mean ± 1 SEM
Strabauchlinn	**SIa** 0.001 – 0.02	540.65	732.75	670.00	601.29	636.17 ± 41.65
(4.1)	**SIb** 0.02 – 0.5	585.78	301.96	506.17	626.58	505.12 ± 72.19
Alwen Reservoir	**SIa** 0.001 – 0.02	496.82	919.75	929.00	686.80	758.09 ± 103.56
(15.8)	**SIb** 0.02 – 0.5	462.35	293.23	416.47	295.06	366.78 ± 42.97
Wythburn Fells	**SIa** 0.001 – 0.02	375.63	1595.00	809.00	402.27	795.48 ± 284.36
(32.8)	**SIb** 0.02 – 0.5	219.13	192.12	250.67	174.95	209.22 ± 16.54

(c) Study site $\mathbf{N}_s$ (kg N ha^{-1} y^{-1})	**System** [glycine] (mM)	Michaelis-Menten	Lineweaver-Burk	Eadie-Hofstee	Hanes-Woolf	Mean ± 1 SEM
Strabauchlinn	**SIa** 0.001 – 0.05	124.47	238.07	231.45	218.03	203.00 ± 26.51
(4.1)	**SIb** 0.05 – 0.5	123.21	151.88	159.82	121.89	139.20 ± 9.75
Alwen Reservoir	**SIa** 0.001 – 0.05	128.16	210.87	205.19	192.12	184.09 ± 19.05
(15.8)	**SIb** 0.05 – 0.5	120.44	153.54	144.96	129.71	137.16 ± 7.44
Wythburn Fells	**SIa** 0.001 – 0.05	194.16	200.00	196.90	196.74	196.95 ± 1.20
(32.8)	**SIb** 0.05 – 0.5	156.64	122.15	146.58	151.75	144.28 ± 7.66

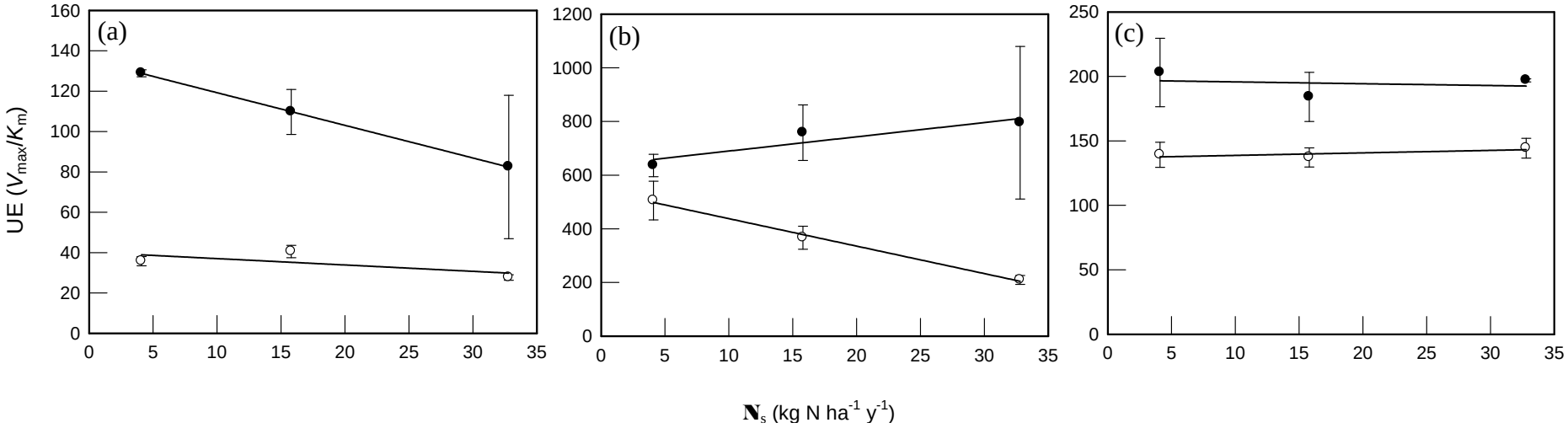


Figure 7.15 Relationship between wet inorganic N deposition and N uptake efficiency (UE, V_{max}/K_m) for high affinity System 1a (●) and System Ib (○) transporter systems in *Cladonia portentosa*: NO_3^- (a, ●, r^2 = 0.990, P = 0.065; ○, r^2 = 0.385, P = 0.574); NH_4^+ (b, ●, r^2 = 0.914, P = 0.189; ○, r^2 = 0.999, P = 0.024); glycine (c, ●, r^2 = 0.098, P = 0.797; ○, r^2 = 0.480, P = 0.513). Uptake efficiencies are calculated from mean K_m and V_{max} values derived from four models (n = 4). Plotted values are means (n = 3 - 4) ± 1 SEM.

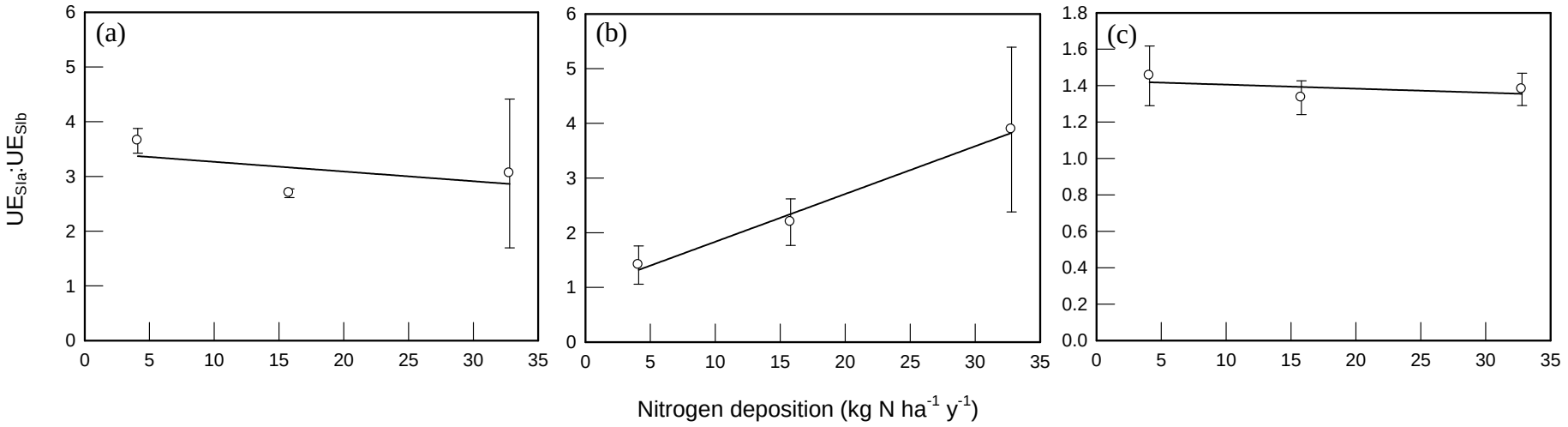


Figure 7.16 Relationships between $\mathbf{N}_s$ and the ratio between uptake efficiency (UE) for System Ia and System Ib (UE_{SIa}:UE_{SIb}) high affinity N transporter systems in *Cladonia portentosa*: NO_3^- (a, r^2= 0.280, P = 0.265); NH_4^+ (b, r^2 = 0.990, P = 0.065); glycine (c, r^2 = 0.270, P = 0.646). Uptake efficiencies are calculated from mean K_m and V_{max} values derived from four models (n = 4). Plotted values are means (n = 3 - 4) ± 1 SEM.

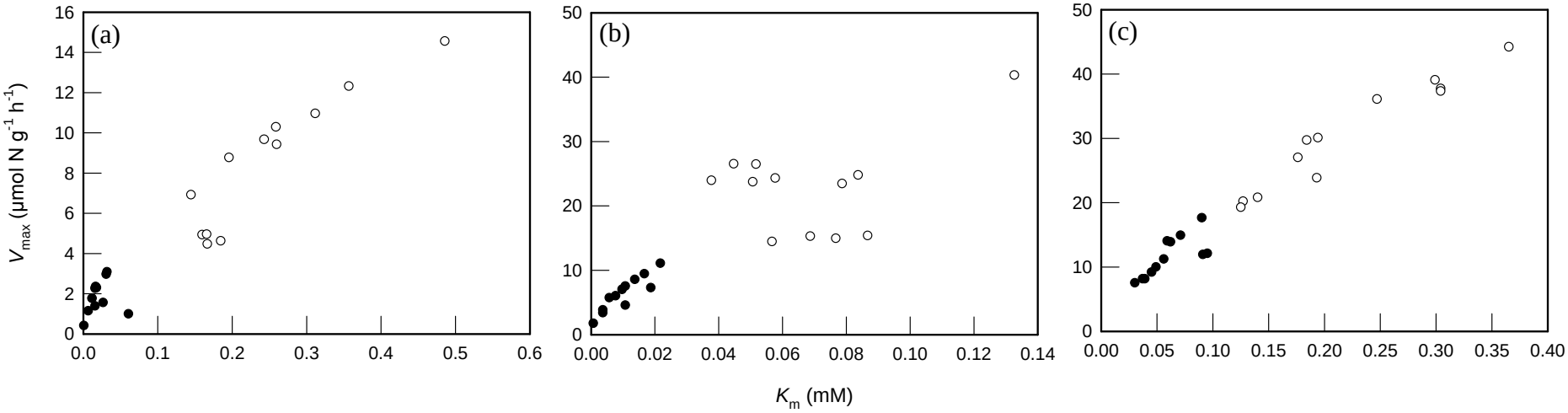


Figure 7.17 Relationships between maximum N uptake rate (V_{max}) and corresponding K_m values (substrate concentration at half V_{max}) for high affinity System Ia (●) and System Ib (○) transporter systems in *Cladonia portentosa*: NO_3^- (a, ●, r = 0.169, P = 0.600; ○, r = 0.914, P < 0.001); NH_4^+ (b, ●, r = 0.902 P < 0.001; ○, r = 0.408, P = 0.188); glycine (c, ●, r = 0.753, P = 0.005; ○, r = 0.968, P < 0.001). Kinetic parameters were determined following fitting of data to the hyperbolic Michaelis-Menten equation and Lineweaver-Burke, Eadie-Hofstee and Hanes-Woolf transformations.

7.3.4 Comparison of linear transformations

In general, fitting PO_4^{3-} and N uptake data to the Michaelis-Menten equation produced K_m and V_{max} values that were similar to those obtained following linear transformation of the data (Tables 7.11 & 7.12). The Hanes-Woolf model gave the best estimates for K_m and V_{max} values when compared against those derived from the Michaelis-Menten model, as evidenced by highly significant coefficient of determination values (Tables 7.11 and 7.12). This was also supported by the non-significant paired sample comparison *P* values, which revealed the null hypothesis (that the mean difference between values for the kinetic parameters determined from either Michaelis-Menten or Hanes-Woolf models was zero) could not be rejected (Tables 7.11 and 7.12). Of the three linear transformations, the Lineweaver-Burk model predicted values of K_m and V_{max} which differed most from those derived from the Michaelis-Menten model.

Table 7.11 Statistical comparison of K_m and V_{max} values for the uptake of PO_4^{3-} by *Cladonia portentosa*, as determined by curvilinear or linearised approaches. Values of K_m and V_{max} were derived from fitting data to the hyperbolic Michaelis-Menten equation (MM) or after Lineweaver-Burke (LB), Eadie-Hofstee (EH) and Hanes-Woolf (HW) transformations. Data were analysed for the effect of PO_4^{3-} concentration (0.002 – 10 mM) on uptake rates in *C. portentosa* collected from 3 sites differing in $\mathbf{N}_s$ by linear regression analysis and paired sample comparison followed by a *t*-test to test the null hypothesis that the mean difference between a kinetic constant determined by two methods was zero ($n = 6$). * Units of K_m are mM, † Units of V_{max} are nmol PO_4^{3-} mg^{-1} h^{-1}.

	Correlation analysis			Paired sample comparison		
	Equation	*P* value	r^2	Mean difference ± 1 SEM	*t*- value	*P* value
K_m*						
MM vs LB	LB = -0.164 + 0.580 x MM	0.132	0.471	-0.080 ± 0.298*	-0.268	0.800
MM vs EH	EH = -0.169 + 0.927 x MM	< 0.001	0.997	0.127 ± 0.036	3.544	0.016
MM vs HW	HW = -0.09 + 0.992 x MM	< 0.001	0.996	0.005 ± 0.025	-0.181	0.863
LB vs EH	EH = -0.315 + 0.784 x LB	0.111	0.510	0.206 ± 0.270	0.765	0.479
LB vs HW	HW = -0.165 + 0.840 x LB	0.111	0.511	0.084 ± 0.283	0.298	0.778
EH vs HW	HW = 0.171 + 1.069 x EH	< 0.001	0.997	-0.122 ± 0.033	-3.674	0.014
V_{max}†						
MM vs LB	LB = 5.066 + 0.848 x MM	0.002	0.925	-0.515 ± 2.906†	-0.177	0.866
MM vs EH	EH = 0.271 + 0.872 x MM	< 0.001	0.997	3.538 ± 1.392	2.541	0.052
MM vs HW	HW = 0.057 + 0.961 x MM	< 0.001	0.995	1.112 ± 0.818	1.360	0.232
LB vs EH	EH = -2.915 + 0.963 x LB	0.001	0.942	4.053 ± 2.160	1.877	0.119
LB vs HW	HW = -3.472 + 1.061 x LB	0.001	0.942	1.627 ± 2.430	0.670	0.533
EH vs HW	HW = -0.237 + 1.101 x EH	< 0.001	0.998	-2.426 ± 1.020	-2.378	0.063

Table 7.12 Statistical comparison of K_m and V_{max} values for the uptake of NO_3^-, NH_4^+ and glycine by *Cladonia portentosa*, as determined by curvilinear or linearised approaches. Values of K_m and V_{max} were derived from fitting data to the hyperbolic Michaelis-Menten equation (MM) or after Lineweaver-Burke (LB), Eadie-Hofstee (EH) and Hanes-Woolf (HW) transformations. Data were analysed for the effect of ^{15}N-NO_3^-, ^{15}N-NH_4^+ and ^{15}N-glycine concentration (0.001 – 0.5 mM) on uptake in *Cladonia portentosa* from 3 sites differing in $\mathbf{N}_s$ by linear regression analysis and paired sample comparison followed by a *t*-test to test the null hypothesis that the mean difference between a kinetic constant determined by two methods was zero (n = 18). * Units of K_m are mM, † Units of V_{max} are μmol N g^{-1} h^{-1}.

	Correlation analysis			Paired sample comparison		
	Equation	*P* value	r^2	Mean difference ± 1 SEM	*t*- value	*P* value
K_m*						
MM vs LB	LB = 0.041 + 1.290 x MM	< 0.001	0.717	0.293 ± 0.100*	2.928	0.009
MM vs EH	EH = -0.158 + 0.972 x MM	< 0.001	0.771	0.126 ± 0.062	2.042	0.057
MM vs HW	HW = -0.043 + 1.000 x MM	< 0.001	0.927	0.043 ± 0.033	1.309	0.208
LB vs EH	EH = -0.378 + 0.623 x LB	< 0.001	0.734	-0.167 ± 0.094	-1.770	0.095
LB vs HW	HW = -0.344 + 0.595 x LB	< 0.001	0.762	-0.25 ± 0.093	-2.695	0.015
EH vs HW	HW = -0.053 + 0.893 x EH	< 0.001	0.907	-0.083 ± 0.039	-2.115	0.050
V_{max}†						
MM vs LB	LB = -0.234 + 0.959 x MM	0.002	0.470	0.275 ± 0.097†	2.838	0.011
MM vs EH	EH = -0.131 + 1.054 x MM	< 0.001	0.936	0.076 ± 0.027	2.831	0.012
MM vs HW	HW = -0.015 + 0.983 x MM	< 0.001	0.955	0.032 ± 0.020	1.585	0.131
LB vs EH	EH = 0.513 + 0.581 x LB	< 0.001	0.556	-0.199 ± 0.889	-2.245	0.038
LB vs HW	HW = 0.611 + 0.508 x LB	0.001	0.498	-0.243 ± 0.094	-2.575	0.020
EH vs HW	HW = 0.125 + 0.914 x EH	< 0.001	0.980	-0.044 ± 0.016	-2.704	0.015

7.4 Discussion

Phosphate uptake was significantly positively associated with $\mathbf{N}_s$ and thallus N:P mass ratio in *C. portentosa*. In agreement with Farrar (1976), two systems for lichen PO_4^{3-} uptake were evidenced. High affinity uptake by System I was characterised by low K_m values and was the dominant system for PO_4^{3-} transport over low substrate concentrations (< 0.05 mM). In contrast, higher K_m values were recorded for System II PO_4^{3-} uptake, indicative of low affinity transport in response to higher substrate concentrations (> 0.05 mM). The uptake efficiency for System I transport increased with enhanced $\mathbf{N}_s$ reflecting an increase in the capacity for PO_4^{3-} uptake (increasing V_{max}) with little change in the affinity for PO_4^{3-} uptake (constant K_m values). Along the gradient in $\mathbf{N}_s$ there was a strong positive relationship between UE_{SI}:UE_{SII}, evidencing that variation in high affinity (System I) PO_4^{3-} uptake is chiefly accountable for the strong positive relationship between PO_4^{3-} uptake and $\mathbf{N}_s$. This is consistent with the findings of Burns and Beever (1977) who reported that P-starvation resulted in a dramatic increase in the capacity for high affinity System I PO_4^{3-} uptake in the fungus *Neurospora crassa*.

Increased N deposition was associated with significant down-regulation of NO_3^-, NH_4^+ and glycine uptake rates in *C. portentosa*. In line with N uptake mechanisms reported for bryophytes and vascular plants (von Wirén *et al.* 1997; Glass *et al.* 2002; Wanek and Pörtl 2008), the range of substrate concentrations selected for the present investigation (0.001 – 0.5 mM) revealed the presence of high affinity uptake. Nonetheless, linear transformation of uptake data evidenced co-operation

between two distinct high affinity uptake systems (System Ia and Ib). For NO_3^- and glycine, change in the rate of uptake occurred at 0.05 mM, whilst for NH_4^+, this was at 0.02 mM.

Values of V_{max} for PO_4^{3-} uptake were positively associated with $\mathbf{N}_s$. This suggests an increased capacity for PO_4^{3-} uptake under conditions of P-limitation (associated with increased N supply), and is in line with the findings of Jackson and Caldwell (1991). These authors reported a 29 % increase in the rate of PO_4^{3-} uptake in roots of *Pseudoroegneria spicata* when N supply was increased from 1 to 20 mM NH_4NO_3. Nitrogen uptake in *C. portentosa* was significantly negatively related to $\mathbf{N}_s$. This is consistent with the findings of Dahlman *et al.* (2004) who reported lower rates of N uptake in N_2-fixing lichens (characterised by high thallus N) compared with non-N_2-fixing species (containing lower thallus N concentrations). Results from the current investigation suggest that *C. portentosa* might experience N saturation at high $\mathbf{N}_s$ sites, and as such reduces investment in the synthesis of N transporter proteins (evidenced by decreased values of V_{max}). Although not investigated in the current set of experiments, there is evidence to suggest that in some lichen species, N uptake is promoted by the addition of PO_4^{3-}. For example, uptake of NH_4^+ from simulated fog water by *Teloschistes capensis* is enhanced by increased availability of PO_4^{3-} (P. D. Crittenden, Pers. Comm). However, this is not a universal trend among lichens, as the addition of P did not influence the rate of NH_4NO_3 uptake in the N_2-fixing species *Nephroma arcticum* and *Peltigera aphthosa* (Dahlman *et al.* 2002).

It is widely reported that P- or N-deprived algae (Tapper 1983), fungi (Marini *et al.* 1997; Maldonado-Mendoza *et al.* 2001; Bücking 2004; López-Pedrosa *et al.* 2006) and vascular plants (Ullrich 1987; Jackson and Caldwell 1991; Ulrich and Novacky 1992; VonWirén *et al.* 1997; Smith 2002; Smith *et al.* 2003; Vance *et al.* 2003) all increase their capacity for PO_4^{3-} or N uptake by transcriptionally up-regulating the number of transporter proteins located in the cell membrane. In *C. portentosa*, values of V_{max} for PO_4^{3-} and N uptake were negatively related to thallus P and N concentrations, respectively. It therefore seems reasonable that the capacity for PO_4^{3-} and N uptake in *C. portentosa* is regulated by negative feedback mechanisms in accordance with the nutrient status of the thallus.

There was compelling evidence for the existence of two distinct systems for PO_4^{3-} uptake in *C. portentosa*. This is in line with Farrar's (1976) suggestion of dual affinity P uptake systems in *Hypogymnia physodes*, based on Michaelis-Menten uptake kinetics. Similar to dual affinity PO_4^{3-} uptake systems reported in fungi (Burns and Beever 1977; Beever and Burns 1980; Straker and Mitchell 1987; Cairney *et al.* 1988; Thomson *et al.* 1990; Bücking 2004) and vascular plants (Reinhold and Kaplan 1984; Schobert and Komor 1987; von Wirén *et al.* 1997; Schweiger and Jackobsen 1999; Glass *et al.* 2002) the two putative systems identified for PO_4^{3-} uptake in *C. portentosa* operate over a large concentration range (0.001 – 10 mM), and probably reflect physiological adaptation to variation in nutrient availability. High affinity PO_4^{3-} uptake is dominant over low substrate concentrations (0.002 – 0.5 mM), and thus is most likely to account for the majority of PO_4^{3-} uptake by *C. portentosa* in the lichen habitat where it is exposed

to trace quantities of P in rainfall. Substrate concentrations markedly higher than those to which *C. portentosa* normally encounters are required to invoke PO_4^{3-} uptake by system II. It is therefore of interest to consider the circumstances under which this might occur. Rainfall and dry deposition events which commonly deposit nutrients to lichen thalli are largely uniform and diffuse. However, temporally and spatially infrequent events such as deposition of leaf litter or animal faeces will potentially deliver local high inputs of N and P (cf. Tomassen *et al.* 2005). It is under these circumstances that low affinity, PO_4^{3-} uptake by System II is most likely to dominate, maximising nutrient capture by an increased capacity for uptake (high values of V_{max} evidencing increased numbers of transporter proteins), whilst minimising the problem of substrate saturation. Kinetic studies on N uptake in fungi and vascular plants has also revealed the presence of dual affinity uptake systems operating in the low (< 0.5 mM) and high (> 0.5 mM) substrate concentration ranges (Wanek and Pörtl 2008). The range of substrate concentrations selected to investigate NO_3^-, NH_4^+ and glycine uptake capacity in *C. portentosa* (0.001 – 0.5 mM) covers the concentration range to which this lichen might be exposed in its natural habitat. However, it did not present the opportunity to examine any uptake by a low affinity (System II) transporter system.

High affinity PO_4^{3-} uptake in *C. portentosa* was characterised by low K_m values ranging from 0.03 – 0.04 mM. In contrast, K_m values for system II were significantly higher, between 1.3 and 2.1 mM. These values can be compared to those reported by Farrar (1976) and Nieboer *et al.* (1984) for the lichens *H.*

physodes (0.03 mM) and *Umbilicaria muhlenbergii* (0.86 mM). The broader range of K_m values calculated for PO_4^{3-} uptake in *C. portentosa* when compared to these other lichen species might be explained by the type of analysis applied to the present data (intended to reveal dual affinity uptake systems) and/or possibly the larger surface area of this fruticose lichen. Where data on dual system PO_4^{3-} uptake in fungi are available, values of K_m for high (0.001 – 0.2 mM) and low (0.1 – 10.2 mM) affinity uptake (Huner 1974; Burns and Beever 1977; Beever and Burns 1980; Clipson *et al.* 1987; Straker and Mitchell 1987; Cairney *et al.* 1988; Thomson *et al.* 1990) are broadly consistent with those documented for *C. portentosa* in the present work. System I in *C. portentosa* showed a limited capacity for PO_4^{3-} uptake, as evidenced by low V_{max} values (6.7 ± 0.26 to 10.87 ± 3.37 nmol PO_4^{3-} mg^{-1} h^{-1}), whereas system II has a larger capacity for uptake (44.8 and 57.7 nmol PO_4^{3-} mg^{-1} h^{-1}) suggesting an increased number of membrane transporters (Straker and Mitchell 1987). Low affinity PO_4^{3-} uptake by system II reflects greater substrate availability and corresponding V_{max} values calculated for *C. portentosa* are intermediate between those recorded for single system PO_4^{3-} uptake in *U. muhlenbergii* (3.54 nmol mg^{-1} h^{-1}) and *H. physodes* (30 µmol PO_4^{3-} mg^{-1} h^{-1}) (Farrar 1976; Nieboer *et al.* 1984). Variation in the values of V_{max} between species might reflect interspecific difference in growth rate and nutrient demand (Dahlman *et al.* 2004). For example, values of V_{max} for PO_4^{3-} uptake in *C. portentosa* are markedly lower than those calculated for non-lichenized fungi, for which estimates of high affinity (System I) V_{max} range from 6 to 300 nmol PO_4^{3-} mg^{-1} h^{-1} and those for low affinity (System II) are between 96 and 900 nmol PO_4^{3-} mg^{-1} h^{-1} (Huner 1974; Burns and Beever 1977; Straker and Mitchell 1987). This

might reflect the higher growth rate of free-living fungi (Turbin 1996) and the frequently greater availability of P in their substrate compared to the growth rate and chemical environment of lichens.

High affinity N uptake in *C. portentosa* is characterised by low K_m values (calculated for both uptake systems); 0.017 - 0.337 mM (NO_3^-), 0.011 – 0.073 mM (NH_4^+) and 0.048 – 0.277 mM (glycine). These values compare favourably with high affinity K_m values reported for ectomycorrhizal fungi (0.046 mM NO_3^-; 0.06 - 0.26 mM NH_4^+) (Martin and Botton 1993; Javelle *et al.* 2003a), bryophytes (0.006 - 0.056 mM NO_3^-; 0.047 – 0.151 mM NH_4^+; 0.008 – 0.211 mM glycine) (Wanek and Pörtl 2008) and vascular plants (0.03 – 6.7 mM NH_4^+; 0.007 – 0.1 mM glycine) (Wang *et al.* 1993; Kielland 1994). High affinity V_{max} values for NO_3^-, NH_4^+ and glycine uptake in *C. portentosa* ranged from 1.04 to 32.8 µmol g^{-1} h^{-1}, consistent with high affinity N uptake in fungi and vascular plants (0.1 – 34.1 µmol substrate hydrolysed g^{-1} dry mass h^{-1}) (Plassard *et al.* 1985; Kronzucker *et al.* 1995; Wallenda and Read 1999; Wanek and Pörtl 2008).

It is instructive to compare mechanisms of nutrient uptake in *C. portentosa* with those reported for other plant/fungal systems, since it is generally accepted that the mycobiont partner dominates nutrient capture (Crittenden 1989; Dahlman *et al.* 2004). Phosphate uptake in unicellular (Bun-ya *et al.* 1991) and filamentous fungi (Harrison and van Buuren 1995; Versaw 1995) along with vascular plants (Dighton *et al.* 1990; Smith 2002; Smith *et al.* 2003) involves $H^+/H_2PO_4^-$ co-transporter proteins belonging to the *Pht1* family. Members of the *Pht1* family of

transporters consist of a membrane-bound transporter through which $H_2PO_4^-$ ions and protons move from the outside of the membrane to the inside of the cell and a H^+-ATPase pump which simultaneously transports protons across the membrane to the outside of the cell. This maintains an electrochemical gradient and energises the uptake process (Ulrich and Novacky 1992; Ferrol *et al.* 2000; Smith 2002; Smith *et al.* 2003). The uptake of NO_3^- and amino acids by algae (Ullrich 1987), fungi (Chalot and Brun 1998; Hawkins *et al.* 2000) and vascular plants (Marini *et al.* 1997; von Wirén *et al.* 1997; Williams and Miller 2001) is also reported to involve several specific H^+-ATPase co-transporters, whilst NH_4^+ uptake is mediated by members of the NH_4^+ transporter family (AMT/MEP) (Marini *et al.* 1997; Javelle *et al.* 2003b; López-Pedrosa *et al.* 2006) involving H^+-coupled symport mechanisms to aid uptake into the cell (Ludewig *et al.* 2002).

Cladonia portentosa has the capacity to take up multiple forms of N, including both inorganic and organic molecules via independent transporter systems. This compares favourably with previous reports of nutrient uptake in this lichen species (Ellis *et al.* 2005) and others (Dahlman *et al.* 2002; 2004; Palmqvist and Dahlman 2006). The capacity to take up multiple forms of N is probably advantageous to lichens in oligotrophic habitats where multiple forms of N may be available simultaneously in trace concentrations (e.g. in rainfall). Values of K_m for NH_4^+ uptake in *C. portentosa* were up to 5 times lower than those for NO_3^- or glycine, implying a greater affinity for this N form. Thus, it appears that under conditions of high N availability, *C. portentosa* has the capacity to preferentially take up NH_4^+. This is in good agreement with measurements made on several lichen

species and filamentous fungi for which higher NH_4^+ uptake rates have been recorded (Jennings and Lysek, 1999). For example, uptake rates for NH_4^+: NO_3^-: amino acids (including glycine) ranged between *c.* 1:0.01:0.33 *in Peltigera membranacea* to 1:1.06:0.462 in *Bryoria fuscescens* (Dahlman *et al.* 2004), and compares with a ratio of 1:0.29:0.25 for *C. portentosa* based on uptake rates recorded in the field (Ellis *et al.* 2005). Preferential uptake of NH_4^+ might also reflect passive uptake via cation exchange sites in hyphal cell walls, which involves less energy than is required for active uptake (Brown *et al.* 1994; Dahlman *et al.* 2004; Palmqvist and Dahlman 2006). However, it is worth emphasising that this apparent selectivity is unlikely to occur in *C. portentosa* growing in oligotrophic lichen habitats where the availability of N in rainfall is low.

Uptake of PO_4^{3-} and N by *C. portentosa* followed Michaelis-Menten kinetics in response to increasing substrate concentration. This is in line with nutrient uptake mechanisms in free-living and lichen-associated microalgae (Syrett 1981; Rowell *et al.* 1985), fungi (Burns and Beever 1977; Beever and Burns 1980; Straker and Mitchell 1987; Griffin 1994; Chalot and Brun 1998; Wallenda and Read 1999), bryophytes (Wanek and Pörtl 2008) and vascular plants (Smith 2002). Nutrient uptake by ion exchange mechanisms follows first-order kinetics, with a linear relationship between rate of uptake and substrate concentration (Büscher *et al.* 1990; Gjengedal and Steinnes 1990). Therefore, production of a hyperbolic curve in response to increasing nutrient concentration suggests that N and PO_4^{3-} uptake in *C. portentosa* involves active, energy-driven processes with catalytic properties

comparable to those observed for enzyme systems (Beever and Burns 1977; Chalot and Brun 1998). The identification of active nutrient uptake mechanisms in *C. portentosa* compares favourably with Farrar's (1976) finding that PO_4^{3-} uptake in *H. physodes* followed Michaelis-Menten kinetics. In addition, Tapper (1983) reported evidence of an active transport system for methylamine (an NH_4^+ analogue) in symbionts of *Cladonia convolute* and more recently, Crittenden (1996) reported that the uptake of NO_3^- in the Antarctic lichen *Usnea sphacelata* is oxygen dependent, and suggested the involvement of active processes.

Phosphate uptake in *C. portentosa* was measurable across a broad range of pH values (2.7 – 6.4), with an apparent optimum at pH 2.9. This is consistent with an apparent optimum pH of 2.5 for membrane-bound PME activity in the same species (Chapter 2). The optimum pH for PO_4^{3-} uptake in fungi and plants is typically around pH 5 (Beever and Burns 1980; Vance *et al.* 2003). However, it is likely to be lower in *C. portentosa* due to the presence of acidic 2^o metabolites, commonly associated with the cell wall (Ahti 1961; Thomson 1967; Houvinen and Ahti 1986; Osyczka 2006).

Phosphorus-starved fungi and vascular plants characterised by low cellular P concentrations rapidly increase their capacity for PO_4^{3-} uptake, and this is frequently combined with marked up-regulation of phosphatase activity (Clarkson and Scattergood 1982; Clipson *et al.* 1987; Thomson *et al.* 1990; Smith *et al.* 2003; Bücking 2004). For example, Kroehler and Linkins (1991) report that up to 70 % of the annual P requirement of *Eriophorum vaginatum* in P-limited tundra

ecosystems could be accounted for by the activity of root-surface phosphatases in association with rapid PO_4^{3-} uptake. Rates of PME activity in *C. portentosa* collected from sites differing in $\mathbf{N}_s$ were *c.* 50 times greater than the maximum recorded PO_4^{3-} uptake rates. This is in line with the findings of Bartlett and Lewis (1973), Chapin III *et al.* (1982) and Kroehler *et al.* (1988) who independently report that rates of phosphatase activity exceed those of PO_4^{3-} uptake in several mycorrhizal fungi. However, it is important to note that rates of PME activity and PO_4^{3-} uptake capacity in *C. portentosa* were measured under substrate saturating conditions in the laboratory. In oligotrophic habitats, *C. portentosa* is typically exposed to trace quantities of P in rainfall, with saturation of PME enzymes unlikely. As such, it seems reasonable to suggest that the rate of enzymatic PO_4^{3-} release rarely (if ever) exceeds the rate of PO_4^{3-} uptake in *C. portentosa* in the lichen habitat, thereby ensuring maximising PO_4^{3-} capture efficiency.

CHAPTER 8. Effect of N and P supply on PME activity in *Cladonia portentosa* in axenic culture

8.1 Introduction

The use of enzyme labelled fluorescence (ELF) as a tool to locate PME in *Cladonia portentosa* provided evidence to suggest that activity in intact thalli is located exclusively on either the fungal membrane or in the inner region of the fungal cell wall (e.g. Chapter 3). Stevenson (1994), using chromogenic staining techniques, also showed PME activity in lichens to be a property of the mycobiont. These results are consistent with the argument that the mycobiont is primarily responsible for nutrient capture in the lichen thallus (Smith 1975; Crittenden 1989; Pavlova and Maslov 2008).

The rate of PME activity in *C. portentosa* has been recorded between 0.6 and 5.5 mmol substrate hydrolysed g^{-1} dry mass h^{-1} (Chapters 4 – 6), consistent with data reported for other lichens, including *Cladonia rangiformis*, (1.2 to 5.0 mmol substrate hydrolysed g^{-1} dry mass h^{-1} (E. Hogan unpublished data)), *Stereocaulon alpinum*, (0.4 to 4.1 mmol substrate hydrolysed g^{-1} dry mass h^{-1}) and *Flavocetraria cucullata*, (0.015 to 2.5 mmol substrate hydrolysed g^{-1} dry mass h^{-1}, see Chapter 9) measured under similar assay conditions. However, PME activity in *C. portentosa* is commonly 10-1000 times greater than activity reported for plants and fungi, especially mycorrhizal symbioses and isolated fungi in culture (see Appendix 1). To date there are no reports of PME activity in lichen-forming

fungi in pure culture. Such data would confirm or otherwise the high capacity for PME in the lichen mycobiont. Given the intrinsically high capacity for PME activity in this lichen, it is desirable to investigate whether mycobiont cultures produce similar rates of PME activity to those recorded in intact lichen thalli. In addition, it is of interest to examine the response of PME activity in *C. portentosa* grown in axenic culture to variation in the availability of P.

Phosphomonoesterase activity in *C. portentosa* responds rapidly to change in nutrient supply. In particular, marked up-regulation of PME activity is associated with N enrichment and a strong negative relationship has been recorded between $[P]_{lichen}$ and PME activity (e.g. Chapters 4 - 5). The positive relationship between PME activity and N enrichment in *C. portentosa* has been attributed to this lichen experiencing a shift from N-limitation to P-limitation, as observed in other components of terrestrial vegetation (Aerts *et al.* 1992; Leith *et al.* 1999; Britton and Fisher 2007a). This proposal is supported by an increase in thallus N:P mass ratio under elevated N supply, and a tight coupling between PME activity and N:P mass ratio (see Chapters 4 - 5). The negative relationship between $[P]_{lichen}$ and PME activity is consistent with the observations of Stevenson (1994) for the lichens *Cladonia arbuscula*, *Peltigera canina* and *P. praetextata* and has been documented in several other plant/fungal systems (Beever and Burns 1980; Press and Lee 1983; Kroehler *et al.* 1988; Pasqualini *et al.* 1992; Joner *et al.* 2000).

The data presented in this chapter aims to address the following hypotheses. First, rates of PME activity in axenic cultures of *C. portentosa* are comparable to those

measured in intact thalli. Second, PME activity in axenic cultures is up-regulated in response to elevated N supply. Third, PME activity is repressed under conditions of elevated P supply and low N:P mass ratio. In addition, information regarding the growth of *C. portentosa* under different nutrient regimes is presented.

8.2 Materials and Methods

8.2.1 Collection of *Cladonia portentosa*

Fertile samples of *C. portentosa* (n = 5) bearing apothecia were selected from lichen cushions growing at The Halsary, Caithness (ND 195 493) on 8th November 2006 by E. J. Hogan, P. D. Crittenden and G. Minnullina. Replicate thalli were collected from distances > 10 m apart to minimise the possibility of sampling genetically identical material. Samples were transported to the laboratory in their naturally hydrated state in polythene bags. Lichens were then air dried for 24 – 48 h at room temperature and stored at -15 ^{o}C for 8 weeks. Powder free latex gloves were worn at all times when handling lichen tissue to minimise contamination.

8.2.2 Isolation and maintenance of cultures

Axenic cultures of *C. portentosa* mycobiont were obtained following the method of Ahmadjian (1993). Thalli were dissected into podetial tips bearing apothecia, and placed in sterile deionised water for 1 h to rehydrate tissue. Approximately

three rehydrated podetial tips from each lichen sample were transferred to a sterile flow-hood where they were blotted dry and attached to the underside of a Petri dish lid using petroleum jelly. This enabled discharge of ascospores onto an underlying surface of malt-extract, yeast-extract medium (MEYE) (Appendix 3). The distance between the lichen and the agar surface was approximately 5 mm. Plates were sealed with Nescofilm and incubated at 18 ^{o}C in the dark. The presence of any spores on the media was checked under a dissecting microscope after 48 h. If spores were observed, the lid of the Petri-dish was replaced with a new sterile lid and plates were returned to the incubation conditions described above. If there was no evidence of spores, this procedure was repeated every couple of days and if after 4 weeks spores were still not visible, plates were discarded. Incubating plates were inspected twice weekly for signs of contamination and where necessary, spores were transferred to fresh MEYE media. The polyspore colonies of *C. portentosa* grew to approximately 2 – 5 mm in diameter within 4 months at which time small agar blocks containing colonies were excised and transferred to MEYE agar slopes in 30 ml universal vials. This gave rise to approximately three slopes from each original lichen sample. The lids of the vials were closed loosely and sealed with Nescofilm permitting gaseous diffusion to occur. Slopes were maintained at 18 ^{o}C in the dark for 8 months, which is within the mycobiont lifetime on agar of 6 months to 1 year, reported by Bloomer *et al.* (1970). Figure 8.1 illustrates slope cultures of *C. portentosa* in axenic culture.

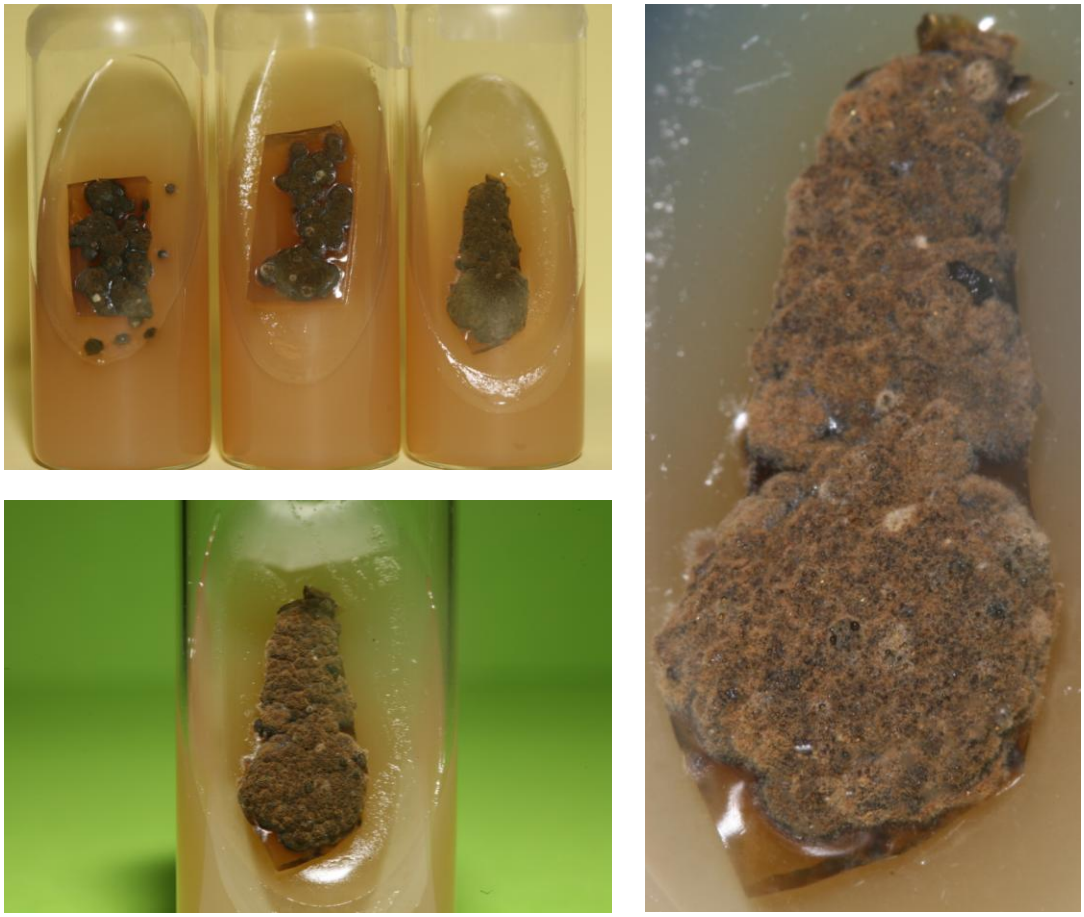


Figure 8.1 Axenic cultures of *Cladonia portentosa* growing on MEYE agar slopes

8.2.3 Growth experiment

Cultures were removed from slopes with a scalpel and cleaned free of any remaining agar. They were then ground in *c.* 12 ml sterile deionised water in a pestle and mortar and 0.5 ml of the resulting macerate was transferred to each of twenty 250 ml conical flasks containing 100 ml of a defined medium with adonitol as the C source (Galun 1988), and different concentrations of N and P. Nitrogen was supplied as NH_4NO_3 at the following initial concentrations ($[N]_{medium}$, $\mu g\ ml^{-1}$): 0.37, 2.72, 20.1 and 148, and P was supplied in the form KH_2PO_4 at

initial concentrations of 0.14, 0.37, 2.72, 20.1 and 148 µg ml^{-1} ($[P]_{medium}$) in full factorial combinations. Thiamine and biotin were supplied in all media along with all other required elements as inorganic salts (Appendix 4). Subsamples of fungal macerate (1, 1.5 or 3 ml) were pipetted into pre-weighed tin capsules and oven dried at 80 ^{o}C for 24 h in order to estimate inoculation mass. Samples were then re-weighed on a Cahn C-31 microbalance. High variation in inoculation masses was attributed to the inclusion of solid agar associated with fungal hyphae. Growth medium pH was initially 5.4 and was selected to be non-buffered in line with the findings of Turbin (1996) who reported reduced growth of mycobiont cultures in buffered liquid medium.

Mycobiont cultures in defined media were maintained at 18 ^{o}C for 15 weeks with weekly observations made for signs of contamination. Following the 15 week incubation period, growth was assessed by measuring the final mass of fungal mycelium in each flask. Cultures were removed by filtration through a nylon mesh filter of 150 µm pore size (Wilson Sieves, Nottingham, UK) with any excess solution removed from hyphal aggregates by blotting with tissue paper. Subsamples were removed for PME analysis (see section 8.2.4) with remaining mycelia centrifuged twice in 5 ml deionised water for 10 min at 3300 rpm (FISSONS Centaur 2 centrifuge). The supernatant was drawn off under vacuum and the mycelial pellet was freeze-dried over night before being oven-dried at 80 ^{o}C for 24 h and weighed. Freeze-dried samples were stored at -15 ^{o}C for up to 5 weeks until chemical analysis (see section 8.2.5).

8.2.4 Calculation of relative growth rate

Relative growth rate (RGR, mg mg^{-1} wk^{-1}) was calculated following Hunt (1982) (Eqn. 8.1).

$$RGR = \frac{(\ln m_{tx+1} - \ln m_{tx})}{(t_{x+1} - t_x)} \qquad \textbf{Equation 8.1}$$

where m is mass (mg) and t is time (wk)

8.2.5 Phosphatase assay

Phosphomonoesterase activity was measured following the *p*NPP method, described by Turner *et al.* (2001) and outlined in section 2.2.4. Hyphal aggregates were added to 2.9 ml 0.01 M citric acid tri-sodium citrate buffered assay medium (pH 5.9 unless otherwise stated) made up in simulated rainfall. Assays were initiated by the addition of 0.1 ml *p*NPP to yield a final concentration of 3 mM (unless otherwise stated) in glass test tubes. Samples were then capped and placed in a shaking water-bath at 15 ^{o}C for 20 min in the dark, after which the reaction was terminated by transferring 2 ml assay medium into 0.25 ml terminator solution (1.1 M NaOH, 27.5 mM EDTA, 0.55 M K_2HPO_4) and the absorbance measured at 405 nm using a NanoDrop ND-1000 spectrophotometer (Labtech International Ltd, Ringmer, East Sussex). Samples were blotted dry, centrifuged and freeze-dried as outlined in section 8.2.3. Enzyme activity is expressed as mmol substrate hydrolysed g^{-1} dry mass h^{-1} using *p*-nitrophenyl to calibrate the

assay. 'No-fungus' controls were performed alongside each set of assays with data corrected for any non-enzymatic hydrolysis of *p*NPP.

8.2.5.1 Influence of assay medium pH on PME activity

The citric acid tri-sodium citrate buffered assay medium was adjusted to the pH values; 2.5, 2.8 (0.02 M), 3.2, 3.9, 4.3, 4.6, 5.3, 5.9 and 6.6 (0.01 M) made up in simulated rainwater following the method outlined in section 2.2.4.1. Assays were performed on cultures grown in liquid MEYE using a final substrate concentration of 0.5 mM. Final assay pH measurements were made using an IQ125 miniLab pH meter (IQ Scientific Instruments, Inc, Carlsbad, USA).

8.2.6 Determination of total N and P concentration

Pre-weighed freeze-dried samples were digested in 0.5 ml 1:1.2 (v/v) mixture of H_2O_2 and H_2SO_4 at 375 ^oC for 1 h following Allen (1989). All digests were diluted to 15 ml with deionised water before analysis. Blank control digests were performed without any lichen material. Ammonium-N in the digest was determined using the fluorometric method of Holmes (1999). 0.3 ml sample digest was diluted with 0.2 ml deionised water, from which 20 or 100 µl subsamples were made up to 500 µl with control blank digest. The resulting 500 µl sample was added to 10 ml borate buffer (40 g sodium tetraborate in 1 l deionised water) or 10 ml working reagent (1 l borate buffer, 5 ml 1 mM sodium sulfite and 50 ml 0.75 mM orthophthaldialdehyde (OPA) in ethanol) and analysed for total N content following the method outlined in Chapter 4, Section 4.2.5.

Phosphorus was assayed by the malachite green variant of the methylene blue method after Van Veldhoven and Mannaerts (1987). 1.8 ml sample digest was diluted with 1.2 ml deionised water following which either 0.1 or 0.5 ml subsamples were removed and added to 2.9 or 2.5 ml blank control digest, respectively. 0.6 ml reagent A (1.75 % (w/v) $(NH_4)_6Mo_7O_{24}.4H_2O$ in 4.25 N H_2SO_4) and 0.6 ml reagent B (0.035 % (w/v) malachite green, 0.35 % (w/v) polyvinyl alcohol (PVA) in 1 l dH_2O) were added to samples at 10 min intervals. Samples were analysed colourimetrically following the method outlined in Chapter 4, Section 4.2.5.

8.2.7 Statistical analyses

Data were initially subjected to normality tests and also to determine homogeneity of variances. As these assumptions were not violated, data were subjected to linear regression analysis or one-way analysis of variance (ANOVA).

8.3 Results

8.3.1 Effect of pH on PME activity

Axenic cultures of *C. portentosa* had measurable PME activity across the entire pH range tested (Fig. 8.2). Although ANOVA revealed no significant effect of pH (P = 0.094), maximum activity was recorded at pH 5.9 (Fig. 8.2), a value similar to the starting pH value of the growth medium (pH 5.4). This value was selected for PME analysis in subsequent investigations.

8.3.2 Effect of nutrient supply on growth

Relative growth rate (RGR) ranged from 0.02 ± 0.02 to 0.14 ± 0.01 mg mg^{-1} wk^{-1}. RGR was significantly positively related to the concentration of N in the media (Fig. 8.3), but no distinct relationships were apparent between RGR and either $[P]_{medium}$ (r^2 = 0.014, P = 0.615) or $([N]/[P])_{medium}$ (r^2 = 0.005, P = 0.760) (data not shown).

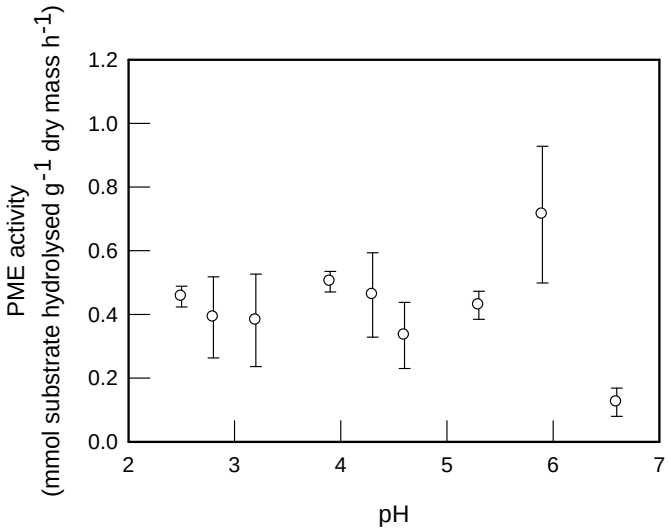


Figure 8.2 The effect of pH on PME activity in axenic cultures of *Cladonia portentosa*. Assays were performed with 0.5 mM *p*NPP for 20 min at 15 ^{o}C in the dark. Samples were isolated from lichen collected at the Halsary, Caithness. Plotted values are means (n = 4 - 5) ± 1 SEM.

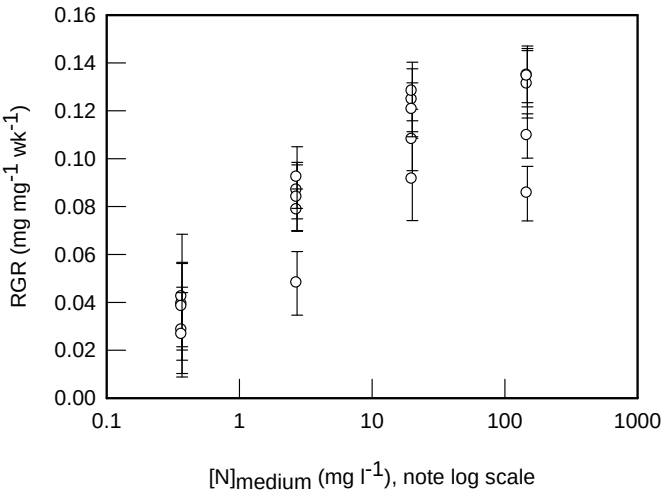


Figure 8.3 Relationship between the initial concentration of N in defined media and relative growth rate (RGR) of axenic cultures of *Cladonia portentosa* during a 15 week period (r^2 = 0.340, P = 0.007). Cultures were grown at 10 ^{o}C in the dark. Plotted values are means (n = 5) ± 1 SEM.

Cultures of *C. portentosa* grown in flasks with the maximum concentrations of N and P (148 µg l^{-1}) achieved 38 ± 4.7 mg new growth and had amongst the highest RGR (0.134 ± 0.01 mg mg^{-1} wk^{-1}) over the 15 week study period. After 15 weeks growth, mycelia grown under such conditions were characterised by large light-brown coloured aggregates (Fig. 8.4b). In contrast, mycelia grown in low nutrient media (0.37 µg l^{-1} N, 0.14 µg l^{-1} P) developed into small, dark aggregates and the media developed a yellow colour (Fig. 8.4a).

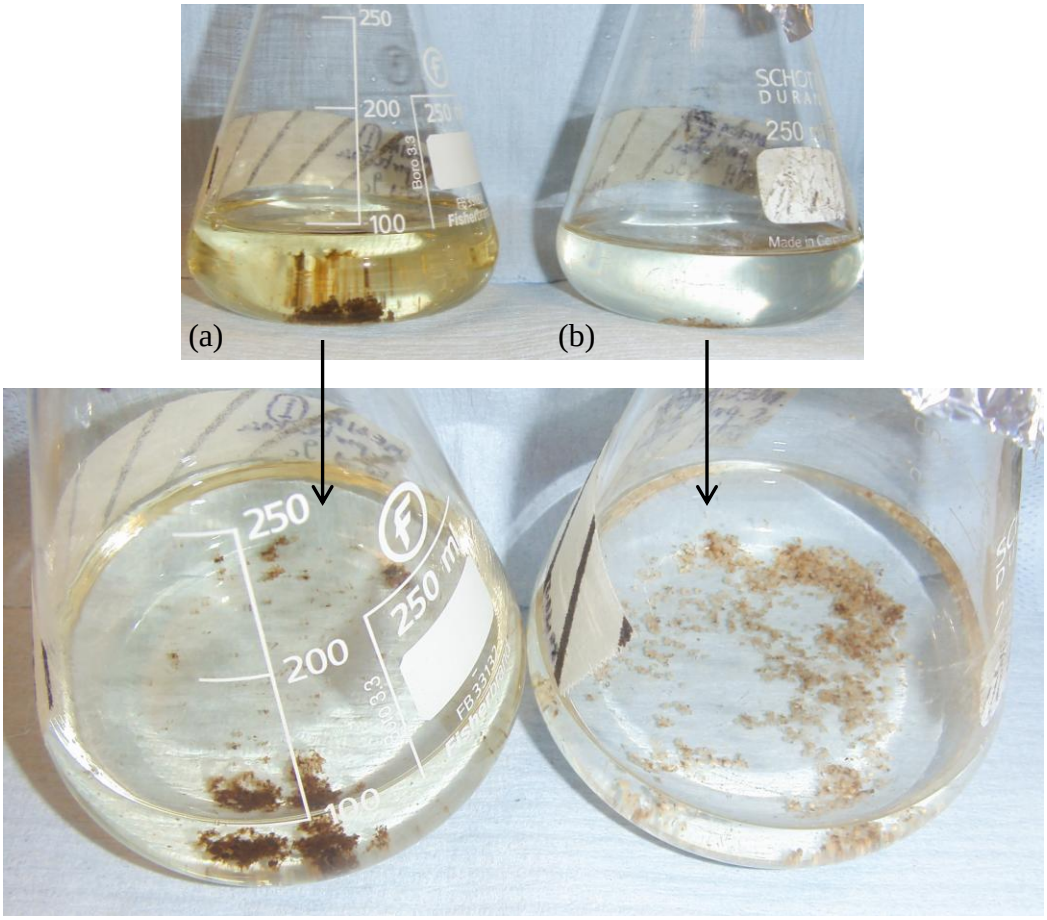


Figure 8.4 Axenic cultures of *C. portentosa* grown in defined media containing 0.37 µg ml^{-1} N, 0.14 µg ml^{-1} P (a) and 148 µg ml^{-1} N, 148 µg ml^{-1} P (b). Samples were grown at 10 ^{o}C for 15 weeks in the dark.

The final pH of the medium was highly negatively related to the initial N concentration, decreasing from 4.9 to 2.8 in flasks receiving 0.37 and 148 μg ml^{-1} N, respectively (Fig. 8.5). There was no relationship between final medium pH and $[P]_{medium}$ or $([N]/[P])_{medium}$. Relative growth rate was significantly positively correlated to mycelial N concentration ($[N]_{myc}$) (Fig. 8.6), but not to either mycelial P concentration ($[P]_{myc}$, $r^2 = 0.073$, $P = 0.249$) or mycelial N:P ratio ($([N]/[P])_{myc}$, $r^2 = 0.014$, $P = 0.614$) (data not shown).

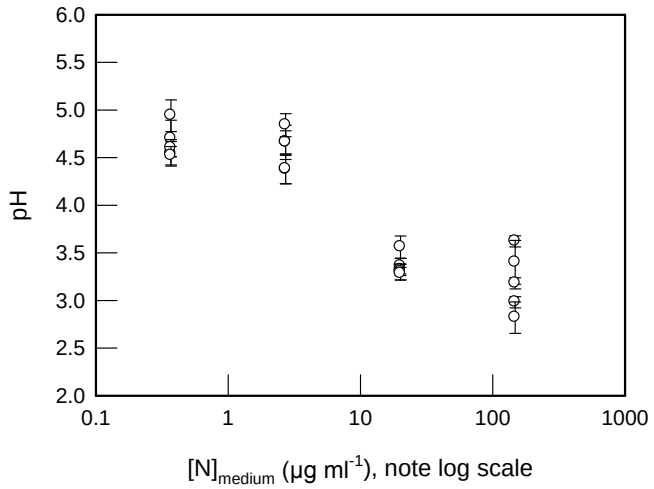


Figure 8.5 Relationship between the concentration of N in defined media and the final pH value of the growth medium ($r^2 = 0.444$, $P = 0.001$). Plotted values are means ($n = 5$) ± 1 SEM.

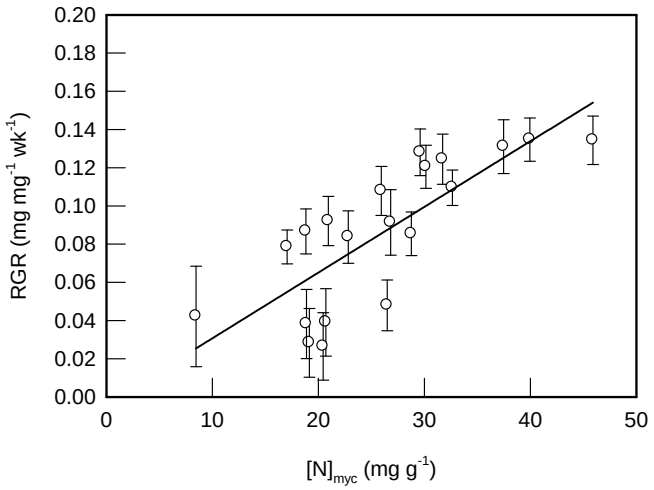


Figure 8.6 Relationship between mycelial N concentration and relative growth rate in axenic cultures of *Cladonia portentosa* (r^2 = 0.674, P < 0.001). Samples were grown at 18 ^{o}C for 15 weeks in the dark. Plotted values are means (n = 5) ± 1 SEM.

8.3.3 Effect of nutrient supply on mycelium nutrient concentration and PME activity

The concentration of N in the defined media was significantly positively related to $[N]_{myc}$ but not to either $[P]_{myc}$ or $([N]/[P])_{myc}$ (Table 8.1). There was no significant relationship between $[P]_{medium}$ and lichen chemistry, and $([N]/[P])_{medium}$ was only significantly related to $([N]/[P])_{myc}$ (Table 8.1).

Table 8.1 Linear regression analyses (r^2) between [N], [P] and ([N]/[P]) in defined media and $[N]_{myc}$, $[P]_{myc}$ and ([N]/[P]) in axenic cultures of *C. portentosa*.

	$[N]_{myc}$	$[P]_{myc}$	$([N]/[P])_{myc}$
$[N]_{media}$	0.609***	0.032	0.025
$[P]_{media}$	0.043	0.123	0.096
$([N]/[P])_{media}$	0.023	0.183	0.270*

* -correlation is significant at the 0.05 level
*** - correlation is significant at the 0.001 level

Phosphomonoesterase activity in axenic cultures of *C. portentosa* ranged between 0.3 ± 0.05 and 7.1 ± 0.5 mmol substrate hydrolysed g^{-1} dry mass h^{-1}. Activity was significantly positively related to $[N]_{medium}$ (Fig. 8.7a), weakly negatively related to $[P]_{medium}$ (Fig. 8.7b) and highly significantly related to $([N]/[P])_{medium}$ (Fig. 8.7c). Activity increased rapidly to *c.* 7 mM substrate hydrolysed g^{-1} dry mass h^{-1} as $([N]/[P])_{medium}$ ratio reached a value of *c.* 54, after which a further increase in $([N]/[P])_{medium}$ ratio to 1057 had no further effect (Fig. 8.7c).

8.3.4 Relationships between mycelium nutrient concentration and PME activity

PME activity was significantly negatively related to $[P]_{myc}$, decreasing by a factor of *c.* 20 between thallus P concentrations of 10.3 ± 0 and 2.1 ± 0.32 mg g^{-1} (Fig. 8.8b). Activity was highly significantly positively related to $([N]/[P])_{myc}$, and maximum PME activity was measured at $([N]/[P])_{myc}$ values > 13 (Fig. 8.8c). However, there was no relationship between $[N]_{myc}$ and PME activity (Fig. 8.8a).

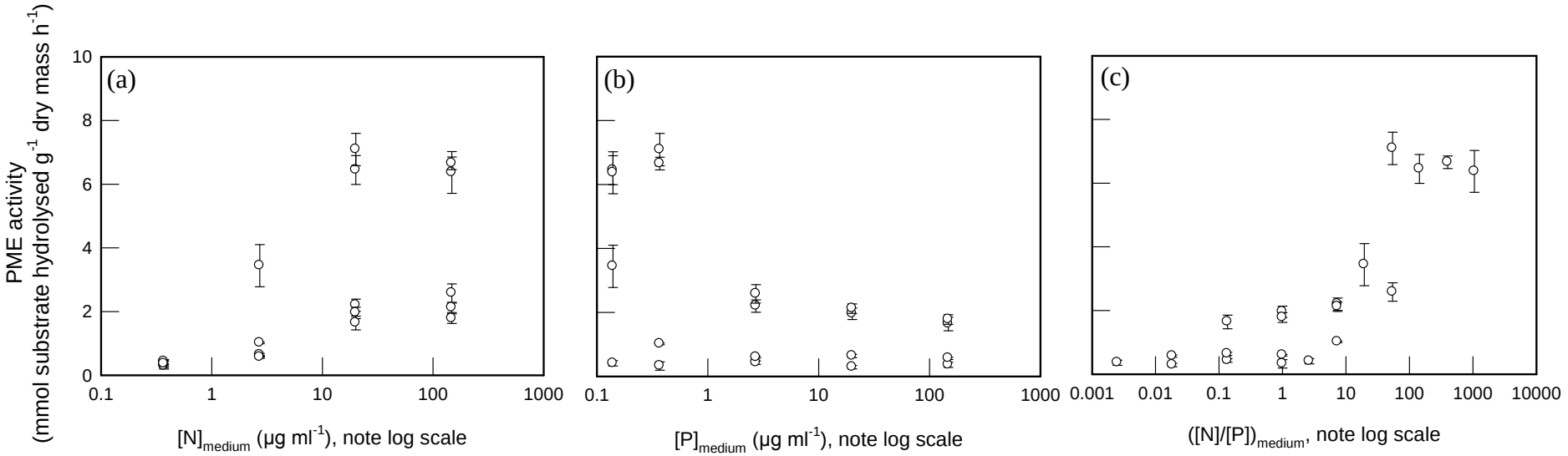


Figure 8.7 Relationships between PME activity in axenic cultures of *Cladonia portentosa* and the growth medium N concentration (a, r^2 = 0.217, P = 0.038); P concentration in medium (b, r^2 = 0.096, P = 0.185); N:P ratio (c, r^2 = 0.398, P = 0.003). Assays were performed with 3 mM *p*NPP for 20 min at 15 ^{o}C in the dark. Plotted values are means (n = 5) ± 1 SEM.

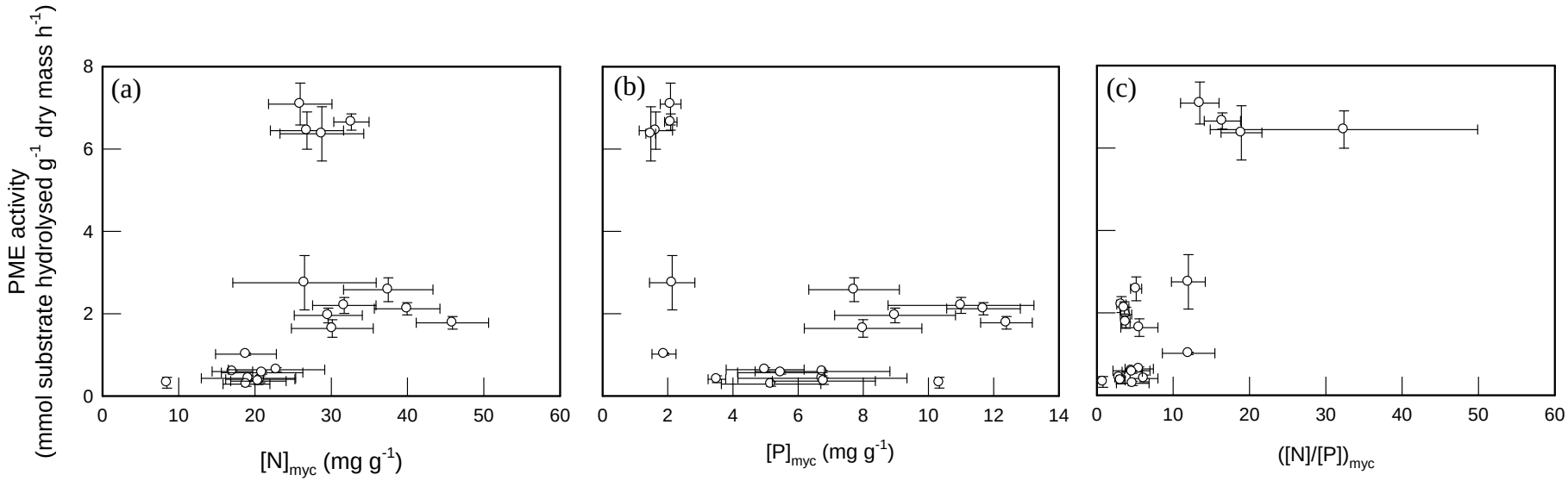


Figure 8.8 Relationships between PME activity in axenic cultures of *Cladonia portentosa* and mycelial N concentration (a, r = 0.380, P = 0.098); P concentration (b, r = -0.506, P = 0.023); N:P mass ratio (c, r = 0.801, P < 0.001). PME assays were performed with 3 mM *p*NPP for 20 min at 15 ^{o}C in the dark. Plotted values are means (n = 5) ± 1 SEM.

8.4 Discussion

Cladonia portentosa in axenic culture is associated with significant PME activity. Activity ranged between 0.3 ± 0.05 and 7.1 ± 0.5 mmol substrate hydrolysed g^{-1} dry mass h^{-1}, depending upon the quantities of N and P supplied. This level of activity is consistent with those measured in intact thalli (under similar assay conditions), which range from 0.6 to 5.5 mmol substrate hydrolysed g^{-1} dry mass h^{-1} (see Chapters 4 – 6). It also compares favourably with an unpublished study in which PME activity in axenic cultures of *C. portentosa* grown under similar conditions ranged between 0.19 and 1.14 mmol substrate hydrolysed g^{-1} dry mass h^{-1} (Z. Mtshali, Pers. Comm.). This high degree of consistency between rates of PME activity measured in intact lichen thalli and in mycobiont cultures provides further support for the proposal that PME activity in *C. portentosa* is dominated by, if not exclusive to, the mycobiont.

Phosphomonoesterase activity was positively associated with $[N]_{medium}$. This is consistent with the results of Kieliszewska-Rokicka (1992) for cultured isolates of ectomycorrhizal fungi, and with data on intact lichen thalli presented in this thesis, where both external N availability and $[N]_{lichen}$ are strongly positively related to PME activity (e.g. Chapters 4 - 6). It is instructive to interpret the present data for *C. portentosa* in axenic culture together with those for intact lichen thalli, where up-regulation of PME activity in response to elevated N supply is associated with increased N:P mass ratio. Following Güsewell (2004), this is consistent with *C. portentosa* experiencing a shift from N-P co-limitation to a P-limited state, which

for terrestrial vegetation, is reported to occur at N:P values > 20. While values of $([N]/[P])_{medium}$ ratio were artificially manipulated between 0.0025 and 1057, values of $([N]/[P])_{myc}$ ratio ranged only from 0.8 to 32. Fungal N:P mass ratio was positively related to PME activity, with maximum activity (7.1 ± 0.5 mmol substrate hydrolysed g^{-1} dry mass h^{-1}) recorded in mycelium with an $([N]/[P])_{myc}$ ratio value of 13.5. As $([N]/[P])_{myc}$ ratio increased to 32, no further significant change in PME activity was observed, suggesting that the maximum rate of PME activity in *C. portentosa* had been achieved under the constraints of this experiment. These data imply that an $([N]/[P])_{myc}$ ratio of 13.5 represents a critical value at which there is a shift from N-P co-limitation to a P-limited state, and that this change corresponds to a much lower value of N:P mass ratio than reported for thalli of *C. portentosa* ($([N]/[P])_{lichen}$ of 20, Chapters 4 – 5). Cultures with the lowest measured $([N]/[P])_{myc}$ ratio (< 3.1) probably represent an N-limited state and were associated with the lowest measured rates of PME activity. Data from this experiment suggests that increased PME activity in axenic cultures of *C. portentosa* is a response to P-deficiency, not to the direct availability of N. This is consistent with data from *C. portentosa* lichen thalli (Chapters 4 - 5), and with other plant-fungal systems (Johnson *et al.* 1999; Phoenix *et al.* 2003).

Phosphomonoesterase activity was negatively related to $[P]_{myc}$ and $[P]_{medium}$, with $[P]_{myc}$ proving the stronger correlate. These data compare favorably with the strong negative relationship recorded between $[P]_{lichen}$ and PME activity in *C. portentosa* collected from several sites in the British Isles with different N deposition values (see Chapter 4). In addition, the data are consistent with the

unpublished data set of Z. Mtshali (Pers. Comm.) who recorded a 32 % decrease in PME activity in line with increasing $[P]_{medium}$ between 10 and 100 mM KH_2PO_4 in axenic cultures of *C. portentosa*. This observation lends support to the suggestion that PME activity in *C. portentosa* is repressible and controlled by internal P concentration. Furthermore, it is in line with similar inverse relationships reported for other fungal species (Gianinazzi-Pearson and Gianinazzi 1978; Beever and Burns 1980; Bousquet *et al.* 1985; Kroehler *et al.* 1988) considered to result from negative feedback mechanisms (Alexander and Hardy 1981). Nonetheless, it is important to consider that Dighton (1983) reported no universal relationship between phosphatase activity and the concentration of P in mycorrhizal fungi isolates, which might imply the presence of constitutive PME enzymes in some fungal species.

The apparent optimum pH for PME activity in axenic cultures of *C. portentosa* (pH 5.9) was *c.* 3 units higher than that recorded for intact thalli (pH 2.5). The low optimum pH value for PME activity in this lichen was attributed to the production of acidic 2^o compounds within the thallus (e.g. Chapter 2). Axenic cultures of lichen-forming fungi usually produce much lower concentrations of 2^o metabolites than those in intact thalli (Cocchietto *et al.* 2002), and it is possible that phenotypic variation in the activity of PME enzymes might explain a higher optimum pH for activity in axenic cultures of *C. portentosa* when compared with lichen thalli. Values of final assay medium pH were up to 2 times lower than the initial pH (5.4), consistent with the findings of Turbin (1996), who reported that

the growth of *Cladonia floerkeana* in axenic culture consistently lowered the pH of liquid media.

Relative growth rates calculated for mycobiont cultures of *C. portentosa* ranged from 0.02 ± 0.02 to 0.13 ± 0.01 mg mg^{-1} wk^{-1}, and can be compared favourably with published growth rates for axenic lichen mycobionts grown in defined liquid media (0.10 ± 0.05 - 0.62 ± 0.05 mg mg^{-1} wk^{-1}, Turbin 1996). Relative growth rates of axenic mycobiont cultures were up to an order of magnitude greater than those calculated for thalli of *C. portentosa* (0.008 ± 0.001 – 0.02 ± 0.001 mg mg^{-1} wk^{-1}, Chapter 6). This is in line with the findings of Turbin (1996) who reported the RGR for *Stereocaulon paschale* mycobiont cultures and intact thalli as 0.28 and 0.042 mg mg^{-1} wk^{-1}, respectively. This variation might reflect a greater nutrient availability for mycobiont cultures. Growth of *C. portentosa* in axenic culture was consistently lower than that reported for cultures of free-living fungi in liquid media, which can achieve RGRs between 1.7 – 11.1 mg mg^{-1} wk^{-1} (Turbin 1996). Slow growth in culture is a feature of fungi which form symbiotic associations, e.g. lichenicolous fungi (Hawksworth and Jones 1981; Lowen *et al.* 1986), ericaceous fungi (Cooke 1977) and vascular plant endophytes (Clay 1989). Low growth rates of lichen mycobionts might be genetically predisposed (as evidenced by the slow growth of lichen thalli and other mycobionts in culture) (Rogers 1990, Ahmadjian 1993, Crittenden *et al.* 1995, Turbin 1996). Alternatively, lichen-forming fungi might invest a larger proportion of energy to physiological processes which do not increase mass, such as cell maintenance or secondary metabolite production (Turbin 1996).

CHAPTER 9. Relationships between N_2-fixing capacity and phosphatase activity in lichens

9.1 Introduction

In chapters 4 and 5, I have shown that lichen N concentration is strongly positively correlated with N availability. In *Cladonia portentosa*, thallus N concentration in the apical 5 mm ($[N]_{apex}$) increased by *c*. 28 % with increasing N deposition from 4.1 to 32.8 kg N ha^{-1} y^{-1}; while under experimental conditions, application of 64 kg N ha^{-1} y^{-1} increased $[N]_{apex}$ to 14.3 mg g^{-1} from 11.9 mg g^{-1} (recorded in control treatments receiving 8 kg N ha^{-1} y^{-1}) (see Chapters 4 – 5). In this lichen, N enrichment is also associated with up-regulation of PME activity and PO_4^{3-} uptake capacity, both of which are considered to result from an increased P demand invoked by high N availability.

Lichens that contain a cyanobacterium as a photobiont fix nitrogen and thus have an internal supply of combined N. N_2-fixing symbioses have an increased P demand compared to non-N_2-fixing equivalents (Kennedy and Cocking 1997; Vance *et al.* 2000), as N_2-fixiation is an 'energy-intensive' process requiring P in the form of *c*. 16 ATP molecules to convert 1 molecule of N_2 to 2 molecules of NH_3 (Sprent and Sprent 1990; Postgate 1998). Phosphorus fertilization has been shown to up-regulate nitrogenase activity in N_2-fixing lichens, and is commonly associated with marked increases in lichen growth and abundance (Weiss *et al.* 2005; Benner *et al.* 2007; McCune and Caldwell 2009). For example, Benner *et*

al. (2007) reported a significant increase in nitrogenase activity in the lichen *Pseudocyphellaria crocata* from 20 to 440 nmol C_2H_2 g^{-1} h^{-1} in response to the addition of 100 kg P ha^{-1} y^{-1}, and McCune and Caldwell (2009) showed that annual biomass growth in the N_2-fixing lichen *L. pulmonaria* doubled when thalli were submersed in 600 mg l^{-1} KH_2PO_4 for 20 min. It was therefore of interest to investigate whether N_2-fixing lichens with elevated thallus N concentrations also have high levels of PME activity as a means of providing sufficient P to support nitrogenase activity.

There is interspecific variation amongst cyanobacterial lichens in the capacity for N_2-fixation, which in turn is reflected in different values of $[N]_{lichen}$ (Palmqvist *et al.* 2002). For example, nitrogenase activity in *Peltigera scabrosa* has been recorded at 3138 ± 182 nmols C_2H_4 g^{-1} h^{-1} and this species contains *c.* 11 times more thallus N than *Stereocaulon alpinum*, a species capable of nitrogenase activity up to 276 ± 66 nmols C_2H_4 g^{-1} h^{-1} (Crittenden 1975; P. D. Crittenden, unpublished data). Accordingly, in this chapter I investigate the relationship between N_2-fixation and PME capacity in several lichens collected from a subarctic environment, selected because it receives low levels of atmospheric pollutants and supports a wide diversity and abundance of N_2-fixing lichen species.

9.2 Materials and Methods

9.2.1 Study sites

Field work was conducted in the vicinity of the Abisko Scientific Research Station, (68°21'N, 18°50'E), an institution belonging to the Royal Swedish Academy of Sciences and which lies approximately 200 km above the Arctic Circle in the Torneträsk region of northern Swedish Lapland (Fig. 9.1) (Malmer and Wallén 1996). The vegetation is classified as subalpine forest which is dominated by *Betula pubescens* ssp. *tortuosa* (Fig. 9.2), with other deciduous trees including *Alnus incana*, *Populus tremula*, and *Salix spp*. (Berglund *et al.* 1996). The forest understorey layer (< 30 cm) is characterised by unevenly distributed shrubs (including *Empetrum hermaphroditum*, *Vaccinium myrtillus*, *V. vitis-idaea*), grasses (*Deschampsia caespitose*, *D. flexuosa*), mosses (*Hylocomium splendens*, *Polytrichum commune*) and lichens (e.g. *Cladonia spp.*, *Peltigera spp.*) (Ovhed and Holmgren 1996).

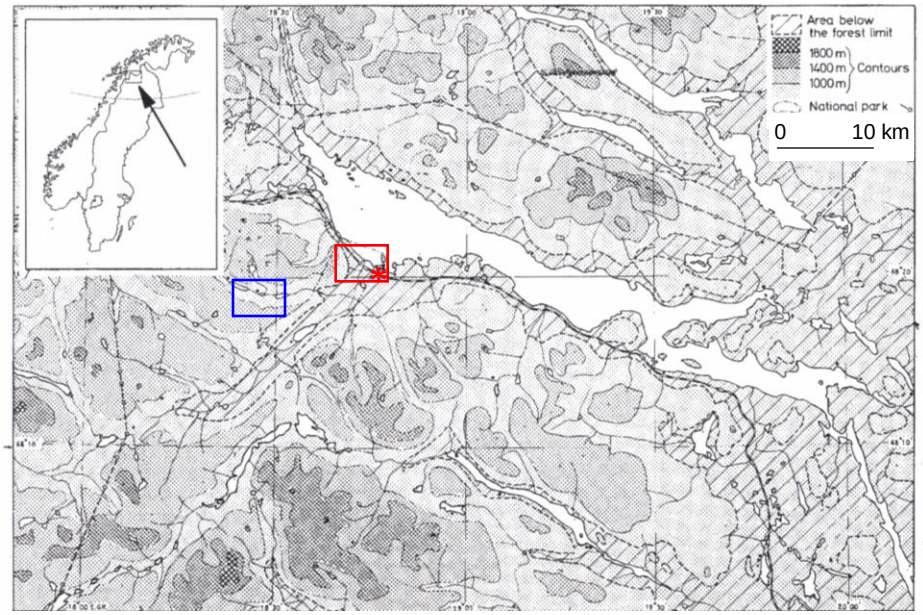


Figure 9.1 Location of the Abisko Scientific Research Station (*) and two study sites: sub-arctic birch forest surrounding the research station (red box) and the slope of Mt. Njulla (blue box). Edited from Andersson *et al.* (1996).

Lichen samples were collected from two study sites. The first was in subalpine mountain birch forest surrounding the research station (*c.* 2 km radius from the station between 67.3733°N, 18.7167°E and 67.3567°N and 18.3333°E, *c.* 380 m asl) (Figs. 9.1 and 9.2). The ground vegetation beneath the birch canopy was characterised by dwarf shrubs, mosses and lichens (see species list above), and could be classified as an *Empetrum-Vaccinium* type (Sonesson and Lundberg 1974). Frequently, the forest was fragmented by patches of tree-less subalpine heath (Fig. 9.3), dominated by shrubs, mosses and lichens. The second was on the eastern slope of Mt Njulla (68°3625'N, 18°7222'E), in a mid-alpine zone above the tree-line at altitudes of *c.* 700-950 m asl (Fig. 9.1). Here the vegetation was alpine-heath with plants including *Arctostaphylos alpinum*, *E. nigrum*, *V. myrtillus*

and *V. vitis-idaea* together with species of gramminoid, moss and lichen. Patches of snow persist into the summer with associated snow-melt patches.

Figure 9.2 Subarctic mountain birch forest dominated by *Betula pubescens* ssp. *tortuosa* surrounding the Abisko Scientific Research Station, Swedish Lapland.

9.2.2 Collection and pre-treatment of lichens

Lichens were collected during two seasons of fieldwork. The first during 28th June – 4th July 2006 where samples were collected with the assistance of P. D. Crittenden and the second during 17th – 23rd August 2007 where collections were made with J. Whiteford. During summer 2006, replicate samples (n = 10) of the terricolous non-N_2-fixing lichens *Cladonia rangiferina*, *Flavocetraria cucullata*, and the N_2-fixing lichens *Lobaria linita*, *Nephroma arcticum*, *Peltigera aphthosa*, *P. scabrosa*, *P. malacea* and *Stereocaulon paschale* (Fig. 9.4) were collected from the forest study site.

Figure 9.3 Area of open heathland in the sub-alpine birch forest surrounding the Abisko Scientific Research Station, Swedish Lapland.

Samples were collected either from inter-tree locations in the forest to minimise canopy through-fall effects or from heathland patches (Fig. 9.3). In all cases, each replicate of the same species was taken from a location ≥ 10 m apart from the others. Replicate collections (n = 10) of *F. cucullata* (non-N_2-fixing), *Solorina crocea, Stereocaulon alpinum* and *S. paschale* (N_2-fixing) (Fig. 9.4) were made at the alpine site. During 2007, all of the afore-mentioned lichen species were collected at both study sites, with the exception of *S. crocea* which was only collected from the alpine site where it occurs in areas irrigated in summer by meltwater from late lying snow patches. For comparative purposes, 10 replicate

samples of the N_2-fixing lichen *S. vesuvianum* were collected from two sites in North Wales: Llyn Llagi (SH 647 481) on 15th September 2006 and a disused slate quarry near Beddgelert Forest (SH 548 505) on 14th February 2008. *Stereocaulon vesuvianum* is a saxicolous species forming cushions on nutrient-poor rock and was selected because rates of N_2-fixation available for this species are comparatively low. Samples of *Peltigera praetextata* (*n* = 5) were collected from Chee Dale Valley, Derbyshire (GR SK124 735) on 04.06.07 for preliminary investigations comparing PME, PDE and 5´-nucleotide phosphodiesterase (5´ ND) activities.

Lichen samples were returned to the laboratory at Abisko (and *S. vesuvianum* to The University of Nottingham) in either sealed 100 ml vials or polythene bags where they were cleaned free of extraneous debris and left to air dry on the open bench for 24 – 48 h. Air-dry thalli were then packaged in either polythene bags or plastic containers and stored at -15 ^{o}C. Powder free latex gloves were worn at all times when handling lichens both in the field and in the laboratory to minimize contamination.

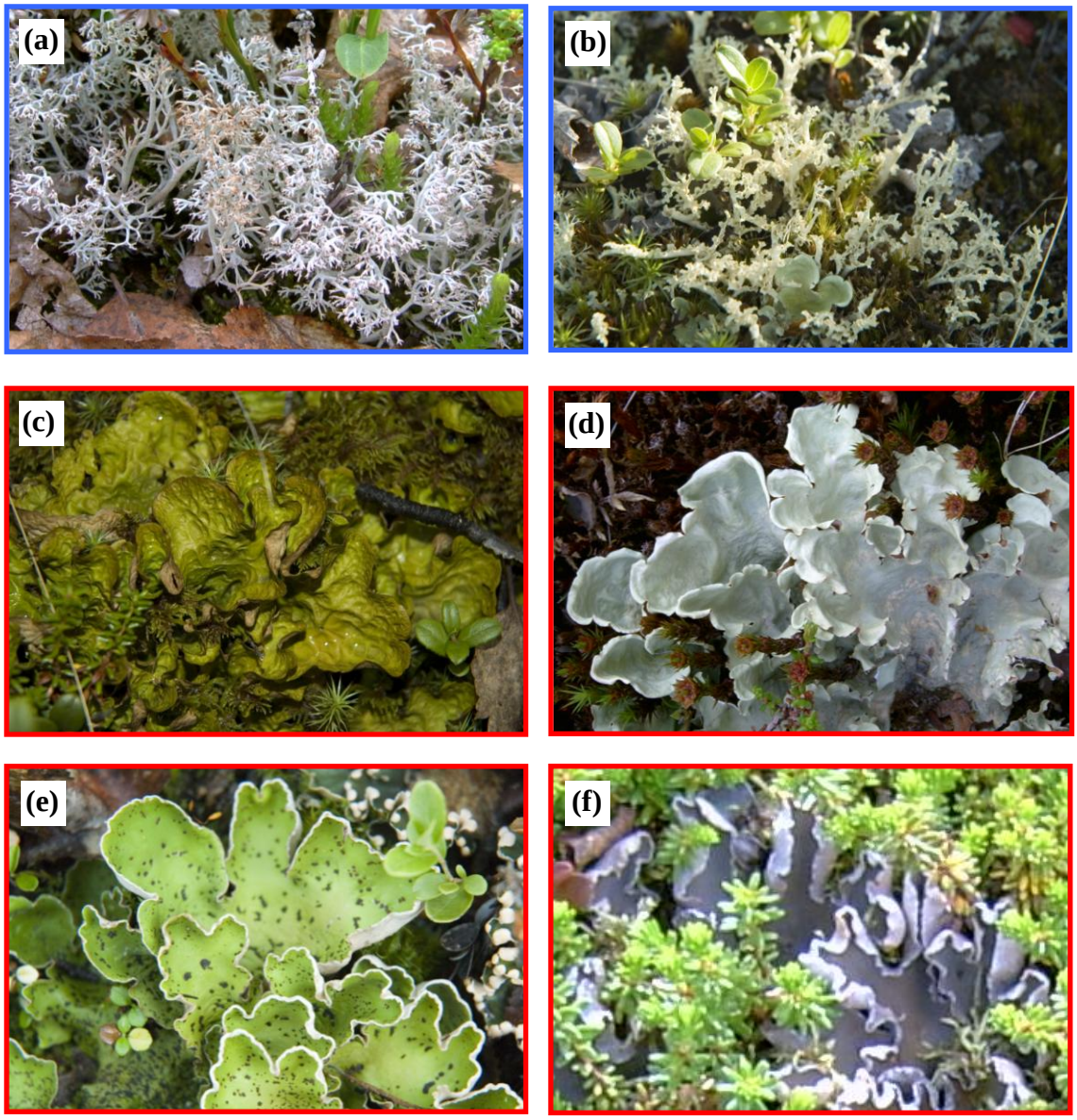


Figure 9.4 Lichen species selected for phosphatase assays: *Cladonia rangiferina* (a), *Flavocetraria cucullata* (b), *Lobaria linita* (c), *Nephroma arcticum* (d), *Peltigera aphthosa* (e), *Peltigera malacea* (f), *Peltigera scabrosa* (g), *Solorina crocea* (h), *Stereocaulon alpinum* (i), *Stereocaulon paschale* (j), *Stereocaulon vesuvianum* (k). Non-N_2-fixing lichens are bordered in blue, whilst N_2-fixing lichens are bordered in red.

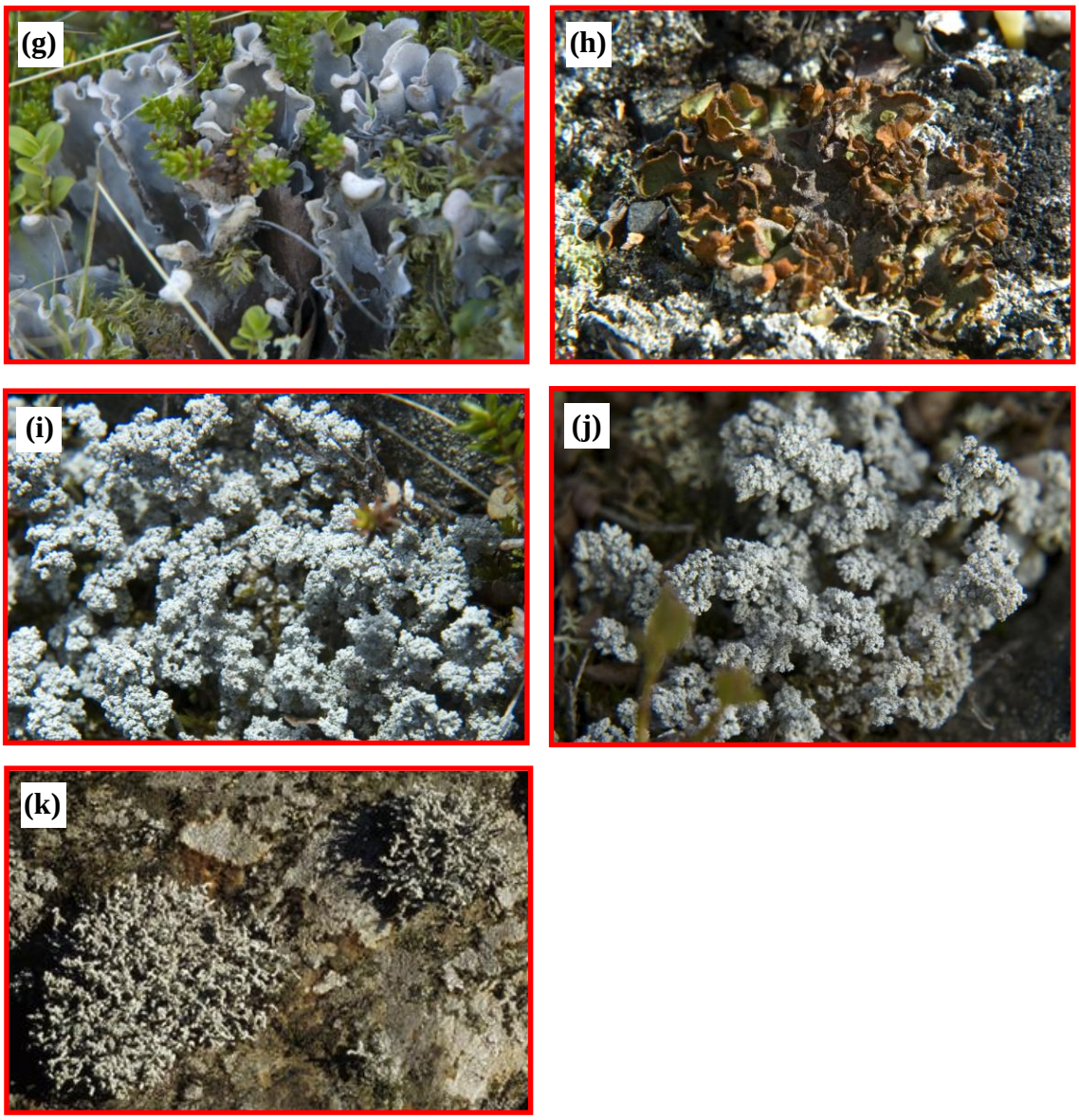


Figure 9.4 continued

9.2.3 Sample preparation

Thalli were rehydrated overnight at 10 ^{o}C in water-saturated air (over water in a desiccator), sprayed lightly with deionised water and cleaned of any remaining debris. In the case of the fruticose species *C. rangiferina*, *F. cucullata*, *S. alpinum*

S. paschale and *S. vesuvianum*, the apical 10 mm of thalli were selected for analysis by cutting with a razor blade at a distance of 10 mm downwards from the apices. In the case of the foliose species *L. linita, N. arcticum, P. aphthosa, P. scabrosa* and *P. malacea*, the marginal region of the thallus lobe was selected for assays by cutting at 10 mm behind the margin. Where required, annuli were also cut at the following distances from the margins inwards to the centre of the thallus: 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 mm. All samples dissected in this way were assayed immediately while still in the hydrated state.

9.2.4 Phosphatase assays

Phosphomonoesterase and phosphodiesterase activities were determined following the *p*NPP and bis-*p*NPP methods respectively, adapted from Turner *et al.* (2001) as outlined in section 2.2.4. Lichen samples were added to 2.9 ml 0.01 M (pH 2.5, 2.8) or 0.02 M (pH 3.2, 3.9, 4.3, 4.6, 5.3, 5.9 and 6.6) citric acid tri-sodium citrate buffered assay medium made up in simulated rainwater as described in section 2.2.4. For PME assays, the reaction was initiated by the addition of 0.1 ml *p*NPP to yield a final concentration of either 0.5 mM or 8 mM or other values where stated. For PDE assays with a final substrate concentration of 0.5 mM, reactions were initiated by the addition of 0.1 ml bis-*p*NPP to 2.9 ml 0.01 M buffered assay medium whilst for assays with a final substrate concentration of 8 mM, 1 ml bis-*p*NPP was added to 2 ml 0.1 M buffered assay medium. The 5´-nucleotide phosphodiesterase assay followed an adapted version of the *p*-nitrophenyl phenylphosphonate (*p*NPPP) method described by Leake and Miles (1996). For

initial investigations of pH effects, lichen samples were added to 2.9 ml 0.01 M or 0.02 M buffered assay medium (as outlined above) with reactions initiated by the addition of 0.1 ml, 0.5 mM *p*NPPP. Assays involving a final substrate concentration > 0.5 mM were performed by adding thalli to 2 ml 0.2 M buffered assay medium and initiating the reaction with 1 ml *p*NPPP. However, one draw-back associated with this method is that at high substrate (saturating) concentrations, there is a marked reduction in the sensitivity of the assay; i.e. blank 'no-lichen' control assays produce very high absorbance values.

To determine the saturating substrate concentration, the rate of phosphatase activity was determined for substrate concentrations ranging from 1 to 10 mM. There was no significant effect of using different citric acid tri-sodium citrate buffer concentrations (0.01, 0.002, 0.1 or 0.2 M; $P = 0.281$) or volumes of assay medium (2.9 or 2 ml; $P = 0.735$) on rates of phosphatase activity. Calculation of the kinetic constants K_m and V_{max} were determined as outlined in sections 2.2.4.6 and 7.2.5 using Michaelis-Menten fit, Lineweaver-Burke, Eadie-Hofstee and Hanes-Woolf transformations.

All assays were incubated for 20 min in a shaking water-bath at 15 ^{o}C in the dark after which the reaction was terminated by transferring 2.5 ml assay medium into 0.25 ml terminator solution (1.1 M NaOH, 27.5 mM EDTA, 0.55 M K_2HPO_4) and the absorbance measured at 405 nm using a NanoDrop ND-1000 spectrophotometer (Labtech International Ltd, Ringmer, East Sussex). Thalli were then blotted dry, oven dried for 24 h at 80 ^{o}C and weighed. Enzyme activity was

expressed as mmol substrate hydrolyzed g^{-1} dry mass h^{-1} using *p*-nitrophenyl (*p*NP) to calibrate the assay.

9.2.5 Determination of total N and P concentration

Oven dried (80 ^{o}C overnight) lichen samples (*c.* 30 mg) were digested by the sulphuric acid-hydrogen peroxide procedure of Allen (1989), as outlined in section 4.2.5. Ammonium-N in the digest was determined using the fluorometric method of Holmes (1999) using a Wallac 1420 Victor^2 multilabel counter (see section 4.2.5). Phosphorus was assayed by the malachite green variant of the methylene blue method after Van Veldhoven and Mannaerts (1987) using a Pye Unicam SP6-350 visible spectrophotometer (as described in section 4.2.5).

9.2.6 Nitrogenase activity

Rates of nitrogenase activity as determined by acetylene reduction, wherever possible under field conditions, were obtained either from the literature or P. D. Crittenden (Pers. Comm.) (Table 9.1).

9.2.7 Thallus surface pH

Thallus surface pH measurements were recorded in the same manner as outlined in section 2.2.5.

Table 9.1 Maximum observed rates of nitrogenase activity in lichens as assayed by acetylene reduction.

Species	Nitrogenase activity ± 1 SEM (nmol ethylene g^{-1} h^{-1})		Reference
Peltigera scabrosa	3138 ± 182*	(*n* = 10)	P. D. Crittenden (Pers. Comm.)
Nephroma arcticum	3000 ± 340‡	(*n* = 9)	Kallio *et al*. (1972)
Peltigera aphthosa	2326 ± 481*	(*n* = 5)	Crittenden (1975)
Peltigera malacea	2205 ± 193*	(*n* = 10)	P. D. Crittenden (Pers. Comm.)
Solorina crocea	1500 ± 200†	(*n* = 12)	Kallio *et al*. (1972)
Lobaria linita	1085 ± 585*	(*n* = 2)	Alexander *et al.* (1974)
S. paschale	1000 ± 230*	(*n* = 10)	Crittenden and Kershaw (1978)
Stereocaulon alpinum	276 ± 66*	(*n* = 5)	Crittenden (1975)
S. vesuvianum	69 ± 17*	(*n* = 5)	Crittenden (1975)

* Measured in the hydrated state under field conditions (typically 12 – 15 ^{o}C)
† Activity measured on undifferentiated thallus pieces at 15 ^{o}C
‡ Activity measured in isolated cephalodia at 15 ^{o}C. A high degree of variability exists among published rates of nitrogenase activity in *N. arcticum* given the frequency of cephalodia included in thallus fragments investigated.

9.2.8 Statistical analyses

All data were tested for normality and homogeneity of variances. If data violated one or both of these assumptions, $\log_{10}$ or square root transformations were performed. If data met both assumptions, one and two-way ANOVA, *t*- tests, linear regression analyses and Pearson's correlation analyses were performed. Significant differences generated with ANOVA were revealed using a post-hoc Fishers LSD test. If data did not fit a normal distribution, non-parametric analyses were performed. These included Kruskal-Wallis and subsequent Bonferroni corrected Mann-Whitney U tests and Spearman's correlation analyses. All statistical analyses were performed using SPSS version 12.0.01 (SPSS Inc., Chicago, IL).

9.3 Results

9.3.1 Influence of storage on PME activity

Samples stored air-dry at -15 ^{o}C were analysed within 12 weeks of the original collection date. However, after 10 months storage at -15 ^{o}C, PME activity in the N_2-fixing lichens *L. linita*, *P. aphthosa* and *S. alpinum* had decreased by up to 63 % (Table 9.2).

Table 9.2 Rates and percentage change in PME activity in N_2-fixing lichens stored at -15 ^{o}C for 8 weeks or 10 months. Assays were performed using 0.5 mM *p*NPP for 20 min at 15 ^{o}C in the dark. Values are means (n = 5) ± 1 SEM.

	PME activity (mmol substrate hydrolysed g^{-1} dry mass h^{-1})		
	Pre-treatment time period at - 15 ^{o}C (air dry)		
Species	8 weeks	10 months	% change
Stereocaulon alpinum	0.655 ± 0.101	0.480 ± 0.032	- 26.83
Lobaria linita	0.720 ± 0.189	0.372 ± 0.062	- 48.32
Peltigera aphthosa	0.215 ± 0.060	0.080 ± 0.004	- 62.83

9.3.2 Relationships between PME activity, pH and thallus location

Phosphomonoesterase activity in all species was readily measurable across the range of pH intervals investigated. In most cases, changes in pH were associated with significant changes in PME activity (Table 9.3). Optimum pH ranges were often broad (Fig. 9.5). Nonetheless there were clear differences between species,

with marked acid PME activity (pH 2.5 – 3.9) evident in all fruticose species and *L. linita* while a more neutral optimum pH (5.9) was evident in other foliose species (Fig. 9.5, Table 9.3). This pH specificity was highly positively related to the thallus surface pH (Fig. 9.6). As shown in earlier chapters, maximum PME activity occurs in the apical 10 mm of mat-forming lichens (Chapter 2, section 2.3, Chapter 6, section 6.3.2). In foliose terricolous species, activity was significantly higher in the central region of the thallus (Fig. 9.7), e.g. PME activity in *N. arcticum* and *P. aphthosa* was *c.* 260 and 70 % higher, respectively, at a distance of 50 – 60 mm from the thallus edge than in the marginal segment (Fig. 9.7). The same response pattern to pH was observed in thallus tissue dissected either from the centre or from the margins of *N. arcticum* (Fig. 9.8). All subsequent enzyme assays were undertaken on either 10 mm apical segments of mat-forming species or central 10 mm annuli of foliose species.

Table 9.3 Apparent optimum pH values for PME activity in 10 mm apical or marginal segments of non-N_2-fixing (*) and N_2-fixing (*) lichen thalli with results of one-way ANOVA to test the significance of the effect of varying pH.

Species	Optimum pH for PME activity	df	*F*-ratio	*P*-value
*Flavocetraria cucullata**	2.5	8	4.24	0.001
*Cladonia rangiferina**	3.2	8	0.70	0.691
*Stereocaulon alpinum**	2.5	8	5.75	< 0.001
*S. paschale**	3.2	8	3.09	0.007
*Lobaria linita**	3.2	8	4.49	< 0.001
*S. vesuvianum**	3.9	8	1.30	0.268
*Nephroma arcticum**	5.9	8	2.55	0.022
*Peltigera aphthosa**	5.9	8	2.56	0.025
*P. malacea**	5.9	8		0.035[†]
*P. scabrosa**	5.9	8	4.96	< 0.001
*Solorina crocea**	5.9	8	5.89	< 0.001

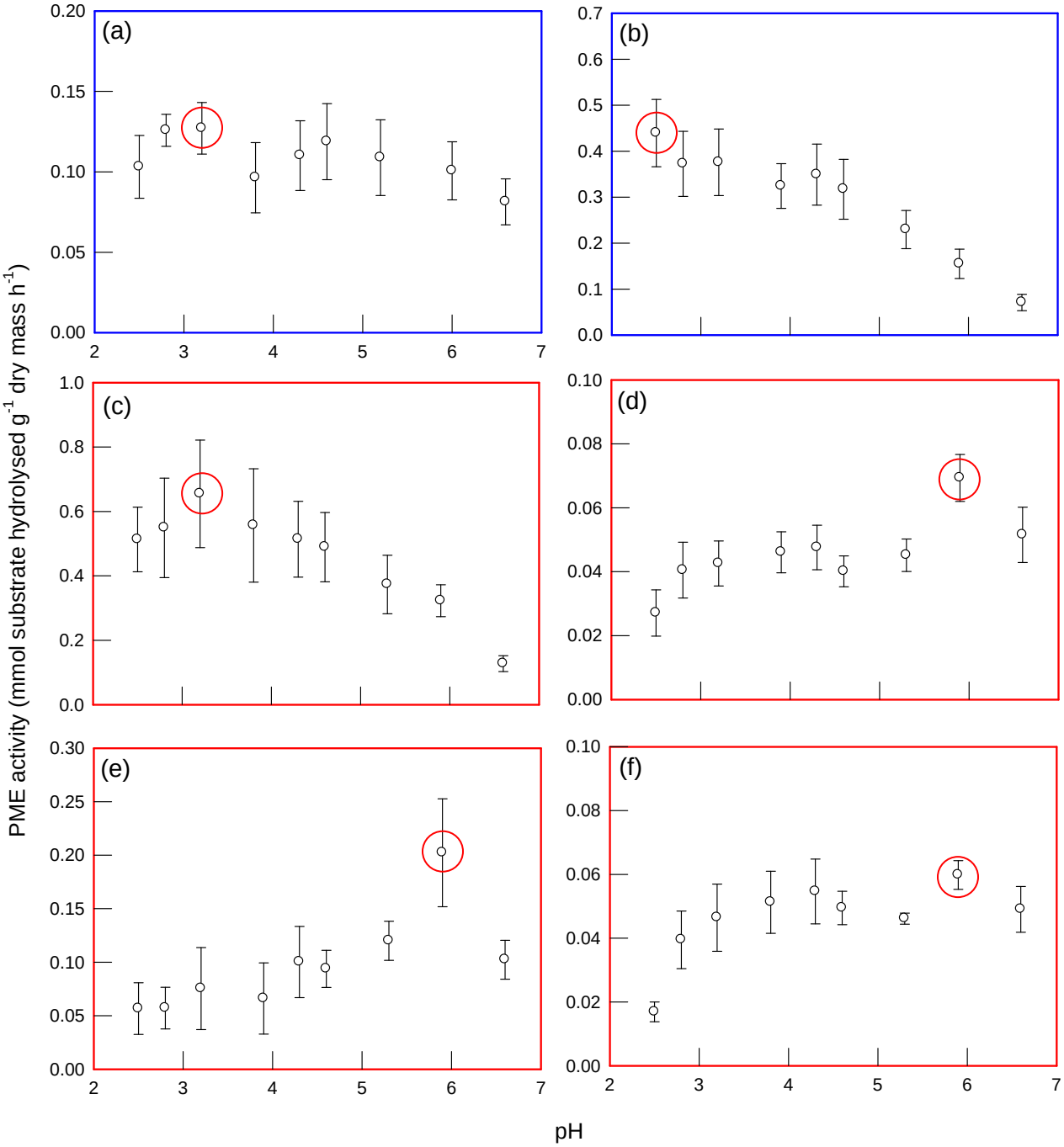


Figure 9.5 Relationship between pH and PME activity in 10 mm apical or marginal thallus sections of *Cladonia rangiferina* (a), *Flavocetraria cucullata* (b), *Lobaria linita* (c), *Nephroma arcticum* (d), *Peltigera aphthosa* (e), *Peltigera malacea* (f), *Peltigera scabrosa* (g), *Solorina crocea* (h), *Stereocaulon alpinum* (i), *Stereocaulon paschale* (j) and *Stereocaulon vesuvianum* (k). Assays were performed with 0.5 mM *p*NPP for 20 min at 15 ^{o}C in the dark. Plotted values are means (n = 10) ± 1 SEM and circled values are those judged to be optimal. Non-N_2-fixing lichens are bordered in blue, whilst N_2-fixing lichens are bordered in red.

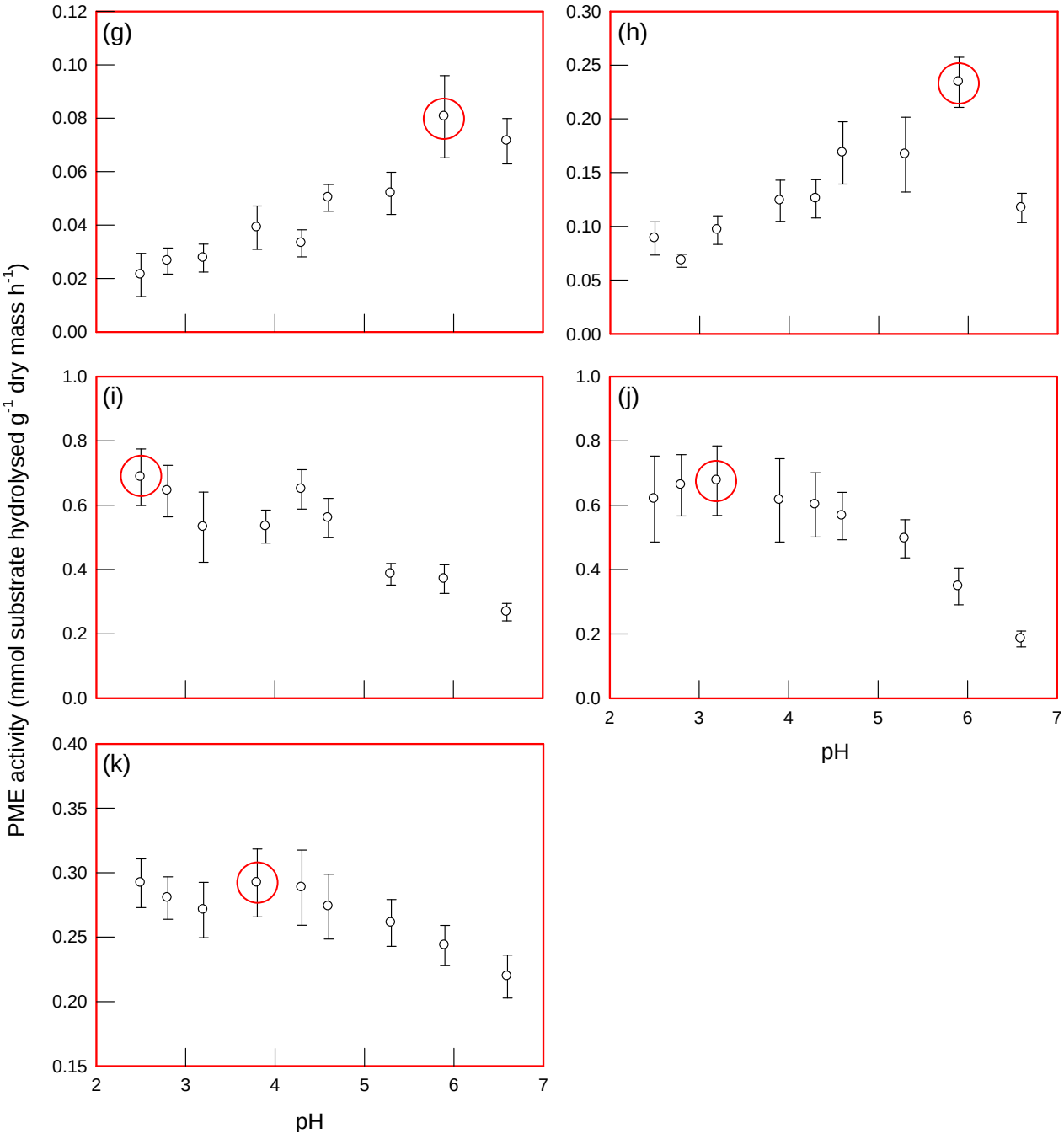


Figure 9.5 continued

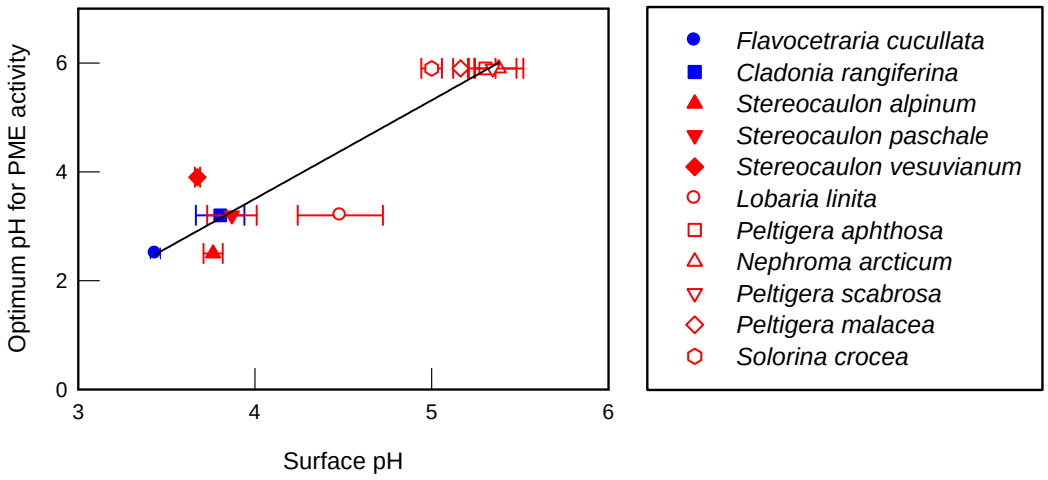


Figure 9.6 Relationship between thallus surface pH and apparent optimum pH for PME activity in a range of lichen species (r^2 = 0.865, P < 0.001). Assays were performed with 0.5 mM *p*NPP for 20 min at 15 °C in the dark. Thallus surface pH measurements were obtained after 1 min with a flat-head electrode in a bathing solution of 0.025 M KCl. Non-N_2-fixing lichens are shown in blue, whilst N_2-fixing lichens are shown in red.

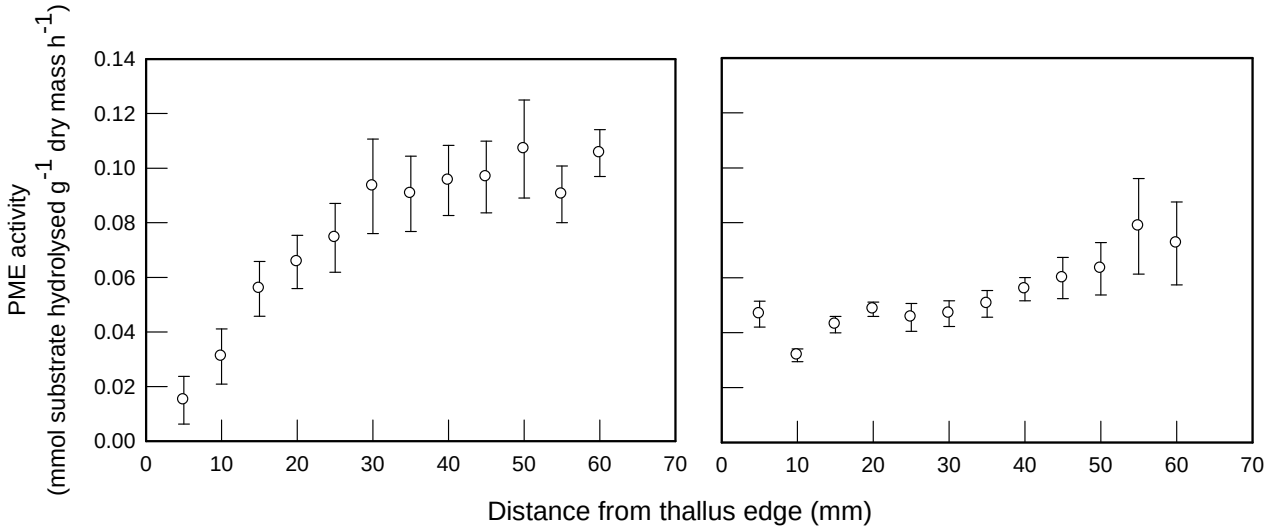


Figure 9.7 Distribution of PME activity across thalli of *Nephroma arcticum* (a, *P* < 0.001) and *Peltigera aphthosa* (b, *P* = 0.003). Assays were performed on 5 mm wide continuous thallus annuli cut at increasing distances from the margins towards the centre of the thallus using 0.5 mM and 20 min long incubations at 15 °C in the dark. Plotted values are means (*n* = 5) ± 1 SEM.

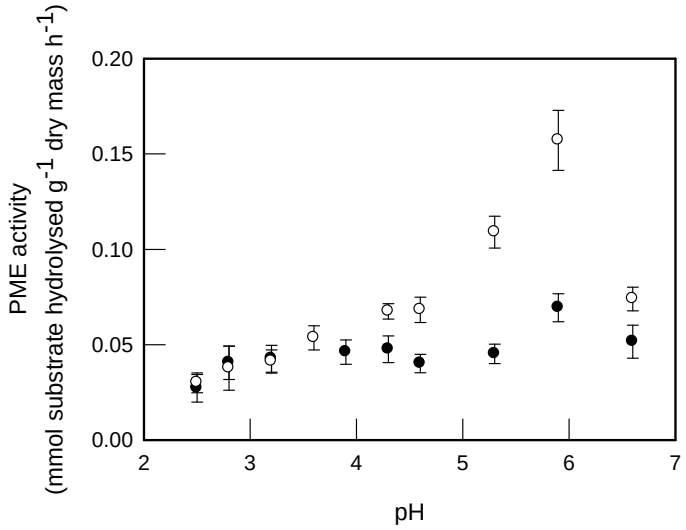


Figure 9.8 The effect of pH on PME activity in central (○) and marginal (●) annuli of *Nephroma arcticum*. Assays were performed with 0.5 mM *p*NPP for 20 min at 15 ^{o}C in the dark. Plotted values are means (n = 6) ± 1 SEM.

9.3.3 Relationships between PME activity and substrate concentration

Preliminary investigations showed that there were large inter-species differences in optimum pH for PME activity. In order to determine maximum PME activity under comparable conditions, the response to increasing substrate concentration was determined for *S. alpinum* (the species which had the highest rate of activity). This produced a hyperbolic curve of enzyme activity, consistent with Michaelis-Menten kinetics (Fig. 9.9a), and it suggested that saturation was obtained at 8 mM *p*NPP, a value used subsequently in assays on other species. Values of K_m and V_{max} obtained from Michaelis-Menten fit and 3 linear transformations of the data (Lineweaver-Burke, Eadie-Hofstee and Hanes-Woolf) also aided selection of this substrate concentration (Table 9.4).

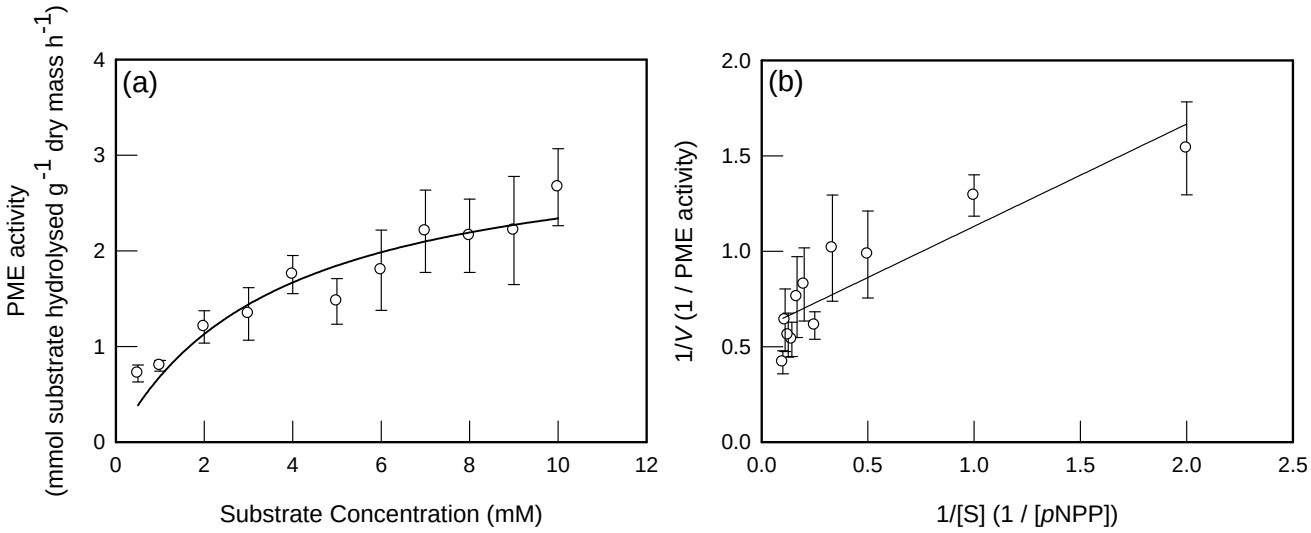


Figure 9.9 Michaelis-Menten plot of PME activity in the 10 mm apical segment of *Stereocaulon alpinum* (a) and Lineweaver-Burke linear transformation (b) where the *x* axis (1/[S]) is the reciprocal of the substrate concentration and the *y* axis (1/*V*) is the reciprocal of the velocity of the reaction (1/PME activity) (r^2 = 0.805, $P < 0.001$). Assays were performed at 15 ^{o}C for 20 min in the dark. Plotted values are means (n = 6) ± 1 SEM.

9.3.4 Phosphomonoesterase activity and nutrient relations in N_2-fixing lichens

Phosphomonoesterase activity was measured for the same set of lichens collected during two consecutive years of study. Broadly, similar rates of PME activity were observed in each lichen species in both years of analysis as illustrated by the significant positive correlation shown in Fig. 9.10. The greatest variation in PME activity was observed in *F. cucullata* (alpine) where activity recorded in 2007 was *c.* 5 times lower than values measured during 2006.

Table 9.4 Kinetic parameters of PME activity in *Stereocaulon alpinum* determined following fitting to the Michaelis-Menten equation and linear transformations as described in section 2.2.4.6. The units of K_m are mM and V_{max} are mmol substrate hydrolysed g^{-1} dry mass h^{-1}. r^2 values are provided to demonstrate the goodness of fit of the straight line following linear transformations. For details of how to calculate kinetic constants, see Chapter 2, section 2.2.4.6.

	Kinetic parameters		
Equation	K_m	V_{max}	r^2
Michaelis-Menten	3.655	3.193	0.346
Lineweaver-Burk	0.901	1.682	0.805
Eadie-Hofstee	1.241	2.204	0.676
Hanes-Woolf	2.867	2.356	0.762
Mean	2.166	2.359	

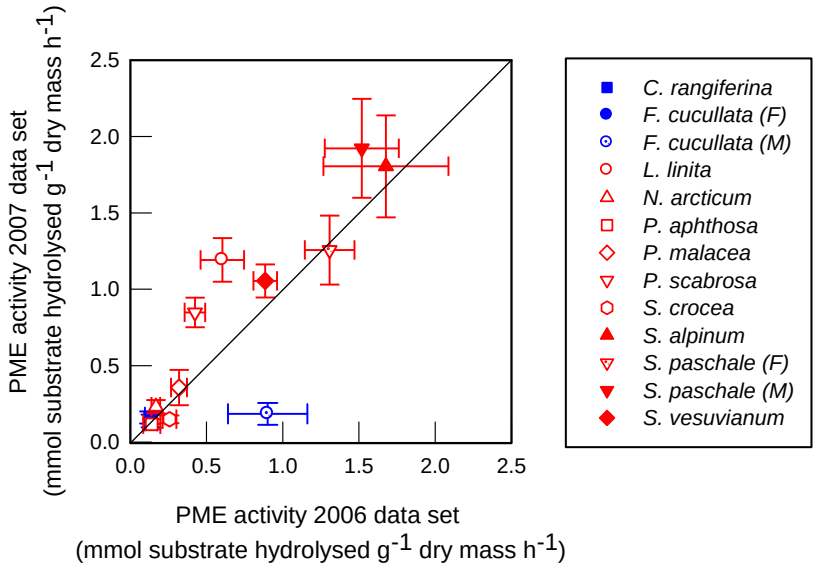


Figure 9.10 Relationship between PME activity recorded in a range of lichen species in consecutive years (2006 and 2007; r = 0.577, P < 0.001). Material selected for analysis was taken from apical or central 10 mm sections of the thallus. Assays were performed at individual species specific apparent optimum pH values and using 8 mM *p*NPP for 20 min at 15 °C in the dark. Plotted values are means (n = 10) ± 1 SEM. Line of 1:1 ratio illustrated. Non-N_2-fixing lichens are shown in blue, whilst N_2-fixing lichens are shown in red.

Phosphomonoesterase activity differed significantly between species (Table 9.5) ranging between 0.115 ± 0.026 and 1.989 ± 0.195 mmol substrate hydrolysed g^{-1} dry mass h^{-1} (Fig. 9.11). Nitrogen fixing species typically had higher rates of activity than non-fixing species (Fig. 9.11), with the highest rates recorded in *Stereocaulon spp.* and *L. linita*.

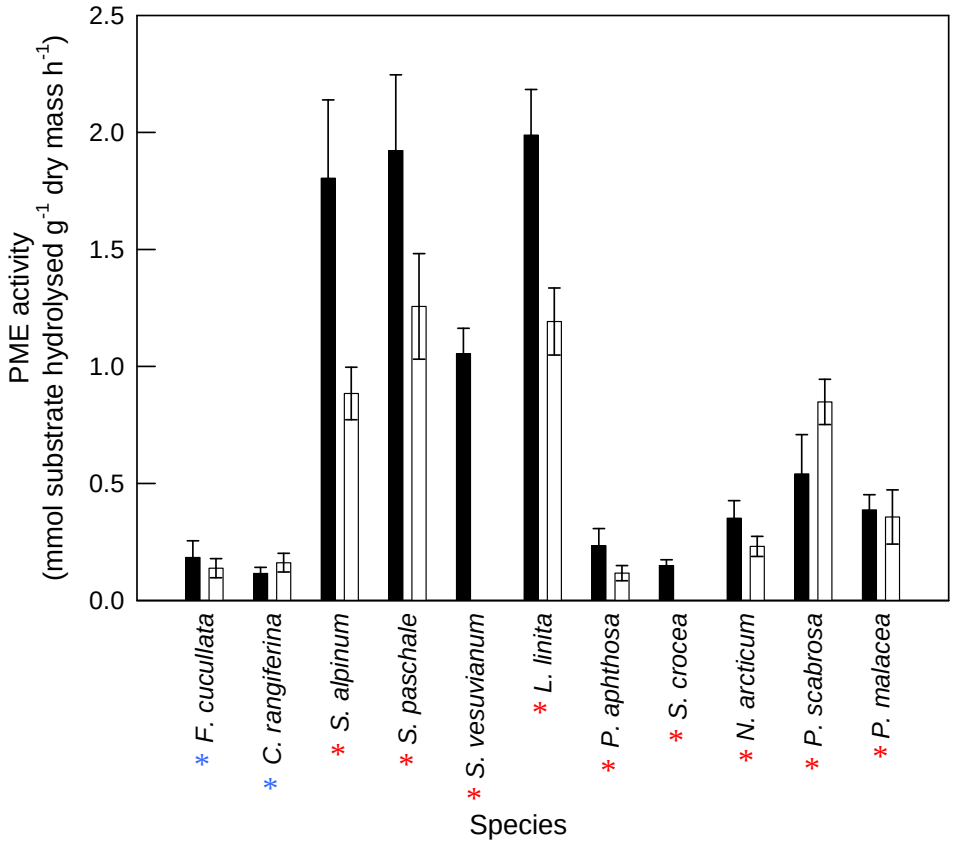


Figure 9.11 Phosphomonoesterase activity in non-N_2-fixing (*) and N_2-fixing (*) lichens collected from alpine (■) and forest (□) habitats in 2007. Material selected for analysis was taken from apical or central 10 mm sections of the thallus. For each species, assays were performed at the apparent optimum pH value for PME activity (see Table 9.3), using 8 mM *p*NPP for 20 min at 15 ^{o}C in the dark. Plotted values are means (n = 10) ± 1 SEM.

Table 9.5 Results of two-way ANOVA comparing PME activity between all lichen species investigated (n = 11) and habitat type (alpine or forest).

(a) Source of variation	df	*F*-ratio	*P*-value
Between species	10	42.49	< 0.001
Between habitat	1	9.31	0.003
Species x habitat	8	3.41	0.001
Error	171		
Total	191		

During preliminary investigations on the lichens collected in 2006, it was noted that the habitat from which a particular lichen species was collected (alpine or forest) significantly influenced the rate of PME activity in both non-fixing (*F. cucullata*) and N_2-fixing (*S. paschale*) species (P = 0.046) (Table 9.6). Data from the 2007 lichen collection confirmed this observation and revealed that lichen growing in an alpine environment had significantly higher PME activity (Table 9.5) than the same species growing in the birch forest (Fig. 9.11).

Table 9.6 Rates of PME activity in 10 mm apical segments of *Flavocetraria cucullata* (non-N_2-fixing *) and *Stereocaulon paschale* (N_2-fixing *) collected from alpine and forest habitats in 2006. Assays performed at species-specific apparent optimum pH values and using 8 mM *p*NPP for 20 min at 15 ^{o}C in the dark. Values are means (n = 10) ± 1 SEM.

	PME activity (mmol substrate hydrolysed g^{-1} dry mass h^{-1})	
Species	Alpine	Forest
*F. cucullata**	0.901 ± 0.261	0.139 ± 0.039
*S. paschale**	1.518 ± 0.243	1.308 ± 0.163

Thallus P concentration was highly significantly correlated to nitrogenase activity (Fig. 9.12). However, there was no relationship between PME and nitrogenase activities (Fig. 9.13).

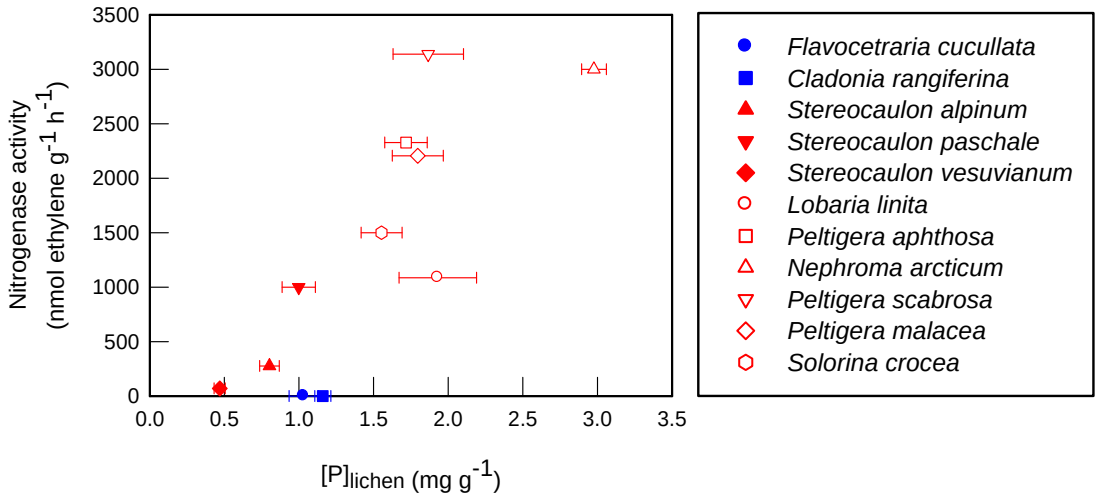


Fig. 9.12 Relationship between $[P]_{lichen}$ and nitrogenase activity in a range of lichen species (r = 0.823, P = 0.002). Chemical analyses were performed on 10 mm apical or central thallus sections of lichen collected in 2006. Plotted values of $[P]_{lichen}$ are means (n = 10) ± 1 SEM. Values of nitrogenase activity are taken from Table 9.1. Non-N_2-fixing lichens are shown in blue, whilst N_2-fixing lichens are shown in red.

Values of $[N]_{lichen}$ and $[P]_{lichen}$ in apical and central 10 mm sections were significantly positively correlated (Fig. 9.14a). Phosphomonoesterase activity was negatively related to $[P]_{lichen}$ and $[N]_{lichen}$, with $[P]_{lichen}$ the stronger correlate. However, neither relationship was significant (Fig. 9.14b, c). When the relationship between PME activity and $[P]_{lichen}$ was investigated for N_2-fixing species alone, the significance increased (correlation not shown, r = -0.835, P = 0.003). There was no relationship between PME activity and thallus N:P mass ratio (Fig. 9.14d).

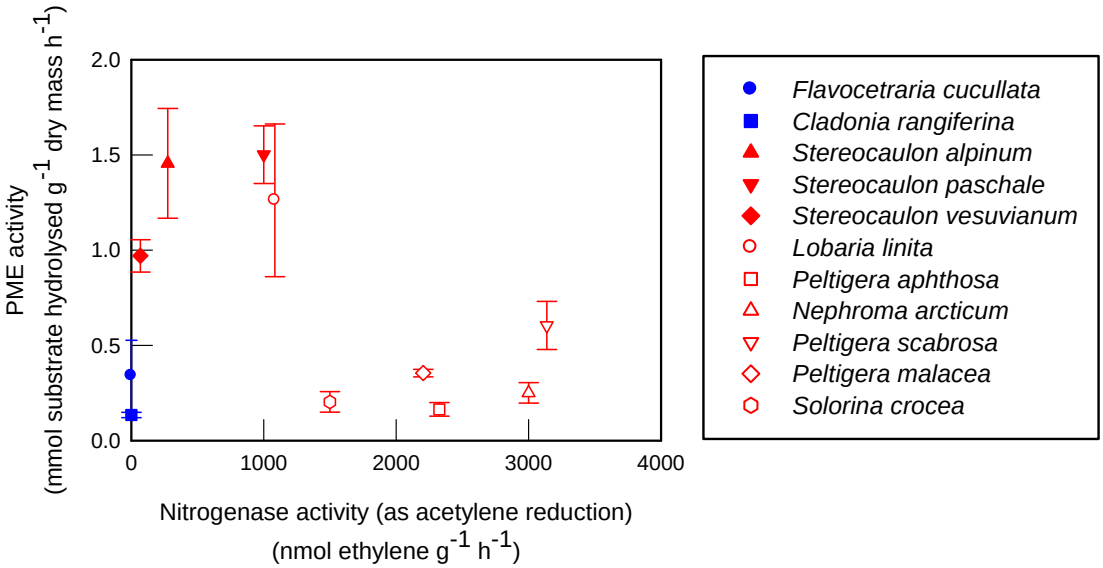


Figure 9.13 Relationship between PME and nitrogenase activities in a range of lichen species (r = -0.514, P = 0.105). Rates of PME activity are mean values for combined 2006 and 2007 data sets (including samples collected from both alpine and forest habitats). Assays were performed on 10 mm apical or central thallus sections at species-specific apparent optimum pH values and using 8 mM *p*NPP for 20 min at 15 ^{o}C in the dark. Plotted values of PME activity are means (n = 20 - 40) ± 1 SEM. Values of nitrogenase activity are taken from Table 9.1. Non-N_2-fixing lichens are shown in blue, whilst N_2-fixing lichens are shown in red.

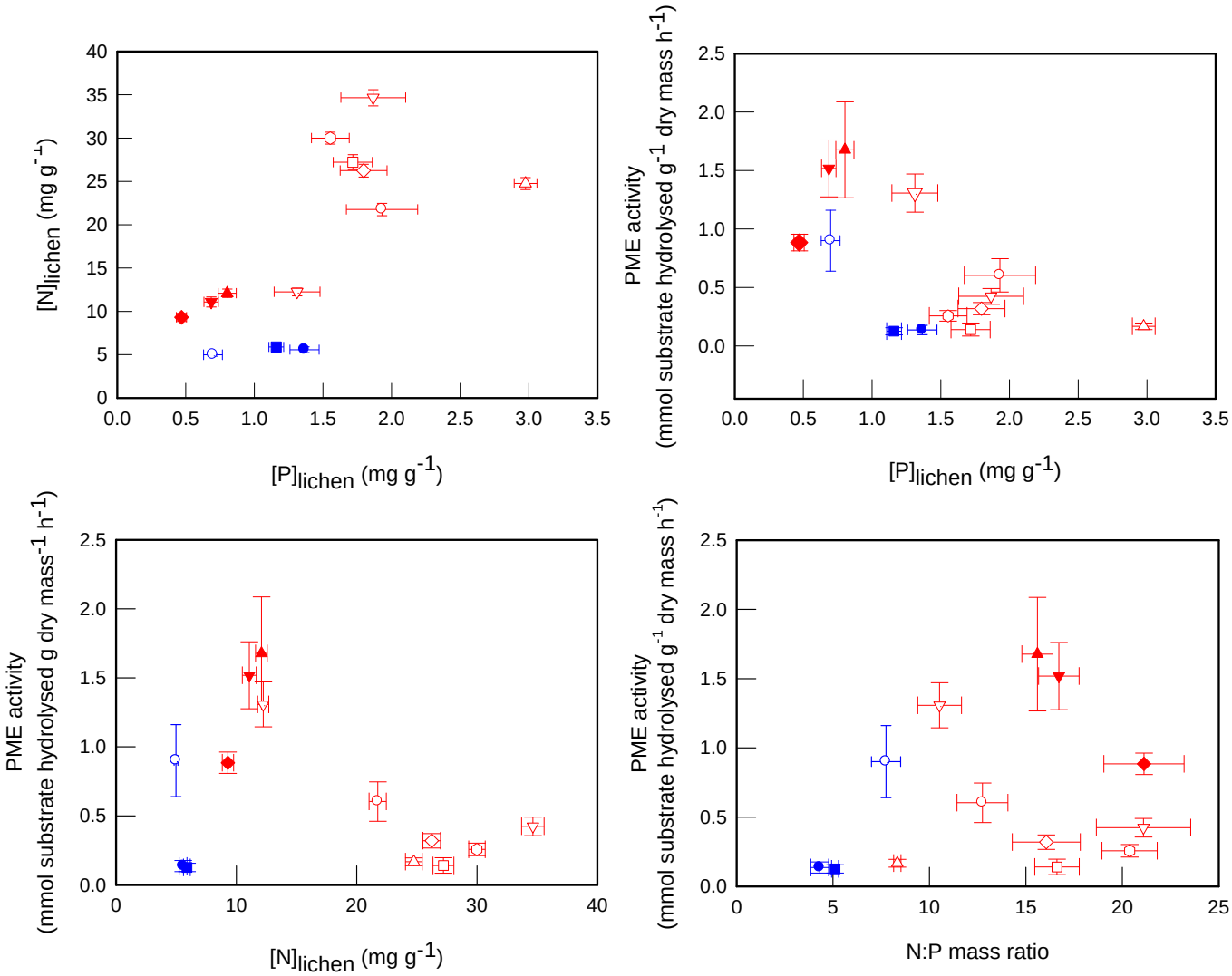


Figure 9.14 Relationships in a range of lichen species between (a) $[N]_{lichen}$ and $[P]_{lichen}$ (r = 0.686, P = 0.010), (b) PME activity and $[P]_{lichen}$ (r = -0.467, P = 0.108), (c) PME activity and $[N]_{lichen}$ (r = -0.137, P = 0.655) and (d) PME activity and N:P mass ratio (r = 0.346, P = 0.247). Lichen samples used in these analyses were apical or marginal 10 mm sections from specimens collected in 2006. PME assays were performed at species-specific apparent optimum pH values, using 8 mM *p*NPP for 20 min at 15 °C in the dark. Plotted values are means (n = 10) ± 1 SEM. Non-N_2-fixing lichens are shown in blue, whilst N_2-fixing lichens are shown in red.

9.3.5 Phosphodiesterase and 5′ ND activities in N_2-fixing lichens

Preliminary investigations on *Peltigera praetextata* revealed large variation between the rates of the three distinct phosphatases examined (PME, PDE and 5′ ND) (Fig. 9.15). The rate of *p*NPPP hydrolysis (5′ ND activity) was significantly higher in central regions of the thallus when compared to activity in the marginal zone, and in addition was significantly higher by a factor of *c.* 7 than PME and PDE activities (Fig. 9.15, Table 9.7). As a consequence of this finding PME, PDE and 5′ ND activities were investigated in lichens collected during 2007. The optimum pH values for PDE and 5′ ND activities were determined on apical or marginal 10 mm sections dissected from specimens collected in 2006 and stored at -15 ^{o}C. This was to minimise any confounding effects due to spatial variation in PME activity throughout the lichen thallus.

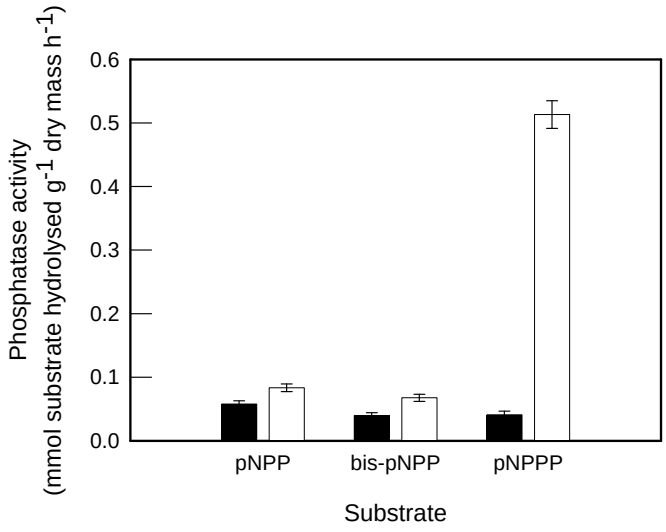


Figure 9.15 Phosphomonoesterase, PDE and 5′ ND activities in marginal (■) and central (□) regions of *Peltigera praetextata* thalli. Assays were performed using a final substrate concentration of 0.5 mM, for 20 min at 15 ^{o}C in the dark. Plotted values are means (n = 5) ± 1 SEM.

Table 9.7 Results of two-way ANOVA comparing the rate of activity between 3 different phosphatase enzymes (PME, PDE and 5′ ND) and between two different regions of the thallus (margins or centre) in *Peltigera praetextata*.

(a)			
Source of variation	df	*F*-ratio	*P*-value
Between enzymes	2	297.98	< 0.001
Between regions	1	444.21	< 0.001
Enzyme x segment	2	318.66	< 0.001
Error	24		
Total	30		

At a substrate concentration of 0.5 mM, 5′ ND and PDE activities were recorded in all species at each pH interval investigated. However, this was frequently low at sub-optimal pH values (Figs. 9.16 and 9.17). For several species (e.g. *F. cucullata*, *P. malacea*, *P. scabrosa*) the effect of pH on the activity of both enzymes was marked, whilst for others (*S. alpinum, C. rangiferina, S. crocea*), activity was maximal across a broad range of pH values (Figs. 9.16 and 9.17).

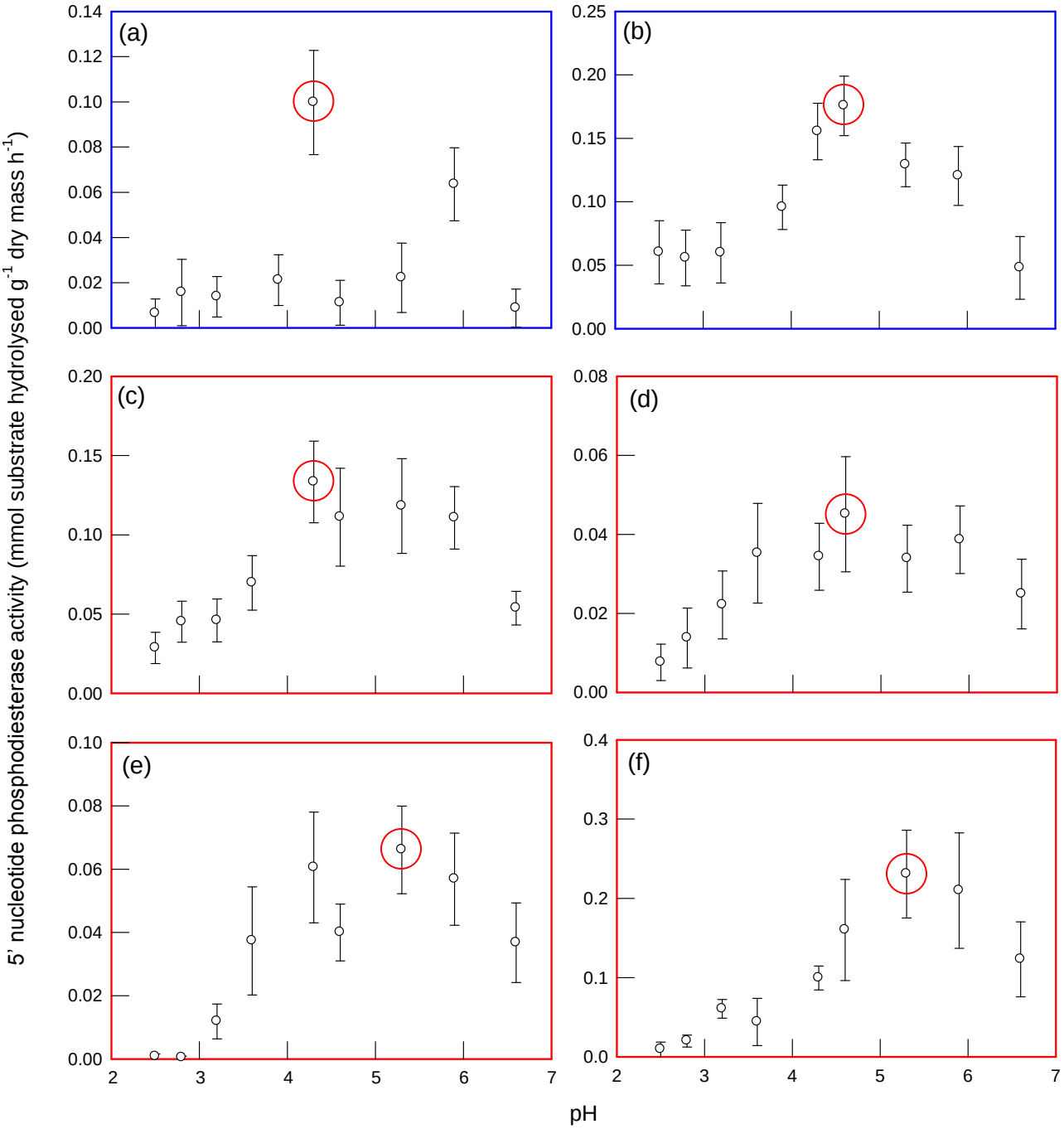


Figure 9.16 Relationship between pH and 5′ ND activity in 10 mm apical or marginal thallus sections of *Cladonia rangiferina* (a), *Flavocetraria cucullata* (b), *Lobaria linita* (c), *Nepthroma arcticum* (d), *Peltigera aphthosa* (e), *Peltigera malacea* (f), *Peltigera scabrosa* (g), *Solorina crocea* (h), *Stereocaulon alpinum* (i), *Stereocaulon paschale* (j) and *Stereocaulon vesuvianum* (k). Assays were performed with 0.5 mM *p*NPPP for 20 min at 15 ^{o}C in the dark. Plotted values are means (n = 6) ± 1 SEM and circled values are those judged to be optimal. Non-N_2-fixing lichens are bordered in blue, whilst N_2-fixing lichens are bordered in red.

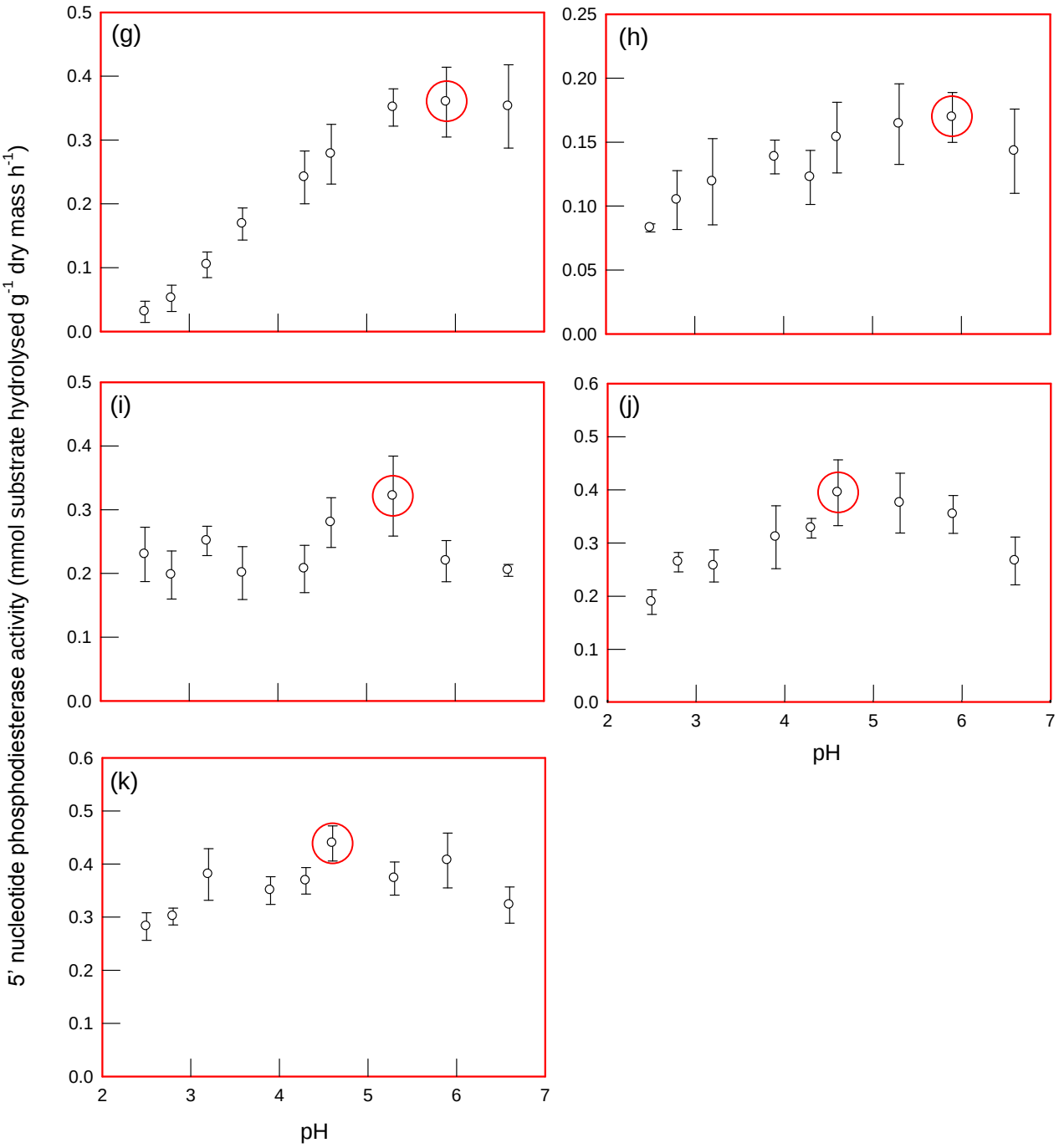


Figure 9.16 continued.

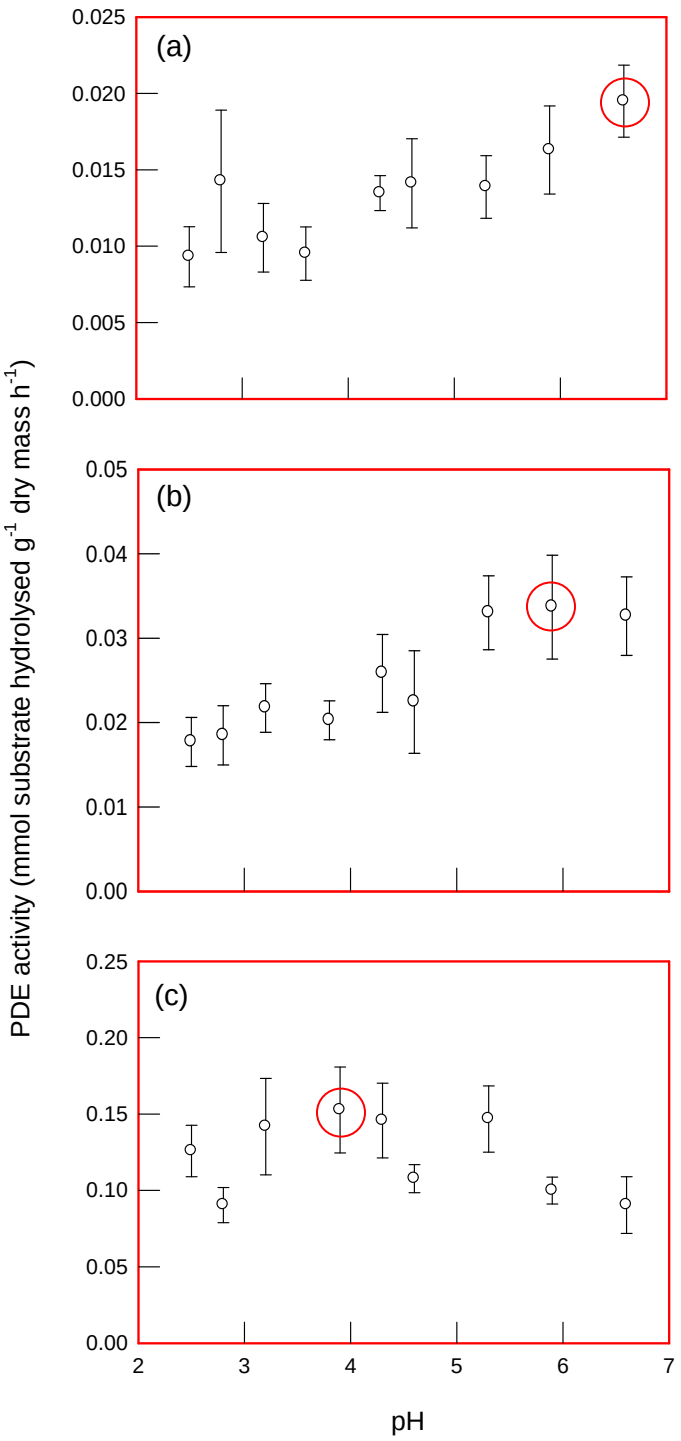


Figure 9.17 Relationship between pH and PDE activity in 10 mm apical or marginal sections of the N_2-fixing lichens *Nephroma arcticum* (a), *Peltigera scabrosa* (b) and *Stereocaulon alpinum* (c). Assays were performed with 0.5 mM bis-*p*NPP for 20 min at 15 ^{o}C in the dark. Plotted values are means (n = 6) ± 1 SEM and circled values are those judged to be optimal.

Maximum 5′ ND activity was recorded across a smaller pH range (between 4.3 and 5.9) than for PME activity (from 2.5 to 5.9), with different apparent optimum pH values frequently identified for the two different substrates (Table 9.8). Only *P. scabrosa* and *S. crocea* had maximum obtainable 5′ ND and PME activities at the same optimum pH value (Table 9.8). In contrast, maximum PDE activities for the 3 species investigated were recorded at pH values highly similar to those for PME activity (Table 9.9). As a consequence, apparent optimum pH values for PDE activity were assumed equal to those of PME activity in all other species.

5′ ND activity in *S. paschale* responded more or less linearly to substrate concentration with no evidence of saturation at 10 mM *p*NPPP, the highest concentration used (Fig. 9.18a). Thus, a Michaelis-Menten hyperbolic curve was not generated and the fitting of this data to the Michaelis-Menten equation gave a very weak coefficient of determination (Table 9.10). The response of 5′ ND activity in *S. paschale* could not be investigated over a higher range of *p*NPPP concentrations due to the constraint of substrate solubility. Linear transformations of the Michaelis-Menten equation applied to the data set (e.g. Fig. 9.18b) therefore resulted in markedly different estimates for K_m and V_{max} (Table 9.10). However, mean values (n = 4) of both kinetic parameters were in agreement with the suggestion that substrate saturation of the 5′ ND enzyme in *S. paschale* is likely to occur at values > 10 mM (Fig. 9.19). This substrate concentration was chosen for all subsequent investigations. Since PDE activity was uniformly lower than measured rates of PME activity, 8 mM bis-*p*NPP was selected for use in subsequent PDE assays.

Table 9.8 Apparent optimum pH values for PME and 5´ ND activities in 10 mm apical or marginal sections of non-N_2-fixing (*) and N_2-fixing (*) lichen thalli together with the results for each species of one-way ANOVA (or non-parametric Kruskal Wallis test*) comparing 5´ ND activity with assay pH.

Species	Optimum pH for PME activity	Optimum pH for 5´ ND activity	df	*F*-ratio	*P*-value
*Cladonia rangiferina**	3.2	4.3	8		0.002*
*Flavocetraria cucullata**	2.5	4.6	8	4.242	0.001
*Stereocaulon alpinum**	2.5	5.3	8	1.194	0.324
*S. paschale**	2.8	4.6	8	2.465	0.026
*Lobaria linita**	3.2	4.3	8	3.600	0.003
*S. vesuvianum**	3.9	4.6	8	2.263	0.040
*Nephroma arcticum**	5.9	4.6	8	3.198	0.006
*Peltigera aphthosa**	5.9	5.3	8		< 0.001*
*P. malacea**	5.9	5.3	8		0.002*
*P. scabrosa**	5.9	5.9	8	13.879	< 0.001
*Solorina crocea**	5.9	5.9	8	1.486	0.189

Table 9.9 Apparent optimum pH values for PME and PDE activity in 10 mm apical or marginal sections of N_2-fixing lichen thalli together with the results for each species of one-way ANOVA comparing PDE activity with assay pH.

Species	Optimum pH for PME activity	Optimum pH for PDE activity	df	*F*-ratio	*P*-value
*Stereocaulon alpinum**	2.5	3.9	8	1.551	0.167
*Nephroma arcticum**	5.9	6.6	8	1.616	0.147
*P. scabrosa**	5.9	5.9	8	4.611	< 0.001

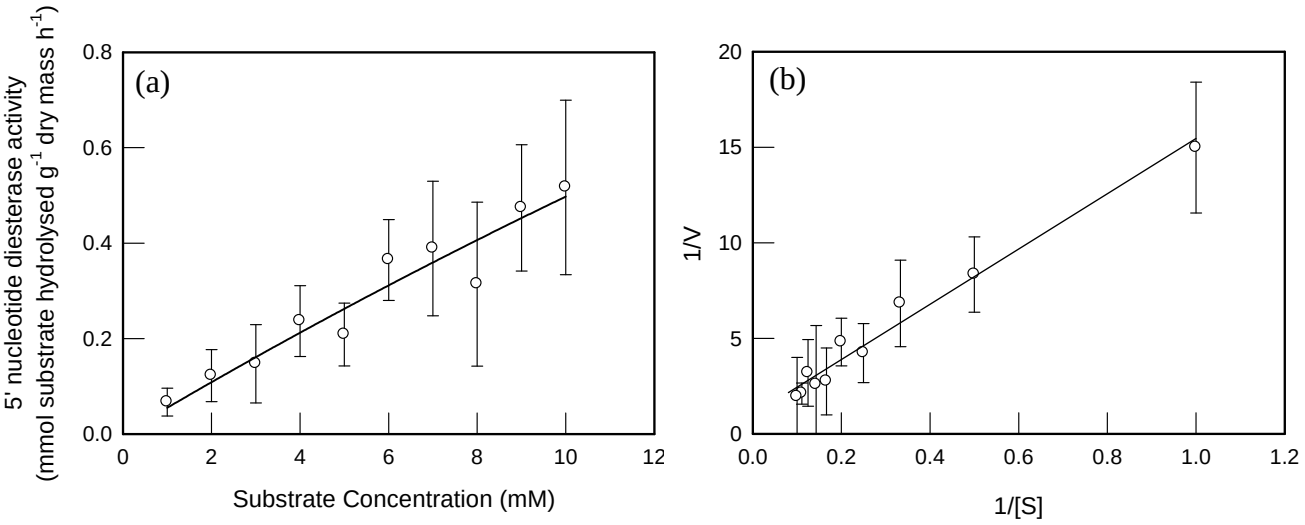


Figure 9.18 Michaelis-Menten plot of 5´ ND activity in the 10 mm apical segment of *Stereocaulon paschale* (a), and Lineweaver-Burke linear transformation (b) where the *x* axis (1/[S]) is the reciprocal of the substrate concentration and the *y* axis (1/*V*) is the reciprocal of the velocity of the reaction (5´ ND activity) (r^2 = 0.979, $P < 0.001$). 5´ ND assays were performed on material collected from Mt Njulla, Abisko 2007 at 15 ^{o}C for 20 min in the dark. Plotted values are means (n = 6) ± 1 SEM.

Table 9.10 Kinetic parameters of 5´ ND activity in *Stereocaulon paschale* as determined following three linear transformations of the Michaelis-Menten equation. The units of K_m are mM and V_{max} are mmol substrate hydrolysed g^{-1} dry mass h^{-1}. r^2 values are provided to demonstrate the goodness of fit of the straight line following linear transformations.

	Kinetic constants		
Equation	K_m	V_{max}	r^2
Michaelis-Menten	86.34	4.79	0.23
Lineweaver-Burk	14.91	1.04	0.98
Eadie-Hofstee	4.48	0.53	0.07
Hanes-Woolf	34.56	2.10	0.18
Mean	35.07	2.10	

5´ ND and PDE activities differed significantly between species (Tables 9.11 - 9.12), ranging from 0 to 1.06 ± 0.1 mmol *p*NPPP hydrolysed g^{-1} dry mass h^{-1} and 0.01 ± 0.01 to 0.17 ± 0.08 mmol bis-*p*NPP hydrolysed g^{-1} dry mass h^{-1} (Figs. 9.19 & 9.20). The lowest rates of PDE activity were measured in non-N_2-fixing lichens (11.6 ± 6.2 – 25.2 ± 14.1 mmol bis-*p*NPP hydrolysed g^{-1} dry mass h^{-1}) while no 5´ ND activity could be detected at the high substrate concentrations (10 mM *p*NPPP) used in these assays (see methods, section 9.2.4).

5´ ND and PDE activities were significantly higher in lichens collected from the alpine site (5´ ND, $P < 0.001$; PDE, $P = 0.054$) (Tables 9.11 – 9.12). In contrast to PME activity, values of PDE and 5´ ND activities for the different species did not appear to form readily explainable groupings (Figs. 9.19 – 9.20). Among the N_2-fixing species, maximum 5´ ND activity was recorded in *S. vesuvianum*, and was found to be significantly higher than activity measured in any of the other species investigated ($P < 0.011$). The lowest rates of 5´ ND and PDE activities were measured in *L. linita* (Figs. 9.19 – 9.20).

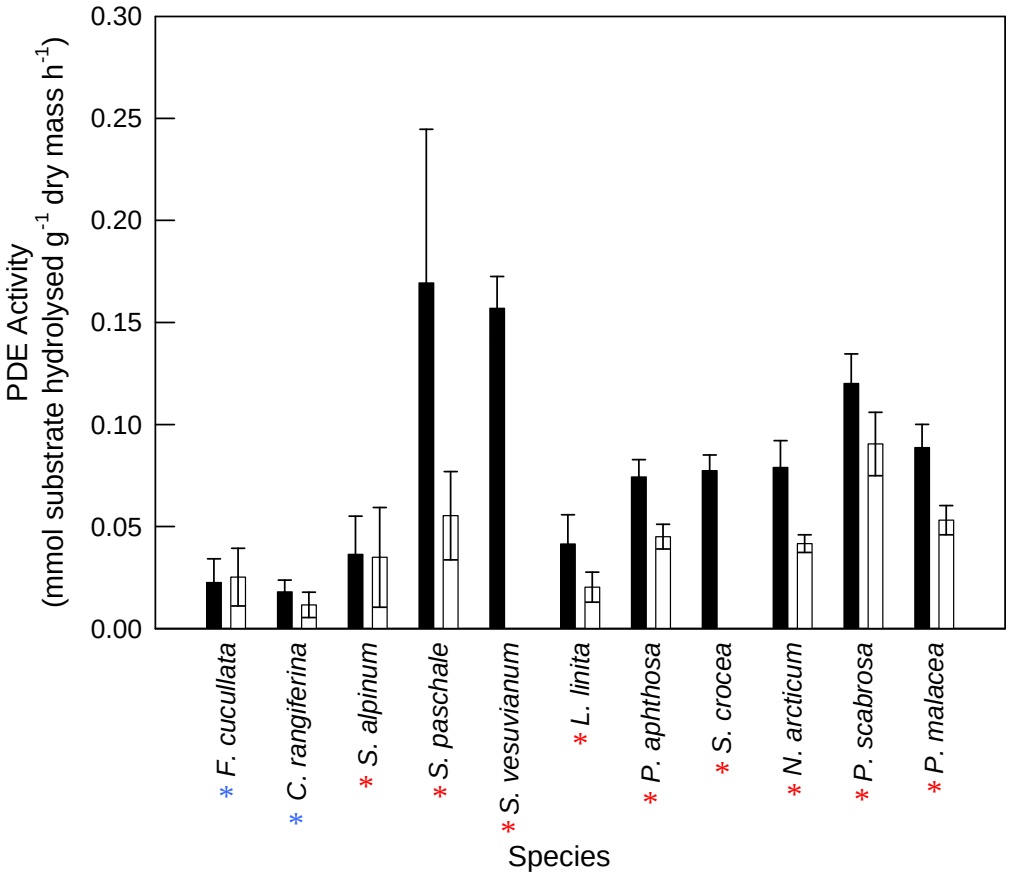


Figure 9.19 Maximum obtainable rates of PDE activity in non-N_2-fixing (*) and N_2-fixing (*) lichens collected from alpine (■) and subarctic forest (□) habitats. Material selected for analysis was taken from apical or central 10 mm thallus sections. For each species, assays were performed at the apparent optimum pH value for PDE activity (see Table 9.8), using 8 mM bis-*p*NPP for 20 min at 15 ^{o}C in the dark. Plotted values are means (n = 10) ± 1 SEM.

Table 9.11 Results of two-way ANOVA comparing PDE activity between all lichen species investigated (n = 11) and between habitat type (alpine or forest).

Source of variation	df	*F*-ratio	*P*-value
Between species	10	12.605	< 0.001
Between habitat	1	3.781	0.054
Species x habitat	8	1.426	0.189
Error	166		
Total	186		

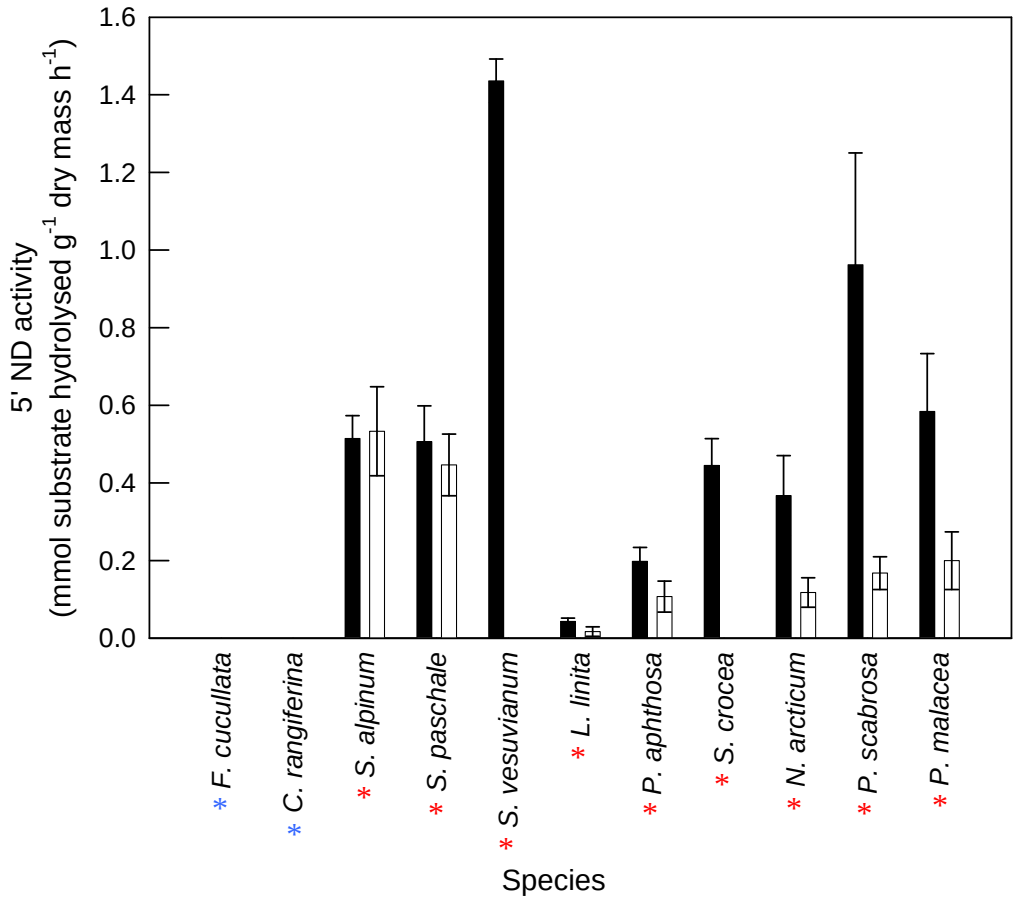


Figure 9.20 Maximum obtainable rates of 5´ ND activity for non-N_2-fixing (*) and N_2-fixing (*) lichens collected from alpine (■) and subarctic forest (□) habitats. Material selected for analysis was taken from apical or central 10 mm thallus sections. For each species, assays were performed at the apparent optimum pH value for 5´ ND activity (see Table 9.9), using 10 mM *p*NPPP for 20 min at 15 ^{o}C in the dark. Plotted values are means (n = 10) ± 1 SEM

Table 9.12 Results of two-way ANOVA comparing 5´ ND activity between all lichen species investigated (n = 11) and between habitat type (alpine or forest).

Source of variation	df	*F*-ratio	*P*-value
Between species	7	13.603	< 0.001
Between habitat	1	28.924	< 0.001
Species x habitat	6	1.977	0.057
Error	127		
Total	142		

In general, lichens with high rates of PME activity also had high PDE and 5´ ND activities irrespective of habitat (Fig. 9.21 and 9.22). However, relationships were not significant and several species did not follow this trend. These included *S. alpinum*, *S. paschale* and *L. linita* which had markedly higher PME activity in comparison to PDE and 5´ ND activities (Fig 9.21a, c, Table 9.12). *Lobaria linita* had amongst the highest values for PME:PDE and PME:5´ ND ratios. 5´ ND activity was significantly positively correlated with PDE activity in lichens from the alpine site, but not in lichens from the subarctic forest (Fig. 9.21c, 9.22c).

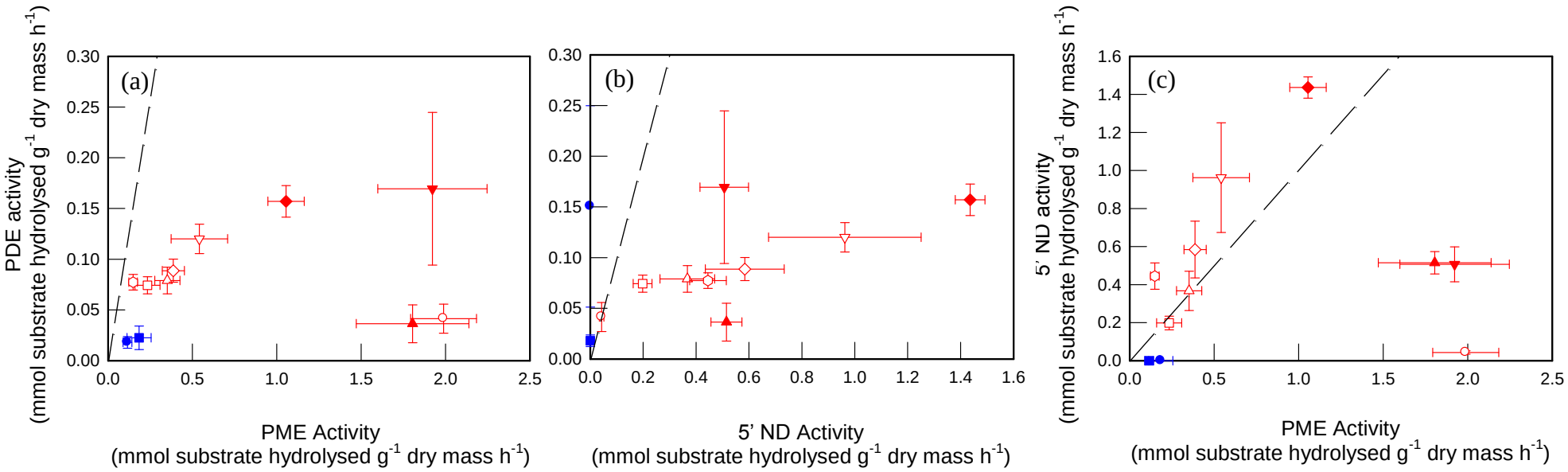


Figure 9.21 Relationships between maximum obtainable PME and PDE activities (a, r = 0.256, P = 0.448); 5′ ND and PDE activities (b, r = 0.766, P = 0.006); PME and 5′ ND activities (c, r = 0.144, P = 0.673) in lichens collected from a subarctic alpine site. Assays performed at 15 ^{o}C for 20 min in the dark, 8 mM *p*NPP and bis-*p*NPP and 10 mM *p*NPPP. Plotted values are means (n = 9-10) ± 1 SEM along with the line of equality. Symbols represent individual species as in Fig. 9.6 with Non-N_2-fixing lichens shown in blue and N_2-fixing lichens shown in red.

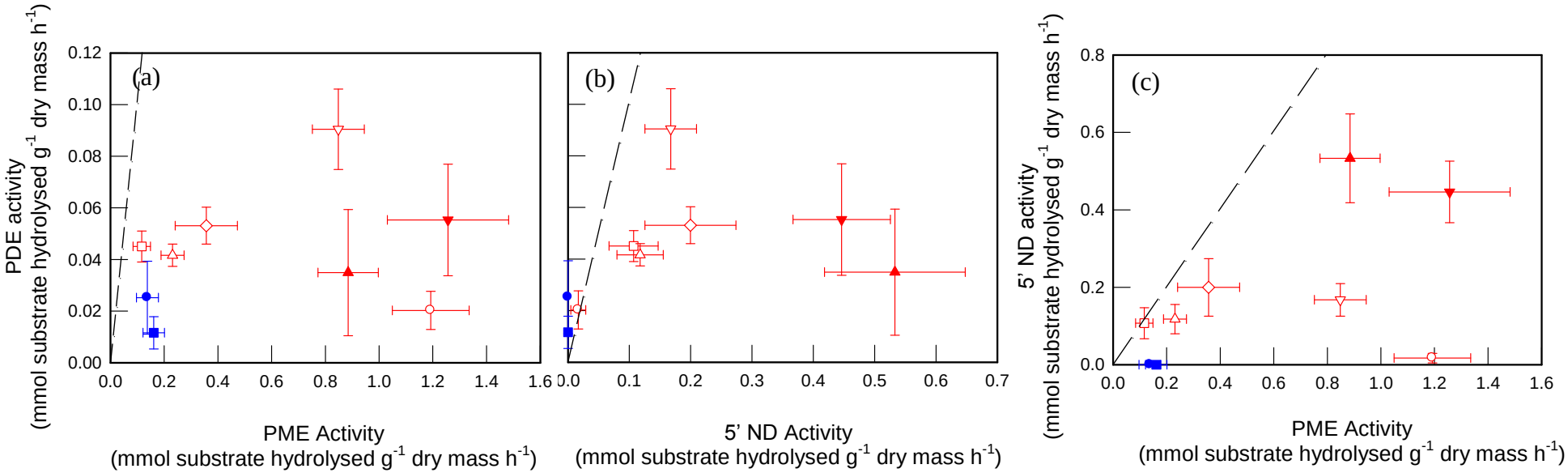


Figure 9.22 Relationships between maximum obtainable PME and PDE activities (a, r = 0.262, P = 0.495); 5′ ND and PDE activities (b, r = 0.346, P = 0.361); PME and 5′ ND activities (c, r = 0.541, P = 0.132) in lichens collected from a subarctic forest site. Assays performed at 15 ^{o}C for 20 min in the dark, 8 mM *p*NPP and bis-*p*NPP and 10 mM *p*NPPP. Plotted values are means (n = 9-10) ± 1 SEM along with the line of equality. Symbols represent individual species as in Fig. 9.6 with Non-N_2-fixing lichens shown in blue and N_2-fixing lichens shown in red.

Table 9.13 Ratios between maximum obtainable PME, PDE and 5′ ND activities in 2 non-N_2-fixing (*) and 9 N_2-fixing (*) lichens collected from alpine and subarctic forest habitats. Assays were performed at 8 mM *p*NPP and bis-*p*NPP, and 10 mM *p*NPPP for 20 min at 15 ^{o}C in the dark (n = 10).

	Alpine site			Forest site		
Species	PME: PDE	PME: 5′ ND	PDE: 5′ ND	PME: PDE	PME: 5′ ND	PDE: 5′ ND
*C. rangiferina**	14.1			21.7		
*F. cucullata**	13.4			11.5		
*S. alpinum**	40.3	2.9	0.7	22.1	1. 4	0.1
*S. paschale**	9.4	3.2	0.3	19.2	2. 4	0.1
S. vesuvianum *	6.7	0.7	0.1			
*L. linita**	40.6	39.0	1.0	50.5	60.4	1.2
*N. arcticum**	4.8	1.0	0.2	6.2	2. 2	0.4
*P. aphthosa**	3.7	1.4	0.4	3.9	1. 6	0.4
*P. malacea**	4.4	0.7	0.2	6.9	1. 8	0.3
*P. scabrosa**	4.3	0.5	0.1	8.7	4. 7	0.5
*S. crocea**	2.3	0.4	0.2			

9.4 Discussion

There was no clear relationship between rates of nitrogenase and PME activities. The highest PME activity was recorded in N_2-fixing species with apparent acid optimum pH for activity and included *Stereocaulon spp.* which have modest rates of nitrogenase activity. Foliose terricolous species such as *Peltigera spp.* with a more neutral pH optima for PME activity had high nitrogenase and low PME activities.

Among all species, $[N]_{lichen}$ and $[P]_{lichen}$ co-varied, with concentrations comparable to those reported previously for the same species (Solberg 1967; Sundberg *et al.* 2001; Dahlman *et al.* 2002; Palmqvist *et al.* 2002). Thallus P concentration was a positive correlate for nitrogenase activity. Nitrogen fixation is an energy intensive process which carries a high metabolic cost, and requires an appreciable supply of P to sustain high rates of activity (Sprent and Sprent 1990; Postgate 1998). High $[P]_{lichen}$ values recorded in lichens with the greatest capacity for nitrogenase activity could not be explained by up-regulated PME activity. It is possible that low rates of PME activity measured in *Peltigera spp.* and other foliose lichens when compared with activity in fruticose species (e.g. *Stereocaulon spp.*) might reflect variation in surface area: volume ratios or different PME enzymes/systems. Alternatively, high thallus P concentrations might repress PME enzyme synthesis (Phoenix *et al.* 2003), however, this argument would require an alternative source of P for uptake. Tamm (1964) observed that the growth of *Hylocomium splendens* was greatest in mats growing at the edge of tree canopies in contrast to those

found in open areas. He linked this increase in growth with the enrichment of rainwater by nutrients leached from tree canopies, and showed that the availability of P increased substantially from 0.1 kg P ha^{-1} y^{-1} in rainfall received by open areas to 1.9 kg P ha^{-1} y^{-1} in through-fall at the edge of *Betula verrucosa* canopies. Lichens collected from the birch forest at Abisko were selected from open areas in order to minimise canopy through-fall effects, and any potential increase in nutrient supply associated with though-fall is unlikely to account for interspecific variation in $[P]_{lichen}$, as foliose N_2-fixing lichens (high $[P]_{lichen}$) grow side-by-side with fruticose N_2-fixing lichens and non-N_2-fixing lichens (low $[P]_{lichen}$). Phosphodiesterase and 5′ nucleotide phosphodiesterase activities were measured to investigate the capacity of lichens to hydrolyse alternative organic P molecules. Interestingly, several foliose terricolous lichens (including *Peltigera spp.* and *N. arcticum*) measured a greater capacity for 5′ ND activity than PME activity. This was most apparent in samples collected from the alpine study site and suggests that lichen growth form and associated ecology might influence the predominant mechanism employed to acquire PO_4^{3-} from organic P sources in this oligotrophic environment.

In the foliose terricolous lichens *P. aphthosa* and *N. arcticum*, phosphatase activity was *c*. 10 times greater in central regions of the thallus associated with rhizines than in marginal zones (with no rhizine development). This compares favourably with the findings of Stevenson (1994) who reports that maximum PME activity in *P. canina* is associated with the under-surface of the thallus, in particular, the rhizines. The development of rhizines enables lichens to firmly

anchor the thallus to the substratum below. As such, these species form an intimate association with the underlying leaf litter and substratum, with the thallus itself frequently being covered by the leaves of deciduous trees. A greater capacity for phosphatase activity in regions of the thallus associated with rhizines might imply that the underlying substratum is a source of hydrolysable P for the lichen. This suggestion is consistent with the observation that many bryophytes (which acquire nutrients from atmospheric deposits, similar to lichens) are capable of obtaining nutrients from underlying substrata via rhizoidal attachments (Bates 1994).

In nutrient poor, alpine soils, it is commonly reported that the largest pool of P comprises organic forms (up to 90 % of total P) (Cosgrove 1967; Turner *et al.* 2004). This is a result of rapid PO_4^{3-} uptake by terrestrial vegetation and low microbial mineralisation rates under low temperatures (Barel and Barsdate 1978). A large proportion of the organic P present in alpine soils exists in the form of phosphodiesters, including nucleic acids and phospholipids (Cosgrove 1967; Turner and Haygarth 2005). Given the abundance of phosphodiesters in alpine soils and the high rates of phosphatase activity measured in rhizine-rich regions of foliose lichen thalli, it seems reasonable to suggest that such lichens maintain high thallus P concentrations by the hydrolysis of phosphodiesters present in the underlying soil and leaf litter. It is generally accepted that lichens absorb nutrients directly from atmospheric deposits (Ellis *et al.* 2004). However, there is recent evidence to suggest that some species of the N_2-fixing genus *Peltigera* directly

alter the nutrient concentration of the surrounding substratum. For example, it has been shown that the availability of N and P in soil rises significantly with increasing proximity to *Peltigera* thalli (Knowles *et al.* 2006; 2008).

The major component of soil organic P is phytic acid (*myo*-inositol hexakisphosphate) (Cosgrove 1967). Despite this molecule containing multiple phosphomonoester bonds, it has been shown that PME enzymes cannot directly hydrolyse phytic acid, although they can catalyse the release of PO_4^{3-} from lower-order inositol phosphates (Cosgrove 1967; Turner and Haygarth 2005). Instead, a substrate specific phytase enzyme is required (Livingstone *et al.* 1983). Although an accurate measure of phytase activity was not identified during the current investigation, it is possible that lichens have a capacity for phytase activity and are thus capable of hydrolysing phytic acid (Beever and Burns 1980); one might expect that this activity will be greatest in foliose terricolous lichens.

Stereocaulon alpinum and *S. paschale* are mat-forming species in which new growth occurs at the apices (Ellis *et al.* 2005). These species are not firmly attached to the underlying substratum, and there is strong evidence to suggest that such lichens do not take up significant quantities of nutrients from underlying soil (Ellis *et al.* 2003; 2004). Instead they are reliant on efficient capture of key nutrients from trace quantities available in atmospheric deposits (Hyvärinen and Crittenden 1998a; 1998c). *Stereocaulon vesuvianum* grows on oligotrophic rocks and is also likely to be predominantly dependent on precipitation for nutrients

such as P. *Stereocaulon spp.* had a much greater capacity for PME activity than 5′ ND activity. In addition, kinetic studies involving *S. alpinum* and *S. paschale* revealed a greater affinity for the hydrolysis of phosphomonoesters over phosphodiester molecules (K_m values for *p*NPP and *p*NPPP hydrolysis were 2.2 and 35.1 mM, respectively). It would be of interest to examine the kinetic properties of PME and 5′ ND activity in foliose terricolous species, where a greater affinity towards 5′ ND hydrolysis might be expected.

5′ nucleotide phosphodiesterase activity could not be detected in non-N_2-fixing lichens under substrate saturating conditions, and PDE activity was low. In addition, rates of phosphodiester hydrolysis were generally reduced when compared to PME activity. This is consistent with Stevenson (1994) who reported up to 5 times greater PME activity than PDE activity in lichen from the genera *Peltigera* and *Cladonia*. It is also consistent with preliminary data presented in this thesis for *C. portentosa* (see Chapter 2, section 2.3) and the same relationship has been recorded in several other groups including algae (Whitton *et al.* 1990; Banerjee *et al.* 2000), fungi (Leake and Miles 1996) and vascular plants (Asmar and Gissel-Nielsen 1997; Turner *et al.* 2001). The need for a greater capacity for PME activity compared with PDE activity might arise because release of PO_4^{3-} from a phosphodiester involves substrate hydrolysis by both PME and PDE enzymes. Phosphodiesterase activity initially releases a phosphomonoester, which must then be further hydrolysed by PME activity in order to release PO_4^{3-} (Turner and Haygarth 2005).

The capacity for PME and PDE activities in all lichen species examined in the present study is consistent with Lane and Puckett (1979) who reported measurable PME activity in 11 species of non-N_2-fixing and N_2-fixing lichens, and with Stevenson (1994) who confirmed PME and PDE activities in *C. arbuscula*, *Peltigera canina*, and *P. praetextata*. Both of the above studies confirm high interspecific variation in lichen PME activity. The present study represents the first report of 5′ ND activity in lichens. However, it is consistent with the literature on filamentous fungi, which documents measurable 5′ ND activity (Leake and Miles 1996) and growth on media containing DNA as the sole source of P (Beever and Burns 1980; Leake and Miles 1996). Phosphomonoesterase, PDE and 5′ ND activities are probably apoplastic, enabling direct uptake of PO_4^{3-} released into the symplast via transporter systems (Turner *et al.* 2004). Lemoine *et al.* (1992) reported that fungal mineralization of nucleic acids occurs in the cell wall matrix, where the highest phosphatase activity was also recorded. However, direct uptake of organic P molecules and subsequent cellular assimilation cannot be ruled out. In mycorrhizal fungal symbioses, it has been suggested that direct assimilation of nucleotides is ecologically advantageous, since by this mechanism N, C and P are all acquired simultaneously (Leake and Miles 1996).

There was marked interspecific variation in the apparent optimum pH for PME, PDE and 5′ ND activities. Species with the highest PME activity (*Stereocaulon spp., L. linita*) had acid pH optima. In contrast, *N. arcticum*, *Peltigera spp.* and *S. crocea* had apparent pH optima for PME activity of 5.9. This is in line with the findings of Lane and Puckett (1979) who recorded maximum PME activity in *C.*

rangiferina at pH 2.2 (c.f. pH 3.2 in the current investigation), and with Stevenson (1994) who measured maximum PME activity in *Peltigera canina* and *P. praetextata* at pH 6. Of the species investigated, pH optima for PME and PDE activities were similar. This is consistent with results obtained for *C. portentosa*, for which the optimum pH for both PME and PDE activities was 2.5 (Chapter 2, section 2.3). Inter-species variation in the optimum pH for 5′ ND activity followed a less obvious pattern. In general, foliose terricolous species had the highest 5′ ND activities with a neutral optimum pH value similar to that for PME and PDE activities. This is an interesting finding given the observation that phosphodiesterase activity and mineralization of DNA in soils is reported to be maximal under alkaline conditions (Browman and Tabatabai 1978; Frankenberger *et al.* 1986). *Stereocaulon spp.* had a higher optimum pH for 5′ ND activity than those for the other phosphatases investigated (by *c.* 1 – 3 units). This was a surprising finding, since it might be expected that all three lichen phosphatase enzymes are located in a similar environment (cell membrane or cell wall) in a manner similar to other filamentous fungi (Leake and Miles 1996). However, Jansson *et al.* (1988) reports that different algal phosphatases show maximum hydrolysing capacity at different pH values, and Whitton *et al.* (2005) states that 'different phosphatase activities do not necessarily respond to pH in the same way'. Few data exist in the literature to explain a species preference for acid, neutral or alkaline phosphatase activity. Several authors have suggested a link between the pH of the underlying substratum and the pH optima for phosphatase amongst components of terrestrial vegetation (Whitton *et al.* 1990; Stevenson

1994; Christmas and Whitton 1998b; Whitton *et al.* 2005). Yet to date, no clear response pattern has been discovered. Acid PME activity in *C. portentosa* (pH 2.5) was attributed to the presence of acidic secondary compounds in the vicinity of the cell membrane and cell wall (Chapter 2).

Lobaria linita differs from other foliose species studied in having a low pH optimum for PME activity. Rates of PME activity were higher than in other foliose species and were comparable to the rates in *Stereocaulon spp.* In contrast, rates of 5′ ND activity were lower than in any of the other nitrogen fixing species studied. *L. linita* is morphologically almost identical to *L. pulmonaria* (Brodo *et al.* 2001), an epiphytic species with acid PME activity (Lane and Puckett 1979), and it is tempting to speculate that *L. linita* represents a relatively recent descendant of *L. pulmonaria* that might not have yet optimized phosphatase activities for a terrestrial lifestyle.

There is a clear effect of habitat on PME, PDE and 5′ ND activities in the lichen species investigated. In the current study, lichens from an alpine habitat had consistently higher phosphatase activities than the same species from a subarctic birch forest. This might reflect differences in nutrient availability, which could be expected to be greater in forest habitats as a result of canopy through-fall, leaf litter accumulation and a greater soil depth. In contrast, the more exposed alpine environment probably receives lower nutrient inputs (as evidenced by low values

of $[P]_{lichen}$ and $[N]_{lichen}$), which in turn might stimulate up-regulation of phosphatase activity.

Evidence from the current investigation implies that lichen phosphatases are remarkably robust. Annual temperatures at Abisko typically fluctuate between -12 and 12 ^{o}C (Andersson *et al.* 1996), with lichens frequently undergoing periods of desiccation followed by subsequent rehydration. In particular during the winter months, thalli are covered by a layer of snow, restricting nutrient, water and light availability. It is therefore essential for phosphatase enzymes located on the outer-surface of the cell to withstand periods of high irradiance, variation in temperature and desiccation, in order to rapidly regain full functionality upon the onset of precipitation. However, a 63 % reduction in PME activity recorded in *P. aphthosa* after storage at -15 ^{o}C for 10 months suggests that at higher temperatures, typical of the summer growing season, there might be a roughly annual turnover of PME units.

CHAPTER 10. General Discussion

Two observations prompted the work reported in this thesis. The first came from the results of a survey by Hyvärinen and Crittenden (1998a, 1998c) that examined relationships between rainfall chemistry and thallus chemistry in the heathland lichen *Cladonia portentosa*. The authors reported a significant positive relationship between wet N deposition (**N**) and N concentration in the thallus (both $[N]_{apex}$ and $[N]_{base}$, Fig. 10.1a) but they also found co-variation between concentrations of N and P. Second, nitrogen-fixing lichens, in addition to frequently containing higher concentrations of N than non-N_2-fixing lichens also contain higher concentrations of P. These observations prompted the initial hypothesis (see Chapter 1) that phosphatase activity might promote P capture in lichens well supplied with N.

In line with the findings of Hyvärinen and Crittenden (1998a), results from the present study confirm that N enrichment is associated with a marked increase in both $[N]_{apex}$ and $[N]_{base}$ in *C. portentosa*. I have shown that PME activity in this lichen is significantly up-regulated in response to elevated N supply, with activity increasing by a factor of *c.* 2 between sites in Great Britain receiving 4.1 and 32.8 kg N ha^{-1} y^{-1}. Analysis of PME activity in thalli growing under artificial wet N treatments at Whim Moss revealed a similar degree of up-regulation and provided evidence of causality. However, elevated PME activity could not explain the co-variation in thallus N and P concentrations observed by Hyvärinen and Crittenden

(1998a) as in the present study, thalli collected from N-rich sites had elevated [N] and PME activity, but not elevated [P] (Fig. 10.1b).

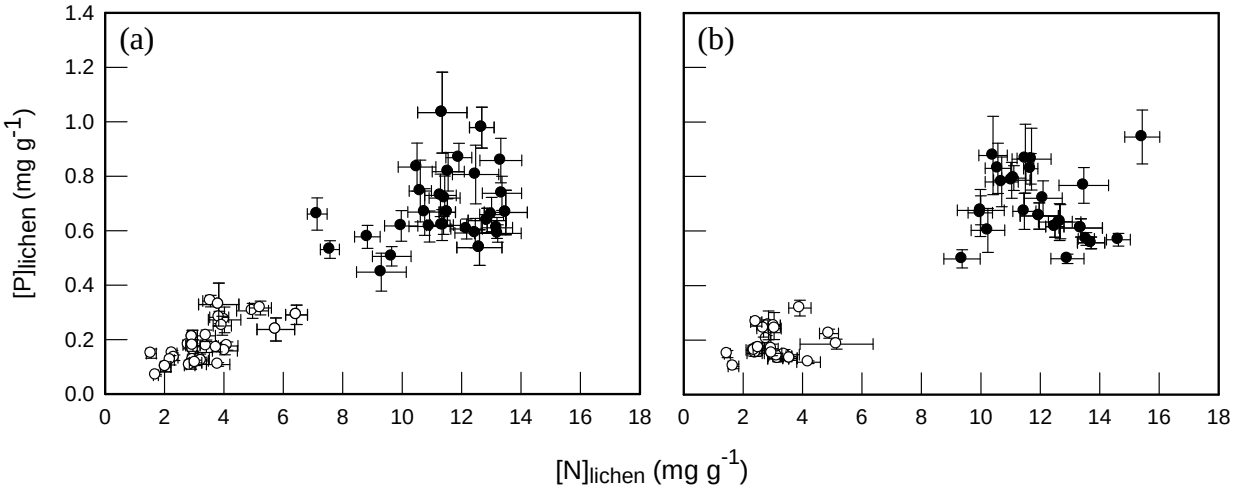


Figure 10.1 Relationships between $[N]_{lichen}$ and $[P]_{lichen}$ in apical (●, $[N]_{apex}$, 0 - 5 mm) and basal (○, $[N]_{base}$, 40 – 50 mm) segments of *Cladonia portentosa* analysed by Hyvärinen and Crittenden (1998a) (a, ●, r = 0.39, P < 0.001; ○, r = 0.65, P < 0.001) or during the current investigation (b, ●, r = -0.146, P = 0.449; ○, r = 0.126, P = 0.532). Plotted values are means (n = 5 - 10) ± 1 SEM.

Up-regulation of PME activity in *C. portentosa* is associated with a marked increase in thallus N:P mass ratio, evidencing a shift from N- to P-limitation. Heathland habitats supporting populations of *C. portentosa* are generally considered to be N-limited (Aerts and Berendse 1988; Aerts and Heil 1993; Britton and Fisher 2007a). However, aerial deposition of N in western Europe increased significantly in the latter part of the 20th century (from background inputs of 1 – 3 kg N ha^{-1} y^{-1} in the early 1900's to maximum values ranging between 30 – 40 kg N ha^{-1} y^{-1} today) (Bobbink *et al.* 2002; Fowler *et al.* 2004), yet

there is little evidence to suggest variation in P supply. Accordingly, it seems plausible that a change in the pollution climate since Hyvärinen and Crittenden's study, published in 1998 but undertaken in 1994 (i.e. falling SO_2 and N emissions (Pitcairn *et al.* 1995; NEGTAP 2001; Fowler *et al.* 2004)), might explain the discrepancies between thallus chemistries reported in the two studies. However, there was no significant difference between the measured N deposition values ($\mathbf{N}_m$) at 8 sites common to both the 1994 and 2006 studies. Alternatively, differences in site selection might account for discrepancies in the results of the two studies, since high N deposition sites also receiving high values of $[N]_{ppt}$ were omitted from the present study (see Chapter 4).

Up-regulation of surface-bound PME activity presumably increases the efficiency with which *C. portentosa* can capture P under conditions of P-limitation associated with N enrichment. Data from the present study suggests that an increased demand for P is probably the primary stimulus for increased PME activity, rather than a direct response to increased N availability. Phosphomonoesterase activity in *C. portentosa* is negatively related to thallus P concentration ($[P]_{lichen}$), a finding consistent with data from axenic mycobiont cultures of *C. portentosa* (see Chapter 8) and other plant/fungal systems (Beever and Burns 1980; Press and Lee 1983; Kroehler *et al.* 1988), and which might represent repression of enzyme synthesis under high tissue P concentrations (McComb *et al.* 1979; Kroehler *et al.* 1988; Whitton *et al.* 2005). It is also possible that this repressive effect might account for low PME activity measured in foliose N_2-fixing lichens (e.g. *Peltigera spp*.) which contain high values of $[P]_{lichen}$. At Whim Moss, application of 16 kg N +

0.57 kg P ha^{-1} y^{-1} and 64 kg N + 4 kg P ha^{-1} y^{-1} resulted in down-regulation of PME activity in *C. portentosa*. This provided further evidence that this lichen encounters P-limitation when highly enriched with N (i.e. 64 kg N ha^{-1} y^{-1}), and that the addition of P (< 4 kg P ha^{-1} y^{-1}) is sufficient to eliminate any deficiency.

Nitrogen fixing lichens which contained high concentrations of N also contained high concentrations of P. High thallus P concentrations in lichens with the greatest capacity for nitrogenase activity might reflect a greater demand for P associated with the maintenance of 'energy intensive' N_2-fixation (Israel 1987; Kennedy and Cocking 1997; Vance *et al.* 2000; Chaudhary *et al.* 2008). It has been calculated that nitrogen fixation requires *c.* 16 ATP molecules to reduce each molecule of N_2 to two molecules of NH_3 (Postgate 1998). High values of $[P]_{lichen}$ in N_2-fixing lichens could not be explained by elevated PME activity, and are considered unlikely to result from direct uptake of inorganic phosphate which is in low supply at subarctic sites such as Abisko. Cosgrove (1967) reports that a large proportion of P in alpine soils is present in the form of phosphodiesters, including nucleic acids and phospholipids. It was therefore of considerable interest that several foliose N_2-fixing lichens (e.g. *Peltigera spp.*) possess a greater capacity for the hydrolysis of the artificial phosphodiester 5´ nucleotide phosphodiesterase (5´ ND) compared to PME activity. This, together with the observation that maximum phosphatase activity in foliose N_2-fixing lichens (e.g. *N. arcticum*, *P. aphthosa*) occurs in rhizine rich regions of the thallus, might indicate that such species maintain high $[P]_{lichen}$ by hydrolysing phosphodiesters available in the underlying leaf litter and soil.

Phosphomonoesterase activity in *C. portentosa* is not distributed uniformly throughout the thallus. Maximum activity occurs in the apices, with a concentration of activity on both the inner- and outer- surfaces of the hollow tube-like thallus branches. Location in the apices and on the outer-most fungal hyphae would serve to maximise enzyme interaction with aerially deposited organic P, while high activity on the inner-most thallus surface might promote additional substrate capture should nutrient deposits reach inside the thallus (e.g. via perforated axils). In addition, Cardinale *et al.* (2006; 2008) have shown the presence of microbial communities on the inner surface of *Cladonia spp*., cell turnover of which could provide a source of organic P for lichen hydrolysis. Alternatively, PME activity on the inner surface of thallus branches could be linked to a role in P conservation via hydrolysis of P-containing metabolites which might leak from the thallus (J. Leake, Pers. Comm.). It is possible that inner surface PME activity contributes little towards total $[P]_{lichen}$, but such activity might serve to maximise nutrient retention which could be ecologically advantageous in nutrient poor habitats. In foliose lichens, maximum PME activity was recorded at the centre of the thallus, in regions associated with rhizines. This finding agrees with Stevenson (1994) who reported that maximum PME activity in *Peltigera spp.* was associated with the under-surface of the thallus and, in particular, with the rhizines. As previously discussed, high phosphatase activity in this region of the thallus might reflect a capacity for foliose terricolous lichens to mine P from underlying leaf litter and soil.

Application of Enzyme Labelled Fluorescence (ELF) revealed that PME activity is exclusively associated with the mycobiont, consistent with high rates of activity recorded in *C. portentosa* grown in axenic culture. Activity in axenic mycobiont cultures was up to an order of magnitude greater than reported for other cultured fungi (see Appendix 1, Ho and Zak 1979; Antibus *et al.* 1986; McElhinney and Mitchell 1993)), suggesting that lichen-forming fungi possess a remarkable capacity for PME activity. This might reflect the relative availability of nutrients to lichen-forming fungi in atmospheric deposits (Crittenden 1989) when compared with that in other fungal habitats, for example, soils (Allen *et al.* 1968; Gore 1968). Phosphomonoesterase activity appears to be highly concentrated around hyphal lumina and might reflect intracellular enzyme systems. However, there is no evidence to confirm that PME activity measured in colourimetric assays results from the actions of enzymes revealed by ELF. It is hypothesised that extracellular phosphatase activity might serve to increase the capture of growth-limiting P in oligotrophic environments, since the release of PO_4^{3-} would occur in the apoplast, in the vicinity of uptake transporters situated in the cell membrane. It might also facilitate recycling of enzymes via endocytosis (Stenberg 2007) which, given the high N content of phosphatases (relative to other proteins) (Treseder and Vitousek 2001), would be an advantageous mechanism in low nutrient habitats. An extracellular location would also provide support for the suggestion by Ellis *et al.* (2005) that the natural turn-over of surface-bound enzymes might explain a loss of ^{15}N from labelled thalli of *C. portentosa* during a 2 year period of growth in the field. Other bound exo-enzymes so far reported in lichens include carbonic

anhydrase (Palmqvist and Badger 1995), laccase (Laufer *et al.* 2006; Zavarzina and Zavarzin 2006) and urease (Pérez-Urria *et al.* 1993).

In a seminal paper, Crittenden *et al.* (1994) explored evidence that nutrient supply (principally N, but also P) might limit the growth of more productive lichens. At the time, a more widely accepted view was that the rate of C assimilation via photosynthesis was the primary limiting factor for lichen growth (Lechowicz 1981; 1983; Kershaw 1985). Several lines of evidence in this thesis support Crittenden *et al*'s (1994) proposal and suggest that both N and P supply might limit the growth of the mat-forming lichen *C. portentosa*. First, the uptake capacities for N and P are finely tuned to the growth-led demand for these elements as indicated by the relative availabilities of N and P. Thus at high modelled N deposition ($\mathbf{N}_s$) sites, transporter affinities for inorganic and organic N forms are lowered, while that for PO_4^{3-} is increased; a counter response was evident at background $\mathbf{N}_s$ sites. Second, there is now abundant evidence that in both plants and micro-organisms, PME activity reflects growth-led demand for P. In *C. portentosa*, application of P in factorial fertilizer treatments suppressed, while addition of N alone, promoted PME activity. A similar response occurred in axenic cultures of the mycobiont. Further, in a field survey, PME activity was maximal at high $\mathbf{N}_s$ sites at which high thallus N:P mass ratios suggest P-limitation in line with Güsewell's (2004) proposals. Third, maximum N and P concentrations measured in the apices of *C. portentosa* ($[N]_{apex}$, 16 mg g^{-1}; $[P]_{apex}$, 1.9 mg g^{-1}) are very similar to the lowest concentrations recorded in axenic mycobiont cultures grown under conditions of severe nutrient-deprivation ($[N]_{myc}$,

20 mg g^{-1}; $[P]_{myc}$, 1.5 mg g^{-1}). This compares favourably with the findings of Crittenden *et al.* (1994) who showed that the maximum concentrations of $[N]_{apex}$ in *Cladonia stellaris* and *Stereocaulon paschale* are similar to the lowest N concentrations measured in N-deprived mycobiont cultures. For *C. portentosa*, this data suggests that growth in oligotrophic heathland habitats is tightly matched to nutrient-income, and is likely to be either N- or P-limited. Fourth, the relative growth rate of *C. portentosa* from a high $\mathbf{N}_s$ site was two-fold greater than that of thalli receiving background N inputs. One explanation is that the higher RGR in lichen from a high $\mathbf{N}_s$ site is linked to higher $[N]_{lichen}$ values. Alternatively, differences in growth rate might be explained by genetic variation. A combination of these two arguments was proposed by McCune and Caldwell (2009) to explain a doubling in the annual growth of *Lobaria pulmonaria* after a single 20 min treatment with 600 mg l^{-1} K_2HPO_4 compared with control thalli receiving no supplementary P. Fifth, nitrogenase activity is strongly positively related to $[P]_{lichen}$, consistent with the suggestion by Crittenden *et al.* (1994) that P limits the growth of N_2-fixing lichens. The authors claim that sufficient N will only be fixed in order to maximise P-use efficiency, and there is much evidence confirming that N_2-fixing organisms increase nitrogenase activity in response to P fertilization (Eisele *et al.* 1989; Smith 1992; Vitousek *et al.* 2002; Weiss *et al.* 2005; Benner *et al.* 2007). For example, Benner *et al.* (2007) reported a 400 % increase in nitrogenase and a 10-fold increase in biomass in the lichen *Pseudocyphellaria crocata* supplied with 100 kg P ha^{-1} y^{-1} for 5.5 years. A comparable increase in nitrogenase activity (from 5.6 to 31.6 nmol ethylene g^{-1} dry mass h^{-1}) was recorded in *Stereocaulon vulcani* when supplied with 1.5 kg P ha^{-1} y^{-1} for 6 weeks (Kurina

and Vitousek 1999) and as mentioned previously, a single 20 min immersion in 600 mg l^{-1} KH_2PO_4 was found to double the annual biomass growth in *L. pulmonaria* (McCune and Caldwell 2009).

Research undertaken during the current investigation has yielded several interesting questions which require further attention. It is hypothesised that foliose terricolous lichens might access a source of organic P from leaf litter and soil via phosphatase activity associated with rhizines. Phytic acid (*myo*-inositol hexakisphosphate), is a major storage form of P in plants (Wyss *et al.* 1998), and as such forms a key component of the organic P pool in soils. Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) is a substrate specific enzyme which catalyzes the sequential hydrolysis of phytic acid to lower inositol phosphates (i.e. penta-, tetra-, tri-, di- and mono-esters of inositol) and inorganic phosphate (McComb *et al.* 1979; Livingstone *et al.* 1983; Vats and Banerjee 2004). There is abundant evidence of phytase activity in fungi (Ullah and Gibson 1987; Colpaert *et al.* 1997; Wyss *et al.* 1998; Yadav and Tarafdar 2003), and it seems plausible to suggest that foliose terricolous lichens might have a high capacity for phytase activity, in particular in rhizine rich regions of the thallus. Conventionally, the release of PO_4^{3-} from phytic acid is used to determine fungal phytase activity (Wyss *et al.* 1998; Yadav and Tarafdar 2003; Henrique *et al.* 2004; Zhou *et al.* 2006), and the most commonly adopted method is the ammonium molybdate method of Heinonen and Lahti (1981). Here, sodium phytate is added to samples incubated in a solution of acetone: 5N H_2SO_4: 10 mM $(NH_4)6Mo_7O_{24}.4H_2O$ (2:1:1 v/v) and enzymatically released orthophosphate is rapidly converted to

phosphomolybdate which forms a yellow colour and can be measured quantitatively on a spectrophotometer at 390 – 420 nm. During the current investigation, an attempt was made to detect phytase activity in *Peltigera praetextata* by measuring PO_4^{3-} released from sodium phytate presented to whole viable thalli. However, no PO_4^{3-} release was recorded. This could indicate either absence of phytase activity, or it might be an artefact due to rapid PO_4^{3-} uptake by the thallus (Farrar 1976; Hyvärinen and Crittenden 1998c). Attempts to kill thalli using gamma irradiation and azide were unsuccessful. There is also a possibility that PO_4^{3-} released became absorbed to the walls of glassware. Clearly, alternative approaches are necessary.

Recently, two novel methods for measuring phytase activity have been documented. First, Chen and Li (2003) reported a high-performance anion-exchange chromatographic method which can separate and identify a large number of the lower-order inositol phosphate isomers released from the hydrolysis of phytic acid. Second, Molecular Probes (Invitrogen) have released a commercially available phytase assay kit based on measurement of the PO_4^{3-} released following a series of enzymatically-linked reactions (see EnzChek Ultra Phytase Assay Kit (E33701) product information sheet, Molecular Probes, Invitrogen for further details). In addition to these two methods, Berry and Berry (2005) and Berry *et al.* (2007) have recently documented a novel method for the detection of phytase activity, which permits direct measurement of the phosphate ester bond cleavage by phytase activity via hydrolysis of the new chromophoric substrate analogue of phytic acid 5-*O*-[6-(benzoylamino)hexyl]-D-*myo*-inositol-1,2,3,4,6-

pentakisphosphate. However, this method remains under development. Nonetheless, these techniques might provide opportunities in the future to test for phytase activity in physiologically active lichens.

Phosphomonoesterase activity in *C. portentosa* appears to be exclusively associated with the mycobiont. However, results presented in this thesis cannot be taken as unequivocal evidence that exogenous PME activity is absent from the photobiont. A comparison of PME activity between freshly isolated photobiont cells and established axenic cultures might confirm the absence of activity in the lichen thallus. It is also of interest to utilise ELF to locate PME activity in foliose terricolous lichens, to test the hypothesis that rhizines are 'hot spots' for PME activity.

Change in PME activity in response to N supply is comparatively rapid and marked in *C. portentosa*. Therefore, analysis of PME activity in this lichen could provide a novel bio-indicator of change in N deposition in heathland habitats. Although up-regulation of PME activity in response to N supply has been detected in other plants/habitats (Johnson *et al.* 1999; Phoenix *et al.* 2003; Bragazza *et al.* 2005; Pilkington *et al.* 2005; Phuyal *et al.* 2008), advantages of using *C. portentosa* include a relatively rapid response time (measured at 6 months, and potentially could be faster) and the direct interception of atmospheric deposits by lichen thalli. If this technique is to be adopted, it might provide a cheap, reliable and highly sensitive bio-indicator for N deposition levels in the British Isles. However, it is worth considering the need to establish a standardised region of the thallus in which to record PME activity, given the vertical variation in activity.

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Appendices

Appendix 1. Maximum obtainable rates of PME activity in a range of bacteria, fungi, lichens, plants and soils.

Species	Group	PME activity (mmol substrate hydrolysed g^{-1} dry mass h^{-1})	Reference
Hymenoscyphus ericae	Mycorrhizal fungus	7.50	Gibson and Mitchell (2005)
Cladonia portentosa	Lichen-forming fungus	7.09	Current investigation
Teloschistescapensis	Lichen	4.52	E. J. Hogan (unpublished)
Cladonia portentosa	Lichen	4.04	Current investigation
Sphagnum capillifolium	Moss	3.96	Phuyal *et al.* (2008)
Cladonia rangiformis	Lichen	3.67	E. J. Hogan (unpublished)
Calluna vulgaris endophyte	Plant endophyte	3.60	Gibson and Mitchell (2005)
Hypnum jutlandicum	Moss	2.79	Phuyal *et al.* (2008)
Unidentified *basidiomycete*	Mycorrhizal fungus	2.40**	McElhinney and Mitchell (1993)
Usnea sphacelata	Lichen	2.00	P. D. Crittenden (Pers. Comm.)
Hebeloma cylindrosporum	Mycorrhizal fungus	1.80	Tibbett *et al.* (1998)
Lobaria linita	Lichen	1.68	Current investigation
Stereocaulon alpinum	Lichen	1.68	Current investigation
Stereocaulon paschale	Lichen	1.60	Current investigation
Agrostis capillaris	Plant (root surface)	1.51*	Johnson *et al.* (1999)
Koelerina macrantha	Plant (root surface)	1.43*	Phoenix *et al.* (2003)
Cladonia portentosa	Lichen-forming fungus	1.38	Z. Mtshali (Pers. Comm.)
Nostoc	Cyanobacteria	1.25	Whitton *et al.* (1990)
Peat bog	Leaf litter	1.08	Bragazza *et al.* (2006)
Stereocaulon vesuvianum	Lichen	1.06	Current investigation

Appendix 1. continued

Species	Group	PME activity (mmol substrate hydrolysed g^{-1} dry mass h^{-1})	Reference
N-fertilized grassland soil	Soil	1.00	Colvan *et al.* (2001)
Flavocetraria cucullata	Lichen	0.90	Current investigation
Unidentified *basidiomycete*	Mycorrhizal fungus	0.90**	McElhinney and Mitchell (1993)
Hymenoscyphus ericae	Mycorrhizal fungus	0.84**	Leake and Miles (1996)
Leotodon hispidus	Plant (root surface)	0.80**	Phoenix *et al.* (2003)
Xanthoria parietina	Lichen	0.80	G. Minnullina (Pers. Comm.)
Peltigera scabrosa	Lichen	0.79	Current investigation
Hordeum vulgare	Plant (root surface)	0.50*	Asmar and Gissel-Nielsen (1997)
Evernia prunastrii	Lichen	0.43	E. J. Hogan (unpublished)
Suillus grevillei	Mycorrhizal fungus	0.41**	McElhinney and Mitchell (1993)
Peltigera malacea	Lichen	0.39	Current investigation
Nephroma arcticum	Lichen	0.38	Current investigation
Laccaria laccata	Mycorrhizal fungus	0.34	Ho and Zak (1979)
Blanket peat	Soil	0.31	Turner *et al.* (2002)
Peltigera aphthosa	Lichen	0.28	Current investigation
Amanita muscaria	Mycorrhizal fungus	0.27	Ho and Zak (1979)
Cladonia rangiferina	Lichen	0.25	Current investigation
Picea sitchensis	Plant (root surface)	0.25*	Alexander and Hardy (1981)
Paxillus involutus	Mycorrhizal fungus	0.24	McElhinney and Mitchell (1993)
Cladonia rangiferina	Lichen	0.23	Lane and Puckett (1979)
Palustriella commutate var. *falcate*	Moss	0.23	Turner *et al.* (2001)

Appendix 1. continued

Species	Group	PME activity (mmol substrate hydrolysed g^{-1} dry mass h^{-1})	Reference
Umbillicara vellea	Lichen	0.21	Lane and Puckett (1979)
Agrostis capillaris	Plant (root surface)	0.20*	Johnson *et al.* (2005)
Upland grassland	Soil	0.19	Johnson *et al.* (1998)
Cladonia arbuscula	Lichen	0.18	LeSueur and Puckett (1980)
Fontalis antipyretica	Moss	0.18	Christmas and Whitton (1998a)
Hylocomium splendens	Moss	0.18	Turner *et al.* (2001)
Hypnum jutlandicum	Moss	0.18	Turner *et al.* (2001)
Thelephora terrestris	Mycorrhizal fungus	0.18	Ho and Zak (1979)
Warnsorfia fluitans	Moss	0.18**	Ellwood *et al.* (2002)
Acid grassland	Soil	0.17	Johnson *et al.* (1998)
Cetraria islandica	Lichen	0.17	Lane and Puckett (1979)
Sphagnum cuspidatum	Moss	0.17	Turner *et al.* (2001)
Glomus intraradices	Mycorrhizal fungus	0.14	Joner and Johansen (2000)
Lobaria pulmonaria	Lichen	0.14	Lane and Puckett (1979)
Plagiothecium nemorale	Moss	0.14	Turner *et al.* (2003)
Plasmatia tuckermanii	Lichen	0.14	Lane and Puckett (1979)
Plantago lanecolata	Plant (root surface)	0.14*	Johnson *et al.* (1999)
Warnsorfia fluitans	Moss	0.14	Turner *et al.* (2001)

Appendix 1. continued

Species	Group	PME activity (mmol substrate hydrolysed g^{-1} dry mass h^{-1})	Reference
Cladonia rangiferina	Lichen	0.13	LeSueur and Puckett (1980)
Lobaria scrobiculata	Lichen	0.13	Lane and Puckett (1979)
Palustriella commutate var. *falcate*	Moss	0.13	Turner *et al.* (2003)
Glomus claroideum	Mycorrhizal fungus	0.12	Joner and Johansen (2000)
Heathland	Soil	0.12	Johnson *et al.* (1998)
Hylocomium splendens	Moss	0.12	Turner *et al.* (2003)
Parmelia spp.	Lichen	0.12	E. J. Hogan (unpublished)
Sphagnum cuspidatum	Moss	0.12	Turner *et al.* (2003)
Sphagnum subnitens	Moss	0.12	Press and Lee (1983)
Cladonia uncialis	Lichen	0.11	LeSueur and Puckett (1980)
Paxillus involutus	Mycorrhizal fungus	0.11	Alvarez *et al.* (2006)
Sphagnum tenellum	Moss	0.11	Press and Lee (1983)
Pisolithus tinctorius	Mycorrhizal fungus	0.10**	Alvarez *et al.* (2004)
Racomitrium lanuginosum	Moss	0.10	(Turner *et al.* 2001)
Rhynchostegium riparioides	Moss	0.10	Christmas and Whitton (1998a)
Sphagnum fuscum	Moss	0.10	Press and Lee (1983)
Acid grassland	Soil	0.09	Turner *et al.* (2002)
Carex flacca	Plant (root surface)	0.09*	Phoenix *et al.* (2003)
N/P fertilized grassland	Soil	0.09**	Spiers and McGill (1979)
Cladonia turgida	Lichen	0.08	LeSueur and Puckett (1980)

Appendix 1. continued

Species	Group	PME activity (mmol substrate hydrolysed g^{-1} dry mass h^{-1})	Reference
Fontalis antipyretica	Moss	0.08	Christmas and Whitton (1998b)
Moorland	Leaf litter	0.08	Pilkington *et al.* (2005)
Peltigera praetextata	Lichen	0.08	Current investigation
Sphagnum capillifolium	Moss	0.08	Press and Lee (1983)
Sphagnum cuspidatum	Moss	0.08	Press and Lee (1983)
Sphagnum papillosum	Moss	0.08	Press and Lee (1983)
Stereocaulon saxatile	Lichen	0.08	LeSueur and Puckett (1980)
Sub-alpine podzol	Leaf litter	0.08	Papanikolaou *et al.* (2006)
Eriophorum vaginatum	Plant (root surface)	0.07*†	Kroehler and Linkins (1988)
Glomus caledonium	Mycorrhizal fungus	0.07*	Gianinazzi-Pearson and Gianinazzi (1985)
Racomitrium lauginosum	Moss	0.07*	Turner *et al.* (2003)
Calcareous grassland	Soil	0.06	Johnson *et al.* (1998)
Peltigera aphthosa	Lichen	0.06	Lane and Puckett (1979)
Sphagnum recurvum	Moss	0.06	Press and Lee (1983)
Umbilicaria papulosa	Lichen	0.06	Lane and Puckett (1979)
Dicranium scoparium	Moss	0.05	Turner *et al.* (2003)
Erica hispidula endophyte	Plant endophyte	0.05*	Straker and Mitchell (1986)
Paxillus involutus	Mycorrhizal fungus	0.05	Alvarez *et al.* (2004)

Appendix 1. continued

Species	Group	PME activity (mmol substrate hydrolysed g^{-1} dry mass h^{-1})	Reference
Peltigera canina	Lichen	0.05	(Stevenson 1994)
Peltigera praetextata	Lichen	0.05	Stevenson (1994)
Rhizopogon vinicolor	Mycorrhizal fungus	0.05	Ho and Zak (1979)
Sphagnum auriculatum var. *innundatum*	Moss	0.05	Press and Lee (1983)
Sphagnum fimbriatum	Moss	0.05	Press and Lee (1983)
Sphagnum palustre	Moss	0.05	Press and Lee (1983)
Autopaxillus boletinoides	Mycorrhizal fungus	0.04**	Alvarez *et al.* (2004)
Cladonia gracilis	Lichen	0.04	Lane and Puckett (1979)
Peltigera canina	Lichen	0.04	Lane and Puckett (1979)
Polytricum commune	Moss	0.04	Turner *et al.* (2003)
Cladonia arbuscula	Lichen	0.03	Stevenson (1994)
Descolea antarctica	Mycorrhizal fungus	0.03	Alvarez *et al.* (2004)
Fontalis antipyretica	Moss	0.03	Turner *et al.* (2001)
Peltigera membranacea	Lichen	0.03	Stevenson (1994)
Calcareous grassland	Soil	0.02	Turner *et al.* (2002)
Peltigera lactucifolia	Lichen	0.02	Stevenson (1994)
Peltigera rufescens	Lichen	0.02	Stevenson (1994)
Piloderma bicolour	Mycorrhizal fungus	0.02	Ho and Zak (1979)
Pinus pinea	Plant (root surface)	0.02†	Pasqualini *et al.* (1992)
Sphagnum squarrosum	Moss	0.02	Press and Lee (1983)
Umbilicaria mammulata	Lichen	0.02	LeSueur and Puckett (1980)

Appendix 1. continued

Species	Group	PME activity (mmol substrate hydrolysed g^{-1} dry mass h^{-1})	Reference
Cenococcum geophilum	Mycorrhizal fungus	0.01	Alvarez *et al.* (2006)
Histosol	Soil	0.01	Drouillon and Merckx (2005)
Pasture soil	Soil	0.01	Turner and Haygarth (2005)
Peltigera polydactyla	Lichen	0.01	Stevenson (1994)
Podzol	Soil	0.01	Drouillon and Merckx (2005)
Sub-alpine podzol	Soil	0.01	Papanikolaou *et al.* (2006)
Phaseolus vulgaris var. *Daisy*	Plant (root surface)	0.001	Helal (1990)
Pisolithus tinctorius	Mycorrhizal fungus	0.001	Jayakumar and Tan (2005)
Gloeocapsa-Tebouxia dominated endolithic community	Endolithic community	5×10^{-4}	Banerjee *et al.* (2000)
Trebouxia dominated endolithic community	Endolithic community	4×10^{-4}	Banerjee *et al.* (2000)
Chroococcidiopsis dominated endolithic community	Endolithic community	6×10^{-5}	Banerjee *et al.* (2000)
Calluna vulgaris	Plant (root surface)	$3 \times 10^{-5*}$	Pilkington *et al.* (2005)
Calothrix parietina	Cyanobacterium	9×10^{-6}	Grainger *et al.* (1989)
Nostoc commune	Cyanobacterium	1.25×10^{-6}	Whitton *et al.* (1990)

* mmol substrate hydrolysed g^{-1} fresh mass h^{-1}

** Approximate value

† Assumed mm^2 surface area to g^{-1} fresh mass ratio of 7000 following Alexander and Hardy (1981)

Appendix 2. **Results of an investigation to compare the growth rates of fertile and non-fertile podetia of *C. portentosa*.**

Method

Naturally hydrated fertile and non-fertile thalli of *C. portentosa* (*n* = 30), typically 2 - 5 interconnected podetia < 20 mm in diameter were collected from the Halsary, Caithness (ND 195 493) and cut to a uniform length of 50 mm (measured downwards from the apex) in the field. Sample collection and preparation followed the method outlined in section 6.2.2. The growth experiment was set up on 25.10.05 following the method described in section 6.2.3. After 12 months' growth in the field (on 08.10.06, T_1), a sub-set of samples (*n* = 27) was collected, with the remaining samples recovered after 2 years on 22.11.07 (T_2). Calculation of RG and RGR follows section 6.2.6 with data analysed as described in section 6.2.7.

Results and Discussion

Both sets of podetia gained significant length and mass during the two year experimental period ($P < 0.001$, Table A2.1). However, no significant differences between the mean growth rates of fertile and non-fertile podetia of *C. portentosa* were found. Thus, it appears that the production of abundant ascomata has no measurable impact on the growth of *C. portentosa*. Between the two years of study (i.e. T_1 and T_2), the RGR growth rate of fertile and non-fertile podetia decreased (Table A2.2), consistent with the findings of Kytöviita and Crittenden

(2007). These authors reported a negative relationship between the RGR and length of podetia of *Cladonia stellaris* and *Stereocaulon paschale* which they attributed to increased N content and N use efficiency in shorter thalli.

Table A2.1 Values of mean mass and length increments in fertile and non-fertile thalli of *C. portentosa* recorded at T_1 and T_2.

Sample	Mean* mass increment (mg) ± 1 SEM		Mean* length increment (mm) ± 1 SEM	
	T_1	T_2	T_1	T_2
Fertile	57.15 ± 8.37	92.06 ± 10.99	18.46 ± 1.69	29.5 ± 2.77
Non-Fertile	42.37 ± 3.86	98.34 ± 10.49	20.69 ± 1.17	36.23 ± 3.83

* n = 13 - 14

Table A2.2 Values of relative growth (RG) and relative growth rate (RGR) for fertile and non-fertile thalli of *C. portentosa* recorded at T_1 and T_2.

	Mean* RG (mg g^{-1}) ± 1 SEM		Mean* RGR (mg g^{-1} y^{-1}) ± 1 SEM	
Sample	T_1	T_2	T_1	T_2
Fertile	511.24 ± 48.41	847.08 ± 75.11	406.80 ± 31.97	301.45 ± 20.32
Non-Fertile	499.67 ± 57.54	1024.95 ± 96.32	395.92 ± 37.68	346.10 ± 23.49

* n = 13 - 14

Appendix 3. Malt-yeast extract medium (from Ahmadjian 1993)

Malt Extract	20 g
Yeast Extract	2 g
Agar	20 g
Water	1000 ml

Appendix 4. Adonitol medium for lichen mycobionts (P. D. Crittenden, Pers. Comm.)

	Per 1000 ml deionised water	ppm
Adonitol	8 g	
NH_4NO_3	0.424 g	148 N
KH_2PO_4	0.44 g	100 P, 126 K
$MgSO_4.7H_2O$	0.5 g	49 Mg
$CaCl_2.2H_2O$	0.2 g	54.6 Ca
$Fe_2(SO_4)_3$	0.2 mg	0.056 Fe
$ZnSO_4.7H_2O$	0.2 mg	0.045 Zn
$MnCl.4H_2O$	0.2 mg	0.001 Cu
$CuSO_4.5H_2O$	-	0.001 Cu
H_3BO_3	-	0.001 B
$Na_2MoO_4.2H_2O$	-	0.001 Mo
Thiamine	100 µg	
Biotin	5 µg	

Stock Solutions

	Aqueous stock solution	Volume of stock per 1000 ml medium
$CaCl_2.2H_2O$	5 g in 250 ml	10 ml
$ZnSO_4.7H_2O$	0.1 g in 500 ml	1 ml
$MnCl.4H_2O$	0.1 g in 500 ml	1 ml
$Fe_2(SO_4)_3$	0.05 g in 250 ml	1 ml
$CuSO_4.5H_2O$	0.0393 g in 1000 ml	0.1 ml
H_3BO_3	0.0572 g in 1000 ml	0.1 ml
$Na_2MoO_4.2H_2O$	0.1262 g in 500 ml	0.1 ml
		per 100 ml medium
Thiamine	0.05 g in 500 ml	0.1 ml
Biotin	0.01 g in 500 ml	0.1 ml