Surface phosphatase activity of peltigera and cladonia lichens

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SURFACE PHOSPHATASE ACTIVITY OF *PELTIGERA* AND *CLADONIA* LICHENS

By

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This thesis is entirely my own work and has not previously been submitted for any other degree

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ABSTRACT

This study examines the effects of environmental factors on the surface phosphatase activities of the lichens *Peltigera canina* (L.) Willd., *P. praetextata* (Flörke. ex Sommerf.) Vain. and *Cladonia arbuscula* (Wallr.) Rabenh. Four plants of each species were collected in the U. K. from low (Middleton Common, Windy Nook) and high (Nenthead) Zn environments. The mean tissue (soil) Zn concentrations in *P. canina*, *P. praetextata* and *C. arbuscula* (high Zn) were 4.3 (18.9), 5.4 (25.4) and 4.1 (8.3) times higher respectively than the same species (low Zn).

Phosphomonoesterase (PMEase) activity was measurable at all pH values in the range 3.0 - (10.3) 11.0 with 250 μM p-nitrophenyl phosphate (pNPP) and 250 μM 4-methylumbelliferyl phosphate (4-MUP) Both pNPP and 4-MUP showed maximum PMEase at pH: 10.3 in *P. canina* (low Zn), *P. praetextata* (low and high Zn); 6.0 in *P. canina* (high Zn), *C. arbuscula* (low Zn); 6.0-7.0 in *C. arbuscula* (high Zn). Maximum PMEase in *P. canina* and *P. praetextata* (high Zn) exceeded maximum PMEase in the same species (low Zn), while the converse was true for *C. arbuscula*. Fluorometric determination of PMEase with 4-MUP yielded lower maximum rates of activity in all species from low and high Zn environments than spectrophotometric methods (pNPP).

PMEase activity in all species from low and high Zn environments exceeded phosphodiesterase (PDEase) activity between 1.5 and 5.1 times. *P. canina* and *P. praetextata* displayed a greater tolerance to Zn than *C. arbuscula*, with inhibitory effects on PMEase activity noticeable at 1 mM in *P. canina*, and *P. praetextata* (high Zn) and 10 μM Zn in all other plants.

Storage for six weeks, rhizinal cellular damage and light all resulted in an increase in PMEase activity with 250 μM pNPP in *P. canina* (high Zn) of less than 4 %. The lower layer (rhizines and veins) of *P. canina* was shown to have greater PMEase activity at all pH values, with 1 μM 4-MUP, 250 μM 4-MUP and 250 μM pNPP. Staining techniques undertaken on *P. canina* (high Zn) suggest that phosphatase activity is probably due to the fungus, especially the rhizines and veins, and not the cyanobacterium or contaminant bacteria. PMEase and PDEase were associated with the cell wall, cytoplasm and most noticeably at hyphal junctions. All plants displayed maximum PMEase activities at pH values which did not correspond with any of the presumed soil pH values.

ABBREVIATIONS

°C	degrees Celsius
ppm	parts per million
d. wt	dry weight (at 105°C)
vol	volume
M	molar
mM	millimolar
μM	micromolar
μmol	micromole
nmol	nanomole
P_i	inorganic phosphate
PMEase	phosphomonoesterase
PDEase	phosphodiesterase
pNP	p-nitrophenyl
pNPP	p-nitrophenyl phosphate
bis-pNPP	bis (p-nitrophenyl) phosphate
4-MU	4-methylumbelliferone
4-MUP	4-methylumbelliferyl phosphate
CAPS	3-(cyclohexylamino)-1-propanesulphonic acid
DMG	3, 3-dimethylglutaric acid
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
HEPES	N-2-hydroxymethylpiperazine-N' -2-ethanesulphonic acid
cAMP	cyclic adenosine monophosphate
AMP	adenosine monophosphate
S.D.	standard deviation

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Phosphorus and zinc are two elements essential for lichen growth (Hale, 1983). Phosphorus occurs in lichens at concentrations of 500 $\mu g\ g^{-1}$ (Farrar, 1976b) and zinc generally at concentrations less than 200 $\mu g\ g^{-1}$ (Beckett & Brown, 1984) in plants found in "background" environments. Many soils throughout the world are P-deficient and even the most fertile soils have P_i concentrations rarely higher than 10 μM (Bieleski, 1973). In order to survive in low phosphorus conditions plants and other organisms must have developed strategies to combat this.

There is an extensive literature on the ability of microorganisms to produce cell-bound (Flynn et al., 1986) phosphatases capable of hydrolyzing organic phosphates from their environment and acquiring at least part of that phosphate. In many cases the phosphatase activities have been shown to be induced under conditions of phosphorus limitation (Whitton, 1991). The ability of cyanobacteria to hydrolyze organic phosphate, and therefore become potentially available for the organisms, is widespread in P-deficient conditions (Whitton et al., 1991). Although the majority of studies have been made on laboratory isolates, there are an increasing number for populations of organisms taken directly from nature. The results indicate that it is likely that the ability to form phosphatases capable of hydrolyzing organic phosphates in the environment is an important strategy for many organisms under conditions of P deficiency.

Lichens have long been recognised as inhabiting environments inhospitable to most organisms and it has been suggested that low nutrient concentrations are likely to be

important determinant factors at many sites. Farrar (1976b) states that *Hypogymnia physodes* may attain its P requirements from about one hour of rain per week and that it is unlikely that P limits its growth in nature. It seems probable that lichens possess highly efficient mechanisms for accumulating a wide range of substances from dilute solutions (Smith, 1961). An ability to scavenge a variety of P sources is likely to be important, especially where there is a relatively high N : P ratio in the environment.

Zinc is essential in plant nutrition because it is involved in a number of metallo-enzymes, it is essential to the stability of cytoplasmic ribosomes, it catalyzes the process of oxidation and the transformation of carbohydrates.

1.11 Phosphorus

Phosphorus is the tenth most abundant element in nature, its average crustal concentration is 0.1% by weight. The average concentrations in continental sediments are 0.07%, soils 0.08%, igneous rock 0.1% and marine sediments 0.12% (Oglesby & Bouldin, 1984). P is present in all forms of life and constitutes approximately 1% of the total dry weight (Corbridge, 1990).

1.12 Occurrence of phosphorus in the terrestrial environment

Phosphorus is not found free in nature and almost always occurs in the fully oxidised state as phosphates or organic P compounds. It is widely distributed in these forms in soil and rocks. Phosphates can be divided into:

i. Orthophosphate

ii. Polyphosphate (chain phosphates)

iii. Metaphosphate (ring phosphates)

iv. Ultraphosphate (branched ring phosphates)

Orthophosphates are generated from biological metabolism, degradation or the weathering of rocks. Polyphosphates and metaphosphates are produced by biological activity. Analytically defined P fractions maybe categorised as:

Tot P = Total phosphorus

↓

PP = Particulate phosphorus > 0.45 μm

TFP = Total filterable phosphorus < 0.45 μm

FRP = Filterable reactive phosphorus

Particulate P includes:

i. Mineral phases of rock and soil, such as hydroxyapatite, in which phosphorus is adsorbed into inorganic complexes such as clays, carbonates and ferric hydroxides.

ii. Phosphorus adsorbed into dead particulate organic matter or in microorganic aggregations.

iii. Phosphorus in organisms as:

a) low-molecular-weight esters of enzymes, vitamins, etc.

b) relatively stable nucleic acids DNA, RNA and phosphoproteins which are not involved in rapid cycling of phosphorus

c) nucleotide phosphate, such as adenosine diphosphate (ADP) and adenosine triphosphate (ATP).

In contrast, TFP is composed of:

i. Orthophosphate (PO_4)

ii. Polyphosphates

iii. Low-molecular-weight phosphate esters

iv. Organic colloids or phosphorus combined with adsorptive colloids

The term filtrable reactive phosphorus is more appropriate than orthophosphate for the phosphorus fraction reacting to give the colour with the molybdenum blue technique.

1.13 Availability of phosphorus in the terrestrial environment

In terrestrial systems there is seldom a deficiency of P, in the sense that all of that in the rooting medium is removed by vegetation. There is usually a large amount of P in the rooting medium which plants cannot utilise because of:

i. Physical burial within a solid matrix

ii. Organic forms which plants cannot assimilate

iii. Slow transfer from the soil solid phase to the plant root, which is inadequate for the needs of the growing plant

Phosphorus is absorbed by plants from soil in the form of phosphate ions HPO_4^{--} and $H_2PO_4^-$. Plant roots tap only a small fraction of the soil's inorganic P store (White, 1987), withdrawn from the available pool, which is made up of:

i. Ions in the soil solution

ii. Exchangeable ions adsorbed by clay minerals and organic matter

Inputs to the available pool occur by:

i. Weathering of soil and rock minerals

ii. Precipitation and dust fallout

iii. Mineralization of organic matter

iv. The application of organic manures, organic and inorganic fertilisers

Orthophosphate that is released by mineralization is rapidly adsorbed by the soil particles where its availability steadily declines with time, a process called phosphate fixation. Phosphate on surfaces that can be readily desorbed, plus phosphate in solution, is called labile P to distinguish it from the P held in insoluble compounds or organic matter, which is non-labile. Many organisms have two uptake systems for phosphate, one being diffusive and the other rapid. The rapid uptake system occurs when the internal phosphorus concentration is low.

1.2 Phosphatases

1.21 Introduction

Phosphatases are enzymes which catalyze the hydrolysis of both esters and anhydrides of phosphoric acid and under some circumstances certain phosphatases act as transferases by catalyzing the transfer of phosphate from one substrate to another. They are often divided into acid and alkaline phosphatase according to their pH optima.

1.22 Classification of phosphatases

The Commission on Enzymes of the International Union of Biochemistry has classified all of these enzymes into five major groups:

i. Phosphoric monoester hydrolases E.C 3.1.3 (phosphomonoesterases)

ii. Phosphoric diester hydrolases E.C 3.1.4 (phosphodiesterases)

iii. Triphosphoric monoester hydrolases E.C 3.1.5

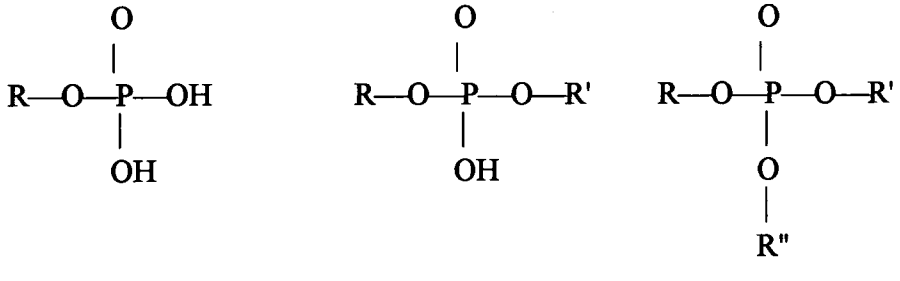
iv. Hydrolases splitting anhydride bonds in phosphoryl-containing anhydrides E.C 3.6.1

v. Hydrolases splitting P-N bonds E.C 3.9 (phosphoamidases)

1.221 Phosphomonoesterases

The phosphomonoesterases catalyze the hydrolysis of monoesters of orthophosphoric acid. Most often the term "phosphatase" is used synonymously with phosphomonoesterases, abbreviated to PMEase. Similar, but functionally different enzymes are the phosphodiesterases, abbreviated to PDEase, which include the nucleases. General formulae for phosphate esters are in Fig 1.1.

Fig. 1.1 Formulae for phosphate esters



PHOSPHOMONOESTER PHOSPHODIESTER PHOSPHOTRIESTER

R represents the organic part of the phosphate esters

The most common catalytic breakdown studied is the breakdown of phosphomonoesters by PMEase. The reaction mechanism (Fig. 1.2) is divided into four steps (McComb et al., 1979):

i. Non-covalent binding of the substrate to the enzyme (EH).

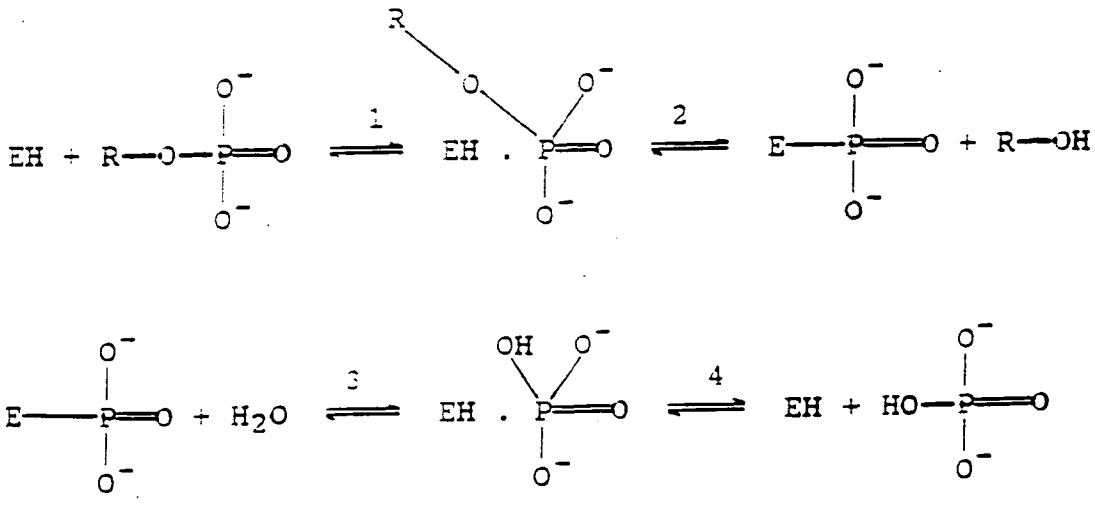
ii. Alcohol release from the complex and P_i become covalently bound to the enzyme forming a phosphoryl-enzyme compound.

iii. Conversion of the phosphoryl-enzyme compound, through uptake of water, to a non-covalent complex.

iv. Release of P_i and regeneration of free enzyme.

Any of the steps ii-iii can be rate-limiting for the overall reaction (McComb et al., 1979).

Fig. 1.2 Reaction scheme for the enzyme catalyzed hydrolysis of phosphate esters as described by McComb et al., (1979)



PMEase activity will primarily depend on the type and concentration of substrate and enzyme. Other factors which affect PMEase actvity are temperature, ionic strength, pH and metal ions (McComb et al., 1979). Alkaline PMEases have been characterized as metallo-enzymes with an essential metal ion, which has been reported to be zinc in many cases (Spiro, 1973; McComb et al., 1979; Torriani-Gorrini et al., 1987).

1.222 Phosphodiesterases

These enzymes catalyze the hydrolysis of a phosphoric diester to yield a phosphoric monoester and an alcohol. PDEases are able to hydrolyze a wide range of nucleotides and are distinguished into two categories on their ability to hydrolyze 3' and 5' nucleotides. PDEase I hydrolyzes nucleic acids to nucleoside 5'-phosphate and PDEase II hydrolyzes nucleic acids to nucleoside 3' phosphates (Kelly et al., 1975). PDEases are typically alkaline and inhibited by EDTA. The activity of EDTA treated PDEases is completely restored by zinc and partially by calcium and magnesium (Ito et al., 1987), which suggests that PDEases are also zinc-metallo enzymes like PMEases. PDEases in all cases are inhibited by P_i and in most cases PDEases are inhibited by ascorbic acid.

1.223 Cyanobacterial and algal phosphatase activity

Phosphatase activity has been found in all major groups and numerous species of Cyanobacteria (blue-green algae) and algae, though not universal (Healey, 1982). Phosphatase activity has been frequently demonstrated in cultured Cyanobacteria and algae (Kuenzler, 1965; Kuenzler & Perras, 1965; Healey, 1973; Doonan & Jensen, 1980; Flynn et al., 1986; Grainger et al., 1989; Whitton et al., 1990, 1991; Islam & Whitton, 1992).

Phosphatase activity has been located on the cell surface and in cell membranes (Brandes & Elston, 1956; Kuenzler & Perras, 1965; Flynn et al., 1986). The release of extracellular enzymes in algal cultures has been reported (Healey, 1973; Wynne, 1981; Siuda, 1984; Grainger et al., 1989; Whitton et al., 1990, 1991; Islam & Whitton, 1992).

1.224 Fungal phosphatase activity

Phosphatase activity has also been found in all major groups and numerous species of Ascomycetes, Basidiomycetes and Oomycetes. Acid phosphatases have been reported in *Saprolegnia monica* (Fevre, 1974), *Achlya bisexualis* (O'Day & Horgen, 1974) and *Schizophyllum commune* (Raudaskoski, 1976; Lilly & Charvat, 1987). Alkaline phosphatases have been reported in *Achlya bisexualis* (Dargent, 1975), *Phytophthora palmivora* (Meyer et al., 1976) and *Schizophyllum commune* (Wilson, 1972).

Phosphatase activity has been demonstrated to be associated within oogonia (Aliaga & Ellzey, 1984), vegetative hyphae (Fevre, 1974; Dargent, 1975; Maxwell et al., 1978; Hill & Mullins, 1980) and in components of the endomembrane system (Kazama, 1973; Fevre, 1974; Dargent, 1975; Meyer et al., 1976; Maxwell et al., 1978; Hill & Mullins, 1980) and deliquescing asci of the Ascomycete *Chaetomium brasiliense* (Rosing, 1984).

1.3 Zinc

Zinc (Zn) is the 24th most abundant element in the earth's crust. It is infrequently present in igneous and metamorphic rocks as sphalerite. Most Zn is distributed as a minor constituent of rock-forming minerals especially those rich in iron, such as magnetite, the pyroxenes, the amphiboles, biolite, spinel, garnet and staurolite (Adriano, 1986).

1.31 Occurrence of Zn in the terrestrial environment

To a large extent the Zn content of soils depends on the nature of the parent rocks, organic matter, texture and pH. As soils develop from the parent material of the earth's surface they acquire, in varying degrees, the elements present in the parent material. Soils formed from basic rocks are richer in Zn, whereas soils from granites and gneisses are poorer.

Table 1.1 Commonly observed zinc concentrations ($\mu g\ g^{-1}$) in various environmental matrices.

Material	Average conc.	Range
Igneous rock	65	5-1070
Limestone	20	<1-180
Sandstone	30	5-170
Shale	97	15-1500
Petroleum	30	–
Coal	50	3-300
Lime	6	<5-8
Phosphate fertilizer	305	40-600
Organic waste	390	8-1600
Sewage sludge	2250	1000-10000
Soil	90	1-900

1.32 Availability of Zn in the terrestrial environment

Zinc in soil that is water-soluble and adsorbed at exchange sites of colloidal materials is considered to be available to plants. Zn in other forms is either not available or not as readily available to plants. Fractions of Zn in the terrestrial environment may include;

i. water-soluble (both ionic and complexed with soluble organic compounds).

ii. exchangeable, from soil surfaces.

iii. extractable, from organic and inorganic sites not released to extractants of exchangeable ions.

iv. occluded by soil hydrous oxides.

v. precipitates.

vi. immobilized in living organisms and biological residues.

vii. constituents of the lattice structures of primary and secondary minerals.

Adsorption of Zn in soil is an important factor governing Zn concentration in the soil solution and Zn availability to plants. Adsorption and fixation in soils are influenced by several factors such as pH, clay mineral, organic matter, soil texture and cation exchange capacity. Severe Zn deficiency is often associated with high soil pH, and Zn is most soluble, and therefore available to plants, under acidic conditions. Zinc availability to plants is generally low in organic soils. Zinc can have important interactions with phosphorus, most noticeably a reduction in Zn uptake in situations where phosphorus is available in large amounts to plants and is subject to luxury uptake resulting in high concentrations in plant tissues (White, 1987).

1.4 Biology of lichen symbiosis

1.41 Introduction

Lichens are complex plants embracing a range of conceptual symbiotic relationships; parasitism, endosaprophytism, mutualism and parasymbiosis. The lichen symbiosis is composed of a mycobiont (fungal component) and a photobiont (algal component). The mycobiont is nearly always an Ascomycete or Basidiomycete; in one case a Phycomycete. The photobiont belongs to the Chlorophyceae or Cyanophyceae (occasionally both) or rarely, in tropical lichens, the Tribophyceae. The dominant nature of the mycobiont has meant that lichens are classified amongst the Fungi (Jahns, 1983).

The lichen symbiosis results in a distinct plant with fungal and algal elements which bear no resemblance to either non-lichenized fungi or algae. Both the fungal and algal partners are significantly modified when lichenized. The mycobiont is apparently not free-living and can only survive when in contact with the appropriate alga. The mycobiont produces the fruiting bodies of the lichen plant, and is the only partner involved in sexual reproduction (Jahns, 1983).

Many lichen photobionts are found free-living as well as occurring within lichens. Vegetative reproduction is undertaken by thallus fragmentation and lichenized structures such as isidia, soredia, squamules and lobules.

Lichens are classified into three basic growth forms, within which there can be many subdivisions. These are crustose, foliose (*Peltigera* type) and fruticose (*Cladonia* type).

Foliose lichens, such as *Peltigera*, have distinct upper and lower surfaces, are leafy in shape and may be attached to their substrate by rhizines. A cross section of the thallus would reveal a paraplectenchymatous upper cortical layer (wholly fungal) 10-40 μm thick

with several layers of cells, comparable to the epidermis of a green plant, but lacking in a cuticle and stomata. Immediately below the upper cortex is the heteromerous algal layer, between 10 and 30 μm thick, and composed of algae and fungi. The remainder of the thallus, the medulla, is composed of weakly gelatinized hyphae forming a layer as much as 500 μm thick. The undersurface of *Peltigera* is not corticate (Hale, 1983).

Fruticose lichens, such as *Cladonia*, have a dimorphic thallus, the primary thallus being squamulose and the secondary thallus fruticose. The primary squamulose thallus is similar to that of *Peltigera*. The secondary fruticose thallus is radial in structure, and consists of a dense outer cortex, a thin algal layer and a medulla surrounding a hollow central axis (Hale, 1983).

1.411 Role of the mycobiont

Only a brief outline of the physical characteristics of lichenized fungi, with reference to the symbiotic role, is considered here. A more detailed account of lichen ecophysiology is addressed in Section 1.42.

The mycobiont envelops the algae with hyphae and therefore provides mechanical protection from the elements, improved water relations, resistance to desiccation and protection from high irradiance (Jahns, 1983).

1.412 Role of the photobiont

The role of the photobiont is superficially a nutritional one, as opposed to the mechanical role undertaken by the mycobiont. The alga photosynthesizes and provides the fungus with organic metabolites, and where the photobiont is a blue-green alga, nitrogen is supplied to the fungus (Jahns, 1983).

Both partners benefit from the symbiotic relationship by the ability to colonise stressed pioneer environments with decisive advantages in competition for space. One disadvantage for the fungus is that although it has a much greater life span than other fungi the price in terms of slow growth is high.

1.42 Taxonomy and classification

1.421 *Peltigera* Willd.

The following details of the genus *Peltigera* are taken from the system proposed by Eriksson (1982). *Peltigera* is one of seven genera within the family Peltigeraceae. This family is characterised by a subfruticose to foliose thallus and a poorly differentiated lower cortex. The photobiont may be green or blue-green, the distribution circumboreal to montane tropical and the habitats include saxicolous, corticolous, terricolous and muscicolous environments. The family Peltigeraceae belongs to the order Peltigerales which in turn is placed in the class Ascomycetes, within which the majority of lichenised fungi are found.

The genus *Peltigera* is described by Duncan (1970) as having a foliose thallus with a loosely arachnoid and veined undersurface. The phycobiont may be *Coccomyxa* or *Nostoc*,

or both when cephalodia are present. *Peltigera* is one of a small number of genera in which some species may have cephalodia, perhaps the best example being *Peltigera aphthosa* (L.) Willd. The foliose habit characterised by *Peltigera* is typified by the development of more or less horizontally spreading leaf-like lobes. The thalli are dorsiventral and have marginal lecanorine apothecia situated on the upper surface. Additional morphological features commonly occurring in *Peltigera* are tomentum and rhizines. Tomentum is a felty or cottony mat of multicellular hyphal strands which lack compaction and can occur on the upper or lower thallus surface. The tomentose upper surface, which lacks lichen substances, prevented thin layer chromatographic studies being undertaken on *P. canina* and *P. praetextata* (White & James, 1987). Rhizine morphology in *Peltigera* is discussed in Section 1.426.

Peltigera is distributed widely throughout the British Isles, occurring chiefly in highland Britain alongside mountain streams growing directly on the soil, over mosses on rocks or at the base of trees (Duncan, 1970). *Peltigera* is also a well represented and important component of the macrolichen flora of heavy metal environments and consequently has been the subject of many ecophysiological studies (Beckett & Brown, 1983, 1984; Goyal & Seaward, 1981, 1982a, 1982b).

Recent taxonomic and nomenclatural changes to the *Peltigera* genus have resulted in an incomplete statement of *Peltigera* in the British Isles. There are approximately 17 species of *Peltigera* in the British Isles (White & James, 1987).

1.422 *Peltigera canina* (L.) Willd.

Peltigera canina may frequently attain a diameter in excess of 15 cm., forming large spreading lobes over mosses on rocks, tree roots, sand dunes, on the ground in woods and on moors (Duncan, 1970).

P. canina was described by Duncan (1970) as being very common. Studies of the lichen flora in N. E. England (County Durham and Northumberland) show that *P. canina* has been recorded in only 3 tetrads (2 km grid squares) in County Durham (Graham, 1988). Graham (1988) describes *P. canina* as very rare, usually found on sand dunes but also inland on heavy-metal-rich sites. *P. canina* is rare, on sand dunes in Northumberland (Gilbert, 1980b).

1.423 *Peltigera praetextata* (Flörke ex Sommerf.) Vain.

Duncan describes the range of preferred habitats of *P. praetextata* as rocks, trees and banks in damp hilly districts especially near water (Duncan, 1970). The distribution of *P. praetextata* is also described by Duncan as being rather common. The studies undertaken in N. E. England, referred to in Section 1.422, record *P. praetextata* in 18 tetrads in County Durham (Graham, 1988), being frequent on mossy tree boles, logs and on mossy woodland floors. *P. praetextata* is described as frequent on rocks, trees, logs and the ground in damp woodland in Northumberland (Gilbert, 1980b).

1.424 *Cladonia* (Hill) Web.

The system of taxonomic classification proposed by Eriksson (1982) referred to in Section 1.421, has been used here as the basis for the classification of *Cladonia*. *Cladonia* is one of four genera within the family Cladoniaceae. All genera within this family are characterised by a crustose or squamolose primary thallus. In addition, these genera have a hollow, erect secondary thallus known as a podetium; fruticose structures, which differ from other fruticose structures by being derived ontogenetically from tissues peripheral to the ascogonial complex in the primary squamules (Hale, 1983). The habitats of members of the Cladoniaceae family are soil and rocks, and the distribution is described by Hale as cosmopolitan. Cladoniaceae belongs to the order Lecanorales which is the largest member of the class Ascomycetes.

Within the genus *Cladonia* the primary, or basal thallus,when present, may be either crustose or squamulose, from which arises the erect secondary thallus, the podetia. Apothecia are borne on the podetia although many species are frequently sterile. When fertile the apothecia are lecideine. The photobiont occurring within *Cladonia* is *Trebouxia* (Duncan, 1970).

Cladonia, like *Peltigera*, has proven to be a problematic genus with regards to taxonomy. The numerous species within this genus include many similar plants distinguished from each other only by chemical criteria. The number of *Cladonia* species in the British Isles is approximately 62 (Duncan, 1970). *Cladonia* spp., probably the most conspicuous of fruticose lichens, are distributed widely throughout the British Isles.

1.425 *Cladonia arbuscula* (Wallr.) Rabenh.

The habitats of *C. arbuscula* include hillsides, sandy and stony heaths, dunes and peat where it grows directly on the soil forming characteristic thick cushions on the ground (Duncan, 1970).

Duncan (1970) describes *C. arbuscula* as being common, sometimes abundant. The studies undertaken in N. E. England, referred to in Section 1.422, record *C. arbuscula* in 19 tetrads in County Durham (Graham, 1988), described as an occasional on high *Calluna*-heath and blanket mire, and as probably decreasing in Northumberland (Gilbert, 1980b). Gilbert provides specific details relating to the distribution of this species, recording it as widespread in the larger more undisturbed acid terricolous habitats, ranging from fixed dunes to blanket bog. It is particularly common on the raised bog peats of Central Northumberland, but less common south of the River Tyne, where it is often associated with lead mines.

1.426 Rhizine morphology in *Peltigera*

Hale (1983) provides a universally accepted definition of a rhizine as an attachment organ of foliose lichens consisting of compressed strands of hyphae. Rhizines superficially resemble roots but the extent to which they are capable of transporting dissolved mineral or organic metabolites from the substrate to the thallus appears to be limited.

Goyal & Seaward (1982a) have reported morphological modifications in *Peltigera canina* and *P. rufescens* from metal-polluted environments. They noted that rhizines from both species from metal-polluted environments were dark brown, short, thick and profusely distributed forming a carpet on the lower surface compared with rhizines from

background material which were whitish or light coloured, elongate and sparsely distributed. They attribute the increased density of rhizines as a result of high metal accumulations. *Peltigera canina* used in this study, from a metal-polluted environment, displayed the same rhizinal modifications.

1.43 Lichen ecophysiology

1.431 Introduction

Lichens absorb water by imbibition, much of which is held externally to the cytoplasm. *Peltigera polydactyla*, when saturated, has been shown to hold 25 % more water per unit dry weight in the medulla than the algal layer and upper cortex. However, in a non-saturated thallus, the algal layer may act as a sink and hold most of the water (Hale, 1983).

The majority of foliose and fruticose lichens have saturated water contents between 100 and 300 % of the dry weight. Air-dry lichen thalli can absorb water very rapidly with the maximum saturated water content achieved only during rain showers and for short periods thereafter (Hale, 1983). Larson and Kershaw (1976) found that broader lobed foliose lichens and clumped lichens have higher resistance to water loss. It is concluded that in nature lichens may achieve better water relations by modifying their morphological structure.

Net assimilation is much lower in lichens than in leaves because, although respiration rates of the two tissue types are comparable, lichens have much lower rates of photosynthesis per unit surface area (Hale, 1983). The chlorophyll content of lichens is only one quarter to one tenth that of green leaves; chlorophyll occurs in the algal cells only

which generally make up less than 10 % of the volume of the thallus. *Peltigera canina*, a shade-tolerant species, has developed a strategy to make maximum use of low incident radiation, by increasing the concentration of phycoerythrin, the accessory photosynthetic pigment (Hampton, 1973). *P. praetextata* may be able to maintain optimal levels of photosynthesis during variable light conditions by acclimatising rapidly to low or high light levels (Kershaw & MacFarlane, 1980).

Richardson (1974) has showed that photosynthetic rates in lichens are correlated with the water content of the thallus, the optimal saturation being 35 - 70 % or more. Farrar (1976a) states that high rates of photosynthesis, dedicated to replenishment of the large polyol pool in the lichen thallus which needs to be maintained, are provided for by the wetting and drying cycles normally encountered in nature.

Although knowledge of respiration rates of variously aged thalli and different areas of the thallus are little known Hale (1983) states that the medulla of *Peltigera polydactyla* has a lower respiration rate than the overlying algal layer and cortex. Hale (1983) has found that with *Peltigera* the respiration rate increases with increasing water content in a more or less linear fashion until it reaches a maximum value, which he concludes may be at a water content as low as 40 % saturation, although the majority of lichens attain maximum rates in the region of 80 to 95 % saturation.

The wetting burst phenomenon studied by Smith (1973) has shown that when an air-dry lichen is wetted there follows an initial rapid loss of CO_2 and a steep rise in respiration rate together with a rapid efflux of organic and inorganic solutes from the mycobiont which gradually declines to normal with the result being a net loss of carbon. Farrar and Smith (1976) suggest this period is a time when the algae can take up the solutes leaked from the mycobiont, and that at the same time the unusually large polyol pool maintained

by the fungus acts as a buffer and guards against damage from frequent rewetting phases normal for most lichens during favourable growing seasons.

1.432 Nutrient cycling in lichens

One of the first attempts at providing a broad and comprehensive account of the physiology of one particular lichen species was undertaken by Smith. His studies resulted in the publication of a number of papers concerned with the physiology of *Peltigera polydactyla*. Smith (1960) reported that *P. polydactyla* can absorb relatively large amounts of phosphate from "dilute" solutions (4.5 mM KH_2PO_2) by an active process.

Using a technique involving dissected discs Smith illustrated that the algal layer absorbed more phosphate than the fungal layer (789 μg P / 100 mg d wt. compared to 461 μg P). The medulla was not believed to form a significant barrier to the diffusion of dissolved substances to the algal layer although the case may be different when the lichen meets much lower concentrations which are expected in nature. The algal layer contains fungal hyphae which are smaller and have thinner walls than those of the medulla. Smith suggests that the algal layer is a region of high metabolic activity, the degree of which is dependent upon water, of which the medulla has a high content, leading to the assumption that the medulla might function as a water reservoir for the algal zone. Additionally, Smith suggests that the relatively strong absorptive capacity of the medulla indicates that it may be able to respire organic substances absorbed from the moisture in it's habitat. In conclusion, the algal zone is considered to be the region of greatest metabolic activity since this is where contact between the symbionts occurs.

Smith (1961) postulates that lichens possess highly efficient mechanisms for accumulating a wide range of substances from dilute solutions, the first stage of which is the absorption of nutrients dissolved in the water passing over the thallus.

Farrar (1976b) reported active uptake of inorganic phosphate (Pi) from very dilute solutions (less than 10^5 nmol Pi dm^{-3}) for *Hypogymnia physodes*, although the Pi was not exchangeable. Pi absorbed from solutions of 10^5 - 10^6 nmol Pi dm^{-3} was exchangeable, being rapidly metabolized to a variety of organic compounds including phospholipids and polyphosphate. Polyphosphate may be used as a store of Pi which can be utilized when environmental supplies fall. Additionally, polyphosphate may represent a means of storing a scarce anion in a non-leaking form since on rewetting dry lichens a considerable leakage of Pi occurs.

Boissière (1982) has demonstrated the presence and ontogeny of polyphosphate granules in *Nostoc* from *Peltigera canina*. Simon (1974) reports that lichens have a rapid initial leakage of phospholipids, largely completed within one minute, followed by a slower rate of leakage. The wetting and drying cycles experienced by lichens in nature result in this loss of solutes which may be re-absorbed by the thallus or they may be washed away and absorbed by other organisms. Farrar (1976b) concludes that lichens may attain their phosphorus requirements from about one hour of rain per week, and that it is unlikely that phosphorus limits the growth of the lichen in nature.

Farrar (1976c) has suggested that the lichen thallus may be viewed as an ecosystem with two trophic levels; algal primary producers and fungal primary consumers. Regarding the flow of nutrients he states that phosphorus is at consistently lower levels in lichens than in non-lichenized algae and fungi, the significance of which remains to be determined.

Harley (1971) states that fungi are adapted physiologically and morphologically to a mode of life where all their nutrient requirements are absorbed as soluble material from their substrates. They produce enzymes which act externally to their bodies; either released free into the substrate or retained on or near their surfaces. Fungi are efficient accumulators of nutrients, possibly even when lichenized.

Smith (1975), in determining whether or not the fungus supplies minerals to the alga, suggests that misconceptions on this point may be based on evidence of the movement of phosphate and other minerals from the fungus to the host in ectotrophic mycorrhizas. He notes that in this case the autotroph is a large tree and has a high demand for nutrients whereas in a lichen the autotrophic symbiont is small in volume compared to the fungus, grows very slowly and presumably has a low demand for nutrients. Smith recognises that lichen fungi have very efficient mechanisms of absorption, and that absorbed minerals probably pass, in some way, to the alga.

1.433 Mineral cycling in lichens

Brown (1976) provides the basis for which mineral cycling in lichens can be understood stating firstly that there are two cation uptake mechanisms; passive, by ion exchange or chelation resulting in ions remaining extracellular or active, whereby ions become located intracellularly. Secondly it must be realised that although the fungal and algal components under normal conditions behave as an integrated association it is important to remember that physiologically they are extremely dissimilar and their response to cations may be quite different.

Studies on metal uptake by lichens demonstrate that most uptake is by a cation exchange process to extracellular sites, presumed to be in the cell walls and on the outer

surface of the cell membrane (Brown, 1991). Brown and Brown (1991) state that the cell wall and exterior of the plasma membrane are able to bind positive metal cations in an exchangeable form because they contain fixed negatively-charged anionic groups. This exchange process, whereby supplied cations will quantitatively displace elements already present on extracellular sites (Brown & Beckett, 1984), may occur naturally and result in an alteration of the metal composition of the cell wall with environmental changes such as rain following seaspray or leaves leaching elements in the autumn (Brown & Brown, 1991).

The precise cellular location of minerals must be known so that precise predictions of environmental supplies or effects on cell metabolism can be made (Brown, 1987). To satisfy this a sequential elution technique allowing quantification of elements bound to the cell wall has been suggested (Brown & Buck, 1979). To quantify extracellular material by cation exchange the plasma membrane must remain undamaged (Brown & Brown, 1991). Without cellular damage Brown and Buck (1985) have shown that Zn is located on the cell wall exchange sites.

Beckett and Brown (1984) have demonstrated that *Peltigera membranacea* from a disused Zn mine had much lower intracellular Cd uptake rates than *P. horizontalis*, *P. membranacea* and *P. praetextata* from uncontaminated sites and *P. hymenina* from a disused Pb mine. However, *P. hymenina* from the Pb mine had similar Cd uptake rates to the lichens from uncontaminated sites. They have also shown that an inverse relationship existed between the thallus content of Zn or Cd and the rate of intracellular Cd uptake with *Peltigera* species. For the same *Peltigera* species used in their study Beckett and Brown suggest that interspecific variation rather than environmentally-induced alteration in Cd-binding capacity was responsible for the differences in extracellular Cd uptake between

lichen samples. Extracellular uptake of Cd was not related to the Zn or Cd content of the thallus.

Beckett and Brown (1983) have reported that experimentally-induced Zn resistance in *P. membranacea*, *P. horizontalis* and *P. hymenina* is related to the concentration of Zn in the thallus, that is the higher the thalline concentration of Zn the more resistant the plant is to reductions in photosynthesis caused by added Zn. They found that *P. membranacea* and *P. hymenina* sampled from the same site had Zn contents of 98 and 186 $\mu g\ g^{-1}$ dry weight respectively and corresponding levels of tolerance to Zn in the laboratory. They suggest that this, together with the fact that Zn tolerance was not related to the concentration of other measured cations in the thallus (Ca, Cu, K, Mg, Mn, Pb), may indicate that thallus Zn content is the most important parameter controlling Zn tolerance.

Brown and Beckett (1983) have shown that there is a differential sensitivity among cyanobacterial and chlorophycean lichens to Zn, Cd and Cu. They have reported that photosynthesis in lichens containing chlorophycean photobionts (*Cladonia convoluta*, *C. rangiformis* and *Peltigera aphthosa*) were less sensitive to brief incubation with 1 mM Zn than were lichens containing cyanobacterial photobionts (*P.hymenina*, *P. membranacea* and *P. horizontalis*). They attribute the sensitivity to Zn to the photobiont and suggest that the mycobiont shows no differential sensitivity to Zn. With the exception of *P. aphthosa* the cyanobacterial lichens were shown to contain slightly more Zn than the chlorophycean lichens.

Seaward et al. (1978) have reported that lichens in the genus *Peltigera* are richer in heavy metals than chlorophycean lichens growing in the same habitats, except where the supply of heavy metals is from aerial fallout. Brown and Beckett (1983) ascribe this to a

contamination by heavy metal-rich soil particles by *Peltigera* lichens, a process not so effectively undertaken by fruticose chlorophycean *Cladonia* lichens.

Goyal and Seaward (1982b) have reported that the rhizines of *P. canina* were capable of absorbing, accumulating, translocating and regulating Cu, Fe, Mn, Ni, Pb and Zn. They demonstrated that metals moved freely from the rhizines to the upper thallus (location of the photobiont) and vice-versa. At high metal concentrations, when the metal uptake capacity of the upper thallus is reduced, the rhizines and medulla were shown to play a significant role in metal accumulation and translocation. Brown (1991) has reported that the Zn concentration in the rhizines of *P. membranacea* increased from 50 to 73 % after the lichen was treated with Zn. Following Zn treatment Zn was mostly located on the cell wall exchange sites of the rhizines.

Experiments on *Cladonia portentosa* (Brown, 1987), where the podetia were divided into 2 cm segments have shown that elemental concentrations of Ca, K, Mg, Na and Zn were in the highest concentrations in the apical segments and were in reduced concentrations towards the basal segments.

Seaward (1974, 1977) has reported that the lichen *Hypogymnia physodes* growing in the immediate area below lengths of barbed-wire fencing on *Fraxinus* trees consistently suffered toxicity due primarily to Zn, presumably through rain down-wash.

1.434 Enzyme characteristics of lichens

Quantitative information regarding the enzyme characteristics of lichens is very sparse. Experiments on the cyanobacterial isolate D800 *Nostoc* sp., the genus of the *Peltigera* photobiont, have recorded PMEase activities of 0.00279, 0.1426 and 0.238 μ mol mg d wt h^{-1} with 1 μM 4-MUP, 250 μM 4-MUP and 250 μM pNPP respectively (BA Whitton, pers. comm., 1992). These are the maximum values recorded at pH 7.5.

Lane and Puckett (1979) have examined the phosphatase activity of *Cladina rangiferina* (pNPP as the artificial substrate), using the same assay principle that is used throughout this study except for the substrate concentration (38 mM), by studying the effects of time, sample size, temperature, light, substrate concentration, pH and a range of cations and anions. They have shown that phosphatase activity increased linearly with increasing substrate concentration (7.6 mM to 137 mM), sample size and incubation time (10 to 120 min). They found no discernible differences in the phosphatase activity of *C. rangiferina* and *Peltigera canina* when incubation was in the light or dark.

The optimal pH for phosphatase activity of *C. rangiferina* and *Lobaria pulmonaria* was between pH 2.2 and 4.0. however, they did not examine the effects of pH above pH 8.9. *C. rangiferina* showed peak phosphatase activity at 61 ± 10 °C. Inhibition studies showed that fluoride and cyanide reduced activity by up to 90 % and azide and tartaric acid produced smaller but significant reductions. Of the cations (as chlorides) tested Ba, Ca, Mg and Na led to < 10 % reductions, while Co and K increased activity by < 10 %. Cu and Ni enhanced activity by 11 and 16 % respectively. Of the cations (as nitrates and sulphates) uranyl and vanadyl reduced activity by > 60 % while ammonia and Li reduced activity by 17 and 15 % respectively. Cu and Pb reduced activity by 2 % while Ag and Sr enhanced activity by 10 and 35 % respectively. The anions biselenite, molybdate,

phosphate and vanadate all significantly inhibited activity. Table 1.2 illustrates the phosphatase activity in other lichen species examined by Lane and Puckett (1979) with 38 mM substrate concentration and at 25 °C.

Table 1.2 Published data of phosphatase activity of lichens, from Lane and Puckett (1979)

Species	Activity (μmol pNP mg h^{-1})
Cetraria islandica (L.) Ach.	0.173
Cladonia gracilis (L.) Willd.	0.0436
Lobaria scrobiculata (Scop.) DC.	0.134
L. pulmonaria (L.) Hoffm.	0.1252
Platismatia tuckermanii (Oakes) Culb. & Culb.	0.144
Peltigera aphthosa (L.) Willd.	0.0624
P. canina (L.) Willd.	0.0378
Umbilicaria papulosa (Ach.) Nyl.	0.058
U. vellea (L.) Ach.	0.205

Mateos et al. (1991) have demonstrated that adenosine levels in *Himantormia lugubris*, from Antarctica, can be taken as an indication of PDEase activity, based on the varying amounts of adenosine and cAMP within the lichen thalli, but the absence of AMP. They suggest that the thalli of *H. lugubris* contains an active alkaline phosphatase, associated with PDEase, able to efficiently catabolize AMP to adenosine.

In studying multiple enzyme forms in lichens Fahselt (1985) has identified that alkaline and acid phosphatases in *Cladonia cristatella* varied within plants collected from the same site. Using zymograms to stain protein extracts of lichen samples she suggests that the basic similarity between fungus and lichen zymograms supports the idea that most of the detectable lichen protein could be produced by the mycobiont. The same study also demonstrated enzyme polymorphism within populations of *Usnea subfloridana.* Fahselt concludes that enzyme polymorphism occurs in esterases, phosphatases or carbonic anhydrases but is not restricted to these enzymes. A similar study on *C. cristatella* (Fahselt, 1986) has demonstrated that variations in acid and alkaline phosphatases exist within and between morphotypes collected from one population.

Fahselt (1987) has recorded variations in alkaline phosphatase enzymes in single spore cultures among mycobiont cultures of *C. cristatella*; no acid phosphatase was detected in any of the cultured mycobionts.

Hageman and Fahselt (1992) have found that alkaline phosphatase enzyme variability within lichen species of the family Umbilicariaceae was too significant to serve as useful taxonomic characters for distinguishing and comparing umbilicate lichen species, while other dehydrogenase enzymes proved to be taxonomically useful.

Fahselt (1992) provides evidence that *Cladonia mitis* affected by heat or gaseous emissions may develop increased enzyme polymorphism within alkaline phosphatases. This phenomenon presents additional problems for physiological studies undertaken on conspecific lichen populations from the same site or separated geographically.

1.44 Lichens in heavy metal environments

Lichens occurring in heavy metal environments have been documented qualitatively by virtue of the interesting plant assemblages that they represent. Within disused mine sites and the associated workings many genera and species representative of all three major lichen growth forms account for a diverse and significant lichen gene pool. The result is that many mineral-enriched environments have featured as an integral part of lichen recording within the capacity of British Lichen Society field meetings.

At Nenthead, one of the sites used in the present study, five *Peltigera* species were recorded as growing on mineral soil, while fourteen *Cladonia* species were found mainly on accumulated surface organic matter (Coppins & Gilbert, 1981). The authors attributed the luxuriant terricolous lichen flora to the open vegetation, light grazing and wide pH range. A similar lichen flora was also recorded at Garrigill (NY 750424).

Goyal and Seaward (1981) have recorded *Peltigera rufescens* and *Cladonia furcata* at two locations, near the Scunthorpe heathlands, where metal concentrations in the soil were considerably higher than in background sites. Gilbert (1980a) identified a pattern among reworked lead rakes in Derbyshire whereby, of the lichen flora *Peltigera* and *Cladonia* species grew most luxuriantly.

Further studies have reported *Peltigera* and *Cladonia* species prominent in an area of lead mines at Minera, Clwyd (Gilbert & Lambley, 1984) and around a lead-zinc mine in Maarmorilik, West Greenland (Hansen, 1991), where the lichen flora is believed to have accumulated significant concentrations of airborne lead and zinc.

1.5 Aims

The overall aim of this study was to survey the ability of representatives of field populations of two lichen genera (*Peltigera* and *Cladonia*) to hydrolyze organic phosphates in their environment. The initial aim was to establish whether there are marked differences in phosphomonoesterase and phosphodiesterase activities between species and / or environments.

Further aims were to examine the influences of: pH; Zn concentrations; the symbionts; storage; light; time; cellular damage; substrate and substrate concentration on phosphatase activities.

An additional aim was to establish the cellular location of phosphomonoesterase and phosphodiesterase activities.

CHAPTER 2

MATERIALS AND METHODS

2.1 Lichen material

2.11 *Peltigera canina* (L.) Willd.

P. canina , as used in this study, was identified by an undulate thallus with broad lobes or with erect lobes when growing among grass. *P. canina* has a characteristic bullate thallus appearing dull brown when wet and grey when dry, and with a conspicuous tomentum on the upper surface. The lower surface has white to brown anastomosing veins and long whitish rhizines. The apothecia are round with a red-brown disc which is appressed to the upper side of the margin at the apex of the lobes (Duncan, 1970).

2.12 *Peltigera praetextata* (Flörke ex Sommerf.) Vain.

P. praetextata was characterised by the foliose isidia which can occur on the margins and cracks of the older parts of the thallus. The grey or red-brown thallus may or may not be bullate, or nearly smooth with a downy upper surface (Duncan, 1970).

2.13 *Cladonia arbuscula* (Wallr.) Rabenh.

Cladonia arbuscula was characterised by being commonly 4-8 cm in height, composed entirely of a secondary podetial thallus, the primary thallus being absent. The verrucose podetia are green to yellow-grey in colour with tetrachotomic branches which are distinctly orientated in one direction (Dahl & Krog, 1973). The podetia are ascyphous

and terminate in slender, yet coarse, attenuated apices which have between 3 and 5 tips. Apothecia are rather rare on *C. arbuscula* (Duncan, 1970). *C. arbuscula* was verified by the thallus colour spot test, a method employed by lichen taxonomists (White & James, 1987). The solution, PD, was applied to a fragment of the podetial cortex which turned red after approximately 30 sec, indicating the presence of fumarprotocetraric acid.

2.2 Collection and storage

All lichen material was collected between 10 February and 10 July 1992. The same criterion was applied to all material for the collection and eventual storage. Each plant was separated from the soil substratum with forceps, together with a sample of soil to a depth of approximately 2 cm. Material was placed into labelled polythene bags and transferred immediately to the laboratories at the University of Durham. In the laboratory each plant was separated from its soil substratum with forceps. The soil component was immediately prepared for pH analysis. Each plant was examined under a low powered binocular microscope so that moss, soil, free living algae and, where possible, detritus was removed with forceps. With *Peltigera* specimens special attention was needed to avoid damage to rhizines. Plants were then washed thoroughly in distilled water and placed on a tray and allowed to dry under ambient laboratory conditions until required for analyses.

2.3 Experimental techniques

All lichen material was washed three times in assay medium prior to an experiment. Four replicates were used throughout every experiment. All material was dried at 105°C for 24 h prior to P and Zn experiments but after all other experiments.

Cladonia material was taken only from the lower podetial thallus, using forceps, excluding apices and excessive secondary branches. *Cladonia* thalli were on average 45 mm tall, of which the basal 20 mm of podetia were sampled. One podetium was used per replicate throughout all experiments. *Peltigera* material was extracted by cutting discs from the thallus, approximately 6 mm in diameter, with a number 2 cork borer. Each disc was taken from an area between 2 and 5 cm from the lobe margins, ensuring that each disc contained a section of the main thallus and rhizines which were in contact with the soil. Discs extracted from *P. canina* and *P. praetextata* from the low metal site constantly had 4-6 rhizines per disc. *Peltigera* material from the high metal site had 5-10 rhizines per extracted thalline disc. *Peltigera* clones were taken from single thalli.

Pyrex glassware bottles, beakers, and Erlenmeyer flasks were used throughout all experiments, together with Volac volumetric flasks and E-MIL glass pipettes. Washing procedures for glassware used in P and Zn analyses involved an initial wash and soak in Decon detergent for a minimum of 2 h, soaking in 4% HNO_3 bath for a minimum of 1 h and then rinsing in tap water 3 times and then distilled water 3 times. Glassware used in phosphatase assays did not come into contact with Decon detergent, as this product was shown to fluoresce. After washing, glassware was dried; at room temperature for approx. 24 h, in a Belling oven for approx. 4 h or at 105°C for approx. 4 h depending on the availability of each.

2.4 Phosphorus analysis

Soil samples and all lichen material was analysed for total Phosphorus (organic and inorganic) only, using the methods of Eisenreich et al. (1975). The method results in a 70% decrease in analysis time for multiple samples and about a 30% increase in sensitivity. Lichen material was prepared for P analysis according to the methods referred to in Section 2.3. Four replicates were used for each plant and soil samples.

2.41 Chemicals

Four stock solutions were prepared for the phosphorus analysis. From these solutions, three reagents were prepared daily for use (Table 2.1).

Table 2.1 Stock solutions and reagents used for phosphorus analysis.

Stock solutions	Reagents
A Phosphate standard solution	Digestion Reagent ($K_2O_8S_2$,D)
B H_2SO_4-Antimony	Mixed Reagent I ($C_6H_8O_6$, B,C)
C Molybdate	
D Digestion acid (conc. H_2SO_4)	

Stock solutions made as communal stocks approx. every two months were stored at 4° C, with the exception of HNO_3. Stock solutions were stored in 1 litre bottles and wrapped in aluminium foil. Additionally, a stock solution of 16M HNO_3, spectrosol grade, was used for the digestion of lichen and soil material.

2.411 Methods

Lichen material was prepared for biomass measurement (dry weight) in advance of P analysis, using 25 ml vol. snap caps, one per replicate, placed in 105°C oven for 24 h. After the dry weights were recorded each replicate was digested in 3 ml of boiling 4M HNO_3 in a test tube boiling rack in a fume cupboard for 1 h. Bubble flasks were placed on snap caps after 10 min digestion. *Peltigera* material was ground with a glass rod prior to digestion because of the fibrous rhizinal component. After digestion, distilled water was used to rinse condensed liquid, which had settled on bubble flasks, into snap caps. Each solution was then transferred to an erlenmeyer flask, diluted to approx. 35 ml with distilled water, and the pH adjusted to pH 7.0 using 1M KOH. Phosphate standard solutions did not have the pH adjusted. Lichen solutions were transferred to a measuring cylinder, diluted to 50 ml with distilled water, and then 25 ml returned to the erlenmeyer flask and 25 ml discarded. Following dry weight measurements soil samples were digested in boiling 4 M HNO_3 for 2 h and the solution filtered through GF/C filter papers prior to P analysis.

Following digestion, phosphorus was analysed using the method of Eisenreich et al. (1975). Solutions, including standards, then had 5 ml of digestion reagent added to them and were then autoclaved. After autoclaving, 5 ml of mixed reagent I was transferred to each solution which were then left for 15 min to allow blue colour to develop. Absorbance was measured at 882 nm on a Shimadzu double beam spectrophotometer (UV 150-02) against a Milli Q water blank. 1cm glass cuvettes were used for each solution, the detection limit for each reading being 0.014.

2.42 Zinc analysis

Zinc analysis was carried out on all lichen and soil material, which was prepared according to the methods in Section 2.3. Four replicates were used for each plant and soil sample, the dry weight of each being ascertained in advance.

2.421 Chemicals

The chemicals used for Zn analysis were 16M HNO_3, BDH spectrosol grade and 1000 mg l^{-1} Zn solutions, also BDH spectrosol grade.

2.422 Methods

Lichen material was prepared for biomass measurement (dry weight) in advance of Zn analysis, according to the methods described in Section 2.3. Each replicate, in a 25 ml vol. snap cap, was placed in a 105°C oven for 24 h. After dry weights were recorded each replicate was digested with 3 ml of boiling 4M HNO_3 in a test tube boiling rack, in a fume cupboard for 1 h. After 10 min digestion bubble flasks were placed over snap caps. *Peltigera* material was ground, with a glass rod, prior to digestion because of the fibrous rhizinal component. After digestion distilled water was used to rinse any condensed water which had settled on the bubble flasks back into the snap caps. Each solution was then diluted to 25 ml using distilled water, and 4 ml was then transferred to 10 ml vol. cylindrical glass cuvettes for flame atomic absorption spectrophotometry. Soil samples were prepared for Zn analysis according to the methods outlined in Section 2.411.

Zn standard solutions were made from 1000 mg l^{-1} Zn solutions (BDH spectrosol). Standards, made in the range 0 to 500 μg l^{-1} using a serial dilution technique, were used to

calibrate the spectrophotometer. All samples were analysed with a PerkinElmer 5000 Atomic Absorption Spectrophotometer and Automatic Burner Control Unit using an air-C_2H_2 flame (gas box settings = 28:35) and deuterium-arc lamp background correction. The sensitivity of the spectrophotometer was enhanced by replacing the flow spoiler with an impact bead. A mean of 3 readings was taken for each sample.

2.43 Phosphatase assay

Phosphatase activity was assayed using 3 substrates (Table 2.2).

Table 2.2 Substrates used for assaying phosphatase activity.

Reagents	Supplier
(p-nitrophenyl phosphate disodium)	Sigma Chemical Co., USA
bis (p-nitrophenyl) phosphate Sodium salt	Sigma Chemical Co., USA
4-Methylumbelliferyl phosphate	Sigma Chemical Co., USA

pNPP and 4-MUP were used to determine phosphomonoesterase activity, while bis-pNPP was used to determine phosphodiesterase activity. pNPP and bis-pNPP may be measured colourimetrically and 4-MUP fluorometrically. The colour of pNP and bis-pNP, the solution formed as a result of hydrolysis of the substrate, was measured at 405 nm against a reagent blank. Phosphatase activity was determined from a calibration curve (Livingstone et al., 1983). pNPP and bis-pNPP were used in a concentration of 250 μM.

Phosphatase assays using 4-MUP were carried out in much the same way as for pNPP and bis-pNPP (Fedde & Whyte, 1990). 4-MUP is a more sensitive substrate and was used in concentrations of 250 μM as well as 1 μM, a more accurate reflection of environmental concentration levels. 4-MU, the fluorescent product of hydrolysis of 4-MUP, was measured at 356 nm excitation and 444 nm emission. Phosphatase activity was determined from a calibration curve of 4-MU. The components of the assay solution were;

buffer	1.6 ml
assay medium	1.5 ml
substrate	0.1 ml

The influence of pH, using all three substrates, was tested in the range 3.0 to 11.0 at intervals of 1 pH unit (plus pH 10.3). The influences of Zn concentrations, substrate concentrations, incubation, storage, light and rhizinal cellular damage were tested at the pH value where the peak activity had already been established. Zn was tested at the following concentrations: 0.001 mM, 0.01 mM, 0.1 mM, 1 mM and 10 mM. 4-MUP was tested at 1 and 250 μM. The influence of the incubation period was tested for 1 μM and 250 μM 4-MUP and 250 μM pNPP at intervals of 10 min in the range 0 to 60 min. The effects of storage, light and rhizinal cellular damage were tested using 250 μM pNPP. Phosphatase activity, with all substrates, is expressed as μmol hydrolyzed mg^{-1} d. wt h^{-1}.

2.5 Development of methodology

2.51 Biomass variability

The biomass variability between the replicate discs, from all sixteen plants used in initial assays examining the influence of pH on PMEase activity, is outlined in Tables 2.3 and 2.4. With *Peltigera* material it is clear that discs from Nenthead plants, which had more rhizines than Windy Nook plants, had a greater average biomass.

Table 2.3 Biomass variability (105°C for 24 h) of *Peltigera canina* and *P. praetextata* replicate discs from Nenthead and Windy Nook, where n = 40.

Site	Species	Plant	d. wt mg	S.D.
Nenthead	*P. canina*	1	4.13	1.20
		2	3.95	1.44
		3	4.44	1.91
		4	3.10	1.21
	P. praetextata	1	6.28	1.69
		2	4.00	1.36
		3	3.87	1.89
		4	2.65	0.68
Windy Nook	*P. canina*	1	3.09	1.30
		2	2.72	0.95
		3	2.29	0.69
		4	2.33	0.76
	P. praetextata	1	2.54	0.79
		2	3.19	0.91
		3	2.79	1.03
		4	2.60	0.77

Table 2.4 Biomass variability (105°C for 24 h) of *Cladonia arbuscula* replicate discs from Nenthead and Middleton Common, where n = 40.

Site	Plant	d. wt mg	S.D.
Nenthead	1	6.32	1.56
	2	7.74	1.71
	3	8.43	1.51
	4	7.60	1.82
Middleton Common	1	7.63	1.74
	2	8.04	1.39
	3	7.81	1.66
	4	8.22	2.08

2.52 Possible auto hydrolysis of substrates

It was observed that pNPP and 4-MUP showed different rates of auto hydrolysis at different temperatures. For the purposes of storage and experimental accuracy sterile pNPP and 4-MUP at concentrations of 250 μM were examined for auto hydrolysis at 4°C and 25 °C over a period of 72 h. Substrates were sterilised in an autoclave at 121°C for 30 min. As a result of this experiment it was considered that pNPP could be kept at 4°C for a maximum period of 72 h, and 4-MUP at 4°C for only 24 h.

2.53 Calibration of substrate standards

A calibration curve plotting concentration against fluorescence was constructed for 4-MU, the standard used for the determination of phosphatase activity using 4-MUP. Concentrations ranged from 0 to 50 μM.

A calibration curve plotting concentration against absorbance was used for a Sigma standard pNP solution (10 μmol ml^{-1}), in connection with Sigma technical bulletin No. 104 (1982).

2.54 Chemicals

The final concentration of 50 mM was chosen as a suitable concentration for buffering physiological media. Each buffer was made to a volume of 100 ml and stored in Pyrex bottles at 4°C. Initial experiments using two different buffers at each pH value showed one set to be particularly poor in holding the required pH value throughout the incubation. One set of buffers were therefore discarded; the buffers used are indicated in Table 2.5.

Table 2.5 Buffers used to investigate phosphatase activity (final assay buffer conc.= 50 mM).

pH	Buffer	Buffering range	pKa at 25°C
3.0	DMG-NaOH	3.2 - 7.6	3.66, 6.20
4.0	DMG-NaOH	3.2 - 7.6	3.66, 6.20
5.0	DMG-NaOH	3.2 - 7.6	3.66, 6.20
6.0	DMG-NaOH	3.2 - 7.6	3.66, 6.20
7.0	HEPES-NaOH	6.8 - 8.2	7.50
8.0	HEPES-NaOH	6.8 - 8.2	7.50
9.0	Glycine-NaOH	8.6 - 10.6	2.35, 9.60
10.0	Glycine-NaOH	8.6 - 10.6	2.35, 9.60
10.3	Glycine-NaOH	8.6 - 10.6	2.35, 9.60
11.0	CAPS-NaOH	9.8 - 11.1	10.40

Reagents used in the preparation of assay media were of Analar grade, obtained from British Drug House Ltd (BDH), Poole, Dorset. The assay media used throughout this research was a modified CHU 10 D medium (low Mn), the elemental composition is indicated in Table 2.6. Assay media was made to a volume of 2 l^{-1}, stored at 4°C and used as a communal stock.

Table 2.6 Modified CHU 10 D medium used to investigate phosphatase activity.

Stocks	g l^{-1}	Volume ml^{-1} of medium	Salt mg l^{-1}	μM
KCl	4.27	0.5	2.14	28.70
$CaCl_2.2H_2O$	35.87	1.0	35.87	243.98
$MgSO_4.7H_2O$	25.00	1.0	25.00	101.43
$NaHCO_3$	15.85	1.0	15.85	188.67
FeEDTA stock		0.125		
$FeCl_3.6H_2O$	9.70		1.21	4.48
NaEDTA	13.35		1.67	4.78
A5 Microelement stock		0.25		
H_3BO_3	2.86		0.715	11.56
$MnCl_2.4H_2O$	0.181		0.045	0.23
$ZnSO_4.7H_2O$	0.222		0.056	0.19
$CuSO_4.5H_2O$	0.079		0.020	0.08
$CoSO_4.7H_2O$	0.042		0.011	0.039
$Na_2MoO_4.2H_2O$	0.027		0.0068	0.028

Table 2.7 Elemental concentrations in assay medium.

Element	mg l^{-1}	μM
Na	4.45	193.59
Ca	9.78	244.01
Mg	2.47	101.63
K	1.12	28.64
S	3.26	101.67
Fe	0.25	4.48
EDTA	1.56	4.78
B	0.125	11.56
Mn	0.012	0.218
Zn	0.013	0.199
Mo	0.003	0.031
Cu	0.005	0.079
Co	0.002	0.034

Phosphatase activity was terminated with 5M NaOH (final conc. = 500 mM) for pNPP and bis-pNPP and 100 mM K_2HPO_4, 100 mM NaOH and 5 mM EDTA (final conc. = 50 mM) for 4-MUP. Activity was terminated after the removal of lichen material; the solution must be raised to about pH 12.5 so that the maximum colour can be obtained and to limit extracellular enzyme or bacteria in the solution which may lead to continued phosphatase activity.

2.541 Methods

Four replicates taken from each plant were used throughout every experiment, prepared according to the methods described in Section 2.3. An assay examining the influence of pH on one plant therefore required 40 discs to be extracted from that plant. All material was washed in assay media 3 times prior to an experiment. The dry weight of each sample was recorded after each experiment by drying at 105°C for 24 h.

A control was used at each pH value alongside the four replicates. Each replicate was assayed in a 25 ml volume snap cap with the following procedure observed.

For pNPP, bis-pNPP and 4-MUP the methodology was largely the same. 1.6 ml buffer was pipetted into each snap cap first, then 1.5 ml assay media was added to this. Lichen material was added to each solution and then bottles were placed in a shaker tank at 25°C and 100 μmol photon m^{-2} s^{-1} illumination from daylight fluorescent tubes. After approx. 10 min 100 μl substrate was added to each solution; incubation starts from this point. After the substrate had been added caps were placed quickly over the tops of the bottles to prevent any uptake of CO_2 and a consequent drop in pH at the higher pH values. The assay was run for 1 hour with all substrates.

For pNPP and bis-pNPP activity was terminated when the lichen material was removed and terminator solution added to the product. With 4-MUP after removal of the lichen 1 ml of the product was transferred to a 1 cm cuvette, which contained 1 ml of terminator solution. The product of pNPP and bis-pNPP were measured on a Shimadzu double beam spectrophotometer UV 150-02 at 405 nm, using 1 cm glass cuvettes, against a reagent blank. The product of 4-MUP was measured on a Baird-Atomic FP100 Fluoripoint Spectrofluorimeter.

Lichen samples were prepared for dry weight analysis immediately after readings were taken, left for 24 h at 105°C in 25 ml volume snap caps.

2.55 Analysis of soil pH

Soil samples collected with lichen material were analysed for pH. Soil was extracted from the lower thallus of each plant with forceps and 5 to 10 g placed in an erlenmeyer flask with 100 ml Milli Q water. Samples were then shaken at 32°C for approx. half an hour. Approximately 20 ml of the supernatant was transferred to snap caps and the pH measured, with an EIL 7050 pH meter, following calibration of the meter with pH 4.01, 7.0 and 9.2 buffers.

2.6 Localization of phosphatase activity

For the purposes of qualitative information on the cellular location of phosphatase activities 3 staining techniques, using organic phosphate substrates, were used on *Peltigera canina* material from Nenthead.

2.61 Localization of phosphomonoesterase activity using 5-bromo-4-chloro-3-indolyl phosphate (BCIP)

Localization of PMEase activity was carried out using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as an organic P substrate (Coston & Holt, 1958; Holt & Withers, 1958). Material was washed three times and resuspended in 250 μM and 1 mM BCIP in assay medium at pH 6.0 in a snap cap. The material was shaken frequently for 15 min at 32°C

and then washed three times in assay medium prior to examination under a high powered microscope (x 400 & x 1000). This assay was carried out in a shaken glass container as O_2 is required for formation of the insoluble blue indigoid.

2.62 Localization of phosphomonoesterase activity using naphthol AS-MX phosphate

Localization of PMEase activity was tested by microscopy using 3-hydroxy-2-naphtholic acid 2, 4-dimethylanilide phosphate sodium salt (naphthol AS-MX phosphate) as the organic P source and diazotized 4-benzylamino-2, 5-dimethoxyanilide zinc chloride (Fast Blue RR diazonium salt) as the coupling agent; the product is a violet insoluble dye. The staining medium consisted of 12 ml assay medium, 0.5 ml naphthol AS-MX phosphate alkaline solution (Sigma Chemical Co, 1984) and 7.5 mg of Fast Blue RR diazonium salt; a magnetic stirrer was used during the preparation. Two concentrations, 250 μM and 1 mM, were tested. The staining medium was adjusted to pH 6.0 using 1 M NaOH and then filtered through a GF/C glass microfibre filter. It was used immediately after preparation. Material was washed three times in assay medium, resuspended in the staining medium, left for 15 min at 32°C, washed three times in assay medium and examined under a high powered microscope (x 400 & x 1000).

2.63 Localization of phosphodiesterase activity using β-naphthyl phenylphosphonate

Localization of PDEase activity was achieved using β-naphthyl phenylphosphonate as the organic P source (Kelly et al., 1975) and diazotized 2-methyl-4-[(2-methylphenyl) azo] benzenediazonium sulphate salt (Fast Garnet GBC sulphate salt) as the coupling agent; the product is an orange insoluble dye. The staining medium consisted of assay medium at pH 6.0, 1 mM naphthyl phenylphosphonate and 0.1 % Fast Garnet GBC sulphate salt. A second concentration of 250 μM was also used. The staining medium was filtered through a 0.22 μM nitro-cellulose filter and used immediately after preparation. Material was washed three times in assay medium and resuspended in the staining medium, left for 15 min at 32°C, washed three times in assay medium and examined under a high powered microscope (x 400 & x 1000). Microtouch medical gloves were used throughout as Fast Garnet GBC sulphate salt is a possible carcinogen.

2.7 Computing

The text for this thesis was processed with Microsoft Word 3.1 running on an Amit system 334. Graphics were processed with Sigma Plot 4.1, also on an Amit system 334.

CHAPTER 3

FIELD SITES AND SAMPLING PROGRAMME

3.1 Nenthead

The study site lies just outside the town of Nenthead (NY 781435), in the County of Cumbria at an altitude of 442 m. The River Nent flows through the site and meets the River South Tyne approximately 5 miles to the west. The geology of Nenthead is very interesting. The area surrounding the study site is underlain by sedimentary rocks, situated on the boundary of Tournaisian & Viséan (Carboniferous Limestone Series) and Namurian (Millstone Grit Series) geological strata. These rocks mark the boundary of the Dinantian and Silesian epochs respectively, laid down in the Carboniferous Period of the late Palaeozoic Era.

Lead mining began at Nenthead in about 1753 and in the 18th and 19th centuries Nenthead was the main centre of lead mining in the North Pennines. By the 1880's lead began a permanent decline but for many years production was replaced by zinc. With the decline in zinc production came a fall in population. Mining ceased in 1905 when the London Lead Company left the area.

Nenthead today is superficially similar to the majority of disused mine sites. The study site is very exposed with the landscape dominated by mine spoil heaps which support a luxuriant lichen flora. *Peltgera* species are mainly confined to the northern bank of the River Nent on horizontal surfaces and on SW facing vertical slopes. *Peltigera canina* and *P. praetextata* are very well represented at Nenthead, in excess of 100 plants of each species. Representatives of both species in excess of 12 cm were frequently encountered. *P. canina* occurred on both horizontal and vertical surfaces, the vast majority on the latter.

This species was most frequently found in association with other plants, most noticeably grasses.

P. praetextata was found only on horizontal surfaces, occurring largely on the exposed mineral soil areas with very short grasses. This species was frequently found more than 15 m from the River Nent.

Cladonia, like *Peltigera*, is a very well represented lichen genus at Nenthead. *Cladonia arbuscula* was found in "cushions", commonly exceeding 20 cm in diameter, the number of these cushions exceeding 50. It was found most frequently within 5 m from the River Nent. *Cladonia arbscula* is most frequent on the southern bank of the river, occurring on horizontal surfaces and N facing vertical slopes. Where *C. arbuscula* occurred on vertical surfaces, directly on spoil heaps, it was rapidly replaced by other *Cladonia* spp. *C. arbuscula* was not found in association with any conspicuous plants, only occasionally among short grasses. The very small number of trees at this site all support a very diverse and rich lichen flora with representative species indicative of Zones 6-7 of the SO_2 air pollution scale (Hawksworth & Rose, 1970). Although the vegetation on the old dressing floors beside the disused mines is dominated by grasses a number of notable metallophytes do occur in some abundance. These include; Scurveygrass, Mountain Pansy, Spring Sandwort, Alpine Pennycress and Moonwort.

3.2 Windy Nook

Windy Nook is situated in County Durham, approximately 1 mile from the centre of Wolsingham. The study site (NZ 065371)is actually a recreation area designated by the Council. It is located on the northern bank of the River Wear in a narrow corridor between the river and the A 689 main road. It is approximately 152 m above sea-level and flanked by the gentle sloping upland moorland of the Wear Valley. Windy Nook is situated rather centrally on the Namurian sedimentary rock strata as described for Nenthead.

The entire study site, which is very exposed, comprises an area of approx. 5 ha which is situated on the flood plain of the River Wear within which there are two major distinctive vegetational habitats. Approximately 60 % of the site is dominated by grasses with occasional *Ulex* and *Cytisus* bushes. Within this area there are frequent areas of bare ground. *Peltigera* species occur in this area on bare ground and among grasses. *Peltigera* is less well represented at Windy Nook, *P. canina* and *P. praetextata* numbering in excess of 25 plants of each species. All *Peltigera* plants encountered were growing on horizontal surfaces, both species growing on the more exposed soil. Separating this area from the River Wear is a strip of deciduous wood running parallel to the river. The wood, no more than 5 m deep at its widest point, supports *Betula*, *Fraxinus*, *Salix*, *Quercus* and *Acer* as the dominant trees, with *Crataegus* and *Sambucus* in association. The corticolous lichen flora is relatively poor. The *Cladonion coniocraeae* federation is almost exclusive; only a few *Salix* and *Sambucus* trees support lichens of the *Physodion* and *Xanthorion* federations. The terricolous lichen flora is dominated by *Peltigera* spp. with only a small number of *Collema tenax* and *Baeomyces rufus* plants present.

3.3 Middleton Common

The study site at Middleton Common (NY 994305) is situated on the eastern bank of Little Eggles Hope, a small tributary (2 m wide, 40 cm deep) of Eggleston Burn which has its confluence with the River Tees approximately 5 miles to the south. The site is approximately 427 m above sea-level on upland moorland dominated by *Calluna* and various grasses. This site is, like Nenthead and Windy Nook, very exposed. The nearest sizeable settlement is Middleton in Teesdale which is roughly 5 miles south-west. Hamsterley Forest lies 2 miles to the east, and represents the only considerable forested area in the region. The geology of Middleton Common resembles that of Nenthead.

Middleton Common supports a large number of *Cladonia* lichens; *C. arbuscula* is represented in numerous communities of the characteristic cushion habit. The range of microenvironments at this site afford *C. arbuscula* with a variety of horizontal and vertical surfaces, supporting a complex mosaic of plant communities. *Cladonia* plants used in this study were sampled from a W facing steep slope immediately adjacent to Little Eggles Hope. *Cladonia arbuscula* was one of nine species of *Cladonia* lichens recorded in the immediate area where plants used in this study were sampled from. *Cladonia* species were by far the most well represented of the terricolous lichen flora. Of the saxicolous lichen flora *Porpidia* and *Rhizocarpon* spp. were most abundant on small siliceous boulders. The almost complete absence of trees, scrub vegetation being dominant, meant that corticolous lichens were very sparse, only *Hypogymnia physodes*, *Parmelia sulcata* and *P. saxatilis* were noticeable. It proved impossible to find an area away from mine spoil with all three species used in this study present so *Peltigera* plants were sampled from one low metal site and *Cladonia* plants from another.

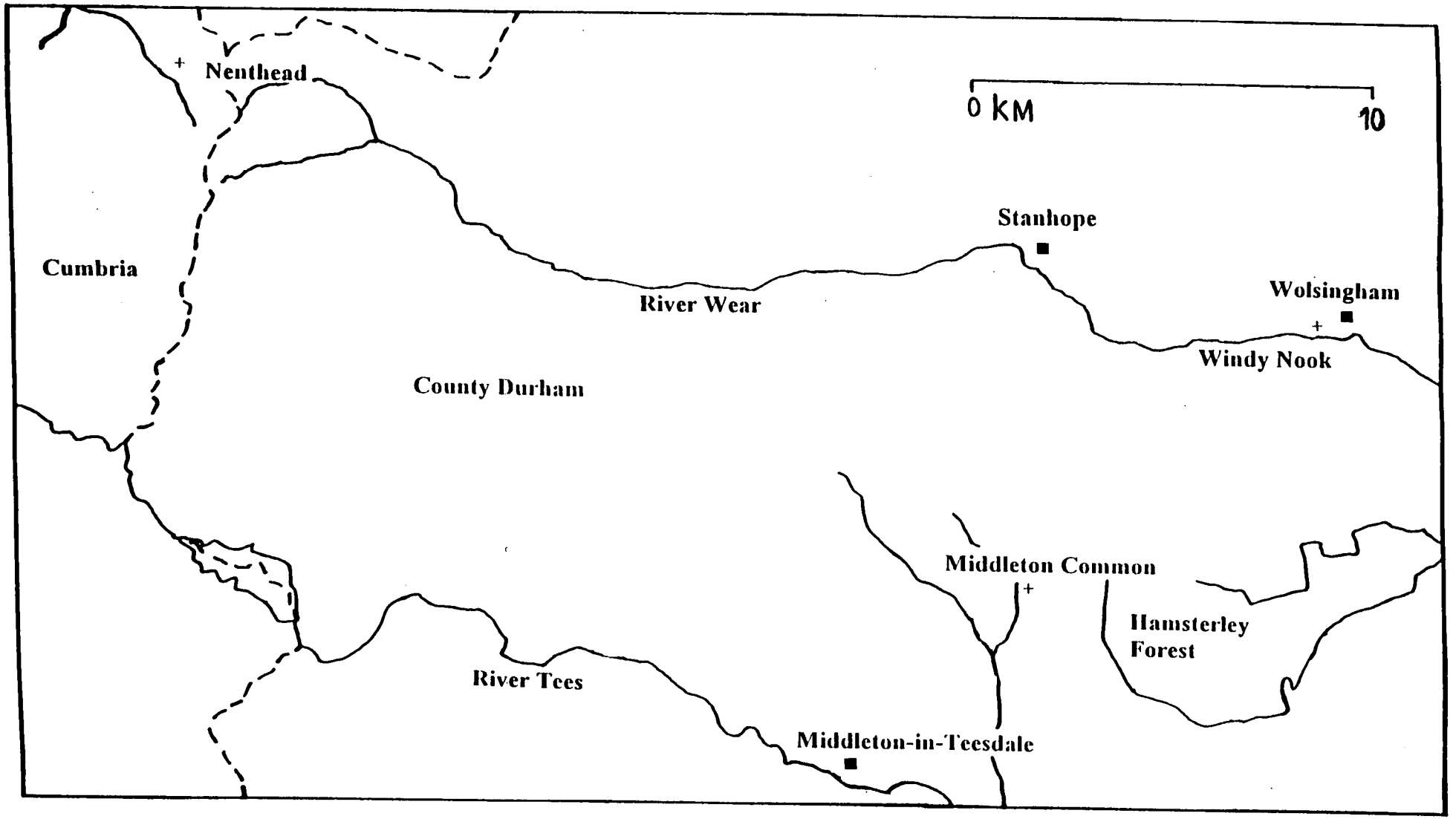


Fig. 3.1 The location of field sites (+), with major towns (■) and river systems.

3.4 Sampling of *Peltigera*

Four plants of each species were collected, each plant from a lichen community which consisted of at least 4 individuals of that particular species. Each plant was selected from a different community, a typical community supported between 5 and 10 individual *Peltigera* plants with as many as 4 different species. The four plants of each species collected represent samples of one population each having a dry weight of between 5 - 10 g. Once collected each plant was placed into a labelled polythene bag for transfer to the laboratories at Durham.

Plants were removed from the habitat with stainless steel forceps, the resultant sample an individual plant containing morphological features and the age range representative of the community. Care was taken not to select material from anomalous populations occurring in conspicuously different habitats.

P. canina and *P. praetextata* material was collected only from horizontal surfaces, in areas between 2 and 6 m from the River Nent.

The same criteria used for sampling *Peltigera* at Nenthead was applied at Windy Nook. All plants, sampled between 10 February and 10 July 1992, were transferred to the laboratories at Durham where they were analyzed and stored according to the methods described in Section 2.2.

3.5 Sampling of *Cladonia*

Four plants of this species were collected, using stainless steel forceps. Each population was sampled from a "typical" community. Individual plants of this species are not as easy to delimit from neighbouring plants, as is the case with *Peltigera*. Once communities were selected for sampling, material was removed from a region midway from the edge and the centre of the plant, ensuring that each plant consisted of all morphological features representative of the species. Each plant had a dry weight of

between 5 - 7 g, approx. one eighth of the host plant. The same criteria for sampling *Peltigera* was applied to *Cladonia*; atypical plants were excluded from sampling.

The four *Cladonia* plants from Middleton Common were selected from areas within 4 m of Little Eggleshope; the same criteria for sampling at Nenthead were applied. Plants were selected from communities on the more exposed areas of soil.

All plants, sampled between 10 February and 10 July 1992, were transferred to the laboratories at Durham where they were analyzed and stored according to the methods described in Section 2.2.

3.6 pH of soil samples

Along with each lichen plant that was collected, a soil sample, to a depth of approx. 2 cm, was included. Each soil sample was separated from the lichen component according to the methods described in Section 2.2. Soil samples were then analyzed for pH according to the methods described in Section 2.55. The pH of each soil sample is intended to represent the pH within the rhizosphere of each plant.

Table 3.1 Soil pH values within the rhizospheres of *P. canina* and *P. praetextata* from Nenthead and Windy Nook.

Site	Species	Plant	Soil pH
Nenthead	*Peltigera canina*	1	7.58
		2	7.60
		3	7.51
		4	7.59
	P. praetextata	1	7.63
		2	7.22
		3	7.93
		4	7.67
Windy Nook	*P. canina*	1	7.88
		2	7.85
		3	7.58
		4	7.22
	P. praetextata	1	7.31
		2	7.36
		3	7.83
		4	7.76

Table 3.2 Soil pH values within the rhizosphere of *C. arbuscula* from Nenthead and Middleton Common.

Site	Plant	Soil pH
Nenthead	1	6.32
	2	6.80
	3	6.91
	4	6.73
Middleton Common	1	7.24
	2	7.65
	3	7.42
	4	7.59

CHAPTER 4

ELEMENTAL CONCENTRATIONS OF LICHENS AND SOIL SAMPLES

4.1 Phosphorus content of *Peltigera* plants and associated soil

The phosphorus content of all *Peltigera* plants and the associated soil samples was determined using the methods of Eisenreich et al. (1975), as described in Section 2.411, following digestion with boiling 4 M HNO_3 (Section 2.411). Total P only, the organic and inorganic fractions, were determined. *Peltigera* material was selected and prepared for analysis according to the methods described in Section 2.3. Discs were extracted from each plant from an area between 2 and 5 cm from the lobe margins using a number 2 cork borer. The resultant discs were approx. 6 mm in diameter. Care was taken to ensure that all discs were comparable. A brief visual examination of each disc identified the main thallus containing the upper cortex, algal layer and medulla and between 4 to 10 rhizines depending on the site from which the plants came (Section 2.3). Soil samples were boiled in 4 M HNO_3 for 2 h and solutions filtered through GF/C filter papers prior to phosphorus analysis (Section 2.411).

Table 4.1 Phosphorus concentration of *Peltigera canina* and *P. praetextata* plants from Nenthead and Windy Nook (n = 4).

Site	Species	Plant	Total P $\mu g\ g^{-1}$ mean	S.D.	Inter-plant mean	S.D.
Nenthead	*Peltigera canina*	1	4200	201	4400	648
		2	4700	327		
		3	5100	278		
		4	3600	269		
	P. praetextata	1	3400	252	3725	275
		2	3600	330		
		3	3900	213		
		4	4000	339		
Windy Nook	*P. canina*	1	4800	229	5150	264
		2	5300	316		
		3	5400	318		
		4	5100	228		
	P. praetextata	1	4400	347	4525	340
		2	5000	128		
		3	4500	176		
		4	4200	219		

Table 4.2 Phosphorus concentration of soil samples associated with *Peltigera canina* and *P. praetextata* plants from Nenthead and Windy Nook (n = 4).

Site	Species	Plant	Total P $\mu g\ g^{-1}$ mean	S.D.	Inter-plant mean	S.D.
Nenthead	*Peltigera canina*	1	4795	225	7958	4466
		2	3795	201		
		3	15200	412		
		4	8040	274		
	P. praetextata	1	2800	197	5330	3467
		2	3090	186		
		3	4160	206		
		4	11270	398		
Windy Nook	*P. canina*	1	3530	100	3880	384
		2	3900	176		
		3	4500	218		
		4	3590	245		
	P. praetextata	1	2780	174	3578	677
		2	3210	182		
		3	3720	192		
		4	4600	208		

Table 4.3 Ratio of lichen : soil P between *Peltigera canina* and *P. praetextata* plants and associated soils from Nenthead and Windy Nook.

Site	Species	Plant	P ratio Lichen : Soil	Inter-plant mean	S.D.
Nenthead	*Peltigera canina*	1	0.86	0.72	0.35
		2	1.24		
		3	0.34		
		4	0.45		
	P. praetextata	1	1.21	0.92	0.34
		2	1.17		
		3	0.94		
		4	0.35		
Windy Nook	*P. canina*	1	1.36	1.34	0.08
		2	1.36		
		3	1.20		
		4	1.42		
	P. praetextata	1	1.58	1.32	0.27
		2	1.56		
		3	1.21		
		4	0.91		

4.2 Phosphorus content of *Cladonia* plants and associated soil

The phosphorus content of all *Cladonia* plants and the associated soil samples was determined using the methods of Eisenreich et al. (1975), as described in Section 2.411, following digestion with boiling 4 M HNO_3 (Section 2.411). As with *Peltigera* only total P, the combined organic and inorganic fractions, was determined. *Cladonia* material was selected and prepared for analysis according to the methods described in Section 2.3. *Cladonia* material was extracted with forceps from the main lower podetial thallus, excluding apices and excessive secondary branches. Each replicate was composed of a section containing the outer cortex, algal layer and medulla. In Tables 4.4 and 4.5 the mean P content, for plants from Nenthead and Middleton Common, without the outlying values have been included in brackets.

Table 4.4 Phosphorus concenration of *Cladonia arbuscula* plants from Nenthead and Middleton Common (n = 4).

Site	Plant	Total P $\mu g\ g^{-1}$ mean	S.D.	Inter-plant mean	S.D.
Nenthead	1	5200	857	2575	1517
	2	1700	394	(1700)	(100)
	3	1800	352		
	4	1600	237		
Middleton Common	1	1500	303	2100	600
	2	1900	435	(1767)	(231)
	3	1900	279		
	4	3100	133		

Table 4.5 Phosphorus concentration of soil samples associated with *Cladonia arbuscula* plants from Nenthead and Middleton Common (n = 4).

Site	Plant	Total P $\mu g\ g^{-1}$ mean	S.D.	Inter-plant mean	S.D.
Nenthead	1	11350	618	5634	3300
	2	3630	104	(3728)	(74)
	3	3810	152		
	4	3745	204		
Middleton Common	1	3100	215	4700	2287
	2	3600	185	(3383)	(209)
	3	3450	204		
	4	8650	304		

Table 4.6 Ratio of lichen : soil P between *Cladonia arbuscula* plants and associated soils from Nenthead and Middleton Common.

Site	Plant	P ratio Lichen : Soil	Inter-plant mean	S.D.
Nenthead	1	0.46	0.46	0.01
	2	0.47		
	3	0.47		
	4	0.43		
Middleton Common	1	0.48	0.48	0.07
	2	0.53		
	3	0.55		
	4	0.36		

Table 4.7 Correlation between lichen and soil P concentrations in *Peltigera canina*, *P. praetextata* and *Cladonia arbuscula* from Nenthead, Windy Nook and Middleton Common. Regression values are where n = 4 and P = 0.05.

Site	Species	*r*
Nenthead	*Peltigera canina*	+ 0.86
	P. praetextata	+ 0.95
	Cladonia arbuscula	+ 0.99
Windy Nook	*P. canina*	+ 0.84
	P. praetextata	- 0.51
Middleton Common	*C. arbuscula*	+ 0.98

4.3 Zn content of *Peltigera* plants and associated soil

The Zn content of all *Peltigera* plants and the associated soil samples was determined according to the methods described in Section 2.422. *Peltigera* material was selected and prepared for analysis according to the methods described in Section 2.3. Discs, approx. 6 mm in diameter were cut from the plant using a number 2 cork borer. As with phosphorus analysis, the discs were taken from an area between 2 and 5 cm from the lobe margins, ensuring that each disc had a section of thallus composed of the upper cortex, algal layer, medulla and between 4 and 10 rhizines, depending on the site from which they came (Section 2.3). Soil samples were boiled in 4 M HNO_3 for 2 h and solutions filtered through GF/C filter papers prior to Zn analysis (Section 2.411).

Table 4.8 Zinc concentration of *Peltigera canina* and *P. praetextata* plants from Nenthead and Windy Nook (n = 4).

Site	Species	Plant	Total Zn $\mu g\ g^{-1}$ mean	S.D.	Inter-plant mean	S.D.
Nenthead	*Peltigera canina*	1	1000	255	1050	208
		2	1300	97		
		3	800	191		
		4	1100	262		
	P. praetextata	1	1400	182	1225	403
		2	1000	246		
		3	1700	254		
		4	800	110		
Windy Nook	*P. canina*	1	270	155	245	26
		2	240	31		
		3	210	8		
		4	260	32		
	P. praetextata	1	210	33	227	35
		2	190	27		
		3	240	82		
		4	270	23		

Table 4.9 Zinc concentration of soil samples associated with *Peltigera canina* and *P. praetextata* plants from Nenthead and Windy Nook (n = 4).

Site	Species	Plant	Total Zn $\mu g\ g^{-1}$ mean	S.D.	Inter-plant mean	S.D.
Nenthead	*Peltigera canina*	1	1120	184	1719	650
		2	1020	157		
		3	2335	217		
		4	2400	186		
	P. praetextata	1	1430	106	1648	452
		2	1075	146		
		3	1785	95		
		4	2300	142		
Windy Nook	*P. canina*	1	100	14	91	5
		2	88	11		
		3	87	9		
		4	90	12		
	P. praetextata	1	65	8	65	7
		2	53	9		
		3	70	4		
		4	70	3		

Table 4.10 Ratio of lichen : soil Zn between *Peltigera canina* and *P. praetextata* plants and associated soils from Nenthead and Windy Nook.

Site	Species	Plant	Zn ratio Lichen : Soil	Inter-plant mean	S.D.
Nenthead	*Peltigera canina*	1	0.89	0.74	0.36
		2	1.27		
		3	0.34		
		4	0.46		
	P. praetextata	1	0.98	0.80	0.26
		2	0.93		
		3	0.95		
		4	0.35		
Windy Nook	*P. canina*	1	2.70	2.68	0.17
		2	2.73		
		3	2.41		
		4	2.89		
	P. praetextata	1	3.23	3.53	0.22
		2	3.58		
		3	3.43		
		4	3.86		

4.4 Zn content of *Cladonia* plants and associated soil

The Zn content of all *Cladonia* plants and associated soil samples was determined according to the methods described in Section 2.422. *Cladonia* material was selected and prepared according to the methods described in Section 2.3. *Cladonia* material was extracted using forceps, from the lower main podetial thallus only, excluding apices and excessive secondary branches. Each replicate consisted of an outer cortex, algal layer and medulla. Soil samples were boiled in 4 M HNO_3 for 2 h and solutions filtered through GF/C filter papers prior to Zn analysis (Section 2.411).

Table 4.11 Zinc concentration of *Cladonia arbuscula* plants from Nenthead and Middleton Common (n = 4).

Site	Plant	Total Zn $\mu g\ g^{-1}$		Inter-plant	
		mean	S.D.	mean	S.D.
Nenthead	1	800	51	1075	320
	2	1400	111		
	3	800	151		
	4	1300	109		
Middleton Common	1	290	40	265	25
	2	270	22		
	3	270	37		
	4	230	21		

Table 4.12 Zinc concentration of soil samples associated with *Cladonia arbuscula* plants from Nenthead and Middleton Common (n = 4).

Site	Plant	Total Zn $\mu g\ g^{-1}$ mean	S.D.	Inter-plant mean	S.D.
Nenthead	1	1740	201	2370	653
	2	2975	274		
	3	1695	184		
	4	3070	158		
Middleton Common	1	310	74	285	31
	2	265	54		
	3	245	37		
	4	320	27		

Table 4.13 Ratio of lichen : soil Zn between *Cladonia arbuscula* plants and associated soils from Nenthead and Middleton Common.

Site	Plant	Zn ratio Lichen : Soil	Inter-plant mean	S.D.
Nenthead	1	0.46	0.46	0.02
	2	0.47		
	3	0.47		
	4	0.42		
Middleton Common	1	0.94	0.95	0.14
	2	1.02		
	3	1.10		
	4	0.72		

Table 4.14 Correlation between lichen and soil Zn concentrations in *Peltigera canina*, *P. praetextata* and *Cladonia arbuscula* from Nenthead, Windy Nook and Middleton Common. Regression values are where n = 4 and P = 0.05.

Site	Species	*r*
Nenthead	*Peltigera canina*	- 0.56
	P. praetextata	- 0.19
	Cladonia arbuscula	+ 0.98
Windy Nook	*P. canina*	+ 0.77
	P. praetextata	- 0.78
Middleton Common	*C. arbuscula*	- 0.33

Table 4.15 P:Zn ratio of *P. canina*, *P. praetextata* and *C. arbuscula* plants and associated soils from Nenthead, Windy Nook and Middleton Common

Site	Species	Plant	P:Zn		Mean (S.D.)	
			Lichen	Soil	Lichen	Soil
Nenthead	*Peltigera canina*	1	4.20	4.28	4.37	4.47
		2	3.62	3.72	(1.20)	(1.22)
		3	6.38	6.51		
		4	3.27	3.35		
	P. praetextata	1	2.43	1.96	3.33	3.02
		2	3.60	2.87	(1.09)	(1.13)
		3	2.29	2.33		
		4	5.00	4.90		
	C. arbuscula	1	6.50	6.52	2.80	2.80
		2	1.21	1.22	(2.17)	(2.18)
		3	2.25	2.25		
		4	1.23	1.22		
Windy Nook	*P. canina*	1	17.78	35.30	21.30	42.81
		2	22.08	44.32	(2.96)	(6.05)
		3	25.71	51.72		
		4	19.62	39.89		
	P. praetextata	1	20.95	42.77	20.40	55.55
		2	26.32	60.57	(3.92)	(8.62)
		3	18.75	53.14		
		4	15.56	65.71		
Middleton Common	*C. arbuscula*	1	5.17	10.00	8.18	16.17
		2	7.04	13.58	(3.15)	(6.46)
		3	7.04	14.08		
		4	13.48	27.03		

Table 4.16 Comparison of Zn for *P. canina*, *P. praetextata* and *C. arbuscula* plants and associated soils between sites.

Species		Mean Zn $\mu g\ g^{-1}$ Nenthead	S.D.	Mean Zn $\mu g\ g^{-1}$ Windy Nook	S.D.	Ratio
P. canina	plant	1050	210	245	26	4.29
	soil	1719	650	91	5	18.89
P. praetextata	plant	1230	400	228	35	5.39
	soil	1648	452	65	7	25.35
		Nenthead		Middleton Common		
C. arbuscula	plant	1080	320	265	25	4.08
	soil	2370	653	285	31	8.32

4.5 Statistical analyses

Statistical analyses were undertaken on *P. canina*, *P. praetextata* and *C. arbuscula* for total phosphorus and zinc concentrations. Analysis of variance was used to examine the interplant variability of P and Zn for each species at each site. This test is designed to see whether differences between samples are more significant than differences within samples. For each species at each site there were 4 plants each with 4 replicates, therefore n = 16. The null hypothesis in each case is that the difference in the intraplant P or Zn concentrations of each species at each site is significantly greater than the interplant

difference. Table 4.17 summarises the results of statistical analyses undertaken for interplant P and Zn concentrations.

Table 4.17 Analysis of variance of interplant P and Zn concentrations in *Peltigera canina*, *P. praetextata* and *C. arbuscula*. P = 0.01

Site	Species	P F ratio		Zn F ratio	
Nenthead	*P. canina*	15.35		2.86	+
	P. praetextata	2.91	+	11.20	
	C. arbuscula	33.32		26.97	
Windy Nook	*P. canina*	2.80	+	2.82	+
	P. praetextata	6.28		2.29	+
Middleton Common	*C. arbuscula*	15.18		1.88	+

A + denotes that the null hypothesis has been accepted and therefore interplant differences in P or Zn concentrations were not significant.

CHAPTER 5

RESULTS

5.1 Preliminary analyses of environmental factors

Preliminary analyses were undertaken on four *Peltigera canina* plants from Nenthead to examine the effects of various environmental factors on phosphatase activity and to establish the analytical procedure which would then be undertaken in the laboratory.

5.11 Influence of storage

To assess the influence of storage, and therefore cellular damage induced by desiccation, on the PMEase activity of *P. canina* from Nenthead 250 μM pNPP was used on thalline discs extracted from the four plants. Thalline discs were divided into 2 categories;

i for immediate use

ii for use after 6 weeks air-drying at room temp. (22 °C)

All material was assayed at pH 6.0 under the conditions outlined in Section 2.541. Table 5.1 illustrates the influence of storage on the PMEase activity of thalline discs of *P. canina* from Nenthead, where n = 4.

Table 5.1 Influence of storage on the PMEase activity of *P. canina*

Plant	PMEase activity of thalline discs μmol pNP mg^{-1} d.wt h^{-1}		Ratio
	fresh	desiccated	
1	0.04236	0.04282	0.99
2	0.04178	0.04223	0.99
3	0.04519	0.04621	0.98
4	0.03962	0.04018	0.99

Table 5.1 illustrates the influence that storage has on the PMEase activity of thalline discs of *P. canina* is not significantly reduced. These results suggested that lichen material could be stored in the laboratory for up to six weeks after removal from the field site.

5.12 Influence of light and dark

Using 250 μM pNPP, at pH 6.0, the effects of light and dark on the PMEase activity were examined for each of the four *P.* canina plants from Nenthead. Replicates analyzed for the effect of light were treated under normal assay conditions, outlined in Section 2.541. Replicates analyzed for the effect of dark were assayed in the same way but in total darkness. The procedure for all experiments undertaken on additional plants follows that outlined in Sections 2.3 and 2.4. Table 5.2 illustrates the results of the effects of light and dark on the PMEase activity of *P. canina* from Nenthead. Included in the table are the concentrations of total P and Zn, where n = 4.

Table 5.2 Influence of light and dark on the PMEase activity of *P. canina*.

Plant	PMEase activity μmol pNP mg^{-1} d.wt h^{-1}		Ratio	P μg g^{-1}		Zn μg g^{-1}	
	light	dark	l:d		SD		SD
1	0.0407	0.0390	1.04	5030	308	1150	166
2	0.0410	0.0384	1.07	5150	1005	1200	384
3	0.0358	0.0354	1.01	3250	177	1780	310
4	0.0388	0.0376	1.03	3630	270	1180	145

Table 5.2 clearly illustrates that PMEase activity in *P. canina* is only slightly reduced in the dark, with activity in the light only 1.04 times on average greater.

5.13 Influence of cellular damage

To assess the influence of cellular damage on the PMEase activity of *P. canina* from Nenthead 250 μM pNPP was used in assays undertaken on excised rhizines only. Rhizines were removed from the thalline discs using a scalpel and then divided into two components;

i intact rhizines

ii macerated rhizines

Following this treatment rhizines were examined under a dissecting microscope and then assayed at pH 6.0 under the conditions outlined in Section 2.541. Table 5.3 illustrates the effect of cellular damage on the PMEase activity of excised rhizines of *P. canina* from Nenthead, where n = 4.

Table 5.3 Influence of cellular damage on the PMEase activity of excised rhizines of *P. canina*.

Plant	PMEase activity of rhizines μmol pNPP mg^{-1} d.wt h^{-1}		Ratio
	intact	macerated	
1	0.03172	0.03189	0.99
2	0.03368	0.03550	0.95
3	0.03270	0.03317	0.99
4	0.03443	0.03502	0.98

Table 5.3 illustrates that the increase in PMEase activity in macerated rhizines of *P. canina* is negligible when compared with intact rhizines. This provides important evidence for the hypothesis that PMEase activity in *P. canina* is cell-bound.

5.2 Phosphatase activity of *Peltigera canina* and *P. praetextata*

5.21 Influence of pH on *P. canina*

Following the experiments described in Sections 5.1, 5.11, 5.12 and 5.13 a series of experiments were undertaken to assess the phosphatase activities of additional plants of *Peltigera canina* and *P. praetextata*. Initial experiments were designed to examine the influence of pH on the PMEase activity of four plants of both species collected from low (Windy Nook) and high (Nenthead) zinc environments. For this purpose standard laboratory conditions, commonly used for these assays, were applied. Therefore, each plant was assayed in the pH range 3.0 - 11.0 at intervals of 1.0 with an additional pH value of 10.3. Four replicates and a control were used at each pH value. The artificial phosphate substrate was pNPP at a final concentration of 250 μM. Duration of the incubation was one hour at a maintained temperature of 25°C and 100 μmol photon m^{-2} s^{-1} illumination from daylight fluorescent tubes.

Fig. 5.1 illustrates the influence of pH on the PMEase activity of *P. canina* from Windy Nook. Throughout all figures the values shown are the mean of four replicates and vertical bars represent the standard deviation of the mean.

Each plant displays a trend of peak PMEase activity in the alkaline pH range 9.0 - 10.3. Intraplant variability is consistently greater in the alkaline pH range.

Fig. 5.1a illustrates the interplant variability of *P. canina* from Windy Nook at each pH value. The maximum and minimum rates of PMEase activity and coefficient of variation are shown by graphs a and b respectively. The area within the lines indicates the degree of variability, the smaller the area indicating less variability. PMEase activity is relatively uniform in the pH range 3.0 - 8.0 but is erratic in the alkaline range.

The graph showing the maximum and minimum coefficient of variation illustrates the degree of replication within the 4 plants of *P. canina*. Although the minimum value at each pH is below 5 %, with the exception of pH 10.0, the maximum values are high enough to indicate a low degree of interplant replication at each pH value.

Fig. 5.2 illustrates the PMEase activity of *P. canina* from Nenthead. The most noticeable difference between the plants from this site and Windy Nook is the shift in the optimum pH of PMEase activity. Plants 1 & 4 have definite peak activities at pH 6.0 while plants 2 & 3 display trends of peak activity at pH 6.0. Alkaline PMEase activity is suppressed with only plant 1 showing a trend of alkaline PMEase activity at pH 10.0. The mean rate of peak PMEase activity of the four plants of *P. canina* for Nenthead is 0.03424 μmol pNPP mg d. wt^{-1} h^{-1} and for Windy Nook is 0.01943 μmol pNPP mg d. wt^{-1} h^{-1}, a ratio of 1.76. Intraplant variability shows no consistent pattern at any pH value.

Interplant variability is illustrated in Fig. 5.2a. There is a considerable degree of variability in the pH range 4.0 - 6.0, as shown by the maximum and minimum rates of activity. Variability within the alkaline pH range is less pronounced and has a more regular pattern of peaks and troughs. Replication, illustrated by graph b, is again poor, particularly within the alkaline pH range 8.0 - 11.0.

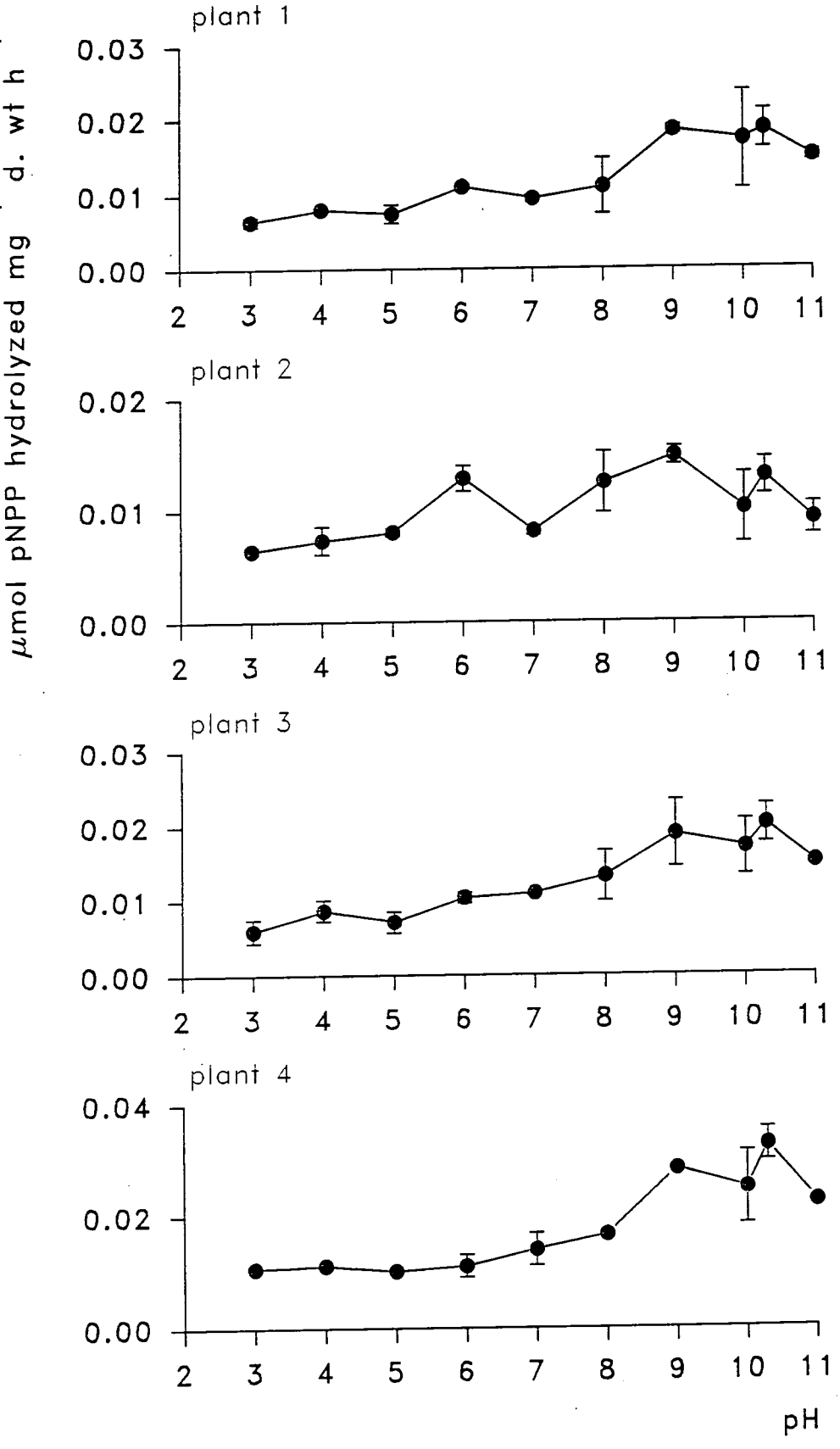


Fig. 5.1 Influence of pH on the PMEase activity of *Peltigera canina* from Windy Nook, using 250 μM pNPP

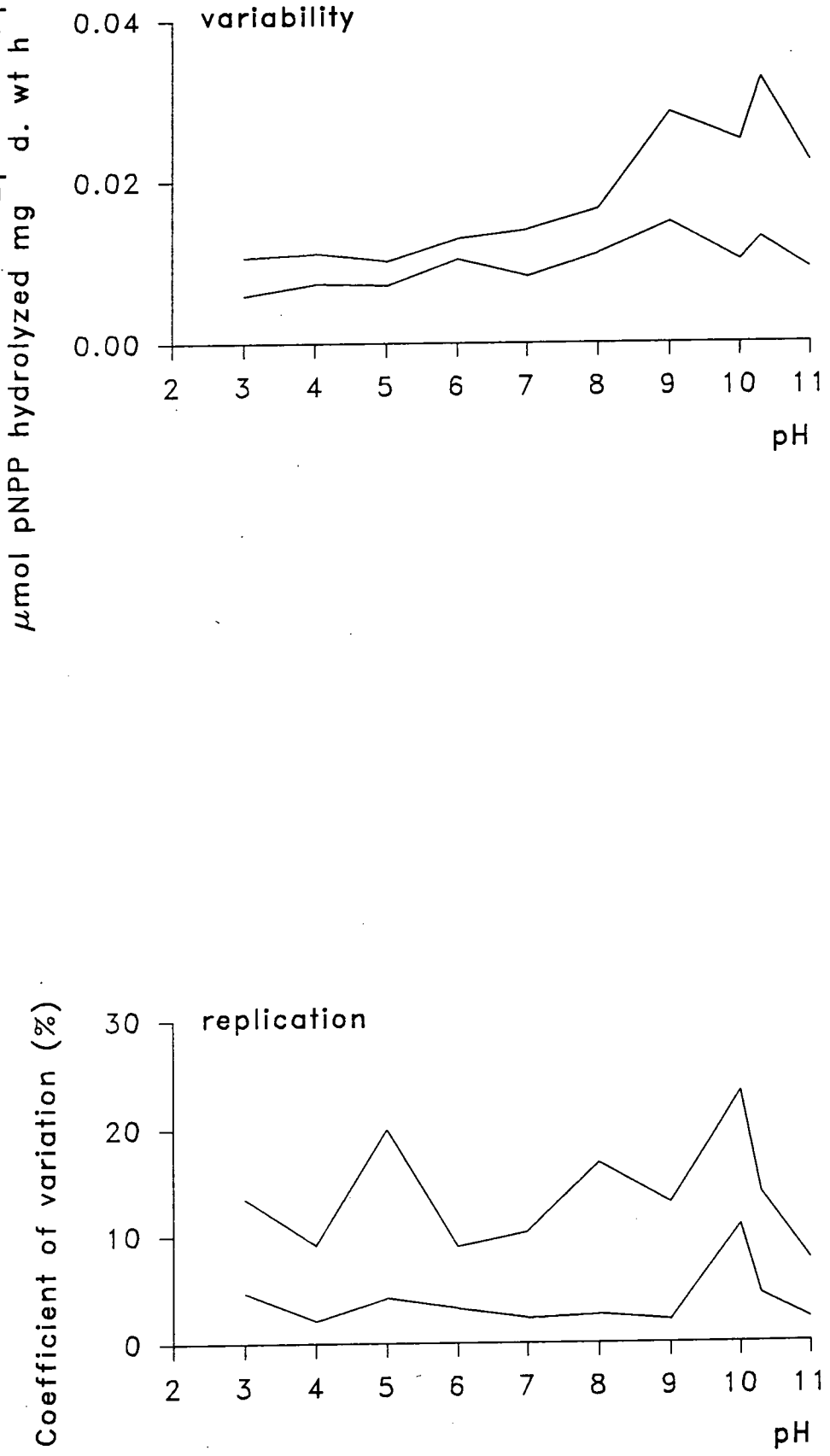


Fig. 5.1a Interplant varability and replication of PMEase activity in *Peltigera canina* from Windy Nook, using 250 μM pNPP

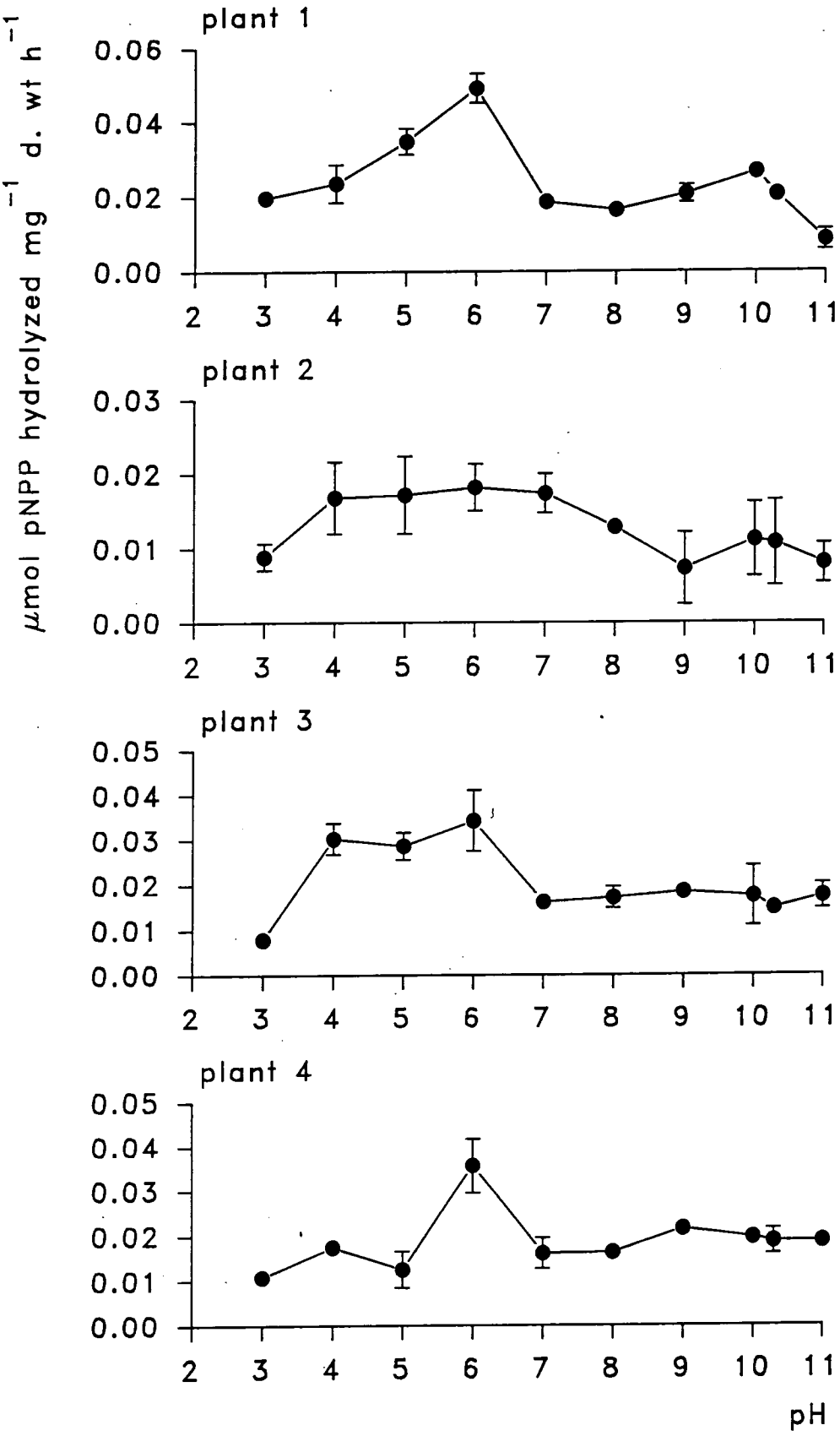


Fig. 5.2 Influence of pH on the PMEase activity of *Peltigera canina* from Nenthead, using 250 µM pNPP

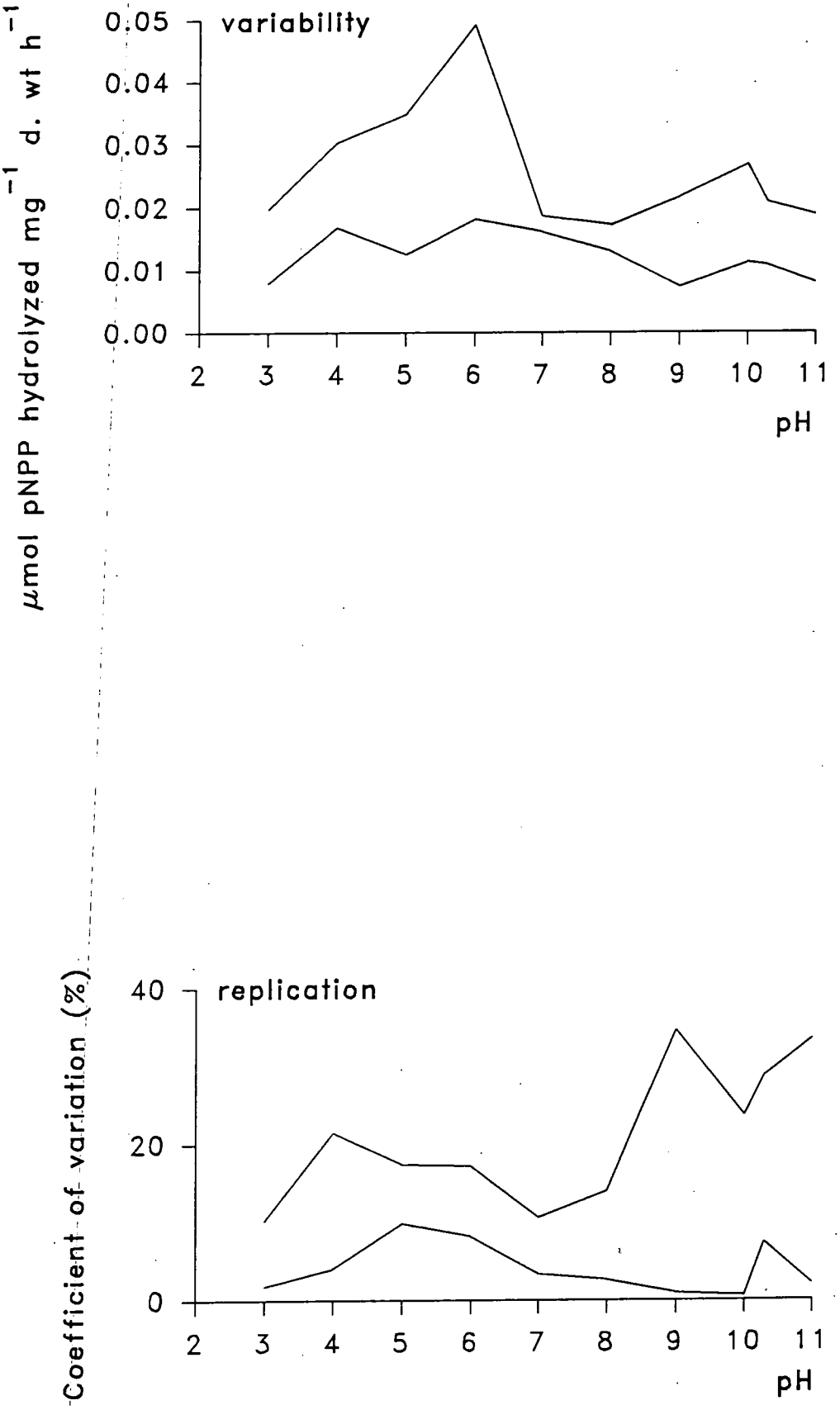


Fig. 5.2a Interplantvariability and replication of PMEase activity in *Peltigera canina* from Nenthead, using 250 µM pNPP

5.22 Influence of pH on *P. praetextata*

Fig. 5.3 illustrates the PMEase activity of *P. praetextata* from Windy Nook. Plants 1, 3 & 4 display bimodal peaks of PMEase activity, but only plant 1 has definite peaks at pH 6.0 & 10.3. Plants 3 & 4 display trends of peak PMEase activity at pH 7.0 & 10.3 and 5.0 & 10.3 respectively. Plant 2 has only a weak discernible peak of PMEase activity in the alkaline pH range 9.0 - 10.3. Intraplant variability displays no consistent pattern at any pH value.

Interplant variability of *P. praetextata* can be gauged by Fig. 5.3a. With the exception of pH 7.0 variability is relatively uniform throughout the pH range examined. Replication is consistently poor at all pH values except pH 10.3 & 11.0.

Fig. 5.4 illustrates the PMEase activity of *P. praetextata* from Nenthead. All four plants display a trend of peak PMEase activity in the alkaline pH range 9.0 - 10.3. The peaks of PMEase activity evident in the acid pH range in plants from Windy Nook are largely suppressed in plants from Nenthead. The mean rate of peak PMEase activity of the four plants of *P. praetextata* from Nenthead is 0.04481 μmol pNPP mg d. wt^{-1} h^{-1} and for Windy Nook is 0.02773 μmol pNPP mg d. wt^{-1} h^{-1}, a ratio of 1.62. This is similar to the difference observed in *P. canina* (Nenthead : Windy Nook ratio 1.76). Intraplant variability shows no consistent pattern at any pH value.

Fig. 5.4a illustrates that maximum and minimum PMEase activities between all plants are relatively uniform throughout the pH range examined. However, interplant variability is still quite marked at all pH values, most noticeably in the alkaline pH range. Replication, as illustrated by graph b, is very poor in the acid pH range and only moderate at pH 10.3 & 11.0.

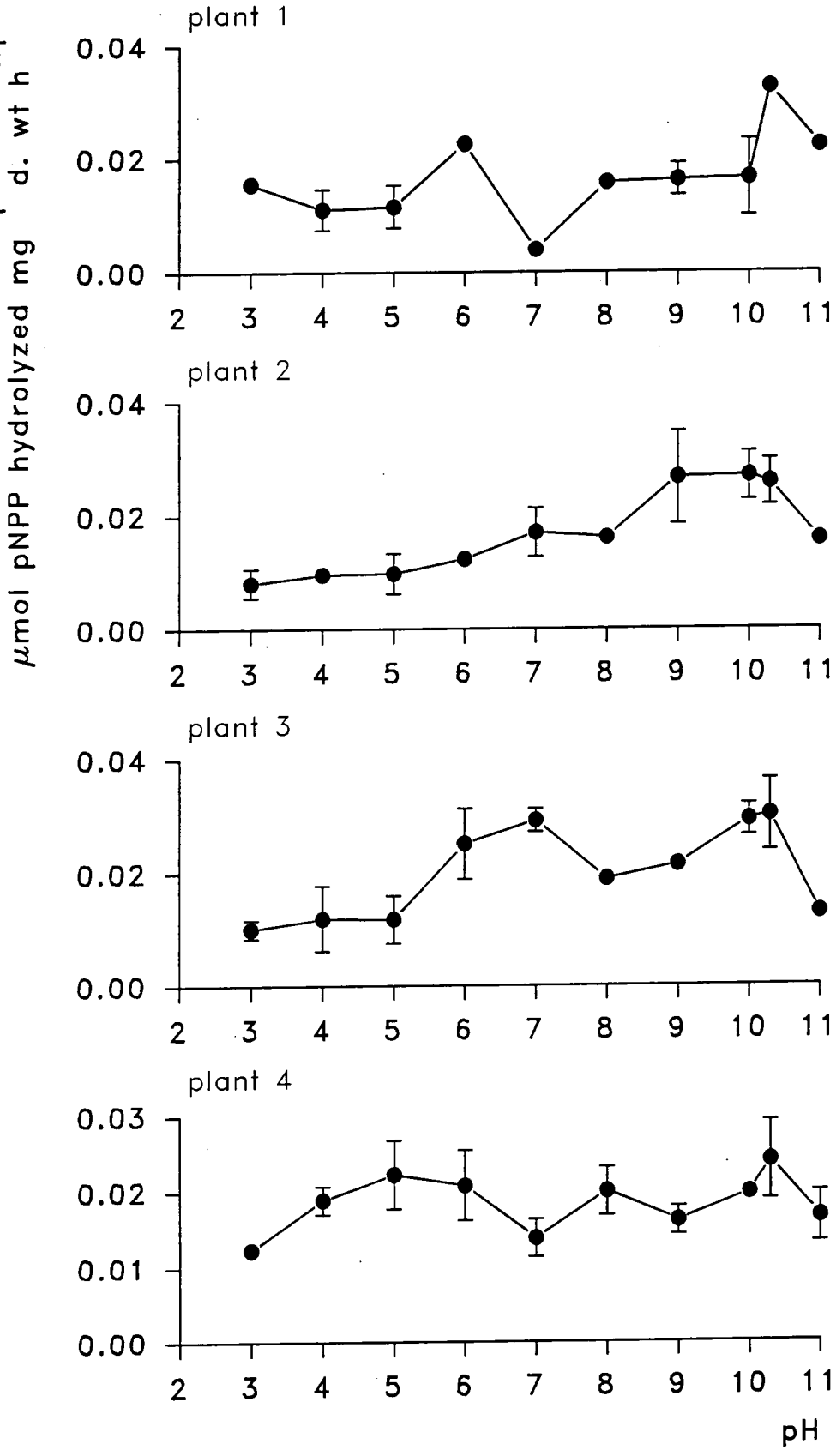


Fig. 5.3 Influence of pH on the PMEase activity of *Peltigera praetextata* from Windy Nook, using 250 µM pNPP

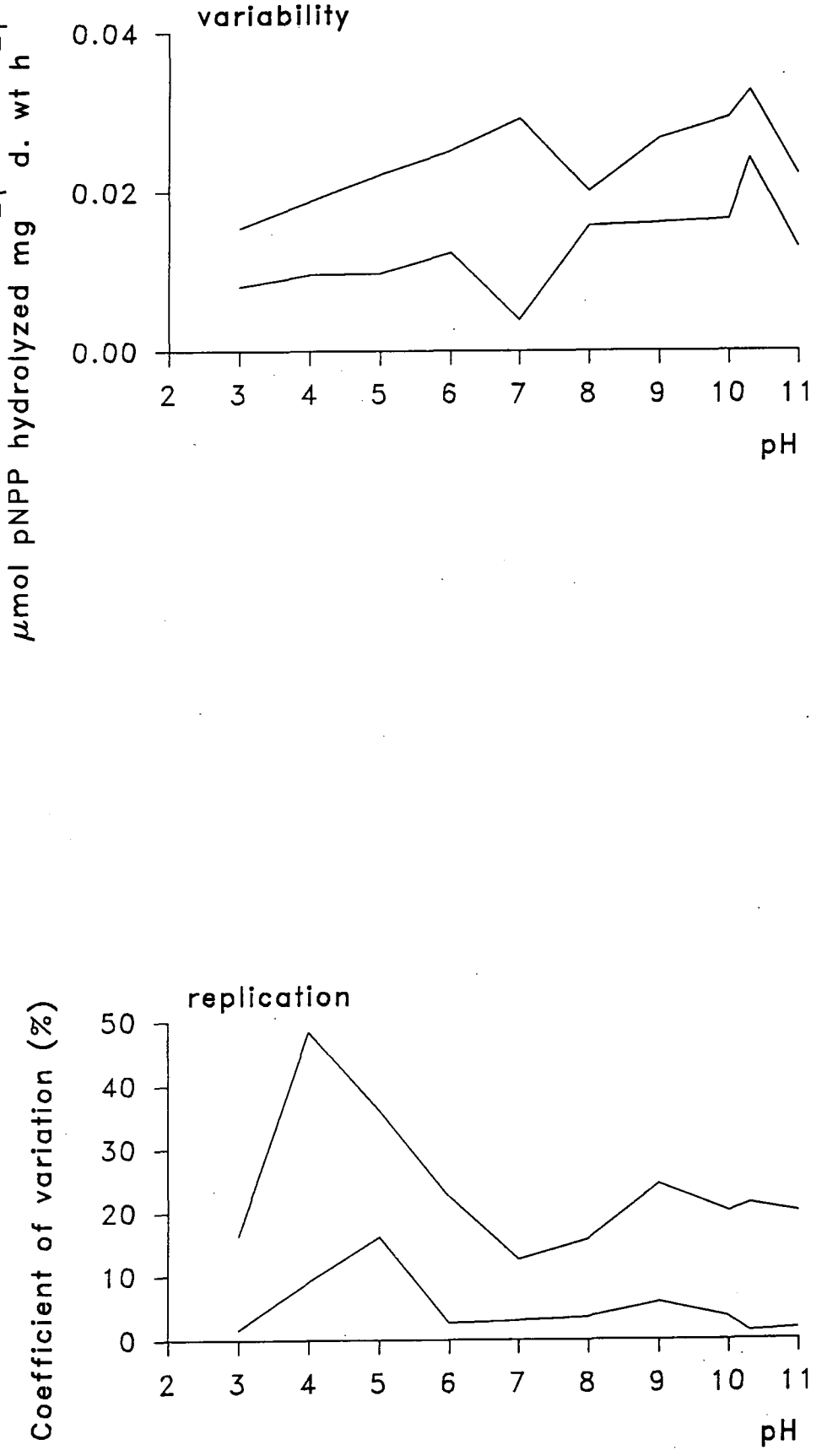


Fig. 5.3a Interplantvariability and replication of PMEase activity in *Peltigera praetextata* from Windy Nook, using 250 μM pNPP

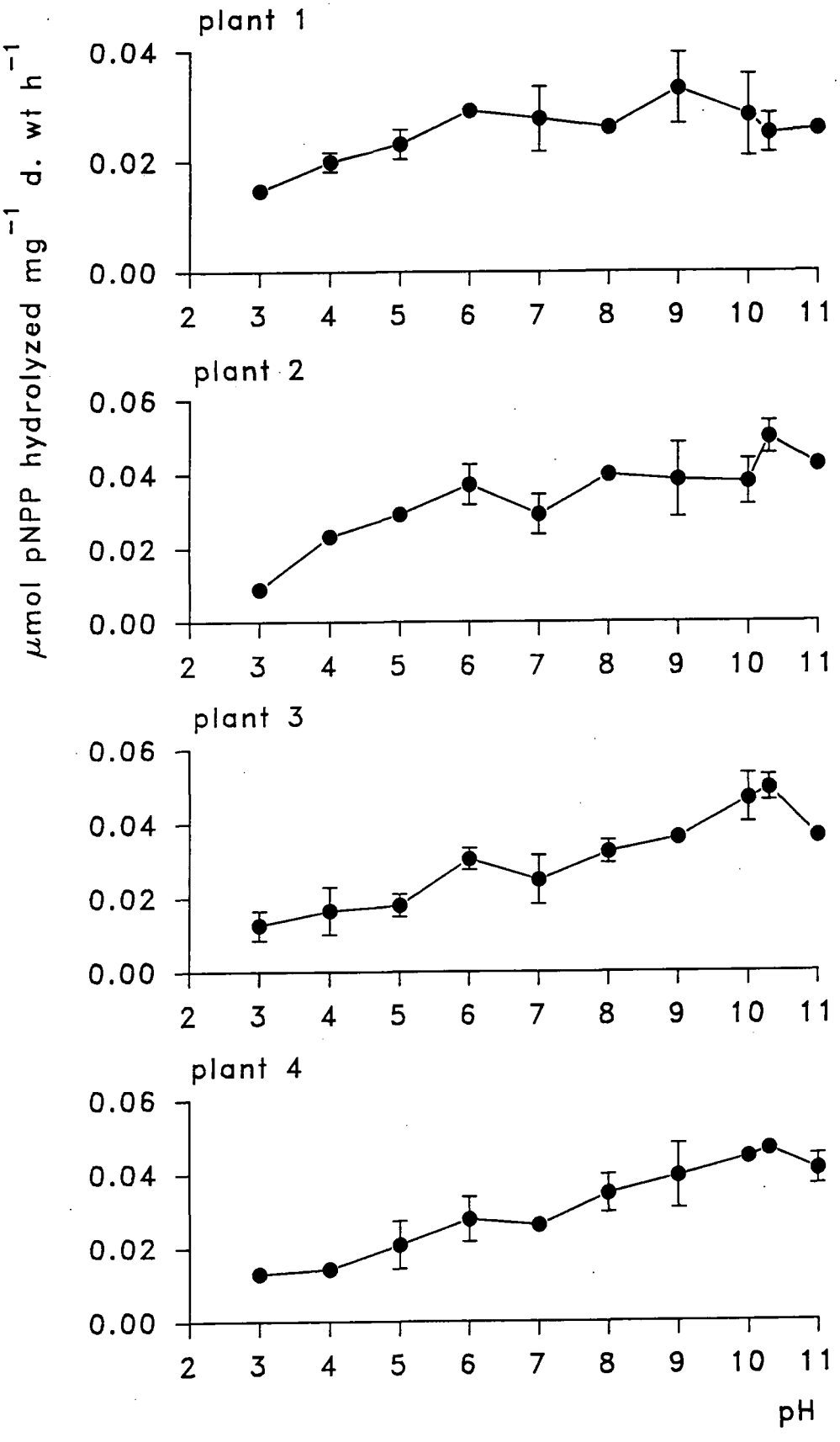


Fig. 5.4 Influence of pH on the PMEase activity of *Peltigera praetextata* from Nenthead, using 250 µM pNPP

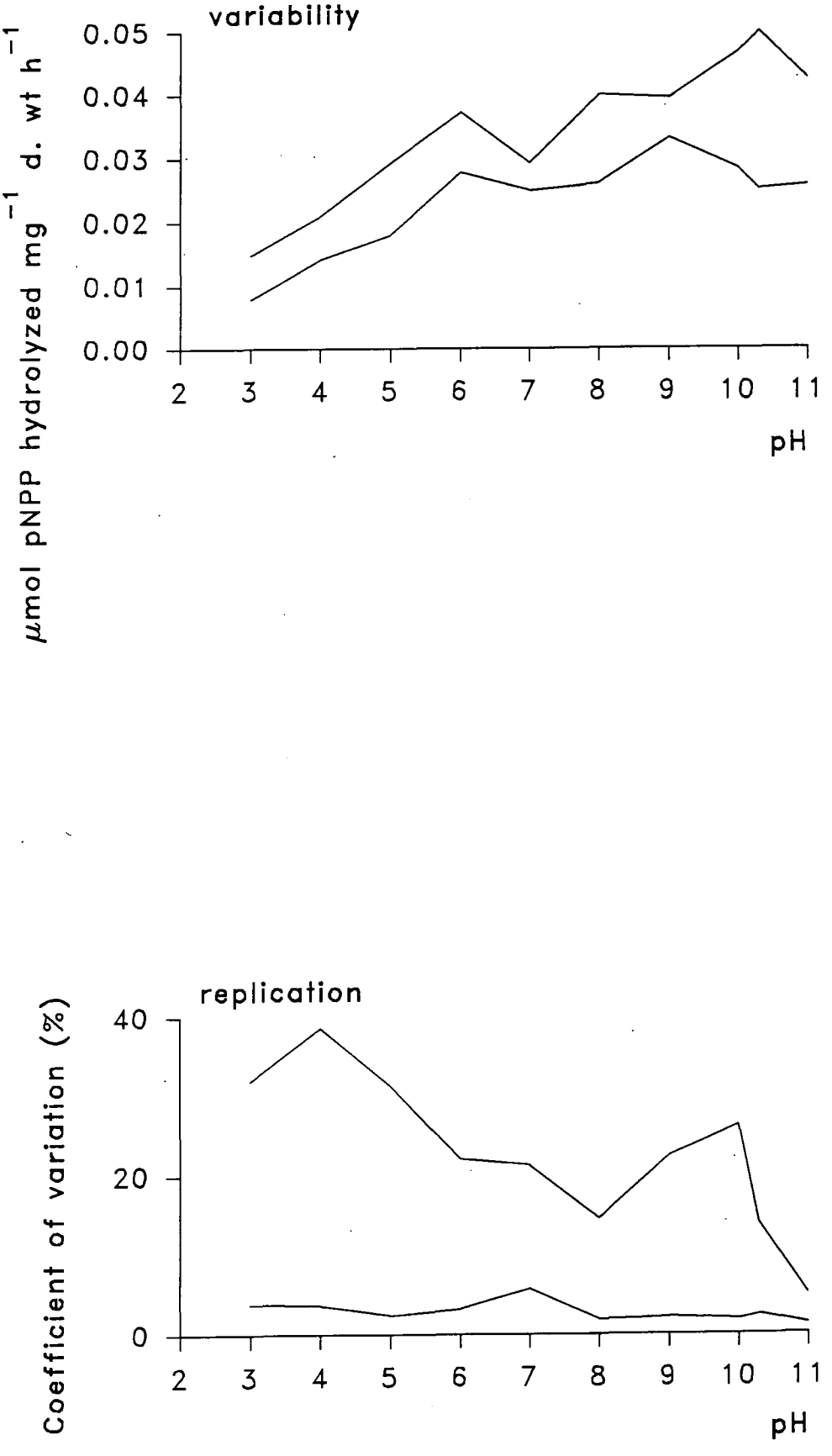


Fig. 5.4a Interplantvariability and replication of PMEase activity in *Peltigera praetextata* from Nenthead, using 250 µM pNPP

Table 5.4 Phosphatase activities at the soil pH value of all *Peltigera* plants as a percentage of the maximum PMEase and PDEase activities.

Site	Species	Plant	Soil pH	% of maximum PMEase	PDEase
Nenthead	*P. canina*	1	7.58	35.2	48.7
		2	7.60	80.6	
		3	7.51	48.7	
		4	7.59	45.5	
	P. praetextata	1	7.63	80.3	
		2	7.22	63.2	74.0
		3	7.93	64.4	
		4	7.67	68.2	
Windy Nook	*P. canina*	1	7.88	58.3	
		2	7.85	79.7	
		3	7.58	60.7	
		4	7.22	44.4	62.4
	P. praetextata	1	7.31	23.3	
		2	7.36	61.9	
		3	7.83	68.7	71.2
		4	7.76	77.0	

5.23 Statistical analyses

Statistical analyses were undertaken to examine the interplant relationship between peak PMEase activities in *P. canina* and *P. praetextata* from Windy Nook and Nenthead. Analysis of variance was used, as outlined in Section 4.5. Results, summarised in Table 5.5, were tested for significance at the 1 % probability level. A + denotes that the null hypothesis was accepted, and therefore interplant differences in PMEase activities were not significant. Also tested were the correlation between mean interplant P concentrations against peak PMEase activity and mean interplant Zn concentrations against peak PMEase activity. These results are outlined in Table 5.5.

Table 5.5 Statistical analyses of maximum PMEase activities and P & Zn concentrations in *P. canina* and *P. praetextata*, where n = 16 and P = 0.01.

Site	Species	PMEase F ratio		P v PMEase *r*	Zn v PMEase *r*
Windy Nook	*P. canina*	24.81		-0.13	+0.28
	P. praetextata	2.11	+	+0.19	-0.41
Nenthead	*P. canina*	31.14		-0.36	-0.60
	P. praetextata	11.48		+0.68	-0.19

5.3 Phosphatase activity of *Cladonia arbuscula*

5.31 Influence of pH on *C. arbuscula*

A series of experiments identical in design to those outlined in Section 5.21 were carried out on four plants of *Cladonia arbuscula* from a low (Middleton Common) and a high (Nenthead) zinc environment. The aim was to assess the influence of pH on the PMEase activity of this species.

Fig. 5.5 illustrates the influence of pH on the PMEase activity of four plants of *C. arbuscula* from Middleton Common. There is a trend of peak PMEase activity at pH 6.0 for all four plants, but there is poor intraplant replication at pH 6.0 with all plants. Plants 1 & 2 display a second peak of activity at pH 10.0 & 10.3 respectively. This bimodal pattern is repeated with plants 3 & 4 with a second peak of activity at pH 10.3 & 10.0 respectively. The peak at pH 10.3 for plant 3 can be described as a definite peak although it is greatly suppressed in comparison with plants 1 & 2. The peak at pH 10.0 for plant 4 is however a trend and is also greatly suppressed.

Fig. 5.5a illustrates the interplant variability and replication of PMEase activity of *C. arbuscula* from Middleton Common. Interplant variability, seen in graph a, is quite low in the acid / circumneutral pH range 3.0 - 8.0 but is considerably higher in the alkaline pH range 9.0 - 11.0. Interplant replication, indicated in graph b, is poor at all pH values except pH 9.0 & 10.0.

Fig. 5.6 illustrates the PMEase activity of *C. arbuscula* from Nenthead. The obvious similarity between *C. arbuscula* from Nenthead and Middleton Common is that the primary peak of PMEase activity is in the acid / circumneutral pH range of 6.0, for plants 2 & 4, and pH 7.0, for plants 1 & 3. These peaks for plants 1, 2 & 4 are definite while the

peak in plant 3 suggests a trend of peak PMEase activity. The pronounced peaks of PMEase activity in the alkaline pH range demonstrated with plant 1 & 2 of *C. arbuscula* from Middleton Common are not evident in material from Nenthead. There are largely suppressed peaks of PMEase activity at pH 10.3 for all plants with only plant 4 having a definite peak. Plants 1, 2 & 3 display trends of secondary peak PMEase activity at pH 10.3. The mean rate of peak PMEase activity of the four plants of *C. arbuscula* for Nenthead is 0.01812 μmol pNPP mg d. wt^{-1} h^{-1} and for Middleton Common is 0.02199 μ mol pNPP mg d. wt^{-1} h^{-1}, a ratio of 0.82. The decrease in peak PMEase activity observed between *C. arbuscula* from the high and low zinc environment is the reverse of the situation observed with both *P. canina* and *P. praetextata*.

Intraplant replication is relatively good across the pH range 3.0 - 6.0 with the exception of plant 3 at pH 3.0. However, in the alkaline pH range only at pH 10.0 & 11.0 does intraplant replication suggest good reproducibility. At all other alkaline pH values replication is erratic and generally poor.

Fig. 5.6a illustrates interplant variability and replication for *C. arbuscula* from Nenthead. Graph a shows that in the extreme acid and alkaline pH ranges interplant variability is very low, but in the pH range 6.0 - 9.0 (except pH 8.0) variability is high. Graph b shows that interplant replication is, to a large degree, inversely related to variability. At the extreme acid and alkaline pH values replication is poor. Replication is similarly poor at pH 8.0, again inversely related to variability at pH 8.0.

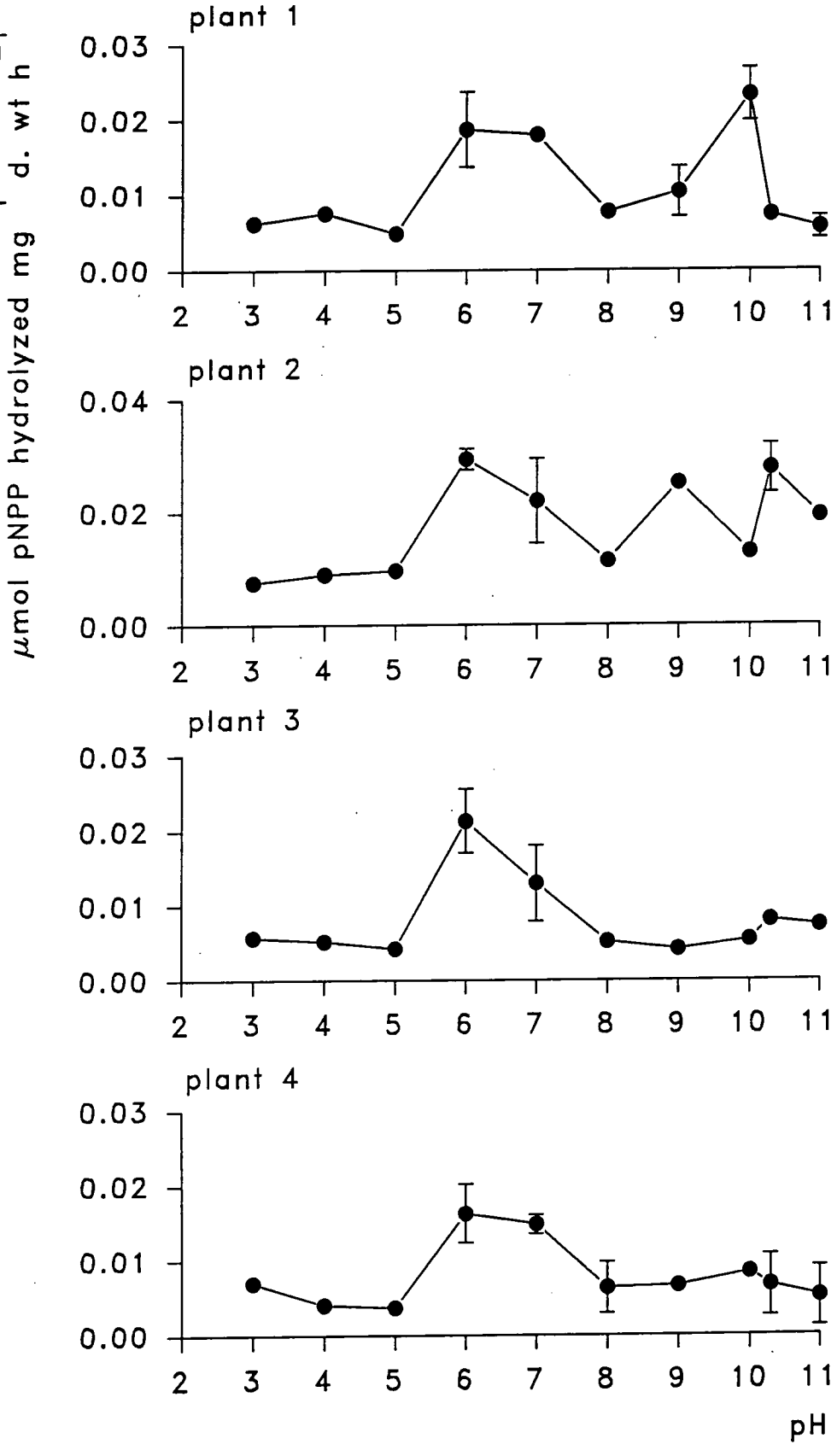


Fig. 5.5 Influence of pH on the PMEase activity of *Cladonia arbuscula* from Middleton Common, using 250 μM pNPP

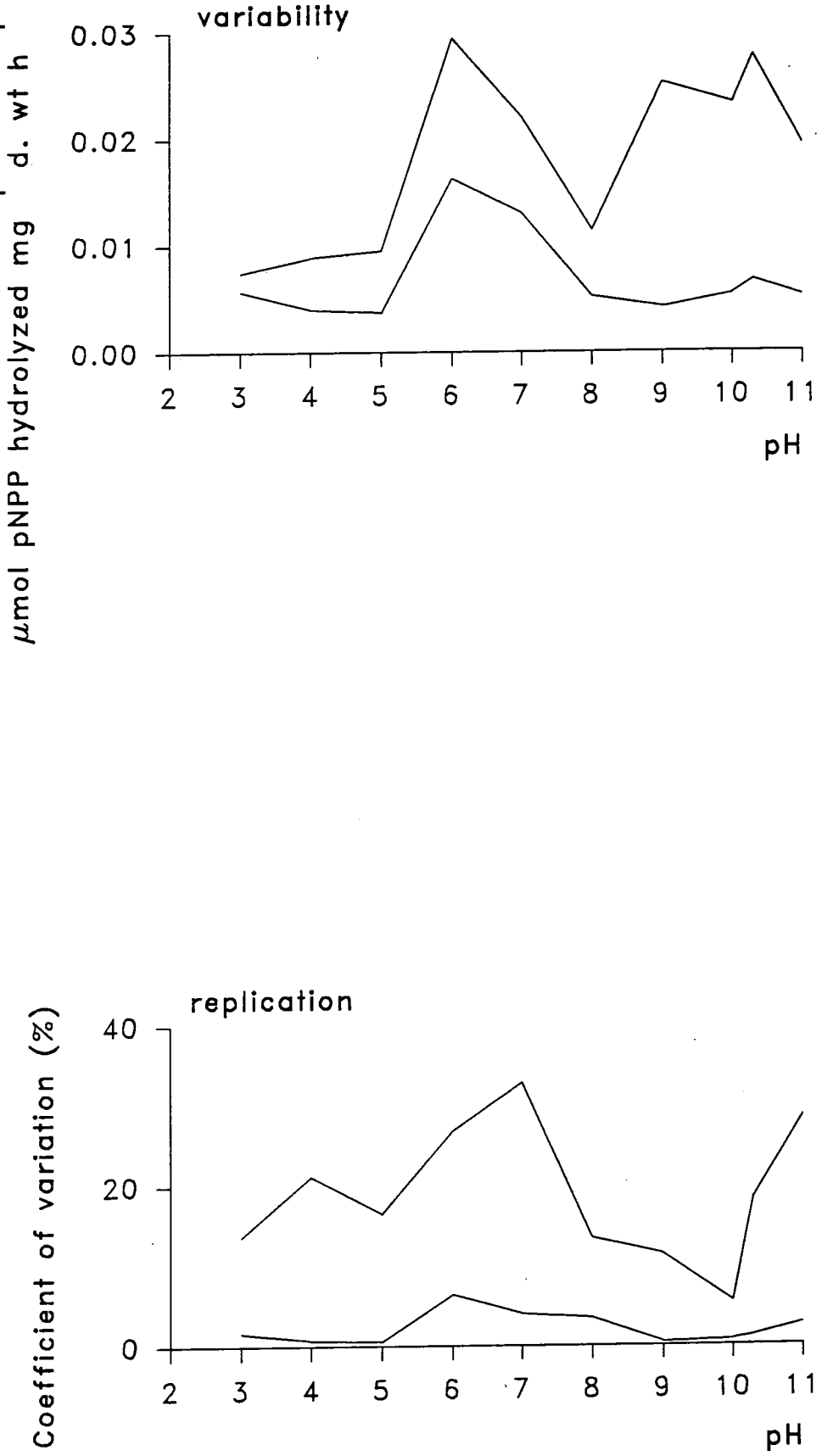


Fig. 5.5a Interplant variability and replication of PMEase activity in *Cladonia arbuscula* from Middleton Common,using 250 μM pNPP

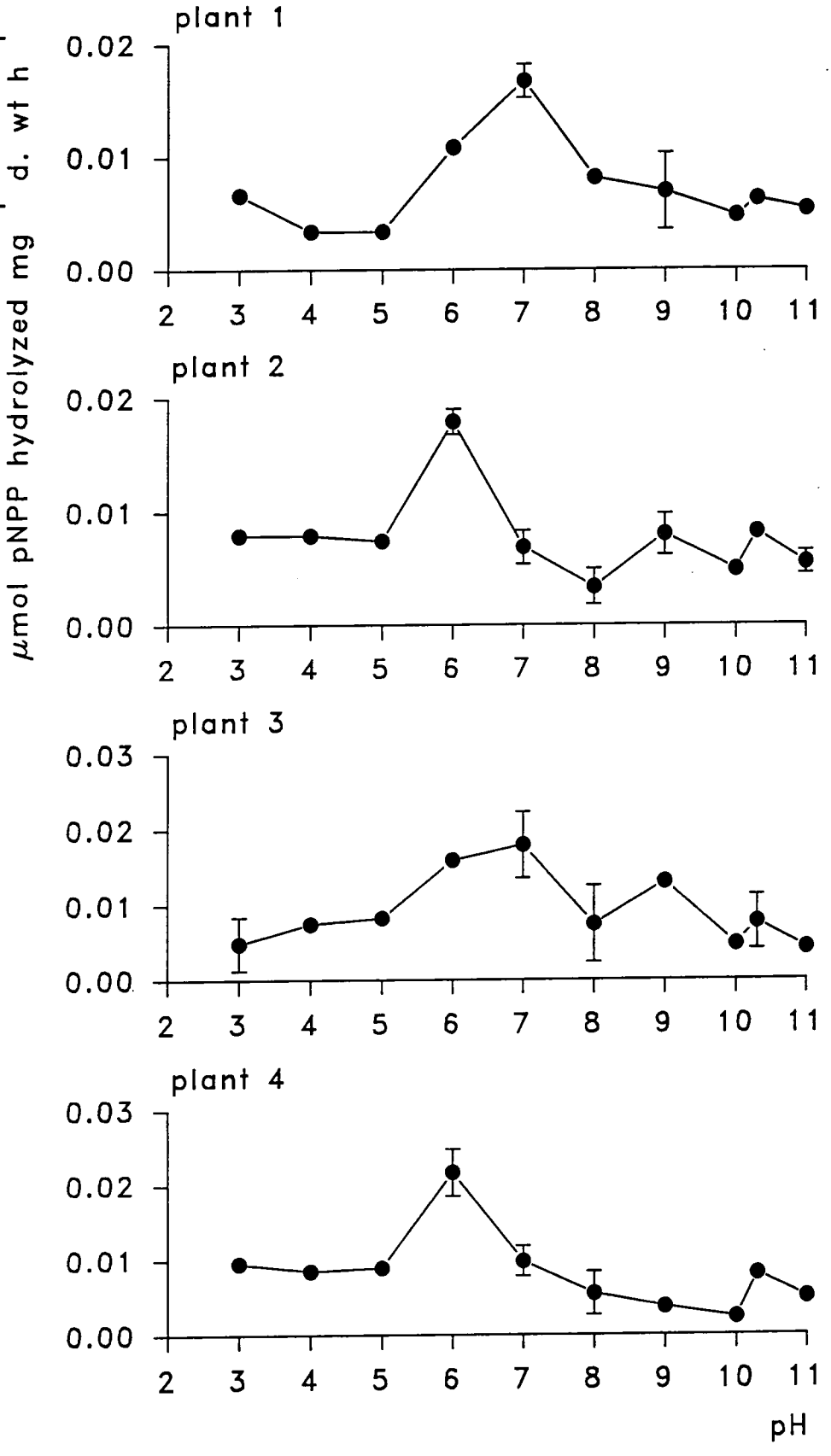


Fig. 5.6 Influence of pH on the PMEase activity of *Cladonia arbuscula* from Nenthead, using 250 µM pNPP

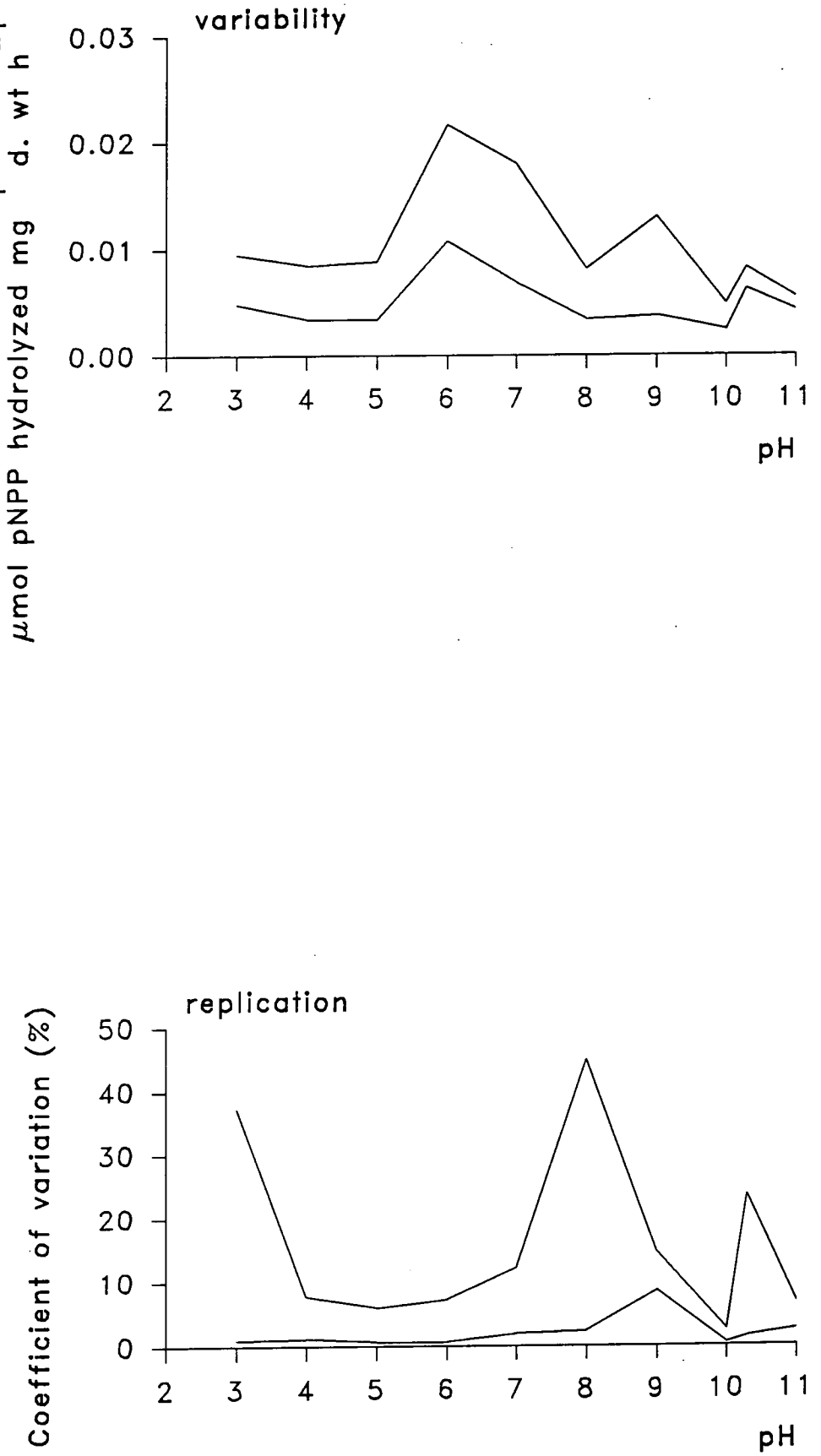


Fig. 5.6a Interplant variability and replication of PMEase activity in *Cladonia arbuscula* from Nenthead, using 250 µM pNPP

Table 5.6 Phosphatase activities at the soil pH value of all *C. arbuscula* plants as a percentage of the maximum PMEase and PDEase activities.

Site	Plant	Soil pH	% of maximum PMEase	PDEase
Nenthead	1	6.32	76.1	
	2	6.80	50.7	18.6
	3	6.91	99.0	
	4	6.73	60.0	
Middleton Common	1	7.24	66.5	
	2	7.65	51.3	
	3	7.42	45.6	63.4
	4	7.59	60.4	

5.32 Statistical analyses

Statistical analyses, identical to those outlined in Section 5.23, were undertaken on *Cladonia arbuscula* from Middleton Common and Nenthead. The results of analysis of variance and correlation are summarised in Table 5.7.

Table 5.7 Statistical analyses of maximum PMEase activities and P & Zn concentrations in *C. arbuscula*, where n = 16 and P = 0.01.

Site	PMEase F ratio	P v PMEase *r*	Zn v PMEase *r*
Middleton Common	26.41	-0.57	+0.51
Nenthead	11.76	-0.79	+0.62

5.4 Phosphatase activity of selected *Peltigera* and *Cladonia* plants

5.41 Influence of pH and substrate

A series of experiments were undertaken on selected *Peltigera* and *Cladonia* plants from Windy Nook, Nenthead and Middleton Common to assess the influence of pH on PMEase activity using 250 μM 4-MUP as the artificial phosphate substrate. Assay conditions were the same as those outlined in Section 5.21. Because of the poor replication and high variability of all *Peltigera* and *Cladonia* plants, discussed in the previous sections, the plants examined here were chosen randomly. In addition to the influence of pH the aim of these assays was to assess the differences in PMEase activity using 4-MUP, a substrate known to hydrolyze phosphatase enzymes less freely than pNPP.

Figures 5.7 and 5.8 illustrate the results obtained from these assays. A summary of the results of the influence of pH and the two substrates is outlined in Table 5.8.

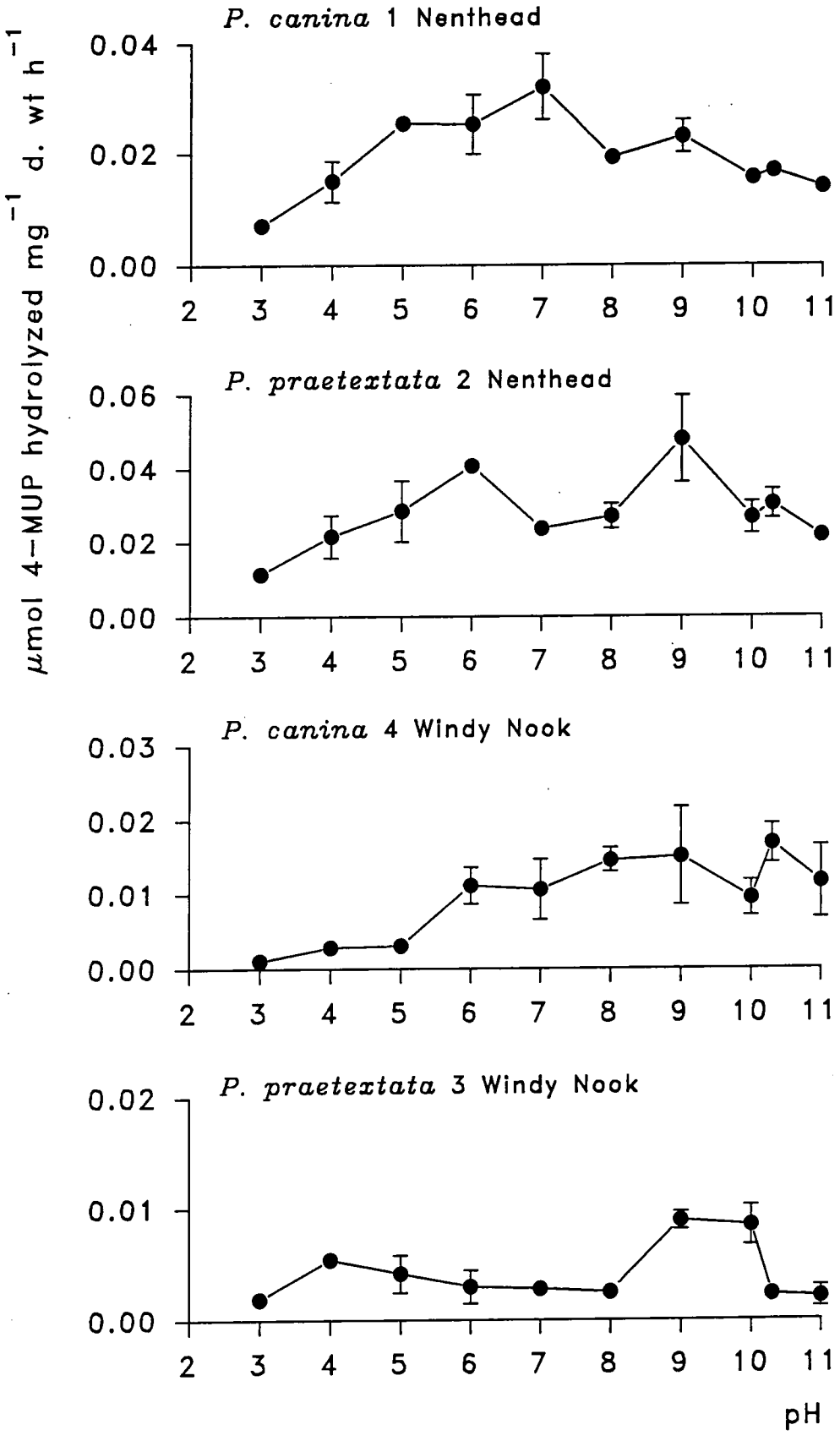


Fig. 5.7 Influence of pH on the PMEase activity of selected *Peltigera* plants using 250 µM 4–MUP

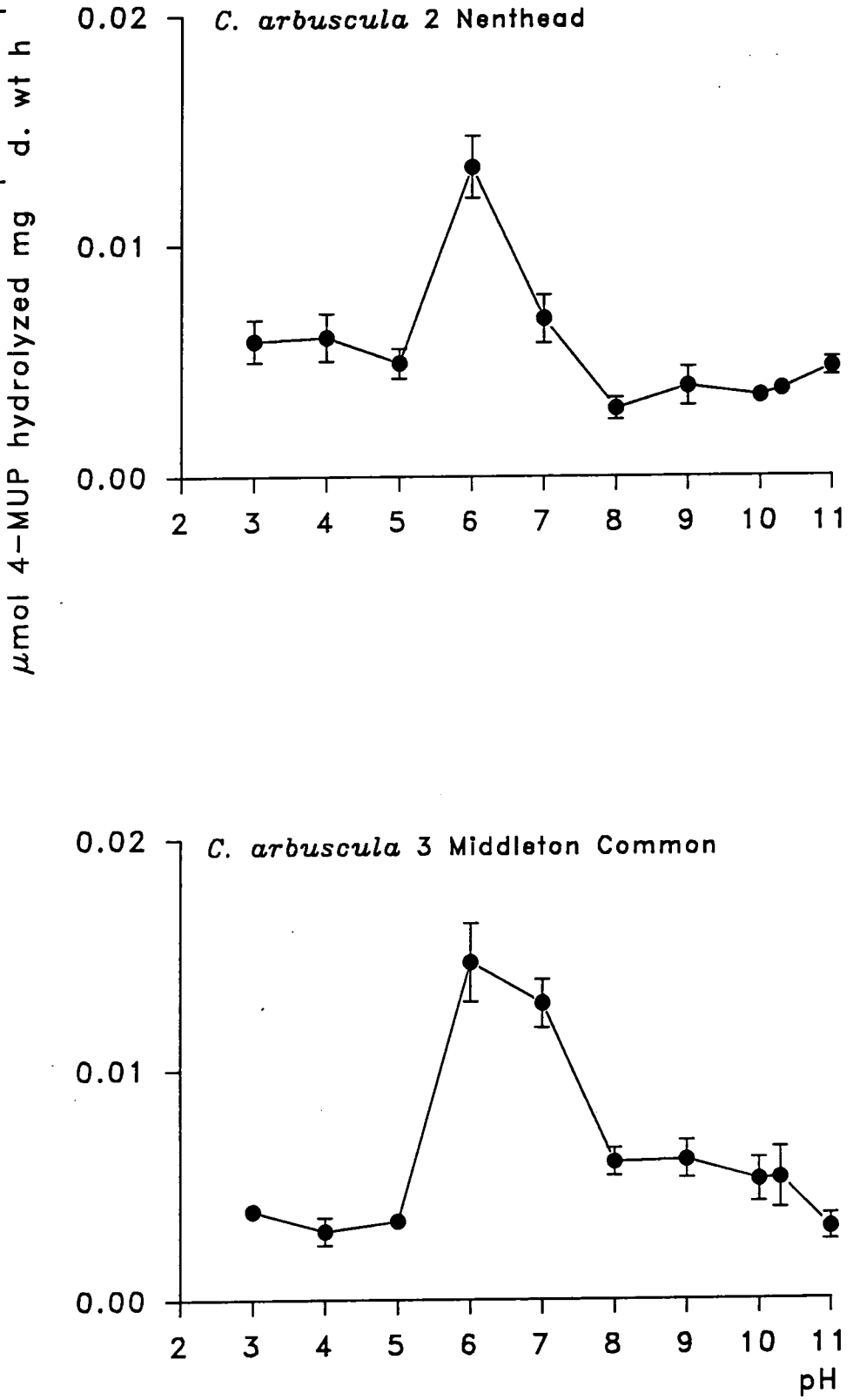


Fig. 5.8 Influence of pH on the PMEase activity of selected *Cladonia* plants using 250 µM 4–MUP

Table 5.8 Comparison between 4-MUP and pNPP in response of *P. canina, P. praetextata* and *C. arbuscula* to PMEase activities.

Species	Plant	Site	Peak pNPP pH	Peak pNPP Rate	Peak 4-MUP pH	Peak 4-MUP Rate	Ratio pNPP:4-MUP
P. canina	1	Nenthead	6.0	0.049	7.0	0.032	1.54
P. praetextata	2	" "	6.0	0.039	6.0	0.041	0.96
			10.3	0.050	9.0	0.048	1.04
P. canina	4	Windy Nook	10.3	0.029	10.3	0.017	1.75
P. praetextata	3	" "	10.3	0.030	9.0	0.009	3.39
C. arbuscula	2	Nenthead	6.0	0.018	6.0	0.013	1.34
C. arbuscula	3	Middleton Common	6.0	0.021	6.0	0.015	1.46

It can be seen from Table 5.8 that PMEase activity is, with the exception of *P. praetextata* 2 Nenthead at pH 6.0, lower with 4-MUP than with pNPP but there is no consistent pattern in the differences of the rates of activity. An important difference with *Peltigera* spp. between the two substrates is the shift in pH of the peak rate of activity. Apart from *P. canina* (Windy Nook) there is a shift in pH for the remaining plants; from 6.0 to 7.0 for *P. canina* (Nenthead) and 10.3 to 9.0 for *P. praetextata* (Nenthead & Windy Nook). *P. canina* from Windy Nook (pH 10.3) and *P. praetextata* from Nenthead (pH 6.0) both have maximum PMEase activity at the same pH values with 4-MUP and pNPP. Peak PMEase activity is at pH 6.0 with 4-MUP and pNPP in *C. arbuscula* from Nenthead and Middleton Common.

Intraplant variability is again inconsistent throughout the whole pH range for all plants. Replication is particularly poor at the pH values of optimum PMEase activity.

5.42 Influence of pH, substrate and substrate concentration

Following the experiments undertaken on *P. canina*, *P. praetextata* and *C. arbuscula* one plant each of *P. canina* and *C. arbuscula* from Nenthead were selected for further studies. Initial assays were carried out to assess the influence of

i pH

ii 1 μM 4-MUP, 250 μM 4-MUP and 250 μM pNPP

Assays were carried out at 25°C with an incubation period of 1 hour. Fig. 5.9 illustrates the PMEase activity of *P. canina* 1 and *C. arbuscula* 2 using 250 μM pNPP and 250 μM 4-MUP. *P. canina* has acid (pH 6.0) and alkaline (pH 10.3) peaks of activity with both substrates. The acid peak is equally prominent with both substrates whereas the alkaline peak is largely suppressed with 4-MUP. At all pH values except 11.0 PMEase activity with pNPP is higher than with 4-MUP. The pNPP : 4-MUP ratio at the optimum pH for PMEase activities is 1.28 at pH 6.0 and 1.82 at pH 10.3.

C. arbuscula has a distinctive acid peak of PMEase activity at pH 6.0 with both substrates. PMEase activity is higher at all pH values with pNPP than with 4-MUP. The pNPP : 4-MUP ratio at pH 6.0 is 1.27.

Fig. 5.10 illustrates the PMEase activity of *P. canina* and *C. arbuscula* using 1 μM 4-MUP. As with 250 μM pNPP and 250 μM 4-MUP the peak of activity for *P. canina* is at pH 6.0, but there is no alkaline peak of activity. The 1 μM : 250 μM 4-MUP ratio at pH 6.0 is 46.02.

C. arbuscula has a trend of peak activity at pH 6.0, the same pH value for optimum activity with 250 μM 4-MUP and 250 μM pNPP. The 1 μM : 250 μM 4-MUP ratio at pH 6.0 is 23.55.

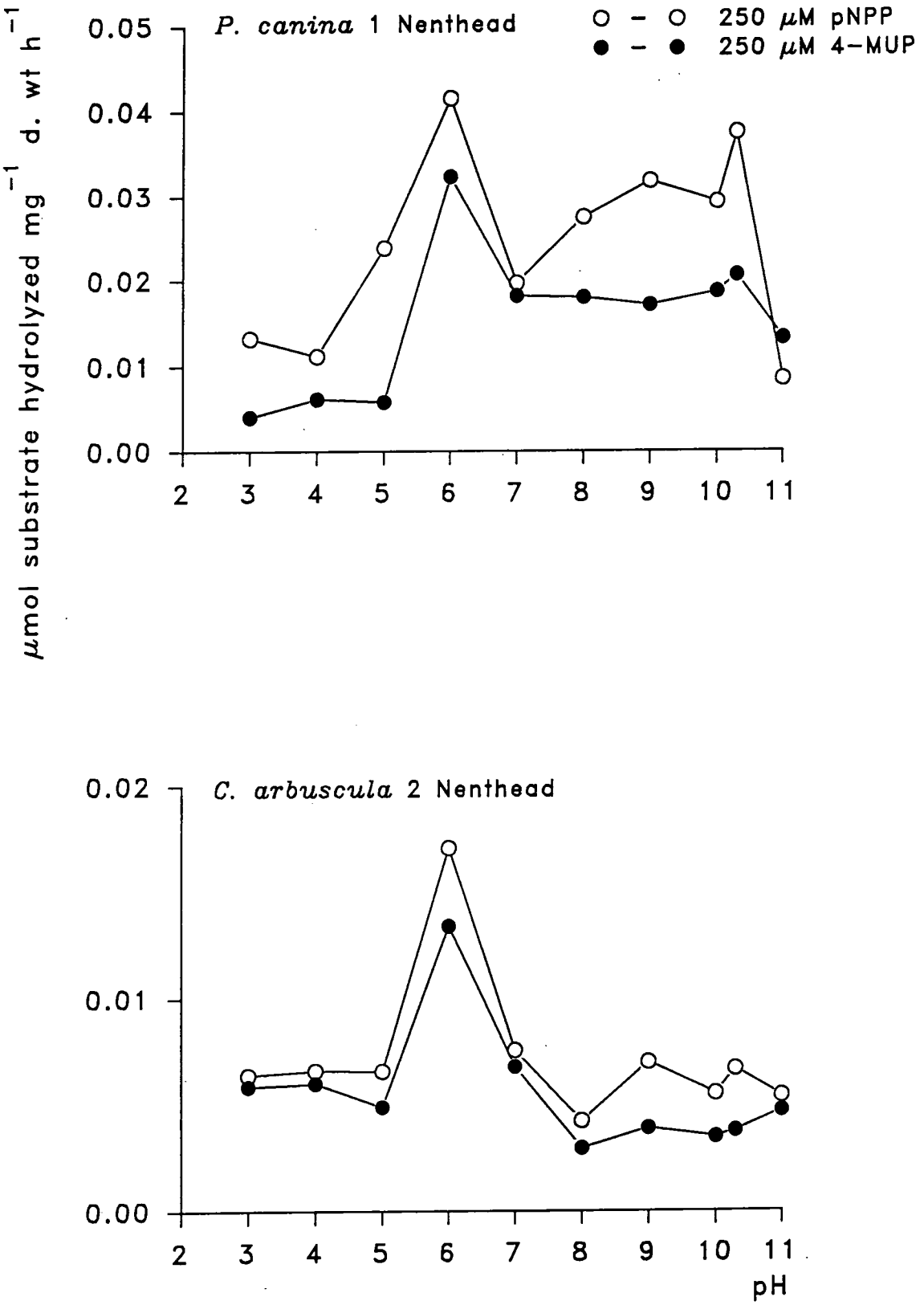


Fig. 5.9 Influence of substrate and pH on the PMEase activity of *Peltigera canina* and *Cladonia arbuscula*

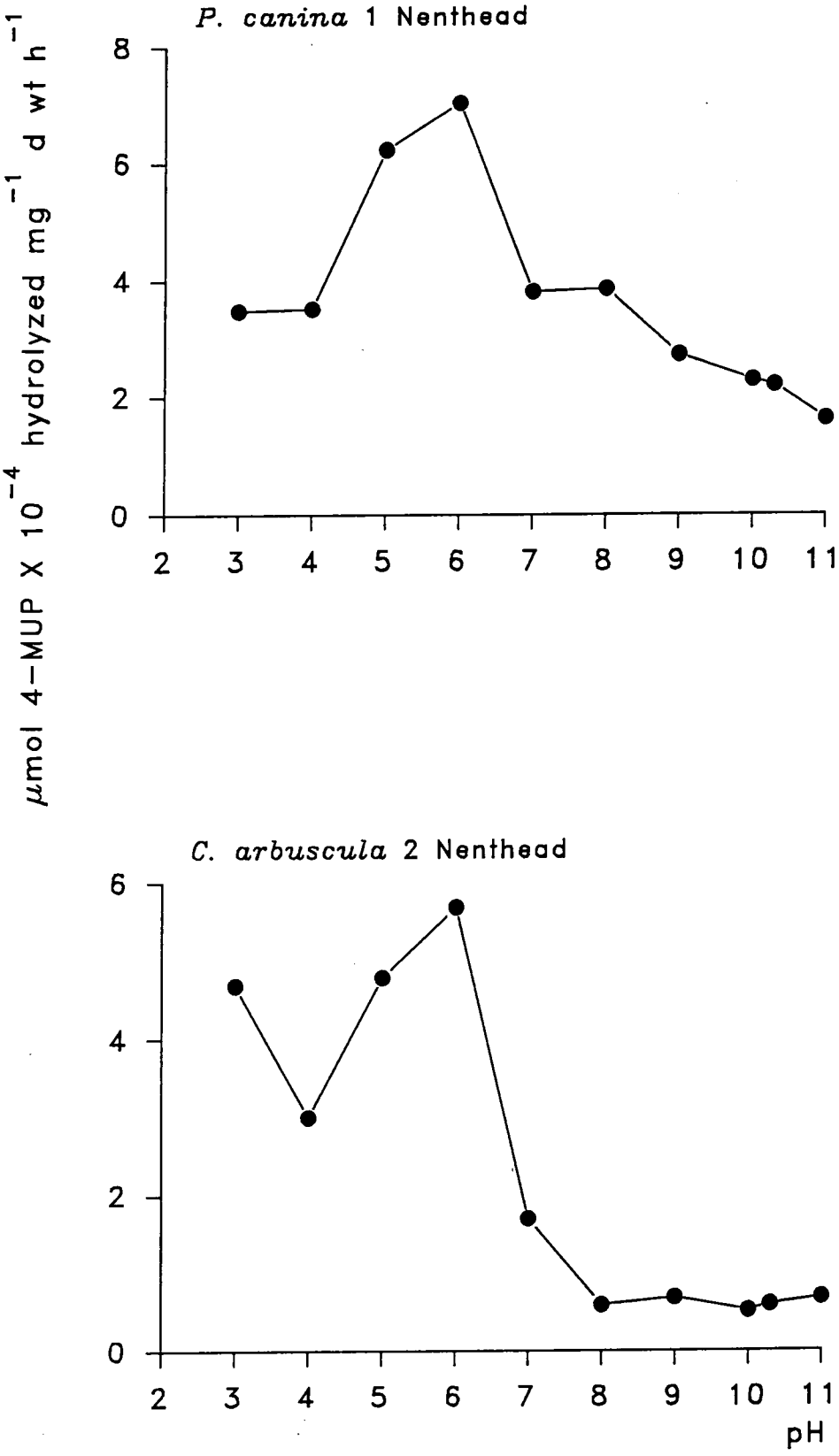


Fig. 5.10 Influence of pH on the PMEase activity of *Peltigera canina* and *Cladonia arbuscula*, using 1 µM 4−MUP

5.43 Influence of pH on phosphodiesterase activity

Assays were carried out on *P. canina, P. praetextata* and *C. arbuscula* to assess the influence of pH on the PDEase activity, using 250 μM bis-pNPP. Assays were undertaken at 25°C and incubated for 1 h. Fig. 5.11 illustrates the influence of pH on the PDEase activity of *P. canina* and *P. praetextata* from Nenthead and Windy Nook.

P. canina from Nenthead displays alkaline peak PDEase activity at pH 9.0, with a second suppressed peak at pH 7.0, while the same species from Windy Nook displays a trend of peak PDEase activity at pH 10.3. Intraplant replication is good at all pH values except pH 9.0 for *P. canina* (Nenthead) and poor at all pH values for *P. canina* (Windy Nook). *P. praetextata* from Nenthead displays a trend of peak PDEase activity at pH 9.0, while the same species from Windy Nook displays acid peak PDEase activity at pH 4.0. Intraplant replication is relatively good for *P. praetextata* (Nenthead) and erratic for *P. praetextata* (Windy Nook).

Figure 5.12 illustrates the influence of pH on the PDEase activity of *C. arbuscula* from Nenthead and Middleton Common. *C. arbuscula* (Nenthead) displays alkaline peak PDEase activity at pH 10.3, with a second suppressed peak at pH 8.0, and a trend of peak PDEase activity in Middleton Common material at pH 10.3. Intraplant replication is particularly good at all pH values for Nenthead material but poor for Middleton Common material. Table 5.9 summarises the relationship between peak PMEase and PDEase activities in *P. canina, P. praetextata* and *C. arbuscula*.

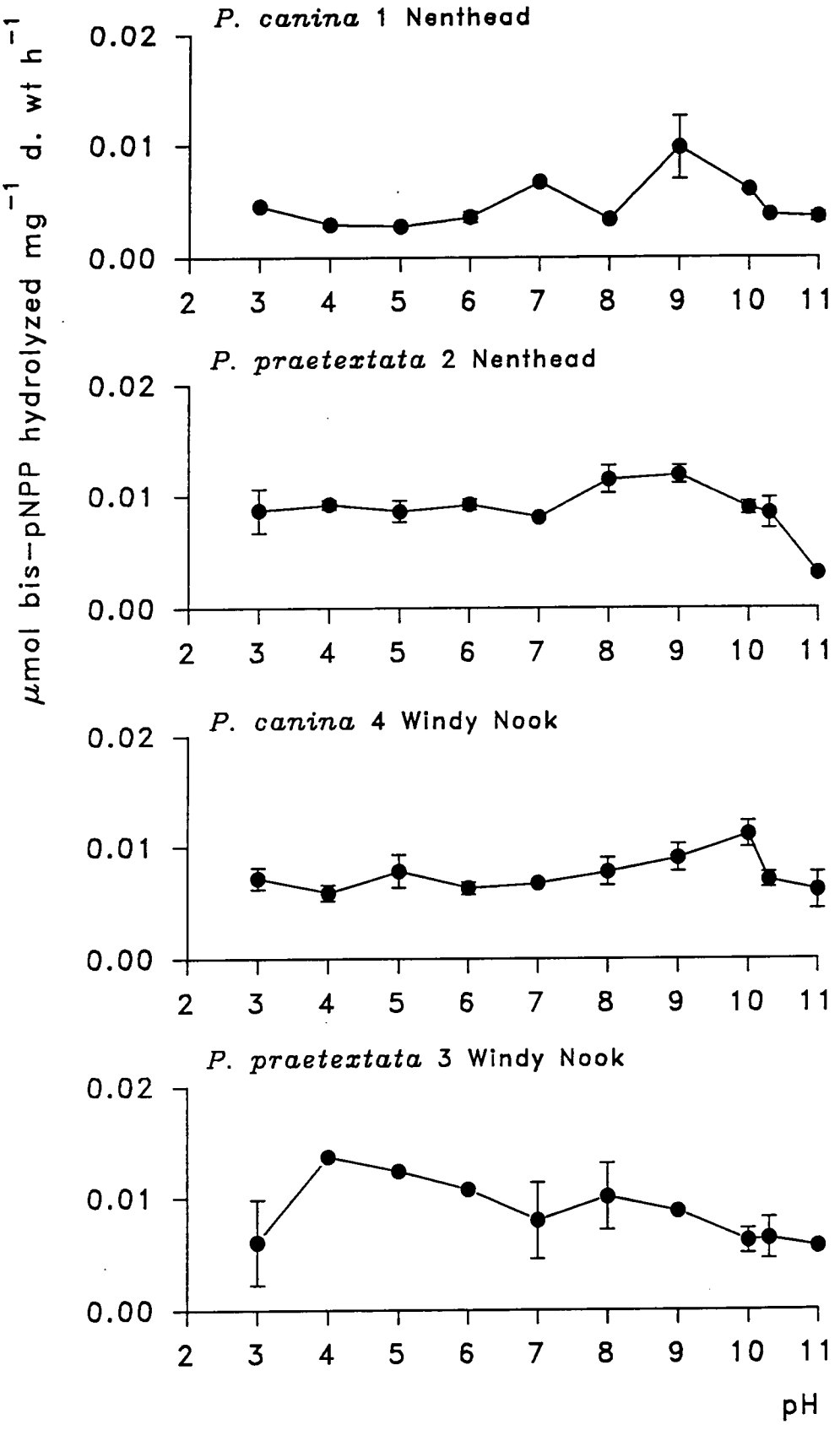


Fig. 5.11 Influence of pH on the PDEase activity of selected *Peltigera* plants using 250 µM bis-pNPP

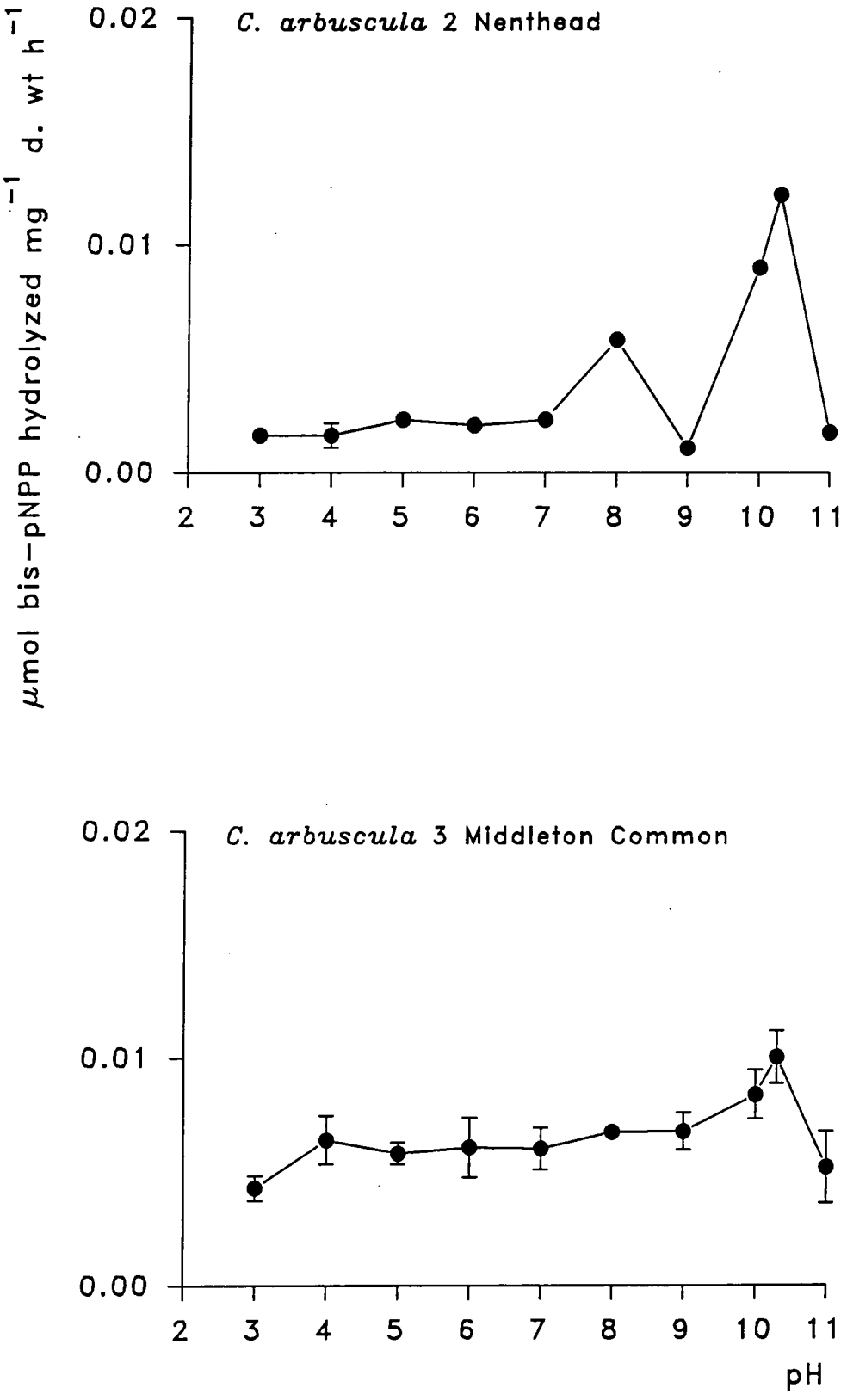


Fig. 5.12 Influence of pH on the PDEase activity of selected *Cladonia* plants, using 250 µM bis-pNPP

Table 5.9 Maximum PMEase and PDEase activities in *P. canina*, *P. praetextata* and *C. arbuscula.*

Site	Species & Plant	Peak PMEase	Peak PDEase	Ratio PMEase:PDEase
Nenthead	*P. canina* 1	0.0493	0.0097	5.06
	P. praetextata 2	0.0500	0.0119	4.20
	C. arbuscula 2	0.0179	0.0122	1.47
Windy Nook	*P. canina* 4	0.0327	0.0111	2.94
	P. praetextata 3	0.0301	0.0137	2.20
Middleton Common	*C. arbuscula* 3	0.0213	0.0100	2.12

5.44 Influence of zinc concentration

Assays were carried out on *P. canina*, *P. praetextata* and *C. arbuscula* from Nenthead, Windy Nook and Middleton Common to assess the influence of zinc concentrations on PMEase activity. Each plant was assayed at pH 6.0 only, using 250 μM pNPP. Zinc was included, as $ZnSO_4$, in the assay medium in the following concentrations; 0.001, 0.01, 0.1, 1.0 & 10 mM. The assays were undertaken at 25°C, and the incubation period 1 h.

Fig. 5.13 illustrates the influence of zinc concentrations on the PMEase activity of *P. canina* and *P. praetextata* from both sites. *P. canina* and *P. praetextata* from Windy Nook have a trend of peak PMEase activity at pH 6.0 with 0.001 mM zinc. *P. canina* and *P. praetextata* from Nenthead have a trend of peak PMEase activity at pH 6.0 with 0.1 mM

zinc. With higher zinc concentrations PMEase activity is reduced linearly. PMEase activity with 10 mM zinc is 66 %, 70 %, 14 % and 10 % of peak PMEase activity for *P. canina* Nenthead, *P. praetextata* Nenthead, *P. canina* Windy Nook and *P. praetextata* Windy Nook respectively.

Figure 5.14 illustrates the influence of zinc concentrations on the PMEase activity of *C. arbuscula* from both sites. *C. arbuscula* from Nenthead has a trend of peak PMEase activity at pH 6.0 with 0.001 mM zinc, while the same species from Middleton Common has a definite peak of PMEase activity with 0.001 mM zinc.

The apparent level of tolerance to zinc in *C. arbuscula* is less than that in *P. canina* and *P. praetextata.* PMEase activity with 10 mM zinc is 63 % and 2 % of peak PMEase activity in *C. arbuscula* Nenthead and *C. arbuscula* Middleton Common respectively.

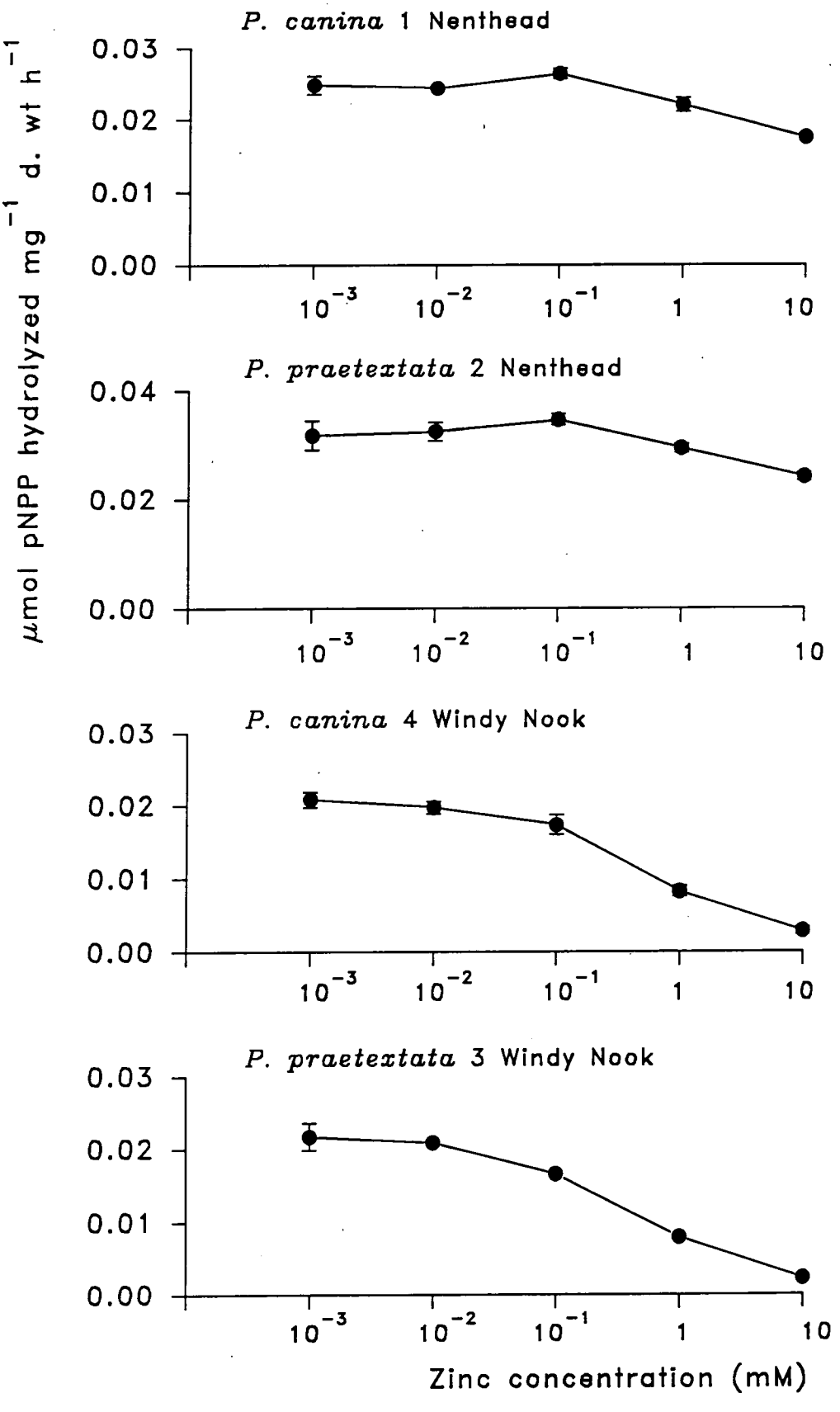


Fig. 5.13 Influence of zinc concentration on the PMEase activity of selected *Peltigera* plants using 250 µM pNPP at pH 6.0

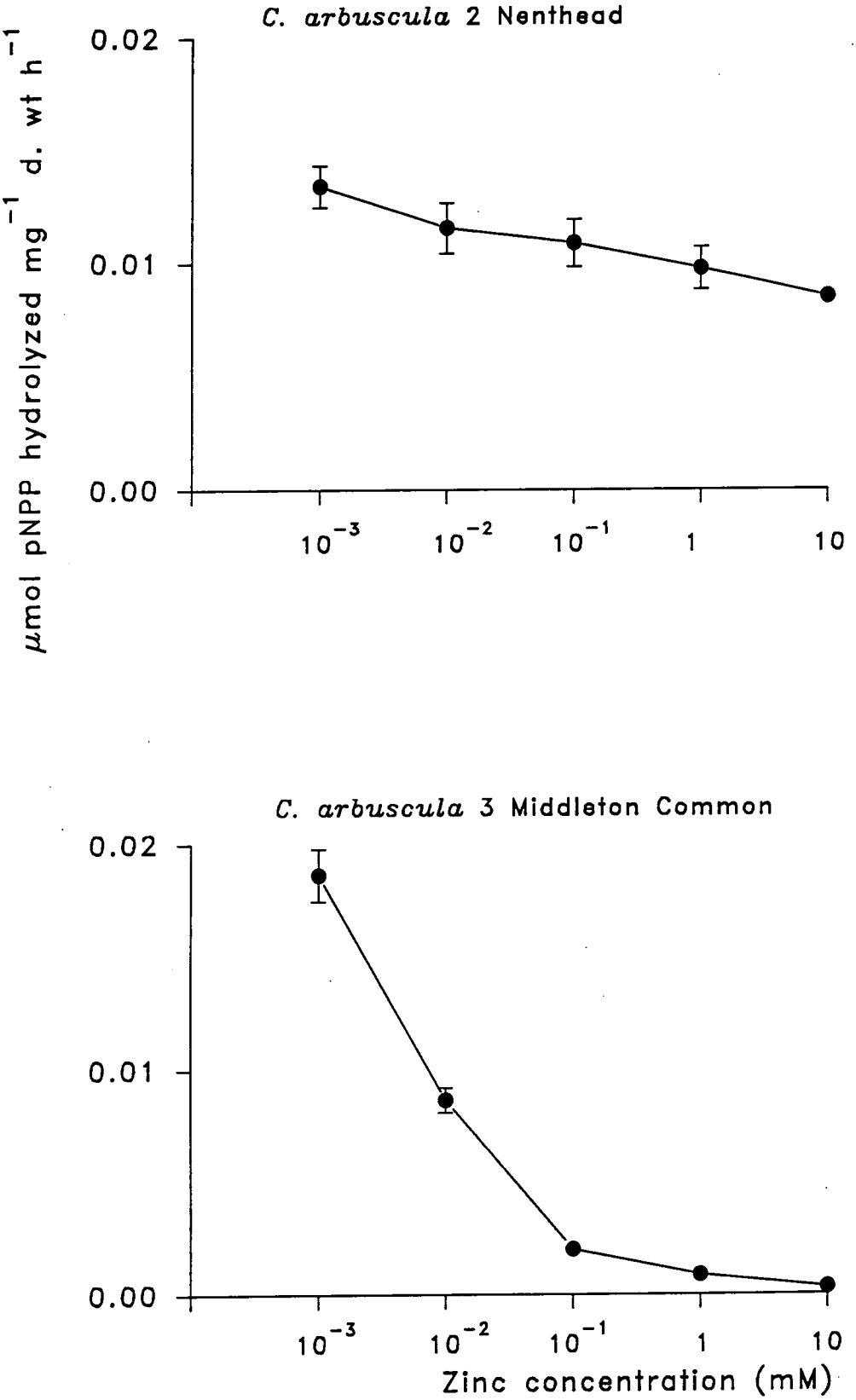


Fig. 5.14 Influence of zinc concentration on the PMEase activity of selected *Cladonia* plants, using 250 µM pNPP at pH 6.0

5.5 Additional analyses of phosphatase activity in *P. canina*

5.51 Influence of the symbionts on phosphomonoesterase activity

A series of experiments were carried out simultaneously on *Peltigera canina* plant 1 (Nenthead) to assess the degree to which each lichen symbiont was responsible for PMEase activity. These experiments may possibly be considered the most important undertaken throughout this study.

Discs were extracted from *P. canina* following the methods outlined in Section 2.3. Each disc was then separated into two horizontal sections;

i upper layer comprising the upper cortex, algal layer and medulla

ii lower layer comprising the rhizines, veins and part of the medulla

Material was assayed with 250 μM pNPP and 250 μM 4-MUP at pH 6.0 & 10.3, and with 1 μM 4-MUP in the pH range 4.5 - 10.0 (at 0.5 pH intervals) with an additional pH value of 10.3.

Fig. 5.15 illustrates the PMEase activity of the symbionts of *P. canina*. The lower layer clearly displays higher PMEase activity at pH 6.0 & 10.3 with 250 μM pNPP and 250 μM 4-MUP. The phenomenon described for Fig. 5.7 whereby *P. canina* had reduced PMEase activity at pH 6.0 & 10.3 with 250 μM 4-MUP in comparison with 250 μ M pNPP is also evident here. With 1 μM 4-MUP the lower layer is largely responsible for PMEase activity with a prominent peak at pH 9.0 and possibly a second peak at pH 7.0. The upper layer shows no marked activity with no discernible peaks of PMEase activity. Table 5.10 shows the values and ratios of optimum PMEase activity between the upper and lower layers as illustrated in Fig. 5.15.

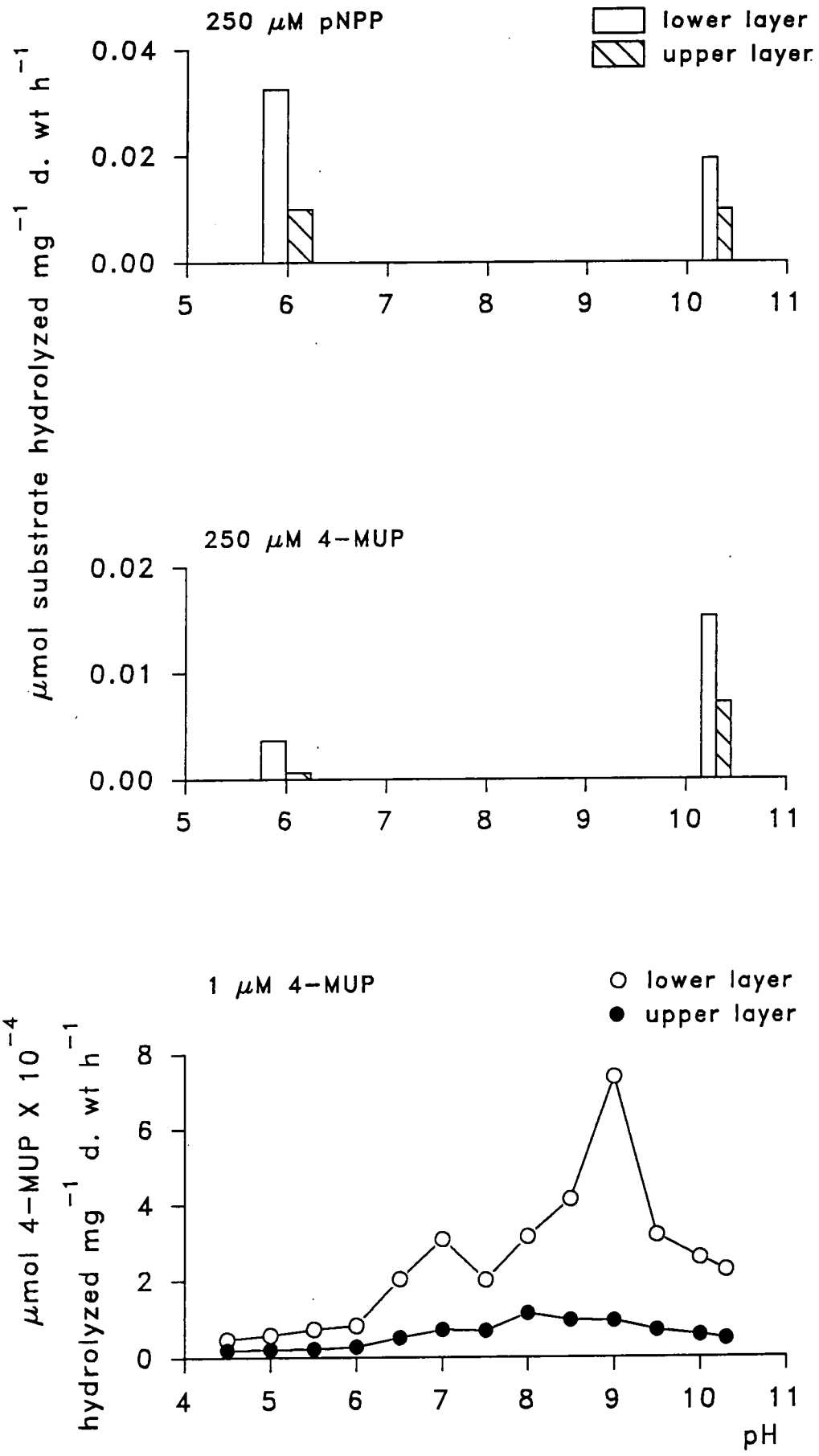


Fig. 5.15 Influence of pH and substrate on the PMEase activity of the symbionts of *Peltigera canina* 1 Nenthead

Table 5.10 Maximum PMEase activity of the separated symbionts of *P. canina* with 1 μM & 250 μM 4-MUP and 250 μM pNPP.

Component	250 μM pNPP		250 μM 4-MUP		1 μM 4-MUP	
	6.0	10.3	6.0	10.3	7.0	9.0
Lower layer (1)	0.032	0.017	0.003	0.015	0.0003	0.0007
Upper layer (2)	0.010	0.010	0.0008	0.007	0.0001*	
1:2 ratio	3.2	1.7	3.8	2.1	3.0	7.0

* Optimum activity occurred at pH 8.0

Figure 5.15a illustrates the major role played by the lower layer in the PMEase activity of *P. canina*, using 250 μM pNPP and 250 μM 4-MUP. In this series of experiments the dry weights and PMEase activities of the lower and upper layers could be correlated with each other by matching the replicates and analysing the results as a percentage of the total for each disc. For each replicate the dry weight and PMEase activity of each symbiont is expressed as a percentage of the total dry weight and PMEase activity of the disc it represents. Fig. 5.15b illustrates the same analyses undertaken on the symbionts of *P. canina*, using 1 μM 4-MUP. Table 5.11 demonstrates the major role played by the lower layer in the PMEase activity of *P. canina*, where the values given are the mean of n = 4.

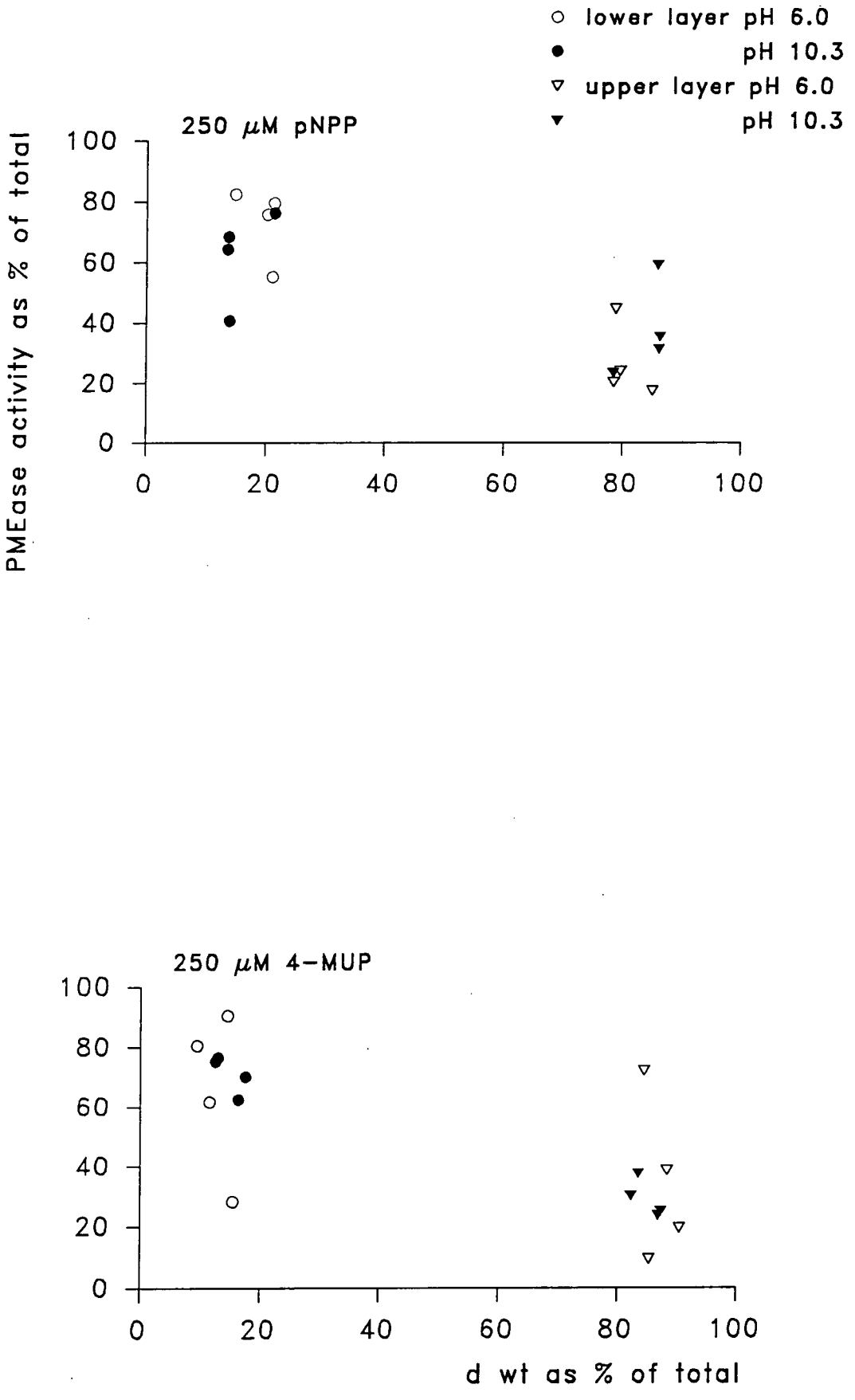


Fig. 5.15a Percentage of PMEase activity and dry weight of each symbiont as a total for each thalline disc of *Peltigera canina*

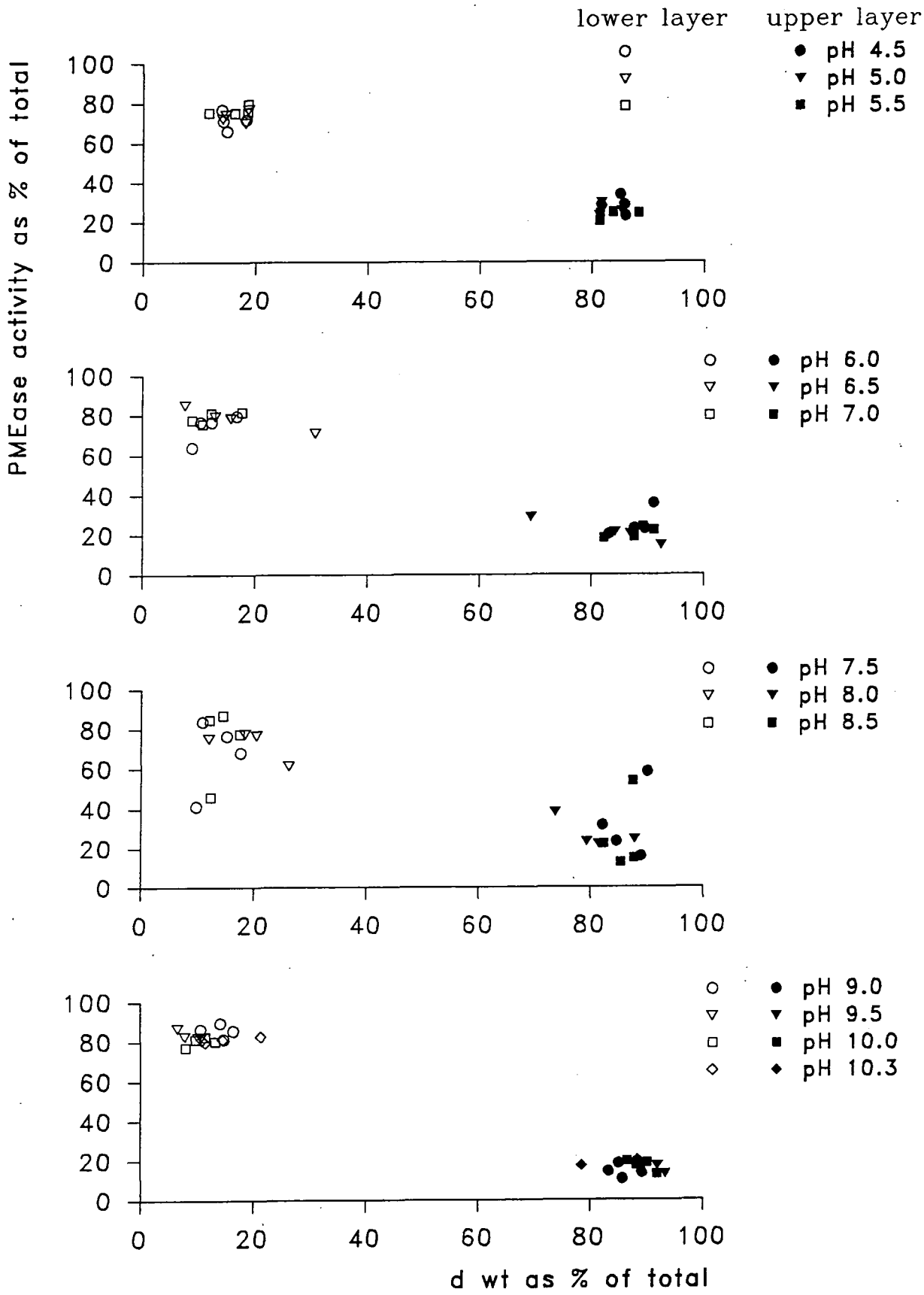


Fig. 5.15b Percentage of PMEase activity and dry weight of each symbiont as a total for each thalline disc of *Peltigera canina*, using 1 μM 4-MUP

Table 5.11 Biomass and PMEase activity of the symbionts in *P. canina*.

Substrate	pH	Lower layer % of total		Upper layer % of total	
		d. wt.	PMEase	d. wt.	PMEase
1 μM 4-MUP	4.5	15.35	71.41	84.65	28.59
	5.0	16.42	73.33	83.58	26.67
	5.5	16.27	76.33	83.73	23.67
	6.0	12.10	74.23	87.90	25.77
	6.5	16.75	78.58	83.25	21.42
	7.0	12.39	78.98	87.61	21.02
	7.5	13.44	67.46	86.56	32.54
	8.0	19.31	72.84	80.69	27.16
	8.5	14.16	73.89	85.84	26.11
	9.0	14.06	85.66	85.94	14.34
	9.5	8.94	83.50	91.06	16.50
	10.0	10.67	80.17	89.33	19.83
	10.3	14.59	81.08	85.41	18.92
250 μM 4-MUP	6.0	12.79	65.19	87.21	34.81
	10.3	14.93	71.07	85.07	28.93
250 μM pNPP	6.0	19.40	73.33	80.60	26.67
	10.3	15.70	62.55	84.30	37.45

5.52 Influence of time and substrate concentration

Peltigera canina plant 1 from Nenthead was selected for a series of assays designed to assess the influence of the incubation period on PMEase activity. Results from previous assays undertaken on this plant showed that peak PMEase activity occurred at pH 6.0. Therefore all 3 assays were carried out at this pH value only. A second aim was to assess the influence of substrate concentration. The experimental conditions were such that this plant was incubated at 10 min intervals from 0 - 60 mins inclusive, using 1 μM 4-MUP, 250 μM 4-MUP and 250 μM pNPP. 1 μM 4-MUP was included because this concentration of phosphorus closely matches those levels that lower plants will encounter in their natural environment. All plants were assayed at 25°C.

Fig. 5.16 illustrates the influence of time and substrate concentration on the PMEase activity of *P. canina* 1 (Nenthead). All three graphs display a strong linear relationship between incubation period and PMEase activity, with the exception of the 50 - 60 min interval for both 4-MUP concentrations. These results suggest that phosphatase assays on lichens may be carried out for 20 or 30 min incubation periods.

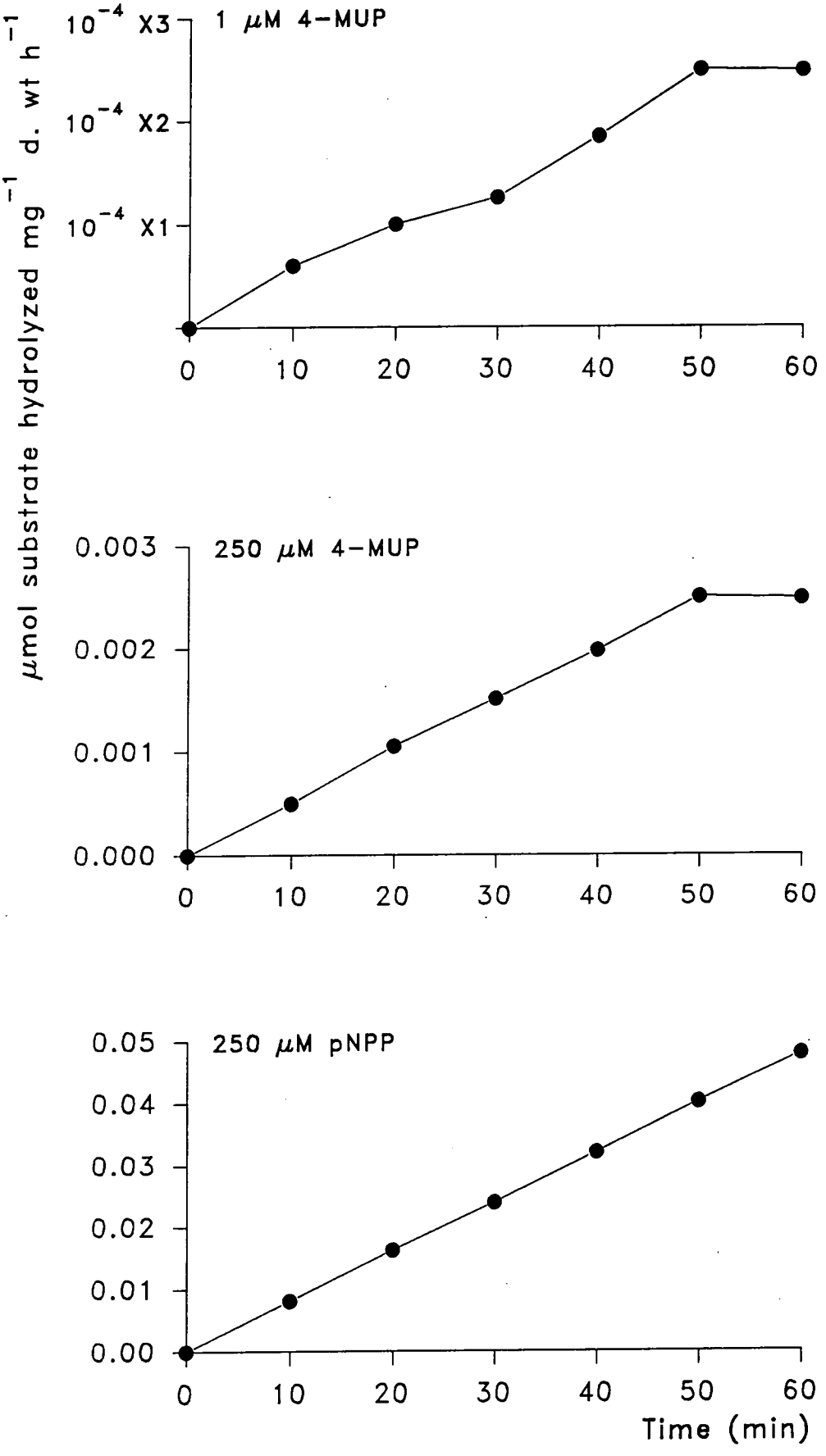


Fig. 5.16 Influence of time, substrate and substrate concentration on the PMEase activity of *Peltigera canina* 1 from Nenthead

Table 5.12 Maximum PMEase and PDEase activities of additional cyanophycean *Peltigera* species from Nenthead, analyzed for rhizine morphology.

Species	pH	PMEase	pH	PDEase	Ratio
P. rufescens	10.3	0.0184	9.0	0.0119	1.55
P. polydactyla	10.3	0.0143	10.0	0.0188	0.76
P. lactucifolia	10.0	0.0200	8.0	0.0117	1.71
P. membranacea	10.0	0.0246	4.0	0.0137	1.80

5.53 Localization of phosphatase activity

Three stains (Sections 2.61, 2.62, 2.63) were used to determine the cellular location of phosphatase activities within *Peltigera canina* from the high metal site. Figures 5.17, 5.18 and 5.19 illustrate the results obtained on the rhizines of *Peltigera canina*. All three stains show that there are no obvious contaminant bacteria responsible for phosphatase activities. It is apparent that the staining, with all three stains is most noticeable in the cytoplasm, but is also evident in the cell wall. One interesting feature was the intense staining noticeable around hyphal junctions, which seemed to be associated with both cytoplasm and cell wall. This was most apparent with Fast Garnet (PDEase). Of the three stains Fast Garnet (Fig. 5.19) produced the most intense colouration, but it must be remembered that the substrate concentrations used for these stains was 1 mM. This is considered to be far in excess of the phosphorus concentration a lichen will meet in nature.

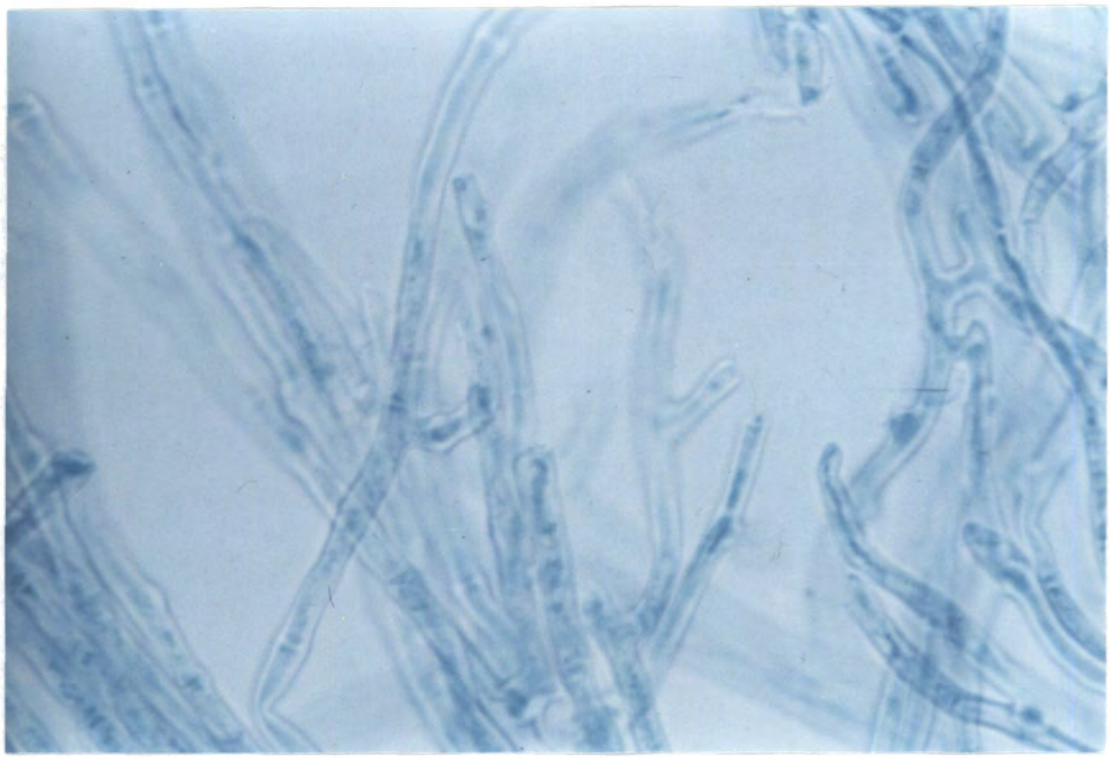


Fig. 5.17 Localization of PMEase activity in the rhizinal hyphae of *Peltigera canina* using BCIP

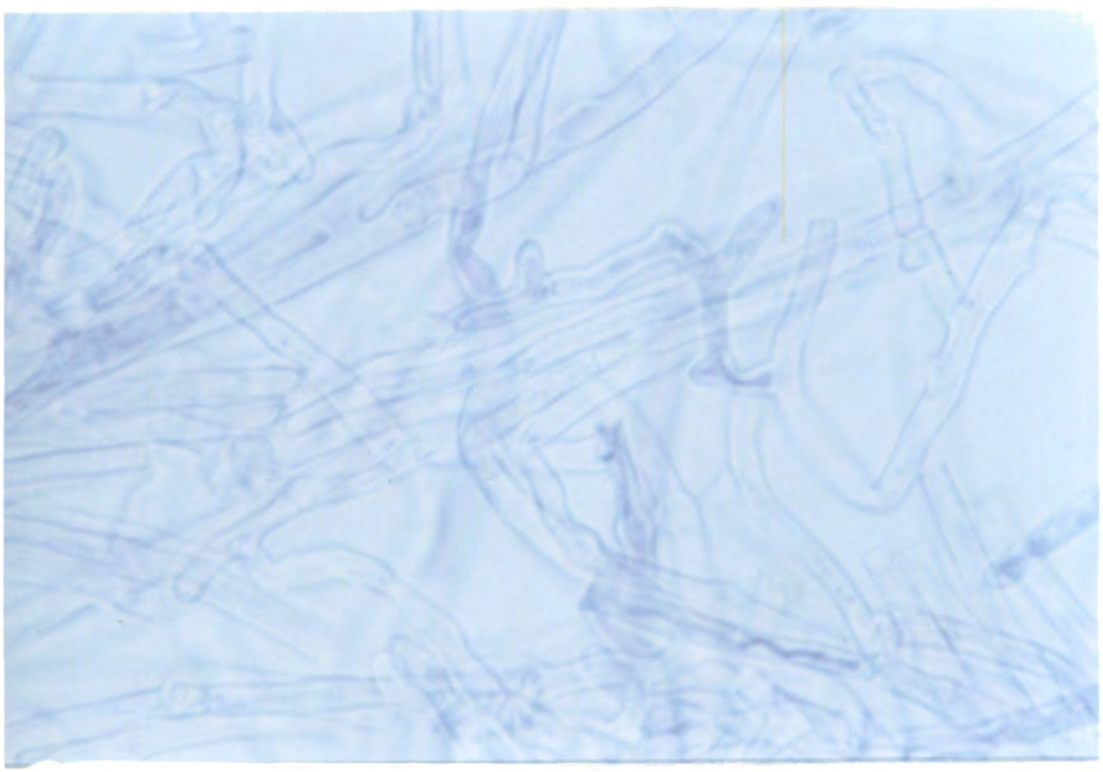


Fig. 5.18 Localization of PMEase activity in the rhizinal hyphae of *Peltigera canina* using naphthol AS-MX phosphate

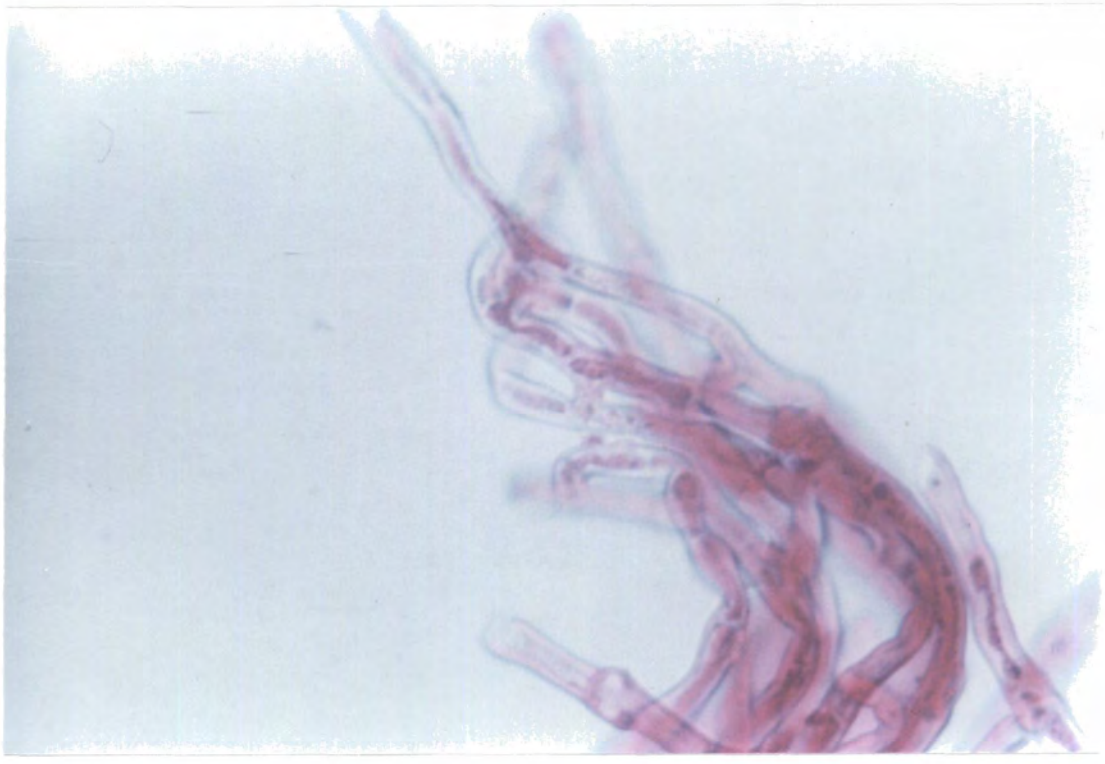


Fig. 5.19 Localization of PDEase activity in the rhizinal hyphae of *Peltigera canina* using Fast Garnet

CHAPTER 6

DISCUSSION

6.1 Phosphatase activities

This study has demonstrated that PMEase and PDEase activities are measurable in *Peltigera canina*, *P. praetextata* and *Cladonia arbuscula* plants, from low and high zinc environments, in the pH range 3.0-11.0. Both acid and alkaline PMEase activities are present in all three species. PMEase (pNPP) activity in populations of *P. canina* and *P. praetextata* from a high zinc environment exceeded that of the same species from a low zinc environment. The reverse was true for *C. arbuscula*.

The zinc concentrations measured in the thalli and associated soil samples of *P. canina*, *P. praetextata* (Tables 4.8, 4.9) and *C. arbuscula* (Tables 4.11, 4.12) confirm that the lichens were not only growing in different zinc environments, but showed an obvious response to the difference. The zinc contents of lichen populations on the mine spoil were between 4.08 and 5.39 (Table 4.16) times higher than those away from it, while the zinc contents of soil samples on the mine spoil were between 8.32 and 25.35 (Table 4.16) times higher than those away from it.*P. canina* from the high zinc environment showed similar responses (Lane & Puckett, 1979) to the effects of time and substrate concentration on PMEase activities. The lower layer of *P. canina* displayed greater PMEase activities than the upper layer at all pH values with 1 μM 4-MUP, 250 μM 4-MUP and 250 μM pNPP. *P. canina* and *P. praetextata* from the low and high zinc environments showed a greater tolerance to zinc than *C. arbuscula* in the response of PMEase activities. Comparisons between the pH values of soil from the rhizosphere of *P. canina*, *P. praetextata* and *C.*

arbuscula and the pH values of maximum PMEase activities for each species suggest that maximum PMEase activities would not be achieved in any of the environments from where each plant was sampled.

The effects of storage (Table 5.1), light (Table 5.2) and cellular damage (Table 5.3), in the laboratory, on the PMEase activity of *P.* canina from the high zinc environment showed only minimal influences.

PMEase activities measured fluorometrically with 4-MUP were shown to be consistently lower than PMEase activities measured spectrophotometrically with pNPP. Similar thalline and soil P contents in *P. canina*, *P. praetextata* (Tables 4.1, 4.2) and *C. arbuscula* (Tables 4.4, 4.5) populations prevented any conclusions regarding these lichens as suitable indicator organisms of the phosphorus status of environments.

6.11 *Peltigera canina* and *P. praetextata*

Analyses for PMEase activity in *Peltigera canina* from the low zinc environment (Fig. 5.1) indicate that PMEase activities are measurable at all pH values in the range 3.0 - 11.0 for all field plants. The optimum pH for all plants is in the range 9.0 - 10.3 and it can therefore be said that *P. canina* from the low zinc environment displays alkaline PMEase activities. In contrast, *P. canina* from the high zinc environment (Fig. 5.2) has optimum pH for PMEase activities in the range 4.0 - 6.0, for all plants. *P. canina* in this environment therefore displays acid PMEase activities, which have been demonstrated as active between pH 2.6 - 6.8 (Siuda, 1984). However, PMEase activity is measurable throughout the pH range 3.0 - 11.0.

Nash (1975) has shown zinc to be damaging to some lichens in concentrations of 200 - 600 μg g^{-1}. This study shows that *P. canina* with a mean tissue zinc content (Table 4.8) of 245 μg g^{-1} has a mean peak PMEase activity of 0.020 μmol pNPP mg d. wt^{-1} h^{-1} while the same species with a mean tissue zinc content of 1050 μg g^{-1} has a mean peak PMEase activity of 0.034 μmol pNPP mg d. wt^{-1} h^{-1}. This shows an increase in PMEase activity of *P. canina* at a rate of 1.8 times where the zinc content exceeds that of those proposed by Nash (1975) as damaging to lichens, by 1.7 times. The much higher concentrations of zinc in the lichen populations from the mine spoil (Table 4.8) indicate that the plants are influenced by the presence of the higher ambient zinc there. This is reflected in the higher tolerance to zinc enrichment shown in laboratory assays of PMEase activity (Fig. 5.13). PMEase activity therefore resembles the photosynthesis of three different *Peltigera* species (Beckett & Brown, 1983) where thalli from Zn-contaminated sites showed higher tolerance to Zn in laboratory assays than thalli from other sites. However, the results of this study differ from the previous one in that the Zn concentrations in the thallus tended to be much higher. The values for the three species from the high metal site in this study were also higher than any of those recorded for seven lichens (including *P. canina*) taken from mine sites by Seaward et al. (1978). Although chemical factors, such as the Ca content of the substratum may have influenced this difference, another factor likely to have favoured the high zinc concentrations in the plants in this study is the selection of very large plants necessary to provide adequate replicates. Although the oldest tissue was avoided, the material used may nevertheless have been relatively old and therefore more likely to have accumulated metals (Brown, 1991).

Rates of maximum PMEase activity of *P. canina* in this study are comparable with those of *P. aphthosa* and *P. canina* demonstrated by Lane and Puckett (1979). The influence of time on the phosphatase activity of *P. canina* (Fig. 5.16) also displays a

similar trend to that expressed by Lane and Puckett (1979) although that study concentrated on *Cladina rangiferina*. The results of that study on organic phosphate hydrolysis by lichens must be treated with caution because the concentrations of substrate used (7.6 and 137 mM) would appear to be a very high environmental concentration for a slow-growing organism such as a lichen.

Analyses of PMEase activities for *P. praetextata* from the low zinc environment (Fig. 5.3) indicate that PMEase activities are measurable at all pH values in the range 3.0 - 11.0 for all field plants. Unlike *P. canina* the optimum pH is not as well defined and occurs in the acid and alkaline pH range. With *P. praetextata* from the high zinc environment (Fig. 5.4) the optimum pH for PMEase activity occurs in the range 9.0 - 10.3, suggesting alkaline phosphatase activities, the converse of the observed phenomena with *P. canina*.

The mean tissue zinc content of *P. praetextata* (Table 4.8) were 228 and 1230 $\mu g\ g^{-1}$ for the low and high zinc environments respectively. Optimum mean PMEase activities were 0.028 and 0.045 μmol pNPP mg d. $wt^{-1}\ h^{-1}$ for the low and high zinc environments respectively. As with *P. canina* there is a similar increase in optimum PMEase activities between the high and low zinc environments (1.6 times). The differences observed in the optimum pH for intraspecific phosphatase activities between the low and high zinc environments may suggest that both symbionts are responsible for phosphatase activities, depending on environmental conditions.

Analyses of PDEase activities in *P. canina* and *P. praetextata* from the low and high zinc environments (Fig. 5.11) displayed a lack of distinct pH optima for both species. PDEase activities were detectable at all pH values in the range 3.0 - 11.0. Peak PMEase activity for *P. canina* (high zinc), *P. canina* (low zinc), *P. praetextata* (high zinc) and *P. praetextata* (low zinc) were 5.06, 2.94, 4.20 and 2.20 (Table 5.9) times higher than peak PDEase activities for the same plants. In the present studies PMEase and PDEase activities

were detectable at all pH values, a result supporting the view of Jansson et al. (1988) who stated that different enzymes have different pH optima and does not mean that they are totally inactive at, or do not tolerate, other pH values. The probability that a number of enzymes are involved applies more to PDEase than PMEase activities, where the responses to pH were relatively flat. The presence of both PMEase and PDEase activities in *P. canina* and *P. praetextata* suggests that these lichens may be able to grow on different organic P substrates.

Acid phosphatase is a non-metallic enzyme which is not activated by divalent cations (Hasegawa et al., 1976). Analyses for PMEase activity in *P. canina* and *P. praetextata* from the high zinc environment at pH 6.0 showed that Zn (Fig. 5.13) had a slight inhibitory effect at high concentrations (1 mM). These results are similar to the results for blue-green algae (Grainger et al., 1989; Whitton et al., 1990). In wheat, it has been reported that Zn inhibited wall enzymes by 23 - 32 % and showed a 36 - 39 % inhibition toward cytoplasmic enzymes (Hasegawa et al., 1976). Zinc is an important micro-nutrient for growth and metabolism in plants (Price et al., 1972) but if applied at high levels it may be toxic (Whitton & Say, 1975; Dhruva et al., 1977). Maximum PMEase activity occurred with 100 μM zinc in *P. canina* and *P. praetextata* with tissue zinc concentrations of 15 μM, suggesting that these species may have optimum PMEase activities in environments of higher Zn status. *P. canina* and *P. praetextata* from the low zinc environment had maximum PMEase activity with 1 μM zinc compared with tissue zinc concentrations of 4 μM and 3 μM respectively. In the presence of 10 mM zinc the activity at pH 6.0 for low versus high metal populations expressed as a percentage of the value for 1 μM zinc was: *P. canina* 13.6 versus 70.6 %; *P. praetextata* 10.4 versus 75.9 %. Both species from the low metal environment therefore clearly showed greater inhibition at a higher zinc concentration than the same species from the high metal environment.

From this study it is clear that these lichens show marked phosphatase activities. Following this, attempts were made at demonstrating which components are responsible: fungus, cyanobacterium / alga or contaminant bacteria. Although the lichen thallus may be expected to have an associated microbial flora, detailed microscopic inspection showed no obvious groups of bacteria or other organisms following pre-treatment (Section 2.4) to experiments. The use of stains (Figs. 5.17, 5.18, 5.19) to demonstrate PMEase and PDEase activities on discs of *Peltigera canina* from the high metal environment showed intense staining of cytoplasm in the rhizines and veins and light staining in the medulla and the fungal part of the upper cortex. The walls of the rhizines showed variable staining, but mostly much less intense than the cytoplasm. However, there were areas of very intense staining near some hyphal junctions (sometimes appearing like a collar), which were probably associated with both the cell wall and cytoplasm. All three stains showed similar effects, though the colouration was most intense with Fast Garnet GBC sulphate salt (PDEase). If naturally occurring organic phosphates and the artificial substrates used for laboratory assays behave like the substrates used for staining, some of the molecules will pass into the cell before hydrolysis takes place. As the laboratory assays are quantified by measurement of the hydrolysis product, this must move out through the cell wall when hydrolysis takes place in the cytoplasm. However, caution is needed in assuming that the cytoplasm is more important than the cell walls, because the concentrations of substrate used for staining (1 mM) are much higher than concentrations of organic phosphates likely to occur in nature.

Analyses for PMEase activities in the lower and upper layers of *P. canina* (Fig. 5.15) showed that with 250 μM pNPP and 1 μM & 250 μM 4-MUP the lower layer, in comparison with the upper layer, had the greatest phosphatase activity at all pH values. For both layers PMEase activities were detectable at all pH values with the optimum pH

value of the lower layer 7.0 & 9.0 with 1 μM 4-MUP, the levels of organic P most likely to be encountered in a lichen's natural environment. Although there was much greater activity (per unit biomass) in the lower than the upper layer, there was also a 69 % reduction in the combined activity of the two discs at pH 7.0 and 60 % at pH 10.3 compared with an uncut disc. With *Peltigera canina,* staining and comparison of PMEase activity in upper and lower horizontal layers indicated that the fungal rhizines and veins are the most important site of activity. However, the results of sectioning must be treated with care because of the overall loss of PMEase activity, suggesting possible inhibition. In addition it should be pointed out that dissection caused a much greater reduction in total PMEase activity (75 %) than the reduction in total phosphate uptake (25 %) found with *Peltigera polydactyla* (Smith, 1960). It is hypothesized that the mycobiont is largely responsible for PMEase activity within the lichen *P. canina*. However, the fact that the photobiont material was only algal-enhanced, these sections unavoidably contained some fungal material, suggest that further studies need to be undertaken to conclude that the photobiont plays a reduced role in lichen phosphatase activity. Phosphatase activity of the isolated photobiont of *Lasallia papulosa* (Ach.) Llano has been monitored by Roberts and Rosenkrantz (1967) while phosphatase activity in cell-free extracts of *Hypogymnia physodes* (L.) Ach. has been demonstrated by Schmid and Kreeb (1975).

This study has demonstrated acid and alkaline phosphatase activities and optimum PMEase activity for the lichen mycobiont at pH 9.0 with 1 μM 4-MUP. The general observation made by Krishnan (1964) is that plant tissues, in contrast to animal cells, exhibit phosphatase activity mainly under acid conditions, however certain fungi do show alkaline phosphatase activity (Nicholas & Commissiong, 1957; Adler, 1978). Acid and alkaline phosphatases have also been observed in the Basidiomycete *Schizophyllum commune* (Wilson, 1972; Lilly & Charvat, 1987).

Preliminary analyses (Section 5.1) were undertaken on four additional *Peltigera canina* plants from the high metal environment to examine the effects of storage (Table 5.1), light (Table 5.2) and rhizinal cellular damage (Table 5.3). There was only a slight influence of light on PMEase activity assayed with 250 μM pNPP at pH 6.0. PMEase activity showed an average increase (n = 4) of 1.04 % in the light compared with 1.03 % for *Cladonia rangiferina* and 1.04 % for *Peltigera canina*, demonstrated by Lane and Puckett (1979). The influence of rhizinal cellular damage showed an average increase (n = 4) in PMEase activity of only 1.03 % which may demonstrate that the contribution of extracellular and intracellular enzymes is negligible. *Peltigera canina* plants stored for six weeks only showed an average increase (n = 4) in PMEase activity of 1.01 %. Although this is negligible for experimental purposes, the close replication suggests that it may reflect a real change, perhaps due to a slight respiratory loss of mass. Brown and Brown (1990) have shown membrane damage induced by desiccation may have marked effects on energy dependent processes in the lichen *Peltigera horizontalis*. However, two important differences between those studies and this one are: phosphatase activity is not energy dependent and the lichens used in this study were collected from very exposed sites where they are presumably prone to longer and more frequent periods of drying out, and were therefore better adapted to these conditions when simulated in the laboratory. The experiments analyzing the effects of storage clearly show that lichens may be kept in an ambient laboratory environment for up to six weeks prior to biossays.

Although the experiments in this study were carried out at 25°C it is unlikely that the temperature in the field would limit phosphatase activity. Lane and Puckett (1979) have demonstrated maximum PMEase activities in the range 61 ± 10°C, with activity dectectable at 10°C. PMEase and PDEase in the moss *Hydrogonium fontanum* (Al Shehri, 1992) have been shown as stable enzymes with cellular activities still detectable at 80°C.

Similar results were reported in *Calothrix parietina* (Grainger et al., 1989) and *Calothrix* strain D764 (Islam & Whitton, 1992).

These results suggest that, with relation to optimum pH and almost certainly temperature, the maximum phosphatase activities of *P. canina* and *P. praetextata* would not be achieved in their natural environments. Table 5.4 illustrates the phosphatase activities at the soil pH values for each *Peltigera* plant as a percentage of the maximum PMEase and PDEase activities with 250 μM pNPP and 250 μM bis-pNPP.

Further studies are necessary to determine if inducible cell-bound phosphatases in lichens occurred that could lead to them being suitable indicator organisms of the phosphorus status of environments, as demonstrated with other plants (Kuenzler & Perras, 1965; Fitzgerald & Nelson, 1966; McComb et al., 1979; Pettersson, 1980, 1985; Healey, 1982; Press & Lee, 1983; Gage & Gorham, 1985).

Following the observations referred to in Section 1.426 and the experiments undertaken on the dissected discs of *P. canina* (Section 5.51; Fig. 5.15) further investigations were made into rhizine morphology in the genus *Peltigera*. In addition to *P. canina* and *P. praetextata* one plant of each of the following species was collected from Nenthead: *P. rufescens*, *P. polydactyla*, *P. lactucifolia* and *P. membranacea*. The diversity in rhizine morphology between the six species of Peltigera was very considerable. The degree of variability in these species was recognised in: rhizine length, colour, density and branching. In view of the results obtained in this study on the rhizines and veins of *P. canina* (Fig. 5.15) and PMEase activity, together with the recognition of rhizines as effective absorbers, accumulators, translocators and regulators of minerals (Goyal & Seaward, 1982b; Brown, 1991) it is hypothesized that rhizines in the genus *Peltigera* may also play an important role in nutrient cycling, and not just act as holdfast systems (Hale, 1983; Dobson, 1992; Purvis et al., 1992).

It is suggested that the diversity in rhizine morphology is a response by the lichen genus *Peltigera* to an efficient cycling and subsequent biochemical processing of minerals and nutrients. If these are solely holdfast systems it would appear that an extraordinary amount of energy has been diverted into creating a wide array of structures designed for one purpose, support, in an environment where the physical nature of the soil was relatively homogenous. In a complex lichen genus such as *Peltigera* it is more likely that the energy resulting in considerable phenotypic plasticity is spent on adapting the lichen to maximising its mineral and nutrient cycling in environments where this genus has been shown as dominant among other lichens (Section 1.44) and in direct competition with bryophytes and grasses.

Table 5.12 shows the maximum PMEase and PDEase activities of the additional cyanophycean *Peltigera* plants analyzed for rhizine morphology, using 250 μM pNPP and 250 μM bis-pNPP. Only one plant of each species was collected.

It is suggested that further quantitative studies are necessary to re-enforce the largely qualitative results of this study regarding the role of rhizines in nutrient and mineral cycling.

6.12 *Cladonia arbuscula*

Results of analyses for PMEase activities in *Cladonia arbuscula* from the low zinc environment (Fig. 5.5) showed that PMEase activities are measurable at all pH values in the range 3.0 - 11.0 for all field plants. All plants display an optimum pH at 6.0 and it can therefore be said that *C. arbuscula*, from the low zinc environment, has acid phosphatase activities demonstrated by Siuda (1984) as active between pH 2.6 - 6.8. However, plants 1 & 2 also showed an optimum pH for PMEase activities at pH 10.0 and 10.3 respectively. This may have been associated with microorganisms still attached to the basal podetia.

These results are in contrast to Lane and Puckett (1979) who found the optimum pH for PMEase activity in *Cladonia* (= *Cladina*) *rangiferina* to be in the range 2.2 - 4.0.

C. arbuscula from the high zinc environment (Fig. 5.6) also has PMEase activities detectable at all pH values. The optimum pH for PMEase activities for all plants was in the range 6.0 - 7.0, again suggesting acid phosphatase activities. Both *P. canina* and *P. praetextata* showed that the increase in PMEase activities between the high and low zinc environments was 1.8 and 1.6 times respectively. Mean maximum PMEase activities recorded in *C. arbuscula* from the low zinc environment were 1.2 times greater than mean maximum PMEase activities in the same species from the high zinc environment. The mean tissue zinc concentrations for *C. arbuscula* (Table 4.11) were 270 and 1075 $\mu g\ g^{-1}$ for the low and high zinc environments respectively.

PMEase activity in *C. arbuscula* from the high zinc environment, assayed at pH 6.0, showed that zinc in concentrations greater than 1 μM (Fig. 5.14) had inhibitory effects, demonstrating less tolerance to zinc by *C. arbuscula* in comparison with *P. canina* and *P. praetextata*. The tissue zinc content of this plant was 22 μM, exceeding the level of tolerance to zinc. PMEase activity in *C. arbuscula* from the low zinc environment also showed that zinc in concentrations greater than 1 μM had inhibitory effects on the plant with a tissue zinc content of 4 μM. In the presence of 10 mM zinc the activity at pH 6.0 for low versus high metal populations expressed as a percentage of the value for 1 μM zinc was 1.8 versus 63.2 %. With 10 μM zinc the PMEase activity in *C. arbuscula* from the low zinc environment was halved.

Analyses for PDEase activities in *C. arbuscula* from the low and high zinc environments (Fig. 5.12) showed that the optimum pH was 10.3 with both populations. PDEase activities were detectable at all pH values in the range 3.0 - 11.0. Peak PMEase

activity in *C. arbuscula* was 1.47 and 2.12 times higher than peak PDEase activity in plants from the high and low zinc environments respectively. This is a similar response that was observed with *P. canina* and *P. praetextata*. As with *P. canina* and *P. praetextata*, PMEase and PDEase activities were detectable at all pH values, enforcing the view of Jansson et al (1988). The presence of PMEase and PDEase activities in *C. arbuscula* suggests that this lichen may be able to grow on different organic P substrates.

These results suggest that, with relation to pH, the maximum PMEase activities of *C. arbuscula* would not be achieved in either of the two environments from where it was sampled. Table 5.6 illustrates the phosphatase activities at the soil pH values for each *C. arbuscula* plant as a percentage of the maximum PMEase and PDEase activities with 250 μM pNPP and 250 μM bis-pNPP.

The observations of Lane and Puckett (1979) regarding temperature suggest that *C. arbuscula* may attain maximum phosphatase activities in environments of considerably higher temperature.

P. canina and *P. praetextata* displayed greater optimum PMEase activity in the high rather than the low zinc environment while the converse was true of *C. arbuscula*.

The pH optima for PMEase activities correspond more closely with the pH of the environment for the high metal population of *C. arbuscula* than with all four *Peltigera* populations and the low metal population of *C. arbuscula*. However, the pH value of the soil environment immediately adjacent to the other populations may have been lower than that measured, so pH optima for PMEase activities at pH 6.0 may not be all that far from the natural environment. The occurrence of maxima at about pH 10.0 has often been recorded for organisms (Whitton, 1991) growing at considerably lower pH values and in many cases no satisfactory explanation has yet been put forward to explain this apparent

anomaly. However, it has been shown (Fedde & Whyte, 1990) for mammalian tissue that reduction of the substrate conentration can lead to a more realistic pH optimum.

Although it is accepted that further studies are necessary (Section 6.11) to determine the extent to which phosphatase enzymes are inducible in lichens this study has demonstrated a negative correlation between total P contents in lichens and PMEase activity. The populations of both *Peltigera* species from the high zinc environment had lower P concentrations and higher rates of PMEase activity compared with the same species from the low zinc environment which had higher P concentrations and lower rates of PMEase activity. However, the rates for individual plants of both *Peltigera* species showed no clear-cut response to the internal P concentration, but this may reflect the fact that there was a relatively narrow range of P concentrations in both these species.

Cladonia arbuscula populations from the low and high zinc environments had lower P concentrations than the four *Peltigera* populations. One plant in each *Cladonia* population had a much higher P concentration than the other three and this was reflected in the soil P concentration. In addition these two plants had the lowest rate of PMEase activity for each population. With *Cladonia* populations, where the range of P concentrations were higher, the hypothesis that plants with a lower P content show higher rates of PMEase activity appears to fit.

6.13 Comparison of phosphomonoesterase activity using two different substrates (pNPP & 4-MUP)

The results (Figs. 5.7, 5.8) of the influence of pH on the PMEase activities of selected *Peltigera* and *Cladonia* plants using 4-MUP as substrate show that, with the exception of pH 7.0 for *P. canina* Nenthead and pH 8.0 and 9.0 for *C. arbuscula* Middleton Common, PMEase activity was higher at all pH values with pNPP in comparison with 4-MUP as substrate. The maximum rate of activity for; *P. canina* Nenthead was obtained at pH 6.0 with pNPP & 7.0 with 4-MUP, *P. praetextata* Nenthead at pH 10.3 with pNPP & 9.0 with 4-MUP, *P. canina* Windy Nook at pH 10.3 with pNPP & 4-MUP, *P. praetextata* Windy Nook at pH 10.0 -10.3 with pNPP & 9.0 - 10.0 with 4-MUP, *C. arbuscula* Nenthead at pH 6.0 with pNPP and 4-MUP and *C. arbuscula* Middleton Common at pH 7.0 with pNPP and 6.0 with 4-MUP. PMEase activity was detectable at all pH values (3.0 - 11.0) using both substrates.

The ratio between maximum PMEase activity , using pNPP, to maximum PMEase activity, using 4-MUP, was 1.54, 1.04, 1.75, 3.28, 1.34 and 1.46 for *P. canina* Nenthead, *P. praetextata* Nenthead, *P. canina* Windy Nook, *P. praetextata* Windy Nook, *C. arbuscula* Nenthead and *C. arbuscula* Middleton Common respectively. These results suggest that 4-MUP is a more sensitive substrate for detecting phosphatase activity than pNPP. The high sensitivity of 4-MUP has been reported previously (Pettersson & Jansson, 1978).

Comparisons between 250 μM pNPP and 250 μM 4-MUP (Fig. 5.9) on *P. canina* and *C. arbuscula* from Nenthead showed that PMEase activity was detectable at all pH values with both substrates. The optimum pH for PMEase activity were the same for both species with pNPP displaying higher PMEase activity at all values except pH 11.0 for *P.*

canina. The ratio between maximum PMEase activity, using pNPP, to maximum PMEase activity, using 4-MUP, was 1.28 & 1.27 for *P. canina* and *C. arbuscula* respectively. When the influence of pH on phosphatase activity was measured using 1 μM 4-MUP (Fig. 5.10) the results indicated that the pH-activity curves were more or less similar in shape and had optimum activity at the same pH for both *P. canina* and *C. arbuscula*. The optimum rate of phosphatase activity was at pH 6.0 indicating that this substrate is suitable for measuring phosphatase activity, since there was no shift in optimum pH values.

Assays with 1 μM 4-MUP, undertaken only on *C. arbuscula* and *P. canina* from the high metal site (Fig. 5.10) illustrate the ratio of PMEase activity at pH 6.0 and 10.3 at 250 μM versus 1 μM was 46 and 81 for *P. canina* and 24 and 63 for *C. arbuscula*. This indicates a proportionately lower rate of increase in activity than rise in substrate concentration.

SUMMARY

1 A study was made on the surface phosphatase activities of the lichens *Peltigera canina* (L.) Willd., *P. praetextata* (Flörke. ex Sommerf) Vain. and *Cladonia arbuscula* (Wallr.) Rabenh. selected from low (Middleton Common & Windy Nook) and high (Nenthead) zinc environments.

2 Four plants of each species were collected from both low and high zinc sites so that the effects of environmental factors on phosphomonoesterase (PMEase) and phosphodiesterase (PDEase) activities could be examined.

3 Mean low and high zinc tissue (soil) contents (d. wt) were 245 (91) and 1050 (1719) (*P. canina*), 228 (65) and 1230 (1648) (*P. praetextata*) and 270 (285) and 1075 (2370) $\mu g\ g^{-1}$ (*C. arbuscula*), respectively.

4 PMEase activities were measured using p-nitrophenyl phosphate (pNPP) and 4-methylumbelliferyl phosphate (4-MUP) and PDEase activities using bis(p-nitrophenyl) phosphate as phosphate substrates.

5 Storage in the laboratory for six weeks showed only a 1 % increase in PMEase activity in *P. canina,* while light and cellular damage resulted in an increase in PMEase activity of 1 and 4 %, respectively.

6 *P. canina* and *P. praetextata* from the low zinc site displayed alkaline PMEase activity (250 μM pNPP) with maximum values in the pH range 9.0 - 10.3. PMEase was measurable at all pH values from 3.0 - 11.0.

7 *P. canina* and *P. praetextata* from the high zinc site displayed acid and alkaline PMEase activity (250 μM pNPP) with maximum values in the pH range 4.0 - 6.0 and 9.0 - 10.3, respectively. PMEase activity was measurable at all pH values from 3.0 - 11.0.

8 *P. canina* and *P. praetextata* plants from the high zinc site had maximum PMEase activities (250 μM pNPP) which exceeded those plants from the low zinc site by 1.8 and 1.6 times, respectively.

9 Maximum PMEase activity (250 μM pNPP) in *P. canina*, *P. praetextata* high zinc and *P. canina*, *P. praetextata* low zinc was 5.06, 4.20, 2.94 and 2.20 times higher, respectively, than maximum PDEase activity (250 μM bis-pNPP). PDEase activity was measurable at all pH values from 3.0 - 11.0.

10 Maximum PMEase activity in *P. canina* from the high zinc site was consistently shown to be with the rhizines and veins at all pH values with 1 μM 4-MUP, 250 μM 4-MUP and 250 μM pNPP. Optimum PMEase activity was observed in the lower layer at pH 9.0 with 1 μM 4-MUP.

11 *C. arbuscula* from the low and high zinc sites displayed acid phosphatase activity (250 μM pNPP) with maximum values in the pH range 6.0 - 7.0. PMEase activity was measurable at all pH values in the range 3.0 - 11.0.

12 Maximum PMEase activity in *C. arbuscula* from the low zinc site was 1.2 times greater than maximum PMEase activity in the same species from the high zinc site

13 Maximum PMEase activity (250 μM pNPP) in *C. arbuscula* from the low and high zinc sites was 2.12 and 1.47 times higher, respectively, than maximum PDEase activity (250 μM bis-pNPP) in the same plants.

14 *P. canina* and *P. praetextata* from both sites showed a higher tolerance to zinc than *C. arbuscula*. Inhibitory effects of zinc were detectable at 1 mM for *P. canina* and *P. praetextata* (high zinc) and 10 μM for *C. arbuscula* (low and high zinc) and *P. canina*, *P. praetextata* (low zinc).

15 PMEase activity with 10 mM zinc was 66, 70, 63, 14, 10 and 2 % of peak PMEase activity for *P. canina*, *P. praetextata*, *C. arbuscula* (high zinc) and *P. canina*, *P. praetextata*, *C. arbuscula* (low zinc), respectively.

16 Staining techniques, using 1 mM organic phosphate substrates, undertaken on *P. canina* from the high zinc site indicated that most phosphatase activity is due to the fungus, not the cyanobacterium or contaminant bacteria, and is associated with the cell wall and cytoplasm, most noticeably at hyphal junctions.

17 4-MUP was a more sensitive substrate than pNPP for analyses of PMEase activities in all lichen species from the low and high zinc sites.

18 *P. canina*, *P. praetextata* and *C. arbuscula* displayed maximum PMEase activities at pH values which did not correspond with any of the soil pH values from the rhizosphere of the natural environment of each plant.

19 *C. arbuscula* displayed a strong negative correlation between P concentration and PMEase activity, supporting the hypothesis that cell-bound phosphatase enzymes are inducible.

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