Nutrient status of emergent macrophytes around Bakethin reservoir, Northumberland

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ABSTRACT

Possible relationships between the acid phosphatase activities of the roots of three emergent macrophyte species and the physical and chemical features of their ambient surroundings were assessed.

Sediments collected from monospecific stands of *Phragmites australis, Equisetum fluviatile* and *Typha latifolia* growing at Bakethin Reservoir, Northumberland, were analysed and as a result microsites having significantly different organic and inorganic properties were selected.

After standardisation of the phosphatase assay procedure it was suggested that the enzyme activity rates of *P. australis* and *T. latifolia* in the field corresponded to levels of P in the environment. It is also suggested that in the case of *P. australis* the amount of available N in the sediments is important. Although significant differences in total P levels were found in the *E. fluviatile* stand the phosphatase activities of the roots collected were non significantly different.

The accuracy of the assay requires that the entire hydrolysis product formed is being released into solution for measurement, and that production of product is entirely due to the activity of extracellular enzymes. When assayed under different pH conditions all three species seemed to release product after termination of the assay or new product was formed from the action of intracellular activity. Any relationship between the amount of product retained or amounts of intracellular phosphatase activity and the pH of the medium was investigated.

It is concluded that more work is needed to attempt to separate the effects of low N and low P concentrations on phosphatase activity rates of higher plants root and that perhaps *P. australis* could be of use as an indicator to the nutrient status of its environment.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
mg	milligrams
μg	micrograms
l	litres
ml	millilitre
cm	centimetre
m	metre
ha	hectares
min	minutes
h	hour
mM	millimolar
μM	micromolar
μmol	micromoles
P	phosphorus
P_i	orthophosphate
TP	total phosphorus
N	nitrogen
NO_3-N	nitrate
NO_2-N	nitrite
NH_4-N	ammonia
PMEase	phosphomonoesterase
4-MUP	4-methylumbelliferyphosphate
pNPP	para-nitrophenylphosphate
CAPS	3-(cyclohexamino)-1-propanesulphonic acid
DMG	3,3-dimethyl glutaric acid
EDTA	ethylenediaminetetra-acetic acid
HEPES	N-2-hydroxymethylpiperazine-N'-2-ethanesulphonic acid
p	probability
L.O.I	loss on ignition
K_m	Michaelis-Menten constant
V_{max}	maximum velocity of reaction
S	standard deviation of sample

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"Every plant is a measure of the conditions under which it grows.
To this extent it is a measure of the soil and climate."

F. E. Clements (1920)

1 INTRODUCTION

1.1 Background to study

The simultaneous advance of ecology and physiology during the first half of this century has resulted in many diverse attempts to classify and understand the complex systems of organisms and their environments. In the present light of continuing enrichment of our waters by the processes of eutrophication there seems to be an urgent need for studies at the littoral margins of lakes and rivers. Information gathered on the biological and chemical processes of unpolluted waters and their littoral zone could be used to formulate management plans, lake restoration projects or biological control programs in the future.

1.2 Phosphorus

1.21 Occurrence and forms

Phosphorus is the 16th most abundant element in our solar system, and the 11th commonest element of the earth's crust (Downing and McCauley, 1992). In nature it is usually present as phosphate, (Holtan, 1988), a compound in which a phosphorus atom is more or less tetrahedrally surrounded by four oxygen atoms, PO_4, (Van Wazer, 1972). Natural soils contain between 0.2-0.4% phosphate, (Patrick and Mahapatra, 1968) most of which is derived from parent minerals from weathering. Phosphates are present as immobilised inorganic compounds (P_i), and it has been suggested that this is the biggest reserve of P in soils and sediments (Russell, 1973). In alkaline soils inorganic compounds include apatite (the commonest form is flurorapatite ($Ca_{10}F_2(PO_4)_6$) and other compounds of calcium. In acid soils it tends to form complexes with iron and aluminium (Bannister, 1976). Organic P has been estimated to be between 3-75% of the total P in the environment (Etherington, 1975).

1.22 Availability of P

It is well known that only part of the total amounts of P in terrestrial and lacustrine environments is available to the biota for the production of organic matter. The relatively insoluble nature of P makes it inaccessible to most plants and algae, yet in the first half of this century there was some controversy over whether it was the limiting nutrient in freshwater systems. This was finally solved in the 1970's with the experiments of Schlinder. He proved by the additions of P, N, and C into freshwater systems that P was in many cases the limiting factor (Schlinder, 1974).

P, unlike N, is available as exchangeable ions absorbed onto soil colloids. This form is readily exchanged with the soil solution, but even then is only present in very small concentrations (0.1 ppm; Etherington, 1975). This soil solution P is readily used by plants and the availability of this fraction is determined by the rate of transfer from the labile pool to the soil solution. Russell (1973) suggests that the concentrations of inorganic P in solution are more or less constant and the availability will actually depend on the uptake of phosphates by the plant roots and the rate of diffusion to the rhizosphere as it is depleted. This in turn will depend on the general characteristics of that soil (grain size, pH, temperature, water content etc.) and the extensiveness of the root system and the amount of root hairs. It also depends indirectly on factors such as climate and topography.

There is a marked increase in the availability of phosphates in waterlogged soil when compared to well drained soils, this being attributed to the reduction of ferric phosphate to a more soluble form (Patrick and Mahapatra, 1968).

1.23 P in the sediments

Various worker have illustrated that terrestrial and emergent species receive most of their P from the sediments (Carnigan, 1982; Granéli and Solander, 1988). The amounts they receive from the sediments also depend on the trophic state of the waters with submerged macrophytes like *Callitriche hermaphroditica* and *Najas flexilis* (Mueller *et al.* 1988) receiving greater than 90% of their P from the sediments if growing in mesotrophic waters and more than 70 % if growing in eutrophic waters (Carnigan and Kalff, 1982). Macrophyte stands are thought to be able to act as permanent P sinks due to the burial of plant litter.

Concentrations of total P vary between 0.01 mg g DM^{-1} or less in sandy, coastal sediments and up to 10 mg g DM^{-1} in iron and carbonate rich sediments (Holtan, 1988). Nurnberg (1987) states that sediments have total P levels in the range of 0.726 to 10.3 mg $^{-1}$ dry weight . Lake sediments act generally as sinks for P (Holtan, 1988), however whether they retain or release P is a complex subject and depends on the physical and chemical properties of sediments. Outside of this, factors such as sedimentation, seepage, diffusion, erosion, mixing, external loading, adsorption and desorption and the oxidation conditions at the sediment water interface also affect P cycling (Olila and Reddy, 1993). The movements of P in freshwaters and sediments are also affected by biological processes such as bioturbation and mineralisation (Huet and de Haan, 1992). If there is a reduction in the external loading of P, sediments tend to release P and release also tends to occur seasonally (Marsden, 1989).

There has been much research into the characterisation of sediment and soil P in the last few decades, especially with respect to amounts of algal-available P (Pettersson *et al.* 1988). Most P determination methods depend on chemical extractions originating from soil science methods. The most important fractions to define have been gradually simplified and empirically separated into three fractions (Holtan, 1988):

1. Non-apatite P;
2. Apatite P;
3. Organic P;

The latter being calculated from the difference of the other two fractions.

Because of the variability of the nature of sediments no general method for the determination of the different fractions has been derived. The results obtained with one method are heavily biased by that method and are often non comparable to others. In studies that aim to determine the input of P to an environment, total P is often measured due to the problems encountered when trying to determine the amounts of biologically available P (Marsden, 1989). As with any chemical characterisation of the environment there is always doubt as to whether the results given do actually reflect the true levels within the environment in question, and it is very difficult to test for accuracy (B. D. Wheeler, pers. comm.). To overcome these problems there is now a move towards more phytometric approaches. Wheeler *et al.* (1992) have suggested that phytometric studies of soil fertility is the best way of comparing the availability of P and N, within the environment, especially with regard to swamps and marshes which are neither soil nor anoxic sediment but lie between these states. This is done by growing test species on

soils under controlled conditions and comparing the standing crop mass. For the purpose of this study this would have taken too long to execute, however future studies may begin to embrace this approach.

1.24 Function in plant metabolism

Inorganic phosphate is fundamental to plant growth - for root production, flowering and seed production (Emsley and Hall, 1976). Subsequently it is classed as a macronutrient. In its orthophosphate form, it plays a fundamental role in the large numbers of plant enzymatic reactions that depend on phosphorylation. It is an essential part of the ATP molecule, the universal energy molecule in higher plant and animal metabolism (Ehrlich, 1981), nucleic acids and phosphorylated sugars. It is a major constituent of the cell nucleus and is essential for cell division and the development of meristematic tissue as shown by radioactive P tracers (Russell, 1973).

Lack of P in the environment or the immobilisation of P lead to P deficiencies. P deficiency can usually be observed by the addition of P by the addition of fertilisers or as a result of accidental eutrophication. The plant community responds to the increased availability by increased growth of the dominant species or conversely by the subsidiary species and tissue concentrations of P will increase. Extreme P deficiencies produce chlorosis and plants shoots and roots become stunted as growth is reduced.

1.3 Nitrogen

1.31 Occurrence and forms

Nitrogen is the 4 th most abundant element (Downing and McCauley, 1992). In gaseous form the inert dinitrogen molecule makes up around 70% of our atmosphere. This molecule is too stable to be affected by the normal assimilation processes of plants, thus they have to rely on nitrogen occurring in more reactive combinations i.e. with hydrogen as ammonium, or with oxygen as nitrite or nitrate. Of this combined N, the majority exists as unavailable organic N of uncertain chemistry (Bannister, 1976).

1.32 Availability of N

Geochemists have estimated that only 0.0025% of the total N in the earth's' crust is available for plant growth (Lewis, 1986). Like P, most plants absorb nitrogen in the

oxidised form as nitrate, NO_3 -N from the rhizosphere. They can do this fairly rapidly, concentrating it within the root tissue or the xylem (Lewis, 1986). However, plant species growing in waterlogged soils or acid soils may utilise ammonium as the absence of oxygen prevents the formation of nitrate (Patrick and Mahapatra, 1968; Bannister, 1976). In general, and where possible NO_3 -N is used over NH_4-N, as it is less toxic, enhances the aborption of K^{2+}, Ca^{2+}, Mg^{2+} and requires no complex translocatory systems (Lewis, 1986). However, once within plant cells, the nitrate must be converted into NH_4-N and this requires energy. Also NO_3-N is easily lost from soils by run off and leaching thus the concentration of NO_3 is several hundred times lower than the total N concentrations.

NH_4-N is a positively charged ion and it is held by the negative forces in the soil. Its toxicity is due to its capability of uncoupling phosphorylation.

1.33 Function in the biota

Nitrogen is a major component of a number of compounds that are essential for the structure and functioning of biological organisms. N is a fundamental part of the purine and pyrimidine bases that make up RNA and DNA. These bases are also integral to nucleosides, nucleotides and nucleic acids. The amino acid structure contains a basic amino group within its structure (Lewis, 1986). These are the building blocks of proteins, that go on to perform a multitude of tasks as enzymes and cell components.

1.4 Phosphatases

1.41 General background

In order for organisms to receive the necessary P for growth they have developed a widespread class of enzymes named phosphatases. These promote the degradation of immobilised and complex phosphates into usable forms of inorganic phosphate. They have been observed in many organisms including, algae (Schmitter and Jurkiewicz, 1981), higher plants (Malcolm and Vaughan, 1979), lichens (Lane and Puckett, 1978), bacteria and bryophytes. There are two classes of phosphatases, the phosphomonoesterases (PMEases) and the phosphodiesterases (PDEases). The PMEases are the most researched and the enzymes studied in this project. They are referred to as phosphatases or PMEases.

1.42 Acid and Alkaline phosphatases

Like most enzymes, phosphatases have been shown to be active as catalysts within a relatively narrow pH range. Therefore traditionally they have been classed as broadly acid or alkaline (Duff *et al.* 1994). Alkaline PMEases have pH optima around pH 7-9 and acid PMEases around 4-6. This follows the current knowledge of enzyme action and there are two theories to explain this. The first is that the enzyme is unstable around the extremes of its range, losing its tertiary structure necessary for conformation of the active site. The second and more probable theory is that the effects of pH on the ionisation of acidic and basic groups of the active centre, thus reducing its efficiency (Wynn, 1973).

1.43 Constitutive and Inducible phosphatases

Enzyme activity can be induced, for example in response in some change in the environment or they can be constitutive and work more or less continually within the cell (commonly described as 'house keeping' enzymes). It is thought that inducible enzymes are switched off by a negative feedback mechanism in response to a by-product or a repressor. It has been suggested that high levels of PMEase activity will be terminated by increases in the P_i concentration of the surroundings (McLachlan *et al.* 1987), however in the literature this has not always been found (Ascencio, 1994). Root acid PMEase activity has also been positively correlated to the P content of vascular epiphytes (Ascencio, 1994).

1.44 Phosphatase activity within the biota

Bacteria exhibit phosphatase activity the majority of studies being done on *E. coli,* (Jansson, 1988). Few have been done on bacteria in fresh waters as there are difficulties in quantifying the amounts of P_i bacteria contribute. Positive correlations have been found between alkaline phosphatase activity and alkaline phosphatase producing bacteria in the uppermost levels of sediments, and the viable counts of populations but more work is needed to determine how much of this P_i is available for plant growth.(Jansson, 1988) It is thought most of the P_i produced will be used in the production of bacterial organic matter and will not be available to other organisms.

Phosphatase activity has also been found in most of the major groups of algae and cyanobacteria, the majority having alkaline activities. Much attention has been given to

the production of hairs which are associated with high PMEase activity and seem to be produced at times of P_i limitation (Whitton, 1988). Subcellular acid PMEase activity has been located within the PAS bodies of the marine dinoflagellate *Gonyaulax* (Schmitter and Jurkiewicz, 1981).

The phosphatase activities of various lichen species have also been described although little is known of their role in lichen metabolism. *Cladonia rangiferina* examined by Lane and Puckett (1978), had acid phosphatase activity independent of light and completely eliminated by boiling. Phosphate addition also inhibited enzyme activity. Stevenson (1994) characterised lichen species growing in low and high Zn environments. *Peltigera praetextata* had alkaline activity and *Cladonia arbuscula* acid activity. *P. canina* was found to have acid and alkaline activity depending on the Zn status of its environment. PMEase activity was subsequently located within the cell wall, cytoplasm, and hyphal junctions. This shift towards alkalinity was reported by Bieleski (1974) in relation to P_i concentrations, rather than metal cation concentration. He found that the pH optima of the phosphatases of various algae, micro-organisms and plants shifted to a more alkaline range when Pi conditions became even more extreme.

Little work has been done on bryophytes, yet they an integral part of mire vegetation where P_i concentrations are notoriously low. Press and Lee (1983) examined the acid PMEase activity of *Sphagnum* species and found that activity was negatively correlated to the amounts of P in the tissues. Laboratory experiments also showed that addition of P decrease enzyme activity, with the opposite also being true.

Lee, (1988) states that a phosphatase with broad substrate specificity, able to reach external substrates without crossing the plasma membrane is an almost universal feature of higher plants. They are found on roots (Pammenter and Woolhouse, 1975), leaves (Ascencio, 1994), and within tissue cultures (Ueki and Sato, 1970). Most alkaline PMEases are believed to be extremely substrate specific, whereas acid PMEases are not (Duff, *et al.* 1994). Some have been shown to be extracellular (Ridge and Rovira, 1971; McLachlan, 1976) their distribution associated with that of ATPase and most commonly found in roots and cell cultures (Duff *et al.* 1994). Environmental determinants that elicit increased enzyme activity of both intra and extra cellular acid PMEases include:

1. Exposure of roots to divalent cations, e.g. Ni^{2+}, Ca^{2+} and Mg^{2+}
2. Salt stress
3. Water deficit stress

However, since PMEases are believed to be involved in the hydrolysis of phosphates then most studies have aimed to show a relationship between PMEase activity and P_i concentration. Studies on *Eriophorum vaginatum* tussocks from Arctic sites in Alaska, have suggested that they receive as much as 65% of their P from root PMEase activity (Kroehler and Linkins, 1988; Moorhead *et al.* 1993). Goldstein *et al.* (1988) reported that cultures of *Lycopersicon esculentum* cells had almost 3 times the activity of the control cultures if in P_i depleted medium.

Recent work also suggests that root phosphatases are induced during controlled conditions of low N (Baloch, pers. comm.) allowing speculation that perhaps increased phosphatase activity is produced in response to both P and N limiting nutrient conditions.

Most of the studies into phosphatases involve examining plant material that has been aseptically grown. This is to remove any discrepancies caused by microbial colonies on plant roots which may well be responsible for increased activity. Very little work has been done on the relationship of phosphatase activity of a higher plant species that has been collected from its own natural environment.

1.45 Methods of determination of phosphatase activity

Phosphatase activity is recorded by measuring the amount of organic product or increase of orthophosphate formed from the hydrolysis of a suitable artificial substrate under suitable conditions. Two of the commonest substrates used are para-nitrophenylphosphate (pNPP; Duff *et al.* 1994) and 4-methylumbelliferylphosphate (4-MUP; Chrost and Krambeck, 1986). Of these 4-MUP is judged to be the most sensitive. As enzyme activity is highly dependent on environmental conditions such as temperature, pH, substrate concentration, and the presence of any inhibitors these must be strictly controlled.

1.5 Background information on species studied

The plants species studied during this research were selected following criteria set in chapters 4 and 5. For clarity and convenience a brief summary of their features are given below.

1.51 *Phragmites australis* Gramineae

This is a stout perennial that with the use of extensive creeping rhizomes can form dense clonal monocultures. It can grow from 1.5-3.0 m tall from the sediment (Muller 1994). From the end of April to the beginning of May, the submerged new stems begin to grow above the water surface and it starts to flower from late July through to September. It is suggested that it has a highly competitive strategy (Grime *et al.* 1988), yet must also have stress tolerating abilities as it can grow in the hostile, anoxic conditions of swamps, fens, marshes and other water margins. In the literature there are many studies on the anatomical features of *P. australis* especially on the production of aerenchymal tissue (Brix, 1988) and detailed studies of the morphology of *P. australis* roots are reviewed in Haslam (1972). Its rhizomes grow horizontally at a depth of around 40-199 cm, from which sparse roots grow. The roots are descibed as being short and narrow, branching several times when they have grown 2-4 centimetres in length to form dense fibrous matts. The density reflecting the conditions they grow in with roots are dense in areas favourable for growth and sparse in poor ones (Haslam, 1972).

1.52 *Equisetum fluviatile* Equisetaceae

The horsetails are a group of herbs with creeping rhizomes. *E. fluviatile* grows to heights of 50-140 cm and are more or less erect in nature. The stems are 2-12 mm in diameter, hollow, green and smooth with 10-30 grooves (Clapham *et al.* 1989). No information on the roots of *E. fluviatile* was found during a search of the literature.

1.53 *Typha latifola* Typhaceae

This is perennial herb with a stout, tall growth form. It can grow to heights at 1.5 to 2.0 and will form dense stands via vegetative spread from rhizomes. It had leathery, rather glaucous, linear leaves of less than 18 mm wide (Clapham *et al.* 1989). It flowers in June-July, but at Bakethin the flowers were not seen until mid July. The flowers are unisexual and are on a tall, terminal spike. Again little information on the morphology of *T. latifolia* roots could be collected, most studies investigate the production of aerenchyma cells and the CO_2 holding capabilities (Constable *et al.* 1991).

1.6 Aims

There have been many studies on the relationships between plants and their substrate in the littoral zone of inland waters, but so far there are few standardised methods that quantify the responses of plants to their surroundings nutrient levels.

This research had the broad aim of using the phosphatase assay to examine the enzyme activity of three emergent macrophyte species from Bakethin Reservoir in relation to the nutrient status of the sediments they grew in. This broad aim consisted of various smaller scale aims. The first specific aim was to select locations within environmental gradients around the shore of Bakethin Reservoir. Physical and chemical features would be determined and microsites from which plants would be collected selected. Then, using standard procedures determined for each species to allow comparison of results, roots from plant species found growing within these locations were to be examined for phosphatase activity. It was hoped that any recorded differences in PMEase activities from plants collected at each site selected could in some way be linked to the environmental parameters measured. It is known that climatic and temporal changes may effect the PMEase activity of organisms (Bresnan, 1994) however, in this study these variables were kept to a minimum. This was done by sampling all the sediments and collecting plant specimens from each site on one day.

From studies by Luff, (1993) problems of the phosphatase assay of roots were highlighted. It seemed the product produced by the hydrolysis of pNPP was in some way retained by the roots assayed and it is questioned whether the true activity was being determined. This problem was examined further using 4-MUP substrate at two concentrations to allow comparison of results.

2 METHODS AND MATERIALS

2.1 Computers and statistics

Data were analysed using a two-way ANOVA without replication to test for significant differences. If there were significant differences the data were analysed further using a Tukey test (Fowler and Cohen, 1990). Linear regressions were fitted using Cricket graph and Excel.

2.2 Laboratory analytical methods

2.21 pH

pH measurements for the buffers and the sediments were determined using an Ingold combination WTW E50 Electrode. The probe was calibrated using BDH standard buffer solutions, prepared with MilliQ water.

2.22 Mass determination

Mass determination of the plant material and the sediments were made using an A&B Company Ltd electronic analytical balance (model ER-182A), to 5 decimal places for root material and 3 decimal places for sediments.

2.23 Fluorimetric and colorimetric analysis

Fluorimetric analysis of the 4-MUP product was carried out using a Baird Atomic Fluoripoint Spectrophotometer. Plastic 1 cm cuvettes were used. In both the N and P analysis the coloured product of the reactions were measured on a Schmiaduz Digital Double-Beam Spectrophotometer (model UV-150-02). Glass cuvettes of 1cm path length were used.

2.24 Light and Fluorescent microscopy

A Nikon type 109 Fluorophot microscope was used to examine the plant material.

2.3 Media

2.31 Stock solutions

Stock solutions for the preparation of the assay media were prepared in MilliQ water and kept at 4°C until needed. They were renewed every three months.

2.32 Phosphatase assay medium

The concentrations of mineral salts in the assay medium are shown in Table 2.1. Fresh assay medium was made up for every assay and allowed to reach room temperature before use.

Table 2.1 The concentrations of mineral salts in assay medium

		Salt (mg l^{-1})	μM
Major elements	$MgSO_4 . 7H_20$	25.000	101.400
	$NaHCO_3$	15.850	188.600
	$CaCl_2.2H_20$	35.830	243.700
	KCl	4.280	57.380
	$Na_2EDTA.2H_20$	1.670	4.170
Microelements	$MnCl_2.2H_20$	0.040	2.280
	$CuSO_4.5H_20$	0.020	0.078
	$CoSO_4.7H_20$	0.010	0.035
	$NiSO_4.7H_20$	0.030	0.030
	$ZnSO_4.7H_20$	0.056	0.019
	Na_2MoO_4	0.007	0.028
	H_3BO_3	0.720	11.560

2.33 pH buffers

pH buffers were prepared with assay medium and had a final concentration of 50 mM. A list of the buffers used is given in Table 2.2. They were prepared in advance and kept at 4ºC until required. Each pH was checked before use. Again, they were left to return

to room temperature until use. All chemicals used were supplied by the British Drug Company Ltd and Sigma Chemical Co. USA.

Table 2.2: A list of buffers used.

pH	Buffer	Buffering capacity
3.0	DMG-NaOH	3.2-7.6
4.0	DMG-NaOH	3.2-7.6
5.0	DMG-NaOH	3.2-7.6
5.5	DMG-NaOH	3.2-7.6
6.0	DMG-NaOH	3.2-7.6
6.5	DMG-NaOH	3.2-7.6
7.0	HEPES-NaOH	6.8-8.2
8.0	HEPES-NaOH	6.8-8.2
9.0	glycine-NaOH	6.8-8.2
10.0	glycine-NaOH	9.8-11.0

2.34 Substrate solution

4-methylumbelliferyl phosphate (4-MUP) substrate solution was used as an artificial substrate to measure PMEase activity. All concentrations of 4-MUP were made up in freshly prepared assay medium on the day as required. Controls with no added plant material were done to allow for non enzymatic hydrolysis of 4-MUP. The highly fluorescent product (methylumbelliferone) was read at 460 nm under 365 nm excitation.

2.4 Cleaning of glass ware and utensils

The glass vials and plastic pipettes used in the phosphatase assay were soaked overnight in 2% Decon detergent. They were then rinsed thoroughly with first tap, then distilled water and left at 100ºC and 40ºC respectively to dry. All glassware used in the P and N determination were soaked in 4% HNO_3 and 10% HCl respectively overnight then rinsed thoroughly.

2.5 Characterisation of sediments

2.51 pH of the sediments

20 g fresh sediment was mixed thoroughly with 20 ml of water. The pH probe was inserted into the slurry mixture and left for 30 min to equilibrate (Allen, 1974). The reading was then recorded.

2.52 Grain size analysis

When determining the grain sizes of soils it is a common procedure to simply shake dry samples of sediment through a column of sieves. The drying procedure has a dramatic effect on the grain sizes and gives results that may not necessarily reflect the true environment. Therefore it was decided that samples of fresh sediment were to be used. Samples were mixed with a little distilled water and gently "puddled" through six brass sieves. The mesh sizes and associated sediment types are shown in Table 2.3.

Table 2.3 Grain size fractions (Parsons *et al.* 1984).

Mesh size	sediment type
4.0 mm	Pebbles
2.0 mm	Gravel
1.7 mm	very coarse sand
600 μm	coarse sand
250 μm	medium sand
150 μm	fine sand
<150 μm	silt

2.53 % water content and % loss on ignition

Water content of the sediments was obtained by drying a known weight of fresh sediment overnight at 105ºC. The percentage water content was then calculated. The samples were put in a Muffle furnace and heated to 550ºC for 1 h. When they had cooled they were re-weighed and the loss of organic matter on ignition was then calculated.

2.54 Determination of P

The collected sediment core was mixed thoroughly and sieved through a 600 μm sieve to achieve uniformity of the sample. This was to allow direct comparison of the different sediments and for any large differences of the bulk densities, which may affect the availability of P to the growing root. Total P (TP) of the sediments was determined by the ignition method (Andersen, 1975). Once digested TP was determined following Eisenreich *et al.* (1975).

2.55 Determination of inorganic N in the sediments

The inorganic N fractions of NO_3-N, NO_2-N and NH_4-N were determined using a 2M KCl extraction (Anon., 1986). After extraction the levels were determined using the methods of Stainton *et al.* (1977).

2.6 Acid phosphatase assay procedure

2.61 Preparation of root material

The root material was carefully washed in assay medium to remove all traces of sediment, cut into 1 cm lengths and placed in clean, labelled glass vials.

2.62 Assay procedure

1.5 ml of buffer and 1.4 ml of assay medium was added to clean glass vials. The glass vials were then transferred to a shaking tray held over a water bath set at 25°C and left to equilibrate for 10 min. Assays were initiated with the addition of the 0.1 ml of the substrate. The vials were then left for the appropriate time. The reaction was terminated by the addition of high alkaline terminator which allowed higher fluorescence. 0.1 ml of the samples were then transferred to cuvettes containing assay medium and terminator and read on the fluorimeter. Each assay had a minimum of four replicates, although often 10 replicates were used.

2.63 Measurement of the apparent and after termination activities

As mentioned in section 1.6, Luff (1993) reported there seemed to be retainment of product from assaying with pNPP. The same response was investigated in the roots using 4-MUP. The initial apparent activity was measured as usual. Then the roots were rinsed thoroughly with assay medium to try and rinse all traces of substrate from them and transferred to new, clean vials containing buffer and assay medium. No substrate was added and the vials were left at 25°C. After 1 h samples were terminated again and were treated exactly as in 2.41. It was hoped that this would allow calculation of the amounts of product retained or elucidate the amounts of product produced by the action of intracellular phosphatases. It must be stressed that the potential of the terminator to halt the formation of product was checked each time it was made up.

2.64 Time trials

Each species was assayed at the highest concentration of substrate used (5 μM 4-MUP) with readings being taken at 10 min intervals for 1 h.

2.65 Substrate concentration

The effect of substrate concentration was examined by assaying each of the species at different substrate concentration over 30 min. To determine K_m (the concentration of substrate where half the active sites are filled) and V_{max} (maximum velocity of reaction) of the enzymes the Lineweaver-Burke plots were drawn (not shown in the results; Stryer, 1988)

2.66 Determination of pH optima

Acid PMEase pH optima were determined by running the assay using buffers of 10 different pH; 3, 4, 5, 5.5, 6, 6.5, 7, 8, 9, 10. This range was used following the work of Luff (1993).

2.67 Measurement of dry weights

Once the assay was complete and any leakage measured the roots were carefully transferred to pre-weighed glass vials and left at 105°C overnight to dry. The next day the samples were removed, placed in a desiccator and then weighed.

2.68 Calculation of the phosphatase activity

The phosphatase activity was calculated using the following equation:

$$\text{PMEase activity in } \mu\text{mol g-1 dry weight hr-1} = \frac{[(\text{Reading on fluorimeter}) - \text{control}] \times 0.0033 \times 30}{57 \times \text{dry weight (g)} \times \text{assay length (min)}}$$

2.7 Root staining techniques

2.71 Toluidene blue

To investigate the effects of the very high and low pH buffers on the integrity of the root cell wall the roots were stained with Toluidene Blue dye and examined under the light microscope.

2.72 Napthol As-MX phosphate

Localisation of PMEase activity was tested by microscopy using Napthol As-Mx phosphate as the organic P source and fast blue RR diazonium salt as a coupling agent as outlined in Grainger (1989). The product is an insoluble violet compound. The roots were prepared in exactly the same way as for the phosphatase assay using the buffer that had recorded the highest PMEase activity of that particular species and left shaking for 30 min at 25°C. The cut sections of root were then washed with assay medium and examined under a light microscope.

2.73 Examination for algal and bacterial colonies

The roots were also examined for surface dwelling bacterial colonies. This was done by placing thin sections of root surface under fluorescent light and examined at a magnification of 100 times which will allow any microbes to be seen.

2.8 Collection and storage of biological material

2.81 Plant specimens

Plant specimens where collected from the chosen sites by carefully digging around the stem base and gradually working the roots free. In many cases it was possible to extract roots fully intact. Touching or handling of the roots was avoided and they were quickly transferred into a polythene bag which was securely tied. The roots were then placed in an ice box (although never directly on ice) and kept at 4°C until the phosphatase activity could be measured.

2.82 Sediments

All sediment samples were collected on the same day to overcome any chemical variations that may occur within the sediments on a temporal scale. Samples were collected from around plant roots in each of the 10 microsites with a 5 cm diameter soil corer. The corer was inserted to a depth of 5 cm, twisted around, then a thin piece of plastic was placed over the bottom and the sample gently removed. The sediment core was placed into a clean, acid washed plastic container and kept at 4°C overnight.

2.9 Water depth

2.91 Water depth

Water depth was measured from the top of the sediment layer to the water surface at all 10 microsites on each visit.

3 BACKGROUND TO STUDY SITE AND LOCATIONS STUDIED

3.1 Bakethin Reservoir

Bakethin Reservoir lies in the district of Tynedale in Northumberland. The reservoir and its associated conservation area are situated at the western end of Kielder Water, the largest man-made water body in Europe. The altitude of the area is 185.2 m AOD, and the entire area is contained within the following grid references: NY642912 and NY 632915. The nature conservation area is covered by sheet 69 of the O. S. Pathfinder, 1:25 000 series.

Bakethin's main inflows include the North Tyne and the Kielder Burn and smaller, less significant inflows include the Capon Burn and Bakethin Burn. Kielder Water is the outflow of the reservoir and the two water bodies are separated for most of the year by a dam, however mixing of the two water bodies does occur after periods of high rainfall. The dam was built to allow Bakethin to have its own naturally fluctuating water levels, the shallow shores providing an ideal refuge for wildlife amongst the relative monoculture of the surrounding coniferous forests (Northern Sports Council 1979). The underlying geology of the area consists of Carboniferous limestone and the waters are slightly alkaline with a pH range of 6.5-8.2 (V. J. Mattin, pers. comm.). The catchment contains unfertilised forestry, moorland, and a mixture of pasture and arable land. The major sources of nutrients are from the Kielder sewage works upstream of the reservoir and agricultural field runoff.

Whilst Kielder has opened its doors to the public, encouraging sailing, windsurfing and other recreational sports, Bakethin has remained relatively quiet and undisturbed. In 1979, the Bakethin Conservation Advisory Group was established and in 1984, the Northumbrian Wildlife Trust drew up a management plan for the area. The designated conservation area covers a total of 123 hectares of which, about half is water surface. In all there are 5 broadly defined habitat types (Bakethin Conservation Report 1982):

1. Open water and the marginal vegetation
2. Semi-natural grassland
3. Calcareous flush
4. Broad-leaved woodland
5. Coniferous plantation

The littoral zone is most important to overwintering wildfowl and in the summer, breeding populations of, for example, goosander (*Mergus merganser*). It is also provides cover and shelter for the elusive otter (*Lutra lutra*). There is active extension of the marginal vegetation at the northern end of the reservoir and the conditions are ideal for the study and collection of a good many wetland species.

This year for the first time, fishing on the reservoir has been stopped completely and the brown trout fisheries have been left unstocked. The main aim of the ban was to try and assess the impact of walkers and fisherman on the reservoirs breeding bird populations, and this move has undoubtedly also been of benefit to the surrounding vegetation. However, this ruling also meant that some areas of the shore were unavailable for examination during this study reducing the scope of the area to be investigated. Therefore it was not possible to lay out a transect from the end of the forest to the limit of the marginal vegetation as hoped. Instead, small areas that appeared to have water depth gradients, grain size gradients (and so possibly N and P differences) were sampled and tested for significant differences. Like most wetland vegetation the species were found in monospecific clumps. Therefore three major sites were selected containing stands of the three species studied.

3.2 Plant species

A preliminary study of the reservoir showed that there were 7 dominant emergent species growing on the shores of the reservoir. These were:

Phalaris arundinacea L.

Carex rostrata Stokes.

Typha latifolia L.

Phragmites australis (Cav.) Trin. ex Steud.

Equisetum fluviatile L.

Sparganium erectum L.

Juncus effusus L.

Next, a survey of where these plant species were growing was done to identify the largest, most heterogeneous environmental gradients available for study. Plant species had to have high PMEase activity and to be at roughly similar growth stages (3-4

leaves). Plants that were found in heterogeneous environments and had easily detectable PMEase activities were chosen. These were: *Phragmites australis, Equisetum fluviatile* and *Typha latifolia.* For a detailed description refer to sections 1.51-1.53

3.3 Description of locations studied

3.31 *Phragmites australis* stand

P. australis was found on the shores of Bakethin in two major stands almost opposite to one another (shown in Fig. 2). Plants were collected from the large bed surrounding the calcareous flush at approximately 0, 5, 7, and 9 m along a randomly positioned transect running into the water.

3.32 *Equisetum fluviatile* stand

E. fluviatile was found to be growing very abundantly in various sites around the reservoir and seems to be advancing on the north side from the viaduct towards the shore of the main body of the reservoir (see Fig. 2). Plants were collected from this bed at 0, 0.5, and 1 m along a randomly selected transect running into the water on a steep bank.

3.33 *Typha latifolia* stand

The access restrictions on various sites around the reservoir meant that the *T. latifolia* transect had to span two areas, as seen in Fig. 2. Plants were taken from a microsite just along from the *E. fluviatile* bed and from deeper water over on the south side.

Fig 1 The north end of Bakethin reservoir

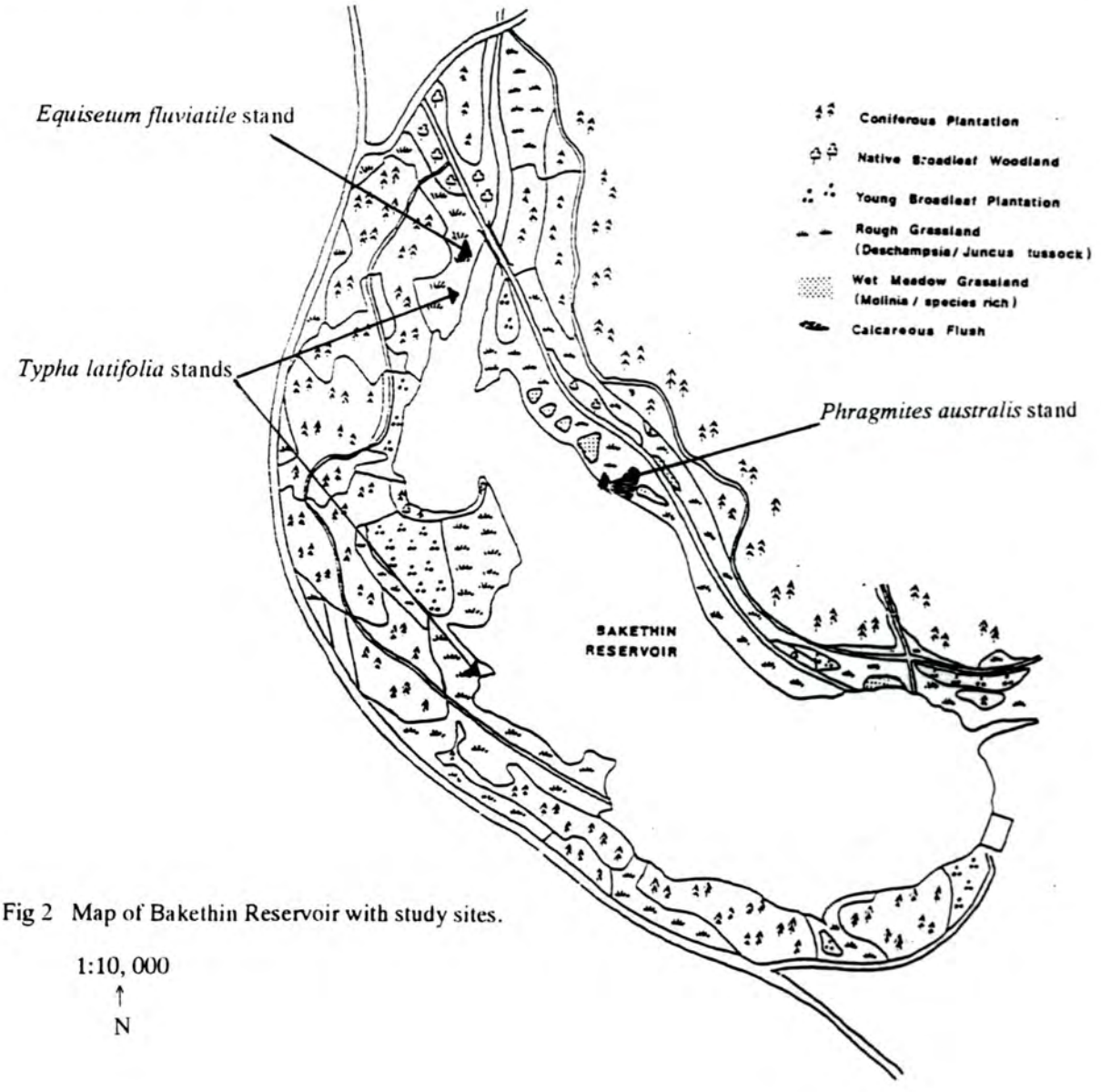

Fig 2 Map of Bakethin Reservoir with study sites.

4 PHYSICAL AND CHEMICAL RESULTS

4.1 Characterisation of physical features of the sediments

The following data were analysed using two-way ANOVA's without replication to test for significant differences between and within microsites. There were no significant differences within microsites for all parameters tested and so for clarity only the between microsite results are shown with the findings of the Tukey tests which identify the source of any significance found.

Table 4.1 Results for physical features of the sediments

Species	Microsite	Water depth (m)	pH	% Water content	% L.O.I	Grain sizes of the sediments (mm)			
						Pebbles >4.0	Gravel 4.0-2.0	Sand 2.0-0.15	Silt <0.15
P. australis	1	0.00±0.00	6.95	89.49	55	2.58	0.00	13.36	84.07
	2	0.17±0.03	6.18	27.90	40	30.38	0.00	69.61	0.00
	3	0.30±0.04	6.54	32.28	35	2.15	8.03	85.01	4.79
	4	0.45±0.04	6.56	33.62	40	5.08	3.40	86.37	5.13
E. fluviatile	1	0.01±0.17	6.78	22.50	15	9.91	23.97	36.27	29.84
	2	0.03±0.08	6.75	22.00	5	5.42	20.29	56.50	17.79
	3	0.45±0.13	6.74	20.90	15	0.75	2.28	78.08	15.13
T. latifolia	1	0.02±0.01	6.34	30.15	25	0.00	0.00	90.21	9.79
	2	0.15±0.01	5.30	21.07	20	11.76	36.76	47.04	4.43
	3	0.40±0.04	6.48	22.06	35	22.95	5.56	36.05	35.30

4.11 Water depth

Within each microsite the water depths measured were averaged to produce mean values for standing water depth (m). The results are shown in Table 4.1. The standing water depths at microsites 1-4 in the *P. australis* bed were significantly different to each other ($F_{3\ 48}$= 879.49 p<0.001; T=8.69 p=0.05).

At the *E. fluviatile* stand the water depths were again highly significantly different between the microsites ($F_{2\ 8}$=1013.268, p<0.001). When tested further however, it was

found that only microsites 1 and 3, and 2 and 3 were significantly different from each other (T=5.49 p=0.05).

The standing water depths in the *T. latifolia* microsites were all significantly different ($F_{2\ 8}$=1295.76, p<0.001; T=4.30, p=0.05).

Plants were collected on the same day and assayed for phosphatase activity the following morning, thus reducing the effect of temporal and water depth variations on the enzyme activity rates measured.

4.12 pH

The pH values of the sediments, based on one determination are shown in Table 4.1. At all the microsites pH was not significantly different ($F_{2\ 6}$=3.05, p>0.05). From these results pH therefore, is fairly uniform and slightly acid ranging from 5.30 to 6.95.

4.13 Grain Size fractions

The results for the grain size analysis from one determination can be seen in Table 4.1. As the four different sand fractions were all similar in proportions all were grouped together. Microsite 1 of the *P. australis* bed has a very large proportion of silt making it very different from the other 3 microsites, whose largest proportions lie in the sand fractions. Microsite 2 had a high proportion of pebbles in its sediments and no silt particles, most probably related to its position on the shore of the reservoir. Outside of the terrestrial microsite (1), as water depth increases the amount of silt increases in accordance to normal particle deposition patterns.

Microsite 1 of the *E. fluviatile* site and microsite 2 contain around 20% gravel in their sediments. These two sites were on the shore of the reservoir. However, contrary to expected, silt fraction amounts do not increase with depth. Instead, sand fractions increase.

Microsite 1 of the *T. latifolia* site, just along from the *E. fluviatile* bed also has sediments composed primarily of sand with no gravel or pebbles, however microsite 2, over on the east shore line is primarily gravel and sand. Microsite 3 (the deepest site) has a large proportion of silt and pebbles in its sediments.

4.14 % water content

The results of the % water content of the sediments can be seen in Table 4.1.

The sediment at microsite 1 of the *P. australis* bed had almost 90% water content. All other sediments at all microsites have less than half this value.

In section 4.37 it can be seen that there is a positive relationship between percentage water content and percentage loss on ignition, which represents the organic component of the sediments. This suggests that the organic fraction has good water holding capacity within the sediment. The results also show that water holding capacity is not necessarily related to grain size, whereas it had been thought that those sediments composed of larger grain sizes would have had greater water holding potential, due to the presence of larger interstitial spaces.

4.15 % loss on ignition

Results for the % loss on ignition of the sediments at all the microsites at the *P. australis* stand, the *E. fluviatile* stand and the *T. latifolia* sites are shown in Table 4.1. Microsite 1 at the *P. australis* site has the greatest amount of organic matter. When compared to the other major sites the amounts of organic matter available in the sediments at the *E. fluviatile* site are lower. Possible reasons for these results are discussed later in section 6. *T. latifolia* microsite sediments have loss on ignition amounts comparable to those for *P. australis* suggesting they have similar decompostion rates and amounts of organic matter.

4.16 Summary

For each species there are too few results to make meaningful comparisons between the physical and chemical features of the microsites. Perhaps more replicates would have provided more meaningful results but they still provide basic information on the types of sediments the roots were growing in.

There seems to be no trends within the *P. australis* data, expect that as water depth increases sediments become more sandy.

Tentatively it could be said that in the *E. fluviatile* stand there seems to be a decrease in the % water content, pH and the amounts of pebbles, gravel and silt in the sediments as water depth increases. However, more samples are needed to make this certain.

T. latifolia sediments seem to have increasing amounts of pebbles and decreasing amounts of sand as water depth increases.

4.2 Chemical analysis

4.21 Total P and N/P ratios

Mean total P levels of all the microsites in the *P. australis, E. fluviatile* and *T. latifolia* stands are shown in Table 4.2.

Analysis of the results for *P. australis* gave highly significant differences in the total phosphorus (TP) of the sediments at microsites 1-4 ($F_{3\ 1}$=12.17.376). As T=57.80 only microsites 1 and 2, 1 and 3, and 1 and 4 at the *P. australis* site are significantly different (p=0.05). All the N/P ratios were extremely low, of the *P. australis* data microsite 2 is shown to be the most P limited.

Mean TP levels at the *E. fluviatile* site increase with increasing water depth and were significantly different ($F_{2\ 2}$=19.51, p<0.05), however the Tukey test showed only microsites 1 and 3 had significantly different levels (T=117.50, p=0.05). It was decided to collect plants for PMEase analysis from these sites even though microsites 1 and 2, and 2 and 3 were not statistically different regarding TP. Again the N/P ratios were very low indicating N is the limiting factor in the sediments, however microsite 2 seems to be the most P limited of the three microsites

There were also significant differences at the *T. latifolia* stand ($F_{2\ 2}$=157.38, p<0.05) with microsites 1 and 2 having significantly different TP levels (T=30.95, p=0.05). N/P values again indicate N-limiting conditions.

4.22 NO_3-N, NO_2-N and NH_4-N

The inorganic fractions of Nitrogen in the sediments gained from one determination are shown in Table 4.2. The data set was too small to test for significant differences, however results are presented to give an indication of the levels of N in the sediments.

All the microsites at the *P. australis* site have NH_4-N as their most abundant source of N. Micro site 1 has the lowest levels of NO_3-N, NO_2-N and NH_4-N available i.e. it is most N limited.

In microsites 1 and 3 of the *E. fluviatile* site, NH_4-N accounts for the majority of inorganic N available for plant growth. Microsite 2 is different in that a large proportion of the inorganic N exists as NO_3-N. These results are from one determination so very little can be concluded from them.

Again, at the micro sites for *T. latifolia* the most abundant N fraction is the NH_4-N. Microsite 1 has the least NO_3-N, whilst the other two microsites have comparable levels of NO_3-N. The NO_2-N fractions are the least abundant in all cases.

Table 4.2 Results of the chemical analyses for mean total P ($\mu g\ g^{-1}$) of the sediments ± standard errors and Mean total inorganic N ($\mu g\ g^{-1}$) / estimated mean TP ($\mu g\ g^{-1}$) ratio for the sediments in microsites 1-10.

Species	Microsite	Total P ($\mu g\ g^{-1}$)	NO_3-N ($\mu g\ g^{-1}$)	NO_2-N ($\mu g\ g^{-1}$)	NH_4-N ($\mu g\ g^{-1}$)	Sum of inorganic N ($\mu g\ g^{-1}$)	N/P
P. australis	1	228.98 ± 0.37	1.24	0.03	7.21	8.49	0.04
	2	6.88 ± 0.18	6.73	0.12	11.58	18.43	2.67
	3	8.44 ± 0.32	6.07	0.17	8.27	14.51	1.79
	4	17.58 ± 0.50	5.15	0.12	14.77	20.04	1.14
E. fluviatile	1	60.69 ± 1.02	3.54	0.12	23.65	27.31	0.45
	2	86.58 ± 0.83	16.15	0.61	13.25	30.01	0.35
	3	193.69 ± 0.57	4.28	0.33	11.32	15.93	0.08
T. latifolia	1	83.54 ± 0.56	13.82	0.33	13.21	27.36	0.33
	2	115.42 ± 0.23	19.34	0.67	12.16	32.17	0.28
	3	86.89 ± 14.15	9.76	0.51	11.08	21.34	0.25

4.3 Correlation analysis.

The whole data set was analysed for any correlations between the physical and chemical data, especially in the case of total P. Percentage water content and percentage loss on ignition were higly correlated ($r= 0.73$, $p<0.05$). Total P and water depth had a r value of 0.28, $p<0.05$. Total P and the % loss on ignition was also unrelated ($r=0.03$, $p<0.05$). Total P and the percentage water content were highly correlated ($r=0.76$, $p<0.05$), however the highest r value was obtained between pH and total P where $r=0.79$ ($p<0.05$).

5 ANALYSIS OF PHOSPHATASE ACTIVITIES

5.1 Phosphatase activity assays

The following results refer to the findings of investigations into the characteristics of the three study species root phosphatase activities and the heights of plants measured at each microsite. To compare the phosphatase activities of the roots it was necessary to standardise the experimental conditions. This was done for time, substrate concentration and pH. The effect of temperature on enzyme activity rates was controlled by running all assays at 25 °C. By-product limitation was not investigated.

5.11 The effect of time on phosphatase activities

The length of time an assay is run for must stay within the bounds of the linear phase of enzyme activity, when rate of reaction is directly proportional to time elapsed.

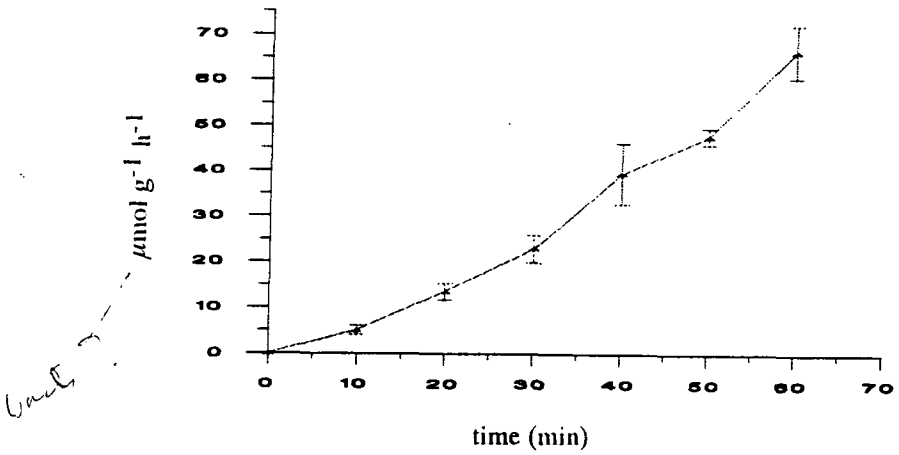

Fig. 5.1 Mean enzyme activity ± standard error (μmol g^{-1} h^{-1}) of *Phragmites australis* roots as a function of time (min).

The effects of time on the mean enzyme activity of *Phragmites australis*, *Equisetum fluviatile* and *Typha latifolia* roots are shown in Figs. 5.1, 5.2 and 5.3 respectively. In all cases there is a linear relationship between time and enzyme activity up to 60 min. The maximum PMEase activity measured after 60 min was for *P. australis* (70.6 μmol g^{-1} h^{-1}). *E. fluviatile* was second (4.6 μmol g^{-1} h^{-1}) and *T. latifolia* third (3.5 μmol g^{-1} h^{-1}). On

the basis of these results all assays with *P. australis*, *E. fluviatile* and *T. latifolia* roots were run for a maximum of 30 min.

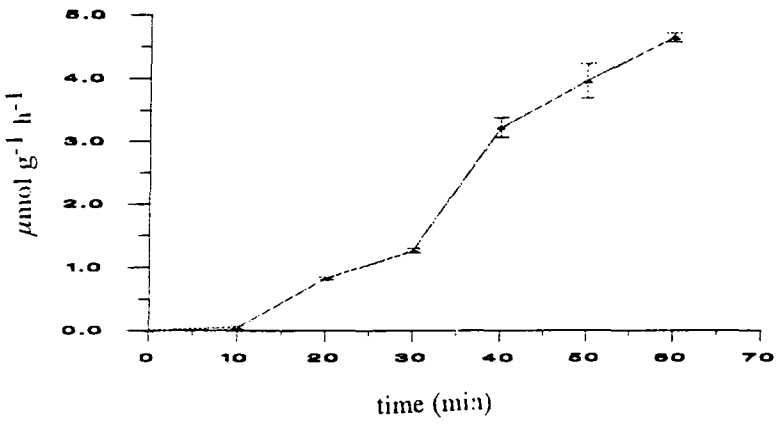

Fig. 5.2 Mean enzyme activity ±standard error (μmol g^{-1} h^{-1}) of *Equisetum fluviatile* roots as a function of time (min).

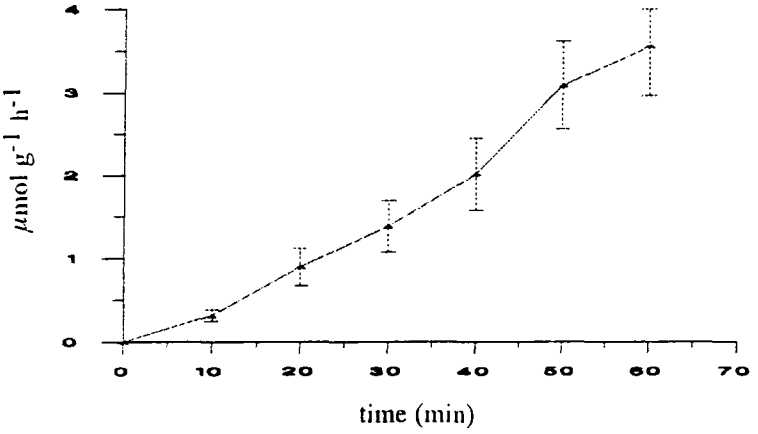

Fig. 5.3 Mean enzyme activity ±standard error (μmol g^{-1} h^{-1}) of *Typha latifolia* roots as a function of time (min).

5.12 Enzyme activity as a function of substrate concentration

Roots from each species were assayed at different substrate concentrations to see which concentration of substrate saturated the enzyme. This was to make sure all assays gave proportional results. All the curves suggest that, assuming there is a constant concentration of enzymes there is an increase in reaction rate with increasing substrate

concentration, which begins to reach an asymptote as maximum velocity is reached. The Michaelis-Menten equation was applied to each data set to calculate the K_m and V_{max} values seen in Table 5.1.

Table 5.1 K_m (μmol) and V_{max} (μmol g^{-1} h^{-1}) values for the phosphatases of the three species.

Species	K_m	V_{max}
P. australis	15.12	73.31
E. fluviatile	8.44	6.53
T. latifolia	9.50	9.53

P. australis has by far the highest maximum velocity of the three species and the greatest number of active sites, thus supporting the results in sections 5.11 and 5.12 where it was found to have the highest activities. The *P. australis* enzyme is therefore most efficient at hydrolysing the artificial substrate into available P_i and its organic moiety. *E. fluviatile* has the lowest V_{max} and K_m values yet in sections 5.11 and 5.12 it has slightly higher activities than *T. latifolia*. These results may reflect the better activity of *T. latifolia* plants at higher concentrations of 4-MUP as all other assays compared were run with 5 μM substrate. Although intriguing, these results were seen as subsidiary to the main aims of the study and so, because of time restraints were not investigated further. These results may help one to clarify and hypothesise on the results of further assays however, little can be extrapolated from these on to the environmental levels of phosphatase activity as it is not known exactly how the enzyme works under natural conditions.

Fig. 5.4 shows the results of changes in the concentration of substrate on the mean enzyme activity of *P. australis* roots. The maximum mean activity was 103.3 μmol g^{-1} h^{-1} at the 250 μM 4-MUP concentration. *E. fluviatile* roots gave results presented in Fig. 5.5. The mean maximum activity is 20.8 μmol g^{-1} h^{-1}, again much lower than the *P. australis* result at 250 μM 4-MUP.

T latifolia root enzyme activity appears to be tailing off at 100 μM 4-MUP (250 μM substrate was not used as not enough suitable roots were available on the chosen plants). The mean maximum activity reached was 9.3 μmol g^{-1} h^{-1} at 100 μM 4-MUP, the lowest activity of the three species at this substrate concentration. Therefore, even though higher levels of maximum activity are not found in *T. latifolia* the initial activity rate is faster, and the number of active sites is greater (from Table 5.1). Further assays for *P.*

australis, *E. fluviatile* and *T. latifolia* roots were assayed at 1 and 5 μM 4-MUP as neither saturated the enzymes and gave as close to the low P levels of the environment that accuracy would allow.

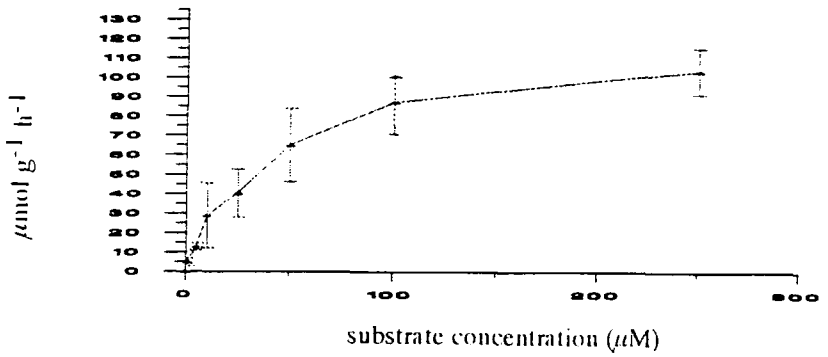

Fig. 5.4 Mean enzyme activity ± standard error (μmol g^{-1} h^{-1}) of *Phragmites australis* roots as a function of 4-MUP concentration (μM).

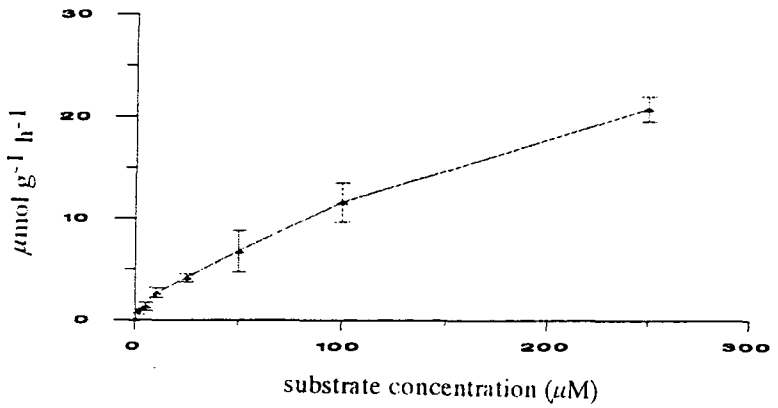

Fig. 5.5 Mean enzyme activity ± standard error (μmol g^{-1} h^{-1}) of *Equisetum fluviatile* roots as a function of 4-MUP concentration (μM).

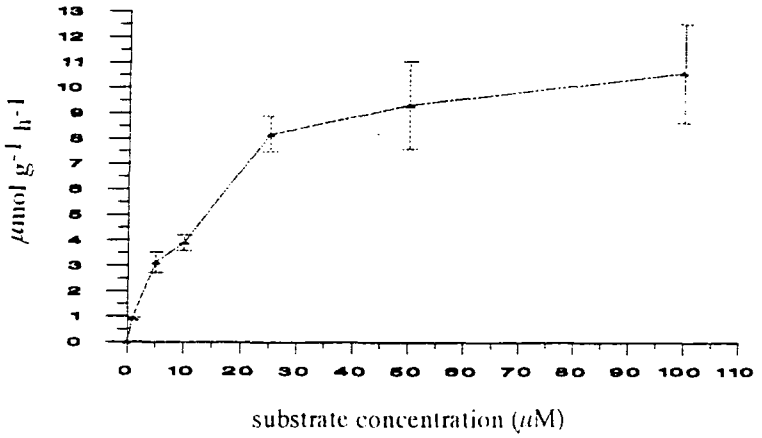

Fig. 5.6 Mean enzyme activity ± standard error (μmol g^{-1} h^{-1}) of *Typha latifolia* roots as a function of 4-MUP concentration (μM).

5.13 Influence of pH on activities at 1 μM and 5 μM substrate

To standardise pH of the medium the optimal pH for each species was determined. Two substrate concentrations were used, 1 and 5 μM 4-MUP to see if differences in amounts of available P affected the optimal pH and to allow comparison of after termination activity on the root surface. The after termination activity is shown as a % of the activity measured during the assay. This was hoped to indicate whether the after termination activity was biologically important to the apparent activity measured during the assay. If the activity measured was very small it was thought it could be regarded as product left on the root surface from the original assay. If however, the after termination activity was a large percentage of the original activity then other forces may be involved. Again, inadequate washing may be the culprit or the increased activity may be due to release of intracellular phosphatases as a result of a wounding response or the slow release of product from the substrate-enzyme complex. In Figs 5.7, 5.8 and 5.9 the after termination activity is shown by the lower line.

Figs. 5.7 (a) and (b) show the effect of pH on mean enzyme activity with 1 μM and 5 μM substrate for *P. australis* roots. The pH optima for both concentrations is pH 5.0 for the apparent enzyme activity (2.5 μmol g^{-1} h^{-1};4.4 μmol g^{-1} h^{-1} for 1 and 5 μM 4-MUP respectively). The mean activity after termination value was also greatest at pH 5.0 for both concentrations (0.3 μmol g^{-1} h^{-1};1.4 μmol g^{-1} h^{-1}). The after termination values as % of the apparent are shown in Table 5.2. In the 5 μM 4-MUP there was extremely high

after termination values at pH 9.0. As this result is not seen in the 1 μM 4-MUP this may be due to inadequate washing of the roots used. All the after termination activities are relatively large. pH 3.0, 5.5 and 6.5, have no subsequent activity in the 5μM substrate concentration assay. This may be due to the washing process rinsing all the substrate and product off. Alternatively it may be that the roots used at these pH's did not release intracellular enzymes or retain and slowly release product.

Table 5.2 Mean activity (μmol g^{-1} h^{-1}) after termination as a % of the apparent activity for *Phragmites australis* roots assayed with 1 and 5 μM of substrate.

pH	3.0	4.0	5.0	5.5	6.0	6.5	7.0	8.0	9.0	10.0
1 μM	10.8	9.4	56.1	44.3	2.5	1.9	55.4	0.5	1.7	0.0
5 μM	0.0	3.1	8.0	0.0	27.3	0.0	26.1	12.0	227.9	5.4

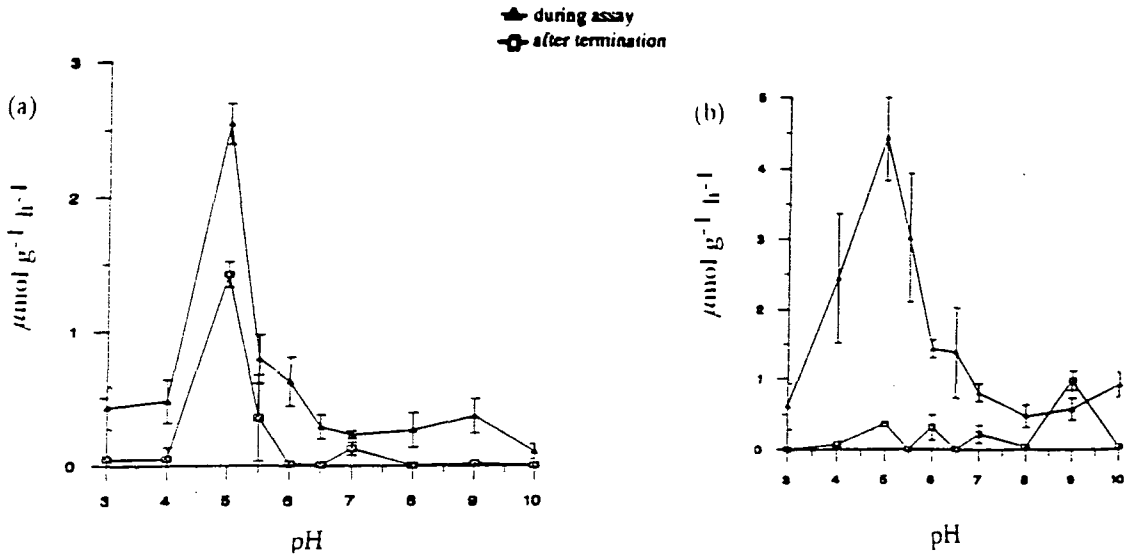

Fig. 5.7 A comparison of the influence of pH on mean enzyme activity ± standard error (μmol g^{-1} h^{-1}) of *Phragmites australis* showing apparent and after termination activities at (a) 1μM 4-MUP and (b) 5 μM 4-MUP.

The mean enzyme activity of *E. fluviatile* roots under these conditions had a pH optima of pH 5.5 (1.2 μmol g^{-1} h^{-1} for 1 μM 4-MUP and 5.9 μmol g^{-1} h^{-1} for 5 μM 4-MUP). The results are shown in Figs. 5.8 (a) and (b) for 1 and 5 μM 4-MUP respectively. At 1 μM 4-MUP the maximum after termination activity was also at pH 5.5 (0.7 μmol g^{-1} h^{-1}). However, it was pH 4.0 that had the greatest after termination value in the 5 μM 4-MUP

assay (0.6 μmol g^{-1} h^{-1}). In the 5 μM 4-MUP assay the activity measured after termination was mostly undetectable (Table 5.3). As the two different substrate concentration assays have such different after termination values it was concluded that the 1μM 4-MUP assay results were due to inadequate washing of the roots before transferral to new glass vials. It seems that *E. fluviatile* roots do not release product or enzymes to hydrolyse traces of remaining substrate after termination. Therefore, with further assays using *E. fluviatile* roots no further after termination activities were measured.

Table 5.3 Mean activity (μmol g^{-1} h^{-1}) after termination as % of the apparent activity for *Equisetum fluviatile* roots assayed with 1 and 5 μM substrate.

pH	3.0	4.0	5.0	5.5	6.0	6.5	7.0	8.0	9.0	10.0
1 μM	44.8	0.0	51.0	52.8	0.0	32.3	42.7	0.0	3.0	41.5
5 μM	23.7	6.3	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0

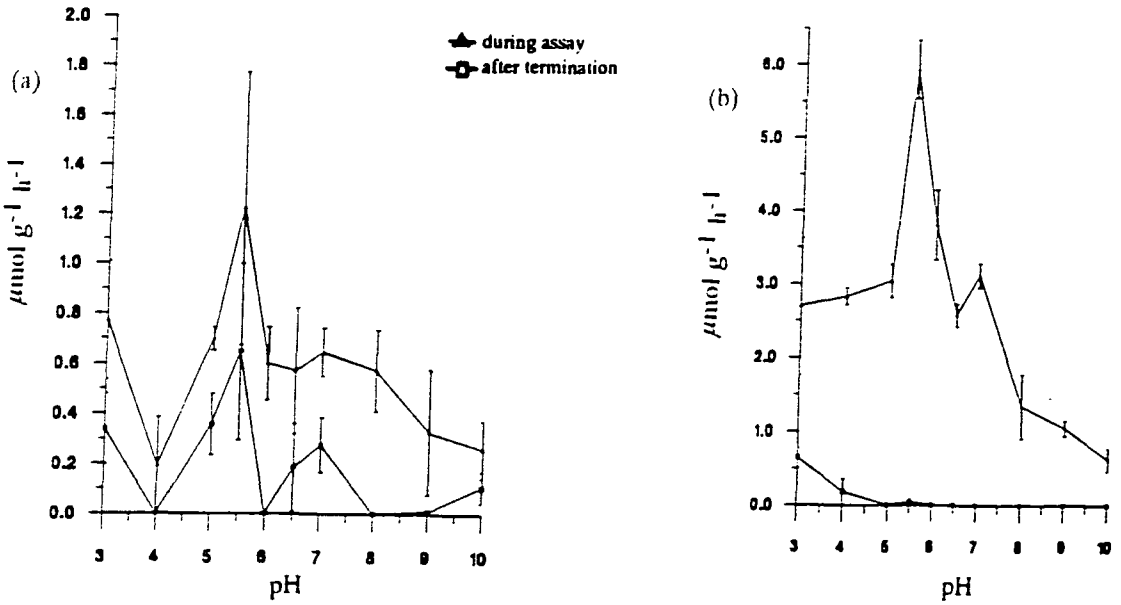

Fig. 5.8 A comparison of the influence of pH on mean enzyme activity ± standard error (μmol g^{-1} h^{-1}) of *Equisetum fluviatile* showing apparent and after termination activities at (a) 1μM 4-MUP and (b) 5 μM 4-MUP.

Figs. 5.9 (a) and (b) show the effect of pH on the enzyme activity of *T. latifolia* roots at both substrate concentrations for 1 and 5 μM 4-MUP respectively. The maximum activity was found at pH 6.0 for both assays (0.9 μmol g^{-1} $h^{-1}$for the 1 μM 4-MUP assay and 2.3 μmol g^{-1} hr^{-1} for the 5 μM 4-MUP assay). *T. latifolia* had the lowest mean activity recorded for the three species. The acid range has a very low activity when compared to the alkaline range. The after termination values were highest at this

optimum pH, indeed all were above 20% (Table 5.4). With 1μM 4-MUP very high activities were found after the termination at pH 3 and 9 and this was seen again when roots were assayed at 5 μM. As the after termination results are such a large proportion of the original activity, doubts were cast as to the accuracy of the assay in determining the true activity of *T. latifolia* roots. These roots were all washed as thoroughly as possible yet still product was released into the medium long after termination of enzyme was carried out. This may be due to the slow release of product from the substrate-enzyme complex or some characteristic of the root surface that 'traps' the hydrolysis product and releases slowly. In order to measure the true activity of *T. latifolia* root phosphatase from field samples the after termination activity would be added to the during assay activity and then tested for significant differences.

Table 5.4 Mean activity (μmol g^{-1} h^{-1}) after termination as % of the apparent activity for *Typha latifolia* roots assayed with 1 and 5 μM 4-MUP

pH	3.0	4.0	5.0	5.5	6.0	6.5	7.0	8.0	9.0	10.0
1 μM	210.8	78.7	57.2	31.7	69.2	32.4	94.3	28.8	124.0	119.0
5 μM	72.1	54.5	66.0	66.0	46.5	24.6	81.2	50.2	55.3	86.7

A linear regression was performed on the mean activity recorded during the 5 μM 4-MUP assay and the subsequent after termination activity. This produced a fairly robust regression coefficient of r=0.82, p<0.05 however no such relationship was found with the 1 μM 4-MUP results (r=0.06, p>0.05).

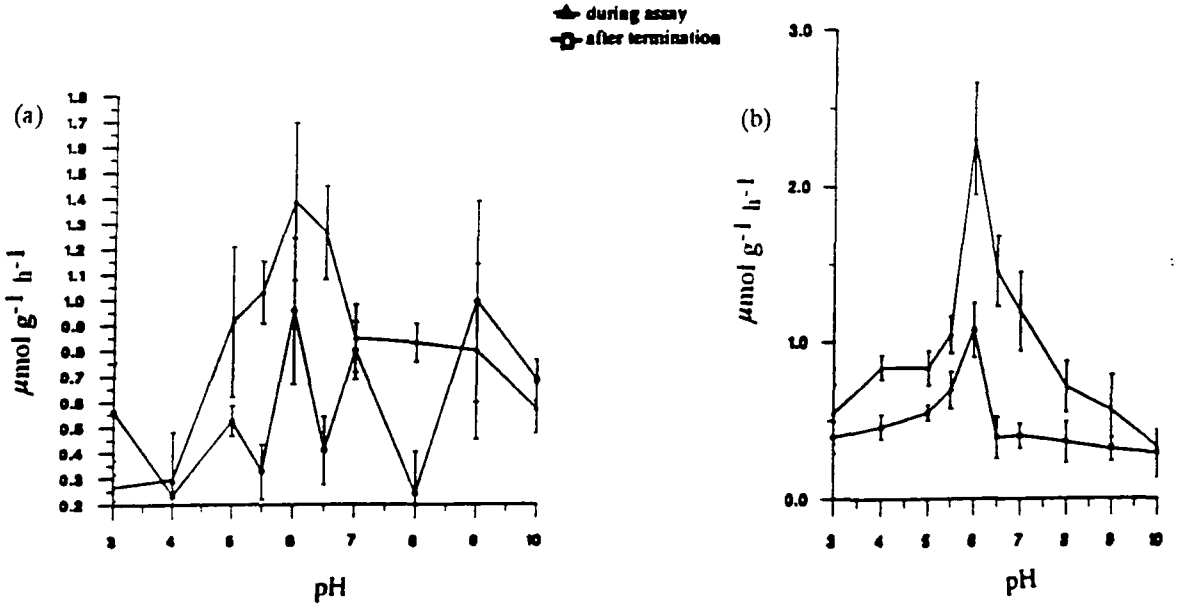

Fig. 5.9 A comparison of the influence of pH on mean enzyme activity ± standard error (μmol g^{-1} h^{-1}) of *Typha latifolia* root showing apparent and after termination activities at (a) 1μM 4-MUP and (b) 5 μM 4-MUP.

5.2 Comparison of phosphatase activities of roots from the 10 microsites assayed with 1 μM and 5 μM 4-MUP

Using the results gained in sections 5.1-5.4 the roots for plants collected in the field were examined for their phosphatase activities. After termination activities were measured for *P. australis* and *T. latifolia*, with 10 replications instead of 5 to reduce variability. Again the assays were done at two concentrations of 4-MUP (1 and 5 μM) to allow comparison. The results for this section can be seen in Table 5.5.

Assays at 1 and 5 μM substrate with roots from microsites 1-4 of the *P. australis* site showed significant differences between the sites regarding the PMEase activities ($F_{3\ 27}$=246.73, p<0.001; T=0.259 for 1 μM 4-MUP and $F_{3\ 27}$=9.33, p<0.001; T=4.67 for 5 μM 4-MUP). The 1 μM 4-MUP assay gave results that showed that all the roots from microsites 1-4 have significant differences but at 5 μM 4-MUP only microsites 2 and 3, and 4 and 2 were significantly different (p=0.05). The mean % after termination values for the roots were 31.25%, 29.31%, 32.00% and 35.13% for the 1 μM 4-MUP assay and 5.32%, 5.61%, 4.15% and 7.06% for the 5 μM 4-MUP for microsites 1-4 respectively. If added on to the apparent activity results the differences in PMEase activities are still significant (p<0.05).

Therefore it is possible to say that on the day of sampling all the microsites at the *P. australis* site have significantly different PMEase activities when assayed with both substrate concentrations.

E. fluviatile specimens had non significant differences in their PMEase activities between the microsites at each concentration ($F_{2\ 18}$=2.04 for 1 μM 4-MUP and $F_{2\ 18}$=0.761 for 5 μM 4-MUP).

Non significant differences between the PMEase activities were also found at the *T. latifolia* sites when assayed at 1 μM 4-MUP ($F_{2\ 18}$=0.834 , p>0.05). However when assayed at 5 μM 4-MUP there were significant differences found between the *T. latifolia* results ($F_{2\ 18}$=4.99, p<0.05). Microsites 1 and 2 and 1 and 3 were significantly different from each other (T=2.30, p=0.05). It was found that the after termination activities for the roots assayed at 5 μM 4-MUP were also significantly different ($F_{2\ 18}$=4.41, p<0.05). Again, T=2.30, making microsite 1 significantly different from microsites 2 and 3 with regars to their after termination activites. The average after termination values as % of the original activity were 41.96%, 31.945 and 37.84% for microsites 1-3 respectively.

The amount of after termination activity measured was added to the amounts measured during the assay and another ANOVA performed. This also showed that when the 'total'

amounts of activity were considered there were significant differences between all the root phosphatase at microsites 1-3 of the *T. latifolia* bed when assayed using 5 μM 4-MUP ($F_{2\ 18}$=9.76, T=2.90, p<0.05).

Table 5.5 Mean enzyme activities ± standard error (μmol g^{-1} h^{-1}) of the roots of plants from microsite 1-10 assayed with 1 μM and 5 μM 4-MUP .

Species	Microsite	Mean activity at 1 μM	Mean activity at 5 μM
P. australis	1	0.89 ± 0.13	3.38 ± 0.37
	2	4.02 ± 0.18	7.74 ± 1.77
	3	0.44 ± 0.04	1.73 ± 0.15
	4	0.33 ± 0.06	1.48 ± 0.26
E. fluviatile	1	0.15 ± 0.04	0.27 ± 0.07
	2	0.08 ± 0.04	0.19 ± 0.04
	3	0.06 ± 0.02	0.12 ± 0.04
T. latifolia	1	2.36 ± 1.06	9.70 ± 2.17
	2	1.23 ± 0.48	2.80 ± 0.86
	3	1.32 ± 0.26	5.00 ± 0.94

5.3 Results of microscopic examination of root tissue

5.31 Effect of pH on cell integrity

Efforts to observe the effect of the extreme high or low pH buffers on the integrity of the root cells apparently showed no cell lysis, even after 1 hour. It seems extreme pH conditions do not induce cell lysis. However all roots used had a minimum of 2 cut edges and the after termination activity recorded may be due to unterminated phosphatases exuded from the wounded surfaces.

5.32 Localisation of root enzyme activity

Staining of the roots using Napthol As-Mx phosphate stain was done on several occasions to locate PMEase activity within the root. However, *P. australis* and *T. latifolia* did not show any uptake of the stain and *E. fluviatile* roots were so highly pigmented no stained areas could be identified.

5.33 Examination of root surface for algal and bacterial colonies

When material was examined under fluorescence, there was no sign of any large bacterial colonies, or large masses of algae or fungal mycelium was seen suggesting that the enzyme activity measured was of eukaryotic, rather than prokaryotic, origin.

6 Discussion

6.1 Properties of the sediments

The *Phragmites australis*, *Equisetum fluviatile* and *Typha latifolia* microsites chosen covered a wide range of environmental features and their implications for plant growth are discussed in section 6.12. The properties of the three species phosphatase enzymes are discussed in section 6.22.

6.11 Nature and chemical properties of the sediments

Water depths on the three stands ranged from 0-0.45 m deep and in most cases were significantly different between microsites.

The 10 microsites also differed in the amounts of organic matter their sediments contained. It is known that soil organic matter is an important nutrient store in wetland soils (Neely, 1994) and must play an important role in plant nutrition. It was hoped it could be related to the TP (total phosphorus) of the sediments. Soil organic matter is derived primarily from living roots and their exudates, microflora and fauna, and decaying plant and animal remains (Malcolm and Vaughan, 1979). The amounts of organic matter deposited will depend on the abundance of living material within that environment and the mortality and decomposition rates. These factors in turn should reflect the physical and chemical parameters of the environment. Microsite 1 of the *P. australis* stand has the highest amounts of organic matter. The low decomposition rate of this species relates to the fact that this stand has the greatest proportion of organic matter (Mason and Bryant 1975). From general observations in the field microsite 1 had greater diversity of flora (and so perhaps fauna) than the other microsites at the P. australis stand as it formed part of a community composed of other wetland plants such as *Filipendula ulmaria*, *Galium palustre*, *Deschampsia cespitosa* and *Potentilla erecta*. These species will add to the litter load thus increasing the organic content of the soil whereas microsites 2-4 will have no additional material. The high pH of the calcareous flush may also effect the organic matter content, perhaps by inhibiting its breakdown. However, this is purely conjecture and requires further study.

The *E. fluviatile* bed had the lowest amounts of organic matter in their sediments which is in accord with the findings of Sjöberg and Danell (1975), who demonstrated this species to have a high decomposition rate due to the high rate of decay of the relatively unligninifed *E. fluviatile* tissue. As *E. fluviatile* was growing in dense monospecific stands there would be no additional plant litter, except perhaps that

blown on to the shore for deposition within the sediments. This finding is further substantiated by the amounts of organic matter found at *T. latifolia* microsite 1. Although only a few metres away from the *E. fluviatile* bed the loss on ignition for this site is higher. *T. latifolia* is reported to have a half life for decay of between 133-704 days (Neely, 1994).

When results from the 10 microsites were pooled however, there was no clear relationship discovered between TP and organic matter, suggesting that TP is affected by other variations in the sediments.

It is known that grain sizes of the sediments will effect the amounts of dissolved nutrients available to plants (Bannister, 1976) and it was hoped that the sediments collected would be generally similar in their grain size fractions and that this variable would be controlled for. However, grain size distribution of the sediments exhibited much variability. At the *P. australis* stand microsite 1 was extremely silty, whereas microsite 2 was pebbly and the other microsites primarily consisted of sand (indicating the normal shore-water distribution). Grain size proportions for the *E. fluviatile* bed mostly fall within the sand-silt fractions though microsites 2 and 3 also have around 20 % of their sediments as gravel. These high amounts of larger fractions may be due to the relative closeness of the Kielder Burn inflow. During times of flood or heavy rain it is possible that a lot of debris is washed into Bakethin and deposited in this region. To overcome this variability subsequent analysis of TP was carried out on sieved sediments.

Microsite 1 of the *P. australis* stand had the greatest amount of TP measured. This result conforms to the findings of other workers, who suggest that a high proportion of fine particles and a lot of organic material will increase the P levels, and its availability and solubility in soils and sediments, through the processes of reduction and chelation (Shapiro, 1958). This is especially true in waterlogged soils where the transformation of organic P into inorganic, usable, P will be negligible without the presence of organic matter. It is important to note that there is a decline in the TP levels at microsite 2 on the shore of the reservoir but this was not correlated to any of the other physical parameters measured.

Total P levels of the *E. fluviatile* site were much higher than those in the *P. australis* sites 2-4, but generally within the range of the TP levels at the *T. latifolia* sites. The *E. fluviatile* site did not have significantly different TP levels, however, there is an increase in total P levels as water depth increases (a trend also found in the *P. australis* data).

Microsite 2 of the *T. latifolia* site has significantly higher concentrations of TP than the other microsites at these stands. This is a slightly confusing result as microsite 1 is the siltier site and has more organic matter when compared to microsite 2. The

differences may lie in the differences in pH between microsite 1 and microsites 2 and 3. Statistical analysis of the pH values have shown them to be non significantly different, but highly correlated to the amounts of TP in the sediments. It may be that a greater number of replicates would indicate significant differences between the microsites that in turn can be used to explain the TP results.

NH_4-N was the most abundant source of inorganic N in the majority of sites. In microsites 1 of the *T. latifolia* and *E. fluviatile* stands, NO_3-N is most abundant indicating that oxidation of inorganic nitrite is still possible around the shores of the reservoir. Similar results are not seen in microsite 1 of the *P. australis* stand, perhaps due to the high water content of that sediment, it also had the lowest amounts of NO_3-N, NO_2-N and NH_4-N of all the microsites.

As seen in section 1.21-1.24 P concentrations in a typical soil are usually very low and the N/P ratio of total inorganic N and total P fractions was intended to determine which was most limited. The results suggest that all the sediments are essentially highly N-limited. Similar results have been found from other sediments collected around Bakethin by V. J. Mattin (pers. comm.). The results may not be entirely accurate as not all fractions of N were measured and TP levels may over estimate the amounts of available P. However, despite this, the N/P ratios calculated were used to compare the relative availability of nutrients at each microsite. Microsite 1 of the *P. australis* stand appears to be the most N-limited microsite and the surrounding vegetation also suggests N-limiting conditions as it consists of two species of carnivorous plants (*Drosera rotundifolia* and *Pinguicula vulgaris*) which can indicate low N environments (Slack, 1979). The N/P ratios of the other microsites at the *P. australis* stand are higher indicating that P was beginning to be more limited, yet the overriding limiting nutrient continues to be N. Microsite 2 has the highest N/P ratio, so it is these plants that have the greatest chance of being P limited.

The *E. fluviatile* site was found to be more N limited than the *P. australis* sites as it has higher amounts of TP. As the N/P ratios change at the *E. fluviatile* stand it was decided to assay the root enzyme activities to investigate the possibility of any differences that may be related in some way to the TP values or with the other environmental parameters measured.

Microsite 1 of the *T. latifolia* bed has the highest N/P ratio of these microsites although the value is still low and suggestive of N-limiting conditions. As the N/P ratio declines from microsites 1-3 it was hypothesised that microsite 1 plants may have the highest PMEase activity.

Enzyme kinetics are highly affected by the pH of their surroundings and so if the pH of the sediments were found to be significantly different then this could be an explanation of any differences in the activity rates of the phosphatase enzymes. However, the selected sites did not differ significantly in pH and this variable cannot be used to explain differences in the PMEase activities.

6.2 Standardisation of phosphatase assay

6.21 Standardisation of assays

Once the microsites had been shown to have significant differences in the physical and, more importantly, chemical variables measured the next task was to begin to relate these differences to the PMEase activities of the three study species.

6.22 Influence of variations in time, substrate concentration and pH

All the species show that if substrate and enzyme concentration is kept constant, enzyme activity increases roughly linearly with time, with no sign of end product inhibition before 60 min. This time scale has also been found in other phosphatase studies on animal derived enzymes (Fernley and Walker, 1965).

The influence of substrate concentration on enzyme activity was examined and the results are seen in section 5.2. Theoretically, the use of the Michaelis-Menten equations to characterise a possible suite of enzymes that utilise the same substrate (as is perhaps the case in the phosphatase assay) is incorrect. The K_m value obtained from the Lineweaver-Burke plot may represent the enzyme with the lowest K_m and the highest saturation level or the properties of 2 or more enzymes (Jansson *et al.* 1988). Nevertheless, though aware of these limitations it is most practical to characterise the group of enzymes for each species by the calculation of a Michaelis-Menten equation.

From the results it can be seen that *P. australis* has the greatest V_{max} and K_m values (73.3 μmol g^{-1} h^{-1} and 15.1 μmol respectively) of the three species assayed, indicating that this enzyme has the lowest affinity for the substrate used and the highest maximum velocity.

If the results are assumed to be a reflection on the properties of the enzymes in nature they indicate that roots of *P. australis* when assayed at these concentrations do not have enzymes with a high affinity for their substrate and so are working in environments with plentiful P. This is followed up by the results in section 4 which suggest N is most limited. Perhaps there is no need for the roots to hydrolyse organic

P to supplement the plants nutrient budget. The other results in section 5 indicate that at all other times *P. australis* also has the highest activity of the three species tested.

For *E. fluviatile* the K_m value (8.4 μmol) is the smallest suggesting that its enzymes have the best affinity for 4-MUP. This is backed up by the fact that the enzymes have the lowest maximum velocity (6.5 μmol g^{-1} h^{-1}). If *E. fluviatile* enzymes are better at combining in an enzyme-substrate complex the velocity of reaction need not be high.

T. latifolia roots have, on this occasion the second lowest K_m and V_{max} values (9.5 μmol and 9.5 μmol g^{-1} h^{-1} respectively).

In the time and substrate assays, *T. latifolia* roots have lower mean enzyme activities when compared to *E. fluviatile,* however, during the standardised assays *T. latifolia* has a greater activity that *E. fluviatile.* The standardised assays were carried out using material collected form microsites 1 of both stands, and comparison of the data show that the roots of *E. fluviatile* have the highest activity on one occasion and on another *T. latifolia* had the highest activity. Subsequently, the conclusion can be made that perhaps seasonal and climatic factors can effect the phosphatase activity of roots. This has been reported by Bresnan (1993) who found that the phosphatase activities of various algal species were affected by temporal and climatic variations.

During this study the possibility of temporal and climatic effects were excluded as far a possible, however it seems their effects could not be totally controlled for.

The Km values for each species (1.5 x 10^{-5} M, 6.2 x 10^{-6} M and 9.5 x 10^{-6} M for *P. australis*, *E. fluviatile* and *T. latifolia* respectively) fall within values quoted for most enzymes (10^{-6}-10^{-4} M; Stryer 1988). Overall the K_m and V_{max} values calculated are higher than others quoted in the literature from assays using 4-MUP as a substrate. Lower K_m values have been recorded by Hantke and Melzer (1993) for the diatom *Synedra acus* (0.09-0.13 μM), and the total amount of alkaline phosphatases in unfiltered water from Lake Gårdsjön, Sweden (0.6 μM; Jansson *et al.* 1981). Triphasic alkaline phosphatases of an oceanic dinoflagellate *Pyrocystis noctiluca* have been shown to have K_m values between 0.1-222 μM (Rivinkin and Swift, 1980).

Healey and Hendzel (1979) noted in a study of algal alkaline phosphatases that using pNPP as a substrate gave much higher K_m values than MFP (*o*-methylfluorescein phosphate) and thus, MUP (Hantke and Melzer, 1993). Examples of K_m values obtained using pNPP are given in Whitton, *et al.* (1990) who calculated the K_m value of *Nostoc commune* UTEX 584 a blue-green alga to be 8.7 μM for cell bound PMEase and 37 μM for cell bound PDEases using pNPP and bis-pNPP as substrates. Grainger *et al.* (1989) calculated K_m values of 43 and 33 μM for cell bound and extracellular activities of *Calothrix parietina* using pNPP.

It is often common when calculating the Michaelis-Menten equation to use two or more substrates for comparison. This would give an indication of the presence of a suite of enzymes and show how different substrates affect K_m and V_{max}. Comparison of the activity measured using other commonly used substrate pNPP was not done due to time restrictions.

All three species have PMEases with optimal pH's in the acid range and this was stable for all species at both 1 and 5 μM 4-MUP. This is in accord with the findings of McLachlan, (1980) who suggests that to play an effective role in the supply of inorganic P phosphatases should be capable of working at the pH of the environment and it is probable that the rhizosphere will be acidic rather than alkaline (Etherington, 1975) due to the net negative charge in the roots, root respiration products and the effects of the uptake of ions. pH optima of enzymes within the acid range have also been found in other work on higher plant phosphatases (Lee, 1988; McLachlan, 1980; Gabbrielli *et al.* 1989).

Although the sediments collected at Bakethin were acidic there seems no clear relationship between environmental pH and the pH optima of the enzymes. This disparity has also been reported in other studies (Luff, 1993; Stevenson, 1994).

Hantke and Melzer, (1993) suggest that changes in pH optima at different low concentrations of substrate may be an adaptation by the organisms to have a readily adaptable phosphatase system whose action is relatively unchanged by variations in environmental pH. The occurrence of 'shifting' pH optima was investigated by the comparison of the enzyme at relatively low concentrations of 4-MUP.

P. australis roots assayed at both substrate concentrations had a pH optima of pH 5.0. Luff (1993) found this species to have a pH optima of 4.0 when assaying roots with 100 μM pNPP however, comparison of these results is difficult as studies on the alkaline phosphatases of rat intestines have shown that the pH optima of the enzymes can change with the substrate concentration, the type of substrate and the buffer used (Ross *et al.* 1951). Also contrary to Luff (1993) there were no alkaline peaks apparent during these assays. Extreme alkaline and acid conditions gave similar activities of the enzyme for both substrate concentrations although the 1 μM 4-MUP graph had a sharper line, with the optima more pronounced.

The optimal pH of *E. fluviatile* roots was found to be pH 5.5 for both the 1 and 5 μM 4-MUP assays. Again, no alkaline peak was seen within the pH spectrum but there was the suggestion that activities measured from the acid range were lower than those in the alkaline range.

T. latifolia plants were found to have an optimal pH of 6.0, at both 1 and 5 μM of 4-MUP. At both substrate concentrations there was very low activity at the below optimal pH. With 1 μM substrate the PMEase activity was relatively high in the alkaline range, but with 5 μM substrate the peak at pH 6.0 was sharply defined. It was thought that this could be due to detection errors at the lower concentrations. Luff (1993) assayed *T. latifolia* roots with 100 μM pNPP and found that the optimal pH was 5.0. This may be as a result of assaying roots with different substrate and higher concentrations but more work is needed totest this hypothesis.

There seems little evidence for a range of pH optima within the phosphatase enzymes at these low concentrations, perhaps smaller concentrations still, need to be used to detect such properties.

Studies by Luff (1993) have questioned the validity of the phosphatase assay technique as there was measurable reaction product being produced after the termination of the experiment (section 1.6, 2.63 and 5.13). Luff (1993) suggested that after hydrolysis of pNPP, the product formed (pNP), is either retained by *T. latifolia* roots through chemical combination with the root surface, or product is being formed as a result of intracellular enzyme activity. It is known that no such retainment or 'leakage' (from now on termed after termination activity) has been found in studies on *Rivularia* (Yelloly pers. comm.) and aseptically grown higher plants (Baloch pers. comm.). However, preliminary experiments showed there was retainment of hydrolysed product after assays with 4-MUP by the roots of the three species. An investigation into the after termination activity was carried out at both 1 and 5 μM 4-MUP in order for comparison of results.

In the case of *P. australis* the mean after termination results for 1 μM 4-MUP peak at pH 5.0 and were generally below 10% of the original activity measured. There was a slight increase in the mean amount of activity at pH 7.0. Both of these results may be the effect of pH, characteristics of individual roots, or that the pH optima conditions produce an excess of product that is released after termination. The results obtained from the 5 μM 4-MUP assay are similar in some respects. Again the mean after termination activity optima is at pH 5.0, corresponding to the greatest mean PMEase activity reached during the assay, however, there was a peak at pH 9.0 that was greater than the original activity, even when standard errors are taken into consideration. It is difficult to attribute these findings to one factor, as all of the ones already mentioned could apply. Discounting this result though, there seems to be some sort of proportional relationship between the original activity and the amounts of

product retained. As it was apparent in both the 1 and 5 μM 4-MUP assays it was concluded that it cannot be due to rogue results or the individualistic properties of the roots used.

Measurable amounts of retained product or further activity from intra cellular enzymes were determined after *E. fluviatile* assays. From section 5.12 it can be seen that *E. fluviatile* roots have the lowest K_m value and thus the highest affinity for substrate. This may mean that at low concentrations the enzyme-substrate complex may be bound very tightly to the root surface and the full amount of product is not released into the medium for measurement. An indication of this is given in the results of section 5.13, as overall the after termination values for *E. fluviatile* were generally small, especially in the 5 μM 4-MUP assay. As very little after termination activity was measured after 1 h and due to time constraints it was concluded that this was not to be investigated further (see section 6.5).

In accordance to the findings of Luff (1993) *T. latifolia* appeared to retain a high proportion of the reaction product. With 1 μM 4-MUP assay, there seems no pattern in the after termination activity except that with one pH it will be high, the next pH it will be low. It may be that the K_m values and thus the affinity of the enzyme to the substrate, vary with pH as found in Hantke and Melzer (1993).

With 1 μM substrate the enzymes appear highly variable and were perhaps showing a selection of pH optima for the purposes suggested earlier. There seems no relationship between the amount of after termination activity and the activity measured during the assay. The extreme pH's had very high activities and it was thought that this could be due to lysis of the cells and release of intra cellular PMEases under the abnormally extreme pH conditions. When examined microscopically, though, there was no sign of any cell break up, even after 2 hours. In the 5 μM 4-MUP assay the after termination activity appears to be directly proportional to the amount of original activity measured, with the largest amounts occurring in roots assayed at pH 6.0. Clearly , as the two substrate concentrations do not show comparable after termination relationships it is difficult to surmise on the predictability of the amounts of substrate retained. More work will have to be done to try and explain this phenomenon.

6.3 Determination of PMEase activities in the field.

When assayed under standard conditions there were found to be highly significant differences between the PMEase activities of *P. australis* plants growing along the environmental gradient selected. When the after termination activity was calculated and added to the original amounts there were still highly significant differences between the results. It was concluded that the differences in enzyme activity must be

due to the plants' responses to the varying environment, as retainment or activity from intracellular enzymes did not change the results.

The amounts of organic matter in soil has been shown to affect PMEase activities. Malcolm and Vaughan (1979) showed that amounts of soil organic matter reduced the amounts of *Hordeum vulgare* (barley) phosphatases, especially the lower molecular weight components of the humic acid fractions. However, microsite 1 has been shown to have the greatest amounts of organic matter in its sediments yet is rated second amongst the 4 microsites. The sequence seems to be reflecting that found for the TP and not that of any of the other environmental parameters measured e.g. water depth or N-availability, so it was concluded that the plants were responding to the relative availability of phosphates. This agrees with the findings that there can be increased activities of PMEases in low P conditions (Boutin *et al.* 1982; Press and Lee 1983; Ascencio 1994). The PMEase activities of the plants at the *P. australis* sites were rated as follows:

Microsite 2 PMEase >>	Microsite 1 PMEase >	Microsite 3 PMEase >	Microsite 4 PMEase
lowest TP and	highest TP and		
highest NO_3-N and NH_4-N	lowest NO_3-N		

The *P. australis* plants with the highest PMEase activities are found in microsite 2, which has the lowest TP concentrations at this site and has a N/P ratio that indicates the most P-limited conditions. In the light of recent findings it seems the levels of N in the environment may also affect the amounts of phosphatase (Baloch, pers. comm.). If this is indeed the case, higher plants may also have high phosphatase activity when grown in low N conditions. This is born out by the results given above as even though microsite 2 of the *P. australis* site has high TP levels plants collected from that microsite also have the second highest mean phosphatase levels. This may be due to the extreme N limiting conditions at this microsite and may indicate phosphatase activity is a widespread response to nutrient limiting conditions and is not linked inextricably to P concentrations.

Clearly more experimental work is required to try and separate the possible interactions of low P and N on levels of phosphatase activity in higher plant roots, perhaps in controlled laboratory conditions.

The three microsites at the *E. fluviatile* stand were found to have non significantly different TP levels and so somewhat unsurprisingly the plants all have non significantly different PMEase activities. It appears that differences in other environmental variables do not alter the phosphatase results. An apparent non significant trend is

seen though in that PMEase activity decreases as water depth increases and P availability will increase (Shapiro, 1958). This suggests that the *E. fluviatile* plants are responding to the levels of TP in the environment but the experimental locations selected were not different enough to show this clearly. Results in Table 4.2 show that microsite 2 has very much higher levels of NO_3-N and so if these phosphatases do respond to low levels of available N then we might expect significant differences between the phosphatase activities determined for microsites 1 and 2 or 2 and 3. However, this is not the case and so we can conclude that *E. fluviatile* phosphatase activity rates cannot be correlated to amounts of NO_3-N in the environment.

If phosphatase activity is linked to amounts of P in the environment alone, then the question should be asked "why should *E. fluviatile* plants be producing enzymes that increase the availability of P when the amounts of P in the environment seem plentiful?" There are a number of possible answers. Firstly the determination of the TP concentrations of the environment may be an over estimation of the amounts actually available and so *E. fluviatile* plants require the action of phosphatases to supplement the amounts of biologically active P in the environment. Secondly it has been shown in some species that heightened PMEase activity is associated with old roots (Boutin *et al.* 1981). If this the case, perhaps this activity can be attributed to old roots that have been exposed to low P conditions earlier in the season. Finally it may be that the phosphatase activity is due to the detection of low level 'background' activity which is used by *E. fluviatile* plants to gain a competitive advantage over the other wetland species growing around the reservoir which have smaller PMEase rates yet to be determined.

For *T. latifolia* there were no significant differences between the microsites at 1 μM 4-MUP, even when the 'total ' values (original plus that activity measured after termination) were used. However, at 5 μM 4-MUP, there were significant differences between microsites 1, 2 and 3. The amounts of NO_3-N are smallest at microsite 3 and if N-limiting conditions induce phosphatase activity then we might expect plants from this microsite to have the greatest amounts of phosphatase activity, however, the greatest activity is found at microsite 1 which has the smallest amounts of TP. These results suggest that phosphatase activity is linked to the levels of TP in the environment and not NO_3-N. Consideration of the total amounts of activity change the result and it was found that there were significant differences between all the microsites at the *T. latifolia* stand. It is not known if the after termination activity is important to the result of the assay or can be discarded as the detection of intracellular activity. The main conclusion is that *T. latifolia* would not be a good species to use as an indicator of nutrient status until the source of this 'extra activity' is traced.

6.4 Use of phosphatase assay as an indicator of nutrient status

Throughout this project various problems have surfaced that have implications on the suitability of the phosphatase assay as an indicator of the nutrient status of the surroundings. The extrapolation of results to nature is difficult as the assay conditions themselves may control the response of the enzymes, and the levels of P provided by artifical substrates used may not be representative of conditions in nature. Extrapolation is also difficult as the assays are carried out at pH levels and temperatures not found in nature and in the presence of light.

The majority of the papers written on the subject which deal mostly crop plants (or other species that are economically important) that have been grown in aseptic, laboratory conditions (Ridge and Rovira, 1971; Ueki and Sato, 1971; Pammeter and Woolhouse, 1974; McLachlan, 1980; Boutin *et al.* 1981; Lee, 1988). There is some doubt that these plants reflect natural levels or conditions.

To overcome this, this current study involved the use of material that was growing in the wild, in the style of Press and Lee, (1983) and Kroehler and Linkins (1988) but this in turn has its associated problems.

There are those that believe that the PMEase activity measured from field specimens is linked to that of epiphytic micro organisms living on the surface of the roots. Muelemans (1988) reported that diatoms and other macroalgae colonise *Phragmites* litter. Evidence for this was seen at the *P. australis* bed as various mats of blue-green algae and *Chaetophora elegans* were seen growing upon the submerged stems. To prove the phosphatase activity is of plant origin all the roots assayed were examined for bacteria and algae after careful washing with assay medium. No evidence of large colonies of micro organisms were seen. Identification of the localisation of the PMEase activity was unsuccessful as it was very difficult to see stained regions in *P. australis*, *E. fluviatile*, and *T. latifolia* roots as all seemed to take up very small amounts of the stain or were highly pigmented.

6.5 Recommendations for further work

The results of this study show the intricacies of attempting to relate root phosphatase activities to their surrounding sediments. *P. australis* seems to be the only species that could be recommended for further study as an indicator of the nutrient status of the sediments, but more research is needed on various aspects of the theoretical and practical features of the assay.

As already mentioned in section 1.24, P deficiencies are often only seen when there has been the addition of that element to the ecosystem. Therefore the addition of P

(and N) compounds on the environmental gradients selected at Bakethin Reservoir is suggested to enable monitoring of the responses of plants to their environment regularly. This would also facilitate the construction of a more complete picture on the relative importance of phosphatase activity in supplementing available Pi concentrations and on the effect of temporal and seasonal variations within the plants and the sediments.

The results obtained concerning pH optima of the enzymes and the pH of sediments are also topics that require further attention. Knowledge on the enzymes ability to adapt to changes in pH has important implications within both practical and theoretical aspects of lake acidification produced by coniferous planting and atmospheric pollution.

Attempts to localise the phosphatase activity in the roots of the three species may be more rewarding if done on specimens grown in vitro to produce cleaner roots.

Finally in spite of the drawbacks already mentioned concerning laboratory experiments they may have a use in determining the properties of phosphatase activities under controlled P and N levels.

SUMMARY

1. The aim of the project was to relate the activities of the roots of representative populations emergent macrophytes with the composition of their associated sediments at a mesotrophic upland reservoir.

2. Three species were chosen for study; *Phragmites australis, Equisetum fluviatile* and *Typha latifolia.*

3. Characterisation of the physical properties of the sediments produced varied results. Percentage water contents were positively correlated to the percentage loss on ignition ($r=0.73$, $p<0.05$) and total P levels ($r=0.76$, $p<0.05$). Total P was also found to be positively correlated to the pH of the sediments ($r=0.79$, $p<0.05$).

4. Total P differed significantly ($p<0.01$) across the microsites at the *P. australis* bed with microsite 1 having significantly higher levels than the others. Microsites 2 had the lowest total P but did not differ significantly from 3 and 4. Total P differed significantly between microsites 1 and 3 ($p<0.05$) at the *E. fluviatile* stand, and at the three *T. latifolia* microsites only microsites 1 and 2 were significantly different($p<0.05$).

5. In most cases NH_4-N was the most abundant source of N indicating waterlogged conditions in the majority of sites.

6. The N/P ratios of the environment were extremely low indicating highly N limited conditions.

7. Problems of accuracy of the phosphatase assay highlighted by other workers were investigated. There seemed to be the release of product after the enzyme reaction was terminated due to either further intracellular enzyme activity or retainment of product by the roots that was slowly released. *P. australis* did not produce consistent results when assayed at two substrate concentrations and there seemed no consistent effect of pH. *E. fluviatile* did not produce high levels of after termination activity. *T. latifolia* roots showed consistently high after-termination activity.

8. *P. australis, E. fluviatile* and *T. latifolia* roots had a pH optima of pH 5.0, 5.5 and 6.0 respectively. The V_{max} and K_m values of the enzymes were determined. *P. australis*

roots had the greatest K_m and V_{max} values (15.1 μM and 73.3 μmol g^{-1} h^{-1} respectively) *T. latifolia* came second (9.5 μM and 9.3 μmol g^{-1} h^{-1}) with *E. fluviatile* third (8.4 μM and
6.5 μmol g^{-1} h^{-1}).

9. Plants from microsite 2 of the *P. australis* stand had the highest phosphatase activity which could only be linked to this site having the smallest amounts of total P. Microsite 2 had the second highest phosphatase activity yet plentiful TP but was very low in NO_3-N. It is suggested that *P. australis* plants produce phosphatase enzymes when under low P or N conditions.

10. There were no significant differences in the species phosphatase activities ($p>0.05$) in plants collected from the three *E. fluviatile* microsites. It is suggested that *E. fluviatile* may use phosphatase activity to its competitive advantage and to always have a low level of phosphatase activity. As the K_m for the enzyme is low, there is high affinity for substrate perhaps explaining its lower activity.

11. *T. latifolia* plants collected showed no significant differences in phosphatase activity when assayed with 1 μM substrate, yet with 5 μM substrate all microsites significantly different phosphatase activities. When the after termination activities were added to the original results all phosphatase activities were significantly different.

12. From these results it is suggested that possibly only *P. australis* could be used as a possible bioindicator of the nutrient concentrations of its substrate. Further work on the properties of after termination activities, along with studies to monitor root phosphatases after the addition of artificial fertilisers was recommended.

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