A study of the root surface phosphatase activities of three species of higher plants: Juncus effusus, Phragnutes australis, and Typha latifolia

Luff, Heather

A study of the root surface phosphatase activities of three species of higher plants: *Juncus effusus*, *Phragmites australis*, and *Typha latifolia*

HEATHER LUFF

A dissertation submitted in partial fulfilment of the requirements for the degree of Master of Science in Advanced Ecology

Department of Biological Sciences

University of Durham

September 1993

ABSTRACT

The aim of this project was to investigate a possible link between environmental phosphorus status and the root surface phosphatase activities of three species of emergent macrophytes, with a view to assessing their potential for use as "bioindicators".

Analyses of water phosphorus concentrations and rates of surface phosphatase activities of *Juncus effusus*, *Phragmites australis* and *Typha latifolia* were carried out on samples from Bakethin Reservoir, Northumberland and Durham University Botanic Garden. Differences were found in water phosphorus concentrations at the sites. Water from Bakethin Reservoir was found to have lower levels of P than water from the Botanic Garden. Interspecific differences at $p = 0.0002$ were discovered in rates of phosphatase activity between *Juncus*, *Phragmites* and *Typha*. *Juncus* and *Typha* were found to exhibit significantly lower rates of phosphatase activity at the Botanic Garden than at Bakethin reservoir ($p = 0.026$ for *Juncus*, $p = 0.037$ for *Typha*). High rates of phosphatase activity in *Juncus* and *Typha* at Bakethin Reservoir corresponded with low concentrations of environmental phosphorus, so it is possible that root surface phosphatases of both species are inducible in conditions of P-limitation. *Juncus* and *Typha* may therefore have the potential for use as bioindicators of environmental phosphorus status.

Several practical problems were encountered, and may be of general significance. Rates of phosphatase activity declined rapidly in 100 μM pNPP assays. As a consequence, assays were terminated after 10 min. It was also observed that roots removed after assays had been terminated often showed a yellow coloration, presumably due to the retention of pNP. The accuracy of the pNPP assay relies upon all the pNP produced by the hydrolysis of pNPP being released into solution. The apparent retention of pNP by roots therefore brings the use of the pNPP assay, as a method for determining rates of phosphatase activity in eukaryotes, under scrutiny. One preliminary experiment carried out showed that more pNP was retained by roots under conditions of low pH.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr B.A Whitton, and Dr Martyn G. Kelly, for their valuable support and advice. I would also like to give special thanks to Vanessa Mattin and Paul Stevensson, for driving me to Bakethin Reservoir on sampling days, and Julia Yelloly, Gul Baloch, Gulham Chandio, and Eileen Bresnan for their help in the laboratory.

ABBREVIATIONS

^{o}C	degrees Celsius
mg	milligramme
µg	microgramme
l	litre
ml	millilitre
m	metre
ha	hectares
h	hour
min	minutes
mM	millimolar
µM	micromolar
µmol	micromoles
P	phosphorus
Pi	orthophosphate
FRP	filtrable reactive phosphorus
TFP	total filtrable phosphorus
FOP	filtrable organic phosphorus
PMEase	phosphomonoesterase
pNPP	*para*-nitrophenylphosphate
pNP	*para*-nitrophenol
BCIP	5-bromo-4-chloro-3-indolyl phosphate
CAPS	3-(cyclohexamino)-1-propanesulphonic acid
DMG	3,3-dimethyl glutaric acid
EDTA	ethylenediamine-tetra-acetic acid
HEPES	N-2-hydroxymethylpiperazine-N'-2-ethanesulphonic acid
pKa	dissociation constant
p	probability
S	standard deviation

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CHAPTER 1 INTRODUCTION

1.1 Phosphorus

1.11 Occurrence and forms of phosphorus

Phosphorus (P) is the eleventh most abundant element in the earths crust. Its average concentration in the environment has been estimated as 0.1% by weight, which results in it being classed geochemically as a trace element. Phosphorus occurs in nature almost exclusively as phosphate (Holtan *et al.*, 1988). Van Wazer (1973) described phosphates as "chemical structures in which a phosphorus atom is more or less tetrahedrally surrounded by four oxygen atoms". Phosphates can be divided into two broad categories : inorganic and organic. In inorganic forms one to three of the hydrogen ions of the phosphoric acid is replaced by metallic cations. In organic forms one or more of the hydrogen ions is eliminated in an ester linkage. The remaining cations are replaced in part or completely by metallic cations.

1.12 Phosphorus in soil

The total content of phosphorus in soils is relatively low, most soils containing between 0.022 and 0.083 % P. Areas prone to prolonged leaching can have P contents lower than these values however. P is released in a soluble form into soils from the weathering of primary P bearing minerals with additions from plant residues and fertilisers. A large proportion of this soluble P is sorbed to soil particles or incorporated into soil organic matter (Holtan *et al.*, 1988).

Most of the phosphorus sorption capacity in soil is due to the soils finest fractions, as the active surface area decreases with increasing particle size. Because of this there is a positive correlation between P sorption and clay content (Holtan *et al.*, 1988). This correlation is also due to the iron and aluminium on the surface of clay minerals (Stuanes, 1982). Humus is not thought to retain much P by itself in soils since it is normally negatively charged. In association with cations such as Fe^{2+}, Al^{3+} and Ca^{2+} however, it is able to sorb significant amounts (Wild, 1950). Organic matter can also act by blocking sorption sites on inorganic particles (Sample *et al.*, 1980), so the importance of organic matter on phosphate absorption is ambiguous.

The pH of soil strongly influences the chemical reactivity of soil constituents causing a negative correlation between phosphate sorption and pH. Soils where only the pH differs are rare however and differences in soil properties are likely to override the effects of pH (Stuanes, 1982). Generally phosphate sorption decreases with decreasing redox potential due to the transformation of Fe^{3+} to Fe^{2+} (Holtan *et al.*, 1988). The amount of P sorbed by soils therefore depends on many factors including geology, soil composition, particle size, pH, redox potential and precipitation (leaching effects) (Holtan *et al.*, 1988).

1.13 Phosphorus in water

Natural phosphorus fractions are defined by Rigler (1973). Total P is divided into particulate P (PP) with particles larger than 0.45 μm, and filtrable P (FP) with particles smaller than 0.45 μm. FP is subdivided into filtrable reactive P (FRP) and filtrable unreactive P (FUP). Particulate P consists of minerals, amorphous precipitates, sorbed reaction products and organic particles. Filtrable P is normally considered to be orthophosphate, inorganic polyphosphates and organic P compounds.

Both organic and inorganic forms of phosphorus are involved in transformations between the solid and liquid phases, either by the release of water-soluble P from the solid phase, or the uptake of dissolved P by the solid phase (Holtan *et al.*, 1988).

1.14 Phosphorus in sediment

The source of sediment phosphorus is partly settled particulate P of allochthonous or autochthonous origin, and partly dissolved phosphate sorbed to the surface sediments and accumulated in the interstitial water (Holtan *et al.*, 1988). The P content of sediment depends on many factors: sediment composition and grain size, pH, redox potential (all determining sorption potential) and the P content of the lake water. As sediments vary so much in composition there is never a consistent relationship between the P content of water and P content of sediment. Lake sediments generally act as sinks for P, especially if there are high P levels in the water. If the P level of the water was to decrease suddenly however previously sorbed P may be released back into solution and into the lake, hence the sediments would act as a source of P (Holtan *et al.*, 1988). This effect means that the

amount of P in sediments is also related to the history of the nutrient status of the water body. No direct correlation can therefore be made between water and sediment phosphorus levels.

1.15 Methods of assaying sediment phosphorus concentration

There are several methods of assaying the phosphorus content of sediment, mostly based on sequential chemical extractions in which P is selectively removed from different compounds in the sediments. In the 1970's researchers became more interested in the algal availability of sediment P and methods were designed to measure this. As yet however no general methods have been developed or accepted for describing the quality of P in sediments, mainly due to the fact that the composition of sediments is highly variable so no scheme can be used generally. Comparisons between different sequential extraction schemes have shown that the results are heavily biased towards the method used (Pettersson *et al.*, 1988).

1.16 Biologically available phosphorus

Schaffner and Oglesby (1978) considered total phosphorus to include some or all of the following fractions: crystalline, occluded, adsorbed, particulate organic, filtrable organic and filtrable inorganic phosphorus. They defined "biologically available phosphorus" as filtrable reactive (inorganic) P, filtrable unreactive (organic) P and labile P. Filtrable reactive P is considered to be entirely biologically available. Filtrable unreactive P is considered to be available by enzymatic hydrolysis (e.g. phosphatase enzymes). Labile P associated with soil particles may dissolve into an aqueous solution (desorption).

1.17 Function of phosphorus in higher plants

Phosphorus in the form of phosphates is removed from soils and remains in this oxidised state within higher plants. Phosphates are extremely important to plants. They are present in many different forms both organic and inorganic, and carry out many diverse functions. Organic forms may be broadly classified as storage compounds, structural compounds or compounds of intermediate metabolism.

Phytin is one example of a storage compound. It is present within seeds and hydrolysed enzymatically during germination liberating inorganic phosphorus (P_i) for growth. Phospholipids also act as storage material in seeds and growing plants, as well as being involved in metabolism and important constituents of cell membranes (Mazliak, 1973). Nucleic acids are compounds of high molecular weight containing phosphoric acid. They are present in genetic material (DNA). In plant metabolism phosphorus plays a direct role as a carrier of energy through high energy phosphate bonds. Adenosine triphosphate (ATP) is the most important energy carrier. Phosphorus also plays an important part in photosynthesis. In the initial reaction light energy is trapped and water molecules split in the presence of P_i , Adenosine diphosphate (ADP) and the coenzyme NAPP (Slatter, 1989).

Phosphorus is so essential to plants that if the level of P in the environment is low, the rate of metabolic processes may be severely limited. Phosphorus is therefore a major factor limiting biomass production. It is classed as a macronutrient.

1.18 Environmental significance of phosphorus concentration

As phosphorus is such an important limiting factor natural ecosystems are "finely tuned" to the level of phosphorus in the environment. Even small changes in phosphorus levels can bring about significant changes in ecosystems including the introduction of new species, loss of original species and alteration of species abundances. Man has disrupted the natural environment by extensively mining phosphate deposits that took millions of years to accumulate, and making them available for the production of agricultural fertilisers facilitating rapid plant growth, and other domestic and industrial products e.g. detergents. Much of this phosphate eventually finds its way into ecosystems, particularly aquatic ones, causing their alteration and disruption (Holtan *et al.*, 1988). Phosphorus is now a major anthropogenic pollutant.

1.2 Phosphatases

1.21 Biological importance

Phosphatases are enzymes which promote the degradation of complex phosphorus compounds into orthophosphate (P_i) and an organic moiety (Jansson *et al.*, 1988). They have been observed in bacteria, algae, fungi, bryophytes and higher plants (Suida, 1984; Press and Lee, 1983). PMEases are the most researched group of phosphatases. They can catalyse the hydrolysis of a rich variety of phosphomonoesters. Similar but functionally different enzymes are the phosphodiesters (Jansson *et al.*, 1988). PMEases were studied in this project. They have been referred to as "phosphatases" for reasons of simplicity.

Phosphatases are extremely important in nature as they hydrolyse filtrable unreactive P into filtrable reactive P (P_i), which can be utilised by organisms. Phosphatases therefore increase the level of P in the environment which is biologically available, enabling organisms to survive in conditions of low environmental P_i.

1.22 Alkaline and acid phosphatases

Phosphatases typically have maximum hydrolysis capacity at different pH values, hence the division in to alkaline and acid phosphatases. Alkaline phosphatases have pH optima above pH 7, most commonly between pH 9 and pH 11. Acid phosphatases express their maximum activity below pH 7, generally between pH 4 and pH 6. Both groups of phosphatase enzymes are broad in specificity hydrolysing a wide range of substrates (Jansson *et al.*, 1988).

Researchers have commented on the contrast in many organisms between the high and low pH optima found experimentally, and the pH conditions in which they live in the field. No clear explanation for this has been provided however.

1.23 Constitutive and inducible phosphatases

Inducible enzymes are those whose synthesis starts in the presence of suitable substrates. Constitutive enzymes are produced independently of an activator i.e. they are more or less constantly synthesised within the cell (Jansson *et al.*, 1988). Repression occurs when a compound, often an end

product of the enzyme-catalysed reaction, turns off the enzyme synthesis. If there is a depletion of the repressor then derepression may occur and the enzyme will be synthesised again. It is thought that high levels of inorganic phosphate may often repress phosphatase synthesis (Cembella *et al.*, 1984).

While alkaline phosphatases tend to occur as extracellular or surface enzymes (Flynn *et al.*, 1986), acid phosphatases have also been located within cells (intracellular). Wynne (1977) found acid phosphatase within cells of *Peridinium cinctum*. As a general rule, extracellular and surface phosphatases (mostly alkaline phosphatases) are regarded as being inducible, produced in conditions of P-limitation. They play an important role in the P nutrition of organisms. Intracellular phosphatases (mostly acid phosphatases) are regarded as constitutive, with the function of serving the internal P metabolism (Jansson *et al.*, 1988).

1.24 Phosphatase activity in bacteria

Bacteria are the most important contributors to environmental phosphatase activity. Most investigations on PMEase structure and function have been done on *E. coli* (McComb *et al.*, 1979). Studies on marine bacteria have shown that PMEase is located in the periplasmic space.

1.25 Phosphatase activity in cyanobacteria and algae

Phosphatase activity has been found in all major groups and numerous species of cyanobacteria and algae (Healey, 1982). A great majority of cyanobacteria reported possess alkaline phosphatase activity, they have probably received the most detailed study (Doonan and Jensen, 1980; Healey 1982). It has been shown that cyanobacteria with trichomes ending in multicellular hairs nearly always have high phosphatase activity associated with the structures (Whitton, 1981). Eukaryotic algae also show predominantly alkaline phosphatase activity which is particularly marked in hair-forming species (Whitton, 1988). Acid phosphatases have been reported in a few species of algae, although predominantly those living in neutral or acidic waters e.g. *Chlamydomonas acidophila* (Boavida and Heath, 1986). Phosphatase activity has been shown to be inducible in all hair-forming species of cyanobacteria showing activity (Whitton, 1988), and most diatoms and green algae showing activity (Fitzgerald and Nelson, 1966; Hino, 1989).

Phosphatase activity has been located on the cell surface and in cell membranes (Flynn *et al.*, 1986). The release of extracellular enzymes in algal cultures has also been reported, (Whitton *et al.*, 1991).

1.26 Phosphatase activity in bryophytes

Little is known about phosphatase activity in bryophytes. Studies have revealed however that acid phosphatases are predominant. Press and Lee (1983) showed that acid phosphatase activity was detectable in all eleven *Sphagnum* species, and that the level of activity was related to their phosphate nutrient status i.e. activity was inducible. Al - Shehri (1992) observed acid phosphatase activity in all fractions (rhizoids, protonema, and leafy shoots) of the moss *Hydrogonium fontanum.* Activity was highest in the rhizoids. Some leafy shoots collected in the field had low phosphorus contents and exhibited high levels of phosphatase activity, while other shoots had high P contents and exhibited low rates of phosphatase activity. This indicates that phosphatase activity of the moss is inducible in conditions of P-deficiency.

1.27 Phosphatase activity in higher plants

It has been reported that most higher plants while rich in non-specific and specific acid phosphatases do not contain appreciable amounts of alkaline phosphatases (McComb *et al.*, 1979; Lee, 1988; Gabbrielli *et al.*, 1989). Acid phosphatase activity has been reported in a number of plant tissues including roots. Acid phosphatases occur intracellularly, are localised in apical meristems and outer surface cells (surface phosphatases) (Shaykh and Roberts, 1974) and are more rarely released extracellularly (Goldstein *et al.*, 1988). Extracellular alkaline phosphatases are synthesised by some species of higher plants. Bieleski (1974) observed extracellular alkaline phosphatases in P-deficient *Spirodela.*

1.28 Phosphatase activity as a bioindicator for the phosphorus status of the environment

As phosphatase enzymes in some organisms are inducible, their production being derepressed under conditions of moderate P limitation, it should be possible to use the rate of phosphatase activity as a guide to the status of phosphorus in the environment i.e. as a bioindicator. This method is easiest

to apply to assess the P status of aquatic environments, by analysing the phosphatase activity of various species of filamentous algae (Whitton, 1991). The use of freshwater algae in this way was first suggested by Fitzgerald and Nelson (1966) who found that cultures of three green algae, four cyanobacteria and two diatoms all showed inducible alkaline phosphatase activity. Other researchers who have suggested that phosphatase activity can be used as an indicator of P status in natural populations include: Reichardt *et al.* (1967), Healey (1978), and Rivkin and Swift (1982), cited by Whitton (1991).

Acid phosphatases have been shown to be inducible in *Euglena gracilis* (Price, 1962) and *Chlamydomonas acidophila* (Boavida and Heath, 1986). Experiments have been carried out on extracellular alkaline phosphatases of some species of aquatic higher plants including *Spirodela oligorrhiza* (Beleski, 1974). It was shown that the phosphatases are inducible as plants grown in media without phosphate showed an increase in activity.

The research carried out has therefore shown that provided suitable precautions are taken, measurement of phosphatase activity from a wide variety of organisms provides a rapid and robust means of assessing the phosphorus status of aquatic environments (Whitton, 1991).

In comparison, the possibility of using higher plant phosphatases as bioindicators for soil and sediment phosphorus status has been little investigated. Previous studies have concentrated on the role of extracellular phosphatases in the P nutrition of a few species of higher plants. It has been shown that extracellular phosphatases are inducible in conditions of moderate P limitation in wheat (McLachlan, 1980) and tomatoes (Boutin *et al.*, 1981). Ueki and Sato (1971) demonstrated that omitting Pi from the medium resulted in an increase in acid phosphatases excreted by tobacco plants. Goldstein *et al.* (1988) showed that under conditions of severe P-starvation acid phosphatases are released extracellularly by tomato plants. These extracellular phosphatases have the potential to be used as bioindicators.

Little research has been done on the effects of P limitation upon the activity of surface and intracellular acid phosphatases of higher plants. Woolhouse (1969) found that the phosphatase activity of cell walls from *Agrostis tenuis* was inducible. Dracup *et al.* (1984) showed that the cell wall bound phosphatases of the roots of *Trifolium subterraneum* increased in activity with imposed P- deficiency. Goldstein *et al.* (1988) demonstrated that Pi-starved tomato plants produced six-times the amount of

phosphatase of control plants in P-sufficient conditions. BCIP staining revealed that increased activity was primarily in root surface phosphatases. Surface-bound acid phosphatases of some species of higher plants may therefore have the potential to be used as bioindicators of P status.

Intracellular acid phosphatase activity is generally thought to be constitutive, with the main function of serving the internal P metabolism (Jansson *et al.*, 1988). Goldstein *et al.* (1988) demonstrated that although rates of activity of surface and extracellular phosphatases in P-starved tomato plants increased, intracellular phosphatase activity remained the same. Research has therefore indicated that intracellular phosphatases of higher plants are not good bioindicators for environmental phosphorus status.

3 Aims

The aim of this project was to investigate the surface phosphatase activities of three species of higher plants: *Juncus effusus*, *Phragmites australis* and *Typha latifolia.* By collecting plant material from two different sites, Bakethin Reservoir, Northumberland, and Durham University Botanic Garden, and carrying out chemical analysis on water samples collected from the sites, it was hoped to assess whether any recorded differences in phosphatase activity of the species could be related to differences in water phosphorus levels; and thus determine whether any of the species would be suitable to use as "bioindicators" of environmental phosphorus status.

CHAPTER 2 MATERIALS AND METHODS

2.1 Computing and statistical treatments

Two computer systems were used for study. Statistical analysis was carried out on Microsoft Excel. Graphic output on Cricket Graph, Word for Windows.

2.2 Sample collection

Small plants or parts of plants were dug up to obtain root material. The plants were then immediately transferred to polythene bags which were tied to retain moisture and labelled. The material was kept cool by immersing it in an ice bucket on the return journey from Bakethin Reservoir and then immediately transferring it to a 4 ^{o}C cool room. The root material was kept for no longer than 24 hours.

Water samples were collected in labelled, acid washed, polypropylene bottles. They were returned to the laboratory in a box filled with ice. Phosphorus analysis was carried out on them immediately.

2.3 Laboratory analytical methods

2.31 pH

pH measurements were carried out using an Ingold combination WTW E50 Electrode and EIL Meter (Model 7050). The probe was calibrated with BDH standard buffer solutions, prepared with MilliQ water.

2.32 Mass determination

Mass was measured on an A&B Company Ltd electronic analytical balance (model ER-182A), to 5 decimal places. All references to weight refer to mass.

2.33 Colorimetric analysis

Colorimetric analysis was carried out using a Schimadzu Digital Double-Beam Spectrophotometer (model UV-150-02). Glass cuvettes with a path length of 1.0 cm were used for all readings of phosphatase activity (pNPP substrate) at wavelength 405 nm. Glass cuvettes with a path length of 4.0 cm were used for phosphorus readings at 882 nm.

2.34 Light microscopy

In the laboratory, material was examined using a Nikon type 109 Fluorphot microscope filtered with a Nikon micrometer eyepiece.

2.4 Media

2.41 Stock Solutions

All stocks were BDH Analar grade stock prepared in MilliQ water and kept in the refrigerator at 4 ^{o}C until required. Stock solutions were renewed every three months.

2.42 Phosphatase assay medium

The concentrations of mineral salts in assay medium are presented in Table 2.1. Assay medium was usually made up fresh for every phosphatase assay.

Table 2.1 The concentration of mineral salts in assay medium

Major elements	**Salt (mg l^{-1})**	**µM**
$MgSO_4 . 7H_2O$	25.0	101.4
$NaHCO_3$	15.85	188.6
$CaCl_2 . 2H_2O$	35.83	243.7
KCl	4.28	57.38

Stock added as Fe Chelate	**Salt (mg l^{-1})**	**µM**
Na_2-EDTA-$2H_2O$	1.67	4.17

Microelements (Trace elements)	**Salt (mg l^{-1})**	**µM**
$MnCl_2 . 2H_2O$	0.04	2.28
$CuSO_4 . 5H_2O$	0.02	0.078
$CoSO_4 . 7H_2O$	0.01	0.035
$NiSO_4 . 7H_2O$	0.038	0.03
$ZnSO_4 . 7H_2O$	0.056	0.019
Na_2MoO_4	0.007	0.028
H_3BO_3	0.72	11.56

2.43 pH buffers

pH buffers were prepared in assay medium to have a final concentration of 50 mM. A list of the buffers is shown in Table 2.2. They were kept in the refrigerator at 4 ^{o}C until required. They were removed from the refrigerator several hours before each assay so that they would warm to room temperature. They were then checked with the pH meter to ensure that they were accurate.

Table 2.2 A list of pH buffers

pH	buffer	buffering capacity	pKa at 20 ^{o}C
3.0	DMG - Na OH	3.2 - 7.6	3.66 + 6.20
4.0	DMG - Na OH	3.2 - 7.6	3.66 + 6.20
4.5	DMG - Na OH	3.2 - 7.6	3.66 + 6.20
5.0	DMG - Na OH	3.2 - 7.6	3.66 + 6.20
5.5	DMG - Na OH	3.2 - 7.6	3.66 + 6.20
6.0	DMG - Na OH	3.2 - 7.6	3.66 + 6.20
6.5	DMG - Na OH	3.2 - 7.6	3.66 + 6.20
7.0	HEPES - Na OH	6.8 - 8.2	7.50
7.5	HEPES - NaOH	6.8 - 8.2	7.50
8.0	HEPES - Na OH	6.8 - 8.2	7.50
9.0	glycine - Na OH	8.6 - 10.6	8.0
10.0	glycine - Na OH	8.6 - 10.6	8.0
11.0	CAPS - Na OH	9.8 - 11.1	10.40

All the chemicals used in the buffers were supplied by British Drug House Ltd (BDH), except HEPES which was supplied by Sigma Chemical Co, USA.

2.44 p-nitrophenyl phosphate disodium (pNPP) substrate solution

100 μM pNPP solution in assay medium was made up fresh for every phosphatase assay.

2.5 Cleaning of glassware and utensils

All glass vials were soaked in tap water with 2% Decon detergent overnight. They were then rinsed three times in tap water and three times in distilled water prior to drying at 100 ^{o}C. All volumetric glassware used for phosphorus analysis was soaked in 4% nitric acid for 20 min, then rinsed three times in tap water and three times in distilled water. It was dried at room temperature. Plastics were also soaked in 2% Decon overnight, rinsed, and then dried at 40 ^{o}C.

2.6 Water phosphorus analysis

Water for phosphorus analysis was filtered immediately on arrival back at the laboratory through a Whatman GF/F filter, washed with MilliQ water. Phosphorus analysis was performed the same day using the method proposed by Eisenreich *et al.* (1975).

2.7 Root Staining

Localisation of PMEase activity was carried out using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as an organic phosphorus substrate (Coston and Holt, 1958; Holt and Withers, 1958). Roots were washed three times in assay medium and then cut into 1 cm lengths. One piece of root was put in to each labelled universal bottle and immersed in 1 mM BCIP in assay medium, at pH 5.0. The bottles were then placed in a 32 ^{o}C water bath for 20 minutes and shaken frequently. After this time the roots were

removed, washed three times in distilled water and analysed under the microscope. Shaking was necessary throughout the staining procedure as oxygen is required to form the insoluble blue indigoid.

2.8 The phosphatase assay

2.81 Preparation of root material

Plant material was removed from the 4 ^{o}C cold room and lengths of root were cut into sterilised plastic petri dishes. The roots were then thoroughly washed in assay medium to remove any dirt. This was done very carefully to ensure that the roots were not damaged.

2.82 Assay procedure for acid phosphatase activity

1.5 ml pH 5.0 solution (DMG and NaOH) and 1.4 ml assay medium were pipetted into snap cap glass vials. 1 cm lengths of root from one particular species were then added to the vials. They were transferred to a shaking tray over a water bath at 25 ^{o}C and left to equilibriate for 10 min. Assays were initiated by the addition of 0.1 ml of 100 μM pNPP substrate. Assays were run for varying lengths of time. Termination of the assay was achieved by the addition of 0.25 ml 5 M NaOH. The roots were then immediately removed from the vials. Each assay was carried out with a minimum of four replicates and two controls but frequently ten replicates were used. The control assay had no root material. Colorimetric readings were taken of the contents of each vial.

2.83 Effect of pH on phosphatase activity

A range of pH buffers from pH 3.0 to pH 11.0 were used to determine the effect of pH on phosphatase activity.

2.84 Measurement of dry weights

Glass vials were labelled, put in the 100 ^{o}C oven overnight and then weighed on the fine balance. After the assay was terminated roots were transferred from the assay vials to the dry weight bottles and they were returned to the oven overnight. The following day the bottles were removed from the oven and placed in a dessicator. The bottles were then each reweighed. The dry weight of each root was obtained by subtracting the initial weight of the bottle from the final weight (of the bottle and root).

2.85 Calculation of phosphatase activity

The phosphatase activity of each root, in μmol per gramme dry weight per hour, was calculated using the equation shown below.

$$\frac{\text{Reading (on spectrophotometer) - Control}}{0.0158} \times \frac{0.0032}{\text{Dry weight (g)}} \times \frac{60}{\text{assay length (min)}}$$

CHAPTER 3 FIELD LOCATIONS AND SPECIES STUDIED

3.1 Field Locations

3.11 Background information on Bakethin Reservoir

Bakethin Reservoir lies 116 Km north west of Durham City, in the county of Northumberland. It is the shallow feeder to Kielder Reservoir and is situated approximately 2 Km south east of Kielder village and to the west of Kielder Water. The two reservoirs are connected by a dam. Bakethin Reservoir functions as a moderately open system with major inputs from the river North Tyne and Kielder Burn, and many smaller inputs including Capon Burn and Bakethin Burn. The output is Kielder Reservoir which is the largest man made lake in Europe.

Bakethin Dam was originally built in the interests of conservation. The Nature Conservancy Council envisaged that the shallower edges of Kielder Water would make the most effective nature reserves, and that naturally fluctuating water levels would be favourable for the development of marginal vegetation and animal communities (Northern Sports Council, 1976). Bakethin Dam separates a shallow water upstream area with fluctuating water levels (Bakethin Reservoir) from the rest of Kielder Water, which is much deeper with more constant water levels. Water recreational activities have been directed towards the main reservoir so that Bakethin has remained quiet except for a few fishermen in rowing boats. Islands and lagoons have been created in an attempt to encourage wildlife. Bakethin Conservation Area is owned by Northumbria Water Plc. It covers an area of 123 ha, approximately half of which are water surface.

The underlying geology of the area consists mainly of Carboniferous limestone. The water of Bakethin Reservoir is slightly alkaline (pH 7.3). It is moderately nutrient poor as most of the catchment area consists of conifer plantations owned by the Forestry Commission, which have never been fertilised. Moorland vegetation also makes up a large proportion of the catchment area. The most important inputs of nutrients to the reservoir come from run off from agricultural land upstream, and outlets from Kielder sewage works.

Figure 3.1 Bakethin Reservoir from the disused viaduct

3.12 Background information on Durham University Botanic Garden

The first botanic garden in Durham was founded in 1925 when the grounds around the science laboratories were laid out as an experimental garden. As the sciences expanded within the university however the gardens decreased in size, so it was decided in 1969 to move the garden to its present site at Hollingside Lane (Sayers, 1975).

Work commenced on the new garden in 1971. It was originally divided spatially into two sections, a north section and a south section. The south section has since been abandoned however and the trees remain growing in a wilderness. The original north section at the top of Hollingside Lane has been developed in to the garden that we know today. It is centred around an old fragment of garden from Hollingside House (Sayers, 1975). The garden slopes towards the south west but the large number of trees planted (over 21,000) protect other plants from the prevailing winds.

The underlying geology of the garden is Carboniferous sandstone (Coal Measures). The average pH of the soil is pH 7.5. Areas of the gardens are fertilised, so it is likely that many plants live in nutrient-rich conditions.

3.2 Plants studied

3.21 Introduction

Initially trial phosphatase assays were carried out on seven species of higher plants collected from Bakethin Reservoir: *Carex binervis, Equisetum fluviatile, Glyceria fluitans, Juncus effusus, Phalaris arundinacea, Phragmites australis* and *Typha latifolia.* All seven species showed positive results in the assays i.e. they all exhibit surface phosphatase activity. On 19th May 1993 there was such heavy rainfall that the water level of Bakethin Reservoir rose over 5 m, totally submerging many of the aquatic pants. The water level remained high for six weeks. The study was then forced to concentrate on a few species of plants that could still be obtained. *Juncus effusus, Phragmites australis* and *Typha latifolia* were chosen as all three species are common, in abundant supply at Bakethin Reservoir and showed relatively high rates of surface phosphatase activity in the trial assays. *Juncus effusus* and *Typha latifolia* also both grow at Durham University Botanic Garden. This provided the opportunity to compare rates of phosphatase activity of these two species at separate localities.

3.22 Description of the species

Juncus effusus (soft rush)

Height : 30 to 150 cm. A densely tufted perennial; stems stiffly erect, leafless, hairless, smooth, glossy; bright green, dark brown or reddish, but not shining at the base. Flowers less than 5 mm, of greenish perianth segments; in loose or compact clusters on the sides of the stems, below the top. June to August. Habitat: damp, frequently acid pastures, marshes, woods. (Whitton, 1979)

Phragmites australis (common reed)

Height : 1.5 to 3m. A stout, erect, perennial grass with extensive creeping rhizomes growing in dense clumps. Leaves are less than 60 cm long and 2 cm wide, tapering to a fine point, greyish green, with a ring of hairs at the base. Flowers awnless, in 2 to 6 flower spikelets, less than 16 mm in loose, silkily hairy paricles, less than 40 cm. August to September. Habitat : swamps, shallow water. (Whitton, 1979)

Typha latifolia (bulrush)

Height : 150 to 250 cm. A stout perennial. Leaves linear, leathery, less than 18 mm wide. Flowers in a terminal spike. June to July. Habitat : margins of ponds, lakes, slow moving water. (Whitton, 1979)

3.3 Location of species at the field sites

3.31 Bakethin Reservoir

All plant material was collected from the northern shore of Bakethin Reservoir, between the disused viaduct and the calcareous flush. *Typha* was collected from the marsh near the viaduct. *Juncus* and *Phragmites* were collected from the edge of the reservoir, below the calcareous flush. See Figures 3.2 and 3.3.

3.32 Botanic Garden

No *Phragmites* grows at the Botanic Garden. *Typha* was collected from a small neglected pond behind the public glasshouses. *Juncus* was collected from the edge of Collingwood College Pond, just outside the Botanic Garden. See Figures 3.4 and 3.5.

Figure 3.4 *Juncus effusus* at Collingwood College Pond

Figure 3.5 *Typha latifolia* at the Botanic Garden

CHAPTER 4 RESULTS

4.1 Phosphorus analysis of water

Phosphorus analysis was carried out on water samples from Bakethin Reservoir, the pond behind the glasshouses at the Botanic Garden and Collingwood College Pond. This was done in order to determine the P-status of the environments of the species studied. The results are shown in Table 4.1

Table 4.1 Results of chemical analyses of phosphorus content ($\mu g\ l^{-1}$) of water collected from Bakethin Reservoir, the Botanic Garden and Collingwood College Pond

Location	**TFP ($\mu g\ l^{-1}$)**	**FRP ($\mu g\ l^{-1}$)**	**ORP ($\mu g\ l^{-1}$)**	**%ORP**
Bakethin Reservoir	10.0	4.4	5.6	55.7
Botanic Garden Pond	56.9	27.3	29.6	52.0
Collingwood College Pond	670.0	667.0	3.0	0.2

4.2 Phosphatase activity assays

4.21 Phosphatase activity of different types of roots from the same species

Phosphatase assays were carried out on different types of roots from the same species to determine whether there was any significant difference in rates of phosphatase activity. By doing this, suitable types of roots exhibiting high rates of activity could be chosen for use in subsequent assays. It was hoped that by standardising the types of roots used, variability between the roots would be reduced thereby producing more accurate results.

4.211 *Juncus effusus*

Phosphatase assays were carried out on two types of root from *Juncus effusus*: white roots and brown roots (containing ferric oxides). Four replicates were used for each assay. The results are shown in Table 4.211

Table 4.211 Phosphatase activity (μmol mg^{-1} h^{-1}) of different types of roots from *Juncus effusus*

Type of root	Mean phosphatase activity (μmol mg^{-1} h^{-1})	Standard deviation
White roots	0.0045	0.0003
Brown roots	0.0045	0.0013

Student t tests carried out on the data for Table 4.211 showed that $p = 0.94$, so there was no significant difference in rates of phosphatase activity between white roots and brown roots of *Juncus effusus*. Despite this, brown roots were used in all subsequent assays to standardise the procedure. Brown roots were chosen because they were more abundant than white roots.

4.212 *Phragmites australis*

Assays were carried out on two types of roots from *Phragmites australis*: roots with root hairs and roots without root hairs. Four replicates were used for each assay. The results are shown in Table 4.212

Table 4.212 Phosphatase activity (μmol mg^{-1} h^{-1}) of different types of roots from *Phragmites australis*

Type of root	Mean phosphatase activity (μmol mg^{-1} h^{-1})	Standard deviation
Roots with root hairs	0.0070	0.0035
Roots without root hairs	0.0094	0.0040

Student t tests carried out on the data for Table 4.212 showed that $p = 0.48$, so there was no significant difference in rates of phosphatase activity between roots with root hairs and roots without hairs of *Phragmites australis*. Despite this, roots with root hairs were used in all subsequent assays to standardise the procedure. Roots with root hairs were chosen because they were more abundant.

4.213 *Typha latifolia*

Phosphatase assays were carried out on two different types of roots from *Typha latifolia*: roots with root hairs and roots without root hairs. Four replicates were used for each assay. The results are shown in Table 4.213

Table 4.213 Phosphatase activity (μmol mg^{-1} h^{-1}) of different types of roots from *Typha latifolia*

Type of root	Mean phosphatase activity (μmol mg^{-1} h^{-1})	Standard deviation
Roots with root hairs	0.0161	0.0015
Roots without root hairs	0.0160	0.0021

Student t tests carried out on the data for Table 2.413 showed that p = 0.94 so there was no significant difference in rates of phosphatase activity between roots with root hairs and roots without root hairs of *Typha latifolia*. Despite this, roots without root hairs were used in all subsequent assays to standardise the procedure. Roots without root hairs were chosen because they were more abundant.

4.22 Phosphatase activity of roots of the same species prepared in different methods

Phosphatase assays were carried out to determine the effect of preparing roots of the same species in different methods. Three methods of root preparation were investigated: rinsing roots, thoroughly washing roots and crushing roots (with forceps and a seeker). Some roots were gently rinsed in order to remove particles of dirt. Some roots were washed more thoroughly and rubbed, in an attempt to remove any bacteria which may be living on the root surface, and could exhibit phosphatase activity. Other roots were severely crushed.

4.221 *Juncus effusus*

Juncus roots were prepared by the three methods mentioned above. Four replicates were used for each assay. The results are shown in Table 4.221

Table 4.221 Phosphatase activity (μmol mg^{-1} h^{-1}) of roots of *Juncus effusus* prepared in different methods

Method of root preparation	Mean phosphatase activity (μmol mg^{-1} h^{-1})	Standard deviation
Rinsing	0.0045	0.0003
Washing	0.0054	0.0007
Crushing	0.0063	0.0019

The results show that increase in preparation time i.e. from rinsing of roots, to thorough washing of roots, to crushing of roots, corresponded with an increase in phosphatase activity. "Analysis of variants" tests carried out on the data for Table 4.221 showed that $p = 0.27$ so there was no significant difference in rates of phosphatase activity between the three methods of treatment.

Phragmites australis

The results of phosphatase assays of *Phragmites australis* roots prepared in the three different methods all ready mentioned are shown in Table 4.222.

Table 4.222 Phosphatase activity (μmol mg^{-1} h^{-1}) of *Phragmites* roots prepared in different methods

Method of root preparation	Mean phosphatase activity (μmol mg^{-1} h^{-1})	Standard deviation
Rinsing	0.0070	0.0035
Washing	0.0071	0.0037
Crushing	0.0182	0.0162

The results show that there was an increase in phosphatase activity corresponding with an increase in preparation (handling) time i.e. rinsed roots, to thoroughly washed roots, to crushed roots. "Analysis of variants" tests carried out showed that $p = 0.34$ so there was no significant difference in rates of phosphatase activity between the three methods of treatment.

4.223 *Typha latifolia*

The results of phosphatase assays carried out on *Typha* roots prepared in the three different methods are shown in Table 4.223.

Table 4.223 Phosphatase activity (μmol mg^{-1} h^{-1}) of *Typha* roots prepared in different methods

Method of root preparation	**Mean phosphatase activity (μmol mg^{-1} h^{-1})**	**Standard deviation**
Rinsing	0.0160	0.0021
Washing	0.0259	0.0026
Crushing	0.0353	0.0160

The results in Table 4.223 show that there was an increase in phosphatase activity corresponding with an increase in preparation time. "Analysis of variants" tests carried out showed that $p = 0.052$ so there was no significant difference in rate of phosphatase activity between the three methods of treatment.

The results displayed in Tables 4.221, 4.222 and 4.223 indicate that in all three species (*Juncus effusus*, *Phragmites australis* and *Typha latifolia*) there was an increase in phosphatase activity corresponding with an increase in handling time of the roots, although statistical analysis has shown that this trend was not significant. This increase in phosphatase activity may have been caused by the release of intracellular phosphatases from the lysed cells of roughly handled roots.

In subsequent assays, roots were rinsed extremely carefully in order to minimise damage to root cells and the release of intracellular phosphatases. These would have obscured the levels of activity of "surface" phosphatases.

4.23 pH spectra

Phosphatase assays were carried out over a range of pH values, from pH 3.0 to pH 11.0 for *Juncus*, *Phragmites* and *Typha*. The purpose of these assays was to determine the optimum pH for phosphatase activity in each species, under the particular assay conditions. The results are shown in Tables 4.231, 4.232, and 4.233, and are displayed in Figure 4.1. Four replicates were used for each assay.

Table 4.231 Phosphatase activity (μmol mg^{-1} h^{-1}) of *Juncus* from pH 3.0 to pH 11.0

pH	Mean phosphatase activity (μmol mg^{-1} h^{-1})	Standard deviation
3.0	0.0079	0.0021
4.0	0.0100	0.0046
5.0	0.0127	0.0048
5.5	0.0089	0.0010
6.0	0.0121	0.0058
6.5	0.0083	0.0076
7.0	0.0080	0.0032
8.0	0.0071	0.0038
9.0	0.0081	0.0037
10.0	0.0086	0.0034
11.0	0.0084	0.0052

The results in Table 4.231 show that pH 5.0 appears to be the optimum pH for phosphatase activity in *Juncus effusus* under the particular assay conditions.

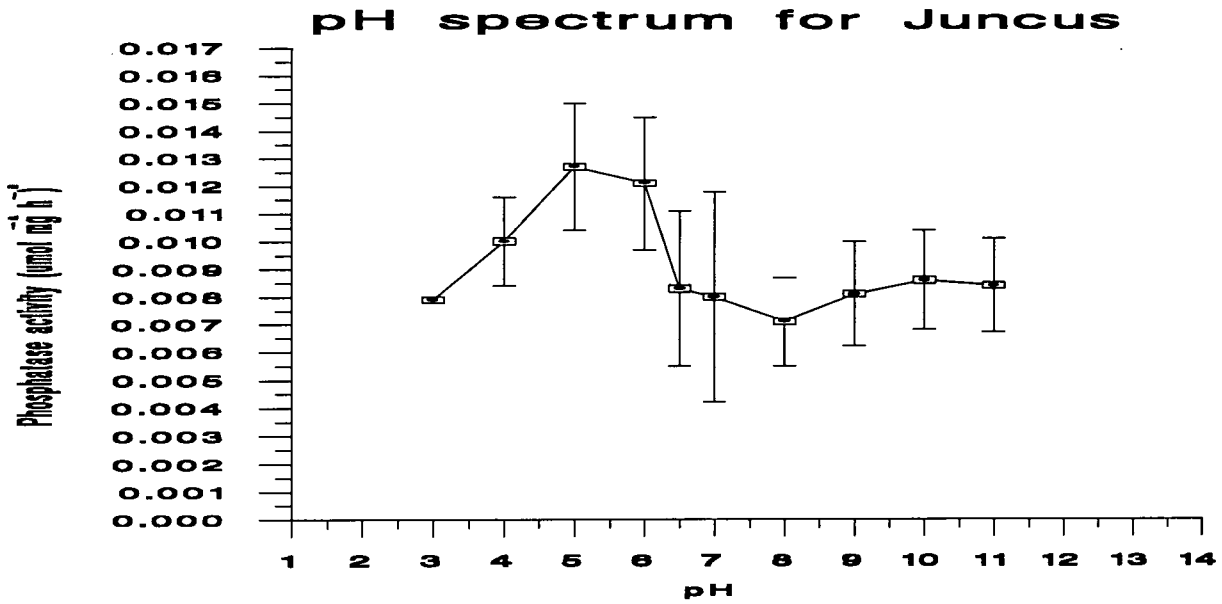
Table 4.232 Phosphatase activity (μmol mg $^{-1}$ h $^{-1}$) of *Phragmites* from pH 3.0 to pH 11.0

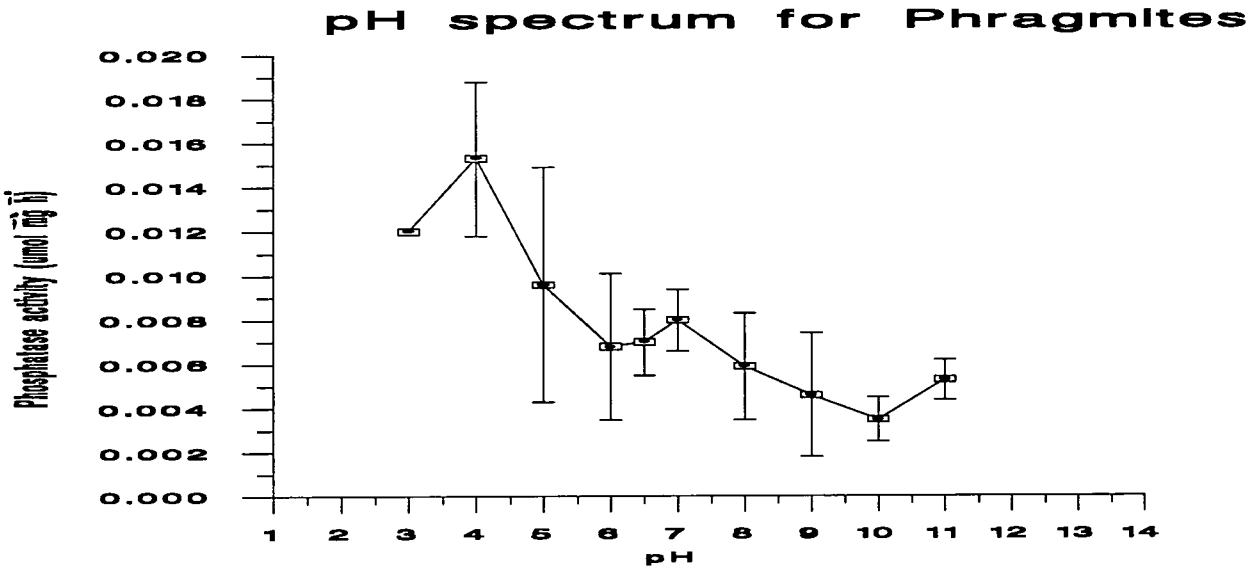
pH	Mean phosphatase activity (μmol mg^{-1} h^{-1})	Standard deviation
3.0	0.0120	0.0070
4.0	0.0153	0.0106
5.0	0.0096	0.0066
5.5	0.0116	0.0070
6.0	0.0068	0.0026
6.5	0.0070	0.0024
7.0	0.0080	0.0041
8.0	0.0059	0.0049
9.0	0.0046	0.0017
10.0	0.0035	0.0016
11.0	0.0053	0.0026

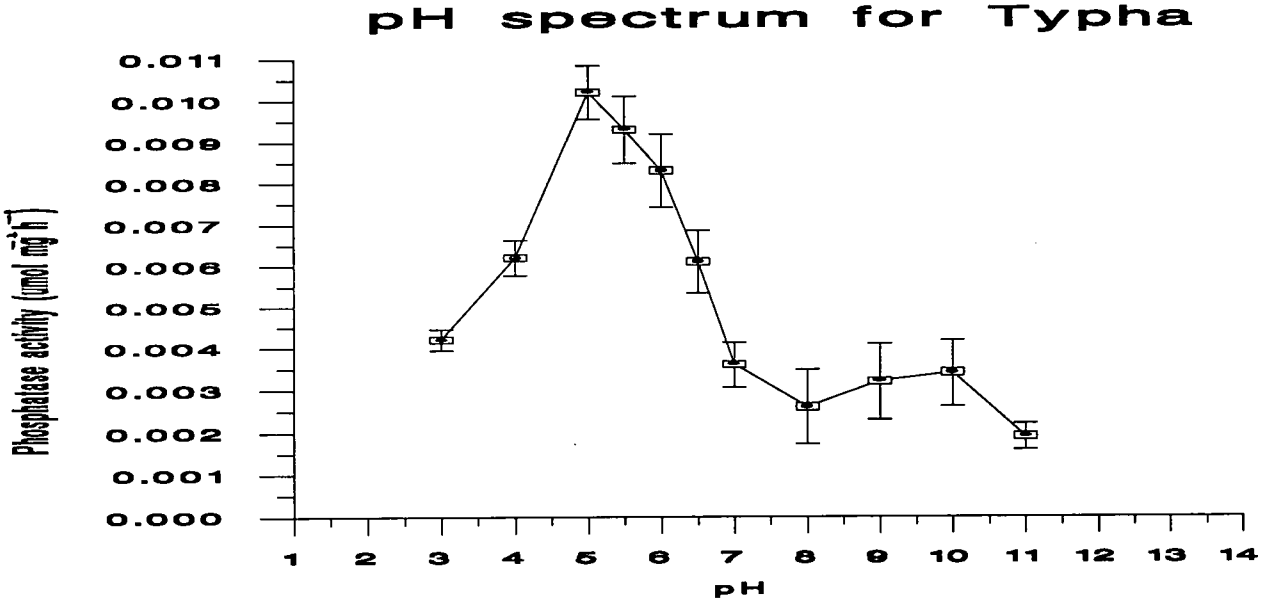
Table 4.233 Phosphatase activity (μmol mg $^{-1}$ h $^{-1}$) of *Typha* from pH 3.0 to pH 11.0

pH	Mean phosphatase activity (μmol mg $^{-1}$ h $^{-1}$)	Standard deviation
3.0	0.0042	0.0051
4.0	0.0062	0.009
5.0	0.0102	0.0013
5.5	0.0093	0.0016
6.0	0.0083	0.0018
6.5	0.0061	0.0015
7.0	0.0036	0.0011
8.0	0.0026	0.0018
9.0	0.0032	0.0019
10.0	0.0034	0.0016
11.0	0.0019	0.0006

Figure 4.1 pH spectra


pH spectrum for Juncus
Phosphatase activity (umol mg-1 h-1)
0.017
0.016
0.015
0.014
0.013
0.012
0.011
0.010
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
1 2 3 4 5 6 7 8 9 10 11 12 13 14
pH


pH spectrum for Phragmites
Phosphatase activity (umol mg-1 h-1)
0.020
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
1 2 3 4 5 6 7 8 9 10 11 12 13 14
pH


pH spectrum for Typha
Phosphatase activity (umol mg-1 h-1)
0.011
0.010
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
1 2 3 4 5 6 7 8 9 10 11 12 13 14
pH

Results in Table 4.222 show that pH 4.0 appears to be the optimum pH for phosphatase activity in *Phragmites australis*, under the particular assay conditions. Results in Table 4.233 show that pH 5.0 appears to be the optimum pH for phosphatase activity in *Typha latifolia*, under the particular assay conditions.

All subsequent assays were carried out at pH 5.0 so that high rates of phosphatase activity could be recorded and compared between species.

4.24 Time trials

In order to determine how rates of phosphatase activity changed during the course of assays, phosphatase assays were carried out for all three species and terminated after different time intervals ranging from 10 min to 90 min. Ten replicates were used for each assay. The results are shown in Tables 4.241, 4.242 and 4.243.

Table 4.241 Phosphatase activity (μmol mg $^{-1}$ h $^{-1}$) of *Juncus* over different time periods

Time (min)	Substrate utilised (μmol mg^{-1})		Phosphatase activity (μmol mg^{-1} h^{-1})	
	mean ±	Standard deviation	mean ±	Standard deviation
10	0.0133	0.0062	0.0798	0.0373
20	0.0134	0.0051	0.0402	0.0264
30	0.0143	0.0065	0.0287	0.0130
60	0.0142	0.0073	0.0142	0.0308
90	0.0144	0.0041	0.0096	0.0012

Table 4.242 Phosphatase activity (μmol mg $^{-1}$ h $^{-1}$) of *Phragmites* over different time periods

Time (min)	Substrate utilised (μmol mg^{-1})			Phosphatase activity (μmol mg^{-1} h^{-1})		
	mean	±	Standard deviation	mean	±	Standard deviation
10	0.0072		0.0024	0.0432		0.0141
20	0.0086		0.0030	0.0258		0.0089
30	0.0118		0.0049	0.0237		0.0098
60	0.0176		0.0067	0.0176		0.0067
90	0.0179		0.0076	0.0119		0.0051

Table 4.243 Phosphatase activity (μmol mg $^{-1}$ h $^{-1}$) of *Typha* over different time periods

Time (min)	Substrate utilised (μmol mg^{-1})			Phosphatase activity (μmol mg^{-1} h^{-1})		
	mean	±	Standard deviation	mean	±	Standard deviation
10	0.0050		0.0015	0.0301		0.0092
20	0.0071		0.0034	0.0214		0.0095
30	0.0076		0.0035	0.0152		0.0070
60	0.0085		0.0028	0.0085		0.0028
90	0.0102		0.0024	0.0068		0.0016

The results in Tables 4.241, 4.242 and 4.423 show that for all three species rates of phosphatase activity were initially high, but declined rapidly after the first 10 min. Phosphatase activity was therefore at its highest during the initial 10 min of the assay. All subsequent assays were terminated after 10 min.

4.25 Comparisons between the phosphatase activity (μmol mg $^{-1}$ h $^{-1}$) of *Juncus*, *Phragmites* and *Typha* at Bakethin Reservoir

Phosphatase assays for all three species from Bakethin Reservoir were carried out. Assays were done at pH 5.0 and terminated after 10 min in order to record high rates of phosphatase activity. Ten replicates were used for each assay. The results are shown in Table 4.25.

Table 4.25 Phosphatase activity (μmol mg $^{-1}$ h $^{-1}$) of *Juncus*, *Phragmites* and *Typha* from Bakethin Reservoir

Species	Phosphatase activity (μmol mg^{-1} h^{-1})	Standard deviation
Juncus effusus	0.0798	0.0373
Phragmites australis	0.0432	0.0141
Typha latifolia	0.0301	0.0092

Results in Table 4.25 show that *Juncus effusus* exhibited the highest rate of phosphatase activity, followed by *Phragmites australis*. *Typha latifolia* exhibited the lowest rate of phosphatase activity. "Analysis of variants" tests carried out showed that $p = 0.0002$ so there was a significant difference in rates of phosphatase activity between the three species.

4.26 Comparisons between the phosphatase activity (μmol mg^{-1} h $^{-1}$) of *Juncus* and *Typha* at Bakethin Reservoir and the same species at the Botanic Garden

Phosphatase assays were carried out for *Juncus* and *Typha* from the Botanic Garden. Assays were done at pH 5.0 and terminated after 10 min. Ten replicates were used for each assay. The results are shown in Table 4.26. Rates of phosphatase activity of *Juncus* and *Typha* from Bakethin Reservoir (shown in Table 4.25) are also included in Table 4.26 for easy comparison.

Table 4.26 Comparisons of the phosphatase activity (μmol mg $^{-1}$ h $^{-1}$) of *Juncus* and *Typha* at Bakethin Reservoir and the Botanic Garden

Species	Location	Phosphatase activity (μmol mg^{-1} h^{-1})	Standard deviation
Juncus effusus	Botanic Garden	0.0377	0.0320
Juncus effusus	Bakethin Reservoir	0.0798	0.0373
Typha latifolia	Botanic Garden	0.0182	0.0084
Typha latifolia	Bakethin Reservoir	0.0301	0.0092

Results in Table 4.26 show that in both species, *Juncus effusus* and *Typha latifolia*, rates of phosphatase activity were significantly higher in plants from Bakethin Reservoir than the same species from the Botanic Garden. Student t tests carried out on the data for Table 4.26 showed that *Juncus* from Bakethin Reservoir and the Botanic Garden had a p value of 0.026. *Typha* had a p value of 0.0037.

4.3 Localisation of phosphatase activity

Staining procedures using BCIP were carried out several times, in an attempt to localise phosphatase activity of the roots. These procedures proved unsuccessful however. *Juncus* roots were too pigmented for the indigoid dye to be observed. *Typha* never retained much dye, and any root coloration was general. No specialised cells for phosphatase activity could be observed under the light microscope.

4.4 Time courses comparing rates of phosphatase activity of *Juncus effusus* between 100 μM and 250 μM pNPP assays

During the course of investigations it was discovered that rates of phosphatase activity in 100 μM pNPP assays declined rapidly after the initial 10 min, for all three species (results are shown in Tables 4.241, 4.242 and 4.423). To compare how rates of phosphatase activity varied over time in 250 μM assays, 100 μM and 250 μM pNPP assays were carried out and terminated at the same times using roots of *Juncus effusus* from Collingwood College Pond. The results are shown in Tables 4.41 and 4.42.

Table 4.41 100μM pNPP assay of roots of *Juncus effusus* collected from Collingwood College Pond

Time (min)	Substrate utilised (μmol mg^{-1})		Phosphatase activity (μmol mg^{-1} h^{-1})	
	mean ±	standard deviation	mean ±	standard deviation
10	0.0063	0.0053	0.0377	0.0320
20	0.0063	0.0036	0.0189	0.0107
30	0.0080	0.0032	0.0160	0.0064
40	0.0093	0.0063	0.0139	0.0094
50	0.0091	0.0047	0.0109	0.0025
60	0.0075	0.0029	0.0075	0.0013

Table 4.42 250 μM pNPP assay of roots of Juncus effusus collected from Collingwood College Pond

Time (min)	Substrate utilised (μmol mg^{-1})			Phosphatase activity (μmol mg^{-1} h^{-1})		
	mean	±	standard deviation	mean	±	standard deviation
10	0.0048		0.0016	0.0289		0.0097
20	0.0075		0.0021	0.0225		0.0064
30	0.0102		0.0064	0.0204		0.0128
40	0.0145		0.0077	0.0181		0.0116
50	0.0151		0.0049	0.0181		0.0059
60	0.0189		0.0089	0.0189		0.089

Results in Tables 4.41 and 4.42 show that the rates of phosphatase activity decreased rapidly after the initial 10 min in 100 μM phosphatase assays whereas they remained more constant, decreasing gradually after the first 10 min, in 250 μM assays.

4.5 Retention of pNP by roots

During the course of investigations it was noted that roots removed after assays had been terminated often had a yellow coloration. This yellow colour was presumably due to the retention of pNP by the roots. To investigate this further, experiments were carried out on *Typha latifolia* from the Botanic Garden in order to determine how much pNP was retained by roots after a phosphatase assay, and how much of the pNP was later released into solutions of different pH values. The results are shown in Table 4.5.

Table 4.5 The release of pNP from roots over a range of pH values

pH	pNP released from roots during the assay (μmol mg^{-1})		pNP released from roots after the assay (μmol mg^{-1})		% pNP released after the assay
	mean	± standard deviation	mean	± standard deviation	
3.0	0.0047	0.0029	0.0002	0.0001	2.99
4.0	0.0050	0.0017	0.0003	0.0002	3.31
5.0	0.0048	0.0027	0.0007	0.0007	8.03
6.0	0.0048	0.0018	0.0005	0.0002	10.31
7.0	0.0050	0.0013	0.0003	0.0001	5.05
8.0	0.0038	0.0014	0.0009	0.0004	20.51
9.0	0.0045	0.0016	0.0008	0.0004	15.93
10.0	0.0050	0.0038	0.0010	0.0004	18.47
11.0	0.0051	0.0028	0.0012	0.0002	21.10

Table 4.5 shows that an increasing amount of pNP is released into solution by *Typha* roots with increasing pH. This indicates that more pNP is retained by roots in acidic conditions.

CHAPTER 5 DISCUSSION

The initial experiments for this project were important to determine which roots from each species were most suitable for use in phosphatase assays, how to prepare them for assaying, and for how long, and under what pH conditions, the assays should be run.

Phosphatase assays were carried out on different types of roots from the same species (results are shown in Tables 4.211. 4.212 and 4.213). Statistical analysis revealed that there was no significant difference in rates of phosphatase activity between the different types of roots of *Juncus*, *Phragmites* or *Typha*. Despite this, in subsequent assays it was ensured that the roots used were as uniform as possible, in size, shape, colour, and number of root hairs, to reduce variations in phosphatase activity. This was particularly important for *Juncus* and *Phragmites*, as results showed that there was a large standard deviation in phosphatase activity between roots, even of the same type. *Typha* roots showed less variation in rates of phosphatase activity.

Phosphatase assays were carried out using three methods of root preparation: rinsing, thorough washing and crushing (results are shown in Tables 4.221, 4.222 and 4.223). Statistical analysis revealed that there was no significant difference in rates of phosphatase activity, for any of the three species. A trend of increasing phosphatase activity corresponding with an increase in handling time, was observed in all three species. This may have been due to the release of intracellular phosphatases from lysed cells of damaged roots. Intracellular phosphatases are not normally thought to have an external function, but are thought to be constitutive enzymes produced mainly to serve the internal P metabolism (Jansson *et al.*, 1988). Intracellular phosphatases were not of interest in this project, the aim of which was to investigate the possible inducible nature of root "surface" phosphatases. As a consequence, roots were prepared extremely carefully in all subsequent assays, to minimise damage to cells and the release of intracellular phosphatases, which would have obscured levels of activity of the surface phosphatases.

Phosphatase assays carried out over a range of pH values from pH 3.0 to pH 11.0, showed that the optimum pH for phosphatase activity in *Juncus* and *Typha* was pH 5.0, under the particular assay conditions. The optimum pH for *Phragmites* was pH 4.0. The results are shown in Tables 4.231, 4.232 and 4.233. The fact that the peak phosphatase activity of the species were found to occur in the acid range of the pH spectrum, agreed with the findings of other research workers who reported that

acid phosphatases are predominant in higher plants (McComb *et al.*, 1979; Lee, 1988 and Gabbrielli *et al.*, 1989).

The pH spectra for all three species also showed second peaks in phosphatase activity in the alkaline range. *Juncus* and *Typha* had second peaks in activity at pH 10.0. *Phragmites* exhibited a second peak at pH 7.0. Second peaks in phosphatase activity have been found by other research workers investigating the phosphatase activity of higher plants. It is open to question whether the alkaline peaks were due to bacteria living on the surface of the roots exhibiting alkaline phosphatase activity, or whether alkaline phosphatases were produced by the roots themselves. Bieleski (1974) found that *Spirodela* normally exhibits acid phosphatase activity, but produces alkaline phosphatases in conditions of P-deficiency. The possibility of *Juncus*, *Phragmites* and *Typha* synthesising alkaline phosphatases in conditions of phosphorus limitation merits further investigation. The aim of this project however was to investigate levels of surface acid phosphatase activity, so subsequent assays were carried out at pH 5.0.

Time trials carried out showed that rates of phosphatase activity of all three species declined rapidly in 100 μM pNPP assays, (results are shown in Tables 4.241, 4.242 and 4.243). Rates decreased more rapidly in assays with higher initial rates of activity e.g. *Juncus effusus* from Bakethin Reservoir. Extra time course assays were carried out on *Juncus* to compare rates of phosphatase activity between assays with 100 μM and 250 μM pNPP, (results are shown in Tables 4.41 and 4.42). They showed that there was a more constant rate of phosphatase activity over time in assays with 250 μM pNPP, which agrees with the findings of other researchers e.g. Al-Shehri (1992).

The rapid decline in phosphatase activity in 100 μM pNPP assays may be due to all of the substrate having been utilised. As more substrate is present in 250 μM assays it will take longer for it all to be hydrolysed, so rates of phosphatase activity will remain constant for longer. Despite the rapid decline in phosphatase activity, 100 μM assays continued to be carried out in this project, as low levels of organic phosphorus are more comparable to conditions in the natural environment. Assays were terminated after 10 min in order to measure high levels of phosphatase activity.

When the preparatory experiments were completed, assays were run to compare the rates of phosphatase activity of *Juncus*, *Phragmites*, and *Typha* at Bakethin Reservoir, (results are shown in Table 4.25). Results showed that *Juncus* exhibited the highest rate of phosphatase activity (0.080 μ

mol mg^{-1} h^{-1}), followed by *Phragmites,* (0.043 μmol mg^{-1} h^{-1}). *Typha* showed the lowest rate of activity, (0.030 μmol mg^{-1} h^{-1}). Statistical analysis showed that there was a significant difference ($p = 0.0002$) between the three species.

Differences in phosphatase activity between the species may have ecological implications. *Juncus effusus* was found to exhibit higher rates of phosphatase activity than *Phragmites australis* and *Typha latifolia*, and therefore may be capable of living in conditions of lower environmental phosphorus. Further investigations need to be carried out before conclusions can be drawn on the relationship between phosphatase activity of species, and the range of environmental P conditions they are capable of living under. That was beyond the scope of this project however.

Phosphorus analysis of water samples from Bakethin Reservoir, the Botanic Garden and Collingwood College Pond revealed marked differences in P concentrations. Bakethin Reservoir had the lowest levels of P (4.43 μg l^{-1} filtrable reactive P, 10.01 μg l^{-1} total P). The pond at the Botanic Garden had higher levels (27.3 μg l^{-1} filtrable reactive P, 56.9 μg l^{-1} total P). Collingwood College Pond had high levels of orthophosphate (667 μg l^{-1} filtrable reactive P, 670 μg l^{-1} total P).

Phosphatase assays were carried out on *Juncus* from Collingwood College Pond, and *Typha* from the pond at the Botanic Garden. Both species were found to exhibit lower rates of phosphatase activity than the same species at Bakethin Reservoir, (results are shown in Table 4.26). Statistical tests revealed that in both species, there were a significant differences in rates of phosphatase activity between the sites.

Low rates of acid phosphatase activity exhibited by *Juncus* and *Typha* from the Botanic Garden corresponded with high concentrations of P_i. This indicates that acid phosphatase activity of both species may be inducible. Low levels of P_i at Bakethin Reservoir would stimulate the production of phosphatases, whereas higher concentrations of P_i at the Botanic Garden and Collingwood College Pond would repress the synthesis of phosphatase enzymes. Inducible surface phosphatase enzymes have been observed in *Agrostis tenuis* (Woolhouse, 1969), *Trifolium subterraneum* (Dracup *et al.*, 1984) and tomato plants (Goldstein *et al.*, 1988).

Another possibility is that there are several ecotypes of each species (Woolhouse, 1969), the ecotypes growing at Bakethin Reservoir being adapted to lower P_i conditions and exhibiting higher rates of phosphatase activity than the ecotypes at the Botanic Garden.

As investigations into whether *Juncus* and *Typha* growing at Bakethin Reservoir and the Botanic Garden belong to different ecotypes were beyond the scope of this project, it is possible to suggest only that if *Juncus* and *Typha* from both sites belong to the same ecotype, or if there are no different ecotypes, then both species may exhibit inducible acid phosphatase activity. If this is the case, then both species could have the potential for use as bioindicators of environmental phosphorus status.

It was hoped that BCIP staining procedures would reveal the locality of phosphatase enzymes in the roots. Staining proved unsuccessful however as *Juncus* roots were too heavily pigmented for the indigoid dye to be observed. *Typha* roots retained only a small amount of dye which appeared to be distributed generally throughout the root. No specialised areas for phosphatase activity were observed under the light microscope.

Several problems with the methodology of this project were encountered throughout the course of investigations. One important problem lay with the actual experiment for measuring phosphatase activity. The " pNPP assay " is most suitable for measuring rates of phosphatase activity in algae, bacteria, and other organisms which produce extracellular phosphatases. The pNPP is hydrolysed to pNP and P_i extracellularly. All the pNP produced is released into solution and its concentration can be measured using a spectrophotometer.

The process is more complicated in the case of eukaryotes however. Acid phosphatases in higher plants are located mostly intracellularly, or as surface enzymes. They are more rarely extracellular (Dracup *et al*, 1984). pNPP is taken by the cells and hydrolysed intracellularly or by enzymes bound to cell walls. For phosphatase activity to determined accurately, all the pNP must be released back into solution so that its concentration can be measured. It was observed however, that in phosphatase assays carried out on *Juncus*, *Typha* and *Phragmites*, roots were often stained yellow after assaying, presumably due to the retention of pNP. As a result, phosphatase assays carried out on the species may not have measured the true rates of phosphatase activity but recorded lower values.

A great deal of investigation needs to be done to determine how capable the pNPP assay is of accurately measuring the phosphatase activity of eukaryotes. If it is proved that a significant amount of pNP is retained by certain species, and not released into solution, then the validity of research carried out previously by many workers will be open to question.

One preliminary experiment investigating the retention of pNP by roots, was carried out on *Typha,* (results are shown in Table 4.5). It was found that more pNP was retained at lower pH values. This may have important repercussions on the results produced in this project. Most of the phosphatase assays were carried out at pH 5.0 so actual rates of phosphatase activity may have been significantly higher than those recorded. Unfortunately the time limit of the project meant that it was not possible to investigate this potential problem further. For the purposes of analysis, it has been assumed that the amount of pNP retained by the roots of *Juncus, Phragmites* and *Typha* was not significant, and that there was no significant difference in levels of retention between species.

Another problem encountered in this project, was that it would have been more accurate to correlate rates of phosphatase activity with sediment P concentrations, than water P concentrations. The P content of lake sediments depends on many factors including sediment composition, grain size, pH and redox potential, as well as the P content of the water (Holtan *et al.*, 1988). As sediments vary so much in composition, there is no consistent relationship between the P content of a water body and P content of the sediments. Unfortunately there was not time to carry out phosphorus analysis of sediments, so for the purpose of this project it has been assumed that sediment P levels can be correlated with water P concentrations. As such marked differences were found in phosphorus concentrations of water from Bakethin Reservoir, the Botanic Garden Pond and Collingwood College Pond, it was hoped that the large differences in P levels of the water bodies would outweigh any differences in the sorption capacity of the sediments.

Another difficulty with this project was relating the rates of phosphatase activity measured in assays to the situation in the natural environment. Phosphatase assays were carried out at the optimum pH for phosphatase activity (pH 5.0), whereas the water was pH 7.3 at Bakethin Reservoir and pH 7.5 at the Botanic Garden. Rates of phosphatase activity occurring in all three species would therefore have been lower in the environment than the values measured in the assays. In this project it has been assumed that the differences in phosphatase activity between the species were not greatly effected by the small difference in pH.

CONCLUDING REMARKS

Despite the problems with the methodology of this project mentioned above, interesting results have been obtained. Phosphatase assays carried out on *Juncus effusus*, *Phragmites australis* and *Typha latifolia* have revealed that the roots of all three species exhibit acid phosphatase activity, and that there is a statistically significant difference in rates of activity between the species.

Assays carried out on root material from Bakethin Reservoir, the Botanic Garden and Collingwood College Pond (sites with markedly different levels of phosphorus), have shown that *Juncus* and *Typha* both exhibit significantly higher rates of phosphatase activity at Bakethin Reservoir (where environmental P_i concentrations are low), than at the Botanic Garden (where P_i levels are higher). This indicates that acid phosphatase activity in both *Juncus* and *Typha* may be inducible in conditions of P-limitation. If further studies reveal that this is the case, then both species may have the potential for use as biondicators of environmental phosphorus status.

SUMMARY

1. Phosphorus analysis of water samples collected from Bakethin Reservoir, the Botanic Garden and Collingwood College Pond showed marked differences in phosphorus concentrations. Collingwood College Pond had the highest concentration of phosphorus, followed by the Botanic Garden. Bakethin Reservoir had the lowest concentration of phosphorus.

2. Phosphatase assays showed no significant difference in rates of phosphatase activity between different types of roots from the same species i.e. between brown roots and white roots of *Juncus effusus* ($p = 0.94$), and between roots with root hairs and roots without root hairs of *Phragmites australis* ($p = 0.48$) and *Typha latifolia* ($p = 0.94$).

3. Phosphatase assays showed that phosphatase activity increased in all three species with increased handing time of roots i.e. from rinsing, to thorough washing, to crushing. This trend was not statistically significant however. The observed increase in phosphatase activity may have been caused by the release of intracellular phosphatases from lysed cells of damaged roots. Phosphatase activity exhibited by bacteria living on the surface of the roots was not significant.

4. Phosphatase assays from pH 3.0 to pH 11.0 showed that pH 5.0 was the optimum pH for phosphatase activity in *Juncus* and *Typha*. pH 4.0 was the optimum pH for phosphatase activity in *Phragmites*, under the particular assay conditions.

5. Time trials showed that rates of phosphatase activity declined rapidly after the initial 10 min in 100 μM assays for all three species. Subsequent assays were terminated after 10 min.

6. Phosphatase assays of all three species from Bakethin Reservoir revealed significant differences ($p = 0.0002$) in rates of phosphatase activity. *Juncus* showed the highest rate of activity followed by *Phragmites*. *Typha* exhibited the lowest rate of phosphatase activity.

7. Phosphatase assays of *Juncus* and *Typha* showed significantly higher rates of phosphatase activity at Bakethin Reservoir than the Botanic Garden ($p = 0.026$ for *Juncus*, $p = 0.037$ for *Typha*). High rates of phosphatase activity at Bakethin Reservoir corresponded with low levels of phosphorus, so it is possible that root surface phosphatases are inducible in conditions of P-limitation. *Juncus* and *Typha* may both therefore have the potential for use as bioindicators of environmental phosphorus status.

8. Time courses on *Juncus* showed that in 100 μM assays, rates of phosphatase activity declined rapidly after the initial 10 min. This may have been caused by rapid hydrolysis of all of the substrate. In 250 μM assays rates of phosphatase activity remained more constant, declining gradually over the course of one hour.

9. Phosphatase assays showed that roots retained more pNP under conditions of low pH. The retention of pNP by roots has serious implications for the validity of the pNPP assay, as a method for determining rates of phosphatase activity in eukaryotes.

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