The status of phosphorus of a calcareous flush at Bakethin Reservoir, Northumberland

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ABSTRACT

In order to assess the status of phosphorus of a calcareous flush at Bakethin reservoir, Northumberland, chemical analyses of the concentration of phosphorus in the water and the phosphatase activity of cyanobacteria, *Nostoc* and *Rivularia*, as well as the bryophytes, *Drepanocladus revolvens* and *Cratoneuron commutatum*, were performed. Concentrations of total filtrable phosphorus ranged from 0.11 to 11.35 $\mu g\ l^{-1}$ and filtrable reactive phosphorus ranged from below detection to 10.55 $\mu g\ l^{-1}$. No statistical difference was determined in concentrations of phosphorus at different sites in the flush, but a significant difference was determined between different sampling dates, at $p < 0.05$. Phosphatase activity in the cyanobacteria, *Rivularia* and *Nostoc*, varied between different sites and different dates. *Nostoc* showed a consistently higher rate of phosphatase activity than *Rivularia*. Activity in both the cyanobacteria showed no correlation between filtrable reactive and filtrable organic content of the water. Phosphatase activity in the bryophytes, *Drepanocladus revolvens* and *Cratoneuron commutatum*, had its pH maximum in the acidic range. Activity also varied between sampling dates and between different species.

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In order to assess the status of phosphorus of a calcareous flush at Bakethin reservoir, Northumberland, chemical analyses of the concentration of phosphorus in the water and the phosphatase activity of cyanobacteria, *Nostoc* and *Rivularia*, as well as the bryophytes, *Drepanocladus revolvens* and *Cratoneuron commutatum*, were performed. Concentrations of total filtrable phosphorus ranged from 0.11 to 11.35 $\mu g\ l^{-1}$ and filtrable reactive phosphorus ranged from below detection to 10.55 $\mu g\ l^{-1}$. No statistical difference was determined in concentrations of phosphorus at different sites in the flush, but a significant difference was determined between different sampling dates, at $p < 0.05$. Phosphatase activity in the cyanobacteria, *Rivularia* and *Nostoc*, varied between different sites and different dates. *Nostoc* showed a consistently higher rate of phosphatase activity than *Rivularia*. Activity in both the cyanobacteria showed no correlation between filtrable reactive and filtrable organic content of the water. Phosphatase activity in the bryophytes, *Drepanocladus revolvens* and *Cratoneuron commutatum*, had its pH maximum in the acidic range. Activity also varied between sampling dates and between different species.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
mg	milligram
µg	microgram
l	litre
ml	millilitre
µl	microlitre
m	metre
cm	centimetre
mm	millimetre
µm	micrometre
h	hour
min	minute
µM	micromolar
µmol	micromole
µS cm^{-1}	microsiemans per centimetre
P	phosphorus
FRP	filtrable reactive phosphorus
FOP	organic reactive phosphorus
TFP	total filtrable phosphorus
p-NPP	*para*-nitrophenylphosphate
MUP	methylumbelliferyl phosphate
CAPS	3-(cyclohexylamino)-1-propanesulphonic acid
DMG	3,3-dimethylglutaric acid
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
HEPES	N-2-hydroxymethylpiperazine-N'-2-aminoethane sulphonic acid
p	probability
MW	molecular weight

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CHAPTER 1

INTRODUCTION

1.1 PHOSPHORUS

Phosphorus is the eleventh most abundant element in nature. Its average concentration in the environment has been estimated to be 0.1% by weight and is thus geochemically classed as a trace element (Holten *et al.* 1988). P is classed as a macronutient. However in comparison to the rich natural supply of the other nutritional components of the biota, P is the least abundant and most commonly limits biological productivity (Wetzel 1983). Orthophosphate is the form of P most easily assimilated by organisms. P plays a predominant and vital role in cellular energetics as a component of adenosine triphosphate (ATP), which is required by all living organisms. It also forms an important part of many structural and biochemically functional components for cell growth (Ahlgren 1988), and is part of the genetic code of DNA (Cole 1979). Hutchinson (1957) claims ' of all the elements present in living organisms, P is likely to be the most important ecologically'.

P is not found free in nature and almost always occurs in the fully oxidised state as phosphate, (Corbridge 1990). The major origin of its supply comes from the weathering of mineral deposits (Fox 1993). Phosphate deposits can be classed into three broad apatite groups, (Ca_5 (PO_4, CO_3)$_3$ (F, Cl, OH) of igneous and metamorphic origin. More than 200 different phosphate minerals are known, but only those in the apatite group occur in significant abundance (Corbridge 1990). Fluoroapatite is the most common (Cole 1979). Weathering of these deposits causes residual minerals to be released into the soils where they can be utilised by organisms, enter the water table, or resorbed into the sediment (Holten *et al.* 1988).

1.2 PHOSPHORUS IN FRESHWATER SYSTEMS

P occurs in natural waters almost always as phosphates. These are classed as orthophosphates, condensed phosphates and organically bound phosphates. They can occur in particles, colloids, in solution, or in bodies of aquatic organisms. Phosphates can also occur in bottom sediments both in inorganic forms and incorporated into organic compounds (American Public Health Association 1989).

Filtration through a 0.45μm pore diameter membrane filter separates dissolved from suspended particulate forms of P (Broberg and Persson 1988). No claim is made that filtration is a true separation of suspended and dissolved forms of P but it is merely a convenient and replicable analytical technique designed to make a gross separation. Phosphates that respond to colorimetric tests without preliminary hydrolysis or oxidative digestion of the water are termed reactive P. While this is largely a measure of orthophosphate, a small fraction of any condensed P present is hydrolysed unavoidably. Reactive P occurs in both dissolved and suspended forms.

1.21 Suspended P

Suspended particulate P is seen as the difference between the total P in filtered and unfiltered water samples (Broberg and Persson 1988). P-containing particles come from three main sources.

(i) Biologically produced cells of plants, bacteria and animals.

(ii) Weathering products such as primary and secondary minerals

(iii) Direct precipitation of inorganic P (authigenic mineral formation) or sorbtion to other precipitates.

In addition degradation and fragmentation of cells provide organic detritus. Flocculation of cells can also provide larger sized aggregates, and inclusion of P by metal- phosphorus binding into organic aggregates can also form particulate forms of P.

1.22 Filtrable P

Filtrable P is conventionally defined as P which passes through a 0.45μm filter (American Public Health Association 1989). It can consist of

(i) Small particles organic or inorganic, not retained by the filter.

(ii) Colloidal material

(iii) Dissolved inorganic P

(iv) Dissolved organic P.

In freshwater ecosystems it is dissolved inorganic and organic P that are of the most importance (Broberg and Persson 1988).

1.23 Colloidal P

Colloids form a badly defined group of compounds between true particles and solids. In true solution, they are present as hydrated inorganic solids, large organic macromolecules or organic-inorganic complexes (Broberg and Persson 1988). They are charged or partly ionised, which by repulsion, prevents their precipitation. In P chemistry, these colloids have been identified as important P carriers (Jackson and Schindler 1975).

1.24 Dissolved inorganic P

Van Wazer (1958) recognises four main categories of dissolved inorganic P. These are,

(i) Orthophosphate

(ii) Polyphosphate

(iii) Metaphosphate

(iv) Ultraphosphate.

Orthophosphate is generated by weathering of rocks, biological metabolism and degradation. Polyphosphates and metaphosphates are produced by biological activity (Broberg and Persson, 1988).

1.25 Dissolved organic P

Dissolved organic P account for a considerable part of the dissolved P in the aquatic environment (Jansson *et al.* 1988). Sources of dissolved organic P are not clearly defined, and it is uncertain as to whether they are released by active cell metabolism or by cell death and decay (Christman and Minear 1971). Many are present as compounds of high molecular weight. Nucleotides and polynucleotides have been observed to consist a major part (Chróst and Overbeck 1987; Chappell and Goulder 1992).

1.26 P in sediments

Sediments can act as a source or sink of P in freshwater systems. P is deposited in the sediment in mineral or organic form. Apatite is the only P mineral which is conserved in it's original form, while others are formed by diagenic processes (Pettersson *et al.* 1988). It is generally believed that P is bound or adsorbed onto iron hydroxides, clay, apatite or in organic matter, either dead or alive (Golterman 1988).

The source of sedimentary P is partly settled particulate P of allochtanous or autochtanous origin and partly dissolved phosphate sorbed to the surface sediment (Holten *et al.* 1988). Total sedimentary P can be divided into dissolved and particulate forms which again can be subdivided into organic and inorganic fractions. Factors affecting the sorbtion and release of sedimentary P are varied.
In general sorbtion is related to a number of physical, chemical and biological factors (Wetzel 1983; Holten *et al.* 1988). Klotz (1985) identifies soil size, iron, aluminium and organic content of the sediment as effecting the rate of sorbtion of P. Release of P can be influenced by pH , redox potential, temperature (Holten *et al.* 1988) O_2 levels, biological activity in sediments and steam velocity (D'Angelo and Webster 1991). The maturity and level of disturbance within the system can also effect the sorbtion/release of sedimentary P (Meyer and Likens 1979).

1.3 THE PHOSPHATASE ENZYME

1.31 Definition of phosphatase enzyme.

Phosphatases are enzymes which hydrolyse ester-bound phosphate from dissolved organic phosphate (Hantke and Melzer 1993). These enzymes release orthophosphate from particulate and dissolved substrates so that PO_4^{3-} becomes available for uptake by organisms in the preferred form (Burkholder and Wetzel 1990). In this study, the term phosphatase refers to phosphomonoesterases, i.e., enzymes that hydrolyse phosphomonoesters. Similar, but functionally distinct classes also occur, including phosphodiesterases (nucleases). Phosphatases have been suggested to be involved in metabolic processes other than the hydrolysis of organic phosphates. These are transport of substances across cell membranes and synthesis of new organic phosphates (McComb *et al.* 1979). The action of phosphatase enzymes are believed to play an important role in the turnover of organic P in the biota (Chróst and Overbeck 1987; Jansson *et al.* 1988; Whitton *et al.* 1990; Hantke and Melzer 1993).

1.32 Action of the phosphatase enzyme

The reaction mechanism for the enzyme catalysed hydrolysis of phosphate esters is divided into four steps (McComb *et al.* 1979).

(i) Noncovelant binding of the substrate to the enzyme (EH).

(ii) Alcohol release from the complex and inorganic P becomes covalently bound to the enzyme forming a phosphoryl-enzyme compound.

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

(iv) Release of inorganic P and regeneration of free enzyme.

The phosphatase activity will mainly depend on the type and concentration of the substrate and enzyme. Other factors which have an effect on phosphatase activity are temperature, ionic strength, pH and metal ions (McComb *et al.* 1979).

1.33 Methods of measuring phosphatase activity.

Phosphatase activity is assayed by hydrolysis of a suitable artificial substrate and detection of the increase of organic product or orthophosphate over time. Two of the most common substrates used are p-NPP (para -nitrophenylphosphate) and 4-MUP (methylumbelliferylphosphate). Of these two substrates 4-MUP is recognised as being the more sensitive, when detecting low levels of phosphatase activity (Jansson *et al.* 1988). Both these assays have a number of drawbacks (Jansson *et al.* 1988).

(i) Substrate concentration used in the assay are higher than natural substrate levels and so, natural rates of hydrolysis may be lower.

(ii) Temperature and pH are standardised in the assay for optimum activity. These standard levels are often not the same in natural waters (Olsson 1990).

(iii) The structure of natural substrates is often not clearly known. The affinity of phosphatases for different substrates differs considerably according to structure, so levels of activity obtained using an artificial substrate may not be representative.

However, in spite of this, phosphatase assays can be used to determine potential phosphatase activity at a particular time. It may also expose differences between rates of phosphatase activity between different nutrient conditions and different taxa.

1.34 Characteristics of phosphatase

Phosphatases all have a broad specificity against different substrates (Whitton *et al.* 1991). In the natural environment phosphatases vary with temperature, pH and orthophosphate concentration

(Chróst and Overbeck 1987). Phosphatases all have maximum activity at specific pH values. Common division in the class of enzymes is into acid or alkaline phosphatase depending on where their maximum activity lies along the pH spectrum. Both acid and alkaline phosphatases have a broad specificity towards the substrate and activity is restricted to the P—O bond. However a number of subtle differences exist between them.

1.35 Acid phosphatases

Acid phosphatases are enzymes of wide specificity which cleave phosphate-ester bonds and play an important role in the mineralization of organic phosphate in the environment. They are non-metallic enzymes, which are not activated by divalent cations and are inhibited by fluoride (Cembella *et al.* 1984). They are found inside algal cells (Siuda 1984).

1.36 Alkaline phosphatases

Alkaline phosphatases require divalent metal ions for their activity. They are inhibited by chelators e.g. EDTA. Alkaline phosphatases are located on the extracellular surface, and are produced or activated in response to the P nutritional status of the environment (Jansson *et al.* 1988).

The potential use of alkaline phosphatase activity as a bioindicator of phosphate deficiency in fresh water has been widely debated. Increased activity in the absence of orthophosphate has led many to suggest that activity at the time of measurement is a good indication of P status in the environment (Whitton 1991). However, correlation between phosphatase activity and the orthophosphate content of the water has not always been observed (Chróst and Overbeck 1987), with phosphatase activity relating more to the internal P content. Therefore the indicator potential may relate more in describing the nutrient status of the organism in relation to the P status of the environment.

1.37 Dissolved phosphatases

Free dissolved phosphatases are defined as enzymes that can pass a 0.45μm membrane filter. They are always found in lakes and can contribute to a substantial amount of phosphatase activity (Jansson *et al.* 1988). These enzymes are provided by excretion from growing algae and zooplankton

and to a lesser extent from bacteria (Aaronson and Patni 1976). Disintegrating cells is also another important source of these enzymes.

1.38 Bacterial phosphatases

Bacteria possess phosphatase activity and it has been extensively studied in *E. coli* (McComb *et al.* 1979). Contribution of bacterial phosphatase to freshwater ecosystems is as yet unclear, with different studies observing the presence and absence of a correlation between bacterial numbers and P activity.

Research into the role of bacteria in sediments is scanty, but past studies have shown that a higher level of bacterial phosphatase activity exists in the sediments than in the overlying water (Jansson *et al.* 1988).

1.39 Algal phosphatases

Phosphatase activity in algae is found in all major groups. It has been frequently demonstrated in cultured cyanobacteria and algae. Activity has been located on the cell surface and on the membrane, and release of extracellular enzymes in algal cultures has been reported (Siuda 1984; Whitton *et al.* 1990; Whitton *et al.* 1991). In cyanobacteria, cell bound surface phosphatase activity is widespread, but by no means universal (Healey 1982). In hair forming strains of *Calothrix* and other members of the Rivulariaceae, phosphatase activity is induced under conditions of low inorganic P, and commences at the same time as hair formation. It has been demonstrated that this phosphatase activity is restricted to these hairs (Whitton *et al.* 1991). Studies on the cyanobacteria *Nostoc* could not determine if different cell or filament types showed different levels of phosphatase activity (Whitton *et al.* 1990).

1.310 Bryophyte phosphatases

Very little research has been performed on phosphatase activity in bryophytes. Press and Lee (1983) observed that in eleven different species of *Sphagnum*, the phosphatase activity was negatively correlated to the tissue level of P in the moss. This correlation has also been observed in the phosphatase activity of *H. fontanum* (Al-Shehri 1992). Al-Shehri (1992) also found that phosphatase

activity in *H. fontanum* was induced at low concentrations of inorganic P. Both these studies showed that the bryophytes in question had maximum activity in the acidic pH range.

1.4 Calcareous ecosystems

Calcareous freshwater ecosystems are rich in dissolved calcium carbonate, an important buffering mechanism for the total inorganic carbon concentration in fresh water (Wetzel 1983). CO_2 solubility is increased markedly in water that contains carbonate. Groundwater of limestone regions, heavily enriched with carbonate can release CO_2 into the atmosphere when it flows to the surface, with much precipitation of $CaCO_3$ (Wetzel 1983). This is summarised in the equation below;

$$Ca(HCO_3)_2 \Leftrightarrow CaCO_3\downarrow + H_2O + CO_2$$

High concentrations of Ca^{2+} in the water can increase the chemical adsorption of phosphate onto sediments. This is due to the soluble Ca^{2+} concentration which causes the product formed (apatite) to co-precipitate with $CaCO_3$ (Golterman *et al.* 1993).

One rare and striking features of calcareous systems is the calcareous patterns formed by the organisms, e.g. algae, in the system. Deposition of carbonate in freshwater systems is caused mainly by calcified autotrophic microorganisms and is relatively uncommon (Rott 1991).

1.41 Calcification in algae

Most calcifying algal regimes are characterised by a water pH of 7.8-9.5. The medium surrounding the calcifying algae is normally supersaturated with respect to mineral phases of calcium carbonate. In water supersaturated with $CaCO_3$, chemical precipitation predominates and many objects become encrusted in carbonate. The kinetics of calcite nucleation are slow and can be inhibited by a number of substances (Pentecost 1991). Communities of cyanophytes often colonise pebbles and shells, rolling over the river bed. As they calcify, a crust grows concentricity starting from the 'nucleus' (Golubic 1973). Pia (1933) called such encrusted algal oncolytes. They were renamed oncoids by Flügel (1982).

Calcified *Rivularia* form oncoid growths. Calcification begins within the extracellular mucilaginous sheath surrounding the trichomes (Pentecost 1987). The process can depend on environmental conditions such as levels of light, low flow, and low current speed. Different calcification patterns can occur within the same genus indicating a high complexity between the structure of the organic matter and development of the calcification (Obenlüneschloss and Schneider 1991). *Rivularia* oncoids were a dominant feature at the calcareous flush at Bakethin reservoir.

1.5 Aims

The aims of this project are

(i) To determine the levels of P in the water at the calcareous flush at Bakethin reservoir

(ii) Examine the phosphatase activity of the cyanobacteria *Rivularia* and *Nostoc.*

(iii) Examine the phosphatase activity of the bryophytes, *Drepanocladus revolvens, Cratoneuron commutatum,* and *Calliergon cuspidatum.*

CHAPTER 2

MATERIALS AND METHODS

2.1 FIELD MEASUREMENTS

2.11 pH

The pH probe, Ingold wtw combination electrode, was calibrated in the field before measurement using BDH standard buffer solutions, which were made up with MilliQ water.

2.12 Conductivity

Conductivity was measured using a WTW meter (model LC 910).

2.13 Temperature

Temperature was measured a WTW meter (model LC 910).

2.2 FIELD SAMPLING

2.21 Collection of cyanobacteria and algal material

Cyanobacteria and algae were collected for analysis using a blunt stainless steel forceps and stored in glass snap-cap vials with a little stream water. The samples were placed on ice until returning to the laboratory, where they were stored at 4°C until analysis the following day.

2.22 Collection of Bryophytes

Bryophytes were collected using a blunt forceps and placed in sterile polyethylene bags. The samples were stored at 4°C until analysis for phosphatase activity. This was performed no more than 48 h after collection.

2.23 Collection of Water

Water samples were collected in acid washed 250ml polypropylene bottles and stored on ice until returning to the laboratory. The samples were then filtered using Whatman GF/F filters . Phosphorus analysis was performed immediately after filtration.

2.3 LABORATORY MEASUREMENTS

2.31 pH

The pH measurements in the laboratory were carried out using an Ingold wtw combination electrode and an EIL meter (model 7050). The probe was calibrated with BDH standard buffer solutions which were prepared in MilliQ water.

2.32 Colourimetric Analysis

Colourimetric analysis was performed using a Shimadzu double beam spectrophotometer (model UV-150-02). Glass cells of 1cm pathlength were used in phosphatase analysis using p-NPP substrate at a wavelength of 405nm. 4cm cells were used for phosphorus analysis (882nm), chlorophyll 'a' analysis (665nm and 750nm), ammonia (640nm) and nitrate and nitrite (543nm)

2.33 Fluorimetric Analysis

A Baird Atomic Fluoripoint Spectrofluorimeter was used for fluorimetric analysis during the phosphatase assay with 4-MUP as substrate. Polystyrene cuvettes with a path length of 1.0cm were used at a wavelength of 440nm emission and 356 excitation.

2.34 Glassware and Utensils

All snap-cap vials used in phosphatase assays were soaked overnight in 2% decon and were rinsed very thoroughly in distilled water. Conical flasks used in the chemical analysis of water were soaked for a minimum of 20 min in 4% sulphuric acid, before being very thoroughly rinsed in distilled water. All glassware was dried overnight at 100 ^{o}C. Volumetric glassware and plastics were dried at room temperature.

2.4 MEDIA AND BUFFERS

2.41 Assay Medium

Chu -10D assay medium was used during phosphatase assays. The medium was freshly made before each assay. The contents of this medium are given in Table 2.1.

Table 2.1. Contents of CHU 10-D assay medium

Major elements	Salt mg l^{-1}	μ M
Mg $SO_4.7H_2O$	25.00	101.40
Na H CO_3	15.85	188.60
Ca $Cl_2.2H_20$	35.83	243.70
KCl	4.28	57.38
Stock added as Fe chelate		
Na_2-EDTA-$2H_2O$	1.67	4.17
Micro elements	**(Trace elements)**	
Mn Cl_2 .$2H_2O$	0.040	2.28
Cu SO_4 .$5H_20$	0.020	0.078
Co $SO_4.7H_2O$	0.010	0.035
Ni $SO_4.7H_2O$	0.038	0.03
Zn $SO_4.7H_2O$	0.056	0.019
Na_2 Mo O_4	0.007	0.028
H_3BO_3	0.720	11.56

2.42 pH Buffers

The different pH buffers used are listed in Table 2.2. The buffers were prepared in assay medium. Buffers were stored at 4°C between assays, but were warmed up to room temperature before the assay was performed.

Table 2.2. The pH buffers used in the phosphatase assay

pH	Buffer	Buffering Capacity	pKa at 20°C
3.0	DMG-NaOH	3.2- 7.6	3.66 + 6.20
4.0	DMG-NaOH	3.2- 7.6	3.66 + 6.20
4.5	DMG-NaOH	3.2- 7.6	3.66 + 6.20
5.0	DMG-NaOH	3.2- 7.6	3.66 + 6.20
5.5	DMG-NaOH	3.2- 7.6	3.66 + 6.20
6.0	DMG-NaOH	3.2- 7.6	3.66 + 6.20
7.0	HEPES-NaOH	6.8- 8.2	7.50
8.0	HEPES-NaOH	6.8- 8.2	7.50
8.2	HEPES-NaOH	6.8- 8.2	7.50
9.0	Glycine-NaOH	8.6-10.6	8.0
10.0	Glycine-NaOH	8.6-10.6	8.0
10.3	Glycine-NaOH	8.6-10.6	8.0
11.0	CAPS-NaOH	9.8-11.1	10.40

2.5 WATER CHEMISTRY

Phosphorus Analysis

The phosphorus content of the water was measured using the method outlined by Eisenreich *et al.* (1975). Two different P fractions were measured, the total filtrable P (TFP), and the filtrable reactive P (FRP), which is equivalent to the dissolved inorganic fraction. The filtrable organic P (FOP) content could be determined as the difference between the FRP and TFP of the sample.

2.6 ASSAY FOR PHOSPHATASE ACTIVITY

2.61 Preparation of cyanobacterial material

2.611 *Rivularia* and *Nostoc*

Cyanobacteria colonies were rinsed in assay medium to remove excess mucilage and debris from the exterior of the colonies. Colonies of roughly the same size of *Rivularia* and *Nostoc* were used to reduce any variation of result due to difference in surface area.

2.612 *Spirogyra*

Spirogyra filaments were rinsed in assay medium and teased to make sure that all debris had been removed from the filaments.

2.62 Preparation of Bryophyte Material

Bryophyte material was were with assay medium to remove any debris. Two shoots of roughly 2cm length were used in each assay.

2.63 PHOSPHATASE ASSAY PROCEDURE USING p-NPP SUBSTRATE

The assay was performed at two different substrate concentrations, 100 μmol and 250 μmol p-NPP. Reagents and buffers were allowed to equilibrate to room temperature before the assay was performed. The reaction was carried out in sterile snap-cap vials. Cyanobacterial and algal assays were performed at the pH maximum of 10.3, while bryophyte assays were performed at the pH maximum of 5.0 to give an indication of the maximum amount of phosphatase activity present. 1.5ml of pH buffer was pipetted into each with 1.4ml of assay medium. The material for analysis was placed in each of these solutions and left to equilibrate for 5 minutes at 25°C. The reaction was initiated by the addition of 0.1ml of p-NPP substrate of relevant molarity. The vials were shaken constantly throughout the assay. As the reaction was observed to be linear with time, it was left to continue for 15 minutes before termination with 3.0ml of 5M NaOH. Material was removed from the vials before termination to prevent lysing of the cell by the terminator.

The solutions were then filtered using Whatman GF/C filters before reading, to prevent any calcite crystals interfering with the light path. The solution was read spectrophotomically at a wavelength of 405nm. Each assay was carried out using four replicates and three controls. Each control was performed with the standard assay procedure except that no algal or bryophyte material was placed in the vials.

The cyanobacterial colonies were frozen until analysis for chlorophyll 'a'. Bryophyte mass was determined by dry weight.

Spirogyra mass was determined by dry weight. This reading was later converted into µmol chlorophyll 'a'$^{-1}$.

This assay gives the phosphatase activity as µmol product produced µmol chlorophyll 'a'$^{-1}$ h^{-1}.

2.64 INFLUENCE OF pH ON PHOSPHATASE ACTIVITY

A pH spectral analyses of phosphatase activity of *Rivularia*, *Drepanocladus revolvens* and *Cratoneuron commutatum* samples was performed. This involved performing the phosphatase assay at different values along the pH spectrum, using 250 µmol p-NPP as substrate.

2.71 ASSAY PROCEDURE USING 4-MUP SUBSTRATE

This assay was performed at two different substrate concentrations, 1µmol and 250µmol 4-MUP. The assay was also performed at the environmental pH rather then the pH maximum. Cyanobacterial material was prepared as in the p-NPP assay. The same procedure was followed except the reaction was initiated with 0.1ml of 4-MUP substrate of the relevant concentration. The reaction was observed to be linear with time and was terminated after a period of 10 min using 0.3ml of a solution containing 2.5 mmol EDTA, 50 mmol NaOH and 50 mmol K_2HPO_4. Solutions were then filtered using Whatman GF/C filters before reading to prevent any calcite crystals interfering with the light path. Cyanobacterial material was removed before termination and the assay solutions were read on the fluorimeter immediately after. The material was then frozen until analysis for chlorophyll 'a'.

This assay gives the phosphatase activity as μmol product produced μmol chlorophyll 'a' $^{-1}$ h^{-1}.

2.8 CHLOROPHYLL 'A' ANALYSIS

Chlorophyll 'a' of the assayed material was analysed using the method outlined by Marker *et al.* 1980.

Samples were thawed on the morning of analysis.

CHAPTER 3

STUDY AREA

3.1 BACKGROUND

The calcareous flush lies on the north-eastern shore of Bakethin Reservoir, Northumberland (Fig 3.1). Bakethin forms part of a two reservoir system with Kielder Reservoir, both owned by Northumbrian Water. The area around Bakethin reservoir has been a designated conservation area since 1979, when the reservoir was built, and has been under an active conservation management plan since 1988.

3.2 LOCATION

The Bakethin area is covered by the following ordnance survey maps:

1: 63,000 sheet 76

1: 50,000 sheet 80

1: 25,000 sheet 69

1: 10,000

1: 2,500

3.3 GEOLOGY

The prevalent geology of the Bakethin area is carboniferous limestone. It is located on the following geological survey maps at;

1: 63,000 sheet 7

1: 10,000 sheet 54

3.4 CATCHMENT AREA

The reservoir is surrounded by Kielder forest, which is owned by the Forestry Commission. No fertilisation has taken place in the area since the reservoir was built. Five farms are located in the area and these are sometimes subjected to a fertiliser regime.

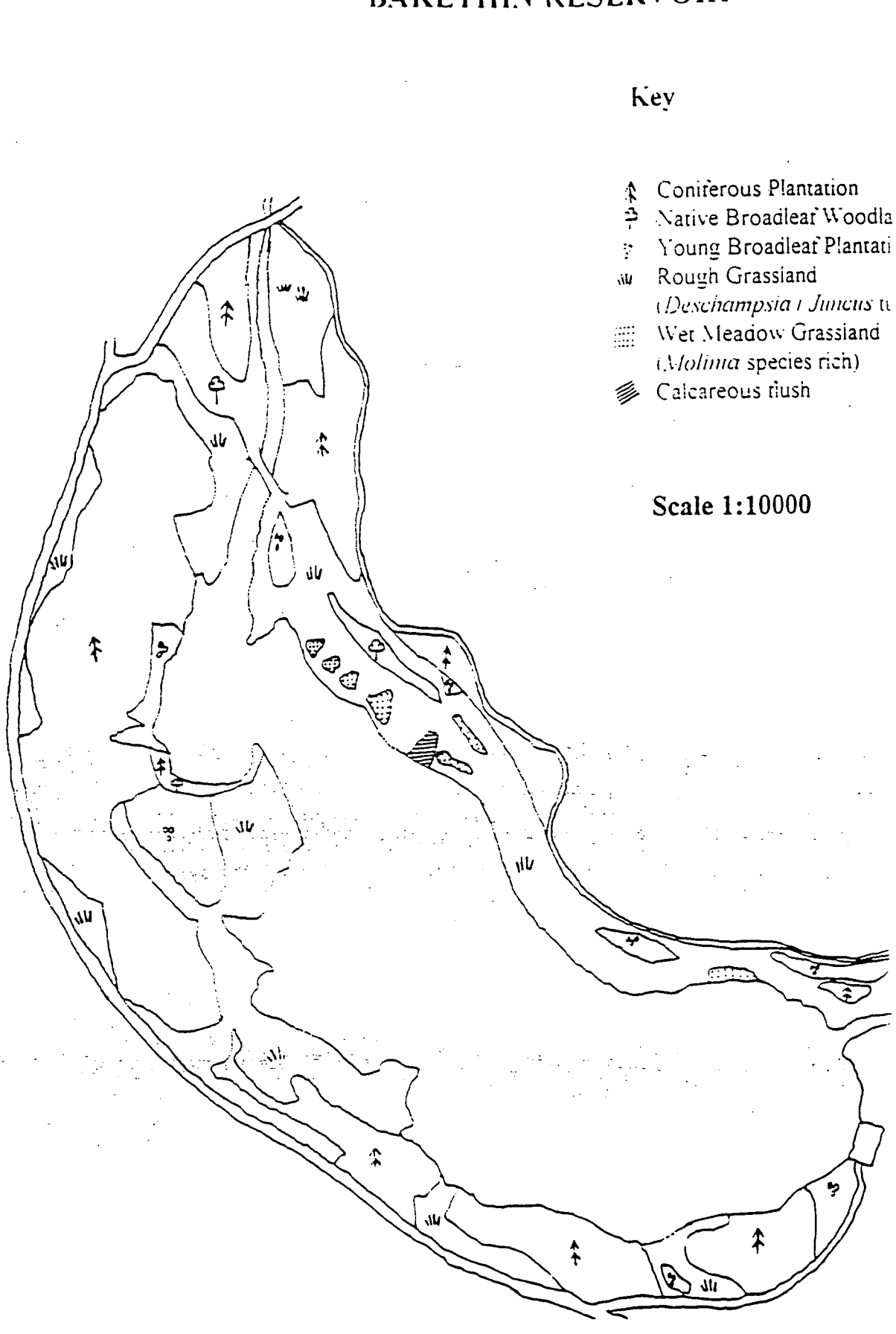


Fig. 3.1 Location of the calcareous flush at Bakethin reservoir

3.5 CLIMATE

The reservoir is subject to climate conditions belonging to the temperate zone, i.e. temperature and insolation change throughout the year give rise to a marked seasonal cycle. Details of monthly rainfall and maximum and minimum temperature, as observed in 1991, are given in below (Table 3.1).

Table 3.1 Rainfall (mm), maximum and minimum temperature °C of the Kielder area.

Month	Total rainfall (mm)	Max temperature (°C)	Min temperature (°C)
January	179.3	9	-8
February	167.3	9	-7
March	103.3	12	-3
April	63.3	14	-2
May	19.7	19	0
June	115.4	20	4
July	83.1	23	9
August	39.3	22	8
September	67.7	21	5
October	93.0	13	0
November	195.5	10	-2
December	106.0	10	-7

3.6 THE CALCAREOUS FLUSH

The flush is located on the North-eastern shore of the reservoir. It is 18m in length and of variable width, ranging from 170 cm to 30 cm. Two separate channels lie about a metre to the left and right of the flush. No calcified algae appear in these channels. The area in which the flush lies is fenced off and does experience any grazing from sheep. Disturbance is kept to a minimum and a wooden walkway was built over the flush to prevent disturbance from anglers on the shore. The source of the flush is not apparent and it appears to be fed by a seepage from the ground. A number of plants were

found growing in the flush itself. These include *Carex disticha, C. hostiana, Equisetum fluviatile* and *Phragmites australis.* A large stand of *P. australis* is located at the head and end of the flush. *Chara vulgaris* was also present in the flush. The site around the flush is the location of a number of orchids *Dactylorhiza incarnata, D. purpurella* and *Gymnadenia conopsea. Pinguicula vulgaris* and *Drosera rotunifolia* were also present.

For sampling purposes the flush was divided into four microhabitats on the basis of physical and floristic features. These features are summarised in Table 3.2.

Table 3.2 Microhabitats of the calcareous flush

Site	Width (cm)	Cyanobacteria	Filamentous green
Site I	170	*Rivularia*	*Spirogyra*
		Nostoc commune	
Site II	150	*Rivularia*	None
		Nostoc commune	
Site III	30	*Rivularia*	None
		Nostoc commune	
Site IV	145	None	None

The dominant algae in the flush were the cyanobacteria *Rivularia* and *Nostoc.* These were found from the top of the flush to the bottom of site III. *Rivularia* were incased in calcium carbonate depositions that formed bodies called oncoids. These oncoids were of variable size and averaged roughly 4 cm in diameter. One unusual aspect of the *Rivularia* oncoids was that large colonies appeared to grow face down in the water. As the water levels observed were not strong enough to cause the oncoids to overturn, further investigation is needed to establish why these flourishing communities grow in such low light levels, and how they become established.

Nostoc was observed to grow in close association with the mosses at the side of the flush, and also independently in the centre, both on it's own, and in close proximity to *Rivularia*. *Rivularia* colonies were completely submerged by the water in the flush. As *Nostoc* colonies were larger, they were only partially submerged. In this study only cyanobacterial colonies that were growing independently at the centre of the flush were sampled.

A colony filamentous green algae was located only in site I. This colony was identified as consisting of *Spirogyra* and diatoms.

A number of bryophytes were observed to grow around the flush. The most common was *Cratoneuron commutatum*. Other mosses sampled were *Drepanocladus revolvens* and *Calliergon cuspidatum*.

CHAPTER 4

RESULTS

4.1 Physical-chemical components

In order to determine the type of environment at the calcareous flush, a number of physical-chemical components were measured. These components, including temperature, conductivity and pH, are important variables that effect both the organisms that live in the water and the chemical reactions that take place there. Values of the physical-chemical components measured are given below (Table 4.1).

Table 4.1. Physical-chemical components of the flush

Variable	Temperature (°C)				Conductivity (μs cm^{-1})				pH			
Sample site	**I**	**II**	**III**	**IV**	**I**	**II**	**III**	**IV**	**I**	**II**	**III**	**IV**
Date of sample												
26.04.93	13.0	13.1	13.3	13.3	450	458	455	461	8.12	8.23	8.22	8.24
07.05.93	14.7	14.5	14.5	14.5	474	473	473	475	8.22	8.24	8.28	8.03
20.05.93	15.6	15.5	15.5	15.4	442	440	441	448	8.25	8.35	8.38	8.29
03.06.93	14.0	15.0	15.5	15.3	478	471	454	460	7.19	8.35	8.39	8.02
22.06.93	16.2	17.2	16.8	16.5	483	472	476	460	8.03	8.35	8.38	8.29
08.07.93	14.0	16.3	16.9	15.2	486	480	476	479	8.22	8.16	8.13	8.14
18.07.93	11.4	15.3	15.9	15.8	484	483	483	478	8.21	8.13	8.08	8.10

The physical-chemical components measured all varied both between the different sites within the flush and between sampling dates. Temperature showed different amounts of inter-site variation on different days, ranging from 0.2°C as on the 26.04.93 to 4.4°C on the 18.07.93. The temperature ranged from 11.4 - 17.2 °C throughout the course of this study. The lowest temperatures recorded were on the 26.04.93, and the highest on the 22.06.93.

Conductivity was also variable between sites, but variation was usually not larger than 10 $\mu S\ cm^{-1}$. pH also varied from site to site. The modal pH value was 8.2, although the range varied from 7.19 - 8.38.

4.2 WATER CHEMISTRY

4.21 Analyses of TFP, FRP and FOP

Concentration of P in water is one of the most important features determining growth of algae and cyanobacteria in freshwater systems. The result of the chemical analyses of TFP, FRP and FOP in the water of the flush is given overleaf (Table 4.2). The concentrations were very variable, both between the sample sites and sample dates.

Table 4.2. Results of TFP, FRP and FOP (μg l^{-1}) analyses of the water of the flush.

Date	Sample Site	TFP	FRP	FOP	%FOP
26.04.93	I	1.82	0.87	0.95	55.2
	II	1.82	1.66	0.16	8.8
	III	2.80	1.66	1.14	40.7
	IV	3.78	1.66	2.12	56.1
07.05.93	I	2.12	0.74	1.38	65.1
	II	2.93	2.19	0.74	25.2
	III	0.11	0.05	0.06	54.5
	IV	0.51	0.31	0.20	39.2
20.05.93	I	4.13	1.47	2.66	64.4
	II	6.55	2.97	3.58	54.6
	III	4.91	0.38	4.53	92.2
	IV	9.35	3.82	5.53	59.1
03.06.93	I	4.11	2.23	1.88	45.7
	II	2.33	2.17	0.16	6.8
	III	2.56	1.94	0.62	24.2
	IV	3.16	2.02	1.14	36.0
22.06.93	I	8.35	4.64	3.71	44.4
	II	2.80	1.46	1.34	47.8
	III	5.73	2.35	3.38	58.7
	IV	11.35	10.55	0.80	7.0
08.07.93	I	3.00	0.91	2.08	69.4
	II	3.69	<detection	3.69	100.0
	III	5.41	0.91	4.50	83.3
	IV	34.75	<detection	34.75	100.0
18.07.93	I	3.33	1.96	1.37	41.4
	II	4.38	1.96	2.42	55.2
	III	2.98	2.39	0.59	19.7
	IV	5.07	3.26	1.81	35.7

The values recorded for TFP ranged from 0.11 to 34.75μg l^{-1}. The high concentration of TFP measured at site IV on 08.07.93 could possibly be due to disturbance, from anglers, prior to the building of the walkway. This disturbance may have released P from the sediment. It is unusual, however, that the FRP fraction in that same sample was below detection, as release of FRP from the sediment would be expected also. The lowest values were recorded on the first two sample visits which were also the lowest in temperature. As the season progressed, the temperature increased by up to 3°C. The TFP value also rose by roughly 2 to 3μg l^{-1}. However no significant correlation between temperature and TFP was observed.

Site IV has the highest concentration of TFP on 5 of the 7 sampling dates. Although this did not prove to be significant, it may be due to external influences on that site. It is possible that flooding from the reservoir, as was observed on two visits, can alter the P concentrations there. Therefore it must be considered that site IV is under a slightly different regulation regime in determining P concentrations than the other three sites.

The concentrations of FRP also varied throughout the study with values ranging from below detection to 10.55 μg l^{-1}. No one place had a constantly higher concentration of FRP but site IV had the highest concentration on four out of the seven visits. The concentrations of FRP remained almost constantly low throughout the summer, with concentrations rarely rising above 3μg l^{-1}. Therefore one can see that low concentrations of easily assimilated P were available to organisms growing in the flush.

The % of FOP recorded throughout the study ranged from 6.8 to 100%. The modal range was roughly 40 to 50 %. On three occasions the values recorded were below 10%. Values for FRP varied on these occasions and therefore it is difficult to determine if any relationship exists between the two.

Anova two way analysis of variance was performed to determine if a statistically significant difference exists between the TFP and FRP measured at different sites and different sample dates. No significant difference was recorded between the concentrations of P in the different sites. The

difference between the different sampling dates proved to be significant for both TFP ($F_{(6,18)} = 4.06$, $p < 0.05$) and FRP ($F_{(6,18)} = 3.13$, $p < 0.05$). This result shows that P is influenced by factors which can cause it to change its P status over time. These influences do not occur in one particular area but influence the concentrations of TFP and FRP throughout the entire flush.

4.22 Relationship between the P concentrations in the flush and rainfall

The rainfall data for the Kielder area were obtained. These data were plotted against the TFP values to determine if a relationship between the two occurs. The results are shown in Fig 4.1-4.4.

Fig 4.1 Relationship between rainfall (mm) and TFP (ug l^{-1}) at site I

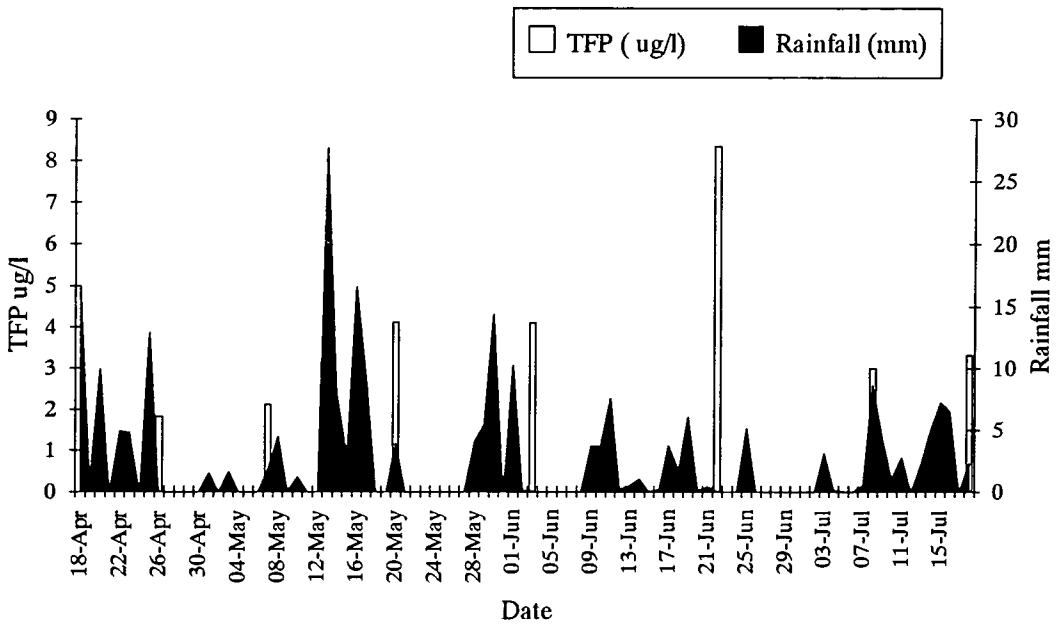


Fig 4.2 Relationship between rainfall (mm) and TFP (ug l^{-1}) at site II

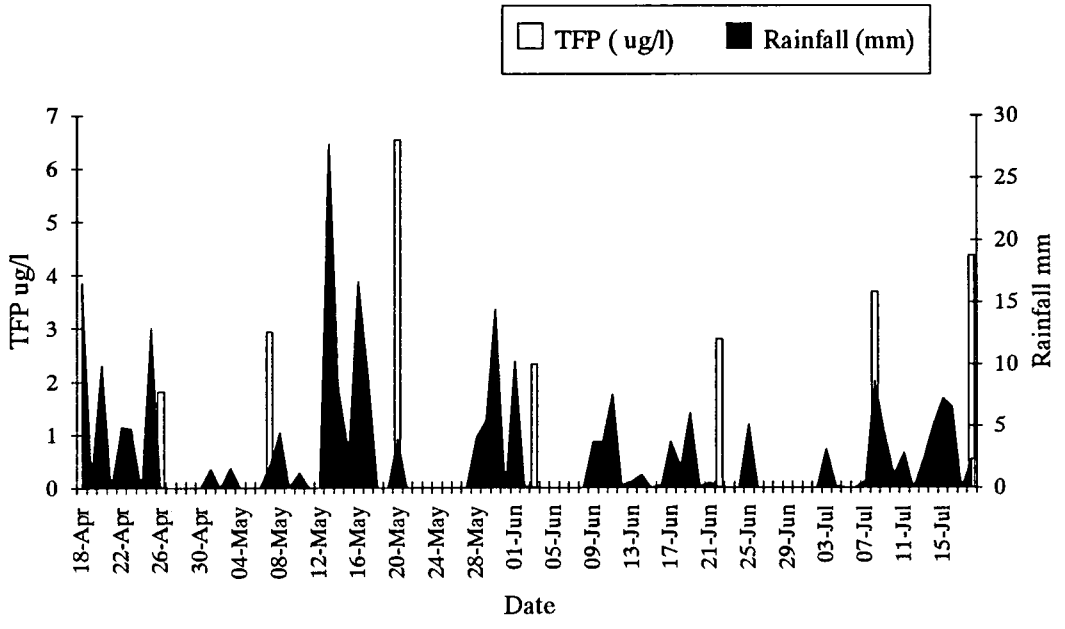


Fig 4.3 Relationship between rainfall (mm) and TFP (ug l^{-1}) at site III

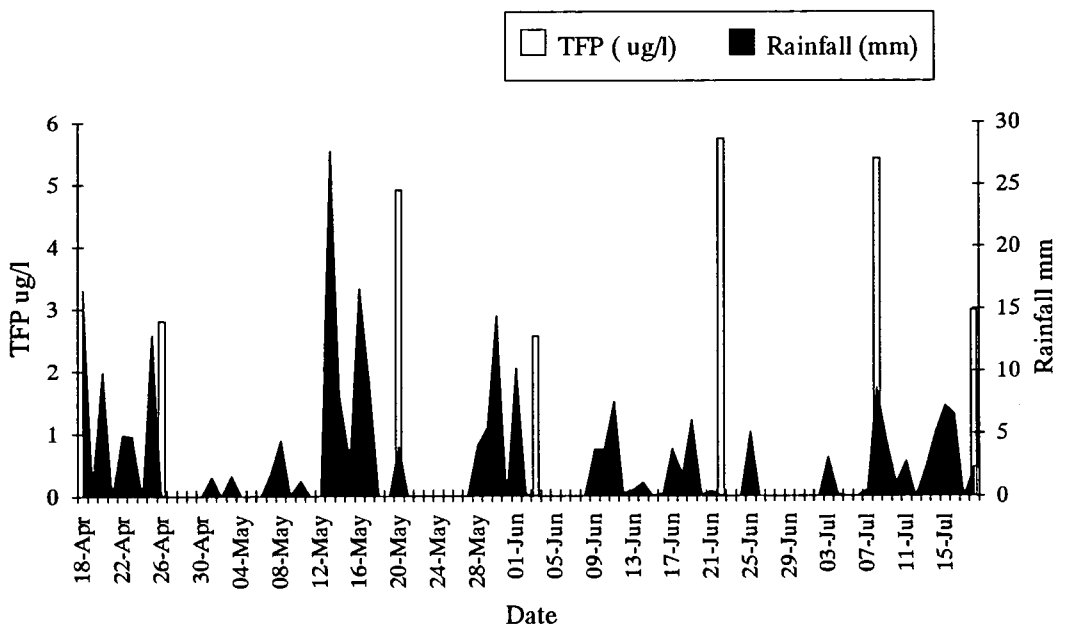
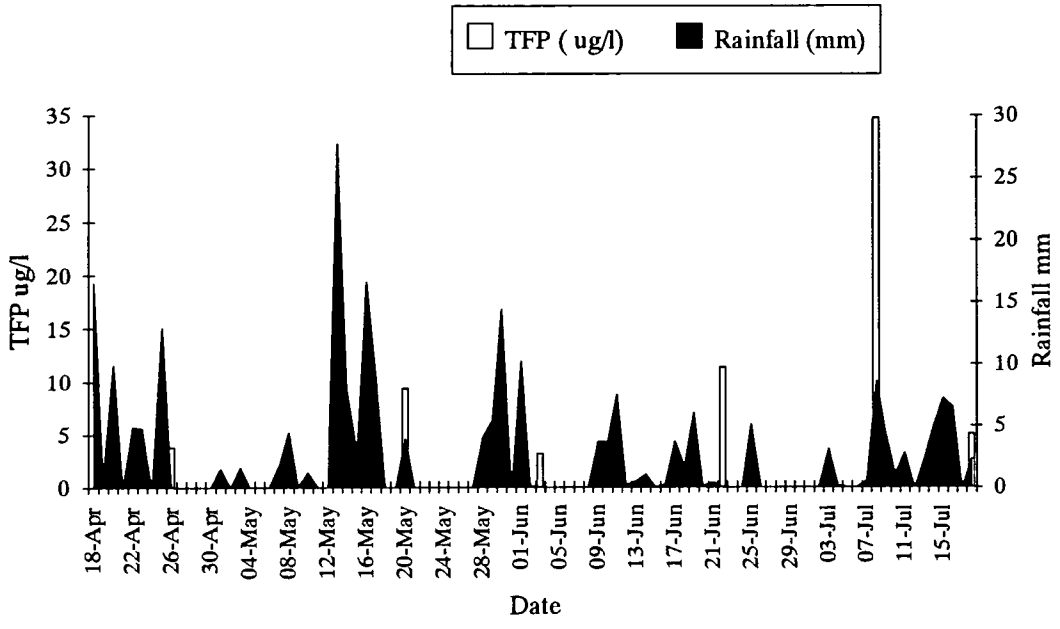


Fig 4.4 Relationship between rainfall (mm) and TFP (ug l^{-1}) at site IV



No relationship can be seen between the concentrations of TFP and rainfall in the flush area. Rainfall varied throughout the course of the study and was greater at the start than at the finish. During periods of low rainfall, TFP was still quite high and therefore, rainfall appears to have little effect on the concentrations of P in the flush.

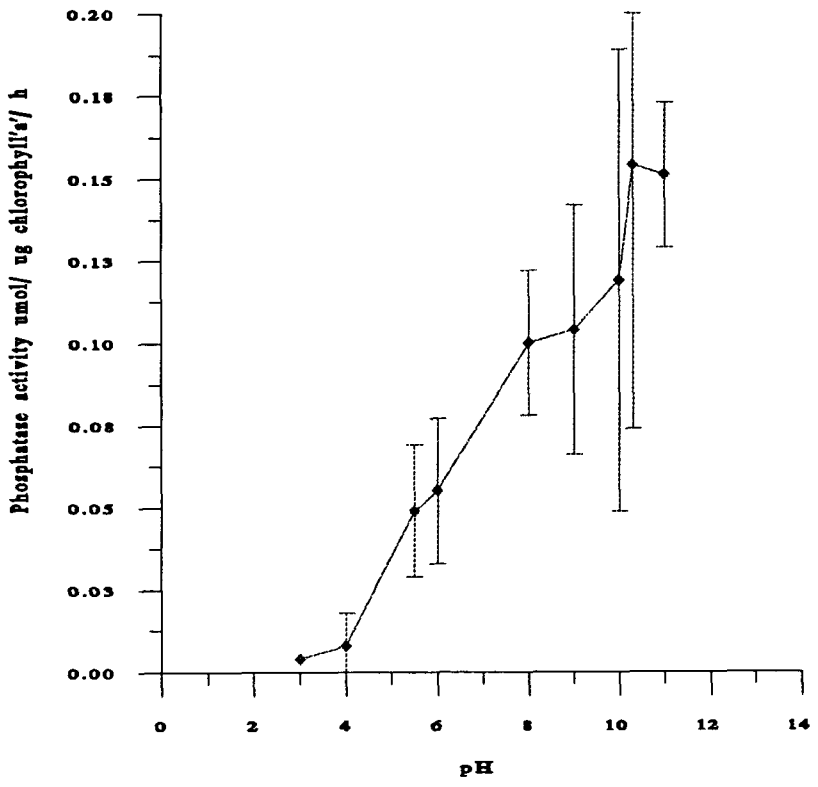
4.3 PHOSPHATASE ACTIVITY OF CYANOBACTERIA

4.31 Influence of pH on the phosphatase activity of *Rivularia*.

Phosphatase enzymes have maximum activity at a specific pH value. To determine this pH value for *Rivularia* a pH spectral analysis was performed using 250 µmol p-NPP substrate. The result is given below (Fig 4.5). The pH maximum was observed to be at pH 10.3. Although activity occurs at pH 3, the rate rises steeply to peak at pH 10.3.

The standard error was observed to be quite large. This could be due to the fact that only four replicates were performed. Limitation in the amount of field material taken prevented a larger number of replicates being performed. Variation could also have occurred due to difference in surface area size of the colonies. Although every effort was made to avoid this, it cannot be ignored.

Fig 4.5 Influence of pH on the phosphatase activity of *Rivularia*



The phosphatase activity of *Rivularia* and *Nostoc* was assayed at pH 10.3 using 100 µmol and 250 µ mol p-NPP substrate. The results of this assay are given below (Table 4.3). The activity of *Rivularia* and *Nostoc* was measured at 250 µmol p-NPP only on the 19.05 and 03.06.93.

Table 4.3. Phosphatase activity (µmol product µg chlorophyll 'a'$^{-1}$ h^{-1}) ± standard deviation) of *Rivularia* and *Nostoc* at 100 and 250 µmol p-NPP substrate

Date	*Rivularia* at 100 µmol	*Nostoc* at 100 µmol	*Rivularia* at 250 µmol	*Nostoc* at 250 µmol
19.05.93				
Site I			0.126 ± 0.100	0.176 ± 0.080
II			0.045 ± 0.014	0.185 ± 0.115
03.06.93				
Site I			0.069 ± 0.022	0.164 ± 0.024
II			0.084 ± 0.049	0.144 ± 0.020
III			0.061 ± 0.047	0.132 ± 0.092
22.06.93				
Site I	0.020 ± 0.009	0.045 ± 0.028	0.113 ± 0.049	0.132 ± 0.036
II	0.013 ± 0.004	0.052 ± 0.017	0.079 ± 0.033	0.101 ± 0.024
III	0.013 ± 0.005	0.053 ± 0.017	0.104 ± 0.038	0.123 ± 0.072
08.07.93				
Site I	0.042 ± 0.005	0.067 ± 0.024	0.144 ± 0.060	0.173 ± 0.081
II	0.024 ± 0.013	0.054 ± 0.021	0.102 ± 0.032	0.151 ± 0.103
III	0.040 ± 0.040	0.054 ± 0.048	0.108 ± 0.041	0.198 ± 0.024
18.07.93				
Site I	0.001 ± 0.001	0.036± 0.025	0.042 ± 0.016	0.142 ± 0.010
II	0.006 ± 0.005	0.125 ± 0.094	0.113 ± 0.053	0.231 ± 0.104
III	0.004 ± 0.007	0.069 ± 0.010	0.045 ± 0.035	0.105 ± 0.048

Activity was variable between sites and between sample dates. In general, the activity measured using 100 µmol p-NPP was less than half the value measured using 250 µmol. The highest activity exhibited by both *Rivularia* and *Nostoc* was measured on 08.07.93. This was observed at both 100 and 250 µmol substrate concentration. FRP levels in the water on this date ~~was~~ were less than 1µg l^{-1} at all the sites.

Nostoc has a consistently higher rate of phosphatase activity than *Rivularia*, both at 100 µ mol and 250 µmol substrate concentrations. Its activity was commonly 1.2 times that of *Rivularia* using 250 µmol and more than twice that using 100 µmol p-NPP. This could be due to a higher affinity for the substrate by *Nostoc*.

The standard deviations measured were also quite large and ranged from 0.25 to 0.9 times the mean. The standard deviation was often a larger proportion of the mean in the 100 µmol p-NPP assay. Four replicates were performed for each assay, and therefore a larger number of replicates may reduce this deviation from the mean. Variation in surface area size of the colonies could also be a factor.

4.32 Correlation between the level of phosphatase activity of *Rivularia* and *Nostoc* and P content of the water of the flush.

To determine if any relationship occurs between phosphatase activity of *Rivularia* and *Nostoc* and P content of the water, Spearman's Rank correlation test was performed. This test showed no statistical significance between the activity of either of the cyanobacteria and the % FOP and FRP at the different sample sites. The result of this correlation is given overleaf (Table 4.4). Spearman's test, could not be applied to site III as an insufficient number of assays had been performed.

Table 4.4 Result of Spearman's test coefficient of correlation between *Rivularia* and *Nostoc* with % FOP and FRP of the flush

Species/Site	Coefficient (% FOP)	Significant (S) Not significant (NS)	Coefficient FRP	Significant (S) Not significant (NS)
Rivularia				
Site I	0.9	NS	0.6	NS
Site II	0.5	NS	0.6	NS
Nostoc				
Site I	0.8	NS	0.9	NS
Site II	0.6	NS	0.3	NS

4.33 Phosphatase activity of *Rivularia* and *Nostoc* using 4-MUP substrate

4-MUP has been recognised as being a more sensitive substrate in phosphatase assays. Activity in *Rivularia* and *Nostoc* was measured using 4-MUP at 1 µmol and 250 µmol concentrations The results are given overleaf (Table 4.5).

Table 4.5 Phosphatase activity (μ mol μg chlorophyll 'a'$^{-1}$ h^{-1}) ± standard deviation of *Rivularia* and *Nostoc* using 1 μmol and 250 μmol 4-MUP as substrate.

Date	Activity of *Rivularia*	Activity of *Nostoc*
1μmol MUP substrate		
08.07.93		
SITE I	$8.0 \times 10^{-4} \pm 1.0 \times 10^{-4}$	$8.0 \times 10^{-3} \pm 2.6 \times 10^{-3}$
II	$5.0 \times 10^{-4} \pm 2.0 \times 10^{-4}$	$5.0 \times 10^{-4} \pm 1.4 \times 10^{-4}$
III	$5.3 \times 10^{-4} \pm 1.5 \times 10^{-4}$	$5.2 \times 10^{-4} \pm 4.2 \times 10^{-4}$
18.07.93		
SITE I	$1.8 \times 10^{-4} \pm 9.3 \times 10^{-4}$	$4.9 \times 10^{-3} \pm 3.6 \times 10^{-3}$
II	$7.7 \times 10^{-4} \pm 3.3 \times 10^{-4}$	$2.6 \times 10^{-3} \pm 1.5 \times 10^{-3}$
III	$9.3 \times 10^{-4} \pm 2.8 \times 10^{-4}$	$5.0 \times 10^{-3} \pm 3.5 \times 10^{-3}$
250 μmol MUP substrate		
08.07.93		
SITE I	$4.5 \times 10^{-2} \pm 1.3 \times 10^{-2}$	$7.2 \times 10^{-2} \pm 3.6 \times 10^{-2}$
II	$1.1 \times 10^{-2} \pm 5.1 \times 10^{-3}$	$3.5 \times 10^{-2} \pm 1.1 \times 10^{-2}$
III	$4.8 \times 10^{-3} \pm 2.2 \times 10^{-3}$	$2.6 \times 10^{-2} \pm 2.0 \times 10^{-2}$
18.07.93		
SITE I	$4.9 \times 10^{-2} \pm 1.8 \times 10^{-2}$	$4.6 \times 10^{-2} \pm 4.5 \times 10^{-2}$
II	$7.8 \times 10^{-2} \pm 6.2 \times 10^{-2}$	$6.3 \times 10^{-2} \pm 3.8 \times 10^{-2}$
III	$5.6 \times 10^{-2} \pm 2.8 \times 10^{-2}$	$9.7 \times 10^{-2} \pm 9.9 \times 10^{-2}$

The phosphatase activity recorded show once more that the phosphatase activity of both *Rivularia* and *Nostoc* was variable between sites on the two dates measured. *Nostoc* again showed a consistently higher rate of phosphatase activity than *Rivularia*, having almost twice its activity at both at 1μmol and 250 μmol substrate concentrations. Activity was higher at 250 μmol concentration than at 1 μmol.

All assays using 4-MUP as a substrate showed lower phosphatase activity than those with the equivalent concentration of p-NPP substrate. The values for 250 μmol 4-MUP were 59% of the activity obtained for the same concentration of p-NPP for *Rivularia* and 36% the activity for *Nostoc.*

4.34 Phosphatase activity of *Spirogyra*

Phosphatase activity of *Spirogyra* was measured using 250 μmol p-NPP only. The results were initially calculated per unit dry weight, but were later converted to unit of chlorophyll 'a'. These results are given below (Table 4.6).

Table 4.6 Phosphatase activity ± standard deviation of *Spirogyra* using 250 μmol p-NPP as substrate

Date	Phosphatase activity(μmol mg dry weight^{-1} h^{-1})	Phosphatase activity (μmol μg chlorophyll 'a'$^{-1}$ h^{-1})
03.06.93	0.080 ± 0.023	0.016 ± 0.006
08.07.93	0.058 ± 0.010	0.011 ± 0.002
18.07.93	0.076 ± 0.028	0.015 ± 0.005

These results show that the phosphatase activity of *Spirogyra* varied from 0.011 to 0.016 μ mol μg chlorophyll 'a'$^{-1}$ h^{-1}on the three dates that activity was assayed. Activity was not as variable between sites and was less than half that measured in the cyanobacteria at the same concentration.

4.4 PHOSPHATASE ACTIVITY OF BRYOPHYTES

4.41 Influence of pH on the phosphatase activity of *Drepanocladus revolvens* and *Cratoneuron commutatum*

A pH spectral analysis of phosphatase activity in the bryophytes, *D. revolvens* and *C. commutatum,* was performed using 250 µmol p-NPP, to determine at which pH value they had maximum activity. These results are shown below and overleaf (Fig 4.6, 4.7). Maximum activity in *D. revolvens* as observed to be at pH 5, while *C. commutatum* was observed to be at pH 4. Both mosses showed activity in the alkaline range.

Error variations were also quite large. Four replicates were performed on each assay, therefore an increase in the number of replicates may reduce the size of these errors.

Fig 4.6 Influence of pH on the phosphatase activity of *Drepanocladus revolvens*

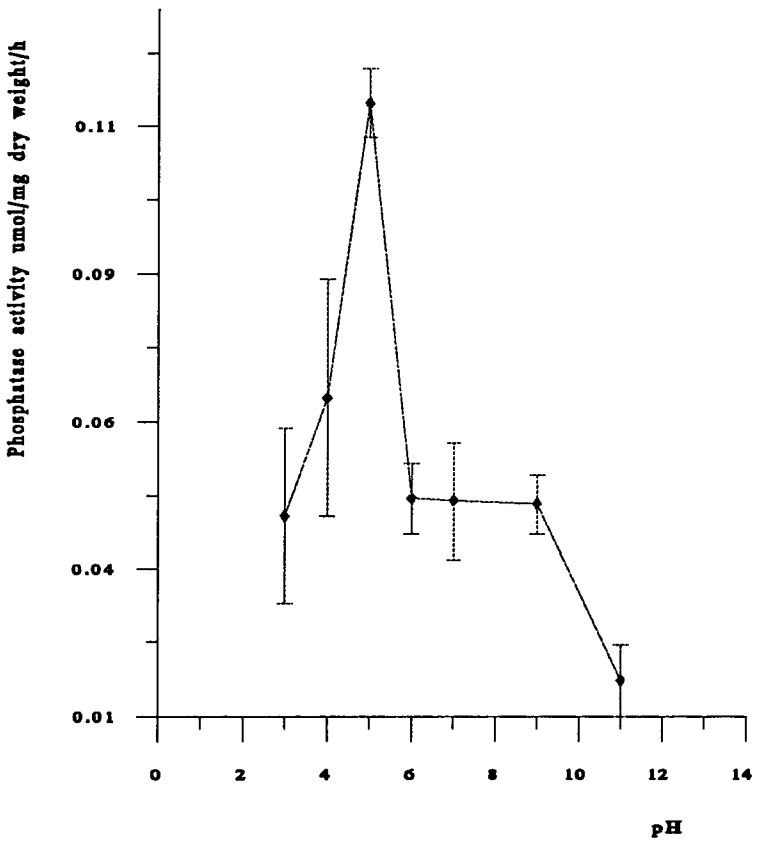
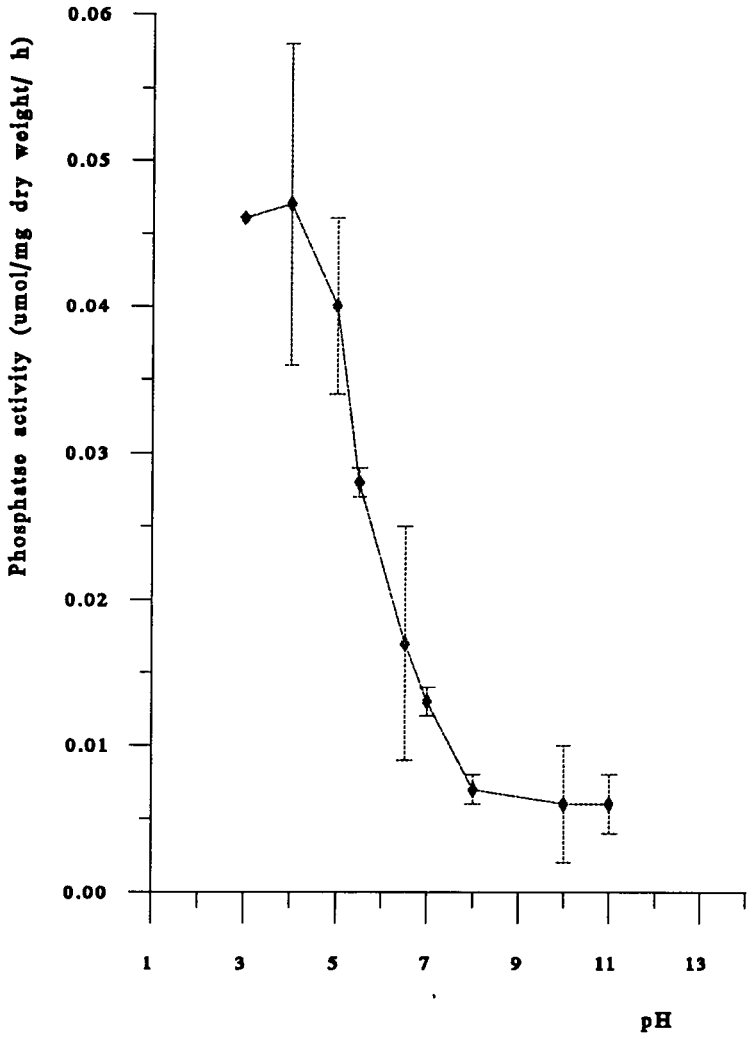


Fig 4.7 Influence of pH on the phosphatase activity of *Cratoneuron commutatum*



4.42 Phosphatase activity of bryophytes

Phosphatase activity in bryophytes was measured using 250 µmol p-NPP only. The assays were measured at the pH maximum for *C. commutatum* and *D. revolvens*. Activity in *C. cuspidatum* was measured at pH 5. Results of the phosphatase assay is shown overleaf (Table 4.7).

Table 4.7 Phosphatase activity (μmol mg dry weight^{-1} h^{-1}) ± standard deviation of bryophytes measured using 250 μmol p-NPP

Date/species Site	Phosphatase activity I	II	III	IV
Cratoneuron commutatum				
23.06.93	0.028 ± 0.010	0.023 ± 0.017	0.038 ± 0.013	
21.07.93	0.064 ± 0.041	0.058 ± 0.034	0.068 ± 0.042	0.055 ± 0.025
Drepanocladus revolvens				
03.06.93		0.031 ± 0.012		
Calliergon cuspidatum				
03.08.93		0.082 ± 0.017		

All bryophytes examined exhibited phosphatase activity. The levels of activity varied between the different species with *C. cuspidatum* having the highest and *C. commutatum,* the lowest. *C. commutatum* was measured on two separate dates. Almost twice the amount of activity was measured on the second date. This variation may indicate that the moss had a greater need for P on the second date and therefore was producing more phosphatase enzyme to provide this. Levels of intracellular P were not measured and so this level cannot be correlated.

Variation in activity between sample sites was as little as 0.10μmol. mg dry weight^{-1} h $^{-1}$at both dates in *C. commutatum. D. revolvens* and *C cuspidatum* also showed phosphatase activity. Activity in *D. revolvens* was lower than that measured during pH spectral analysis. Again, this difference could not be correlated to intracellular P. Activity in *C. cuspidatum* was only measured on one occasion and therefore no variation in phosphatase activity was investigated.

CHAPTER 5

DISCUSSION

5.1 WATER CHEMISTRY

Chemical analyses of the flush shows that concentration of TFP and FRP in the water changed between sites and between sampling dates. The difference between sites was not statistically significant, but the difference between samples proved significantly different at the $p < 0.05$ level. This difference between sampling dates indicates that the P cycle in the flush is a dynamic system that changes with time. P in freshwater systems is determined both by supply from the drainage basin, and by processes within the stream (D'Angelo *et al.* 1991). Any one, or combination of these factors, could account for the difference in P between the sampling dates.

Transfer of P within the freshwater system can be caused by biologically mediated transfer mechanisms and by abiotc complexing reactions such as sorbtion. Increased demand for P during the spring and summer seasons can cause removal of P from the water by algae and bacteria (Jansson 1988). The measurement of phosphatase activity in the cyanobacteria assayed indicates that the algae are relying on organic P as well as inorganic P as a P source. Recycling of P may be rapid, especially in unpolluted freshwater systems with FRP < 1 $\mu g\ l^{-1}$ (Chróst and Overbeck 1987).

Invertebrate fauna can also can also play a role in the cycling of P through the system. Ingestion of food particles by zooplankton at certain times during the year can be quite large. Through egestion, these creatures can release nutrients in the form of soluble P ions and some organic P compounds, back into the environment, which can be reassimilated (Lehman 1980).

Although the concentration of FRP varied over the course of the study, the concentration of TFP increased. Biological mechanisms may not have the only effect on the P concentration. Other factors, chemical and physical in nature, may also play a part.

Many studies on stream systems have established the importance of sediments in P dynamics (Meyer and Likens 1979; Hill 1982; Newbold *et al.* 1983; Klotz 1985; D'Angelo and Webster 1991; D'Angelo *et al.* 1991). Sediments can act as a source or sink for P. Retention of P by sediments is strongly influenced by physical, chemical and biological factors (Hill 1982). Examples of these include stream velocity, temperature, biomass, (D'Angelo and Webster 1991), particle size and speciation (Stone and English 1993), redox potential and pH (Klotz 1985). Owing to the fragility of the calcareous flush, sediment samples were not taken. This is unfortunate as P analysis of the sediment, may have determined its input to the P cycle in the flush. D'Angelo *et al.* (1991) cites stream velocity as one of the most important physical factors effecting P concentration in the water. The contribution of this factor could not be determined as the level of water in the flush was never more than a few cm, which was too shallow for velocity to be measured using conventional methods. No correlation between air and water temperature was performed to determine what relationship exists between them and if it can account for the fluctuation in water temperature observed.

The effects of adsorption of P onto calcite should also be considered. House and Donaldson (1986) found that this process was temperature and pH dependant. As both the water temperature and pH varied over the course of the study, they could effect the rate of adsorption. This could contribute to the differing concentration of P.

The results show that P in the flush system is dynamic. Changing concentrations of TFP and FRP were observed throughout the course of this study. A more detailed investigation into the biological, chemical and physical effects is needed to fully understand this cycle further. The method of chemical analysis of water employed in this study does have limitations. American Public Health Association (1989) claim that filtration through a 0.45μm filter provides a rough separation of dissolved from suspended forms of P. In this study a 0.7μm filter was used. Therefore it is possible that the values for TFP, FRP and FOP contained reactive P from both particles and colloids that passed through the filter. It can not be assumed that the proportion of particles and colloids that pass through the filter in each sample are constant. Therefore, the accuracy of the results as an actual representation of the amount of TFP, FRP, and FOP in the water must be given consideration.

To determine if an external source of P had any effect on the system, the amount of rainfall was examined. Rainfall can effect the level of P in a water system in two ways, by direct input into the water system itself and by leaching of nutrients from the surrounding soil area (Ahl 1988; Holten *et al.* 1988). The results (Figs 4.1-4.4) show no real relationship between the P and level of rainfall. Rainfall levels varied greatly and were considerably lower throughout the latter part of the study. Determination of the concentration of P in the surrounding soil would give a greater indication of the level of P that may be available for leaching and thus may clarify the contribution of rainfall. It is also possible that rainfall may have an effect only above a certain level. A more long term study is required to investigate this. The effects of rainfall may be mitigated by other climatic variables such as wind and temperature. Therefore a detailed study into all aspects of the climate in the flush area would clarify this factor.

Distribution of the cyanobacteria in the flush in relation to the nutrient content of the water is not clear. *Nostoc* and *Rivularia* , as cyanobacteria, have the ability to fix N from the atmosphere and so P is their major limiting nutrient. Both of these algae were observed to be distributed equally along the flush in sites I, II, and III. They were found growing in close association with mosses along the sides of the flush as well as independently in the centre. No cyanobacteria were found growing in site IV. Although the P concentrations at this site were often higher than in the other three pools, the absence of the algae is probably due to the frequent flooding that occurs at this site from the reservoir when water levels increase. On two occasions the entire site was observed to be flooded by the reservoir after periods of heavy rain.

The filamentous green algae *Spirogyra*, was found only at site I. This algae is more limited in its distribution as it cannot fix atmospheric N and therefore needs a certain concentration in the water before it can establish. Detailed investigation into the N:P ratio at this site could determine this. *Spirogyra* was observed to have a lower rate of phosphatase activity then either of the cyanobacteria studied. Its occurrence at site I only was probably due to nutrient limitation at the other sites. It was interesting to observe that the filamentous mat of algae was at its largest on 22.06.93, the date on which site I had its highest concentration of TFP and FRP.

5.2 PHOSPHATASE ACTIVITY OF CYANOBACTERIA

All organisms assayed showed phosphatase activity. As studies have shown that phosphatase activity is induced by low concentrations of available P (Whitton *et al.* 1991), it is probable that these organisms are living in a P limited environment. The organisms studied in detail were the cyanobacteria *Rivularia* and *Nostoc*. Study of their phosphatase activity highlighted a number of interesting factors. pH spectral analyses of the phosphatase activity of *Rivularia* showed it to have it's maximum activity at pH 10.3. Its activity at the environmental pH was 65% of its maximum, indicating that *Rivularia* is adapted to scavenge organic P from the environment.

Comparison of the phosphatase activity of *Rivularia* with those measured from Upper Teesdale by Livingstone and Whitton (1984), showed that the colonies from the flush exhibited three times the phosphatase activity of the colonies from Upper Teesdale even though P concentration measured in the flush were higher. This difference could be attributable to the fact that activity was measured at environmental pH rather than the pH maximum, as well as the fact that a different assay method was used.

Comparisons between the activity of *Nostoc* and *Rivularia* show that *Nostoc* has a consistently higher rate of phosphatase activity, using p-NPP and 4-MUP substrate at different concentrations. Studies have shown that the internal phosphorus status of the organism is one of the more crucial factors determining the activity of alkaline phosphatase (Chróst and Overbeck 1987; Whitton 1991). The higher rate of phosphatase measured, indicates that *Nostoc* may have a higher requirement for P then *Rivularia*. This higher requirement may be due to higher metabolic needs. It could also be possible that *Nostoc* is less efficient at storing P in polyphosphate granules when P is in excess. Analysis of the internal P concentrations of the algal colonies would elucidate this point further.

Colonies of *Rivularia* were submerged in the water in the flush, but *Nostoc*, due to its larger size, was not submerged fully. It is possible that *Nostoc* experiences a different P regime to *Rivularia*,

as not all of its surface area is in contact with the water. This could account for its higher rate of phosphatase activity

Substrate affinity can also be seen as a factor that influences the rate of phosphatase activity. If *Nostoc* has a higher affinity for the p-NPP and 4-MUP substrates than *Rivularia*, this could explain the consistently higher results. The difference in phosphatase activity between the two cyanobacteria was greater at 100 µmol p-NPP then 250 µmol. This again could indicate that *Nostoc* had a greater affinity for the artificial substrate used. Investigation in the enzyme kinetics of these two cyanobacteria is needed if this point is to be determined.

5.21 Correlation between phosphatase activity and FRP and % FOP of the water

Many studies have shown that the level of phosphatase activity in organisms is inversely proportional to the level of FRP in the water (Berman 1970; Aaronson and Patni 1976; Gage and Gorham 1985; Olsson 1990). Spearman's rank correlation tests performed on the phosphatase activity of *Rivularia* and *Nostoc* proves the relationship to be not significant, both with the concentration of FRP and % FOP in the water. This lack of correlation could be due to a number of factors. For example, as stated earlier, studies have found the that the internal P concentration is more important in determining the level of phosphatase activity and therefore a measure of this may give a better correlation.

Chróst and Overbeck (1987) observed a time delay between the effect that the level of P in the water had on the rate of phosphatase activity, due to internal P stores in the algae. This could also have been a factor in this study. Depending on the speed at which P is cycled in the flush system, such an effect would not be evident in data sampled only once every two weeks. Analysis of water chemistry and phosphatase activity of the algae in the flush over a set period of time, taking hourly samples, would determine if any such time lag was present.

As stated earlier, the amount of P available to *Nostoc* is different to that available to *Rivularia*, as it is not fully submerged by the water. Therefore, other factors could detract from the

correlation between phosphatase activity and concentration of FRP. These factors include size of colony and proportion of colony submerged by water.

The FRP measured may also contain P from condensed phosphates, colloids and particles. These P compounds would not be available for hydrolysis by the phosphatase enzyme and therefore would detract from the correlation.

Similarly, no correlation was found between phosphatase activity and the % FOP present. The true nature of filtrable organic P is uncertain. It is probable that most of it is truly associated with organic molecules. On many occasions the water in the flush contained up to 50% filterable organic P. However it cannot be said that all of these compounds are equally available for hydrolysis by the phosphatase enzyme. Olsson and Jansson (1984) observed that phosphatase did not break down either colloidal P (MW> 50 000 daltons) and XP (termed by Lean 1973) (MW > 250 daltons). Hino (1989) showed that no organic P compounds with MW > 1500 were hydrolysed by phosphomonoesterases alone. A combination of these enzymes with nucleases increased the range over which they were active. Therefore, the action of the phosphatase enzyme is limited to the number of particles of organic P which are available for it to hydrolyse.

Measurement of phosphatase activity of bacteria in the water of the flush would also give insight into the role of competition in the system for P. Studies have shown that under periods of P limitation, bacteria are far more efficient scavengers of P then algae (Currie and Kalff 1982). They do not sustain this increased need as they are unable to store P in their cells (Roloff and Nicklisch 1993). However as phosphatase activity has been recognised as playing an important part in the cycling of P in fresh waters (Jansson *et al.* 1988; Whitton *et al* 1991; Hantke and Melzer 1993) identification of the role played by bacteria would be important.

5.3 PHOSPHATASE ACTIVITY OF BRYOPHYTES

Very little research has been performed on the phosphatase activity of bryophytes. However previous studies have shown that the phosphatase activity of the bryophytes was inversely proportional

to the level of P in their tissues (Press and Lee 1983; Al- Shehri 1992). Al-Shehri (1992) also found that phosphatase activity in *H. fontanum* was induced by low concentrations of inorganic P. *Drepanocladus revolvens* and *Cratoneuron commutatum* both showed phosphatase activity. Activity was variable between species, and analysis of *C. commutatum*, showed that activity was variable between dates. It was not determined if phosphatase activity was induced by low concentrations of inorganic P. Analysis of the tissue P content was not performed and therefore it cannot be determined if this change in activity could be directly related to a change in tissue P content.

The pH spectrum analysis of the phosphatase activity of *D. revolvens* and *C. commutatum* revealed that both these mosses have their pH optimum in the acid range. *D. revolvens* has at its pH optimum at pH 5 and *C. commutatum* at pH 4. This is a surprising result considering the calcareous environment which the mosses inhabit. Although phosphatase activity was still present at pH 8, it was 40% of the optimal activity for *D. revolvens* and only 17% for *C. commutatum.*

This poses a number of questions. If mosses are producing a phosphatase enzyme in response to low concentration of P, then producing an enzyme with a pH optimum different to the environment, thus reducing its efficiency is not very effective. Previous studies on *Sphagnum* (Press and Lee, 1983) showed it to have phosphatase activity with an optimum pH of 4.2. Al-Shehri, 1992, shows *H. fontanum* to have phosphatase activity with an optimum pH of 5.0, although activity was still measured in the alkaline range. A more thorough investigation into the phosphatase activity in bryophytes is merited to clarify this point. It is possible that the enzyme hydrolyses sufficient amounts of P for the moss, even working at 17% of its efficiency.

Measurement of the rhizoids of the moss may be merited also. Rhizoids show higher sensitivity to the phosphatase enzyme, and to pH (Al-Shehri 1992). However large quantities of moss material are needed to harvest enough rhizoids to perform a routine phosphatase assay, and a substantial amount is required to obtain enough to perform a pH spectrum. Therefore in this study the activity of the shoots was measured.

Investigation into the phosphatase activity of higher plants that grow in the flush, would be merited. It would be interesting to discover if higher plants, which usually develop acid phosphatase activity, would adapt to P limitation in such a calcareous environment. This needs careful consideration, as removal of enough plant root material to perform phosphatase assays can be quite destructive to the environment and therefore was not attempted during the course of this study.

5.4 CONCLUSIONS

Concentrations of TFP and FRP in the flush changed significantly over the course of the study. More investigation into the biological, physical and chemical mechanisms that control the P cycle in the flush system is needed.

Phosphatase assays revealed that both the cyanobacteria and bryophytes living in the calcareous flush produce phosphatase enzymes which hydrolyse some forms of organic P, thus it is probable that they live in conditions of inorganic P limitation. The cyanobacteria measured, *Rivularia* and *Nostoc*, were observed to have almost ten times the phosphatase activity of the filamentous green algae, *Spirogyra*. *Nostoc* had a consistently higher rate of activity than *Rivularia*. This could be due to a genetic difference between the two species or differences in P limitation. It is also possible that *Nostoc* has a higher affinity for the artificial substrates used in the assay.

The phosphatase activity of bryophytes was observed to be variable both between species and between sampling dates. Investigation into the influence of pH on this activity in two species of moss, *Drepanocladus revolvens* and *Cratoneuron commutatum*, showed that maximum activity occurred in the acidic pH range for both species. Activity was only 40% and 17% of the enzyme maximum respectively at the environmental pH. It was not determined if phosphatase activity in the bryophytes was inducible.

SUMMARY

(i) A study was performed to determine the status of P in a calcareous flush at Bakethin reservoir, Northumberland.

(ii) Chemical analysis of the water in the flush showed levels to be low, with concentrations of TFP ranging from 0.11 to 11.35 $\mu g\ l^{-1}$ and FRP ranging from below detection to 10.55 $\mu g\ l^{-1}$. Statistical analysis showed that no difference occurred in the P levels at different sites along the flush, but that P levels varied between different sample dates at the $p < 0.05$ level.

(iii) The levels of TFP in the flush were compared with rainfall in the Kielder area. No relationship was determined between the two.

(iv) Phosphatase activity in the cyanobacteria *Rivularia* and *Nostoc* was examined. *Rivularia* showed maximum activity at pH 10.3. Activity varied between sites and between sample dates. Both cyanobacteria showed a higher activity using p-NPP substrate, than with 4-MUP substrate. *Nostoc* showed a consistently higher rate of phosphatase activity than *Rivularia*.

(v) No correlation was observed between the phosphatase activity of *Rivularia* and *Nostoc* and the FRP and FOP of the water.

(vi) Phosphatase activity in Spirogyra was also measured using p-NPP. This algae had only 10% of the phosphatase activity observed in *Rivularia* and *Nostoc*.

(vii) The influence of phosphatase activity in the bryophytes, *Drepanocladus revolvens* and *Cratoneuron commutatum*, was examined. *D. revolvens* showed to have maximum activity at pH 5.0 and *C. commutatum* showed maximum activity at pH 4.0. Activity in *C. commutatum* was shown to vary between different sites along the flush, and between different dates. No correlation between phosphatase activity and tissue P content of the bryophytes was performed.

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