## *Distribution and Alkaline phosphatatase activity of filamentous conjugales in an upper Teesdale stream*

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# Distribution and Alkaline Phosphatatase Activity of Filamentous Conjugales in an Upper Teesdale Stream

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## ABSTRACT

Two small streams in Upper Teesdale National Nature Reserve were examined with respect to water chemistry and filamentous Conjugales present. Phosphate, nitrate and ammonia concentrations in the water were determined, to investigate a possible link between the nutrient status of the water and phosphatase enzyme activity of the algae. It has been shown that phosphatase activity is inducible in some algae under conditions of low inorganic phosphorus availability. Phosphatase enzymes enable the algae to hydrolyse organic phosphorus, converting it into a more easily utilised form. A connection was found at Red Sike between nutrient, especially organic phosphorus, levels and phosphatase activity.

Algae were also sampled from subjectively determined microhabitats in Red Sike and Slapestone Sike, to investigate if particular community assemblages were more common in certain microhabitat sites.

## ACKNOWLEDGEMENTS

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## ABBREVIATIONS

| | |
|---|---|
| ° C | Degrees Celsius |
| mg | milligramme |
| µg | microgramme |
| l | litre |
| ml | millilitre |
| µl | microlitre |
| m | metre |
| cm | centimetre |
| mm | millimeter |
| µm | micrometer |
| nm | nanometre |
| h | hours |
| min | minutes |
| µM | micromolar |
| µmol | micremole |
| meq $l^{-1}$ | milliequivalents per litre |
| µS $cm^{-1}$ | microsiemens per centimetre |
| P | phosphorus |
| FRP | filtrable reactive phosphorus |
| TFP | total filtrable phosphorus |
| FOP | filtrable organic phosphorus |
| *p*-NPP | *para*-nitrophenylphosphate |
| *p*-NP | *para*-nitrophenol |
| EDTA | ethylenediamine tetra-acetic acid |
| HEPES | N-2-hydroxymethylpiperazine-N'-2-ethanesulphonic acid |
| p | probability |
| S.E. | standard error |

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# CHAPTER 1 INTRODUCTION

## 1.1 GENERAL INTRODUCTION

Observations of filamentous Conjugales in the Upper Teesdale National Nature Reserve have suggested that the species dominating occasional blooms may change from year to year (B. Whitton, pers. comm.). The reason for this variation in algal dominance is unclear. It may be related to changes in phosphorus concentrations in the streams and may therefore be linked to the differing abilities of the three main genera involved, *Mougeotia*, *Zygnema* and *Spirogyra*, to hydrolyse organic phosphorus. If this is the case, a survey of phosphatase activity of the algae, in association with an analysis of stream water chemistry, might give an insight into the reasons for different algal dominance at different times.

It is also possible that the algae are competitively separated on the basis of adaptation to particular physical conditions of the environment. In a very dry year, for example, a species which was capable of withstanding desiccation may flourish, while in a year with very high rainfall, a species which was able to stand up to strong currents could dominate. It is therefore important to look at the physical situation of the algae in the stream, to determine if any of the species tends to dominate in any particular microhabitat.

## 1.2 NITROGEN

There are four main dissolved inorganic nitrogen pools: ammonium, nitrite, nitrate and elemental nitrogen, of which only the first three are available for uptake by most algae. Elemental nitrogen is quite unreactive and can only be used by nitrogen- fixing blue-green algae (Cyanobacteria) and some bacteria. Available forms of nitrogen are very soluble and are therefore very easily transported into waterways. Decomposition of plant and animal protein yields mainly ammonia. Nitrite bacteria use the oxidation energy of ammonia in metabolism according to the formula

$$(NH_4)_2CO_3 + 3O_2 = 2NO_2 + CO_2 + 3H_2O + \text{Energy}$$

This oxidation is continued by nitrate bacteria:

$$2HNO_2 + O_2 = 2HNO_3 + \text{Energy}$$

This nitrate is readily given up to ground water, so ground and spring waters uncontaminated by human activity generally contain nitrogen mainly in the form of nitrate. In contrast to phosphate, nitrate is easily leached from the soil to ground water, so spring waters tend to contain higher levels of nitrate than phosphate.

## 1.3 PHOSPHORUS

### 1.3.1 Forms of phosphorus

The average amount of phosphorus in the earth's crust has been estimated at 0.1% by weight; phosphorus is therefore classed geochemically as a trace element (Holten *et al*, 1988). Compounds of phosphorus have an important influence on production in fresh waters. Phosphorus is, however, one of the least abundant of the elements required for algal growth in fresh water; oligotrophic lakes may contain less than1 mg $l^{-1}$ inorganic phosphorus (Ruttner, 1953). Phosphorus is also relatively insoluble, and will readily precipitate as a complex of iron, aluminium and other metals in rocks and soils (Moss, 1980). Phosphorus is present in water bodies in the form of inorganic phosphate ions ($PO_4^{3-}$, $H_2PO_4^-$ and $HPO_4$), inorganic polymers, organic compounds and in living organisms and detritus. Many forms of phosphorus are not readily available for plant growth, but may become available through microbial action in the water.

Phosphates are defined by Van Wazer (1973) as "chemical structures in which each phosphorus atom is more or less tetrahedrally surrounded by four oxygen atoms"; in condensed phosphates, one two or three corners of the phosphate tetrahedra are shared with other tetrahedra through P-O-P bonds. Orthophosphate, made up of a single $PO_4$ group, is the principal form of phosphorus used in plant nutrition, being the form which is biologically mobile, and the main state in which phosphorus is interchanged between the various biological components of an ecosystem. Some studies of the dynamics of phosphorus exchanges in freshwater, however, have suggested that $PO_4$ is a transient state, and there may be rapid cycling of phosphorus between several large phosphorus pools within any system (Hutchinson, 1957). Much of the earth's phosphorus resource is in organic form, but the continual

cycling of phosphorus in organic systems means that the size of the organic phosphorus pool is constantly changing as the biota respond to changes in their physical and chemical environment (Hooper, 1973).

Phosphorus fractions termed dissolved are nominally those that could pass through a filter of pore size 0.45 mm. Compounds reacting with molybdate to form heteropoly blue without digestion or hydrolysis are termed soluble (filtrable ) reactive phosphorus. Dissolved fractions which require digestion and/or hydrolysis before reaction, are termed filtrable unreactive phosphorus and are assumed to be mostly organic.

### 1.3.2 Importance in biological systems

Phosphorus-containing compounds are important in algae, as in all living organisms, playing a role in metabolism and energy transfer reactions. Phosphorylated compounds are also important in photosynthesis (O' Kelly, 1973).

In general, the extent to which the potential productivity of a freshwater system is realised is limited by the phosphorus compounds available. However, addition of phosphorus to a water system will only cause an increase in primary productivity if all other necessary elements are present in plentiful supply. The form of phosphorus enrichment also affects the biological response. The response to organic phosphorus addition will depend on the alkaline phosphatase activity of the taxon involved, that is, the ability of the organism to break down and utilise organic phosphorus compounds.

## 1.4 PHOSPHATASE

### 1.4.1 Occurrence of Phosphatases in Plants

Phosphatase activity has been recorded from all major groups of algae (e.g. Gibson and Whitton, 1987; Bone, 1971).

In animal cells, phosphatase enzymes are located in the lysosome. However, plant cells do not have

lysosomes and the vacuole may take their place. Electron microscopy has shown that much of the phosphatase of a mature plant cell is located in the vacuole (Bieleski, 1973). Phosphatases of phosphorus deficient organisms, however, have a different location. The enzymes associated with an increase in phosphatase activity are located on the external cell membrane, where they are accessible to substrates outside the cell. As cell membranes are relatively impervious to phosphate esters, it is obviously advantageous to the alga to hydrolyse complex organic compounds of phosphorus outside the cell and then take up the necessary inorganic phosphate. Kuenzlar (1965) found that marine algae with the ability to hydrolyse glucose-6-phosphate in phosphate-deficient medium did not take up the whole molecule; they hydrolysed the ester extracellularly and took up the phosphate ion that resulted from hydrolysis. Those algae without phosphatase activity did not take up glucose-6-phosphate, became phosphorus deficient and stopped growing. Walther and Fries (1976) reported evidence that glycerophosphate was not taken up directly by *Aerocystis* and *Ectocarpus*, but was split by external enzymes, and phosphate ions assimilated. This conclusion resulted from two observations:

(a) Glycerophosphate was not taken up immediately, but remained in the medium until enzyme production had begun.

(b) They detected the development of orthophosphate in the glycerophosphate medium.

### 1.4.2 Bioindicator Potential

The potential use of alkaline phosphatase activity as an indicator of phosphate deficiency in freshwater has been widely debated. Increased phosphatase activity in many species in the absence of orthophosphate has led to the suggestion that measurement at time of harvesting would be a good indication of phosphorus status of the environment and the way in which organisms would be likely to respond to changes in nutrient status (Whitton, 1991). The reversal of natural water eutrophication depends on limiting the nutrients available for algal growth, so a critical analysis for plant limiting nutrients could be of great importance (Fitzgerald, 1969). An assay for the presence or absence of surplus phosphorus in algae from a particular environment could also be valuable in evaluating the nutritional status of algae mass-produced for economic purposes. Fitzgerald & Nelson (1966) assayed enzyme activity in phosphorus-limited media of four species of blue-green algae, three species of green

algae and two species of diatoms and concluded that an investigation of alkaline phosphatase activity was a useful test for conditions of phosphorus-limited growth in algae. Berman (1970) found a reciprocal relationship between the level of enzymatic activity per phytoplankton biomass unit and the concentration of total phosphorus; Wynne (1977) examined phosphomonoesterase activity of *Peridinium westii* during a bloom in Lake Kinneret, Israel and found that activity reached a maximum after the peak of the bloom, when inorganic phosphate became limiting; Ihlenfeldt & Gibson (1975) recorded the onset of phosphate-limited growth and increase in alkaline phosphatase activity in *Anacystis nidulans* (*Synechococcus*) and found that phosphorus limitation was followed within hours by derepression of alkaline phosphatase.

Healy (1973) summarised the necessary characteristics of a reliable bioassay for nutrient limitation in algae:

(i) The response measured should be specific to one or few nutrient deficiencies.

(ii) It should be widespread in occurrence among algae.

(iii) It must change rapidly at the onset and termination of nutrient limitation.

(iv) The response should have a low susceptibility to artefacts arising from experimental manipulation.

Alkaline phosphatase activity measurement in algae has the advantage of using a relatively simple reaction, with a stable enzyme. The stability and short incubation time required should reduce the occurrence of experimental artefacts. The increase in activity appears to be specific to phosphorus limitation in many algae (for example, *Anabaena variabilis*, Healy, 1973). However, in some taxa activity may be absent (e.g. *Dunaliella*, Kuenzlar, 1965) or unaffected by phosphorus deficiency (e.g. *Rhodomonas*, Kuenzlar, 1965). In addition, in some cases high alkaline phosphatase activity may persist after readdition of inorganic phosphate. Healy (1973) found that in *Anabaena variabilis*, activity was only lost by dilution as a result of renewed growth after addition of inorganic phosphate. This is not a universal situation, however: Bennun & Blum (1966) found that alkaline phosphatase activity in *Anacystis nidulans* disappeared almost immediately on readdition of inorganic phosphorus.

Provided that suitable preliminary studies are made for each individual case, assay for phosphomonoesterase activity may have considerable value in monitoring nutrient limitation in fresh

waters. Once the relationship between phosphatase activity of the species present and their phosphorus status has been determined, assays for phosphatase activity may be used to describe the nutrient status of the environment at different times. It is not a universally useful method, however, and care must be taken to verify the applicability of the assay for each particular situation.

### 1.4.3 Types of Phosphatase Enzymes

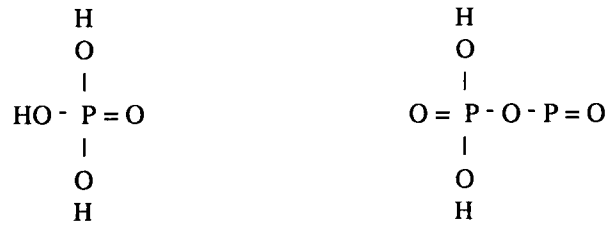
Phosphatases are a widely distributed group of hydrolytic enzymes, which catalyse the hydrolysis of esters and anhydrides of phosphoric acid (Feder, 1973). Phosphatases are classified, according to the substrate on which they act, into (i) phosphoric monoester hydrolases, (ii) phosphoric diester hydrolases, (iii) phosphoric triester hydrolases, (iv) enzymes acting on phosphoryl-containing anhydrides and (v) enzymes such as phosphoamidases, which act on P-N bonds (Feder, 1973).

Included in the phosphoric monoester hydrolases (phosphomonoesterases) are the acid and alkaline phosphatases, which are distinguished by the pH dependence of their catalytic activity (Fernley, 1971). They catalyse P-O bond cleavage and have a wide range of substrate specificities. phosphoric diester hydrolases catalyse the hydrolysis of phosphoric diesters, to yield a phosphoric monoester and an alcohol. The group is further sub-divided according to the specific substrates catalysed. The least substrate-specific are the phosphodiesterases.

### 1.4.4 Action of Alkaline Phosphatases

#### 1.4.4.1 Substrates

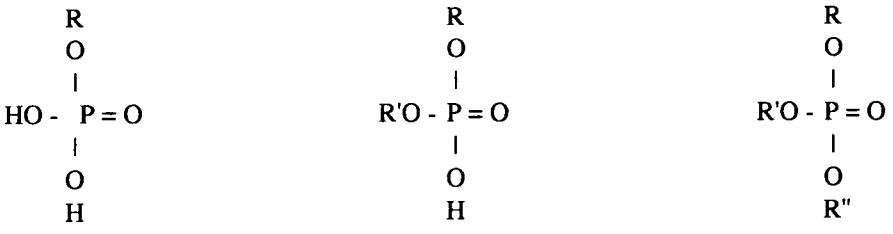
There is a variety of phosphorus compounds which can be acted upon by alkaline phosphatases; the basic compound is orthophosphoric acid (Fig. 1.41a). One of the attached oxygen atoms is represented as binding to phosphorus with a double bond, but in reality, all the P-O bonds are equal, and the double bond is shared between them. Orthophosphoric acid may be condensed with the elimination of water to form pyrophosphoric acid (Fig. 1.41b).



(a) Orthophosphoric Acid (b) Pyrophosphoric Acid

Fig. 1.41 Forms of phosphoric acid (from M$^c$ Comb *et al*, 1979)

Figure 1.42 shows the structures of some esters of orthophosphoric acid, the hydrolysis of which is catalysed by phosphomonoesterases, phosphodiesterases and phosphotriesterases respectively.



(a) Phosphomonoester (b) Phosophodiester (c) Phosphotriester

Fig. 1.42 Esters of orthophosphoric acid (M$^c$ Comb *et al*, 1979).

1.4.4.2 Reaction Mechanism

The enzyme-catalysed hydrolysis takes place in four main steps, (as outlined in figure 1.43):

(a) Michaelis Complex: The substrate becomes bound non-covalently to the enzyme (EH).

(b) Phosphoryl-Enzyme Compound: An alcohol (R-OH) is cleaved and the phosphate moiety is covalently bound to the enzyme.

(c) The phosphoryl-enzyme compound takes up water and is converted to a non-covalent compound.

(d) Orthophosphate is released and the free enzyme regenerated.

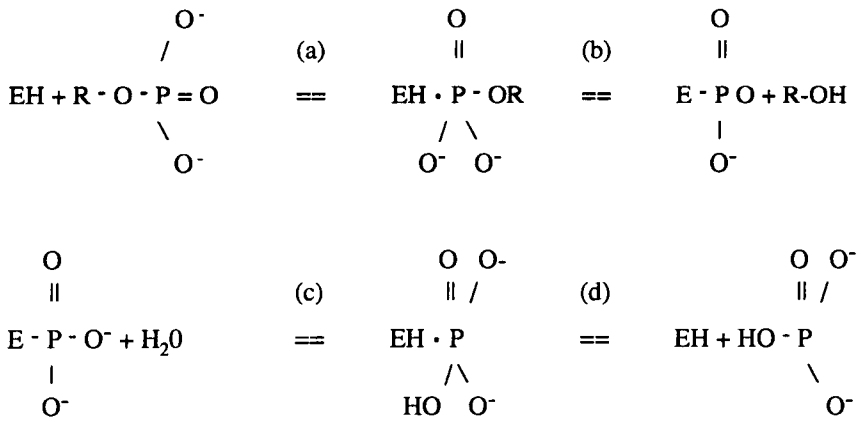


Fig. 1.43 Steps in the enzyme-catalysed hydrolysis of phosphate esters as described by Mᶜ Comb *et al*, 1979)

1.4.4.3 Production of Alkaline Phosphatases

It has been widely suggested that cells produce phosphomonoesterase in response to low levels of phosphate in their environment. Horiuchi, Horiuchi and Mizuno (1959) reported a linear increase in phosphomonoesterase activity in *Escherichia coli* for several hours after inorganic phosphate in the medium was exhausted, while no phosphatase activity was recorded in a phosphorus rich medium. There is abundant evidence that alkaline phosphatase is produced by algae in response to limitation of growth by deficient inorganic phosphate (e.g. Fitzgerald and Nelson, 1966; Rhee, 1973).

It seems that while acid phosphatases are constitutive, i.e. they are constantly produced for internal cell metabolism, alkaline phosphatases are produced or activated in response to phosphorus nutritional status of the environment (Jansson, Olsson and Pettersson, 1988). Induction, the synthesis of alkaline phosphatase in the presence of a suitable substrate, appears to be relatively uncommon. There are two other ways in which phosphate supply could regulate enzyme action:

(i) Repression: The end product of the enzyme-catalysed reaction shuts off further synthesis of the enzyme. Derepression, the resumption of synthesis, occurs when the repressor is depleted. Aaronson & Patni (1976) found that the phosphomonoesterase activity of phosphorus depleted *Ochromonas danica* could be partially repressed by inorganic phosphate.

(ii) Inhibition: The regulator acts on the enzyme itself and stops its activity. Phosphate can compete with

phosphoric esters for place on the active site of the enzyme, and so inhibit reaction (Jansson *et al*, 1988). There are several examples in the literature of algae with alkaline phosphatases regulated by inhibition, (e.g. Pettersson ,1980, Wynne, 1977).

### 1.4.5 Assay for Phosphatase Activity:

Phosphatase activity may be measured using a synthetic substrate, *para*-nitrophenylphosphate (*p*-NPP). The rate of hydrolysis of this substrate can be determined by measuring the rate of appearance of the product of hydrolysis, *para*-nitrophenol (*p*-NP). Jansson *et al* (1988) list several problems associated with this assay method:

(1) Substrate concentrations used in the assay are higher than natural substrate levels, so natural rates of hydrolysis may be much lower.
(2) Temperature and pH are standardised in the assay for optimum activity; these standard levels are often not the same as in natural waters (Olsson, 1990).
(3) The structure of natural substrates is often not clearly known. The affinity of phosphatases for different substrates (expressed as the Michaelis constant, Km), differs considerably according to structure, so levels of activity obtained using an artificial substrate may not be representative.

While these problems are important, assays using synthetic substrates are useful in determining potential phosphatase activity at a particular time, and variations in the maximum potential activity between different taxa and under different nutrient regimes.

## 1.5 MICRO-HABITAT

The three genera of filamentous green algae found in Red Sike and Slapestone Sike tend to grow together in mixed aggregations. Any species that was better adapted to the environment in the stream would tend to dominate over the others. This adaptation could be, for example, a higher phosphatase activity. Some investigators have suggested that increased enzyme activity under conditions of low phosphate concentration could enhance algal competitive ability, by enabling algae to use complex

organic substrates as alternative sources of orthophosphate (Francko, 1984; Kuenzlar, 1970). Berman *et al* (1991) report that differential ability in algal species to exploit dissolved organic sources of phosphorus and nitrogen could be critical in determining both the amounts and population composition of algae in freshwater.

It is also possible, however, that a competitive advantage might be gained by a species by physical adaptation to a specific micro habitat within the stream. The filamentous green algae found in Red Sike (*Zygnema*, *Mougeotia* and *Spirogyra)* are more usually found in pools and at the edges of ponds (Hynes, 1970). They are not firmly attached and are generally temporary and sporadic in occurrence, as slight changes in the water level may remove them. Any species which was better equipped to withstand strong currents may therefore be at an advantage. This would be evident in a microhabitat survey, as this species would be more common at more exposed microhabitats.

Several micro habitats with different environmental conditions were chosen in Red Sike and Slapestone Sike, and algal samples taken. It may be expected that, if water analyses showed different nutrient concentrations at the spring in Red Sike and further downstream, the filamentous Conjugales assemblages at these points may differ. Similarly, different conditions in Slapestone Sike, a small distance away from Red Sike, might significantly affect the algal assemblages in this stream.

## 1.6 AIMS

(1) To examine physical and chemical parameters of Red Sike over a period of four months, to record any trends and note their effects on the communities of filamentous Conjugales.

(2) To examine of phosphatase activity of filamentous Conjugales from different sites in Red Sike, to determine whether the alkaline phosphatase activity of the algae is related to nutrient levels, specifically inorganic phosphorus concentration, in the stream water.

(3) To investigate the presence and distribution of filamentous Conjugales at different microhabitats in Red Sike and Slapestone Sike with respect to possible temporal and spatial variation.

# CHAPTER 2 MATERIALS & METHODS

## 2.1 Physical Parameters

On each sample date, several physical parameters of Red Sike were measured. These included current, stream width and depth and temperature, conductivity, pH, and alkalinity of the water.

Current was measured using a calibrated Ott meter, positioned perpendicular to the direction of flow at the point of fastest current in the stream. The revolutions measured per minute were converted to m $s^{-1}$ (Patterson, 1983).

Water temperature and conductivity were measured using a wtw meter (model FC 910). A wtw meter (model pH 91) was also used to measure pH.

Alkalinity was measured by titration of a sample of stream water against 0.02 N HCl, to pH 4.2. Total alkalinity (meq $l^{-1}$) was calculated according to Golterman *et al* (1977).

## 2.2 Water Chemistry

Water was collected for chemical analysis from Red Sike at monthly intervals over a four-month period (4 May, 8 June, 6 July, 3 August). The water was collected in acid-washed 250 ml polyethylene containers at 15 minute intervals over the course of an hour. The bottles were kept on ice, in the dark until return to the laboratory, where the water was filtered using GF/F (0.7 μm) filters.

### 2.2.1 Nitrate

Water for nitrate analysis was frozen after filtration and thawed on the morning of analysis. Nitrate analysis was carried out according to the procedure outlined in Stainton *et al* (1977): nitrate ($NO_3$) in alkaline buffered solution was reduced to nitrate ($NO_2$) in a cadmium-copper reduction column. The solution was then treated with sulphanilamide and N-1-naphthylethylenediamine-dihydrochloride (NNED), to yield a pink azo dye, the optical density (543 nm) of which was proportional to nitrate concentration. This method assumes that nitrite levels in the samples were negligible relative to nitrate concentrations.

### 2.2.2 Ammonia

As with samples analysed for nitrate, water samples for determination of ammonia were frozen after filtration. The thawed samples were treated with phenol and hypochlorite under alkaline conditions to form indophenol blue, as outlined by Bolleter, Bushman & Tidwell (1961). The intensity of coloration was proportional to ammonia concentration and was measured specrophotometrically at 640 nm.

### 2.2.3 Phosphorus

Water samples were analysed, on the day of collection, for total filterable phosphorus (TFP) and filterable reactive phosphorus (FRP), using the method of Eisenreich, Bannerman & Armstrong (1975). This method is a simplified version of the "molybdenum blue" technique of Murphy and Riley (1961). FRP was determined colorimetrically without further treatment. The water for TFP determination was treated with acid persulphate, which digests organic and condensed phosphates. The difference between TFP and FRP was taken as an estimate of organic phosphorus. A standard curve of total and reactive phosphorus against absorbance was constructed (Appendix A) and used to calculate phosphorus concentrations in water samples.

## 2.3 Phosphatase Activity Assay

### 2.3.1 Collection of Algae

Algae were collected from several sites in Red Sike (May - August) and from Slapestone Sike (July and August) using blunt forceps, and transferred to glass snap-top containers with a small amount of stream water. These were kept on ice until return to the laboratory, where they were refrigerated.

### 2.3.2 Algal Culture

Algae were grown in sterile conical flasks. The growth medium used was CHU 10-F, a modification of the No. 10 medium described by Chu (1942), buffered at pH 7.5 with HEPES. The concentrations of elements in this medium are listed in table 2.1. Growth medium (30 ml) was transferred to labelled flasks, which were then autoclaved at 15 PSI (121°C) for 20 min. Algae were transferred to the cooled medium using a sterile loop, in a laminar flow cabinet. The culture flasks were kept in shaker tanks at 25°C.

As bacteria may show phosphatase activity, the presence of bacteria on algal filaments during assays may affect the activity recorded. To allow for this possibility, an axenic strain of *Mougeotia* was cultured and phosphatase activity measured. If the field material was significantly affected by the presence and activity of bacteria, it would be expected that the phosphatase activity recorded would be higher than for the axenic strain.

The filamentous Conjugales in Red Sike and Slapestone Sike tend to grow closely together in mixed communities. This means that material collected from the field contains filaments from several different species, so any enzyme activity cannot be directly attributed to any one species. To examine whether any algal species in Red Sike had a greater potential phosphatase activity (and so a greater potential competitive ability), single filaments of each species present were separated out under sterile conditions and grown in CHU-10F. The cultures arising from this separation were unialgal and free from visually obvious bacterial contamination. However, it is not possible to state that there was no bacterial contamination without the use of nutrient agar plates.

Table 2.1 Concentration of elements in CHU 10 F growth medium and CHU 10 D assay medium.

| Element | CHU 10 F Growth Medium | | CHU 10 D Assay Medium |
|---|---|---|---|
| | mg $l^{-1}$ | µM | µM |
| Na | 55 | 2391 | 2280 |
| K | 1.25 | 41 | 57.3 |
| N | 14 | 9921 | 0.14 |
| Mg | 2.5 | 100 | 100 |
| Ca | 6.7 | 169 | 243 |
| Fe | 0.5 | 9.0 | 4.47 |
| P | 1.0 | 32 | - |
| EDTA | 3.34 | 9.0 | 4.47 |
| B | 0.125 | 11.5 | 11.5 |
| Mn | 0.12 | 2.3 | 2.28 |
| Zn | 0.01 | 0.019 | 0.193 |
| Mo | 0.002 | 0.028 | 0.00276 |
| Cu | 0.005 | 0.079 | 0.0789 |
| Co | 0.002 | 0.043 | 0.0373 |

2.3.3 Treatment

Before the assay, the algae were centrifuged in distilled water and in assay medium (CHU-10D, containing all the elements necessary for growth, except phosphorus; see Table 2.1) for 10 minutes each, to remove excess mucilage and debris. They were then suspended in approximately 25 ml assay medium and stirred

and teased gently with forceps, to form a homogenous suspension. It was found that passing the algae through successively smaller needles, using a syringe, tended to damage or kill the filaments, so this procedure was not used.

### 2.3.4 Assay

Algae were suspended in 1.5 ml CHU 10 D assay medium buffered at pH 10.3 with Glycine-NaOH. At t = 0, the substrate, *para*-Nitrophenyl Phosphate (*p*-NPP), was added to a final concentration of 250 µM. A time course experiment showed that the hydrolysis of *p*-NPP was linear (i.e. not substrate limited) over a period of 50 minutes (Appendix B), so subsequent assays were generally carried out over a period of 45 minutes. The bottles containing the assay were capped and shaken at 25 °C for the course of the assay. At the end of the assay, the solution was filtered (GF/C) to remove the algae and the assay terminated with 5.0 M NaOH. The absorbance of the filtrate was measured immediately on the spectrophotometer at 405 nm.

A calibration curve was generated using standard dilutions of *para*-Nitrophenol (Appendix C), to calculate a calibration value (absorbance $_{405nm}$ for 1 µmol *p*-NP). This was used to calculate phosphatase activity of the algae (µmol *p*-NPP hydrolysed mg-1 h-1), in the equation:

[Reading / Calibration Value] x Assay Volume (l) x [1 / Dry Weight (mg)] x [1 / Time (h)].

### 2.3.5 Dry Weight Determination

The algal filaments from each assay container were washed into porcelain crucibles and dried at 105°C. The crucibles were transferred to a desiccator after 24 h and weighed.

## 2.4 Microhabitat Analysis

### 2.4.1 Collection of Algae

Microhabitats were selected in Red Sike and Slapestone Sike (July and August) on a subjective basis, according to environmental variables such as moisture, light, current speed and substrate. Algae were

removed from a 1 $cm^2$ patch and transferred to snap-top containers with a small amount of stream water. These were then stored on ice until return to the lab, where they were refrigerated.

### 2.4.2 Preparation and Mounting

Small sub-samples were removed from each sample and suspended in 10 ml distilled water. These were then shaken gently to homogenise the suspension and one drop placed on a glass microscope slide and covered with a coverslip.

### 2.4.3 Algal Identification

The algae were examined whilst alive in all cases, as identification was based mainly on chloroplast features. As none of the algae produced zygospores, it was not possible to carry out identification to species level. The species recorded were distinguished instead using width classes.

### 2.4.4 Estimation of Abundance of each Taxon

The slides were examined at x400 magnification and the abundance of each species was scored using a point-transect method. The eye-piece graticule (divided into 100 parts) was used as a transect, with major divisions (10, 20...) used as the points along it. For each field of view, any filament crossing a point on the transect was scored. The slide was moved regularly along until all fields of view had been examined. At least three sub-samples from each sample were examined microscopically and the mean number of filaments and standard error (S.E.) for each taxon present calculated. If the S.E. was high, more sub-samples were examined, until a low standard error (< 5% of the mean) was achieved.

Absolute numbers of each taxon at each site and proportion of the total made up by each taxon were plotted on histograms.

# CHAPTER 3 STUDY AREA

## 3.1 Upper Teesdale

Widdybank Fell (National Grid Reference NY 820 300) is included in the Upper Teesdale National Nature Reserve, and is of great scientific interest, mainly because of the number of rare plant species found there (Clapham, 1978). The high altitude of the nature reserve tends to cause low average temperatures and high precipitation (Fig. 3.1, Table 3.1)

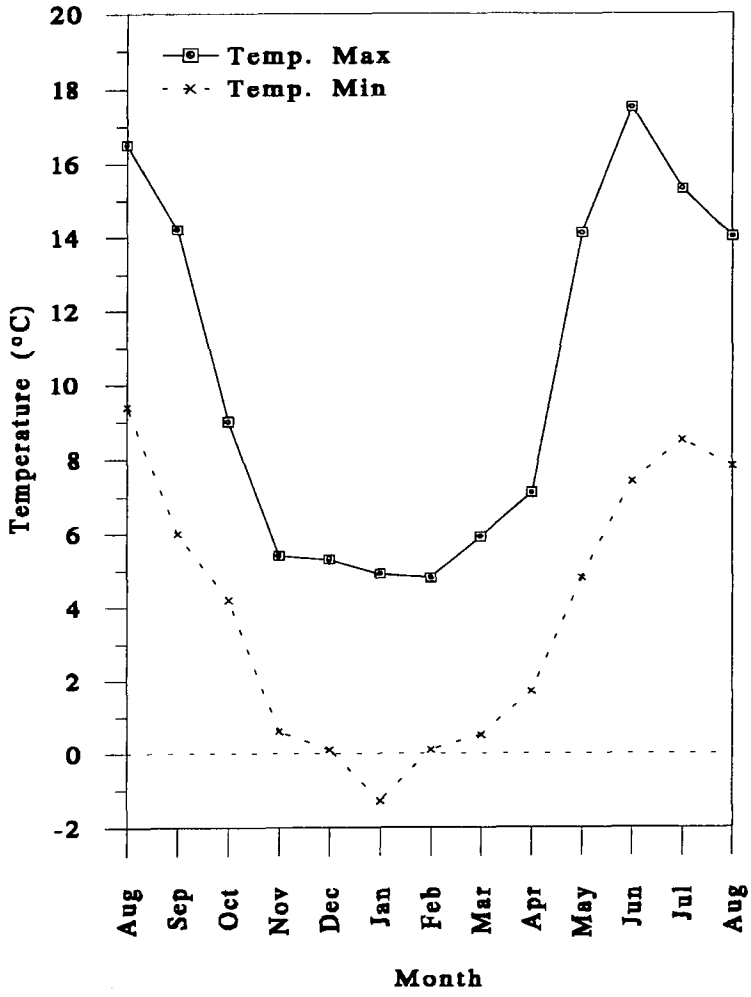



Fig. 3.1: Monthly maximum and minimum air temperature on Widdybank Fell, August 1991 - August 1992 (I. Findlay, pers. comm.).

Table 3.1 Meteorological data from Cow Green, April 1991 - August 1992 (I. Findlay, pers. comm.).

| Month | Temperature Maximum (°C) | Temperature Minimum (°C) | Total Rainfall (mm) | Total Snowfall (mm) | Sunshine (h $day^{-1}$) |
|---|---|---|---|---|---|
| **1991** | | | | | |
| April | 7.3 | 0.9 | 90.4 | 3.0 | 4.3 |
| May | 10.3 | 3.5 | 24.8 | - | 4.2 |
| June | 11.8 | 4.7 | 133.2 | - | 4.8 |
| July | 17.6 | 10.0 | 61.5 | - | 5.3 |
| August | 16.5 | 9.4 | 64.9 | - | 5.9 |
| September | 14.2 | 6.0 | 99.7 | - | 5.9 |
| October | 9.0 | 4.2 | 189.7 | - | 2.4 |
| November | 5.4 | 0.6 | 228.3 | 21.0 | 2.0 |
| December | 5.3 | 0.1 | 213.1 | 8.0 | 1.5 |
| **1992** | | | | | |
| January | 4.9 | -1.3 | 58.6 | 6.0 | 2.9 |
| February | 4.8 | 0.1 | 112.5 | 27.0 | 2.3 |
| March | 5.9 | 0.5 | 176.2 | 16.0 | 2.7 |
| April | 7.1 | 1.7 | 119.2 | - | 3.6 |
| May | 14.1 | 4.8 | 57.4 | - | 8.0 |
| June | 17.5 | 7.4 | 10.7 | - | 7.9 |
| July | 15.3 | 8.5 | 90.6 | - | 5.1 |
| August | 14.0 | 7.8 | 167.1 | - | 5.5 |

The vegetation of the area is also influenced by the geology. An important feature of the geology of the area is the Great Whin Sill quartz-dolerite intrusion. The intrusion of hot magma into Carboniferous

sediments in the late Carboniferous period caused extensive contact metamorphism (Johnson, 1978). Pure limestone underwent recrystallisation to form coarse-grained marble ("sugar limestone"). There are large areas of outcrop of this granular marble on Widdybank Fell.

Most of the upper area of Widdybank Fell is covered with blanket mire and small areas of dry heath; much of the remaining hillside has well drained acidic or calcareous soil (Livingstone and Whitton, 1984).

### 3.2 Red Sike

Red Sike (National Grid Reference NY 817 293) is a small stream with a mean discharge of 0.04 $m^3s^{-1}$ (Crisp & Howson, 1982). It has a south-westerly aspect and is mainly fed by limestone springs. It cuts through glacial drift, to expose limestone beds, separated by bands of shale and sandstone (Johnson, 1978)

There are three genera of filamentous conjugales present in the spring: *Zygnema*, *Spirogyra*, and *Mougeotia* (three species). Several diatom genera were recorded, including *Cymbella*, *Fragillaria*, *Achnanthes*, *Gomphonema*, *Navicula* and *Meridion*. The dominant cyanobacterial genus present was *Rivularia*.

Most of the water analysis and algal sampling was done on a 10 m stretch of stream adjacent to the road (referred to as the downstream reach). Sampling was also carried out in July and August from a spring approximately 350 m upstream. While the filamentous green algae were prevalent at the downstream reach near the road in early Summer, they were drastically reduced in July and August. Green algae were present in abundance, however, at and near the upstream spring examined in July and August.

### 3.3 Slapestone Sike

Slapestone Sike, like Red Sike, drains a metamorphosed limestone area. The stream is larger than Red Sike and, although water level tends to fluctuate, it is unlikely that it ever dries up completely (Hughes & Whitton, 1972). The upper reaches of the stream occupy a slight hollow, in which peat has developed

(Turner, 1978).

The main algal vegetation of Slapestone Sike is, as in Red Sike, filamentous conjugales, *Rivularia* and diatoms (especially *Cymbella* spp.). Only two species of *Mougeotia* were found here, although Hughes & Whitton (1972) report the presence of three, separated by differing widths. It has been suggested (Hughes & Whitton, 1972) that an algal complement such as is found in this stream is restricted to highly calcareous sites which are free from any enrichment from fertilisers or organic wastes.

# CHAPTER 4 RESULTS

## 4.1 Physical Parameters

A total of eight stream parameters were measured at Red Sike on each sample date. The results of these measurements are summarised in table 4.1. Purely physical measurements, such as stream width, depth and current speed would be expected to depend primarily on rainfall and vary in much the same way. The stream response to rainfall seems to be slightly delayed: Table 3.1 (Chapter 3)shows high rainfall levels in March and April, declining rainfall in May and June, and an increase in July and August. Stream depth (at the water sample point) and flow rate peaked in May and declined in June and July, with a slight increase in August. Stream width rose to a maximum in June, but the decreased in July and showed a similar increase in August (Fig. 4.1.1). However, as the stream was only sampled on one day each month, it is not possible to judge whether the as real trends or just sampling coincidences.

Table 4.1 Field measurements from Red Sike, May - August, 1992.

| Parameter | May | June | July | August |
|---|---|---|---|---|
| Stream width (m) | 2.50 | 3.15 | 1.20 | 1.50 |
| Depth (water sample point, cm) | 9.5 | 8.0 | 5.1 | 6.2 |
| Current speed (m $s^{-1}$) | 0.327 | 0.116 | 0.077 | 0.124 |
| Water Temperature (° C) | 7.7 | 13.3 | 20.2 | 15.7 |
| Conductivity (µS $cm^{-1}$) | 239 | 283 | 282 | 279 |
| pH | 7.20 | 8.29 | 8.49 | 8.84 |
| Alkalinity (meq $l^{-1}$) | 11.98 | 6.29 | 6.86 | 8.48 |
| O.D. of Water (440 nm) | 0.015 | 0.004 | 0.009 | 0.024 |

Water temperatures rose to a maximum (20.2 °C) in July, a month behind air temperatures, which peaked in June (see Fig. 4.1.2).

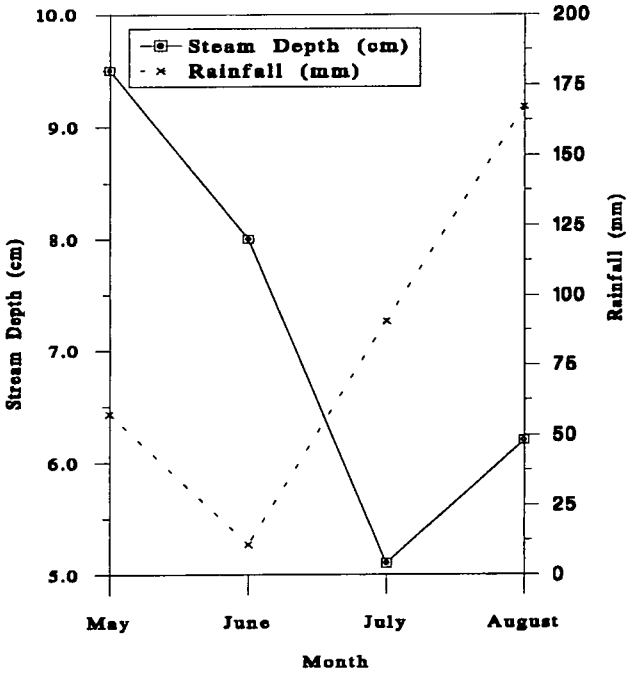



Fig. 4.1.1 Comparison between depth of water in Red Sike and rainfall, May - August, 1992.

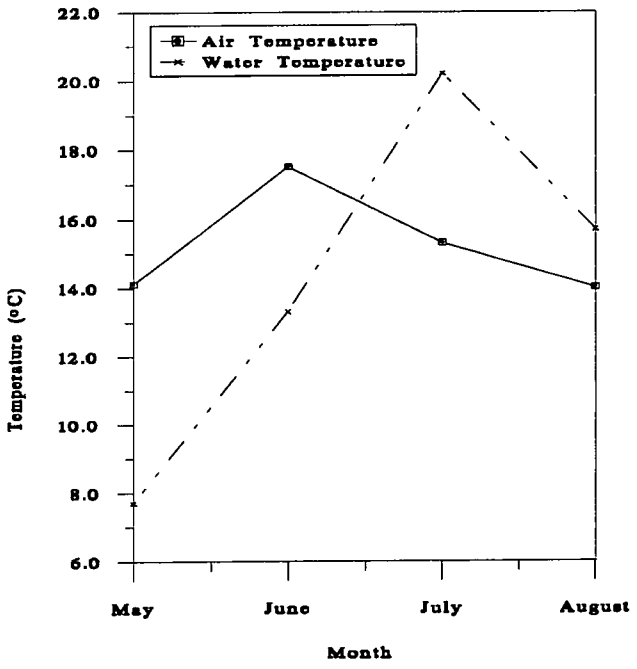



Fig. 4.1.2 Comparison between water temperatures in Red Sike and air temperatures at Cow Green, May - August, 1992.

The pH and conductivity ($\mu S\ cm^{-1}$) of the water behaved in a similar manner over time - both were lowest in May and rose to a more or less steady level over June - August (Fig. 4.1.3). The alkalinity ($meq\ l^{-1}$) of the water varied quite considerably over time, with a peak of 11.95 $meq\ l^{-1}$ in May falling to a minimum of 6.29 $meq\ l^{-1}$ the following month. The alkalinity rose in July and continued to rise in August.

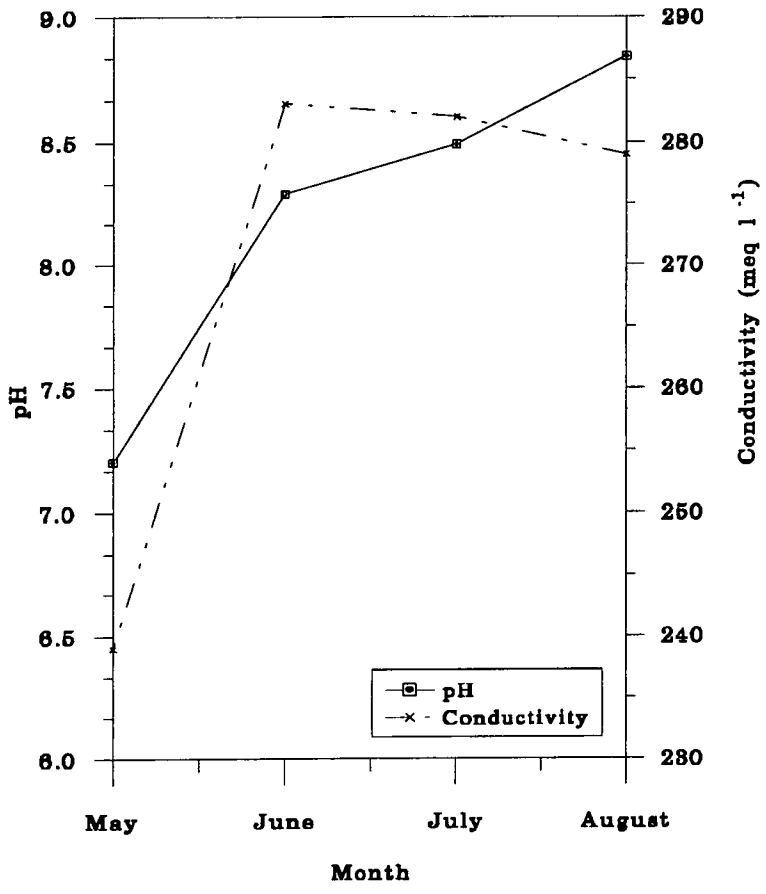



Fig. 4.1.3 Conductivity and pH of water in Red Sike, May - August, 1992.

## 4.2 Water Chemistry

### 4.2.1 Nitrogen

Two compounds of nitrogen were measured: nitrate ($NO_3$) and ammonia ($NH_3$). Nitrate is the fully

oxidised form and would therefore be expected to be present in higher concentrations in a small, unpolluted stream such as Red Sike.

Concentrations of nitrate and ammonia recorded at the downstream reach of Red Sike (April - August) are shown in table 4.2.1 and figure 4.2.1.

Table 4.2.1 Concentration (mg $l^{-1}$) of nitrate and ammonia in water from Red Sike, April - August, 1992 (reach = downstream reach, adjacent to road; spring = upstream spring site)

| **Month** | | **Nitrate** | | **Ammonia** | |
|---|---|---|---|---|---|
| | Site | Concentration | S.E. | Concentration | S.E. |
| April | reach | 0.639 | 0.031 | 0.244 | 0.020 |
| May | reach | 0.439 | 0.016 | 0.057 | 0.015 |
| June | reach | 0.545 | 0.036 | 0.070 | 0.003 |
| July | reach | 0.555 | 0.013 | 0.037 | 0.006 |
| | spring | 0.775 | 0.009 | 0.109 | 0.008 |
| August | reach | 0.362 | 0.004 | 0.034 | 0.004 |
| | spring | 0.670 | 0.011 | 0.062 | 0.002 |

Both nitrate and ammonia showed similar trends over time, with highest levels in April and lowest in August. The trends shown in nitrogen levels are similar to phosphorus trends: phosphorus concentrations were also lowest in August; water phosphorus was not measured in April, but was highest in July, which also showed the second highest nitrogen levels.

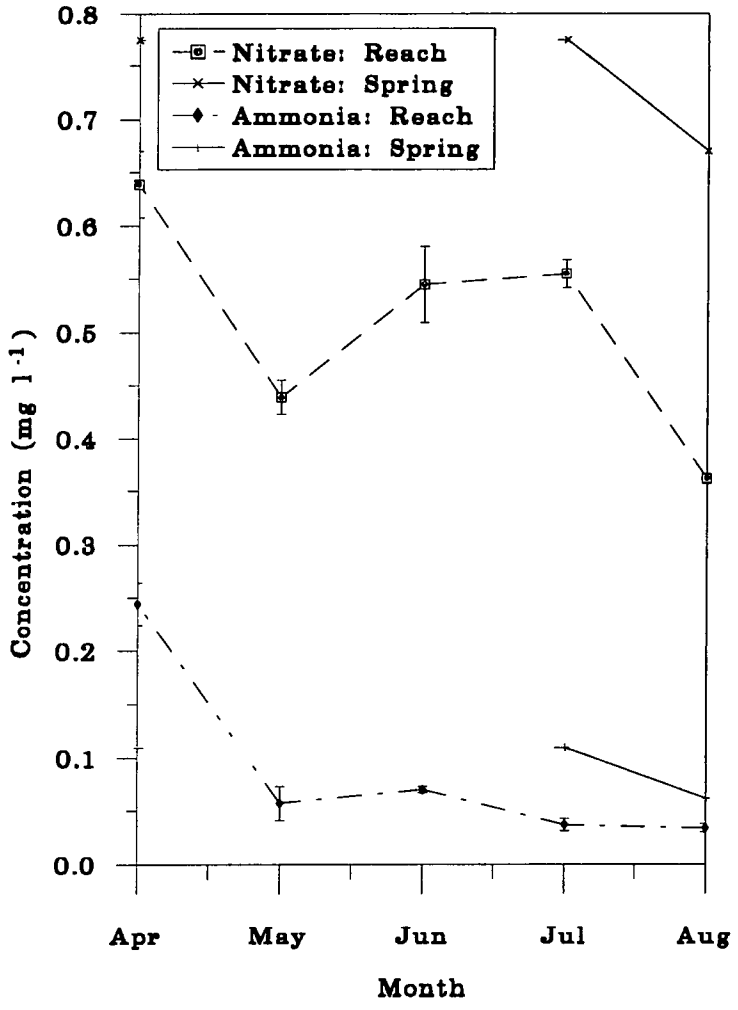



Fig. 4.2.1 Monthly variation in nitrate and ammonia concentrations (mg $l^{-1}$ ), April - August, 1992 (Spring & downstream reach).

### 4.2.2 Phosphorus

The phosphorus concentrations in water at Red Sike were measured at three positions in the stream: a pool in the 10m downstream reach adjacent to the road (May - August); a spring approximately 300m upstream (July & August) and a point midway between these positions (July & August).

The total filtrable phosphorus (TFP) at the downstream reach varied from a minimum of 2.13 µg $l^{-1}$ in August to a maximum of 6.77 µg $l^{-1}$ in July (Table 4.2.2, Fig. 4.2.2). The filtrable reactive phosphorus

(FRP) also peaked in July (3.75 $\mu g\ l^{-1}$) and fell to below the detection limit (< 0.001 $\mu g\ l^{-1}$) in August. The filtrable organic phosphorus (FOP) concentration in the water was estimated by subtraction of FRP from TFP, and the percentage organic phosphorus calculated (Table 4.2.1, Fig. 4.2.3). The proportion of phosphorus in the water made up by the organic fraction did not show the same trend as TFP and FRP; the maximum percentage FOP occurred in August, when both TFP and FRP were at a minimum, while the % FOP in July, when TFP and FRP peaked, was at a minimum.

Table 4.2.2 Phosphorus concentrations (Total, Filtrable & Organic, $\mu g\ l^{-1}$) and % Organic phosphorus in water from Red Sike, May - August, 1992

| **Month** | **Site** | **TFP** | | **FRP** | | **FOP** | | **% Organic** |
|---|---|---|---|---|---|---|---|---|
| | | Concentration | S.E. | Concentration | S.E. | Concentration | S.E. | |
| May | reach | 3.59 | 0.20 | 1.91 | 0.54 | 1.67 | 0.63 | 46.52 |
| June | reach | 6.35 | 0.20 | 0.14 | 0.06 | 6.25 | 0.20 | 98.43 |
| July | reach | 6.77 | 0.11 | 3.75 | 0.16 | 3.02 | 0.20 | 44.61 |
| | mid | 7.43 | - | 4.66 | - | 3.01 | - | 40.51 |
| | spring | 11.57 | - | 8.56 | - | 2.77 | - | 23.94 |
| August | reach | 2.13 | 0.15 | < 0.001 | - | 2.13 | 0.15 | 100.00 |
| | mid | 4.05 | 0.28 | < 0.001 | - | 4.05 | 0.28 | 100.00 |
| | reach | 7.04 | 0.03 | 5.20 | 1.25 | 1.85 | 1.22 | 26.28 |

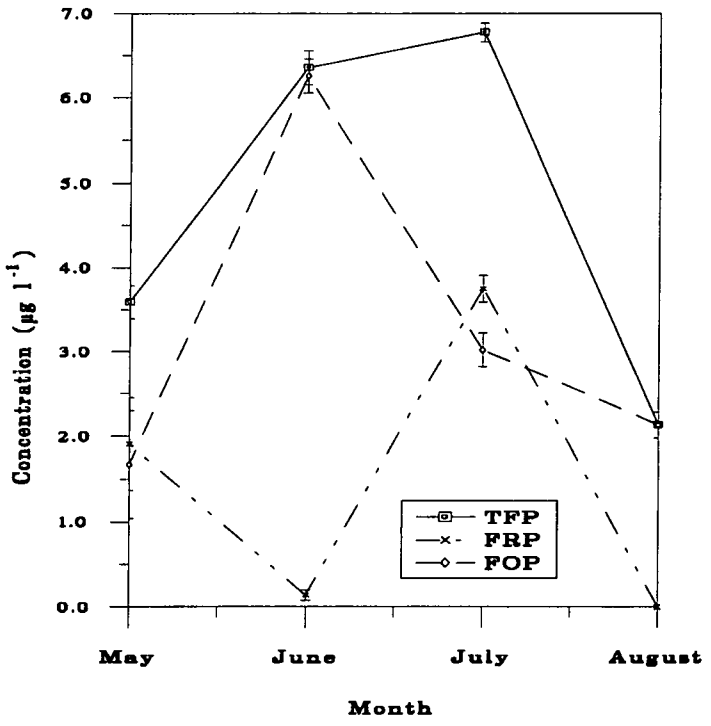



Fig. 4.2.2 Monthly variation in phosphorus levels (TFP, FRP & FOP, µg $l^{-1}$ ) in Red Sike, May - August.

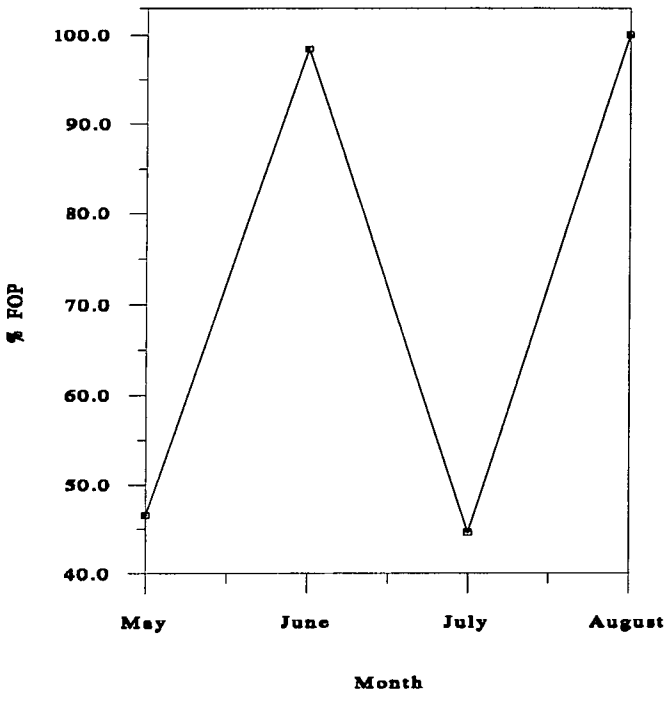



Fig. 4.2.3 Monthly variation in % organic phosphorus in water from Red Sike, May - August, 1992.

Phosphorus levels, both TFP and FRP, were considerably higher at the spring than at the downstream reach (Table 4.2.1). Spring water was assayed for phosphorus content in July and August only, but the general trend shown by the downstream reach is shown here also: the TFP and FRP concentrations were higher in July and the % FOP was higher in August. A major difference, however, between the spring and the downstream reach was the proportion of the water phosphorus made up by organic compounds: this was lower on both occasions at the spring than downstream. In fact, the % FOP in the water in August was only 26.28% at the spring, while the water phosphorus was made up of 100% organic compounds downstream. This must have important implications for the algal communities at the two sites .

The site mid-way between the spring and downstream reach had phosphorus levels between the two, as might be expected. However, the phosphorus concentrations, and in particular the % FOP were considerably closer to values recorded downstream than to the spring values. In August the % FOP at the mid-way point was 100%, like the percentage at the downstream reach, and compared with 21.2% at the spring. This result implies that phosphorus and in particular FRP is removed from the water quite quickly. This is in agreement with observations of algal communities in the stream, which were always more abundant at the spring, declining rapidly downstream, especially in August.

As in the case of nitrogen, concentrations of available phosphorus at the upstream spring were higher than at the downstream reach (July and August), with higher % FOP in July, declining in August (Fig. 4.2.4).

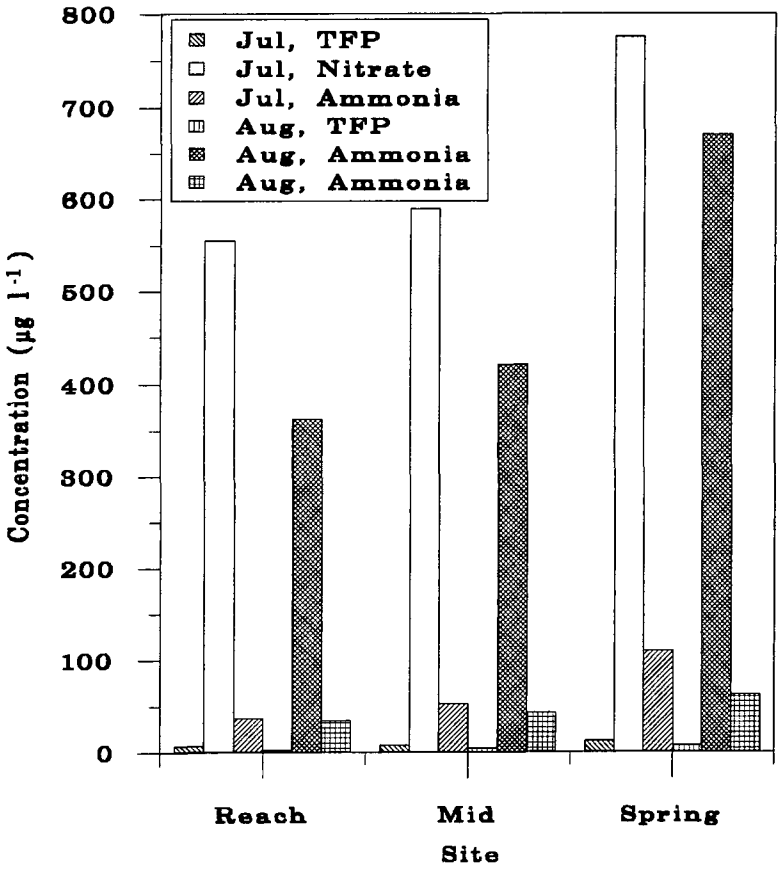



Fig. 4.2.4 Comparison between spring and downstream reach nutrient levels, Red Sike, July & August.

Overall nutrient levels in Red Sike were higher in mid-summer, with marked decreases in both phosphorus and nitrogen in August. This, again, is in agreement with observations at the stream: filamentous Conjugales were reduced at the spring in August and almost absent at the downstream reach.

## 4.3 Phosphatase Activity

Mixed aggregations of filamentous Conjugales were collected from the stream each month from May to August. These were assayed for phosphatase activity without further treatment beyond centrifuging and washing to remove mucilage and debris, to give an indication of the phosphatase activity of the algal community *in situ*. Samples from the downstream reach were collected and assayed May - August, while

algae from the spring were sampled in July and August.

The mean phosphatase activity (μmol $mg^{-1}$ $h^{-1}$) of the mixed community each month is shown on table 4.3.1.

Table 4.3.1 Phosphatase activity (μmol $mg^{-1}$ $h^{-1}$) of mixed community of filamentous green algae from Red Sike, (May - August).

| **Month** | **Site** | **Mean Activity** | **S.E.** |
|---|---|---|---|
| May | reach | 0.064 | 0.0048 |
| June | reach | 0.141 | 0.0040 |
| July | reach | 0.016 | 0.0004 |
| | spring | 0.008 | 0.0004 |
| August | reach | 0.064 | 0.0023 |
| | spring | 0.026 | 0.0008 |

The activity of the algae at the downstream reach, like water nutrient concentrations, showed quite a wide variation, from a maximum in June of 0.141 μmol $mg^{-1}$ $h^{-1}$ to a minimum the following month of 0.016 μmol $mg^{-1}$ $h^{-1}$. Phosphatase activity and water phosphorus concentrations at the downstream reach were closely related (Fig. 4.3.1). When the % FOP of the water was high (and therefore the proportion contributed by FRP was low), phosphatase activity was high, and vice versa.

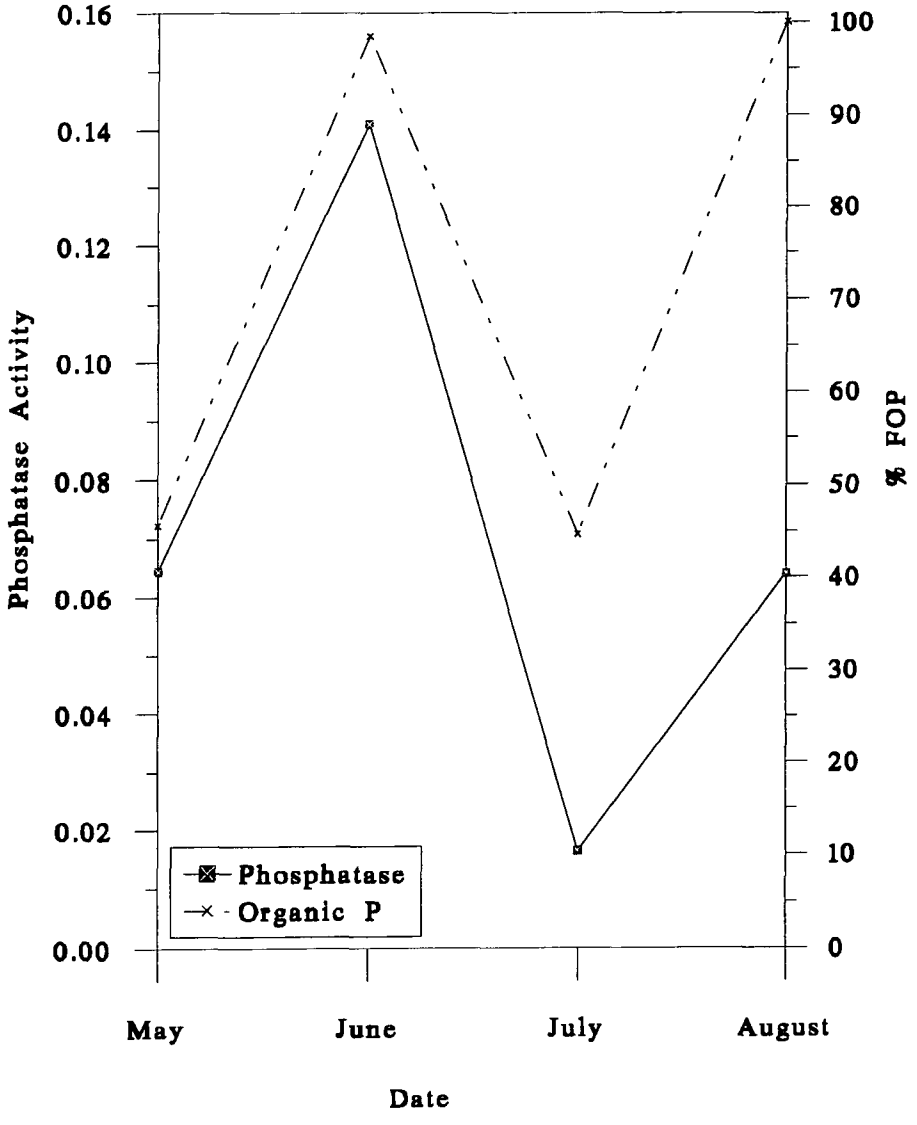



Fig. 4.3.1 Monthly variation in organic phosphorus of stream water and phosphatase activity of mixed community of filamentous Conjugales from the downstream reach of Red Sike, May - August, 1992.

The filamentous Conjugales at the spring showed lower enzyme activity than the algae further downstream (Table 4.3.2, Fig. 4.3.2). This lower activity correlates with recorded phosphate concentrations, which were higher at the spring than downstream (Fig. 4.2.4)

Table 4.3.2 Comparison between phosphatase activity of algae collected from the spring and downstream reach of Red Sike, July and August.

| Month | Activity ($\mu$mol mg$^{-1}$ h$^{-1}$) | | |
|---|---|---|---|
| | **Spring** | **Reach** | **Spring/Reach x 100** |
| July | 0.0082 | 0.016 | 51.25 |
| August | 0.0260 | 0.064 | 40.63 |

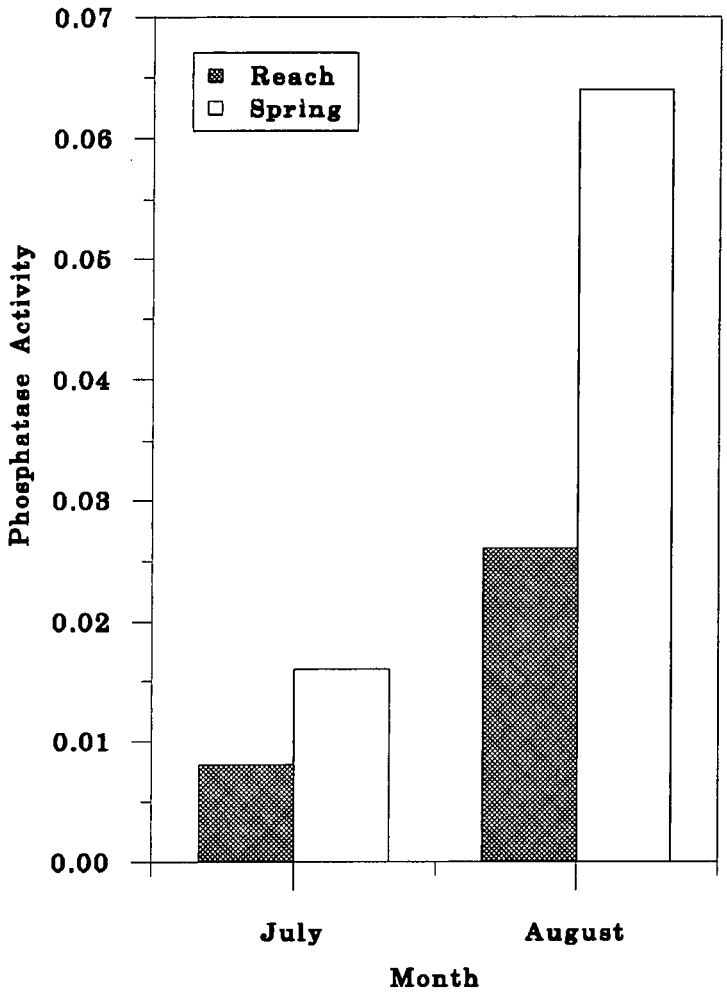



Fig. 4.3.2 Comparison of phosphatase activity of mixed filamentous Conjugales from the downstream reach and upstream spring of Red Sike, 6 July & 3 August, 1992

The phosphatase activity of the algae was found to be significantly related to the amount of phosphorus in the water; phosphate concentration ($\mu g\ l^{-1}$) was negatively correlated with phosphatase activity ( $r = 0.789$, $p < 0.01$) (Fig. 4.3.3). Phosphatase activity was positively correlated with the proportion of the water phosphorus made up by organic compounds ($r = 0.784$, $p < 0.01$) (Fig. 4.3.4).

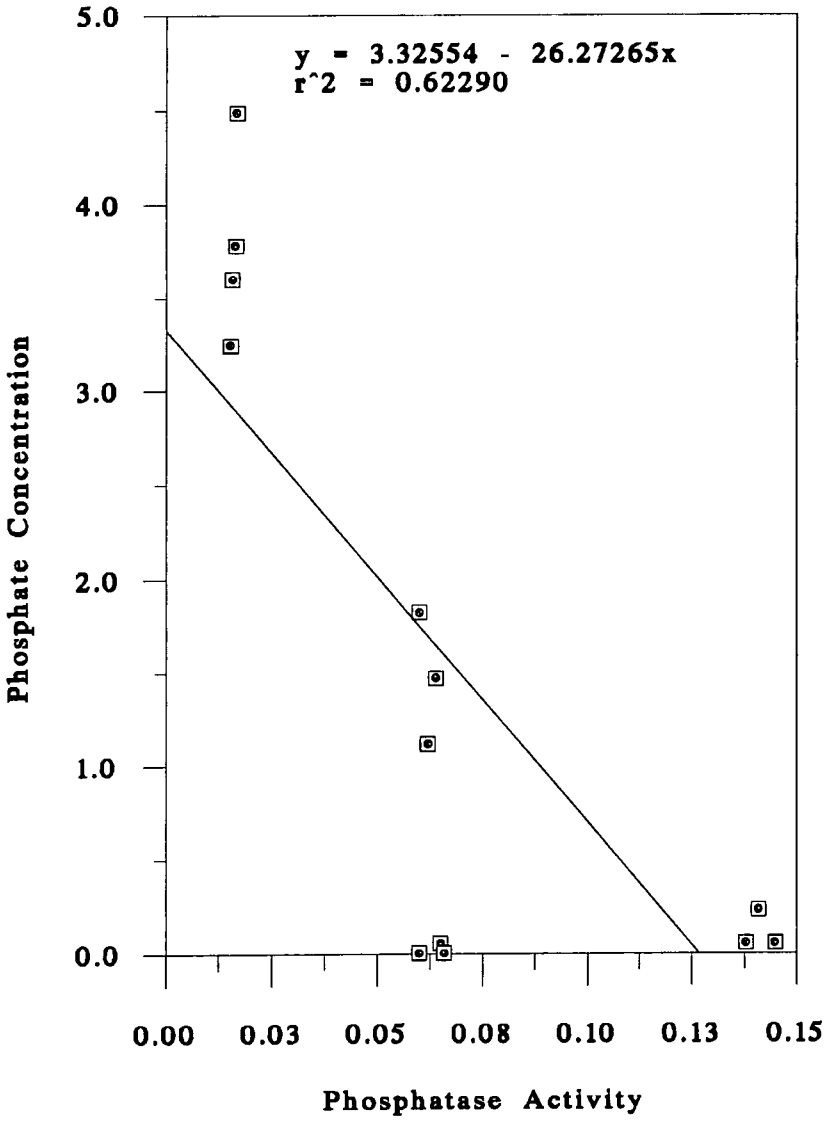



Fig. 4.3.3 Relationship between water phosphate concentrations and algal phosphatase activity at Red Sike, May - August, 1992.

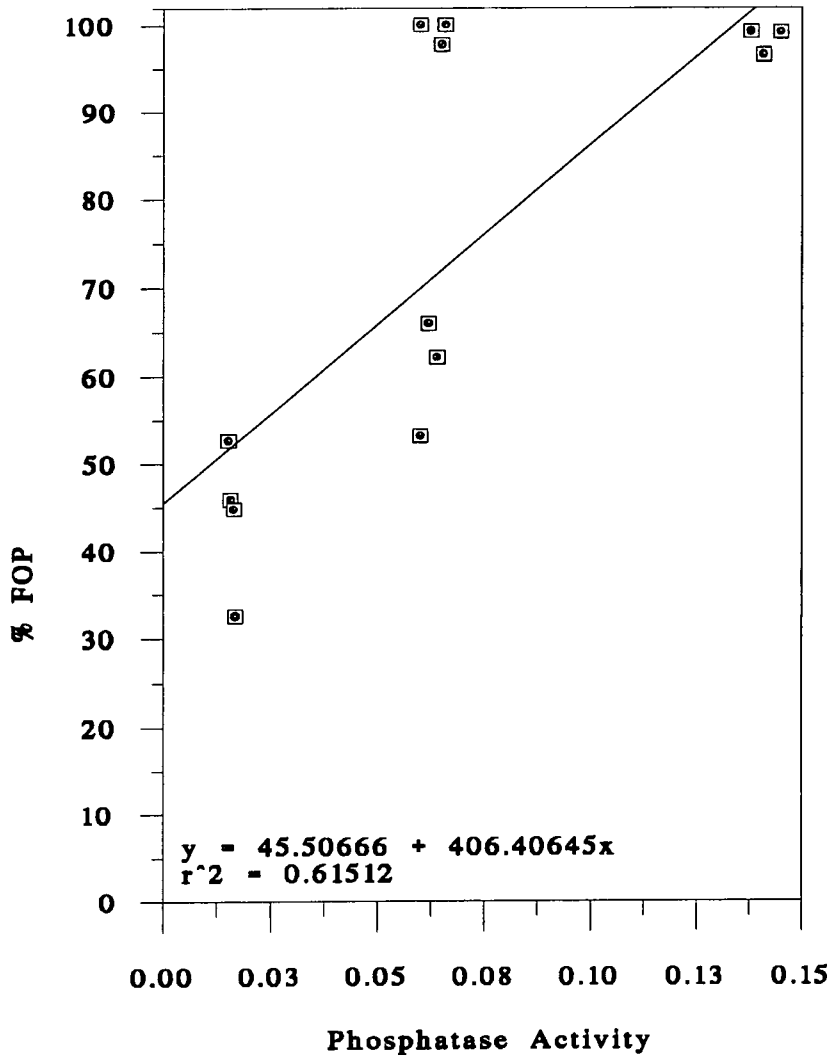



Fig. 4.3.4 Correlation between % FOP of stream water and phosphatase activity of algae at Red Sike, May - August, 1992.

When an axenic laboratory culture of *Mougeotia* (*Mougeotia* D536) assayed, the strain showed consistently low phosphatase activity (mean 0.0193 μmol $mg^{-1}$ $h^{-1}$). In addition, filaments from Red Sike were transferred under sterile conditions to growth medium and assayed for phosphatase activity after 14-18 days growth. Results of the assays using three different species for two positions in the stream in

June and July are shown in table 4.3.3. (*Spirogyra* sp. from Red Sike was also cultured, but failed to grow sufficiently for assay).

Table 4.3.3 Phosphatase activity of laboratory cultured filamentous Conjugales: axenic *Mougeotia* and *Mougeotia* spp. and *Zygnema* sp. isolated from field (grown in CHU-10F + Vitamins).

| **Date** | **Site** | **Taxon** | **Mean Activity** | **S.E.** |
|---|---|---|---|---|
| June | Reach | *Zygnema* | 0.0953 | 0.0032 |
| | | *Mougeotia* 6 -8 μm | 0.0871 | 0.0010 |
| | | *Mougeotia* 2 - 4 μm | 0.0769 | 0.0019 |
| July | Reach | *Zygnema* | 0.0498 | 0.0082 |
| | | *Mougeotia* 6 - 8 μm | 0.0385 | 0.0027 |
| | | *Mougeotia* 2 - 4 μm | 0.0216 | 0.0024 |
| | Spring | *Mougeotia* 6 - 8 μm | 0.0074 | 0.0009 |
| | | *Mougeotia* 2 - 4 μm | 0.0043 | 0.0011 |
| Axenic Culture | | *Mougeotia* 536 | 0.0193 | 0.0025 |

The algal taxa collected in June, when tested as unialgal cultures after 14 days growth in CHU 10F + Vit., all showed slightly lower activity than the mixed populations assayed the day after collection (Table 4.3.3). *Zygnema sp.*, which was the dominant species at the downstream reach, showed activity closest to the mixed population activity (0.141 μmol $mg^{-1}$ $h^{-1}$).

Of the algae collected from the downstream reach site in July, *Mougeotia* 2-4 μm and *Zygnema* had slightly lower activity than the mixed population activity (0.0135 μmol $mg^{-1}$ $h^{-1}$)and 0.0120 μmol $mg^{-1}$ $h^{-1}$ respectively ). *Mougeotia* 6-8 μm, however, showed substantially higher activity (0.0498 μmol $mg^{-1}$ $h^{-1}$ ), though not as high as the activity shown by any of the species in June.

Only two species were cultured from the spring in July: *Mougeotia* 6-8 μm and *Mougeotia* 2-4 μm.

*Mougeotia* 6-8 μm was present in higher proportions and showed higher phosphatase activity than either *Mougeotia* 2-4 μm or the mixed community (Fig. 4.3.5).

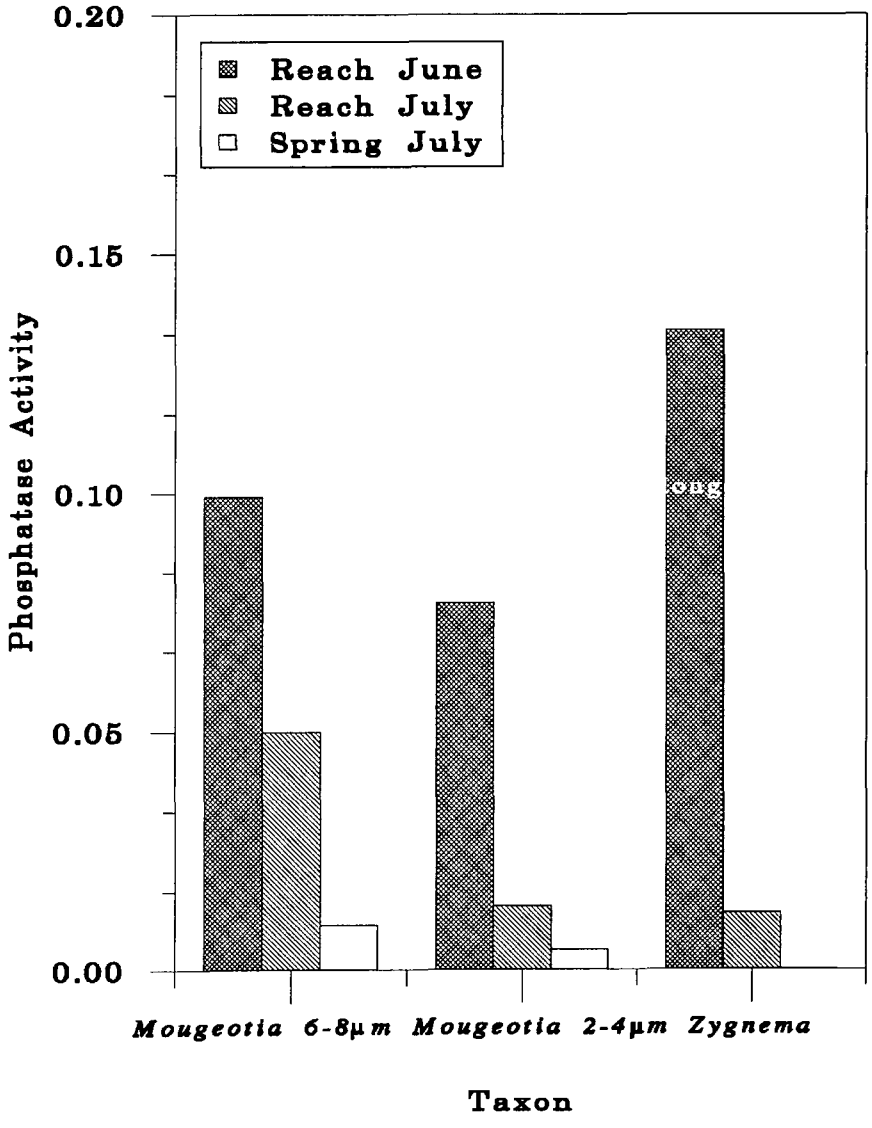



Fig. 4.3.5 Phosphatase activity of communities of filamentous Conjugales, axenic *Mougeotia*, and filaments (*Mougeotia* spp. & *Zygnema*) isolated from the field, grown in CHU-10F.

## 4.4 Microhabitat

Five species of filamentous Conjugales were recorded: 3 species of *Mougeotia* , 1 *Spirogyra* (4-6 μm) and 1 *Zygnema* (6-8 μm). The three species of *Mougeotia* were distinguished as *Mougeotia* 2-4 μm, *Mougeotia* 4-6 μm and *Mougeotia* 6-8 μm.

Algal samples were taken from several different microhabitats on each sample date; the subjectively determined environmental variables at each site are listed on table 4.4.1. Site numbers on the following tables refer to sample dates and variables recorded on this table.

Table 4.4.1 Environmental variables at the sites sampled, Red Sike (8 June, 17 June, 6 July) and Slapestone Sike (6 July)

| Date | Site | Sample Number | Moisture | Light | Depth (cm) | Current |
|---|---|---|---|---|---|---|
| 8 June | Red Sike | 1 | medium | high | < 1 | medium |
| | | 2 | medium | high | < 1 | weak |
| | | 3 | wet | high | > 5 | strong |
| | | 4 | dry | high | < 2 | medium |
| | | 5 | wet | low | < 2 | medium |
| | | 6 | medium | low | < 1 | medium |
| | | 7 | wet | high | | |
| | | 8 | dry | high | | |
| 17 June | Red Sike | 9 | dry | high | | |
| | | 10 | wet | high | | |
| | | 11 | wet | low | | |
| | | 12 | wet | low | | |
| 6 July | Red Sike - Reach | 13 | wet | high | | v. strong |
| | | 14 | wet | high | | v. strong |
| | | 15 | dry | high | > 5 | strong |
| | | 16 | dry | high | < 1 | weak |
| | | 17 | wet | low | < 5 | weak |

continued.
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Table 4.4.1 (cont.) Environmental variables at sites sampled, Red Sike and Slapestone Sike, 3 August.

| Date | Site | Sample Number | Moisture | Light | Depth | Current |
|---|---|---|---|---|---|---|
| 6 July | Red Sike - Spring | 18 | wet | high | > 5 | strong |
| | | 19 | dry | high | < 1 | weak |
| | | 20 | wet | high | > 5 | v. strong |
| | | 21 | wet | low | < 2 | medium |
| 6 July | Slapestone Sike | 22 | dry | low | < 1 | weak |
| | | 23 | wet | low | < 1 | weak |
| | | 24 | wet | low | < 1 | strong |
| | | 25 | wet | high | > 10 | v. weak |
| 3 August | Red Sike - Reach | 26 | wet | high | | weak |
| | | 27 | dry | high | | weak |
| | | 28 | wet | low | | weak |
| 3 August | Red Sike - Spring | 29 | wet | high | | strong |
| | | 30 | dry | high | | weak |
| | | 31 | wet | high | | strong |
| | | 32 | wet | low | | medium |
| 3 August | Slapestone Sike | 33 | dry | high | | v. weak |
| | | 34 | wet | high | | weak |
| | | 35 | wet | low | | weak |
| | | 36 | wet | high | | v. weak |

The downstream reach was sampled on 8 June, 17 June, 6 July and 3 August, while the spring was sampled in July and August only. *Zygnema* and two species of *Mougeotia* (2-4 μm and 6-8 μm) were present at all sites sampled on 8 June. *Spirogyra* was present at all but one (Table 4.4.2). *Zygnema* sp. was the dominant species at all sites, making up more than 70% of filaments present in each case.

The most obvious difference in species distribution was at site 5 (see Table 4.4.1), where *Spirogyra* made up 40.1% of the filaments present. This site was one of very low light intensity; the algae were growing near to the edge of the stream, overhung almost entirely by heather.

Table 4.4.2 8 June: summary of algal abundance counts: mean number of filaments at each site and % each species makes up of total filaments.

| Site | *Zygnema* | | *Mougeotia* 2-4 μm | | *Mougeotia* 6-8 μm | | *Spirogyra* | |
|---|---|---|---|---|---|---|---|---|
| | Mean | % | Mean | % | Mean | % | Mean | % |
| 1 | 42.00 | 73.7 | 2.30 | 2.6 | 10.60 | 16.2 | 4.00 | 7.4 |
| 2 | 94.30 | 89.9 | 4.30 | 5.1 | 4.30 | 3.7 | 1.00 | 1.2 |
| 3 | 93.30 | 75.8 | 13.30 | 10.8 | 7.70 | 6.1 | 9.30 | 7.6 |
| 4 | 960 | 73.3 | 21.00 | 1.6 | 3.70 | 2.7 | 25.30 | 7.9 |
| 5 | 3.79 | 51.3 | 7.70 | 4.4 | 6.70 | 4.2 | 67.70 | 40.1 |
| 6 | 43.30 | 81.2 | 1.00 | 2.0 | 5.00 | 9.0 | 3.70 | 7.9 |
| 7 | 46.00 | 82.2 | 1.00 | 1.8 | 5.30 | 9.5 | 3.70 | 6.5 |
| 8 | 39.00 | 77.3 | 4.30 | 7.9 | 7.30 | 14.8 | 0.00 | 0.0 |

All four species were present at each site sampled on 17 June (Table 4.4.3). On this occasion, the community was dominated by *Zygnema*, even more so than before, with *Zygnema* making up more than 85% of the filaments present at three of the four sites examined. At site 11, a wet site with low light intensity, *Zygnema* was less prominent and 32.9% of the filaments present were of *Spirogyra*. Site 12, also a low light site had a higher proportion of *Spirogyra* than the two remaining sites, which were both high light, but the difference was less marked than at site 11.

Site 9, a low moisture site, had slightly more *Mougeotia* 6-8 μm than the other sites, as did site 8 (sampled 8 June), which also had low moisture.

Table 4.4.3 17 June: summary of algal abundance counts: mean number of filaments at each site and % each species makes up of total filaments.

| Site | *Zygnema* | | *Mougeotia* 2-4 μm | | *Mougeotia* 6-8 μm | | *Spirogyra* | |
|---|---|---|---|---|---|---|---|---|
| | Mean | % | Mean | % | Mean | % | Mean | % |
| 9 | 173.3 | 86.1 | 3.7 | 1.9 | 14.0 | 7.2 | 11.3 | 4.8 |
| 10 | 114.0 | 90.7 | 1.7 | 1.3 | 5.0 | 4.1 | 4.7 | 4.0 |
| 11 | 84.0 | 60.4 | 2.3 | 1.7 | 3.7 | 5.0 | 45.7 | 32.9 |
| 12 | 116.7 | 89.6 | 1.0 | 0.7 | 1.0 | 0.5 | 11.7 | 8.9 |

In July, microhabitats at two different positions within Red Sike and another stream, Slapestone Sike, were examined. The area near the spring in Red Sike had an algal community that was considerably different from the community further downstream (Table 4.4.4). A third species of *Mougeotia* (*Mougeotia* 4-6 μm) was recorded at a site < 5 cm from the spring (site 8). This species was not found at any other site, near the spring or further downstream. It was present in approximately the same numbers as the other two *Mougeotia* species at that site. Site 19 was a small, stagnant pool approximately 30 cm from the spring. The algal community in this pool was dominated by *Mougeotia* 6-8 μm (92.1%), with small amounts of *Mougeotia* 2-4 μm.

Five microhabitats in the downstream reach were sampled on 6 July. The first two examined, sites 13 & 14, had fast flow; *Mougeotia* 6-8 μm was absent from both. *Mougeotia* 2-4 μm was absent from one and present in low numbers in the other.

As on previous occasions, it was found that *Spirogyra* sp. was present at higher levels at the low light site. At all the other sites examined, *Zygnema* was the predominant species.

The second stream examined on 6 July, Slapestone Sike, (Table 4.4.5), had one very distinctive microhabitat (site 25), a small pool off the main stream which contained only *Mougeotia* 6-8 μm. As in the downstream region of Red Sike, *Zygnema* sp. predominated at the other three sites examined. *Mougeotia* 2-4 μm was present in low numbers and absent from one wet site. *Mougeotia* 6-8 μm was also absent from one site, site 24, at which the current was slightly higher than elsewhere.

Table 4.4.4 6 July, Red Sike: summary of algal abundance counts: mean number of filaments at each site and % each species makes up of total filaments at each site.

| Site | *Zygnema* | | *Mougeotia* 2-4 µm | | *Mougeotia* 6-8 µm | | *Mougeotia* 4-6 µm | | *Spirogyra* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | % | Mean | % | Mean | % | Mean | % | Mean | % |
| 13 | 42.3 | 92.7 | 0.7 | 1.4 | 0.0 | 0.0 | 0.0 | 0.0 | 2.7 | 5.9 |
| 14 | 48.0 | 91.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 4.7 | 8.8 |
| 15 | 41.0 | 90.2 | 0.0 | 0.0 | 1.3 | 2.8 | 0.0 | 0.0 | 3.3 | 7.1 |
| 16 | 35.0 | 71.5 | 0.7 | 1.4 | 3.6 | 7.4 | 0.0 | 0.0 | 9.7 | 19.7 |
| 17 | 32.0 | 47.8 | 2.3 | 3.2 | 6.0 | 8.8 | 0.0 | 0.0 | 26.7 | 40.2 |
| 18 | 0.0 | 0.0 | 52.3 | 33.8 | 51.7 | 33.5 | 50.7 | 32.9 | 0.0 | 0.0 |
| 19 | 0.0 | 0.0 | 7.3 | 7.9 | 85.0 | 92.1 | 0.0 | 0.0 | 0.0 | 0.0 |
| 20 | 0.0 | 0.0 | 37.3 | 52.6 | 33.7 | 47.4 | 0.0 | 0.0 | 0.0 | 0.0 |
| 21 | 0.0 | 0.0 | 32.3 | 61.6 | 21.0 | 38.4 | 0.0 | 0.0 | 0.0 | 0.0 |

Table 4.4.5 6 July, Slapestone Sike: summary of algal abundance counts: mean number of filaments at each site and % each species makes up of total filaments at each site.

| Site | *Zygnema* | | *Mougeotia* 2-4 µm | | *Mougeotia* 6-8 µm | | *Spirogyra* | |
|---|---|---|---|---|---|---|---|---|
| 22 | 47.7 | 94.7 | 2.0 | 4.0 | 0.7 | 1.4 | 0.0 | 0.0 |
| 23 | 42.7 | 98.5 | 0.0 | 0.0 | 0.7 | 1.5 | 0.0 | 0.0 |
| 24 | 48.7 | 80.3 | 4.0 | 6.7 | 0.0 | 0.0 | 8.0 | 13.2 |
| 25 | 0.0 | 0.0 | 0.0 | 0.0 | 50.0 | 100.0 | 0.0 | 0.0 |

These sites were marked and resampled on 3 August. The results were mostly similar, the major difference being that a large proportion of the algal community had died by August, so the numbers recorded from most sites were lower. The proportions of each species present, however, were broadly comparable (Tables 4.4.6 & 4.4.7).

Table 4.4.6 3 August, Red Sike: summary of algal abundance counts: mean number of filaments at each site and % each species makes up of total filaments at each site.

| Site | *Zygnema* | | *Mougeotia* 2-4 μm | | *Mougeotia* 6-8 μm | | *Mougeotia* 4-6 μm | | *Spirogyra* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | % | Mean | % | Mean | % | Mean | % | Mean | % |
| 26 | 52.4 | 83.4 | 0.0 | 0.0 | 3.7 | 5.9 | 0.0 | 0.0 | 6.7 | 10.7 |
| 27 | 39.7 | 74.6 | 1.3 | 2.4 | 3.6 | 6.8 | 0.0 | 0.0 | 8.6 | 16.2 |
| 28 | 37.0 | 49.3 | 1.7 | 2.3 | 5.0 | 6.7 | 0.0 | 0.0 | 31.4 | 41.8 |
| 29 | 0.0 | 0.0 | 38.7 | 30.23 | 47.2 | 36.9 | 42.1 | 32.9 | 0.0 | 0.0 |
| 30 | 0.0 | 0.0 | 19.7 | 18.3 | 87.7 | 81.7 | 0.0 | 0.0 | 0.0 | 0.0 |
| 31 | 0.0 | 0.0 | 62.7 | 52.3 | 57.3 | 47.7 | 0.0 | 0.0 | 0.0 | 0.0 |
| 32 | 0.0 | 0.0 | 52.7 | 48.5 | 24.3 | 22.4 | 0.0 | 0.0 | 31.7 | 29.2 |

Table 4.4.7 3 August, Slapestone Sike: summary of algal abundance counts: mean number of filaments at each site and % each species makes up of total filaments at each site.

| Site | *Zygnema* | | *Mougeotia* 2-4 μm | | *Mougeotia* 6-8 μm | | *Spirogyra* | |
|---|---|---|---|---|---|---|---|---|
| | Mean | % | Mean | % | Mean | % | Mean | % |
| 33 | 35.3 | 76.6 | 4.0 | 8.7 | 1.0 | 2.2 | 5.8 | 12.6 |
| 34 | 36.3 | 87.3 | 3.3 | 7.9 | 2.0 | 4.8 | 0.0 | 0.0 |
| 35 | 44.0 | 67.7 | 3.0 | 4.6 | 0.0 | 0.0 | 18.0 | 27.7 |
| 36 | 0.0 | 0.0 | 0.0 | 0.0 | 61.0 | 100.0 | 0.0 | 0.0 |

# CHAPTER 5 DISCUSSION

## 5.1 WATER CHEMISTRY

Analysis of nutrient concentrations in the water at Red Sike showed quite wide fluctuations in monthly phosphorus and nitrogen levels, with high concentrations in June and July, decreasing in August (Tables 4.2.1 & 4.2.2). A longer sampling period, perhaps with more samples taken per month, could provide a more detailed look at the water chemistry of the stream and may suggest reasons for the fluctuations seen in this study.

The spring sites sampled had consistently higher phosphorus concentrations than the downstream reach, so there were always lower concentrations of nutrients available to the algae growing downstream. It appears, then, that much of the phosphorus in the stream water comes from ground-water entering the stream via small springs. It is likely, however, that not all of the phosphorus in the stream comes from these sources as phosphorus levels may be changed by run-off of surface water from the catchment, animal deposits and the release by micro-organisms of nutrients from dead plant and animal matter in the stream, (Moss, 1980) as well as by co-precipitation with iron (Davison & Tipping, 1984) and calcium (House, 1984).

Nitrate and ammonia concentrations were also higher at the spring than further downstream. No attempt was made, however, to quantify the requirements or use of nitrogen compounds by filamentous Conjugales. The blue-green alga *Rivularia* was present in great abundance in the stream throughout the sampling period. *Rivularia* has the ability to both fix atmospheric nitrogen and utilise organic phosphorus and so may have different requirements for N:P ratio in the water. Livingstone & Whitton (1984) recorded enzyme activity of 0.032 μmol (μg chl *a*)$^{-1}$ h$^{-1}$ for *Rivularia* colonies in Red Sike in April. A comparative study of the nutrient requirements of these two groups, which thrive in the same small stream, could prove useful in allowing greater understanding of the ecology of the stream.

The filamentous green algae in Red Sike, which were most abundant in mid-Summer, were obviously affected by nutrient levels in the water, and may exhibit cyclical patterns of abundance, related to nutrient availability. As phosphorus is not always available in excess, it would be advantageous for a species to have the capacity to utilise as many forms of phosphorus as possible. This capacity was

investigated in examination of the phosphatase activity of the filamentous Conjugales present.

## 5.2 PHOSPHATASE ACTIVITY

Alkaline phosphomonoesterase activity exhibited by the mixed communities of filamentous Conjugales at the downstream reach of Red Sike was found to be negatively correlated with phosphate concentration and positively correlated with the proportion contributed by organic compounds to the total phosphorus content of the water (Section 4.3; Fig. 4.3.3 & Fig. 4.3.4). Gibson & Whitton (1987) also reported a significant relationship between phosphatase activity and water phosphorus concentration in *Stigeoclonium* in a northern England streams. Chróst & Overbeck (1987) report that internal phosphorus concentrations influence the enzyme activity more than levels in the external environment. However, Gibson & Whitton (1987) found only slightly better correlations between phosphatase activity and internal phosphorus than between enzyme activity and phosphorus concentrations in water.

Algae sampled from the spring region had lower phosphatase activities than those downstream (Fig. 4.3.2), which is in accordance with the fact that they were growing under conditions of greater phosphorus availability. As the species composition of the spring and downstream sites were so different (See Chapter 4.4), it is possible that the maximum potential phosphatase activity of the *Mougeotia* species growing at the spring is lower than the maximum potential of *Zygnema*, which dominated downstream, where phosphorus concentrations were lower. In order to test this hypothesis, single filaments of each species were isolated under sterile conditions, cultured and assayed individually for phosphatase activity. Upon individual assay, *Zygnema* sp. from the downstream reach of Red Sike showed the highest activity (Table 4.3.3). As this was the species that was dominant at all sites examined in the downstream reach (see section 5.3), it would be expected that it would show some advantage over the other species present. It appears that *Zygnema* is able to dominate at the downstream reach because it has a higher potential phosphatase activity, thus allowing it to make more efficient use of what phosphorus is available. *Mougeotia* 6-8 μm, which was generally more common at the downstream reach than *Mougeotia* 2-4 μm, showed higher phosphatase activity, though not so high as that shown by *Zygnema*. It seems, then, that the distribution of these two species is also at least partially

affected by potential phosphatase activity. This is in agreement with the suggestion of Berman *et al* (1991) that phosphatase activity could be an important factor in influencing plant community composition in fresh waters. If stream water frequently contains low levels of FRP, the algae that will survive and dominate will be those which can utilise alternative sources of phosphorus.

A laboratory assay to fully examine the different potential phosphatase activity of the algal species present in Red Sike would involve the isolation of single filaments of each species from the field and the growth under phosphorus-limited conditions of bacteria free unialgal cultures. This procedure, carried out with control cultures under conditions of phosphorus abundance would allow more meaningful comparison of the enzyme activity of the algae.

The unialgal cultures assayed for phosphatase activity all showed lower activity than mixed communities from the field. This may be due to the fact that the algae in culture assays spent 14 - 18 days growing under conditions of plentiful phosphorus supply, so phosphatase enzymes would not be active. Such a period was, however, essential to allow sufficient biomass for an assay to grow.

The axenic laboratory strain of *Mougeotia* also showed consistently lower phosphatase activity than the algae collected from Red Sike (Table 4.3.3). It is possible the activity recorded from the field material was due more to bacteria present on the samples than to enzymes produced by the algae; however, there was no visually obvious bacterial contamination. Also, as the laboratory strain was not growing under phosphorus-limited conditions for any period of time, it is more likely that the low activity recorded was due to the fact that the enzyme was not induced or derepressed, as phosphate was in plentiful supply. It is also possible that the parent population of the strain was adapted to different environmental conditions. In order to fully examine the effects of bacterial activity on the assay results, it would be advantageous to grow field material and axenic strains under conditions of similar phosphorus limitation for a period of time before the assay. However, this still would not allow a direct comparison with the field activity.

## 5.3 MICROHABITAT ANALYSIS

Three major areas were sampled with respect to spatial differences in the distribution of filamentous Conjugales: a downstream reach of Red Sike, a spring upstream of the reach and a mid-stream stretch in Slapestone Sike. Before examination of specific microhabitats in these three areas, an overall pattern was evident. *Zygnema* (6-8 μm) was the dominant species at the downstream reach in Red Sike and in Slapestone Sike, making up more than 90% of the filaments present in some cases. Two species of *Mougeotia* (2-4 μm & 6-8 μm) and one of *Spirogyra* (4-6 μm) were also present at most sites in these two areas, in varying proportions. Algal communities from the upstream spring area of Red Sike were considerably different. *Zygnema* was entirely absent and *Spirogyra* was absent from all except one site (sample 32). The most obvious reason for this difference in algal assemblages is nutrient concentration. Total phosphorus concentration was consistently higher at the spring than further downstream in Red Sike, and FRP made up a greater proportion of the phosphorus present (Fig 4.2.4). It seems that *Zygnema* is better equipped to deal with nutrient shortage than *Mougeotia* and so dominates the algal communities where phosphorus concentrations are lower. It is possible that *Zygnema* grows better under conditions of nutrient limitation because it is better able to utilise organic phosphorus compounds in nutrition (see section 5.2). Under conditions of higher phosphorus concentration, such as at the spring in Red Sike, *Mougeotia* spp. appears to out-compete *Zygnema* and comes to dominate the algal community.

A third species of *Mougeotia* (width class 4-6 μm) was recorded from one site near to the spring. This species had not been recorded in Red Sike by previous authors (e.g. Livingstone & Whitton, 1984). As it was found only very close to the spring, where phosphorus concentrations were highest, it is possible that this species is only able to survive where there is abundant phosphorus. No assay of alkaline phosphatase activity was performed, however, it is likely that an assay of the activity of this species might indicate that it has a lower potential activity and is therefore restricted to areas in the stream where phosphorus concentrations are high. If the species is limited in its distribution by nutrient availability, a short transect running downstream from the spring could give a reliable estimate of how rapidly phosphorus is removed from the water after its addition at the spring.

Two stagnant pools were examined in July and August, one at the spring area of Red Sike, the other in

Slapestone Sike. Both pools, although widely separated, showed similar assemblages of filamentous Conjugales, considerably different from other sites in either stream. The pool in Slapestone Sike contained only *Mougeotia* 6-8 μm, which also made up the largest proportion (92.1%) of the algae found in the pool in Red Sike. A small amount of *Mougeotia* 2-4 μm was also growing in the small pool in Red Sike. This implies that *Mougeotia* 6-8 μm grows better under conditions of very low flow. This inference is corroborated by the observation that *Mougeotia* 6-8 μm is reduced at sites examined with strong current. Hynes (1970) states that filamentous Conjugales, although occasionally found in streams, are more common in standing water, as they are not adapted to strong currents (e.g. they are not firmly attached to the substratum). This study tends to agree with this, and suggest that *Mougeotia* 6-8 μm is less well suited to running water than either *Zygnema* or *Spirogyra*.

The phosphorus availability in a small pool would be expected to differ from the main stream; as there would be less turnover, a pool would be more likely to be phosphorus-deficient. As the species found in both pools examined was found to be restricted elsewhere to areas of higher nutrient concentrations, its presence in large numbers in the pools implies that phosphorus is not limiting. An assay of phosphatase activity of the algae in the pools would determine if phosphorus is in plentiful supply, or, alternatively, if the strain of *Mougeotia* 6-8 μm in the pools is better adapted to low phosphate levels.

Several sites with low light intensity were examined in Red Sike. These sites tended to have a higher proportion of *Spirogyra* than other sites in the stream. This may reflect a physical adaptation of this species to low light conditions, where other species cannot grow so well. The low light sites tended, however, to be somewhat sheltered and situated towards the edges of the stream, so it is also possible that the increase in *Spirogyra* at these sites was not due to low light intensity, but to the lack of disturbance (*Spirogyra* tended to be the most easily damaged when handling). Perhaps this species can only grow in low current sites, but tends to respond to competition from *Mougeotia* spp. by occupying lower light intensity areas, where *Mougeotia* spp. are at less of an advantage.

## 5.4 CONCLUDING COMMENTS

This study attempted to view the overall ecology of filamentous Conjugales in Red Sike, by looking at the enzymatic and physical responses of the algae to nutrients in the stream water. Although the communities of filamentous Conjugales may appear at first to be uniform in distribution and species composition, there appears to be a real pattern of occurrence, based at least partially on patterns of nutrient availability. Those species (*Mougeotia* spp.) with less ability to hydrolyse organic phosphorus compounds are restricted to the areas in the stream where inorganic phosphorus is abundant, while the species with higher phosphatase activity (*Zygnema sp.*) is able to extend downstream and colonise areas not open to the more nutrient-restricted organisms.

## SUMMARY

(1) There is a considerable difference in phosphate, nitrate and ammonia concentrations between the spring and the downstream reach of Red Sike, which is reflected in differences in the algal communities at these sites.

(2) Phosphorus concentrations in the stream water varied over time, and this variation was reflected in changes in the phosphatase activity of the filamentous Conjugales. Phosphatase activity of the algae showed a significant negative correlation ($p < 0.01$) with phosphate concentrations in the water.

(3) The alkaline phosphatase activity of the algae from the downstream reach of Red Sike was higher than the activity found for the algae growing at and near the spring. This difference is in keeping with the different nutrient concentrations: reactive phosphate levels were higher at the spring and the proportion of the total phosphate which was organic was lower. That the algae under conditions of higher organic phosphate show higher phosphatase activity suggests that the algae can utilise organic phosphate when reactive phosphate is present at low concentrations.

(4) Axenic cultures of *Mougeotia* grown in CHU-10F medium showed lower phosphatase activity than field cultures examined. This could suggest that the high phosphatase activity of the algae collected from Red Sike was due to bacterial enzyme activity and not the algae. It is more likely, however, that the enzyme activity of the laboratory cultures was lower because they were not growing under conditions of phosphorus stress.

(5) Microhabitat analysis showed some differences in the habitat preferences of the species of filamentous Conjugales present. *Zygnema* sp. dominated at the downstream reach, where phosphorus and nitrogen concentrations were lower. At the spring, where nutrient levels were considerably higher, *Zygnema* was absent and *Mougeotia* spp. 2-4 μm and 6-8 μm were co-dominant.

(6) *Spirogyra* sp. tended to be found in greater numbers at sites with low light intensity. It is unclear, however, whether this was due to low light or some other factor which was correlated with this.

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# APPENDICES

Table A Concentrations and absorbances used in the construction of phosphorus (TFP & FRP) standard curve.

| Concentration | TFP | | FRP | |
|---|---|---|---|---|
| | Mean | S.E. | Mean | S.E. |
| 0.0 | 0.000 | - | 0.000 | - |
| 5.0 | 0.022 | 0.019 | 0.039 | 0.011 |
| 25.0 | 0.120 | 0.020 | 0.048 | 0.014 |
| 50.0 | 0.237 | 0.020 | 0.287 | 0.012 |
| 150.0 | 0.758 | 0.032 | 0.851 | 0.035 |

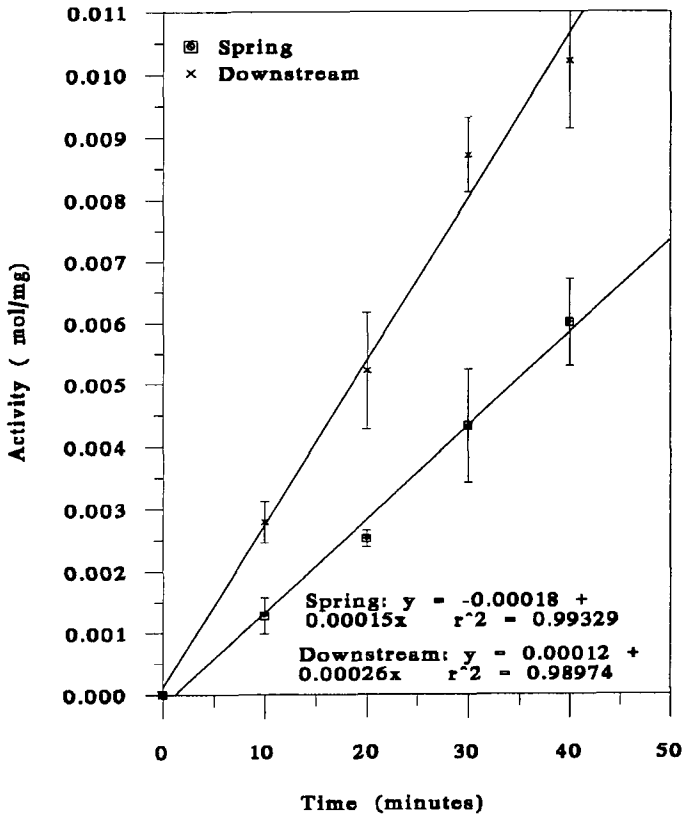


Fig. A Standard curve of absorbance against phosphorus concentration ($\mu g\ l^{-1}$).

Table B *p*-NPP time course data: phosphatase activity (µmol *p*-NPP hyrolysed $mg^{-1}$ dry weight) at 10 min intervals over 40 min, Spring and downstream reach, Red Sike (n = 6).

| Time (min) | Spring | | Downstream Reach | |
|---|---|---|---|---|
| | Mean | S.E. | Mean | S.E. |
| 0 | 0.000000 | - | 0.000000 | - |
| 10 | 0.001283 | 0.0003 | 0.002795 | 0.0003 |
| 20 | 0.002525 | 0.0001 | 0.005218 | 0.0009 |
| 30 | 0.004326 | 0.0009 | 0.008715 | 0.0006 |
| 40 | 0.005996 | 0.0007 | 0.010215 | 0.0010 |

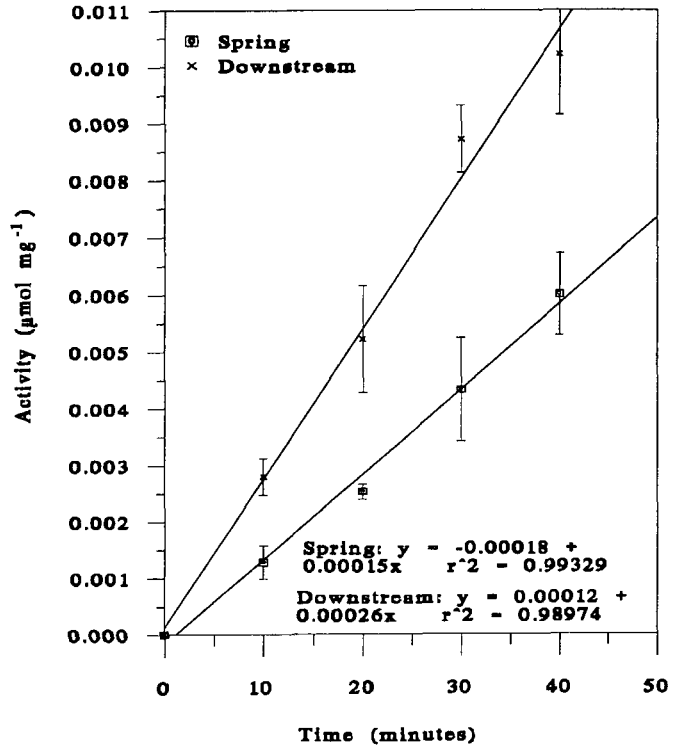


Fig B *p*-NPP time course experiment: demonstrating linearity of activity to 40 minutes.

Table C Concentrations (μM) and absorbances used in the generation of *p*-NP calibration value (0.017).

| Concentration (μM) | Absorbance |
|---|---|
| 4.50 | 0.091 |
| 9.00 | 0.178 |
| 18.00 | 0.363 |
| 27.00 | 0.546 |
| 36.00 | 0.723 |
| 40.95 | 0.811 |

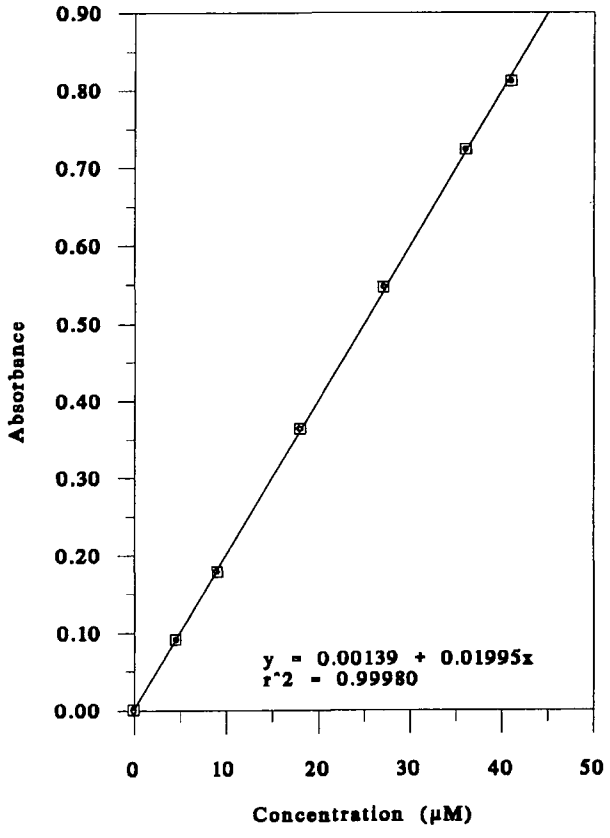


Fig. C Standard Curve of *p*-NP concentration (μM) against absorbance.