PHYTOPLANKTON MUCILAGE — QUANTIFICATION, CHARACTERISATION, AND DETERMINATION OF METAL-BINDING ACTIVITY

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ABSTRACT

Freshwater phytoplankton consists of a variety of algae, some of which produce large amounts of mucilage. Some mucilage may be released into the water column but most remains on the surface of the cell where it is thought to play an important role in algal biology, including regulating the uptake of trace metals. Three aspects of the structure and function of mucilage have been studied in laboratory cultures and environmental samples from a eutrophic freshwater lake (Rostherne Mere, Cheshire, U.K.).

1. Seasonal changes in total cell-associated mucilage and soluble extracellular polysaccharides in Rostherne Mere.

   Five particular lake algae (*Anabaena spiroides, Anabaena flos-aquae, Anabaena circinalis, Microcystis aeruginosa* and *Eudorina elegans*) were found to have large amounts of cell-associated mucilage. Calculation of the total amount of cell-associated mucilage in the phytoplankton samples showed that it occupied 0.0001% to 0.007% (the latter during a bloom of *Microcystis*) of lake water volume within the epilimnion.

   Colorimetric determination of the concentration of soluble extracellular polysaccharides (dissolved in lake water) revealed concentrations of between 2.5 and 60 mg l^{-1}, with peak levels during the bacillariophyceaen bloom and late clear water phase. No direct correlation occurred between soluble and algal-associated mucilage over a single annual cycle.

2. Characterisation of surface sugars on the surfaces of phytoplankton cells and laboratory-cultured algae by lectin labelling.

   Qualitative and quantitative studies using the lectins ConA, PHA-E, PWM and PNA showed marked differences in the pattern of labelling during the growth

cycle of a laboratory-cultured alga, *Anabaena cylindrica*. Clear differences were also observed in the pattern of labelling of different species of laboratory-cultured algae (stationary phase) and different phytoplankton samples. These studies indicated the presence of particular (target) sugars on algal surfaces, with high levels of mannose, glucose, and N-acetyl glucosamine and relatively low levels of galactose and N-acetyl galactosamine.

Determination of the sugar and protein content of extracted mucilage demonstrated great diversity in its composition. Relatively low levels of sugar and protein were detected in culture media and in lake water.

3. Analysis of metal binding capacity. The role of surface mucilage in the binding of copper to algal cells was examined. Clear adsorption (binding to the cell surface) but not absorption (uptake into the cell) occurred over 1-2 hours, after which absorption took place. Copper binding to both laboratory-cultured and phytoplankton cells fitted a Freundlich but not a Langmuir isotherm. These data are consistent with cation adsorption to a heterogeneous but not a homogeneous monolayer. Copper adsorption occurred in algal cells with and without mucilage. Determination of Freundlich adsorption constants showed a high adsorption capacity for cells containing mucilage compared to cells without mucilage. The ability of mixed phytoplankton samples to bind copper varied with species composition. X-ray microanalysis of copper-treated *Anabaena spiroides* showed clear adsorption to cell surface mucilage.

DECLARATION

That no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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CHAPTER 1. GENERAL INTRODUCTION

The purpose of this project was to study various aspects of the surface chemistry and biological properties of a range of laboratory-cultured algae and phytoplankton (algal) cells from the lake environment. A full description of aims and objectives is given in Section 1.4.

1.1 Cell-associated and soluble mucilage from naturally-occurring algae

1.1.1 General occurrence

Mucilage in the freshwater environment was first reported as a green slimy material present in ponds and ditches (Lindley, 1845) and as a turbid, slimy fluid (Paxton, 1849), quoted in Boney (1981). Canter-Lund and Lund (1995) described mucilage as "watery colloidal matter of a diverse chemical composition but generally consisting largely of carbohydrates". The term "mucilage" is often considered synonymous with "mucus" (any viscous, sticky or slimy secreted fluid), "mucinous substances" (viscid secretions of high water-binding capacity), "mucins" (mucoprotein and glycoprotein-like substances which form mucus in solution) and "mucoproteins" or "mucoids" (compounds of polysaccharide with protein) (Leppard, 1995). Decho (1990) used the term "exopolymer" to describe mucilage produced by microbial flora.

In the aquatic environment, mucilage occurs associated with both pelagic and benthic biota. It is produced by almost every major group of aquatic organism including vertebrates and invertebrates (e.g. fish and corals), plants (e.g. kelp) and microorganisms (e.g. phytoplankton and bacteria) (Decho, 1990 ; Decho & Herndl, 1995). However, microorganisms are considered the major source of mucilage in the aquatic environment. This thesis examines the mucilage produced by one particular

group of microorganisms - freshwater (lake) phytoplankton and related laboratory-cultured algae.

Phytoplankton.

The association of mucilage with the algal cell wall was discussed by O'Colla (1962), who noted that mucilages of algae are water-soluble polysaccharides with great diversity in molecular architecture and serve as constituents of the continuous amorphous phase of the cell wall. This amorphous phase can be released into the surrounding water in the form of dissolved polysaccharides without cell damage (Ramus, 1972 ; Leppard et al., 1977 ; Mingazzini & Thake, 1995). It is thus likely that mucilage is present as two forms: (1) an insoluble sheath or capsule forming a thick and solid outer envelope, and frequently enclosing several cells or a thin, slime layer; (2) a soluble form which is excreted and dispersed away from the organism into the surrounding water (Drews, 1973 ; Leppard et al., 1977 ; Drews & Weckesser, 1982 ; Leppard, 1995).

Bacillariophyceae. According to Innamorati (1995), pelagic mucilage is largely secreted by planktonic species, mostly diatoms, while the benthic mucilage is produced largely by multicellular-filamentous algae. Diatoms are thought to be of particular importance as mucilage producers in the marine environment, especially *Nitzschia closterium, Chaetoceros affinis* and *Skeletonema costatum*. Naumann (1925) (quoted by Hutchinson, 1967) found *Cyclotella, Fragilaria* and *Stephanodiscus* have gelatinous capsules.

Cyanobacteria. Cyanobacteria are important producers of mucilage in both the marine environment (Mingazzini & Thake, 1995) and freshwater environment (Grossart & Simon, 1993). Desikachary (1959) pointed out that all cyanobacteria secrete mucilage, but they differ in the amount of mucilage produced and the consistency of the

mucilage. Naumann (1925) noted that all the planktonic cyanobacteria have gelatinous sheaths except some members of filamentous genera *Oscillatoria, Lyngbya,* and *Aphanizomenon.*

Martin and Wyatt (1974) described three types of cell-associated mucilage in cyanobacteria: (1) in the form of a sheath, which is generally uniform, reflects the shape of the algal unit and is visible without staining; (2) as broad, amorphic slimy shrouds which evenly surround the algal cell and appear less dense toward the outer margins; and (3) intermediate slimy sheaths which are cloud-like with sharp outlines. Type (2) and (3) can only be seen following staining with indian ink.

The sheath surrounding cyanobacteria appears to consist of fibrils reticulately arranged within a matrix to give a homogeneous appearance (Desikachary, 1959 ; Morris, 1967 ; Schrader et al., 1982). Roughly parallel microfibrils with a 3-10 nm diameter were found, for example, in the thick mucilaginous envelope around the cylindrical trichome of *Oscillatoria chalybea* using electron microscopy of serial longitudinal sections (Lamont, 1969). Other studies (Jürgens & Weckesser, 1985 ; Adhikary et al., 1986 ; Panoff et al., 1988 ; Weckesser et al., 1988 ; Pritzer et al., 1989), on cyanobacteria *Calothrix, Chlorogloeopsis, Chroococcus, Fischerella, Gloeothece,* and *Synechocystis,* demonstrated fine structure of mucilage is fibers orientated in parallel to the cell surface.

Chlorophyta. Many of the nonflagellate chlorophyta such as desmids (e.g. *Staurastrum* and *Staurodesmus*) secrete sheaths, which can be shown to have a fibrillar structure when stained with cresyl blue (Canter-Lund and Lund, 1995). Some of the flagellated planktonic algae (e.g. *Eudorina* and *Pandorina*) may produce mucilage.

The form of the planktonic mucilage varies considerably. Exopolymer secretion by bacteria and microalgae was investigated by Decho (1990), who indicated that these

exopolymers exist in 'dissolved' (<0.5 μm) and 'particulate' (>0.5 μm) forms and suggested that they influence a wide range of marine processes such as aggregate formation in the water column, benthic larval settlement, micro-scale biogeochemical processes, sediment stability and metal sequestering by hydrothermal vent bacteria. Mucilage which passes through a 0.45 μm filter is normally defined as the dissolved form (Leppard, 1995). Decho and Herndl (1995) indicated that under natural conditions, mucilage often disperses away from the organisms into the surrounding seawater where it can undergo aggregation or dissolution. They considered that this mucilaginous material ranges from colloidal dissolved organic matter (DOM) in water to particulate amorphous material associated with marine snow as massive highly visible mucous macro-aggregates (e.g. clouds, stringers, and flocs). Small mucilage aggregates (e.g. marine snow) are commonly found in marine systems such as in the Adriatic Sea and have been intensively investigated recently (e.g. Mingazzini & Thake, 1995). However, few studies (e.g. Grossart & Simon, 1993) have examined the formation of macroscopic organic aggregates (lake snow) in freshwater systems. Grossart et al. (1997) found the abundance and composition of aggregates (lake snow) had a pronounced seasonal and vertical pattern which closely related to phytoplankton and zooplankton dynamics and wind conditions in Lake Constance (Germany). Leppard et al. (1977), using electron microscopy, found electron-opaque fibrils of approximately 3-10 nm in diameter not only present on the surfaces of lake algae and bacteria but also free in the water column and free on the surfaces of the lake bottom.

In this study, mucilage production by planktonic algae was investigated in a eutrophic freshwater system, Rostherne Mere (U.K.).

1.1.2 Chemical composition

Mucilage consists of a gel formed by cross-linked polysaccharides. The hydrophilic chains of mucilage form a network which can immobilize a large volume of water (Walsby and Reynolds, 1980). Approximately 99% of the volume of the mucilaginous coat is water (Reynolds, 1993).

There is a great diversity in the chemical composition of mucilage between species (O'Colla, 1962). This has been particularly well studied in marine mucilage for commercial reasons (O'Colla, 1962 ; Aspinall & Stephen, 1973 ; Lewis et al., 1990 ; Lee, 1997). The mucilages of marine macroalgae of the division Phaeophyta (brown algae) and Rhodophyta (red algae) contain alginates, carrageenans and agars which possess unique functional properties that can thicken, gel, emulsify and stabilize many food (e.g. ice cream) and industrial products (e.g. adhesives) (Lewis et al., 1990). However, little chemical analysis of mucilage from freshwater algae has been carried out.

Most types of mucilage consist of two major components: polysaccharides which make up the bulk of the biomass of most mucilage, and proteins which usually occurs in lesser amounts and often in the form of glycoproteins (Decho & Herndl, 1995). The carbohydrate components of mucilage have been identified as pectin and acid mucopolysaccharides because of their strong affinity for both ruthenium red and alcian blue stains (Desikachary, 1959 ; Leak, 1967 ; Fuhs, 1973). The detailed chemical analysis of some algal species using thin layer and gas-liquid chromatography, summarized in Table 1.1, emphasizes the variation in chemical composition of mucilage. Mucilage composition varies not only with algal species but also with different culturing conditions (Tease & Walker, 1987).

Table 1.1 Composition of extracellular mucilaginous polysaccharides and proteins from various algal cells

Algae	CPS or EPS	Glu.	Man.	Gal.	Xyl.	Fuc.	Rib.	Ara.	Rha.	UroA.	GalA.	ManA.	GluA.	Carbo-hydrate	Protein	Refer-ence
Rivularia	CPS	+						+		+?						(a)
Phormidium tenue	CPS	+	+?	+?					+			+				(a)
Nostoc	CPS	+		20-30%	25%				10%	30%						(a)
Anabaena flos-aquae	CPS	67%			30%		2%						1%			(a)
Anabena cylindrica (Fogg strain)	CPS	31%	0	6%	25%			6%	6%				25%			(a)
Anabena cylindrica (Wolk strain)	CPS	47%	25%	6%	21%	Trace		0	0				0	66%	5%	(b)
Microcystis aeruginosa K-3A	CPS	8%	5%	8%	20%	6%		5%	24%		28%			67%	13%	(c)
Microcystis aeruginosa K-3A	EPS	80%		Trace	Trace	Trace		Trace	Trace							(c)
Microcystis flos-aquae C3-40	CPS	2%	5%	2%	3%				6%		83%			72-90%	<1%	(d)
Microcystis spp.	CPS									12-15%				35-47%	18-25%	(e)
Synechocystis PCC6803 (young cultures)	CPS	23%	14%	3%	12%	21%			13%						40%	(f)
Synechocystis PCC6803 (old cultures)	CPS	29%	15%	5%	10%	18%			12%						40%	(f)
Synechocystis PCC6714 (young cultures)	CPS	59%	7%	10%	5%	4%		9%	5%						20%	(f)
Synechocystis PCC6714 (old cultures)	CPS	3%	4%	7%	14%	21%			29%						60%	(f)
Chroococcus minutus SAG B.41.79	CPS	8-15%												23-46%	13%	(g)

Gloeothece PCC6501	CPS	11%	8%	14%	2%				4%					44%	3%	(h)
Calothrix parietina D184	CPS	17%	6%	13%	3%	1%		3%	1%		4%			50%	16%	(i)
Chlorogloeopsis PCC6912	CPS	14%	7%	5%	3%			3%					3%	38%	23%	(j)
Fischerella sp. PCC7414 (nmol/mg)	CPS	1062	625	595	725	283								60%	4%	(k)
Gloeothece ATCC27152[(1)](ratio)	CPS	8	6	9	1				3.5		0.5	0.06	0.09	41%	6%	(l)
Gloeothece ATCC27152[(2)](ratio)	CPS	8	6.5	10	1				3		0.4	0.14	0.11	55%	11%	(l)
Cyanospira capsulata (ratio)	CPS	1	1			1		1			2					(m)
Cosmocladium saxonicum	CPS		Trace	+	+	+		+	+	+?			+			(n)
Closterium sp. (ug/mg)	CPS	11	3	10	39	57		16	2				77			(o)
Spondylosium panduriform (mol%)	CPS	2		40	16.5	39		2.5	Trace				Trace		4%	(p)
Spondylosium panduriforme (mol%)	EPS	5		30	8	30		3	Trace				24		6%	(p)

- *Abbreviation:*

CPS: capsule polysaccharides; EPS: extracellular polysaccharides; Glu.: glucose; Man.: mannose; Gal.: galactose; Xyl.: xylose; Fuc.: fucose; Rib.: ribose; Ara.: arabinose; Rha.: rhamnose; UroA.: uronic acid; GalA.: galacturonic acid; ManA.: mannuronic acid; GluA.: glucuronic acid.

- *Reference:*

(a) reviewed by Wolk, 1973 ; (b) Dunn and Wolk, 1970 ; (c) Nakagawa et al., 1987 ; (d) Plude et al., 1991 ; (e) Amemiya and Nakayama, 1984 ; (f) Panoff et al., 1988 ; (g) Adhikary et al., 1986 ; (h) Weckesser et al., 1987 ; (i) Weckesser et al., 1988 ; (j) Schrader et al., 1982 ; (k) Pritzer et al., 1989 ; (l) Tease & Walker, 1987 [(1) and (2) mean cells collected from cultures fixing N_2 and grown with $NaNO_3$, respectively] ; (m) Vincenzini et al., 1990 ; (n) Surek and Sengbusch, 1981 ; (o) Domozych et al., 1993 ; (p) Paulsen and Vieira, 1994.

(a)~(m): Studies of mucilage of cyanobacteria. (m)~(p): Studies of mucilage of chlorophyta.
(a)~(d) & (f): Monosaccharides are shown as percentage of total sugars.
(e)&(g)~(j): Monosaccharides are shown as percentagae of dry mass of extracted material.
Carbohydrate and protein content is shown as percentage of dry mass of extracted material.

- Table compiled from various sources.

1.1.2.a Characterisation of sugars using lectins

Lectins are powerful tools for the study of carbohydrates and their derivatives, both in solution and on cell surfaces, and have been used in the present study to characterise the surface sugars of algal cells.

I. Lectins

The term 'lectin' (from the Latin '*legere*': to select, to choose, or to pick out) was first proposed by Boyd and Shapleigh in 1954 to explain the blood group specificity of plant extracts. A widely accepted definition of lectin was made by Goldstein et al. (1980): "A lectin is a sugar-binding protein or glycoprotein of non-immune origin which agglutinates cells and/or precipitates glycoconjugates". Lectins have a number of characteristics: (1) They should bear at least two sugar-binding sites with which to agglutinate animal and plant cells (most commonly unmodified or enzyme-treated erythrocytes) and/or precipitate polysaccharides, glycoproteins and glycolipids. (2) The specificity of a lectin is defined in terms of the mono-saccharide(s) or simple oligosaccharides that inhibit lectin-induced agglutination (or precipitation or aggregation) reactions. (3) Lectins may be soluble in biological fluids (e.g. concanavalin A) or membrane-bound (e.g. rabbit lectins). (4) Under some conditions, sugar-specific enzymes (e.g. glycosidases and glycosyltrasferases) with multiple combining sites agglutinate cells (and /or precipitate glycoconjugates) and so act as lectins (Goldstein et al., 1980). Barondes (1988) redefined a lectin as a carbohydrate-binding protein other than an enzyme or an antibody. This broadened definition helps to focus attention on the evolution of lectins and on their adaptation for a variety of functions in biological systems, although it may be criticized for being so inclusive (Barondes, 1988). Thus, proteins bearing only one sugar-binding site would not be

recognized as a lectin according to the definition of Goldstein et al. (1980), but would be categorized as lectins by Barondes (1988).

The use of lectins in biological research began in 1888, when Stillmark noticed that *Ricinus communis* seed extracts (which he called ricin) were able to agglutinate erythrocytes. Extracts from the seeds of other plant species were also subsequently shown to exhibit the same agglutinative property, though the chemical basis for this was unknown (Kocourek, 1986). However in 1936, Sumner and Howell observed precipitates on mixing concanavalin A (Con A), the lectin from *Conavalia ensiformis* beans, with glycogen and starch solutions and inhibition of agglutination of erythrocyte by the same molecule in the presence of sucrose. From these observations they suggested that agglutinins of plant origin (phytohemagglutinins) may bind to sugar residues located at the erythrocyte surface to achieve agglutination. Watkins and Morgan (1952) showed that monosaccharides were capable of inhibiting lectin activity and Morgan and Watkins (1959) further demonstrated that the sugar binding specificity of lectins was the direct reason for their blood group specificity. The specificity of a lectin is usually expressed in terms of the simple monosaccharide which best inhibits its effect; however, the binding specificity of lectins is far more complex than this simple inhibition test would suggest. Binding is thought to involve three monosaccharides, terminal and sub-terminal in the oligosaccharide chain, in a particular spatial arrangement; and sometimes part of the protein or lipid structure to which the oligosaccharide is attached. In addition, hydrophobic and electrostatic interactions which are not located at the sugar binding site may also influence the binding of a lectin (Levene et al., 1994 ; Brooks et al., 1997). For instance, Mn^{2+} and Ca^{2+} are essential for saccharide-binding activity of ConA and the binding specificity of ConA is not only dependent on the nature of the terminal sugar present but also on

the nature of the protein moiety (Goldstein, 1972 ; Chowdhury, 1974 ; Goldstein, 1974 ; Reeke, et al., 1974). However, lectins isolated from marine macroalgae did not require divalent cations for their binding activity (Rogers & Hori, 1993). Lectin binding to carbohydrates (glycosyl groups) is reversible without altering the covalent structure of the recognized glycosyl ligands (Kocourek & Hořejši, 1983).

Most lectins were originally derived from higher plants, but they were discovered subsequently to be present in viruses, bacteria, algae, fungi, the blood and fluids of invertebrates, and in vertebrate tissues including man (Driessche, 1988 ; Brooks et al., 1997). Today, over 100 lectins have been purified and partially characterised with respect of their structures and specificities. The seeds of legume plants have proven to be especially excellent lectin sources, as up to 15% of the total protein content of these seeds may consist of lectin (Driessche, 1988).

II. Applications of lectins

General applications

Lectins are able to interact sensitively with glycoproteins and glycolipids owing to their carbohydrate-binding specificity. This interaction makes lectins useful tools in studying glycoconjugate structure and function, and involves the fields of immunobiology, histochemistry, membrane biochemistry and cell biology. (Rüdiger, 1981 ; Roberts & Goldstein, 1983 ; Lis & Sharon, 1986 ; Maki & Mitchell, 1986 ; Doyle, 1994 ; Brooks et al., 1997)

A very fruitful field of lectin application is the characterisation of specific carbohydrate structures on cell or membrane vesicle surfaces and inside cells and tissues. For this the lectins are mostly used in a conjugated form to allow fluorescence- or electron microscopical examination of receptor distribution. This cytochemical marker may be fluorescein isothiocyanate (FITC), colloidal gold or ferritin. By the

detection of lectin receptors (glycoconjugates), lectins become extremely useful tools for mapping cell surface architecture, and to monitor structural changes associated with cell behaviour, development and disease (Maki & Mitchell, 1986 ; Lis & Sharon, 1986 ; Doyle, 1994 ; Brooks et al., 1997).

Lectins have been used to probe glycosylation changes in developing, normal and diseased cells and tissues in medical research. For instance, lectins have been used to distinguish normal from cancer cells and to identify cancer cell behaviour (reviewed by Brooks et al., 1997).

Doyle (1994) reviewed the applications of lectins in microbiology and some specific applications of lectins in diagnostic microbiology and epidemiology. He indicated numerous microbial structures that serve as receptors for lectins, including components in the capsule and cell wall of bacteria and fungi.

Use of lectins in phycology

Various studies have demonstrated glycoconjugates (lectin receptors) associated with a wide range of algae by using lectins conjugated with cytochemical markers (Maki & Mitchell, 1986). In a study of the desmid *Cosmocladium saxonicum*, Surek and Sengbusch (1981) compared the data obtained from the fluorescent probes with those obtained by thin layer chromatography of hydrolysed mucilage. They demonstrated that the application of specific fluorescent probes is an appropriate method for gaining information about the molecular structure of plant cell surfaces and changes during morphogenesis. Sengbusch et al. (1982) indicated that the expression of glycoconjugates might depend upon the developmental stage and the physiological state of the algae. Sengbusch and Müller (1983) showed that the lectin binding sites at the algal surface were species-specific not group-specific in analysing molecular diversity and morphological variation on the cell surface. They suggest that lectin

receptors are involved in intracellular communication between algae from the same and from different species and in the competence of the cell to discriminate between self and non self. Lectins may also be a tool in algal taxonomy. For example, Costas and López Rodas (1994) used fluorescent lectins to distinguish toxic and nontoxic species of marine dinoflagellates which are difficult to classify by morphological criteria and López-Rodas and Costas (1997) identified different *Microcystis* species with the same morphology using the similar approach. Also, Waite et al. (1995) investigated cell-surface sugars on diatoms using FITC-conjugated concanavalin A (FITC-ConA) labelling and suggested that cell-surface sugar accumulation may be related to diatom stickiness, based on a correlation between FITC-ConA measurements and stickiness estimates in the literature in the several species. Hori et al. (1996) suggested that lectins and lectin receptors are present on the cell surfaces of several species of microalgae belonging to the Dinophyceae, Cryptophyceae, Bacillariophyceae and Raphidophyceae and these lectin receptors may function in cell recognition processes, adherence to the cell surfaces of macroalgae, symbioses with marine invertebrates or other algae, or phagocytosis of virus, bacteria, or other algae.

As phytoplankton species in the natural environment are diverse, the use of lectins with mixed phytoplankton samples in this investigation represents a highly specific and sensitive approach to study cell surface chemistry of particular species within the sample.

1.1.3 Function of mucilage

Mucilage has a number of functions that confer benefits to the organism secreting it. Examples of biological functions of mucilage range from the reduction in water friction by fish slime to the maintenance of symbiotic relationships in plants and animals (Decho, 1990).

The precise function of algal mucilage is not clear, but there have been several suggestions as to its biological role.

(1) Protection from grazing and digestion: The increase in cell or colony size brought about by the presence of mucilage may be advantageous in reducing the susceptibility of ingestion by filter-feeding zooplankton (discussed by Reynolds, 1993 ; Horne & Goldman, 1994 ; Canter-Lund and Lund, 1995). This is because the feeding behaviour of filter-feeding zooplankton often depends on the size of particles (Richman et al., 1990). *Keratella quadrata,* for example, can only filter and ingest cells with a size less than 18 μm (Pourriot, 1977). Mucilage may also protect cells from digestion by consumers as it can resist digestive enzymes. It has been observed that diatoms with capsular coats can pass through the gut, still intact and viable in the faecal pellets of polychaetes such as *Streblospio benedicti* (Decho, 1990).

(2) Homeostasis: The mucilage 'bathing' the cells may have special homeostatic properties in providing an immediate environment of a relatively steady state which can provide cells with a favourable microenvironment. The presence of mucilage around the cell may, for example, buffer cells against rapid ionic and environmental changes arising from shift in pH and salinity. However, it is not clear how the efficient exchange of gases, nutrients and wastes is facilitated (Decho, 1990 ; Reynolds, 1993). Chang (1980) found that the CO_2 uptake by *Oscillatoria rubescens* with the sheath removed was higher than that in cells with sheaths suggesting that the mucilage behaves as a barrier to CO_2 diffusion.

(3) Determination of buoyancy: As mentioned in Section 1.1.2, mucilage is a network of hydrophilic polysaccharides which can hold large volumes of water. This characteristic decreases the overall cell density, bringing it closer to the density of the suspending medium water (Reynolds, 1993 ; Lancelot, 1995). According to the

estimate of Reynolds et al. (1981), the density of the *Microcystis* mucilage is about (ρ + 0.7) kgm^{-3} where ρ is the density of water. It cannot be less than that of the water as the density of polysaccharides (~1500 kgm^{-3}: Reynolds, 1993) is higher than that of water. Therefore, the effect of mucilage is to decrease overall density of algal cells towards to the density of water. According to the Stokes equation (1), this will tend to decrease sinking velocity. Thus, the importance of mucilage is to decrease sinking rates rather than providing positive buoyancy.

where $Vs = 2gr^2 (\rho'_c-\rho)/9\eta$ .....(1)

and Vs: sinking velocity of a spherical cell (m s^{-1}).
g: gravitational acceleration (m s^{-2}).
r: radius of a spherical cell (m).
ρ'_c: density of a spherical cell (kg m^{-3}).
ρ: density of the surrounding water (kg m^{-3}).
η: coefficient of viscosity of the surrounding water (kg m^{-1} s^{-1}).

However, decreasing cell density by secreting mucilage also increases its effective radius (r) which has the opposite effects on sinking rates. Under certain circumstances, mucilage production may actually increase the sinking rates (Walsby & Reynolds, 1980). Hutchinson (1967) has defined the critical conditions (equation 2), which was derived from the hypothesis that the sinking velocity (V_{c+m}) of cell with mucilage is less than that (V_c) of cell without mucilage.

$$\frac{\rho'_c - \rho'_m}{\rho'_m - \rho} > a(a+1) \quad \text{..... (2)}$$

where,
ρ'_c : density of a spherical cell.
ρ'_m : density of mucilage.
ρ : density of water.
a : an increasing factor of cell radius.

By the addition of a mucilage sheath, "r" (radius of a cell without mucilage) will increase to "ar" (radius of a cell with mucilage), where "a" will be always ≥ 1, so the difference of density between cell and mucilage must be at least double that between mucilage and water. If this condition is achieved, there will be a range of thickness of

the mucilaginous coat, from zero up to a critical maximum, which will provide a decreased sinking rate. Beyond this maximum the increase in size is no longer compensated for by the decrease in density (Walsby & Reynolds, 1980 ; Reynolds, 1993).

(4) Control of movement: Some benthic algae have the capacity for oriented movement through the localised extrusion of mucilage. For instance, the thin discoid cells of certain species of *Micrasterias* may 'stand on end' by producing mucilage, like a coin placed on its edge, to avoid excessive irradiation (Canter-Lund and Lund, 1995). For most benthic algae, the important movement is gliding which has been well investigated for Cyanobacteria (review by Drews, 1973 ; Wolk, 1973 ; Drews & Wickeder, 1982) and Chlorophyta (especially desmids: Domozych et al., 1993 ; Canter-Lund and Lund, 1995). Although it has been proposed that sheath or mucilage extrusion was the cause of motility of Cyanobacteria (e.g. *Oscillatoria spp.*), recent evidence suggests that the slime production accompanies movement but does not cause it (Drews, 1973 ; Drew & Weckesser, 1982). Canter-Lund and Lund (1995) noted that the desmid *Tetmemorus* can produce broad strings of mucilage to enable the cell to change position. These mucilage trails have been used to test desmid viability by staining with Alcian blue (Brook & Williamson, 1988).

(5) Protection from desiccation: Mucilage is hydrophilic and contains large amounts of water. This may prevent cells from drying out in dry environments (Fay, 1983 ; Decho, 1990).

(6) Attachment: The sticky nature of mucilage may help cells attach to a suitable substratum. Experiments on *Closterium* attached to glass showed that considerable force was necessary to remove them (Canter-Lund and Lund, 1995).

(7) Attachment of associated bacteria, fungi and other algae:

Various authors have noted the presence of bacteria in the mucilage around algae (reviewed by Round, 1981). For example, a ring of bacteria were observed in the mucilage of *Dictyosphaerium sp.*, *Spondylosium sp.*, *Volvox aureus*, *Gomphosphaeria sp.*, and *Chrysostephanosphaera sp.*. Such rings of bacteria are usually situated about halfway between the alga and the margin of the mucilage envelope (Geitler, 1948 and Hamburger, 1958 quoted by Round, 1981 ; Canter-Lund and Lund, 1995). Fisher and Wilcox (1996) found that bacteria within the sheaths of *Spondylosium pulchrum* were more evenly distributed, but those associated with *Hyalotheca dissiliens*, *Desmidium majus* and *D. grevillii* tended to be concentrated in small pockets in the sheath material located near the isthmus and the region between adjacent cells in the filament. In the case of *Volvox,* it seems that the bacteria (strains of *Pseudomonas fluorescens*) are essential to the algae since daughter colonies separated from the centre of the parent colony are not viable unless supplied with bacteria. Moreover, specificity of symbiosis between these bacterial strains and *Volvox* species was also observed (reviewed by Round, 1981). Epiphytic bacteria appear to have adapted to the micro-environment of the mucilage envelope by growing on the extracellular organic products and O_2 released by the algae. Carbon dioxide produced by bacteria could be immediately available to the algae in photosynthesis after assimilation of organic substrates by bacteria (Whitton, 1973 ; Fay, 1983).

There have been a large number of studies showing that cyanobacterial exudates (mucilage) provide an excellent growth substrate for bacterial and protozoan colonizers (reviewed by Paerl, 1992). In the case of the N_2-fixing genera *Anabaena* and *Aphanizomenon,* bacteria are commonly attached to heterocysts (Decho, 1990). Paerl (1984) demonstrated direct, rapid transfer of nitrogen fixed by heterocysts of cyanobacteria into associated bacteria. For non-N_2-fixing species (e.g. *Microcystis*),

both mucilaginous excretions and associated bacteria appear more randomly distributed along the periphery of colonies and filaments (Paerl, 1992). Some bacteria (usually aerobic, gram-negative rods, non-flagellate but motile and pigmented) attached to cyanobacteria are parasitic and has been shown to lyse 'bloom' producing genera, such as *Anabaena, Aphanizomenon* and *Microcystis* (Daft & Steward, 1971). A variety of ciliates and flagellates (protozoans) are also frequently observed in the mucilaginous coat of photosynthetically-active *Microcystis aeruginosa.* These protozoans live in the mucilage and consume bacteria and individual *Microcystis aeruginosa* cells (Paerl, 1992).

Algal mucilage is also important for the attachment of the zoospores of chytrid fungi. These zoospores subsequently produce germ tubes to penetrate the algal cells (Canter & Lund, 1969 ; Canter, 1979 ; Round, 1981 ; Canter-Lund and Lund, 1995).

Surface mucilage may also be a major site for epiphytic algae, co-existing on the surface of host algae. Carter-Lund and Lund (1995), for example, found *Chlamydomonas* cells attached to or occurring within the mucilage surrounding colonies of *Dictyosphaerium* and suggested that *Chlamydomonas* cells may simply be occupying the space as commensal organisms.

(8) Carbohydrate source: As mucilage is composed mainly of polysaccharides, it is a rich carbohydrate source for heterotrophs such as bacteria and fungi (Martin & Wyatt, 1974). Lange (1976) found the gelatinous sheath of cyanobacteria served as food for bacteria in the absence of organic matter. Decho (1990) also noted that a large number of enzymes capable of degrading mucilage have been isolated from bacteria.

(9) Chelating agents: Dissolved mucilage may act as chelators for cations. For example, Fogg & Westlake (1955), Khailov (1964), Swallow et al. (1978) and Kaplan

et al. (1987) found that mucilaginous extracellular products excreted by algae could form complexes with inorganic ions to reduce the toxicity of these ions to algae.

(10) Metal adsorption: Mucilage also plays an important role in metal adsorption. A detailed discussion of this can be found in Section 1.2.3.

1.2 Metal interactions with aquatic microorganisms

1.2.1 Metal chemistry in the aquatic environment

1.2.1.a Heavy metals and trace elements

A metal is an element that will form a cation when in solution, and the oxide of which will form a hydroxide rather than an acid with water (Lapedes, 1974). The term 'heavy metal' is generally held to refer to those metals having a specific gravity greater than 5 g cm^{-3}, and includes about 40 elements in all (Passow et al., 1961). 'Heavy metals' include trace elements which are present at very low concentrations in most parts of the biosphere, and include metals which are both essential and non-essential. Nieboer and Richardson (1980) proposed a classification in which metal and metalloid ions are separated into Class A (oxygen-seeking), Class B (sulphur-seeking) and borderline (intermediate between A and B). Class A ions include the alkali metals and alkaline earths, notably the biologically essential K^+, Na^+, Mg^{2+}, and Ca^{2+}, plus the non-essential Al^{3+}. Class B ions include Cu^+, Hg^{2+}, Ag^+ and Pb^{2+} which are extremely toxic and for the most part nonessential. Borderline ions include Fe^{2+}, Fe^{3+}, Mn^{2+}, and Cu^{2+}, which have biological roles but which may also exhibit toxic effects.

1.2.1.b Sources of metals in the aquatic environment

In the aquatic environment, metals originate from two main sources: natural sources and anthropogenic influences.

I. Natural sources: Rocks and soils directly exposed to surface waters are the most important natural source of metals. The quantity of metals present varies greatly with the rock and soil type and their mineral content (Williams et al., 1974 ; Förstner, 1979). The weathering of rocks by rain or surface waters releases significant amounts of metal bound up in rocks which are then transported by various physical processes (Fergusson, 1990). High background levels of As and Hg in streams are mainly from weathering of rocks and soil (Förstner & Wittmann, 1981).

Fine air-borne particles derived from volcanic eruption, from the evaporation of sea spray or other water droplets (aerosols), or from the erosion and weathering of rocks and soils in the atmosphere may contain some heavy metals. Although the particles are very small, they can introduce relatively large quantities of heavy metals to the aquatic environment by precipitation, rain, and snow (Williams et al., 1974). It has been recorded that volcanic action is a major natural source of Cd (Hutton, 1987). Fergusson (1990) indicated that 90% of the lead and 50% of the cadmium came from the particles in the atmosphere.

II.Anthropogenic sources (human-induced contamination):

Owing to human activities, there is a substantial input of heavy metals into the aquatic environment. Some of the anthropogenic sources of heavy elements to the hydrosphere result from the industrial production such as mining and smelting and use of compounds and materials containing heavy metals, plus leaching from waste dumps, urban run-off, sewage effluent, and agricultural run-off (Fergusson, 1990).

Mining and exposed mine tailings have introduced high levels of heavy metals, such as arsenic and lead, into waterways as a result of subsequent weathering. Mann and Lintern (1983) found that the leaching of tailings in West Australia elevated

concentrations of lead in water close to the source to > 1000 μg l^{-1}, and cadmium to 680 μg l^{-1}. The lead concentration in sediments rose to > 9600 μg g^{-1}.

Using metal-containing products is another source of heavy metal pollution. Lead pollution from petroleum combustion is the most widely recognized problem, although this source of lead is now decreasing with its removal from petrol. Waste dumps leach high concentrations of heavy elements as they contain significant quantities of metal-containing wastes.

Fertilizers are the main source of heavy elements in agricultural run-off. Although the concentrations of metals may vary in specific fertilizers, they have been shown to contain chromium, copper, iron, manganese, nickel, and zinc (Williams et al., 1974).

1.2.1.c Metal speciation in natural aquatic systems

Detailed metal speciation based on the particle size fractions has been described by Stumm and Bilinski (1972) and Williams et al. (1974) and Guy and Chakrabarti (1975). According to these authors, the forms of metal speciation in aquatic systems could be divided to two groups by whether they can be filter through a 0.45 μm pore size membrane filter or not: (1) filterable metal species: (a) dialysable species: free aquated ions, metal ions complexed by inorganic anions such as $CuCO_3$, and metal ions complexed by organic ligands such as amino, fulvic and humic acids. (b) colloids: metals bound to high molecular weight organic material and metals sorbed on colloids. (2) unfilterable metal species: precipitates, mineral particles, organic particles, and metals present in live and dead biota such as algae

The tendency of metals to move from the suspended to the dissolved phase or vice versa depends not only on the properties of the element but also on the size and nature of the particulate material, other competing cations and the pH and E*h*

(oxidation-reduction potential) of the medium (Stokes, 1983). In naturally acidified waters, metal levels (especially Al, Mn and Fe) usually increase, as the low pH induces leaching of metals from the surrounding rock and soil (Dillon et al., 1984). Jackson (1979) found that the relative affinities of Hg, Cu, Zn, Cd, and Fe for different binding agents in selected freshwater sediments vary systematically with the properties of the metal and that during transport from Ross Lake to Schist Lake (Canada), metals of large ionic radius, such as Hg, are more efficiently trapped in the sediment owing to its higher sulphide and organic content. Thus, situations for metal competition between the microbial surface and the sediment and other non-biological surfaces is complicated and unpredictable in natural environments. Ramamoorthy et al. (1977) showed that bacteria could accumulate mercury more effectively than sediment. However, Gibson et al. (1984) demonstrated that the ability of marine bacteria to accumulate the metals was masked by uptake of natural sediments and clays.

Chelating agents such as EDTA (a synthetic chelator) and humic acid (a naturally occurring chelator) play an important role in the stability of complexes. The toxic effect of metals was reduced in the presence of chelating agents (Jackson & Morgan, 1978 ; Huntsman & Sunda, 1980 ; Rai et al., 1981 ; Stokes, 1983). Huntsman and Sunda (1980) reviewed studies in deep or upwelling sea water and indicated that the addition of EDTA markedly reduced cupric ion activity and decreased toxic effect of cupric ion on phytoplankton growth. In natural waters, there exist many forms of dissolved organic matter (e.g. fulvic acid, humic acid and amino acids) which may complex with heavy metals. This complexed heavy metal is unavailable to algae and decreases its toxicity (Rai et al., 1981). Kosakowska et al. (1988) found that amino acids could decrease the toxicity of ions of copper, cadmium and mercury to

phytoplankton. Twiss (1996) emphasized the importance of copper speciation by proline in reducing the toxicity of copper to cyanobacterium *Anacystis nidulans.*

Organisms have the ability to control the speciation and bioavailability of trace metals in the aquatic environment by excreting extracellular organic compounds such as polypeptides and polysaccharides (Giesy, 1981). Fogg and Westlake (1955) first suggested that extracellular polypeptides released by *Anabaena cylindrica* could complex and detoxify copper in the medium. Cyanobacteria can produce extracellular products which are as effective as EDTA in keeping ion and trace metal nutrients from precipitating at pHs above 8. These extracellular products may be important in maintaining the availability of trace metal nutrients (Hunter, 1972 ; Lange, 1974). Production of siderophores (an organic compound) by cyanobacteria is well known. This powerful iron chelator makes cyanobacteria have a competitive advantage over other phytoplankton under low iron conditions (Murphy et al., 1976 ; Mcknight & Morel, 1980 ; Hutchins et al., 1991 ; Horne & Goldman, 1994). Mcknight and Morel (1979) examined 21 algal species and found that cyanobacteria and most of eucaryotic algae produce weak organic acids that are strong copper-complexing agents and suggested these agents may dominate the speciation of soluble copper in freshwater lakes during algal blooms. Kaplan et al. (1987) found that the dissolved polysaccharides produced by *Chlorella* species exhibit metal-complexing capacity. Therefore, by releasing extracellular materials that complex metals, algae could modify metal speciation in the medium and effectively control metal availability or toxicity in their external environment.

1.2.2 Responses of microorganisms to heavy metals

The biological responses of microorganisms such as bacteria and algae to heavy metals represent a balance between two factors: toxicity (the chemical inactivation of

enzymes) and tolerance (detoxification) (Albergoni & Piccinni, 1981). Wood & Wang (1983), Stokes (1983), Gowrinathan & Rao (1990) and Cho et al. (1994) have proposed a range of potential tolerance mechanisms in microorganisms including:

(1) binding of metal ions to cell surfaces,

(2) precipitation of insoluble metal complexes at the cell surface by excretion of extracellular ligands,

(3) development of energy-driven efflux pumps that keep the concentration of the toxic element low in the interior of the cell,

(4) oxidation or reduction of metals enzymatically and intracellularly to convert a more toxic form of an element to a less toxic form,

(5) biosynthesis of intracellular polymers that serve as traps to remove metal ions from solution.

All mechanisms of detoxification of microorganisms to heavy metals can be simply described as exclusion of metals from cells [mechanisms (1)~(3)] and extrusion of metal after uptake [mechanisms (4) and (5)]. Therefore, the uptake of heavy-metal ions by biologically active microorganisms can occur in two principal ways: passive uptake due to surface binding (metabolism-independent biosorption: adsorption) followed by slower active uptake into the cytoplasm (metabolism-dependent intracellular accumulation: absorption) (Khummongkol et al., 1982 ; Gadd, 1988 ; Ting et al., 1989 ; Cho et al., 1994).

1.2.3 Metal adsorption on the surface of algae in the freshwater environment

Adsorption is important in a wide range of physical, chemical and biological processes in the natural environment. It involves the accumulation of charged particles at a surface or interface between any two phases (liquid-liquid, gas-liquid, gas-solid

and liquid-solid ; Weber, 1972). In considering the adsorption of metal ions on microorganisms (liquid-solid phase), the structure of the surface determines the adsorption behaviour. There are many functional groups present on the surfaces of microorganisms that are involved in metal adsorption. These include charged groups such as carboxylate, phosphate, sulphhydryl, sulphate and hydroxyl, amine and imidazole and uncharged groups such as peptide N atoms. Although certain functional groups (amines and imidazoles) are positively charged when protonated and may bind negatively charged metal complexes, generally speaking, cell surfaces are anionic. Therefore, the surface of microorganisms has polyfunctional metal-binding sites for both cationic and anionic metal complexes, and exposure to metals results in the rapid binding of metals to a range of functional binding sites on the cell surface (Hughes and Poole, 1989 ; Greene & Darnall, 1990). Microorganisms have a wide range of charge and geometry as they have different structures of cell surfaces. Therefore, the adsorption phenomenon on different species may be quite different. This study mainly investigated the adsorption of metal ions onto algal surfaces.

In the majority of algal cells, the outermost part of the cell consists of a cell wall and sometimes mucilage (its composition has been described in Section 1.1.2). The main organic constituents of the cell walls are carbohydrates although lipids, proteins and silica may also be present. The cell wall of prokaryote algae (Cyanobacteria) is composed of a number of layers and is basically the same as that of Gram-negative bacteria (Bellinger, 1992 ; Lee, 1997). The major component of the cyanobacterial cell wall is peptidoglycan (synonymous with murein, glycopeptide, mucopeptide), constituting up to 50% of the dry weight of the cell and containing diaminopimelic acid, muramic acid and N-acetyl glucosamine (Frank et al., 1962 and Höcht et al., 1965 are quoted by Lee, 1997 ; Bold et al., 1980). In eucaryotic algae,

there is great diversity in cell wall structure. Cellulose is usually the major component of the cell wall except in the Chrysophyceae and Bacillariophyceae where there is a marked tendency for silica deposition (Bellinger, 1992), and in some groups of Chlorophyta and Rhodophyta where xylans and mannans replace cellulose (Greene & Darnall, 1990 ; Lee, 1997). Both the cell wall and mucilage contain extracellular ligands (ionic and non-charged chemical functional groups, reviewed by Greene & Darnall, 1990) that can complex metals and so prevent their cellular uptake. The major polysaccharides of the cell wall and mucilage are acidic due to the presence of uronic acid units or half ester sulphate groups and are therefore associated with the cations present in waters (Percival and McDowell, 1981).

Since there are wide structural and chemical variations in the algal cell wall and mucilage as mentioned above, the metal-binding properties of algal cells may vary. Metal-binding to the cell wall and mucilage of living and non-living algal cells has been widely investigated (Greene & Darnall, 1990 ; Wehrheim & Wettern, 1994a,b,c ; Gonzalez-Davila et al., 1995 ; Kim et al., 1995 ; Volesky & Holan, 1995 ; Knauer et al., 1997). The study of nickel binding by seven different strains of nickel-tolerant algae showed cyanobacteria and green algae could concentrate nickel primarily at the cell surface to over 3000 times over the concentration in the culture medium (Wood & Wang, 1983). Steemann Nielsen et al. (1969), studying copper toxicity in *Chlorella pyrenoidosa,* found that over a short time interval only a little of the Cu was attached to the plasmalemma and entered into the cytoplasm of the cell, and suggested that the major proportion of the copper must be bound in the cell wall and the "slime" envelope (mucilage). Khummongkol et al. (1982) demonstrated that adsorption of metals to algal cells was rapid and that equilibrium was reached after about 10 minutes. Glooschenko (1969) found that formalin-treated *Chaetoceros costatum* cells adsorbed

more mercury than untreated cells and assumed that formalin treatment increased the positive charge on the cell surface as mercury is found as a negatively charged complex in sea water. However, Burkett (1975) found live *Cladophora* was much more effective than the dead alga in taking up methylmercury, which does not occur in the ionic state. Darnall et al. (1986) reviewed the work of Horikoshi et al. (1979) which found that heat-killed cells have a binding capacity for U^{6+} three times greater than that measured for living cells, and of Ferguson and Bubela (1974) which suggested that there is selective adsorption of Pb^{2+} or Cu^{2+} on the freeze-dried preparations of *Ulothrix*, *Chlamydomonas* and *Chlorella vulgaris*. Greene & Darnall (1990) reviewed the work on metal-ion binding to the cell walls of algae and concluded that the surface of algae is a mosaic of distinct metal-ion binding sites which are created by its carbohydrate, protein and lipid components and that differ in affinity and specificity. The potential metal-binding sites on the surfaces of algal cells have been elucidated by Crist et al. (1981), Greene & Darnall (1990), Crist et al. (1992), Cho et al. (1994), Fourest & Volesky (1996) and Kiefer et al. (1997). Crist et al. (1992) indicated that carboxylate and sulphate groups accounted for most of the metal adsorption on algal cell surfaces. Darnall et al. (1986) carried out metal-binding site experiments on *Chlorella vulgaris* and suggested that an algae-silica matrix can be used as a "biological", mixed-bed ion-exchange resin because the diversity of the binding sites gave a broad applicability not found in conventional ion-exchange resins.

1.2.3.a Determination of metal adsorption processes using adsorption mathematical models--Langmuir model and Freundlich model

Adsorption models have been developed on the basis that the quantity of adsorbate which can be taken up by an adsorbent is a function of its characteristics and adsorbate concentration and the temperature. At a constant temperature, the amount of

material adsorbed is determined as a function of adsorbate concentration and the resulting function is called an adsorption isotherm (Tchobanoglous & Burton, 1991). Two mathematical models, the Langmuir and Freundlich models, are most commonly to describe adsorption isotherm data.

The Langmuir model (Langmuir, 1918) is a single-layer (monolayer) adsorption model for homogenous surfaces. This model makes three assumptions: (1) there are a fixed number of accessible sites on the adsorbent surface and maximum adsorption occurs when adsorbate molecules bind in a single layer to those accessible binding sites; (2) the energy of adsorption is constant; and (3) there is no transmigration of adsorbate molecules on the surface plane. The Langmuir equation is:

$$x/m = \frac{QbC}{(1+bC)} \quad \text{.....(3)}$$

Where, (presuming the adsorbent as algae and adsorbate as metals),

x: the amount of metal adsorbed (mg).

m: the dry weight of cells (g).

x/m: the amount of metal adsorbed per unit dry weight (mg g^{-1}).

Q: the amount of metal adsorbed per unit dry weight in forming a complete monolayer on the surface (maximum adsorption) (mg g^{-1}).

b: a constant related to the energy or net enthalpy, ΔH, of adsorption. It serves as an indicator of the isotherm in the region of lower residual concentrations and reflects the 'strength' or 'affinity' of the absorbent for the metal (Holan, et al., 1993).

C: the metal concentration remaining in solution at equilibrium (mg l^{-1}).

There are two linear forms of the Langmuir equation:

$$\frac{C}{(x/m)} = \frac{1}{bQ} + \frac{C}{Q} \quad \text{.....(4)}$$

or

$$\frac{1}{(x/m)} = \frac{1}{Q} + \left(\frac{1}{bQ}\right)\left(\frac{1}{C}\right) \quad \text{.....(5)}$$

Either of these forms could be used for linearization of data that accord with the Langmuir equation, depending on the range and spread of the data (Weber, 1972). Values of Q and b can be calculated by the linear regression relationship between C and C/(x/m) or 1/C and 1/(x/m) as shown in Fig. 1.1 and Fig. 1.2.

The Freundlich (or van Bemmelen) model (Freundlich, 1926) also relates to monolayer adsorption, but is specifically for a heterogeneous surface. It assumes that the energy of adsorption which is the term 'b' in the Langmuir equation (equation 3-5) is variable and that the heat of adsorption varies with the surface binding sites. The Freundlich equation is defined as:

$$x/m = K_f C^{1/n} \quad \text{.....(6)}$$

where K_f and n are constants and n>1.

The Freundlich equation is empirically derived but is often useful as a means for data description. Data can be fitted to the logarithmic form of the equation:

$$\log (x/m) = \log K_f + (1/n) \log C \quad \text{.....(7)}$$

This equation gives a straight line with a slope of 1/n and an intercept equal to the value of log K_f (Fig. 1.3). The intercept (log K_f) is an indicator of adsorption capacity and the slope, 1/n, of adsorption intensity (Adamson, 1967 ; Weber, 1972).

The Freundlich equation generally agrees well with the Langmuir equation and experimental data over specific ranges of concentration, C (Weber, 1972).

The Langmuir and Freundlich models have been commonly used for comparison of powdered activated carbon or microorganisms as absorbents in water

treatment applications, especially the Freundlich model (Ting et al., 1989 ; Mullen et al., 1992 ; Holan et al., 1993 ; Özer et al., 1994 ; Volesky & Holan, 1995). Mullen et al. (1989) indicated that the Freundlich model may be useful for describing bacterium-metal interactions with metals such as Cd and Cu but it becomes inadequate when precipitation of metals occurs. Wehrheim and Wettern (1994a) also suggested that both the Langmuir and Freundlich isotherm models were suitable for describing the short-term adsorption of cadmium, copper and lead by the cell wall of *Chlorella fusca* and cadmium and copper adsorption by whole *Chlorella* cells. In this study, both Langmuir and Freundlich equations were used to analyse and determine the metal-binding capacity of different algal species.

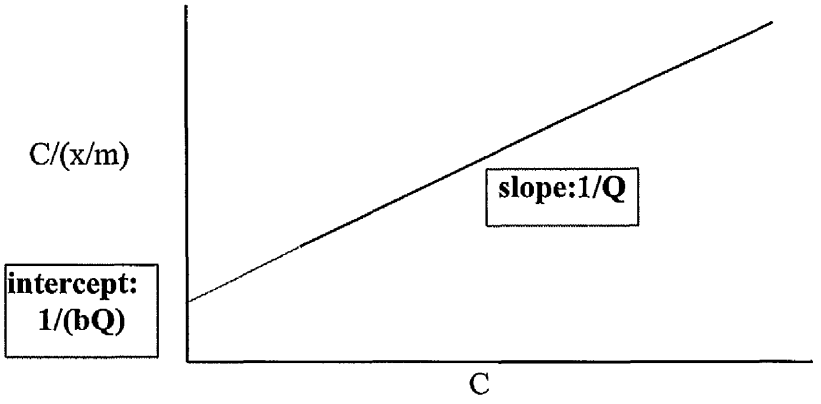


Fig. 1.1 Langmuir plot from equation (4).

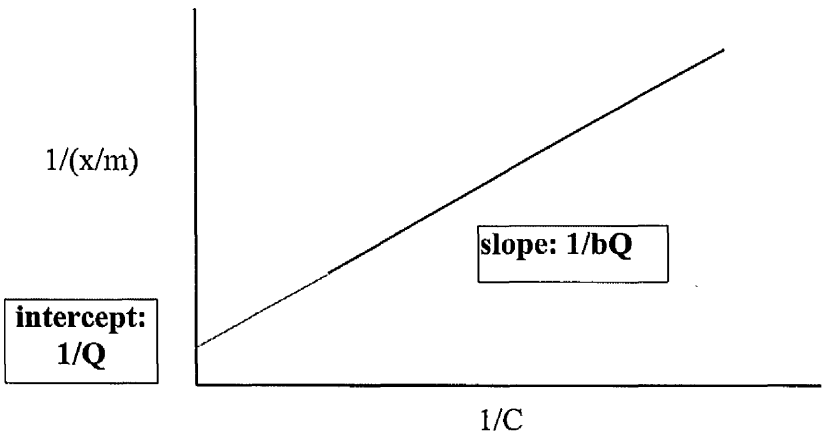


Fig. 1.2 Langmuir plot from equation (5).

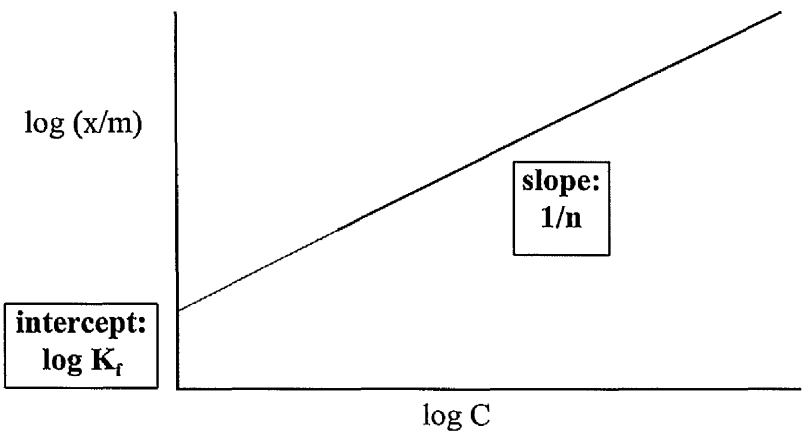


Fig. 1.3 Freundlich plot from equation (7).

1.3 Seasonal periodicity of phytoplankton

Seasonal periodicity of phytoplankton is relevant to this study, since this will influence seasonal changes in lakewater mucilage content, changes in phytoplankton-bound mucilage, and lectin-binding activities of phytoplankton from different sample times.

The species composition of the phytoplankton community changes continuously in time and space, often in a predictable seasonal manner for a particular lake (phytoplankton "succession"). The seasonal succession in abundance and composition of phytoplankton in a number of lakes with varying trophic status was reviewed by Hutchinson (1967) and more recently by Reynolds (1993). Generally speaking, there are three major characteristics of the seasonal cycle in phytoplankton in lakes from temperate regions: the large spring diatom bloom, the smaller irregular summer peaks of various flagellates and cyanobacteria, and the large autumnal bloom of diatoms, cyanobacteria, and dinoflagellates (e.g. Horne & Goldman, 1994). For a particular alga to be a successful component of the phytoplankton, it must maintain a place in the epilimnion and must be capable of assembling the materials necessary for growth in the face of physical, chemical and biotic environmental constraints (Reynolds, 1993). Various environmental factors are known to cause seasonal periodicity of phytoplankton. They are summarized briefly below:-

I. Light, temperature and stratification (physical factors): It has been shown that different phytoplankton have different light and temperature requirements and been suggested that a particular species predominates at a particular season because the prevailing light and temperature conditions favour it (Fogg, 1965 ; Prescott, 1969). Algae require light energy for photosynthesis and thus they can alter their pigment concentration to utilize the light available at the depth where they live. Post et al.

(1984) found that during the transition from low-to-high growth-irradiance levels, the concentration of chlorophyll a in the marine diatom *Thalassiosira weisflogii* is diluted by cell division, but during transition from high-to-low growth-irradiance levels there was a sharp drop in growth rate and a slow increase in the amount of chlorophyll a per cell. Some algal species can produce extra pigments to protect them from cellular damage in an environment with high light levels. For example, benthic cyanobacteria on the bottom of shallow Antarctic lakes where solar radiation is intense have a layer of orange carotene-rich cells above the green photosynthetically active ones (Horne & Goldman, 1994). In *Microcystis aeruginosa* surface blooms, photosynthetic efficiencies increased with carotenoid concentration which has an absorption peak in the near-UV region of the spectrum and may play a photoprotective role (Thompson & Rhee, 1994). Temperature also influences seasonal variation in phytoplankton biomass. *Asterionella formosa* is known as a cold-water organism as it can grow faster in low temperature and low irradiation than the other warm-water species. Thus, it blooms in spring and autumn in temperate lakes (Hutchinson, 1967 ; Horne & Goldman, 1994). Ibelings (1996) investigated the influence of irradiation and temperature on photosynthesis in cyanobacteria and found that an increase in temperature enhanced photodamage to cells in the top layer of the surface water bloom and low photon irradiances in subsurface layers would offer protection against thermal injury. He suggested that if the high temperatures extend to the deepest, dark layers of the bloom, damage in those layers is likely to occur. Light and heat inputs to the water cause temperature changes in the water column and induce lake stratification which is the important physical event for lake biota. The wax and wane of the spring and autumn bloom of diatoms is an obvious biological change directly associated with thermocline formation (e.g. Horne & Goldman, 1994). Ibelings et al. (1994) found that

Scenedesmus showed a more flexible response in acclimating to high and fluctuating photosynthetic photon flux densities and had a higher growth rate than *Microcystis,* so the former may outcompete the latter in the environment. However, under stable conditions (stratification), *Scenedesmus* would be subject to increased sedimentation losses, decreasing the net gain in biomass. As *Microcystis* has the ability to regulate its buoyancy by gas vacuoles, this could explain the success of *Microcystis* in a stable lake.

II. Nutrients (Chemical factors): Pearsall (1923) was the first worker to examine the relationship between phytoplankton composition and water chemistry. He found that (1) diatoms tend to increase when the water is rich in phosphate, nitrate, and silica; (2) desmids favour water with a low calcium content and a low nitrate/phosphate ratio, and along with other chlorophyta, occur during the summer depletion of nutrients; (3) the abundance of cyanobacteria is correlated with high concentrations of dissolved organic matter in the water. These algae are able to increase at very low concentrations of inorganic nutrients. The nutrients required for the growth of phytoplankton include macronutrients (e.g. C, O, H, N, P, S, K, Mg, Ca, Na and Cl) and micronutrients (e.g. Fe, Mn, Cu, Zn, B, Si, Mo, V and Co). However, Si (silicon) is a major component of the cell wall of chrysophyta and diatoms and hence a macronutrient (Reynolds, 1993). Kilham (1990) suggestes that elemental ratios can be useful tools for characterising phytoplankton communities in lakes and are relevant to the seasonal succession of phytoplankton. For example, a decline in the Si:P ratio resulted in a change in dominance from *Synedra* to *Stephanodiscus* (Tilman et al., 1982). Rhee (1978) found there were differences in the optimum N:P ratio for chlorophyta and cyanobacteria and thus these influenced their mutual competition along a N:P gradient. Schindler (1977) demonstrated that low N:P ratios (~5) promoted the dominance of nitrogen-fixing

cyanobacteria. Likewise, Reynolds (1978a) found *Anabaena* and *Aphanizomenon* (nitrogen-fixing species) became dominant when nitrate concentration in water decreased in the Shropshire meres. Stoermer (1990) reviewed the works carried out on the Great Lakes and concluded that increased phosphorus loading first resulted in secondary silicon limitation which caused a shift from dominance by diatoms and chrysophytes to dominance by species that did not require silicon, and that nitrogen-fixing cyanobacteria became dominant when nitrogen became limiting. *Microcystis* and *Ceratium* have the ability to tolerate low nutrient concentrations in the late summer epilimnion of stratified lakes because their high motility allows them to exploit hypolimnetic nutrient reserves unavailable to their competitors (Reynolds, 1993). Both abilities could explain the dominance of *Microcystis* and *Ceratium* during the summer that is characteristic of many temperate lakes. Carrillo et al. (1995) found the seasonal succession of phytoplankton is characterised by a shift in dominance from eukaryotic species to prokaryotic cyanobacteria in oligotrophic Lake La Caldera (Spain) when environmental conditions result in low or intermediate concentration of released (zooplankton) phosphorus and irrespective of the grazing pressure. Non-cyanobacterial growth was conversely stimulated by the absence of predators or high levels of released phosphorus. Sakshaug and Olsen (1986) found that when the nutrient supply is continuous the desmid *Staurastrum luetkemuellerii* outcompetes the cyanobacterium *Microcystis aeruginosa* in a chemostat, but when nutrient supply is pulsed the opposite happens. They suggested that interspecific competition in nutrient-limited communities depends on the nutrient requirement of individual species and the mode of nutrient uptake. Similarly, Reinertsen et al. (1986) found *Anabaena flos-aquae* was able to maintain considerably higher uptake rates of P when exposed to

pulses of nutrient (e.g. P released by fish) than did *Staurastrum luetkemuelleri*, so the former can either coexist with or outcompete the latter in heterogenous systems.

III. Selective grazing and parasitism (biological interaction): Selective grazing of algae by animals is known to influence the composition of phytoplankton (Fogg, 1965 ; Canter and Lund, 1969 ; Bailey-Watts, 1978 ; Round, 1981 ; Reynold, 1993). James et al. (1995) found that there was a significant correlation between total ciliate numbers [mostly small ciliates (<20μm) capable of consuming particles <2μm] and picophytoplankton in Lake Taupo (New Zealand) due to the size-selective grazing behaviour of zooplankton. Conversely, the species composition of phytoplankton may effect zooplankton composition in a lake. Carney and Elser (1990) examined the interactions of zooplankton and phytoplankton in lakes with different trophic status. They found in the oligotrophic Lake Tahoe (U.S.A.), "macrofiltrator" zooplankton are dominant as they can feed on edible phytoplankton, but eutrophic Lake Michigan (U.S.A.) contained a high proportion of small fine-mesh cladocerans because large, inedible phytoplankton increase and bacteria become the major part of the zooplankton diet.

Fungal parasites are highly host-specific and infection of a particular algal species may favour successional development of other competing algae. Therefore, such fungi may play a significant role in the control of planktonic algal succession (Van Donk and Bruning, 1992 & 1995). Canter and Lund (1951) and Van Donk and Ringelberg (1983) showed a *Asterionella formosa* population highly inflected by the chytrid *Rhizophydium planktonicum* was replaced by the other diatoms such as *Fragilaria crotonensis.*

1.3.1 Rostherne Mere

Rostherne Mere (National Grid Reference SJ 745843, with geographical coordinates: 53°20'N; 2°23'W) is one of the largest, deepest and least disturbed lowland lakes in the Shropshire-Cheshire Plain. Since 1961, it has been a National Nature Reserve managed by English Nature as an undisturbed wildfowl refuge. It is also a site of special scientific interest (SSSI). The mere was formed by subsidence caused by dissolution of salt-bearing rocks and has an area of 48.7 ha., a maximum depth of 30 metres and a volume of 6.6×10^6 m^3 (Tattersal & Coward, 1914 ; Reynolds, 1978b ; Woof & Wall, 1984 ; English Nature, 1995). It is surrounded by pasture, woods and arable land, whilst the eastern margin is covered by reed beds (Pearsall, 1923).

Rostherne Mere is a eutrophic lake, the major inflow being Rostherne Brook and the largest outflow Blackburn's Brook (Fig. 1.4). Rostherne Brook has been identified as a source of pollution of the Mere as it carried nutrient-rich water from a local sewage treatment works directly into the mere. Since the Water Company installed a pipeline to divert sewage effluent around the mere in 1992, this source of pollution has ceased (English Nature, 1995). Fertilisers or herbicides from the surrounding farmland and the faecal matter of birds that roost on the mere are also thought to be sources of enrichment of the waters (Brinkhurst and Walsh, 1967 ; Livingston, 1979).

The phytoplankton of Rostherne Mere has been studied for nearly a century. Pearsall (1923) analysed samples collected by Tattersall and Coward (1914) and showed a seasonal succession in the composition of phytoplankton. Bacillariophyceae were the dominant element in winter and spring, followed by *Ceratium* in summer and *Aphanizomenon* in autumn. By comparing the seasonal succession of the composition

of phytoplankton with the physical and chemical features of the Mere described by Tattersall and Coward (1914), Pearsall (1923) first suggested that the chemical composition of the material dissolved in a lake water determines the nature of the phytoplankton community, including its quantity and its seasonal distribution. A similar succession of phytoplankton was observed by Griffiths (1925) and Lind (1945). However this seasonal succession has changed since Belcher and Storey (1968) noted that *Microcystis aeruginosa* has become increasingly dominant during the summer and autumn. According to Livingston's investigation (1979), this increase in *Microcystis aeruginosa* occurred around 1958. Nelms (1984) also confirmed that a change in the phytoplankton community occurred around this time. This change is thought to be attributable to increased eutrophication (Reynolds, 1979).

In recent years phytoplankton periodicity in Rostherne Mere has involved dominance by the diatoms *Melosira spp., Stephanodiscus spp.* and *Asterionella formosa* in winter and spring, by the filamentous cyanobacteria *Anabaena spp.* and *Aphanizomenon flos-aquae* in the early summer, followed by the colonial cyanobacteria *Microcystis aeruginosa* in the mid-late summer (Reynolds, 1978b ; Booth, 1988 ; Clay, 1992 ; Carvalho, 1993). Apart from the phytoplankton, invertebrates, vertebrates and zooplankton have also been investigated by Hynes (1964), Coward (1914) and Carvalho (1993), respectively.

The chemical composition of water has been analysed by Tattersall and Coward (1914), Lind (1945), Gorham (1957), Grimshaw and Hudson (1970) and Carvalho (1993). Carvalho (1993), comparing his nitrate-nitrogen data with Grimshaw and Hudson's (1970), found a similar pattern and suggested that there has been little change in nitrate loading since 1965-1967.

Because of good access and sampling facilities, good background information and high phytoplankton productivity (including a significant number of mucilage-producing algae), Rostherne Mere was chosen as the monitoring site to investigate temporal and spatial changes in mucilage production in this study, it was also used as a source of naturally occurring phytoplankton for the investigations of surface sugars and metal adsorption.

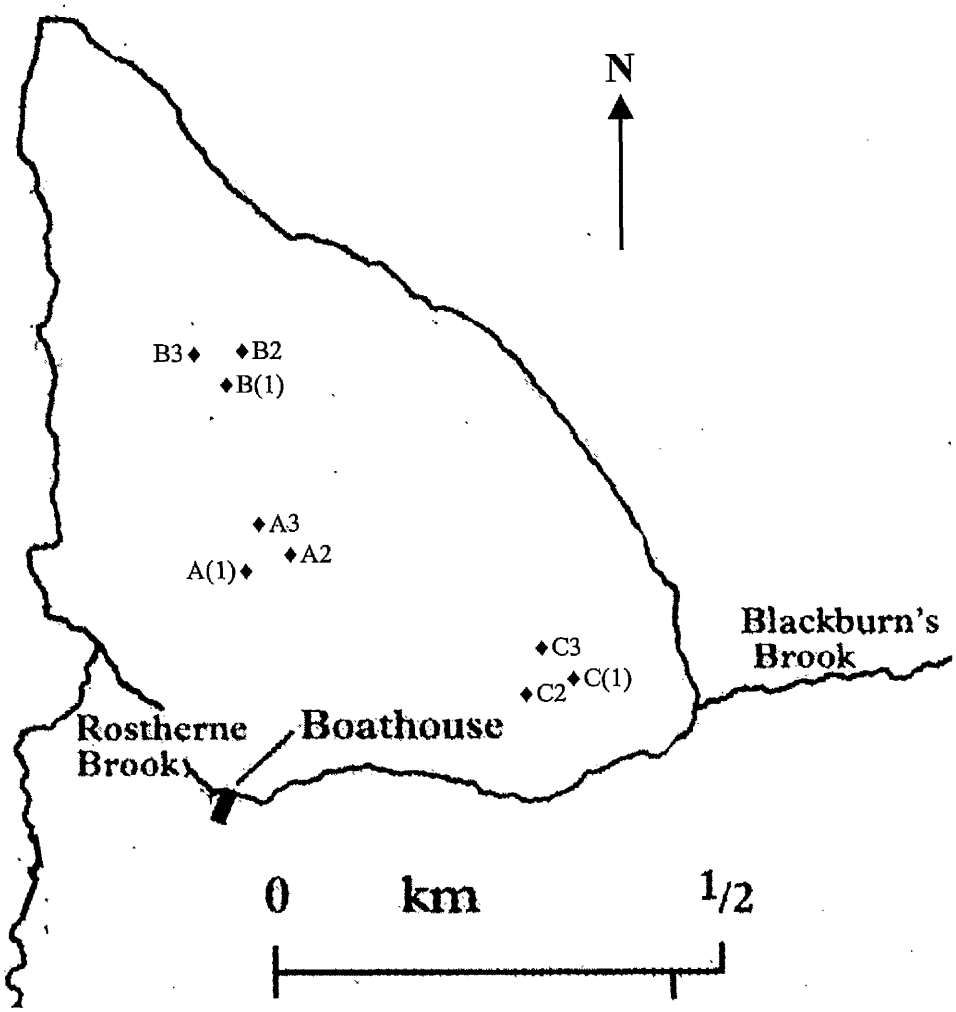


Fig. 1.4 A map of Rostherne Mere Nature Reserve showing the position of the sampling sites.

1.4 Aims and objectives

The general aim of this project was to study various aspects of the occurrence and biological role of surface mucilage in lake phytoplankton and laboratory-cultured algae. A subsidiary aim was to examine the degree to which cell-associated mucilage is released into the environment.

Three major inter-related aspects were investigated:

1. How does the occurrence of algal surface-associated mucilage vary throughout the season, and what influence does this have on the quantity of dissolved mucilage in lakewater? (Chapter 3)
2. How does the presence of mucilage affect the surface chemistry (sugar composition) of algal cells and are there major changes in surface sugars during the seasonal changes in phytoplankton? (Chapter 4)
3. Does the presence of surface mucilage influence the ability of cultured algae and phytoplankton cells to bind cations? (Chapter 5)

CHAPTER 2. MATERIALS AND METHODS

2.1 Laboratory cultures

Eight species of algae (Table 2.1 ; Plate 1, 2) having different amounts of associated mucilage were selected for the laboratory studies. These comprised three species of cyanobacteria (with different amounts of mucilage), one species of dinophyceae (no mucilage, see Plate 1), two species of chlorophyta (one with mucilage and the other without mucilage) and two species of bacillariophyceae (no mucilage, see Plate 2).

2.1.1 Culture of cyanobacteria and eukaryotic algae

Various cyanobacteria and eukaryotic algae were routinely grown in 100 ml sterile growth medium in sterile 250 ml Ehrlenmeyer flasks. Cultures were typically incubated at 23 °C under fluorescent light (22 μmol photon m^{-2} s^{-1}, 15 hours light / 9 hours dark cycle) on a shaker at 90 rpm. *Microcystis aeruginosa* was cultured without shaking and *Ceratium hirundinella* was grown under ambient daylight. Subculturing was conducted every 3-4 weeks to prevent total exhaustion of nutrients in the growth medium.

2.1.2 Determination of changes in algal biomass

Three methods were used to estimate algal biomass:

2.1.2.a Measurement of optical density:

Ten ml aliquots of culture were pipetted into a glass test tube. Readings were taken by inserting the test-tube into a Corning Eel portable colorimeter with a red filter (Chance Tricolour Filter "OR1" : maximum transparency in the wavelength range

Table 2.1 Sources and growth media of laboratory-cultured algae

Species	Source	Growth medium
Cyanobacteria (blue-green algae)		
Anabaena cylindrica Lemmermann	Prof. N.G. Carr, Warwick, axenic	BG-11
Anabaena spiroides Klebahn	NIES-76, axenic	CT
Microcystis aeruginosa Kutzing emend. Elenkin	Sciento LA 905, non-axenic	BG-11
Chlorophyta (green algae)		
Eudorina elegans Ehrenberg	Sciento LA 85, non-axenic	BBM
Chlorella vulgaris Beijerinck	Sciento LA 155, non-axenic	BBM
Bacillariophyceae (diatoms)		
Asterionella formosa Hassall	CCAP 1005/8, non-axenic	DM
Melosira varians Agardh	Sciento LA 750, non-axenic	BM
Dinophyceae		
Ceratium hirundinella fo. *furcoides* Schroeder	CCAP 1110/4, non-axenic	MWC

NIES : National Institute for Environmental Studies, 16-2, Onogawa, Tsukuba, IBARAKI 305, Japan.
CCAP : Culture Collection of Algae and Protozoa, Institute of Freshwater Ecology, the Windermere Laboratory, Far Sawrey, Ambleside, Cumbria LA22 0LP.
Sciento : Sciento Educational Services, 61 Bury Old Rd, Whitefield, Manchester M45 6TB.
The composition of the growth media is shown in Appendix 1.

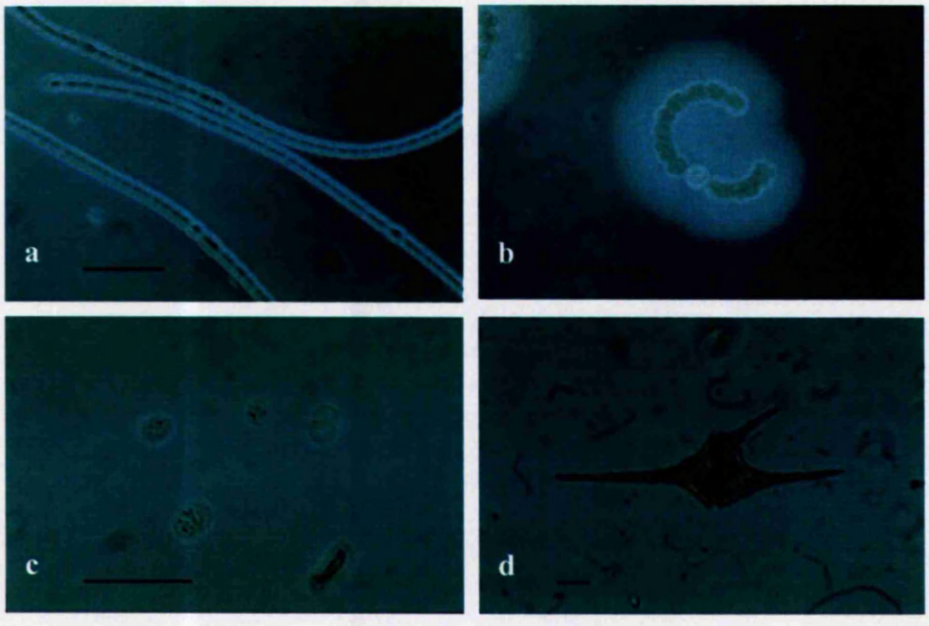


Plate 1. Morphology of laboratory-cultured Cyanobacteria: (a) *Anabaena cylindrica,* (b) *Anabaena spiroides,* (c) *Microcystis aeruginosa,* and Dinophyceae: (d) *Ceratium hirundinella.*

Anabaena cylindrica and *Anabaena spiroides* have filamentous colonies with different amounts of mucilage. *Microcystis aeruginosa* is a single cell species with a thin layer of mucilage. *Ceratium hirundinella* is a single cell species without mucilage.

(Scale bar = 20 μm)

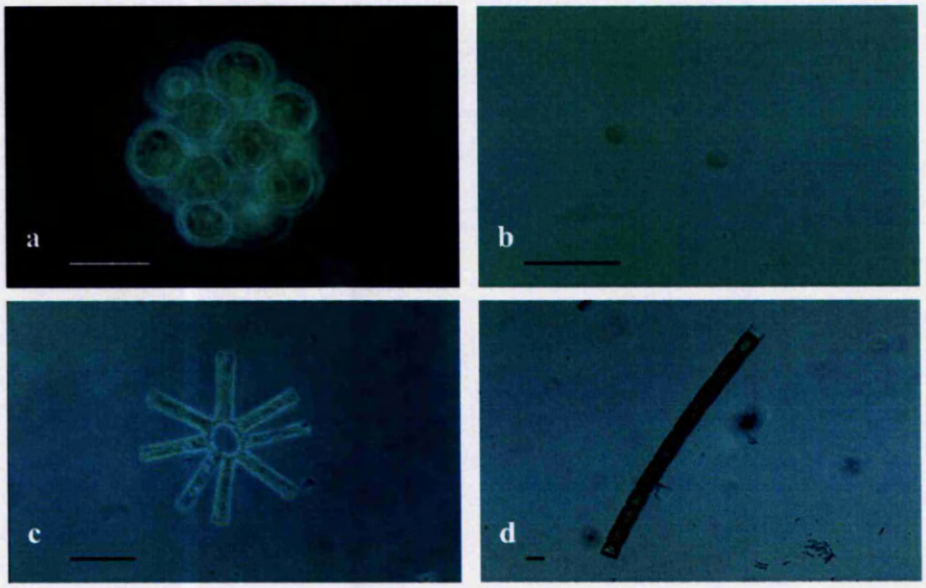


Plate 2. Morphology of laboratory-cultured Chlorophyta: (a) *Eudorina elegans*, (b) *Chlorella vulgaris*, and Bacillariophyceae: (c) *Asterionella formosa*, (d) *Melosira varians*.

Eudorina elegans is a spherical colonial species with mucilage. *Chlorella vulgaris* is a single cell species without mucilage. *Asterionella formosa* and *Melosira varians* are colonial algae without mucilage.

(Scale bar = 20 μm)

>660nm). The colorimeter was set to zero using distilled water before taking the measurement.

2.1.2.b Estimation of dry weight

Whatman GF/C filters (47mm in diameter) were dried at 70 °C for 2 days and weighed prior to use. Known volumes (about 10 ml) of sample were filtered through the preweighed filter, then dried at 70 °C for 2 days to a constant weight and weighed. Prior to weighing, care was taken to ensure that the filters did not absorb moisture from the environment by keeping them in a desiccator.

2.1.2.c Cell/colony counts and measurement of the length of filaments

I. Direct counts

A Sedgwick-Rafter counting chamber (consisting of a grid of 1000, 1 mm^2 squares in a well 1 mm deep) with a total volume of 1 ml was used for counting cells/colonies with a diameter greater than 5 μm. A Haemocytometer with an improved Neubauer scale (0.1 mm deep, 2 chambers, each one consisting of 25 squares 0.2 mm on a side, with a total volume of 0.0001 ml per chamber) was used to count cells with a diameter smaller than 5 μm. With pure cultures, counts were obtained from 10 squares containing at least 100 individuals. This is in accordance with the protocol of Lund et al. (1958) who recommended that at least 100 individuals should be counted to achieve ± 20% accuracy. With environmental samples, counts were normally made from 10-20 squares to give total counts of at least 100 individuals for major species. Lake sample means were derived form at least 3 sites. Counts were undertaken with a Leitz Dialux 20 phase-contrast light microscope.

II. Determination of the total number of cells for colonial and filamentous algae

The number of cells per colony or filament was counted using a Leitz Dialux 20 light microscope with the magnification of ×400 or ×1000. The number of colonies required to obtain an accurate estimate of mean cell number was determined by frequency plots [Fig 2.1(a) & (b)]. These showed that at least 20 colonies of *Eudorina elegans* and at least 50 filaments of *Anabaena cylindrica* were required for an adequate level of accuracy (SE<10% mean). The number of colonies or filaments per ml was counted as described above. Therefore, total number of cells was calculated as follows:

$$Tc = Ac \times Nc \quad \text{.....(8)}$$

where, Tc: total number of cells per ml

Ac: average number of cells per colony

Nc: number of colonies per ml

III. Determination of filament length for multicellular filamentous algae

As an alternative to cell counts, biomass was also determined as total filament length using a measure-mouse image analysis system (Analytical Measuring System, Pamisford, Cambridge) with a Leitz Dialux 20 light microscope. One ml of sample was pipetted into a Sedgwick Rafter slide, observed under the light microscope and the image of filaments displayed on a computer screen. A line was drawn along each filament using a mouse and the length of the line (filament) was automatically recorded. The minimum measuring frequency was also determined by a frequency plot. An example of the frequency plot is shown in Fig. 2.1(c) and reveals that measurement of at least 40 filaments gives an adequate level of accuracy (SE<10% mean) in estimating average length per filament of *Anabaena cylindrica.* Total length of the filaments was calculated as follows:

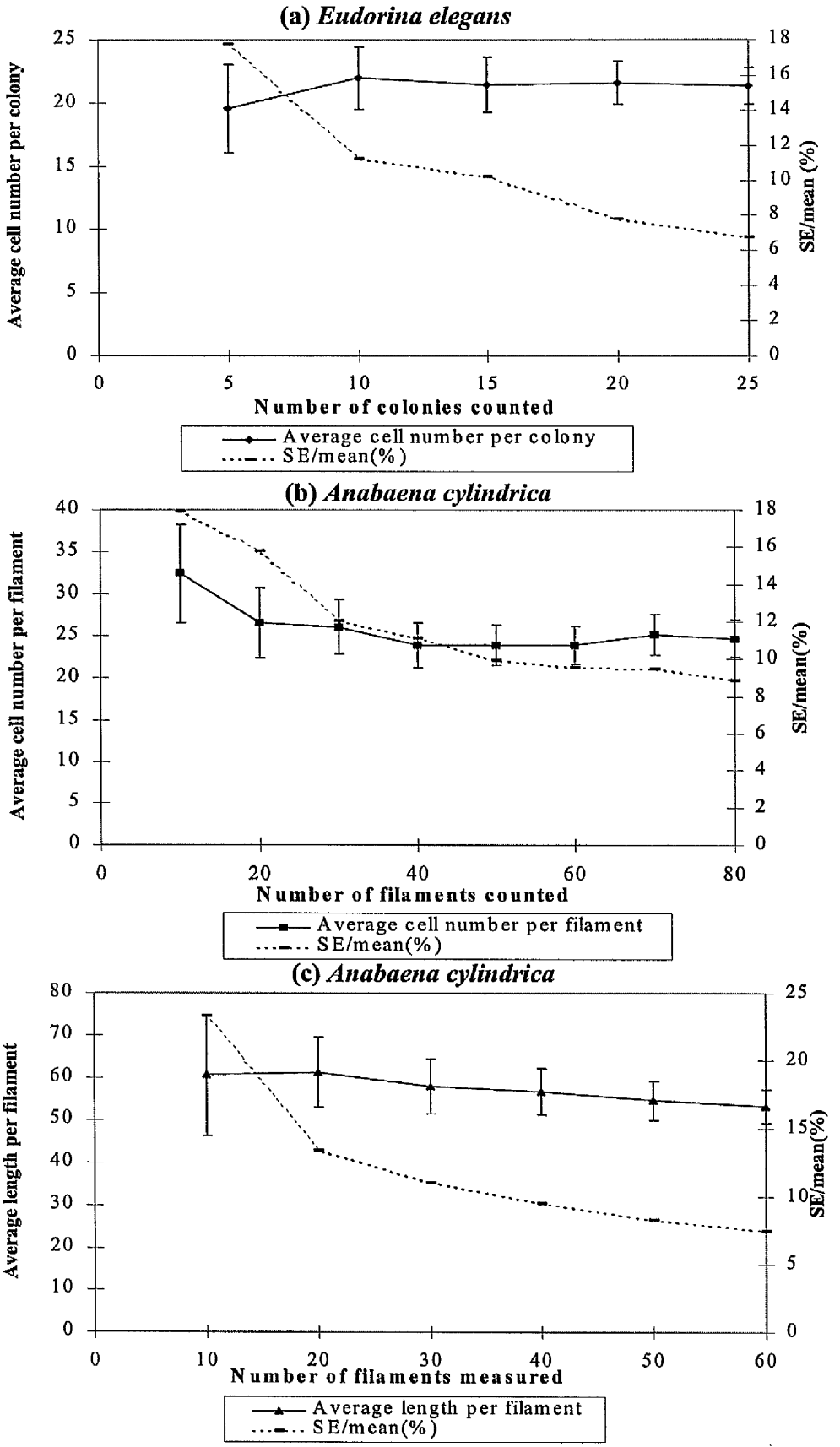


Fig 2.1 Frequency plots: (a) and (b) represent mean cell number per colony of *Eudorina elegans* and per filament of *Anabaena cylindrica* with increasing counting effort. (c) represents mean filament length per filament of *Anabaena cylnidrica* with increasing measuring effort. (Error bars represent ±standard errors).

$Tl = Al \times Nf$ .....(9)

where, Tl: total length of the filaments (μm) per ml

Al: average length per filament (μm)

Nf: number of filament per ml

2.2 Field studies

Field studies were carried out at Rostherne Mere (see Section 1.3.1). Samples were taken mainly at three sites (A, B, C) on the lake by boat (shown in Fig. 1.4) for cell counts and determination of the concentration of mucilage. A number of physico-chemical parameters were measured immediately in the lake water sample taken on boat.

2.2.1 Measurement of physico-chemical parameters in Rostherne Mere

Physico-chemical parameters in Rostherne Mere were measured at site A, B and C from February 1996 to November 1997. The readings were taken on approximately a monthly basis during 1996 and 1997. Measurements were taken as follows:

2.2.1.a Temperature and dissolved oxygen

A pHOX Model 62 meter (pHOX Systems Ltd.) with a Model 620 probe was used to measure temperature and dissolved oxygen. Readings were taken in an integrated lakewater sample (taken by a weighted 5-meter long polythene hose) from all 3 sites in the lake, and a depth profile (1 m intervals) was also recorded at site B. The meter user manual quotes a precision of ± 0.5 °C for temperature and ± 3% saturation for dissolved oxygen.

2.2.1.b Secchi depth

Secchi depth was measured at each sampling site in the lake using a black-and-white Secchi disc. At each site, two separate readings were taken, one when the Secchi disc disappeared from view upon being lowered through the water column and the other when the disc appeared again after pulling up. The two values were then averaged to calculate a mean.

2.2.1.c pH

Readings were taken in the integrated lakewater samples from sites A,B,C using a pHOX Model 42 pH meter (pHOX system Ltd.). The meter was calibrated at pH 7 and pH 10 before taking the readings. The user manual quotes a precision of ± 0.1 pH unit.

2.2.1.d Conductivity:

Conductivity was determined in the integrated samples using a pHOX Series 52 conductivity meter (pHOX system Ltd.).

2.2.2 Collection of phytoplankton samples at Rostherne Mere

Phytoplankton samples were collected from the three sites between October 1995 and November 1997. Two types of sample were taken every 2-4 weeks.

(A). Concentrated phytoplankton (trawl-net) samples: These were collected by trawling a phytoplankton net (mesh size: 53 μm) for 3 minutes across the lake surface. Two sets of samples were obtained:

(1) A 250 ml aliquot was preserved with 2.5% (final concentration) glutaraldehyde solution (TAAB Laboratories) for phytoplankton identification and estimation of mucilage volume (Section 2.3). Comparison with fresh samples showed that fixation in 2.5% glutaraldehyde solution did not result in any change in cell or mucilage volume.

(2) Mucilage extraction: Cell-associated mucilage was extracted from a concentrated phytoplankton suspension using a procedure modified from Nakagawa et al. (1987). A 500 ml aliquot was filtered through a 47mm Whatman GF/C filter using a vacuum pressure of approximately 15 cm Hg. Cells were resuspended with 20 ml of distilled water using a whirlimixer (Fisons Scientific Ltd. Serial No. NEL 4991) for 3 min and again filtered through a 47 mm Whatman GF/C filter. This cycle was repeated 2 times to shed mucilage from the cell. Mucilage-containing filtrate was collected and freeze-dried in a Modulyo freeze-dryer (Edwards High Vacuum, B.O.C Ltd., Crawley, England). The dried mucilage was stored in a universal tube at -80 °C for subsequent carbohydrate and protein analysis (Section 2.5).

(B) Integrated lake water samples: Integrated lake water samples were used for phytoplankton counts and soluble extracellular polysaccharide analysis.

(1) Phytoplankton counts: 250 ml of the integrated sample was fixed on site with 5-6 ml of Lugol's iodine (150 g KI, 50 g I_2, 20 ml glacial acetic acid and 1000 ml distilled water ; Bellinger, 1992) for subsequent cell counts. Lugol's iodine samples were taken from site A, B, C in Rostherne Mere on each occasion. In addition, on 29 August 1996, samples were taken from 9 sites (A1, A2, A3, B1, B2, B3, C1, C2, C3) to investigate horizontal variation in mucilage volume within the lake. As shown in Fig. 1.4, there was a 10 m distance between sites A1, A2, A3, between sites B1, B2, B3 and between sites C1, C2, C3. Prior to counting, samples were placed in measuring cylinders and stored in a dark cupboard at 20 °C for at least 24 hours to allow the phytoplankton to settle. 225 ml supernatant was siphoned off carefully and discarded. After shaking the 25 ml concentrated sample, 1 ml was pipetted into a Sedgwick-Rafter counting chamber. The number of cells, colonies and filaments was counted as described in Section 2.1.2.c.I. The number of individuals per ml (N) was determined:

$$N = \frac{A \times 1000 \times 25}{250} \quad \text{.....(10)}$$

Where, A: average number of individuals per square in Sedgwick Rafter counting chamber.

The abundance of multicellular filamentous cyanobacteria (e.g. *Anabaena spp.* and *Aphanizomenon flos-aquae*) was measured as total filament length using the method described in Section 2.1.2.c.III. All observations and counts were undertaken with a Leitz Dialux 20 phase-contrast light microscope.

(2) Soluble extracellular polysaccharide analysis: 180 ml of the integrated sample was used for soluble extracellular polysaccharide determination (Section 2.2.3) and carbohydrate and protein analysis (Section 2.5). Samples were collected in acid-washed plastic bottles and filtered through a 47 mm diameter 0.45 μm pore size Whatman cellulose nitrate filter membrane. Nine universal tubes, each containing 20 ml of filtrate, were obtained from the above sample allowing triplicate samples for soluble extracellular polysaccharide determination and six tubes for carbohydrate and protein analysis. Samples were freeze-dried in a Modulyo freeze-dryer (Edwards High Vacuum B.O.C Ltd., Crawley, England), then stored at -80 °C until analysis.

2.2.3 Quantification of soluble extracellular polysaccharides in lake water

Freeze-dried samples from filtered integrated lakewater samples [Section 2.2.2.(B).(2)] were reconstituted with 2.5 ml of ultra high purity (UHP) water, then sonicated for 15 min using a KS 100 sonicator (Kerry's ultrasonics Ltd.). One ml of the reconstituted sample was placed into a universal tube after complete mixing, and incubated in a 37 °C water bath for 10 min. A 0.1 ml aliquot of 92 mM sodium

periodate (Sigma Chemicals, Poole, UK) in 0.5 M H_2SO_4 (0.396 g of sodium periodate in 20 ml of 0.5 M H_2SO_4) was added. The sample was incubated for a further 30 min in the 37 °C water bath with shaking every 10 min to dissolve the solid-phase extracellular polysaccharides. A 0.2 ml aliquot of 2.7% sodium arsenite (Sigma Chemicals) (0.27 g in 6.8 ml 1N HCl + 3.2 ml UHP water) was added to remove excess sodium periodate. After shaking, the samples were incubated in a 37 °C water bath until the yellow colour (due to the formation of iodine) disappeared. Half a ml (0.5 ml) of Schiffs reagent (BDH) was added to the sample and left for 30 min in a 37 °C water bath (with shaking every 10 min). The sample was cooled in an ice bath (to approximately 4 °C) then centrifuged at 4 °C for 5 min at 3000×g. Absorbance was measured at the optimum wavelength (λ) of 565nm within 1 hour using an ultraviolet spectrophotometer (Pye Unicam SP8-100). Standards were made from purified porcine stomach mucin (Type III, Sigma Chemicals) at concentrations between 0 mg l^{-1} to 1000 mg l^{-1}. Standards were also included with each series of measurements to confirm consistency. (Jugdaohsingh et al, 1998)

2.3 Determination of the surface area and cell-associated mucilage volume of laboratory-cultured algae and environmental phytoplankton cells

Fresh or glutaraldehyde-fixed cells from laboratory cultures or the concentrated environmental samples [Section 2.2.2.(A)] were placed into a nanoplankton chamber made from coverslips and having an area of 22 mm × 10 mm and a depth 0.4 mm. This chamber prevented mucilage being compressed (which would cause errors in estimating the amount of mucilage) and allowed higher magnification for a more accurate assessment. Indian ink was added and mixed with the sample to visualize cell-associated mucilage. Measurements were done by taking photographs of cells or

colonies and calibrated graticules (50×2 μm and 100×10 μm, Graticules Ltd, Kent, England) using a phase-contrast Leitz Dialux 20 light microscope equipped with an automatic camera. Plates 3 and 4 show how measurements were taken from photographs of the different algal species.

The volume of mucilage associated with single cells and whole colonies was determined by subtracting the volume of the individual cell/colony from the total volume (cell/colony + mucilage). The total volume of a cell or a colony including mucilage was calculated from the mean dimensions of a cell or a colony, assuming that its shape approximates to a geometrical solid such as a sphere ($4/3\pi r^3$), a cone ($1/3\pi r^2 h$), or a cylinder ($\pi r^2 h$) (Bellinger, 1974 ; Reynolds, 1993). Cell/colony volume without mucilage was calculated by the same method. For each sample, the average mucilage volume for each cell type or colony was obtained from at least 30 cells or colonies. This was then multiplied by the number of cells, filaments, or colonies per unit volume of sample to give an estimate of the total mucilage volume per unit volume of sample for that particular species.

The surface area of a cell or a colony was also calculated from a geometrical approximation using the equation for a sphere ($4\pi r^2$) or a cylinder [$2\pi r$ (r + h)] (Bellinger, 1974 ; Reynolds, 1993). Total surface area per unit volume of sample was obtained by multiplying the average surface area per unit cell or colony (obtained from at least 30 cells or colonies) by the total number of cells or colonies per unit volume of sample.

The geometrical shapes used in the estimation of surface area and mucilage volume of the eight laboratory-cultured algae are shown in Table 2.2.

For the environmental samples, there were five main mucilage-producing species observed in Rostherne Mere from October 1995 to November 1997. They are

Anabaena spiroides, Anabaena flos-aquae, Anabaena circinalis, Microcystis aeruginosa, and *Eudorina elegans*. Table 2.3 shows the geometrical shapes used in calculating the mucilage volume of these five main mucilage-producing species. Further details on individual species are given in the result section (Section 3.2.3). Variations in total mucilage volume between sites A, B, C areas on 29 August 1996 were investigated by sampling in 9 sites within the lake as described in Section 2.2.2.(B).(1) and analysed by the nonparametric Mann-Whitney U-Wilcoxon Rank Sum W Test (two independent samples, with the significance level of $p<0.05$).

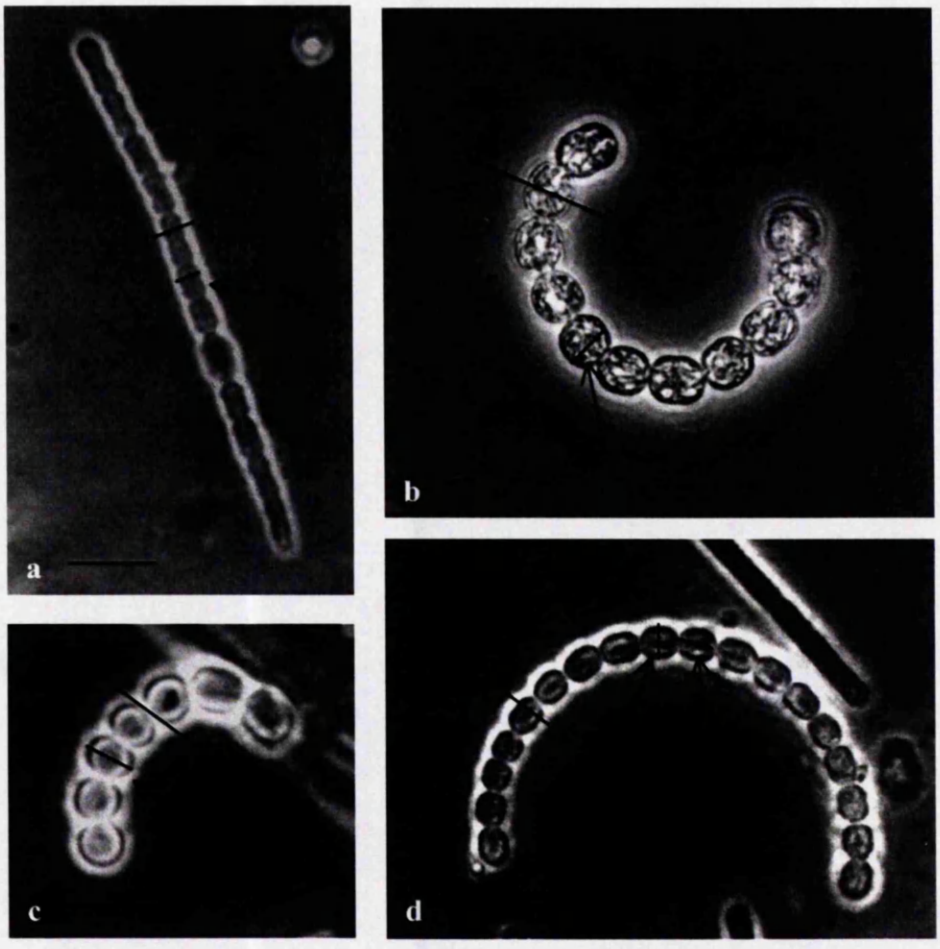


Plate 3. Illustration of how measurements were made of mucilage volume and/or cell surface area and volume from photographs of (a) *Anabaena cylindrica,* (b) *Anabaena spiroides,* (c) *Anabaena circinalis* and (d) *Anabaena flos-aquae.*

R: radius of the colony, including mucilage.

Values for r exclude mucilage:

where, r: radius of a spherical cell.

$2r_1$: width of a cylindrical cell.

$2r_2$: length of a cylindrical cell.

(Scale bar = 10 μm)

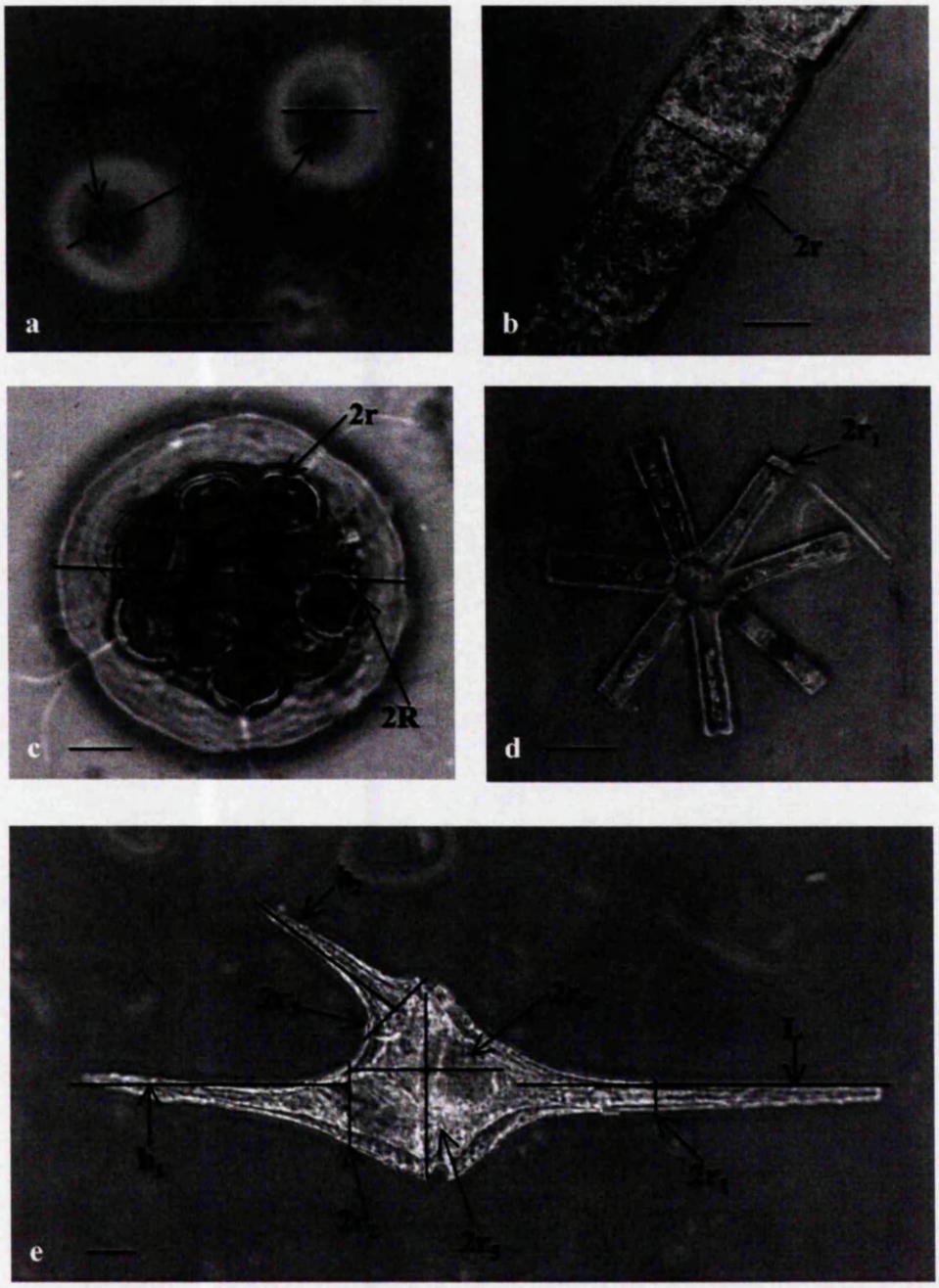


Plate 4. Illustration of how measurements were made of mucilage volume and/or cell surface area and volume from photographs of (a) *Microcystis aeruginosa,* (b) *Melosira varians,* (c) *Eudorina elegans,* (d) *Asterionella formosa* and (e) *Ceratium hirundinella.*

R: radius of a colony (*Eudorina elegans)* or a cell (*Microcystis aeruginosa*) including mucilage.

All other parameters exclude mucilage:

Where, r: radius of a single spherical or a cylindrical cell.

L: length of a cylindrical cell.

$r_{1\text{-}5}$: radius of a cylinder, cone or ellipsoid.

$h_{1\text{-}2}$: height of a cone.

(Scale bar = 10 μm)

Table 2.2 Geometrical shapes used to estimate surface area and mucilage volume of laboratory-cultured algae.

Species	Colony	Cell
Anabaena cylindrica*	cylinder	cylinder
Anabaena spiroides*	cylinder	sphere
Microcystis aeruginosa* (non-colonial form)		sphere
Eudorina elegans*	sphere	sphere
Chlorella vulgaris		sphere
Asterionella formosa		cylinder
Melosira varians	cylinder	
Ceratium hirundinella		apical horn: a cylinder lateral horn: a cone body: an ellipsoid

'*' denotes species with cell-associated mucilage.

Table 2.3 Geometrical shapes used to estimate the colony and cell volume and mucilage volume of five environmental main mucilage-producing species from Rostherne Mere.

Species	Colony	Cell
Anabaena spiroides	cylinder	sphere
Anabaena flos-aquae	cylinder	oblate spheroid(ellipsoid)
Anabaena circinalis	cylinder	sphere
Microcystis aeruginosa	sphere	sphere
Eudorina elegans	sphere	sphere

2.4 Characterisation of surface sugars by lectin binding

Characterisation of surface sugars in laboratory and environmental samples was carried out by lectin assay. Four lectins were used, each with different sugar binding specificities (Table 2.4). Binding of FITC-labelled lectins to algal cells was progressively investigated using three types of microscopy: qualitative fluorescence microscopy, confocal microscopy and quantitative fluorescence microscopy.

2.4.1 Qualitative fluorescence microscopy

One drop of lectin-labelled and non-lectin-labelled cell suspension was mounted on a glass slide with a coverslip, sealed with rubber solution and observed directly under a Leitz Dialux 20 microscope equipped with an automatic camera, phase contrast optics, and an epifluorescence attachment (with a FITC filter set--Excitation in the blue region, 450-500 nm wavelength, of the spectrum). Microphotographs were taken using Kodak EL400 colour films.

2.4.1.a Lectin binding activities of laboratory-cultured *Anabaena cylindrica*

I. Autofluorescence

Preliminary observation indicated that autofluorescence occurs in unfixed (fresh) samples. Various treatments were therefore employed to reduce this.

Anabaena cylindrica stock cultures (stationary growth phase) were treated as follows:-

(A) Control : no treatment.

(B) Glutaraldehyde fixation: Cells that had been pelleted by centrifuging at 3000×g, 4 °C for 15 minutes were suspended in 2.5% glutaraldehyde solution [1 ml

Table 2.4 Specificity of lectins (Sigma Chemicals) used in the study.

Lectin	Abbreviation	Specificity*
Concanavalin A	ConA	mannose, glucose, GlcNAc
Phytohaemagglutinin-erythroagglutinin	PHA-E	galactose, GlcNAc, mannose, complex carbohydrate
Pokeweed mitogen	PWM	GlcNAc
Peanut agglutinin	PNA	galactose, GalNAc

GlcNAc: N-acetyl glucosamine.
GlaNAc: N-acetyl galactosamine.

'*': Goldstein & Poretz, 1986 and Brooks et al., 1997.

25% glutaraldehyde solution (TAAB Laboratories) + 9 ml 0.01M sodium cacodylate buffer solution] for 2 hours at room temperature, then stored in a 4 °C refrigerator before analysis.

(C) Acetic ethanol fixation: Cells were concentrated by centrifuging at 3000×g, 4 °C for 15 minutes and fixed in acetic ethanol (3:1 ratio of ethanol and glacial acetic acid) for 1 hour at room temperature. Cells were then centrifuged (3000×g 4 °C for 15 minutes), resuspended in pure ethanol and stored at 4 °C before analysis. This cell suspension was centrifuged at 3000×g, 4 °C for 15 minutes, then suspended in 50 % ethanol for 5 min prior to analysis.

Each treatment was washed with 0.01M sodium phosphate buffer (pH 7.4) containing 0.15M NaCl (PBS, Sigma Chemicals) and examined for autofluorescence.

II. FITC-ConA lectin labeling (comparison of cell treatments)

Cells of *Anabaena cylindrica* were tested without any treatment (control) and with glutaraldehyde and acetic ethanol preservatives as described above, then washed with 0.01M sodium phosphate buffer (pH 7.4) containing 0.15M NaCl (PBS). Cells were resuspended in FITC-ConA solution (1 mg FITC-ConA, see Table 2.4, in 1 ml 0.01M PBS) and incubated for 1 hour at room temperature in the dark. Unbound lectin was subsequently removed by two washing cycles in 0.01M PBS to decrease the interference of background fluorescence.

III. Sugar-binding activity on the cell surface with different FITC-conjugated lectins (comparison of lectins)

Four FITC-conjugated lectins were used (listed in Table 2.4). Each FITC-lectin was dissolved in PBS to give a concentration of 1 mg ml^{-1}. *Anabaena cylindrica* fixed with acetic ethanol was treated with each fluorescent lectin for 1 hour at room temperature in the dark and then washed twice in PBS.

2.4.1.b Assessment of lectin binding activities on environmental phytoplankton from Rostherne Mere

I. Autofluorescence

Living concentrated phytoplankton [trawl net samples, see Section 2.2.2.(A)] from Rostherne Mere were fixed separately with buffered 2.5% glutaraldehyde solution and acetic ethanol (3:1 ethanol and glacial acetic acid, then pure ethanol and 50% ethanol) as described above.

The degree of autofluorescence was assessed on phytoplankton cells which were fixed with two different preservatives after washing with PBS as described above.

II. FITC-conjugated lectin labeling

FITC-Conjugated lectins (listed in Table 2.4) were dissolved in PBS (see above). Cells from Rostherne Mere fixed with acetic ethanol were treated with each lectin for 1 hour at room temperature in darkness then washed two times in PBS.

2.4.2 Determination of lectin binding using confocal microscopy

Laboratory-cultured *Anabaena cylindrica,* and environmental *Anabaena spiroides* and *Anabaena flos-aquae* from Rostherne Mere were fixed in acetic ethanol (see Section 2.4.1.a.I.), washed in PBS, treated with FITC-ConA for 1 hour at room temperature in the dark, and then washed twice in PBS. FITC-ConA binding activity on cells was immediately observed using a Bio-Rad MRC-600 Laser Scanning Confocal Microscope filtered with an Argon 25mW laser and equipped with Filter A1 (wavelength detection: 488 nm) and Filter A2 (wavelength detection: 512 nm) for double label visualisation. These filters allowed collection of different fluorescence images on each optical section. The confocal microscope only recorded focussed fluorescence on a specific deep plane of the specimen and eliminated out of focus images. Therefore it reduced blurring of the image and increased effective resolution.

A series of optical images (Z series) were taken at successive higher or lower planes along a Z axis (depth of specimen), each separated by a distance (Z step) of 1 μm.

2.4.3 Analysis of lectin binding to different growth phases of Anabaena cylindrica using quantitative fluorescence microscopy

Quantification of lectin binding was carried out using a Leica Dmrxa fluorescence microscope, equipped with phase contrast optics and fitted with an epifluorescence attachment with an FITC filter set--excitation in the blue (450-500nm wavelength) part of the spectrum. The microscope was also equipped with an automatic camera (photometrics slow scan CCD on Mac platform) connected to IP Lab Spectrum software which can record images and analyse the intensity of fluorescence in the recorded images.

In order to reduce fading of fluorescence during microscopy, an antifade reagent was prepared by mixing 0.1 ml of reagent 1 (1 mg ml^{-1} of p-phenylenediamine, Sigma Chemicals) and 0.9 ml of reagent 2 [9:1 of glycerol (BDH) and PBS buffer solution, pH = 8-9). Reagent 1 and Reagent 2 were stored in the dark in a -20 °C freezer (Johnson & Araujo, 1981).

Five ml of *Anabaena cylindrica* stock culture was inoculated into 250 ml Ehrlenmeyer flasks containing 100 ml sterile BG-11 medium and incubated at 23 °C with shaking at 90 rpm and at a light intensity of 22 μmol photon m^{-2} s^{-1} (a 15 hour light / 9 hour dark cycle). Triplicate flasks were used. At day 2, 6, 9, 13, 16, 19, 23 and 26, 10 ml was taken from well mixed flasks to measure optical density as described in Section 2.1.2.a to determine the changes in biomass. All measurements made from three cultures. At day 2, 9, 16 and 26, another 10 ml was taken and fixed in acetic ethanol as described in Section 2.4.1.a.I for lectin binding analysis. Samples were stored at -4 °C before analysis.

These acetic ethanol fixed samples were treated with the four FITC-lectins (listed in Table 2.4, at a concentration of 1 mg ml^{-1} of PBS) for 1 hour at room temperature in the dark, then washed two times in PBS. Cells were resuspended in the antifade reagent, and observed under a Leica Dmrxa fluorescence microscope. Three images were captured with an exposure time of 1 second to obtain comparable results. The short exposure time was to reduce the loss (bleaching) of fluorescence. The intensity of fluorescence was measured from a specific area on both the cell and mucilage of *Anabaena cylindrica* using IP Lab Spectrum software as shown in Plate 5.a. At least 5 areas were measured on the cell and on the mucilage.

Differences in binding activity between cells in different growth phases and between different lectins and samples were analysed using the non-parametric Mann-Whitney U-Wilcoxon Rank Sum W Test (two independent samples) and the Kruskal-Wallis One-Way ANOVA (more than two independent samples, taking $P<0.05$ as the significance level).

2.4.4 Analysis of lectin binding to laboratory-cultured and environmental algae using quantitative fluorescence microscopy

Eight stationary phase cultures of laboratory-cultured algae (Table 2.1 and Plate 1,2) and trawl-net phytoplankton samples collected during the spring and summer 1996 from Rostherne Mere [see Section 2.2.2.(A)] were fixed in acetic ethanol fixation as described in Section 2.4.1.a.I, then washed with PBS. FITC-conjugated lectins (Table 2.4) were added and samples were incubated for 1 hour at room temperature in the dark. Unbound lectins were removed by two washing cycles in PBS, and cells were suspended in the antifade reagent (described in Section 2.4.3). Cells were observed under a Leica Dmrxa fluorescence microscope as described in Section 2.4.3. Three images were captured with an exposure time of 1 second from each species to obtain

comparable results. The intensity of fluorescence was measured from a specific area on both the cell and mucilage using IP Lab Spectrum software. At least 5 measurements of fluorescence intensity were taken from the cell and mucilage on each image. Plate 5 shows two examples of how fluorescence intensity was taken from the marked area on the cell and mucilage of different species.

The non-parametric Mann-Witney U-Wilcoxon Rank Sum W Test and Kruskal-Wallis One-Way ANOVA ($P<0.05$ as the significance level) were used to analyse differences in binding activity of different lectins and algae.

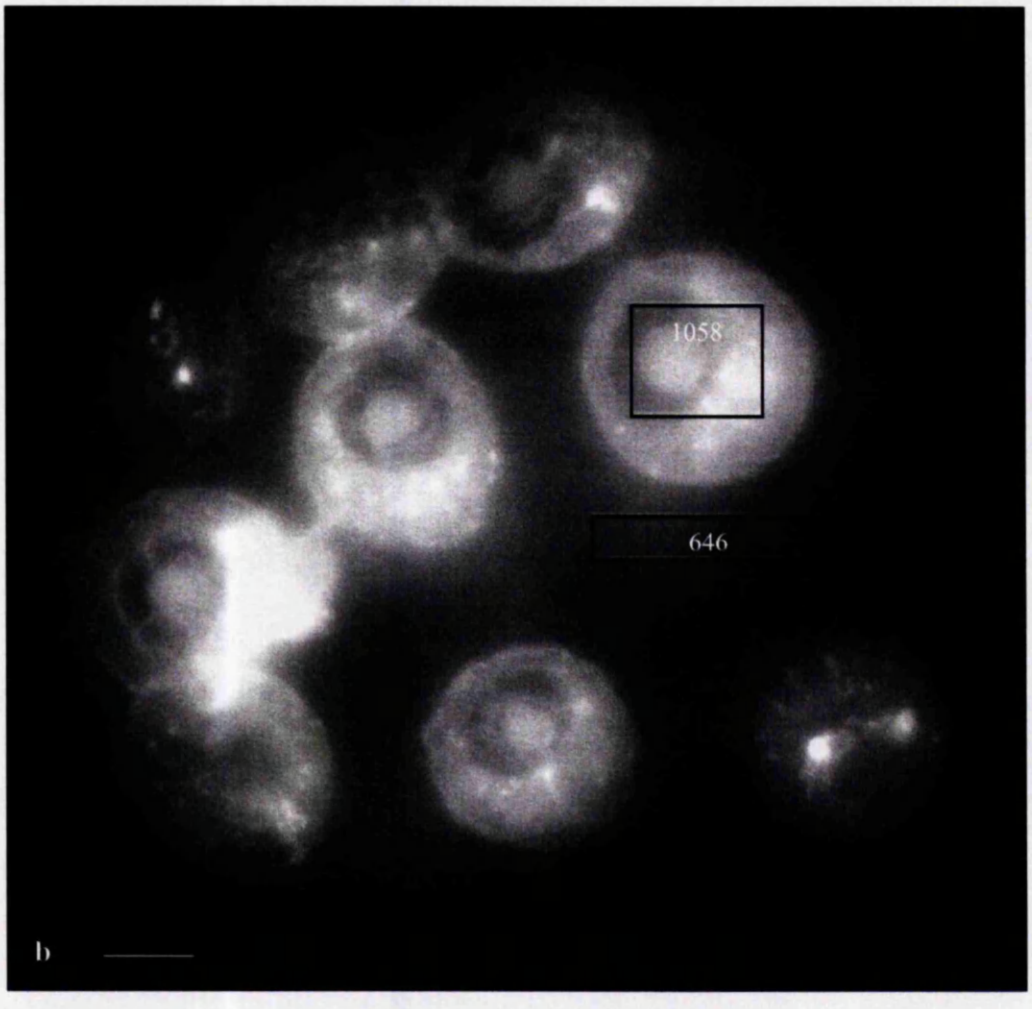


Plate 5. Fluorescent images of FITC-ConA labelled laboratory-cultured (a) *Anabaena cylindrica* and (b) *Eudorina elegans* taken by a photometrics slow scan CCD camera. The black box and white number show measured area and average light intensity within the area respectively. (Scale bar = 5 μm)

2.5 Carbohydrate and protein analysis of extracted cell-associated mucilage and soluble extracellular products

The carbohydrate and protein content was analysed from two types of sample. One was extracted cell-associated mucilage which was obtained from four laboratory-cultured mucilage-producing algae (*Anabaena cylindrica, Anabaena spiroides, Microcystis aeruginosa, Eudorina elegans*) and mixed environmental phytoplankton. The other was soluble (<0.45 μm) extracellular products obtained from cultured media and lake water.

Seven cultured algae (*Anabaena cylindrica, Anabaena spiroides, Microcystis aeruginosa, Eudorina elegans, Chlorella vulgaris, Asterionella formosa, and Melosira varians*) were harvested in stationary growth phase (after about 24 days incubation) by filtering through 47mm diameter GF/C Whatman filter membranes. The filters, containing concentrated mucilage-producing algae (*Anabaena cylindrica, Anabaena spiroides, Microcystis aeruginosa, Eudorina elegans*), were processed using the method described in Section 2.2.2.(A).(2) to obtain freeze-dried cell-associated mucilage. The filtrate (cultured medium without algae) from each species was again filtered through a 47mm diameter 0.45 μm pore size Whatman cellulose nitrate filter membrane, then freeze-dried using a Modulyo freeze-dryer (Edwards High Vacuum B.O.C. Ltd., Crawley, England) to obtain freeze-dried extracellular products.

For environmental samples, freeze-dried extracted cell-associated mucilage and soluble extracellular products were obtained as described in Sections 2.2.2.(A).(2) and 2.2.2.(B).(2).

2.5.1 Carbohydrate analysis

A known weight of freeze-dried sample (extracted cell-associated mucilage or soluble extracellular products from laboratory-cultured algae and environmental samples) was added to 1 ml of distilled water and mixed with 1 ml of phenol reagent (5% w/v) in 10-ml glass vials. One ml of distilled water was used as a blank. Five ml of concentrated H_2SO_4 was added slowly (over 15-20 sec) to the surface of the liquid in the vial. The end of a 5-ml pipette tip was cut off to deliver the concentrated H_2SO_4 to promote mixing and heat development necessary for the assay. The samples were incubated at room temperature for 30 minutes. Absorbance was measured at 485 nm wavelength in an ultraviolet spectrophotometer (Pye Unicam SP8-100). Analysis was carried out in triplicate. [developed from Dubois et al. (1956) and Kochert (1978)]

Glucose was used as standards at the range of 5-500 μg. The carbohydrate content calculated from the standard curve should be corrected by a factor of ×1.3 as the polysaccharide in extracted mucilage and extracellular products is a heteropolysaccharide which produces a different intensity of colour development than that obtained from glucose (Nakagawa et al., 1987). All carbohydrate determination was expressed as μg with reference to this calibration.

2.5.2 Protein analysis

The BCA (bicinchoninic acid) protein assay (Brown et al., 1989) was used to quantify protein content of extracted cell-associated mucilage from laboratory-cultured and environmental phytoplankton and soluble extracellular products in culture media and lake water. The BCA working reagent was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B (Pierce Chemical company). The working reagent is stable for at least 1 day by storing in a closed container at room temperature. Disposable 1.5 ml microcentrifuge tubes were used during the assay. All the containers

used were acid-washed with 10% HNO_3. Bovine serum albumin (BSA) (Pierce Chem.) was used as a protein standard. The 2 mg ml^{-1} BSA stock standard was diluted to obtain concentrations between 2.5 and 150 $\mu g\ ml^{-1}$.

Known weights of sample, together with 1 ml of distilled water or 1 ml of a known concentration of standard, were treated with 0.1 ml of sodium deoxycholate (DOC, Sigma chemicals, 0.15%, w/v) for 10 min at room temperature. After adding 0.1 ml of trichloroacetic acid (TCA, BDH, 72%, w/v), the samples were vortexed and centrifuged at 16000×g for 1 min at room temperature. The supernatant was aspirated off using a 0.2 ml pipette tip attached to a vacuum flask. Pellets were solubilized by mixing with 0.05 ml of sodium dodecyl sulphate (SDS, BDH, 5%, w/v) containing 0.1N NaOH using a whirlimixer (Fisons Scientific Ltd. Serial No. NEL 4991). A 0.05 ml aliquot of SDS was used as a blank. Immediately after adding 1 ml of BCA working reagent, each sample was whirlimixed and incubated at 37 °C for 30 min. The optical density was measured at 562nm wavelength using an ultraviolet spectrophotometer (Pye Unicam SP8-100). Measurements were carried out in triplicate.

2.6 Determination of metal-binding activity on algal surface

Metal binding activity was investigated mainly in relation to laboratory-cultured algae, particularly *Anabaena cylindrica* (Section 2.6.2-2.6.4). Studies were also carried out on metal binding to environmental samples (Section 2.6.7). Copper was used as the test cation to assess metal binding activity. To avoid the copper binding to growth medium constituents, and to eliminate trace metal present in the medium, a modified BG-11 medium was used. This medium contained no citric acid, ferric ammonium citrate, EDTA and trace metals (see Appendix 1). The ability of algae to grow in this medium was tested in a preliminary experiment (Section 2.6.1).

2.6.1 Assessment of the growth of Anabaena cylindrica *in modified BG-11 medium*

Three 250 ml Ehrlenmeyer flasks containing 100 ml modified BG-11 medium and 5 ml stock *Anabaena cylindrica* (in exponential growth phase), plus controls containing *Anabaena cylindrica* in normal BG-11 medium (composition shown in Appendix 1) were incubated at 25 °C, in an orbital incubator at 100 rpm and 22 μmol photon m^{-2} s^{-1} continuous fluorescent light. Every 2-3 days, the optical density of each culture (described in Section 2.1.2.a) was measured and 0.1 ml of 25% of glutaraldehyde solution (TAAB Laboratories) was added to 1 ml of sample for observation of cell types under a Leitz Dialux 20 Phase-contrast light microscope.

Statistical analysis was conducted using the nonparametric Mann-Whitney U - Wilcoxon Rank Sum W test with a significance level of $p<0.05$.

2.6.2 Assessment of copper uptake by Anabaena cylindrica *in modified BG-11 medium*

Three groups of 1L Ehrlenmeyer flasks (in triplicate) were treated as follows:

Experimental group and control group 1: each group of three 1L Ehrlenmeyer flasks contained 500 ml of modified BG-11 medium. They were inoculated with 5 ml *Anabaena cylindrica* stock culture (in exponential growth phase).

Control group 2: This group of three 1L flasks contained 500 ml of modified BG-11 medium without algae.

After 18 hours incubation under identical conditions to those described in Section 2.6.1, copper sulphate ($CuSO_4.5H_2O$, 'Analar' grade, BDH) was added to experimental group and control group 2 to give a final concentration of 100 μg l^{-1} of copper.

After addition of copper, 25 ml samples were removed from each flask at 0, 1h, 2h, 4h, 8h, 12h, 24h, and 32h and subsequently centrifuged at 32500×g, at 4 °C for

15 minutes. Five ml of the supernatant was taken to determine the copper concentration. The pellet was dried at 70 °C for 2 days and weighed. One ml of concentrated HNO_3 and 1 ml of concentrated HCl were added to digest the dried pellet. The concentrated acid was evaporated to dryness on a hot plate at a constant temperature (80 °C) and the sample was redissolved in 5 ml of 0.1 M nitric acid and analysed for copper using atomic absorption spectrophotometry (Smith-Hieffe spectrophotometer model 11).

The pH was measured at time 0 and 24 hours after adding copper. All containers used were acid-washed in 10% nitric acid for at least 24 hours and rinsed with distilled water beforehand. All chemicals used were of 'Analar' grade (BDH).

Statistical analysis was conducted using the nonparametric Mann-Whitney U - Wilcoxon Rank Sum W test with a significance level of $p<0.05$.

2.6.3 Copper adsorption onto Anabaena cylindrica *in distilled water*

In order to further minimise the effect of growth medium, Cu-adsorption was investigated by exposing cells to Cu in distilled water. Starting from an initial actively-growing culture of algae, three treatments (experimental, control 1, control 2) were prepared as follows (also see Fig. 2.2):

Experimental group: Three ml of stock *Anabaena cylindrica* (exponential growth phase) was pipetted into six polycarbonate tubes, washed with distilled water, centrifuged (32500×g, 4 °C, 15 min) and resuspended in 30 ml distilled water containing 100 $\mu g\ l^{-1}$ copper (added as copper sulphate in distilled water). After a known period of incubation, the samples were centrifuged (32500×g, 4 °C, 15 min) and 5 ml of the supernatant taken for measurement of copper concentration using atomic absorption spectrophotometry. The pellets from three tubes were dried and digested as described in Section 2.6.2. The remaining three pellets were resuspended in 30 ml 10

mM EDTA (disodium magnesium salt), sonicated for 1 minute in a sonicator (Model W-225R), and then centrifuged (32500×g, 4 °C, 15 min). The clear supernatant was analysed for copper and the pellets were dried, weighed and digested following the procedures in Section 2.6.2.

Control group 1 (no Cu added): Three ml stock of *Anabaena cylindrica* (in exponential growth phase) was washed, centrifuged (32500×g, 4 °C, 15 min), resuspended with 30 ml distilled water and then incubated for a known time. Five ml of the supernatant was analysed for copper and the pellet was resuspended in 30 ml 10mM EDTA (disodium magnesium salt), sonicated for 1 minute, centrifuged (see above), and the supernatant was analysed for copper following drying and digestion of the pellet (Section 2.6.2). Three replicates were used.

Control group 2 (no algae): Three polycarbonate tubes containing distilled water with a final concentration of 100 $\mu g\ l^{-1}$ copper was incubated for a known time, then centrifuged (see above). Copper concentration was measured in the supernatant.

The incubation periods were 30 minutes, 1 hour, 2 hours, and 4 hours respectively for all three groups. All containers used were acid-washed beforehand as described in Section 2.6.2 and chemicals were of 'Analar' grade (BDH).

For all data, statistical analysis was carried out using the nonparametric Mann-Whitney U - Wilcoxon Rank Sum W test with a significance level of $p<0.05$.

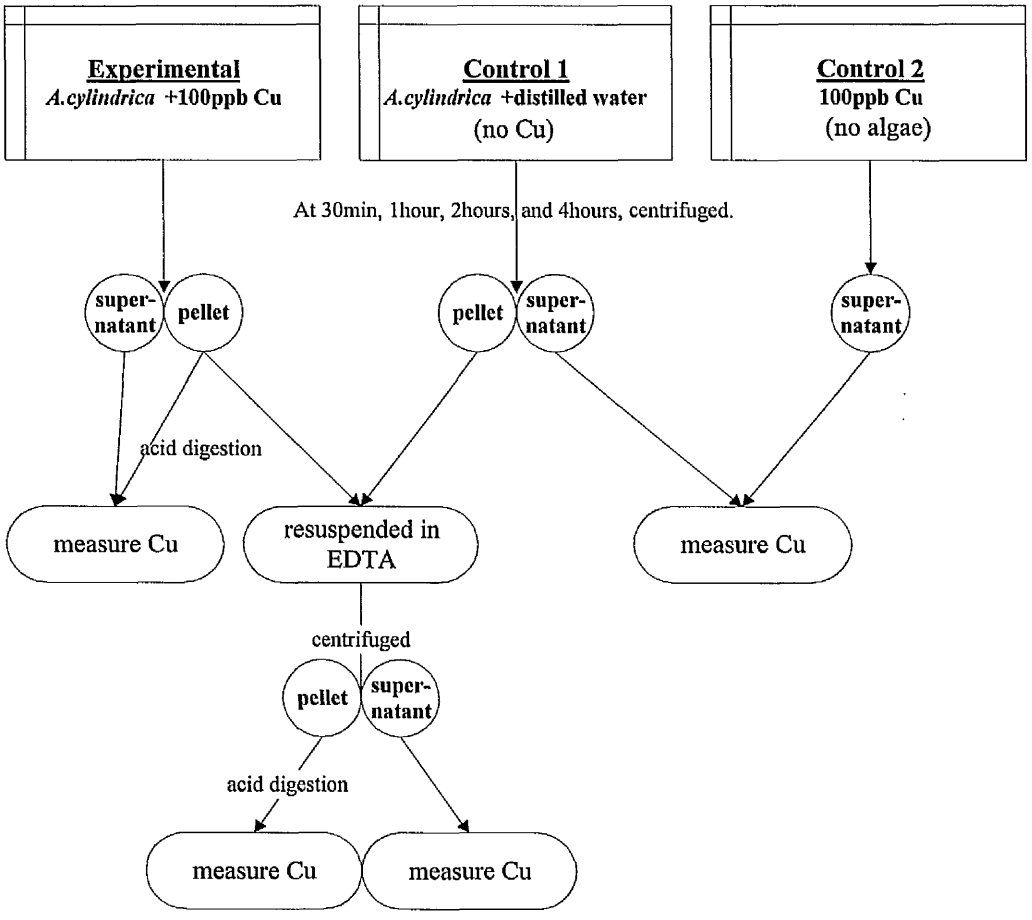


Fig. 2.2 A flow diagram of the experiment assessing copper adsorption onto *Anabaena cylindrica* in distilled water

2.6.4 Comparison of copper adsorption by living and dead (glutaraldehyde-fixed) cells of Anabaena cylindrica

Three treatments were carried out to assess copper adsorption by living and dead cells of *Anabaena cylindrica*:

Experimental group 1: A 2 ml stock of *Anabaena cylindrica* (exponential growth phase) was washed with distilled water (in triplicate), then resuspended in 30 ml 450 $\mu g\ l^{-1}$ copper for 30 min, 1 hour, 2 hours and 4 hours.

Experimental group 2: A 2 ml stock of *Anabaena cylindrica* (exponential growth phase) was treated with 2.5 % glutaraldehyde solution for 15 minutes, then washed with distilled water. Three replicates of glutaraldehyde-fixed (dead) cells were resuspended in 30 ml 450 $\mu g\ l^{-1}$ copper and incubated for the same period as experiment group 1.

Control group (no algae): Three replicates without algae, each consisting of 30 ml distilled water containing 450 $\mu g\ l^{-1}$ copper, were set up and incubated as experimental groups 1 and 2.

All three groups were centrifuged (32500×g, 4 °C, 15 min) after a known incubation time (30min, 1 hour, 2 hours and 4 hours) and the clear supernatant was analysed for copper using atomic absorption spectrophotometry. The pellets from two experimental groups were resuspended and sonicated in 30 ml 10mM EDTA, then centrifuged (see above). The supernatant was also analysed for copper (see above).

All containers were acid-washed as described in Section 2.6.2 beforehand and chemicals were of 'Analar' grade (BDH). Statistical analysis was conducted using the nonparametric Mann-Whitney U - Wilcoxon Rank Sum W test with a significance level of $p<0.05$.

2.6.5 Assessment of copper biosorption in four laboratory-cultured species Anabaena cylindrica, Anabaena spiroides, Eudorina elegans and Chlorella vulgaris

Anabaena cylindrica, Anabaena spiroides, Eudorina elegans and Chlorella vulgaris (Plates 1 and 2) were harvested after 20 days (exponential growth phase) incubation by centrifugation at 3000×g for 20 min at 4 °C. Cells were resuspended in distilled water containing 250 μg l^{-1} copper and 100 ml of this suspension placed in 150 ml plastic beakers. Controls (no algae) were also set up, with 100 ml of distilled water containing 250 μg l^{-1} of copper. Each treatment plus controls were replicated three times. After respective incubation periods of 15 minutes, 45 minutes, 75 minutes, 2 hours, and 4 hours, 5 ml was filtered through a 0.4 μm Cyclopore™ polycarbonate membrane (Whatman) and the copper concentration of the filtrate was measured using atomic absorption spectrophotometry.

Immediately after resuspending cells with copper solution, 10 ml aliquots were taken for determination of dry weight using the method described in Section 2.1.2.b. A 5 ml aliquot was fixed with 0.5 ml of 25% glutaraldehyde solution (TAAB) for determination of surface area and mucilage volume using the methods described in Section 2.3, and a 5 ml aliquot was fixed with 1 drop of Lugol's iodine solution for cell counts using the method described in Section 2.1.2.c. All measurements were conducted in triplicate.

All containers used were acid-washed as described in Section 2.6.2 beforehand and the chemicals were of 'Analar' grade (BDH). Statistical analysis was carried out using the nonparametric Kruskal-Wallis One-Way ANOVA and Mann-Whitney U - Wilcoxon Rank Sum W test (taking $p<0.05$ as the level of significance).

2.6.6 Determination of copper biosorption in living and dead (freeze-dried) laboratory-cultured algae using adsorption mathematical models

The experimental procedures used in this section are based on the Freundlich and Langmuir adsorption models discussed previously (Section 1.2.3). At a constant temperature, the amount of metal adsorbed varies with different concentrations of metal. Adsorption isotherms were determined in relation to two major parameters: the amount of metal adsorbed per unit dry weight (x/m) and the metal concentration remaining in solution at equilibrium (C), see equation 4, 5 and 7.

Eight species of laboratory-cultured algae (Plates 1 & 2) were harvested during exponential growth phase by centrifugation at 3000×g for 20 min at 4 °C. Cells were resuspended in 300 ml of distilled water. An 105 ml aliquot of cell suspension was freeze-dried using a Modulyo freeze-dryer and resuspended with the same amount (105 ml) of distilled water (dead cell suspension). Five ml of cell suspension (either a living or dead cell suspension of each species) was added separately to 5 ml of specified concentrations of copper (giving the final copper concentrations of 1, 5, 10, 50, 100, 500 and 1000 mg l^{-1}) in a polycarbonate tube and was incubated on a 100 rpm shaker for 1 h at 25 °C. Five ml of distilled water was added to 5 ml of each copper concentration as controls. All experiments were carried out in triplicate. All samples were filtered through a 0.4 μm Cyclopore™ polycarbonate membrane (Whatman) and the filtrate analysed for copper using atomic absorption spectrophotometry.

The dry weight of cells was measured in triplicate using the method described in Section 2.1.2.b. Five ml of living cell suspension was fixed with 0.5 ml of 25% glutaraldehyde solution (TAAB) for estimation of surface area and mucilage volume (calculated by the methods described in Section 2.3) and another 5 ml of living cell suspension was fixed with 1 drop of Lugol's iodine solution for cell counts as

described in Section 2.1.2.c. Five ml of freeze-dried cell suspension was examined immediately after resuspending in distilled water upon freeze-drying under a Leitz Dialux 20 phase-contrast light microscope to check cell integrity after this procedure.

All containers used were acid-washed as described in Section 2.6.2 beforehand and the chemicals were of 'Analar' grade (BDH). Statistical analysis involved linear regression and correlation analysis (with the significance level of $p<0.05$).

2.6.7 Determination of the copper binding capacity of phytoplankton samples from Rostherne Mere

2.6.7.a Preliminary assessment of copper binding by mixed phytoplankton cells from Rostherne Mere

Copper binding to mixed phytoplankton cells was initially assessed using both integrated lake water and trawl-net samples from Rostherne Mere, both collected on 07 May and 11 June 1997. One hundred ml of fresh integrated lakewater and fresh trawled-net samples (both containing algae) were treated with copper at a final concentration of 200 $\mu g\ l^{-1}$. One hundred ml of integrated lakewater was filtered through a 0.2 μm Cyclopore™ polycarbonate membrane (Whatman) (without algae) and exposed to 200 $\mu g\ l^{-1}$ of copper as a control. All samples were in triplicate. Samples collected on 07 May were incubated with $CuSO_4$ solution for periods of 10 min, 20 min, 30 min, 45 min, 60 min and 120 min. At each time interval, a 5 ml aliquot was removed and filtered through a 0.2 μm Cyclopore™ polycarbonate membrane (Whatman). Samples collected on 11 June were processed similarly, except for incubation periods of 10 min, 25 min and 40 min. Copper concentrations in the filtrate were measured using atomic absorption spectrophotometry. The pH of the filtrate and the dry weights of cells (in triplicate, as described in Section 2.1.2.b, but following

filtration through 0.45 μm cellulose nitrate Whatman filter membranes) were measured.

Five ml of the concentrated trawled-net samples was fixed with Lugol's solution and cell counts carried out as described in Section 2.1.2.c. Phytoplankton counts of the integrated lake water used here were the same as site B results on 07 May and 11 June, 1997.

The nonparametric Kruskal-Wallis One-Way ANOVA and Mann-Whitney U - Wilcoxon Rank Sum W test (with the significance level of $p<0.05$) were used to examine differences between treatments.

2.6.7.b Assessment of copper biosorption in living and dead (freeze-dried) phytoplankton samples from Rostherne Mere using adsorption mathematical models

This experimental procedure is also based on the Freundlich and Langmuir adsorption models discussed in Section 1.2.3. It is therefore similar to the experiment described in Section 2.6.6.

Mixed phytoplankton samples were collected by trawling a phytoplankton net for 3 min on selected sampling dates (09 July, 05 August, 02 September, and 27 November 1997) at Rostherne Mere. Lake water containing the concentrated algal cells was filtered through a 0.45 μm cellulose nitrate filter membrane and the cells resuspended in 300 ml of distilled water (living cells). An 105 ml aliquot of cell suspension from July and September was freeze-dried in a Modulyo freeze-dryer and then resuspended with the same amount (105 ml) of distilled water (dead cells). Five ml of cell suspension (either a living or dead cell suspension) was added to 5 ml of a specified concentration of copper (giving the final copper concentrations of 1, 5, 10, 50, 100, 500 and 1000 mg l^{-1}) in a polycarbonate tube and incubated on a 100 rpm

shaker for 1 h at 25 °C. Five ml of distilled water was added to 5 ml of the desired copper concentration as controls. All experiments were carried out in triplicate. Five ml of incubated sample was then filtered through a 0.2 μm Cyclopore™ polycarbonate membrane (Whatman) and the concentration of copper in the filtrate analysed using atomic absorption spectrophotometry.

The dry weights of the algal biomass were measured as described in Section 2.1.2.b, except that the 0.45 μm cellulose nitrate filters were used. Five ml of cell suspension was fixed by the addition of 1 drop of Lugol's iodine solution. Phytoplankton cells were counted using a Sedgwick Rafter counting chamber as described in Section 2.1.2.c. Five ml of freeze-dried cell suspension was examined immediately after resuspending in distilled water upon freeze-drying under a Leitz Dialux 20 phase-contrast light microscope to check cell integrity.

Linear regression and correlation analysis (with a significance level of $p<0.05$) were used to statistically analyse the data.

2.6.8 Examination of copper binding to cell-associated mucilage using X-ray microanalysis

Cultured cells of *Anabaena spiroides* (shown in Plate 1) were collected during exponential growth phase by centrifugation at 3000×g for 20 min at 4 °C. Cells were resuspended with 100 ml of distilled water in a 150 ml plastic beaker to give an optical density of 0.5. A $CuSO_4$ solution was added to give a final copper concentration of 50 mg l^{-1}. After incubating at 25 °C with gentle magnetic stirring for 30 min. Five ml samples were filtered through 0.4 μm Cyclopore™ polycarbonate membranes (Whatman) then washed with distilled water to remove excess copper on the filter membrane. The filter membrane with deposited *Anabaena spiroides* was immediately frozen in nitrogen slush, freeze-dried for 12 hours in an Edwards tissue-drier at -60 °C,

mounted on aluminum SEM stubs using double sided sellotape and carbon DAG, and carbon coated. Samples were stored in a desiccator before examining by scanning electron microscopy.

Carbon-coated samples were analysed using a Cambridge 360 Stereoscan Scanning Electron Microscope associated with a Link AN10000 analyser. For X-ray microanalysis, the probe current was adjusted to give a count rate of approximately one thousand counts per second. X-ray emission spectra were obtained from the cell, mucilage and the filter membrane (to check if there was residual copper present on the filters) at an accelerating voltage of 25 kV and 100 s live time.

Quantification of the elements in the samples was carried out using the Link ZAF/PB program, with cobalt as reference element because it has a well defined peak of known energy. The 'fit index' which is a measure of the quality of fit of the stored standard peak profiles to the unknown spectrum was calculated and was kept between 0 and 2 in this study. Within this range of 'fit index', copper concentration was obtained as elemental mass fractions (g/100g dry weight). Five measurements were taken on the cell and mucilage respectively.

CHAPTER 3. QUANTIFICATION OF CELL-ASSOCIATED AND SOLUBLE MUCILAGE IN ROSTHERNE MERE

3.1 Introduction

Mucilage aggregates are a well recognized phenomenon in coastral waters throughout the world (Lancelot, 1995 ; Leppard, 1995) where they may prevent fishing activities due to the clogging of nets (Calvo et al., 1995), reduce the recreational suitability of beaches (Degobbis et al., 1995) and cause suffocation of benthic organisms such as molluscs and crustaceans (Rinaldi et al., 1995). Mingazzini and Thake (1995) found that the periodicity of occurrence of marine mucilage was irregular and is influenced by many factors. Phytoplankton composition is an important factor since exudate production in marine waters seems to be qualitatively and quantitatively species-specific (Migazzini & Thake, 1995).

Increasing eutrophication of the freshwater environment tends to increase the numbers of mucilage-producing species such as the cyanobacteria *Anabaena spp.* and *Microcystis spp.*. Much of the mucilage remains on the surface of the algal cell but some is released into the water (reviewed in Section 1.1). Grossart and Simon (1993) first documented the occurrence of macroscopic organic aggregates ('lake snow') in Lake Constance (Germany). Grossart et al. (1997) further found that the abundance and composition of lake snow showed a pronounced seasonal and vertical pattern and bore a close relationship to phytoplankton and zooplankton dynamics and wind conditions. Thus, the amount of cell-associated and soluble mucilage in the water may be correlated with the abundance of the mucilage-producing species and will thus be influenced by the seasonal succession in abundance and species composition of phytoplankton (described in Section 1.3).

The objectives of this section of the study were to quantify the amounts of cell-associated and soluble mucilage in a freshwater lake (Rostherne Mere) over time and to relate these to changes in the physico-chemical and biotic (phytoplankton composition) environment. A detailed description of Rostherne Mere can be found in Section 1.3.1.

3.2 Results

3.2.1 Variation in physico-chemical parameters at Rostherne Mere

A number of physico-chemical parameters were recorded in Rostherne Mere during 1996 and 1997. Mean values were derived from records obtained from sites A, B, C, as described in Chapter 2, Materials and Methods.

3.2.1.a Temperature and dissolved oxygen

I. Temperature

The mean recorded water temperature of the integrated samples (taken from the first 5 m of the water column) ranged from 6 °C (March, 1997) to 20 °C (mid-August 1996) and 22 °C (early July 1997) [Fig. 3.1(a)].

Seasonal variations in temperature with depth in 1996 and 1997 can be seen in Fig. 3.2(a), which plots isotherms at 2 °C intervals. In 1996, there was a distinct thermocline formed between 6 m and 10 m on 30 May with a fall in temperature from 12 °C to 9 °C. Stratification continued until October. In 1997, there was clear stratification between May and September. Fig. 3.3(a) illustrates the stratification of the water column between 6 m and 10 m on 05 August 1997.

II. Oxygen

In the integrated lakewater sample, oxygen saturation ranged mostly from 68% to 120% except for two occasions when the highest levels were recorded, the first in August 1996 (139%) and the second in July 1997 (144%) [Fig. 3.1(b)].

Oxygen profiles with depth showed clear evidence of stratification during the summer months [Fig. 3.2(b)] with a well developed oxycline between 6 m and 10 m in September-October 1996 and August-September 1997. At these times the hypolimnion had an oxygen saturation of < 20%. For example, on 05 August 1997, an oxycline formed between 6 m and 10 m and oxygen saturation fell from 96% to 26% [Fig. 3.3(b)].

3.2.1.b Secchi depth

The greatest Secchi extinction depth occurred during May 1996 and between June and July 1997 [Fig. 3.1(d)]. Secchi depth and chlorophyll a concentration (the latter analysed by V. Krivtsov in samples taken in 1996 and by E. Levado in samples taken in 1997) were negatively correlated ($R = -0.66$, $p = 0.002$, Fig. 3.4).

3.2.1.c pH

The mean pH of the integrated water samples ranged from 6 to 9.3 and was mostly within the range 7 to 9. Between April and May 1996, August and September 1996, May and July 1997, and during September 1997, the pH was above 8 [Fig. 3.1(c)] coinciding with the spring and summer blooms of phytoplankton (Section 3.2.2). A significant ($p=0.017$) correlation between pH and chlorophyll a concentration was observed ($R = 0.53$, Fig 3.5).

3.2.1.d Conductivity

Lake water conductivity was highest between June and August 1996 and between June and August 1997 [Fig. 3.1(e)]. Conductivity and the pH of the integrated lake water samples were strongly ($p=0.003$) correlated ($R = 0.61$, Fig. 3.6).

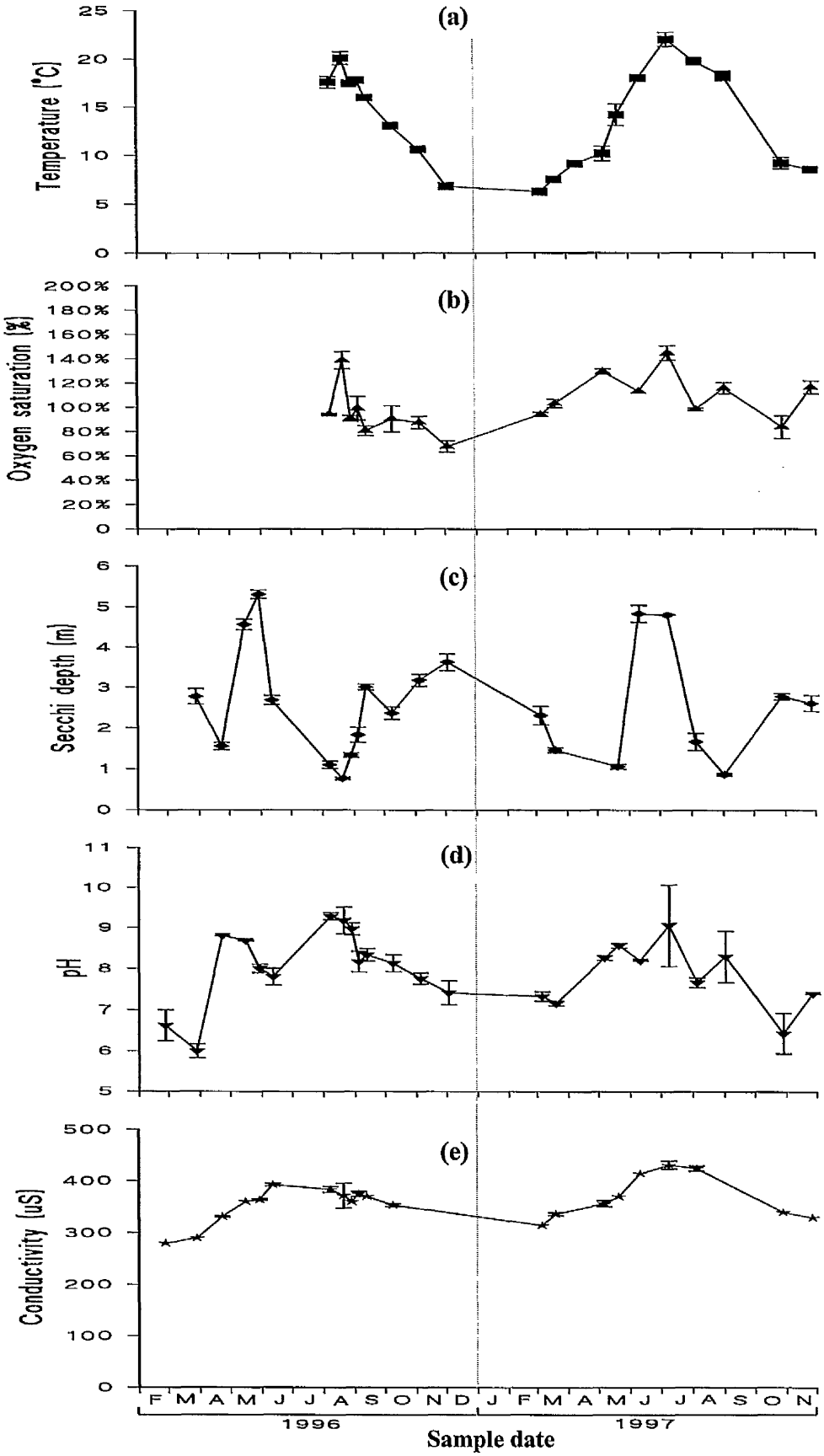
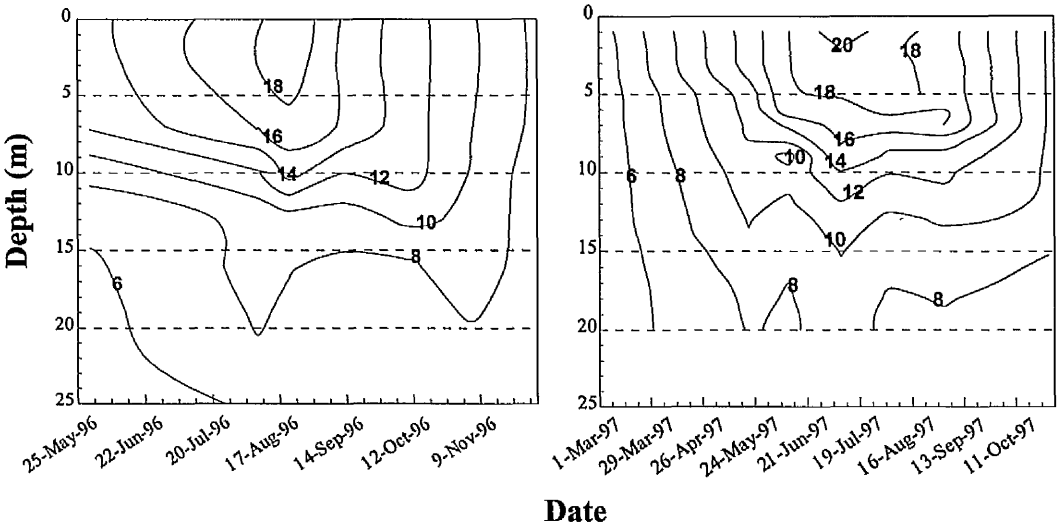


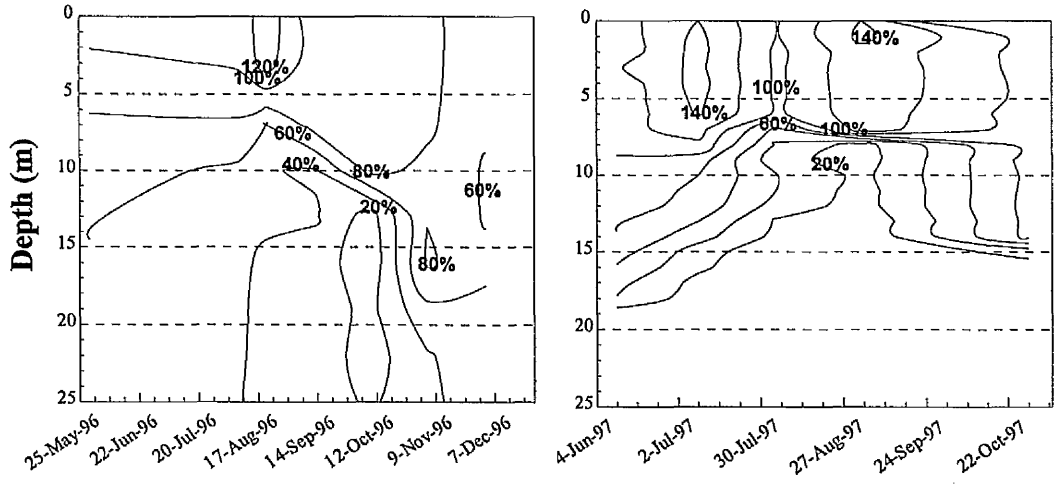
Fig. 3.1 Seasonal changes in (a) temperature, (b) oxygen saturation, (c) Secchi depth, (d) pH and (e) conductivity in Rostherne Mere from February 1996 to November 1997. Values are the mean ± SD derived from a single measurement of the integrated sample from three sites.

(a) Temperature isotherms



Date

(b) Oxygen saturation isotherms



Date

Fig. 3.2 Depth-time diagrams for (a) temperature (isotherms at 2 °C intervals) and (b) oxygen saturation (isotherms at 20% intervals) in Rostherne Mere in 1996 and 1997.

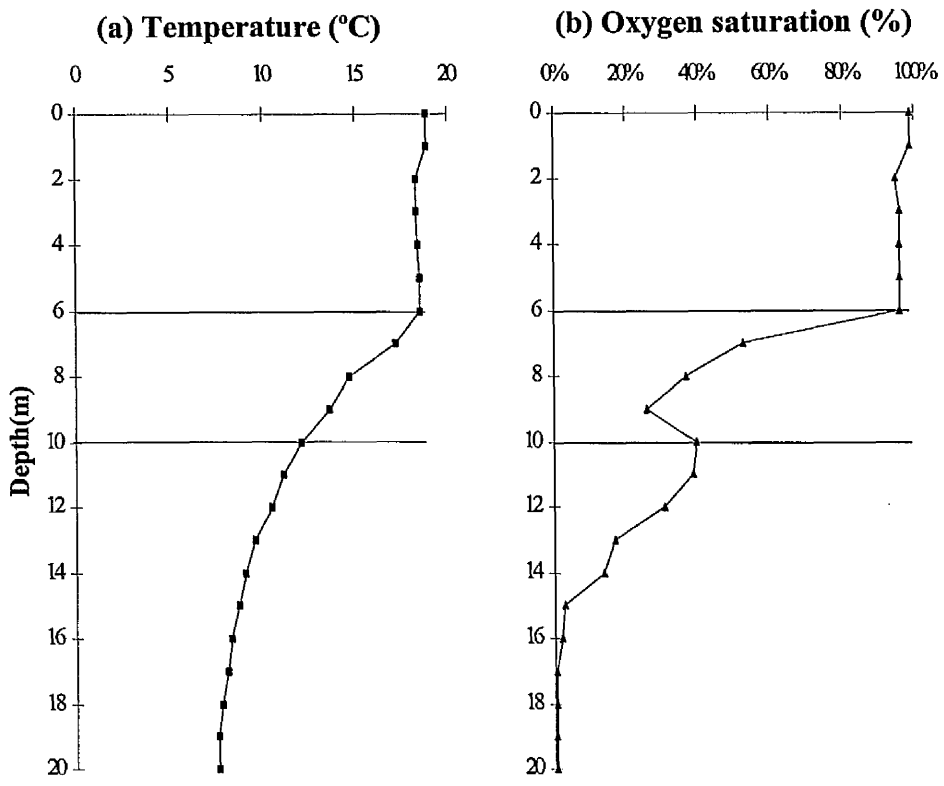


Fig. 3.3 Vertical depth profiles of (a) temperature (b) oxygen saturation at 05 August 1997 at site B in Rostherne Mere.

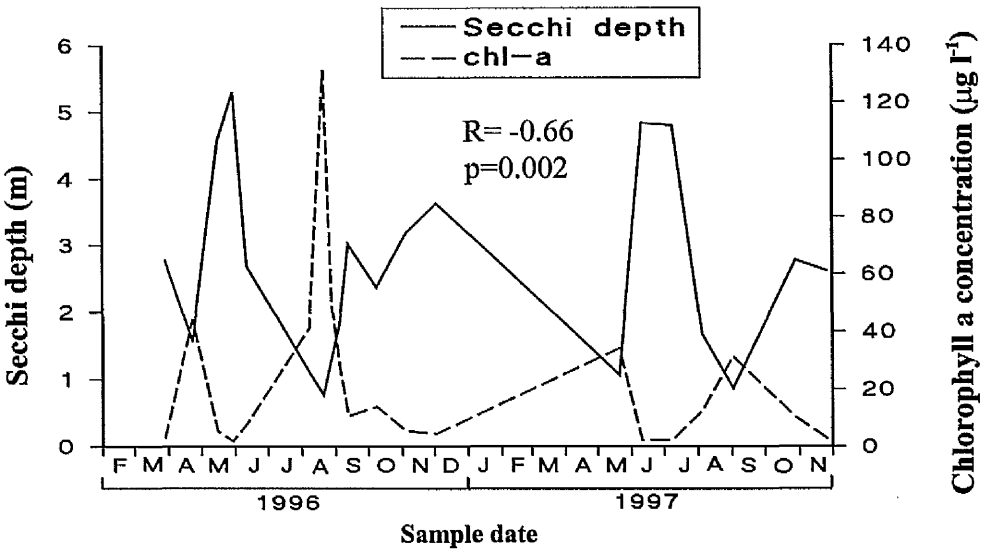


Fig. 3.4 Secchi depth and chlorophyll a concentration in the integrated lake water samples from Rostherne Mere. (R= correlation coefficient of the relationship between Secchi depth and chlorophyll a)

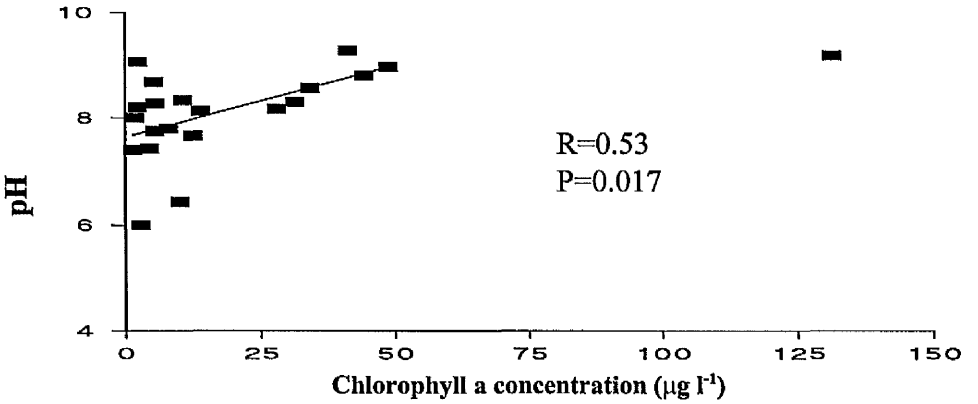


Fig. 3.5 Relationship between pH and chlorophyll a concentration (analysed by V. Krivtsov and E. Levado) in the integrated lake water samples from Rostherne Mere. (R= correlation coefficient)

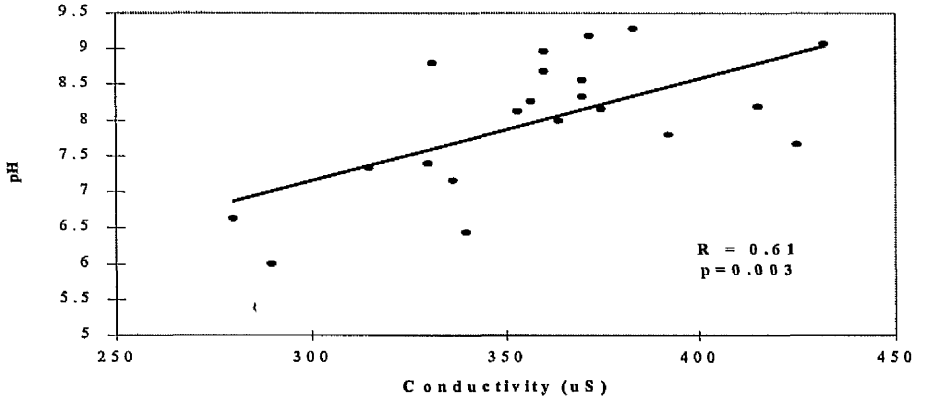


Fig. 3.6 Relationship between conductivity and pH in the integrated lake water samples from Rostherne Mere. (R= correlation coefficient)

3.2.2 Species composition of phytoplankton

The algal species recorded at Rostherne Mere during the course of this study are listed in Table 3.1, with a total of 63 species (49 genera). Less than half this number was present on any sampling date, with a maximum 22 species (4390 individuals ml^{-1}) at site B in July 1996 and a minimum 3 species (760 individuals ml^{-1}) at site C in June 1997. Seasonal changes in the total algal population and individual species in Rostherne Mere from October 1995 to November 1997 are shown in Fig. 3.7 and Fig. 3.9- 3.13.

The total algal population and the populations of different algal groups are shown in Fig. 3.7. High population counts were recorded in spring (April 1996 and May 1997), summer (July and August 1996) and autumn (October 1996 and September 1997) [Fig. 3.7(a)]. Population counts were closely correlated with chlorophyll a concentration ($R= 0.6$, $p= 0.003$) and with 1/Secchi depth ($R= 0.79$, $p< 0.001$, Fig. 3.8).

Over the period October 1995 to November 1997, Bacillariophyceae (diatoms) were the dominant algae in the spring (April 1996 and 07 May 1997) and in the 1996 autumn (October) bloom. Cryptophyta dominated the late spring (21 May) and autumn (September) bloom during 1997 and Cyanobacteria were dominant during the summer (August) 1996 bloom [Fig. 3.7(b)&(c)].

The most abundant bacillariophyceae (diatoms) during the sampling period were *Asterionella formosa, Stephanodiscus spp., Melosira spp.* and *Nitzschia acicularis* (Fig. 3.9). Of these four major diatom species, *Asterionella formosa* was the most numerous, reaching peaks of 2600 cells ml^{-1} in April 1996, 3200 cells ml^{-1} in October 1996 and 1000 cells ml^{-1} on 07 May 1997. Three species of *Stephanodiscus* were observed, *Stephanodiscus rotula, Stephanodiscus minutula* and *Stephanodiscus*

hantzschii. None exceed 35 cells ml^{-1}, except for *Stephanodiscus rotula* which achieved a maximum of 63 cells ml^{-1} in March 1996. Filaments of *Melosira spp.* were found during the autumn and winter (maximum: 130 filaments ml^{-1} on 29 August 1996). *Nitzschia acicularis* was present at a maximum of 100 cells ml^{-1} on 30 May 1996.

Cryptophyta were abundant with population counts exceeding 20 cells ml^{-1} in all samples. In 1997, large populations occurred in May (5500 cells ml^{-1}) and September (1700 cells ml^{-1}) [Fig. 3.7(c)]. *Cryptomonas spp.*(maximum: 3100 cells ml^{-1}, 21 May 1997) and *Rhodomonas minuta* (maximum: 2800 cells ml^{-1}, 26 July 1996) were the most common species (Fig. 3.10).

Eudorina elegans, Ankyra spp., and *Chlamydomonas spp.* were the dominant species of Chlorophyta (green algae) (Fig. 3.11). *Eudorina elegans* attained a maximum population of 130 colonies ml^{-1} in April 1996 but less than 37 colonies ml^{-1} for the rest of the time. *Ankyra spp.* only appeared in summer with a maximum of 720 cells ml^{-1} in June 1997. *Chlamydomonas spp.* occurred frequently during July 1996 and November 1997. The highest population (1100 cells ml^{-1}) of *Chlamydomonas spp.* was recorded in May 1997.

Filamentous cyanobacteria became abundant in late spring and summer in 1996. The main species were *Anabaena flos-aquae, Anabaena spiroides, Anabaena circinalis, Aphanizomenon flos-aquae,* and *Oscillatoria spp.*. The filament length of the five major filamentous cyanobacteria is shown in Fig. 3.12. *Anabaena spiroides* only appeared in May and June 1996, reaching a filament length of 3 mm ml^{-1} (equivalent to 20 filaments ml^{-1} and 600 cells ml^{-1}) and 70 mm ml^{-1} (equivalent to 270 filaments ml^{-1} and 15000 cells ml^{-1}) respectively [Fig. 3.12(b)]. In 1996, *Anabaena circinalis* grew rapidly between early July and August with a maximum filament

length of 12 mm ml^{-1} (equivalent to 120 filaments ml^{-1} and 2800 cells ml^{-1}) on 26 July. In 1997, it was found between April and June (maximum: 3 mm ml^{-1}, equivalent to 20 filaments ml^{-1} and 600 cells ml^{-1} in May) [Fig. 3.12(c)]. The length per filament of *Anabaena circinalis* was greatest during the middle bloom with an average length of 100 μm per filament in 1996 ($p<0.05$), but it was similar throughout 1997 ($p>0.05$). *Anabaena flos-aquae* developed rapidly after 30 May 1996, reaching a peak of filament length of 340 mm ml^{-1} (equivalent to 1800 filaments ml^{-1} and 78900 cells ml^{-1}) on 09 July 1996, followed by a rapid decline, then increased again by 21 August 1996 (110 mm ml^{-1}, equivalent to 910 filaments ml^{-1} and 23000 cells ml^{-1}) and decreased afterwards [Fig. 3.12(a)]. Growth of *Anabaena flos-aquae* populations showed large fluctuations in 1997 but filament length did not exceed 10 mm ml^{-1}. Large clumps of *Anabaena flos-aquae* were found during the middle of the bloom (on 09 July 1996) with a maximum length of 1400 μm per filament.

Aphanizomenon flos-aquae was initially detected in July 1996 and then bloomed, reaching a maximum density of 800 mm ml^{-1} (equivalent to 5300 filaments ml^{-1}) on 08 August 1996 [Fig. 3.12(e)]. However, in 1997, *Aphanizomenon flos-aquae* occurred in low densities (< 1mm ml^{-1}, equivalent to 7 filaments ml^{-1}) during late spring. *Oscillatoria spp.* were found during the late spring and summer in 1996 and 1997 but at less than 7 mm ml^{-1} (equivalent to 50 filaments ml^{-1}), except in late August 1996 when 40 mm ml^{-1} (equivalent to 140 filaments ml^{-1}) were observed [Fig. 3.12(d)]. Among the five dominant filamentous cyanobacteria recorded during the sampling period, the highest total filament length (840 mm ml^{-1}) appeared on 08 August 1996. *Aphanizomenon flos-aquae* accounted for most of the biomass (Fig. 3.12).

Microcystis aeruginosa was the most dominant colonial coccoid cyanobacteria throughout the study period. It was present during the summer and autumn. The

population attained the highest densities on 21 August 1996 (100 colonies ml^{-1}, equivalent to 83300 cells ml^{-1}) and on 02 September 1997 (180 colonies ml^{-1}, equivalent to 679100 cells ml^{-1}) [Fig. 3.13(a)].

Large numbers of *Ceratium hirundinella* were observed at the same time as the *Microcystis aeruginosa* bloom. *Ceratium hirundinella* reached peaks of 560 cells ml^{-1} on 26 July 1996 and 620 cells ml^{-1} on 21 August 1996, but in 1997, only achieved a density of 130 cells ml^{-1} in September [Fig. 3.13(b)].

Table 3.1 Phytoplankton taxa observed in Rostherne Mere from October 1995 to November 1997.

Species	Species
Cyanobacteria	**Chlorophyta**
Microcystis aeruginosa	*Eudorina elegans*
Anabaena flos-aquae	*Pandorina morum*
Anabaena spiroides	*Closterium spp.*
Anabaena circinalis	*Scenedesmus obliquus*
Gloeocapsa rupestris	*Scenedesmus acutus*
Chroococcus turgidus	*Scenedesmus acuminatus*
Merismopedia punctata	*Scenedesmus quadricauda*
Oscillatoria spp.	*Scenedesmus abundans*
Gloeotrichia pisum	*Scenedesmus arcuatus*
Aphanizomenon flos-aquae	*Chlorella vulgaris*
Coelosphaerium naegelianum	*Staurastrum planctonicum*
Bacillariophyceae	*Selenastrum spp.*
Stephanodiscus rotula	*Pleurococcus spp.*
Stephanodiscus minutula	*Ulothrix aequalis*
Stephanodiscus hantzschii	*Crucigenia tetrapedia*
Cyclotella spp.	*Tetraedron minimum*
Asterionella formosa	*Kirchneriella obesa*
Frustulia vulgaris	*Kirchneriella lunaris*
Frustulia rhomboides	*Haematococcus lacustris*
Navicula phyllepta	*Ankyra spp.*
Navicula tripunctata	*Coleochaetae spp.*
Navicula cincta	*Botryococcus spp.*
Navicula cryptocephala	*Chlamydomonas spp.*
Synedra ulna	*Ankistrodesmus spp.*
Nitzschia acicularis	*Golenkinia radiata*
Melosira spp.	*Tetrastrum staurogeniaeforme*
Amphora pediculus	*Pediastrum spp.*
Neidium spp.	*Stichococcus spp.*
Cryptophyta	*Zygnema spp.*
Cryptomonas spp.	*Sphaerocystis spp.*
Rhodomonas minuta	*Chodatella spp.*
Dinophyceae	**Euglenophyceae**
Ceratium hirundinella	*Euglena spirogyra*
Xanthophyceae	
Tribonema bombycinum	

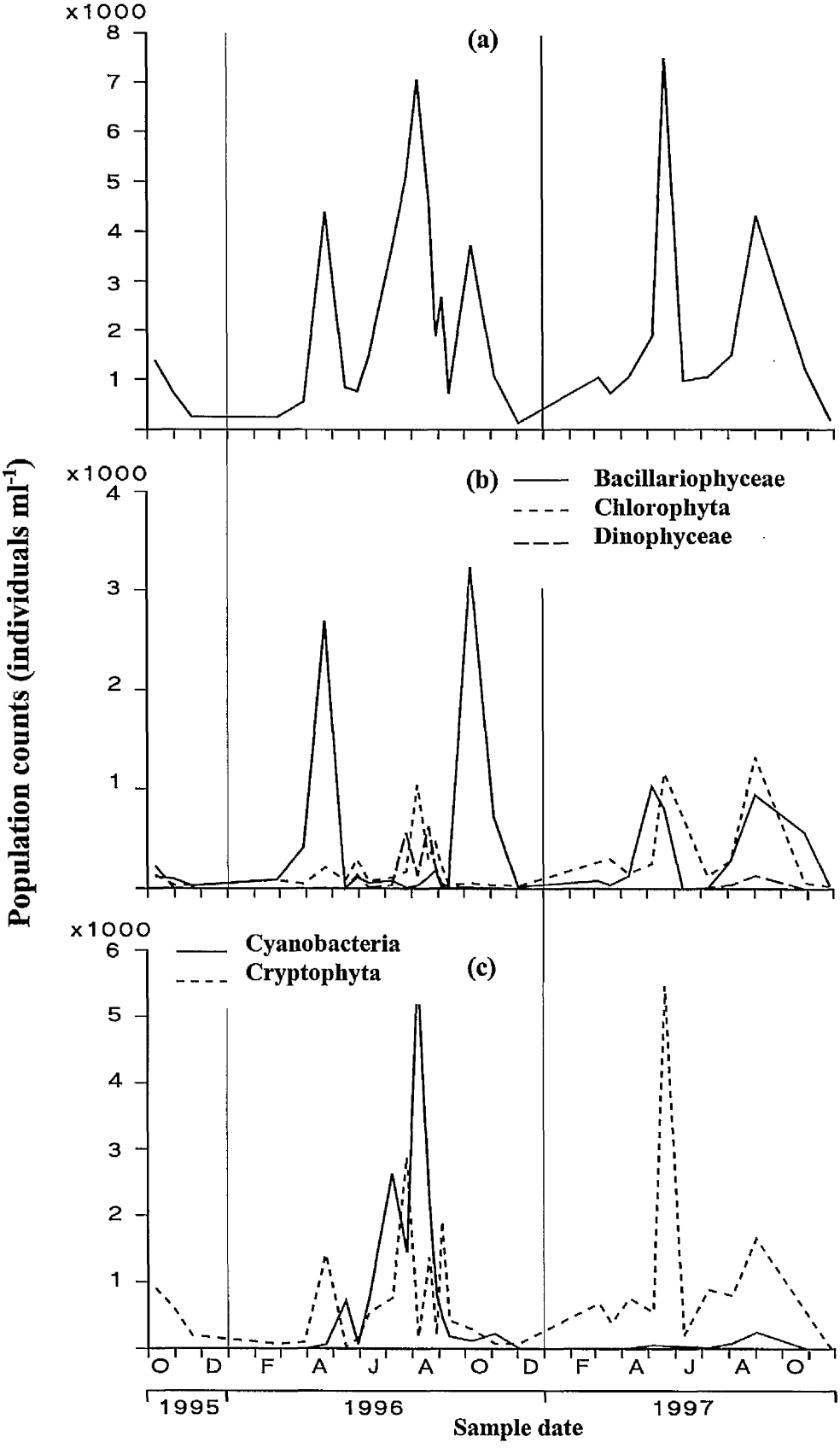


Fig. 3.7 Seasonal changes in (a) total algal population, (b) population of Bacillariophyceae, Chlorophyta and Dinophyceae, and (c) population of Cyanobacteria and Cryptophyta in Rostherne Mere from October 1995 to November 1997.

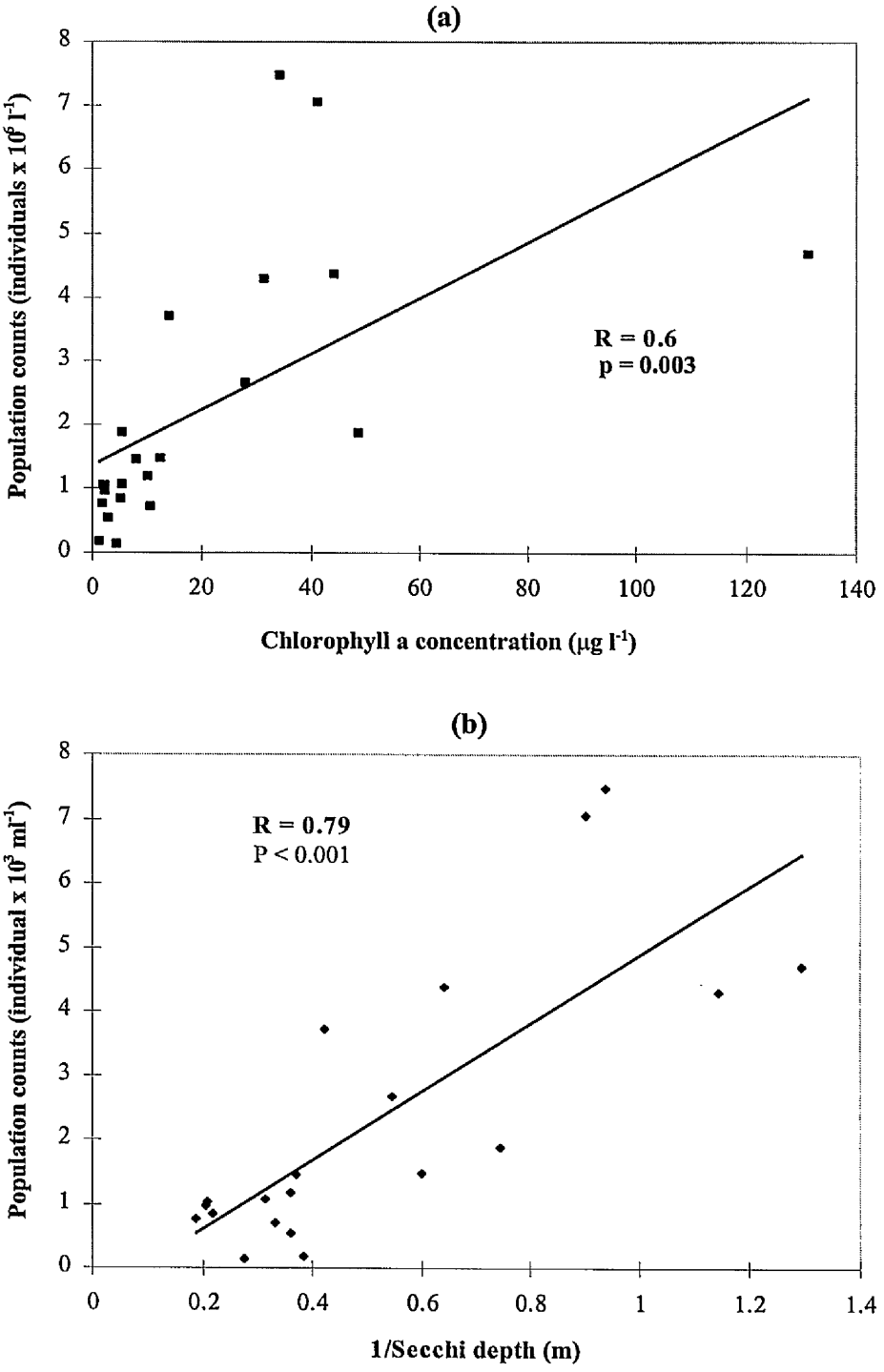


Fig. 3.8 Relationship between (a) population counts and chlorophyll a concentration, and (b) population counts and 1/Secchi depth in Rostherne Mere. (R= correlation coefficient)

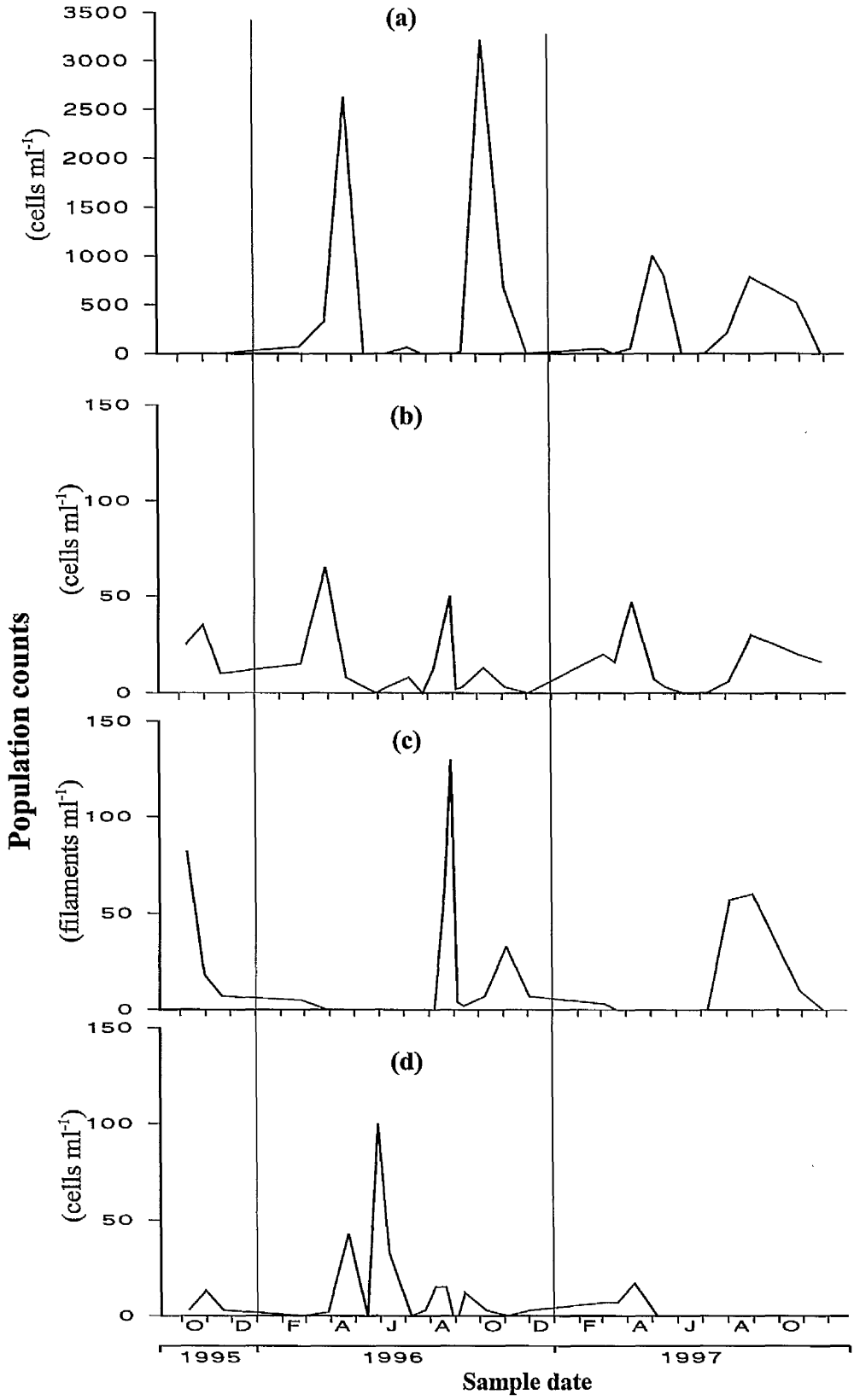


Fig. 3.9 Changes in the population of Bacillariophyceae: (a) *Asterionella formosa,* (b) *Stephanodiscus spp.,* (c) *Melosira spp.* and (d) *Nitzschia acicularis* in Rostherne Mere from October 1995 to November 1997.

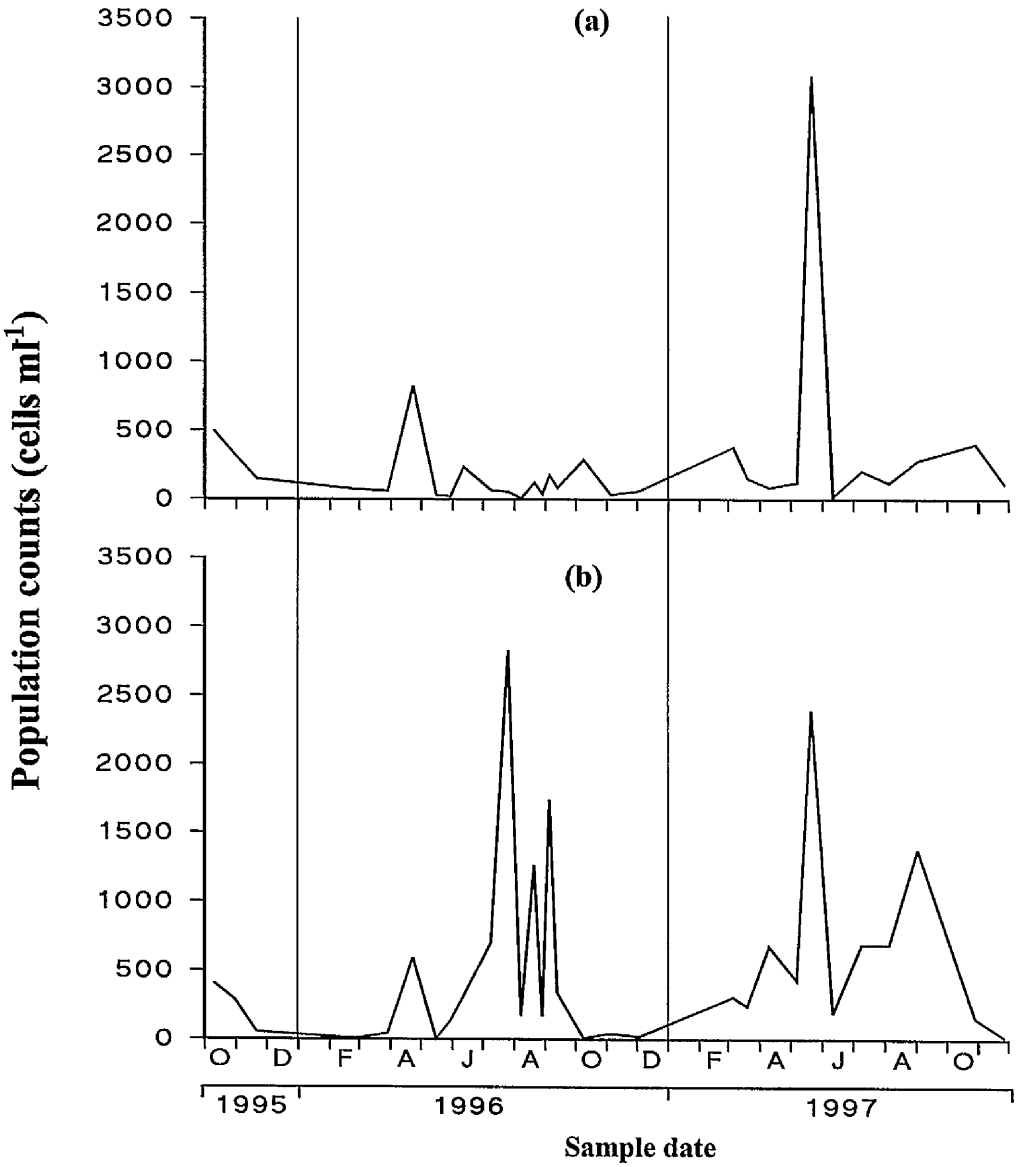


Fig. 3.10 Changes in the population of Cryptophyta: (a) *Cryptomonas spp.* and (b) *Rhodomonas minuta* in Rostherne Mere from October 1995 to November 1997.

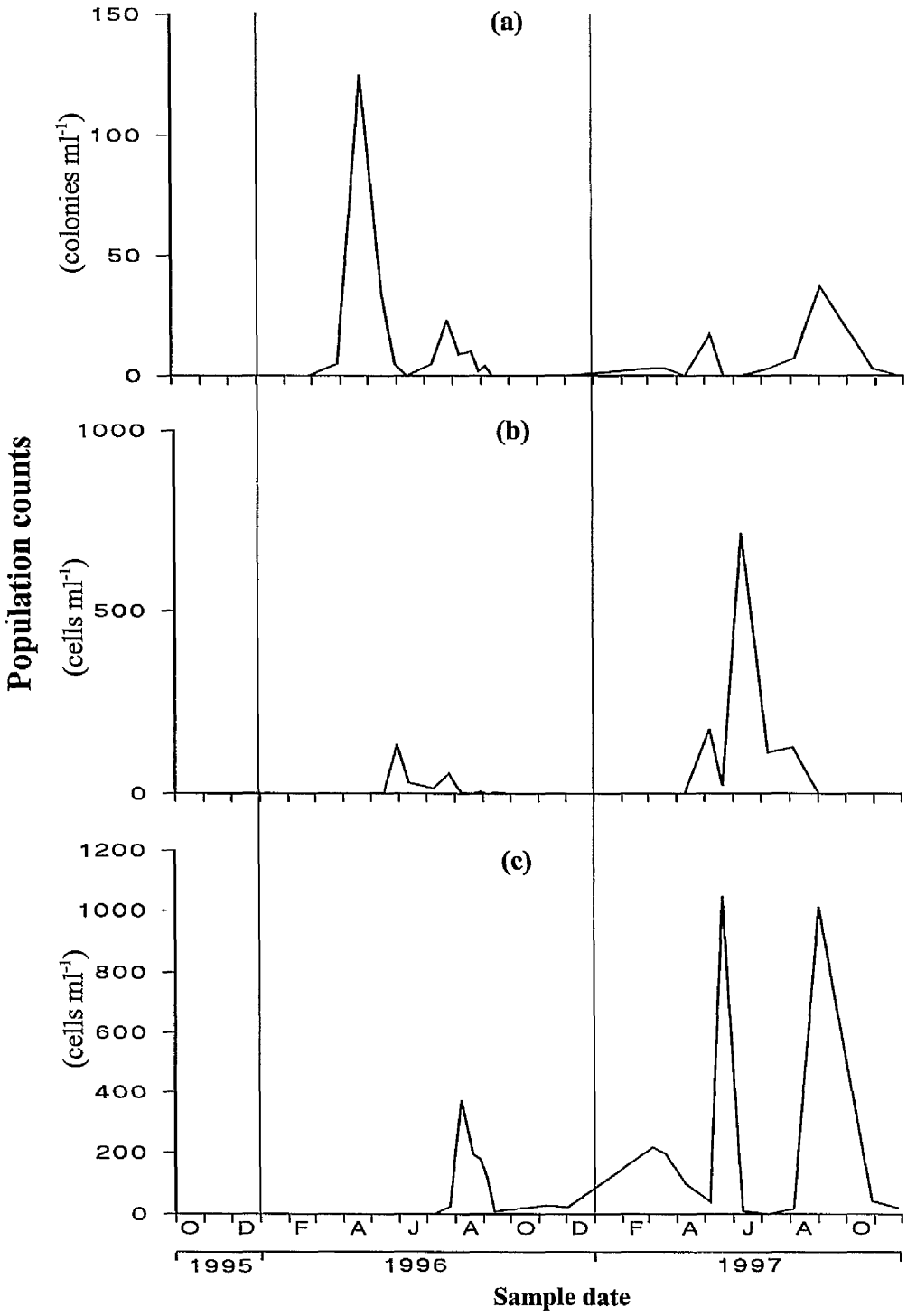


Fig. 3.11 Changes in the population of Chlorophyta: (a) *Eudorina elegans,* (b) *Ankyra spp.* and (c) *Chlamydomonas spp.* in Rostherne Mere from October 1995 to November 1997.

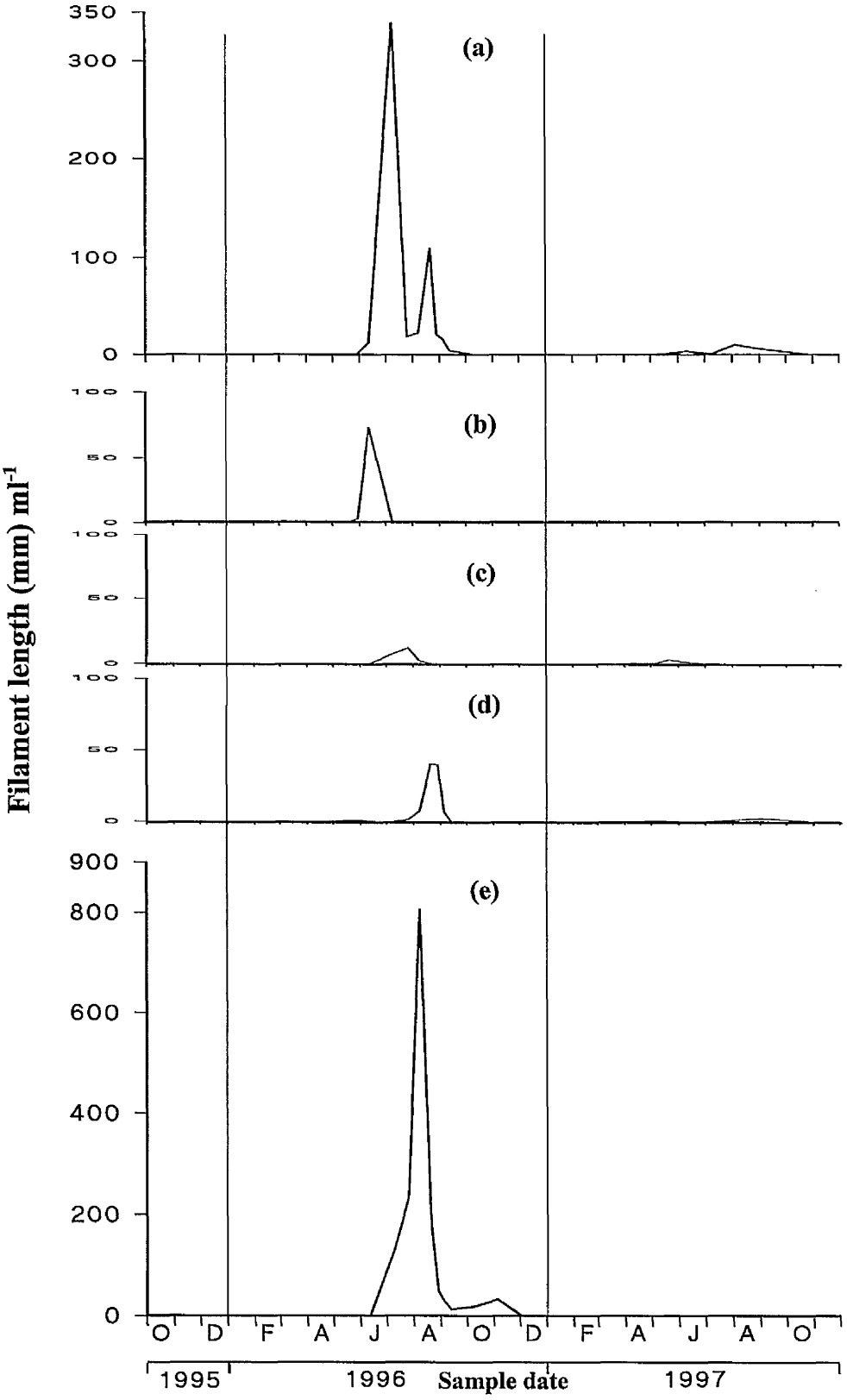


Fig. 3.12 Changes in the population of Cyanobacteria: (a) *Anabaena flos-aquae,* (b) *Anabaena spiroides,* (c) *Anabaena circinalis,* (d) *Oscillatoria spp.* and (e) *Aphanizomenon flos-aquae* in Rostherne Mere from October 1995 to November 1997.

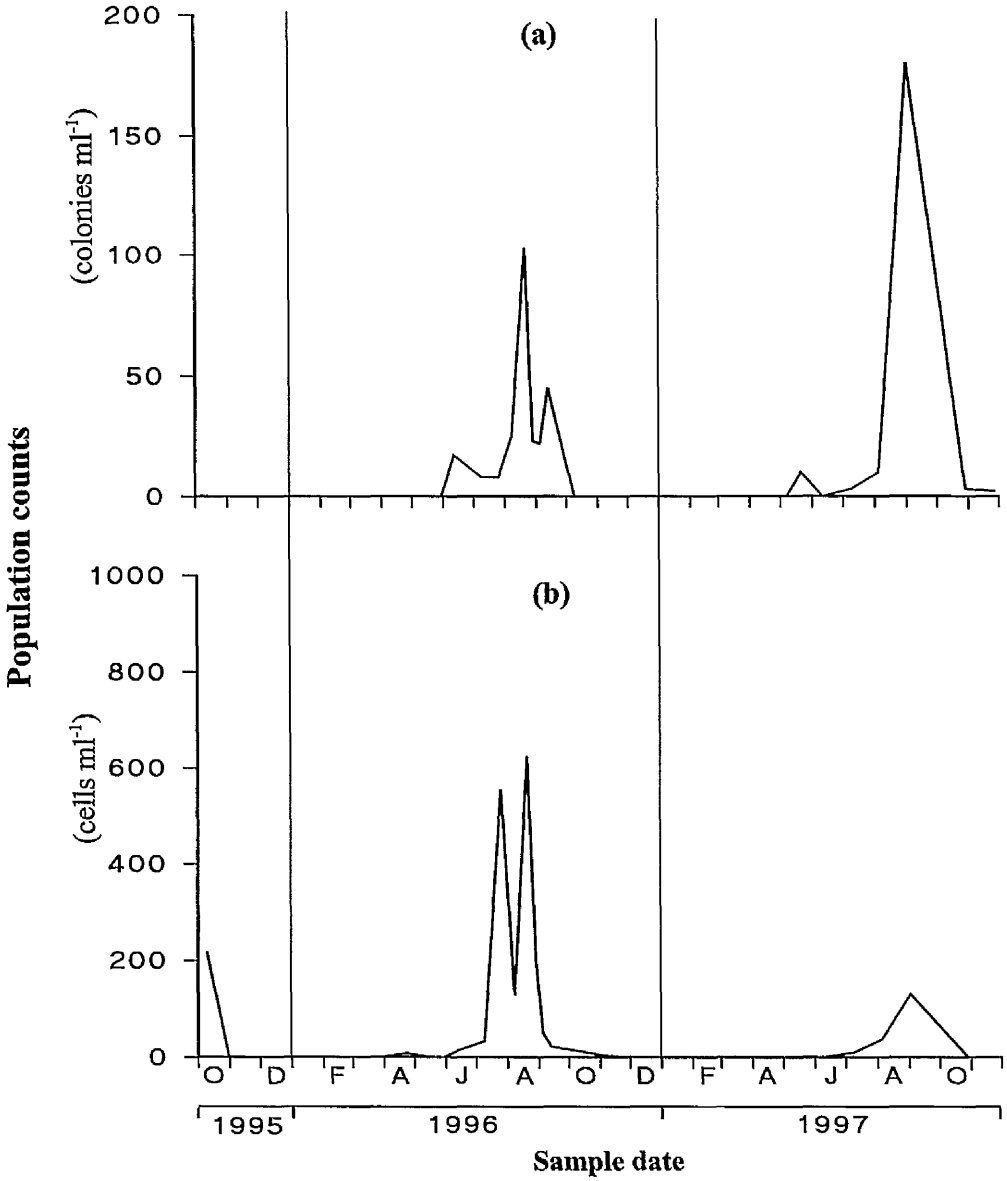


Fig. 3.13 Changes in the population of (a) Cyanobacteria *Microcystis aeruginosa* and (b) Dinophyceae *Ceratium hirundinella* in Rostherne Mere from October 1995 to November 1997.

3.2.3 Estimation of the volume of cell-associated mucilage in the phytoplankton cells from Rostherne Mere

Anabaena flos-aquae, Anabaena spiroides, Anabaena circinalis, Microcystis aeruginosa and *Eudorina elegans* were the main species having cell-associated mucilage in Rostherne Mere from October 1995 to November 1997. Mucilage volume was estimated for each species as follows:

(1) *Anabaena spp.*: Three *Anabaena* species (see above) were observed in Rostherne Mere. The total mucilage volume of these three filamentous *Anabaena* species on a particular date was calculated by subtracting the volume of a chain of spherical or elliptical cells (obtained from total cell number multiplied with cell volume without mucilage) from the volume of a long cylindrical tube. Fig. 3.14 shows an example of the detailed calculation for estimating mucilage volume of *Anabaena spiroides* collected on 12 June 1996. *Anabaena spiroides* had a single cell volume without mucilage of 62 μm^3 and a total number of 1.5×10^4 cells per ml. Thus, the total volume of a chain of spherical cells was 9.3×10^5 μm^3 ml^{-1} (Fig. 3.14.A2). The volume of the cylindrical tube was 1.2×10^7 μm^3 ml^{-1} (Fig. 3.14.A1). Therefore, the total mucilage volume of *Anabaena spiroides* on 12 June 1996 was 1.1×10^7 μm^3 ml^{-1}. Fig. 3.17 shows the mucilage volume of each *Anabaena* species over the sampling period.

(2) *Eudorina elegans*: Mucilage volume per colony of *Eudorina elegans* was obtained by subtracting total cell volume per colony (A3) from colony volume (A4) as shown in Fig. 3.15. The total mucilage volume of *Eudorina elegans* on a particular sampling date was obtained by multiplying the number of colonies on the sampling date by the average mucilage volume per colony. Fig. 3.15.A5 shows an example of the

calculation of total mucilage volume per ml of *Eudorina elegans* on 23 April 1996 and Fig. 3.18(a) shows the mucilage volume of *Eudorina elegans* over the sampling period.

(3) *Microcystis aeruginosa*: The average mucilage volume per colony was obtained by multiplying the average mucilage volume per cell with the average cell number per colony. Fig. 3.16 shows the detailed calculation. Mucilage volume per cell was calculated by subtracting the cell volume without mucilage from the cell volume with mucilage (Fig. 3.16.A6). Average mucilage volume was obtained from 50 cells, giving the mean value of 105 μm^3 per cell on 21 August 1996. The average colony volume was also calculated from 50 colonies, giving a value of 1.2×10^5 μm^3 for the 21 August 1996 sample. As the cells of *Microcystis* were densely packed together within the mucilage, it is presumed that there is no additional space between them. Therefore, the average cell number per colony was calculated by dividing the average colony volume by the mean cell volume with mucilage (as shown in Fig. 3.16.A7). The average cell number per colony was approximately 800 cells on the above date, giving an average mucilage volume per colony of 8.4×10^4 μm^3. The total mucilage volume of *Microcystis aeruginosa* on a particular sampling date was obtained by multiplying the number of colonies per ml on the sampling date by the average mucilage volume per colony. On 21 August 1996, this was 8.7×10^6 μm^3 ml^{-1} (Fig. 3.16.A8). Fig. 3.18(b) shows the total mucilage volume of *Microcystis aeruginosa* on each sampling date.

Overall changes in cell-associated mucilage

Total changes in cell-associated mucilage are shown for the five main mucilage-producing species in Fig. 3.17 and 3.18. Values are expressed as mucilage volume per ml lake water, as determined by the above calculations.

A high cell-associated mucilage volume occurred during the late spring and summer, with a maximum of 7×10^7 μm^3 ml^{-1} in September 1997. Total mucilage volume occupied only less than 0.0018% of lake water volume for most of the sampling period, except in September 1997 when it rose to 0.007% of the lake water volume [Fig. 3.18(c)].

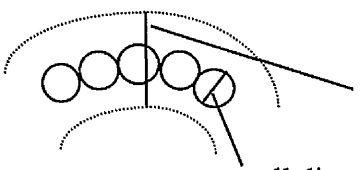
In 1996, total mucilage volume fluctuated between 1.17×10^6 μm^3 ml^{-1} (occupying 0.0001% of the lake water volume) and 1.76×10^7 μm^3 ml^{-1} (occupying 0.0018% of the lake water volume) from April to September. The highest surface mucilage biomass occurred in April, and was due to the abundance of *Eudorina elegans* [Fig. 3.18(a)]. *Anabaena spiroides* and *Microcystis aeruginosa* accounted for most of mucilage biomass in June (86% algal mucilage) and September (98% algal mucilage) respectively as they bloomed at this time [Fig. 3.17(b) and Fig. 3.18(b)]. Although *Anabaena flos-aquae* dominated the population community during the summer, it did not have a high mucilage volume [Fig. 3.17(a)] reaching a maximum of 65% of total algal mucilage on 09 July.

In 1997, *Microcystis aeruginosa* was the most abundant mucilage-producing species, giving the highest mucilage biomass (6.7×10^7 μm^3 ml^{-1}) and accounting for most (96% algal mucilage) of the total mucilage biomass in September [Fig. 3.18(b)&(c)]. *Anabaena flos-aquae, Anabaena circinalis* and *Eudorina elegans* were also present, but contributed only a small amount to the total mucilage volume, with less than 2×10^6 μm^3 ml^{-1} for these species [Fig. 3.17(a)&(c) and Fig. 3.18(a)].

Horizontal variation

Horizontal variation of cell-associated mucilage volume was investigated on 29 August 1996 with samples collected from 9 sites (A1, A2, A3, B1, B2, B3, C1, C2, C3). Three main mucilage-producing species were found: *Anabaena flos-aquae,*

Microcystis aeruginosa and *Eudorina elegans.* As shown in Fig. 3.19, site C1 had the highest total cell-associated mucilage volume (4.6×10^6 μm^3 ml^{-1}) and site B1 had the least (4.5×10^5 μm^3 ml^{-1}). The highest mean total mucilage volume (3.8×10^6 μm^3 ml^{-1}) was found within the site C area (derived from sites C1, C2, C3), followed by that (2×10^6 μm^3 ml^{-1}) within the site B area and that (1.7×10^6 μm^3 ml^{-1}) within the site A area. *Eudorina elegans* only appeared in sites A3 and C1, contributing 1.4×10^6 μm^3 ml^{-1} of mucilage volume. The mucilage volume of *Anabaena flos-aquae* between the 9 sites within the lake fluctuated between 1.5×10^5 μm^3 ml^{-1} to 6×10^5 μm^3 ml^{-1}. High volumes of mucilage resulted from the presence of *Microcystis aeruginosa,* accounting for at least 60% of total mucilage volume from each site, except sites A3 and B1 where no *Microcystis* colonies were present. The total mucilage volume was similar ($p>0.05$ using the nonparametric Mann-Whitney U-Wilcoxon Rank Sum W test) between the site A (A1, A2, A3) and the site B (B1, B2, B3) areas, and between the site B and C areas, but different ($p<0.05$) between the site A and C areas.



Filament diameter, 2R = 14.2 μm

cell diameter without mucilage, 2r = 4.9 μm

Total filament length (h) measured on 12 June 1996 = 7.4×10^4 μm ml^{-1}

So, total filament volume with mucilage:

$\pi R^2 h$ (a cylindrical tube)= $\pi(7.1)^2(7.4 \times 10^4) = 1.2 \times 10^7$ μm^3 ml^{-1}.....(A1)

Cell volume: $4/3\pi r^3$ (a sphere) = $4/3\pi(2.45)^3 = 62$ μm^3

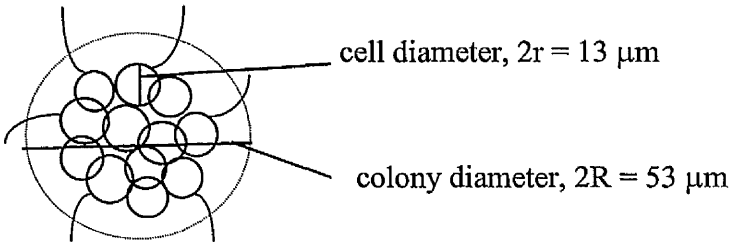
Total cell number counted on 12 June 1996 = 1.5×10^4 cells ml^{-1}

So, total volume of the chain of spherical cells=$62 \times (1.5 \times 10^4) = 9.3 \times 10^5$ μm^3 ml^{-1}...(A2)

Therefore, the total mucilage volume of *Anabaena spiroides* on this date:

A1-A2 = 1.1×10^7 μm^3 ml^{-1}

Fig. 3.14 Calculation of the mucilage volume of *Anabaena spiroides* (data from samples collected on 12 June 1996).



cell volume = $4/3\pi r^3$ (a sphere) = $4/3\pi(6.5)^3 = 1.15 \times 10^3$ μm^3

cell number per colony = 12

So, total cell volume per colony = $(1.15 \times 10^3) \times 12 = 1.38 \times 10^4$ μm^3 .....(A3)

Colony volume = $4/3\pi R^3 = 4/3\pi(26.5)^3 = 7.8 \times 10^4$ μm^3.....(A4)

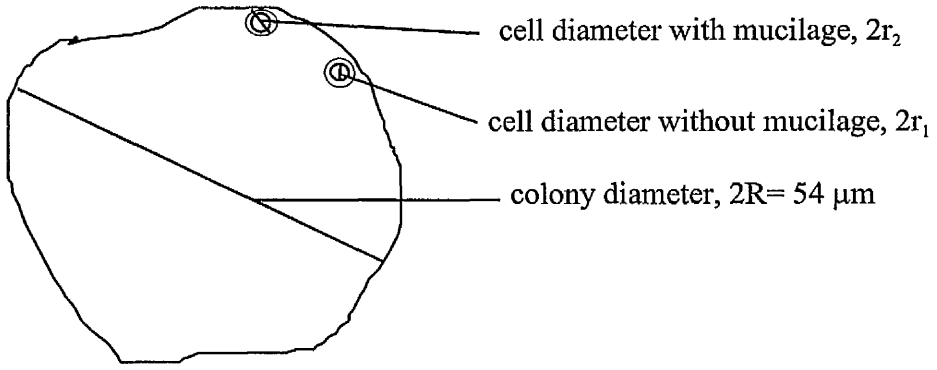
Thus, mucilage volume per colony = A4-A3 = 6.42×10^4 μm^3

On 23 April 1996, the mean mucilage volume per colony was 1.4×10^5 μm^3, obtained from 30 colonies.

Therefore, total mucilage volume ml^{-1} on this date

= 125 colonies $ml^{-1} \times 1.4 \times 10^5$ μm^3 $colony^{-1} = 1.75 \times 10^7$ μm^3 ml^{-1}.....(A5)

Fig. 3.15 Calculation of the mucilage volume of *Eudorina elegans* (data from samples collected on 23 April 1996).



- For one particular cell: $2r_2 = 7\mu m$, $2r_1 = 5\mu m$

So, cell volume with mucilage = $4/3\pi(3.5)^3 = 180\ \mu m^3$

cell volume without mucilage = $4/3\pi(2.5)^3 = 65\ \mu m^3$

Mucilage volume per cell = 180-65 = 115 μm^3.....(A6)

- For one colony:

Average mucilage volume per cell (n=50) = 105 μm^3

Colony volume = $4/3\pi(27)^3 = 8.2 \times 10^4\ \mu m^3$

- Population of colonies on 21 August 1996:

Average colony volume (n=50) = $1.2 \times 10^5\ \mu m^3$

Average volume of cell + mucilage (n=50) = 149 μm^3

So, average cell number per colony = $(1.2 \times 10^5)/149 \cong 800$.....(A7)

Thus, average mucilage volume per colony = $105 \times 800 = 8.4 \times 10^4\ \mu m^3$

Therefore, total mucilage volume on 21 August 1996

= 103 colonies $ml^{-1} \times 8.4 \times 10^4\ \mu m^3\ colony^{-1} = 8.7 \times 10^6\ \mu m^3\ ml^{-1}$.....(A8)

Fig. 3.16 Calculation of the mucilage volume of *Microcystis aeruginosa* (data from samples collected on 21 August 1996).

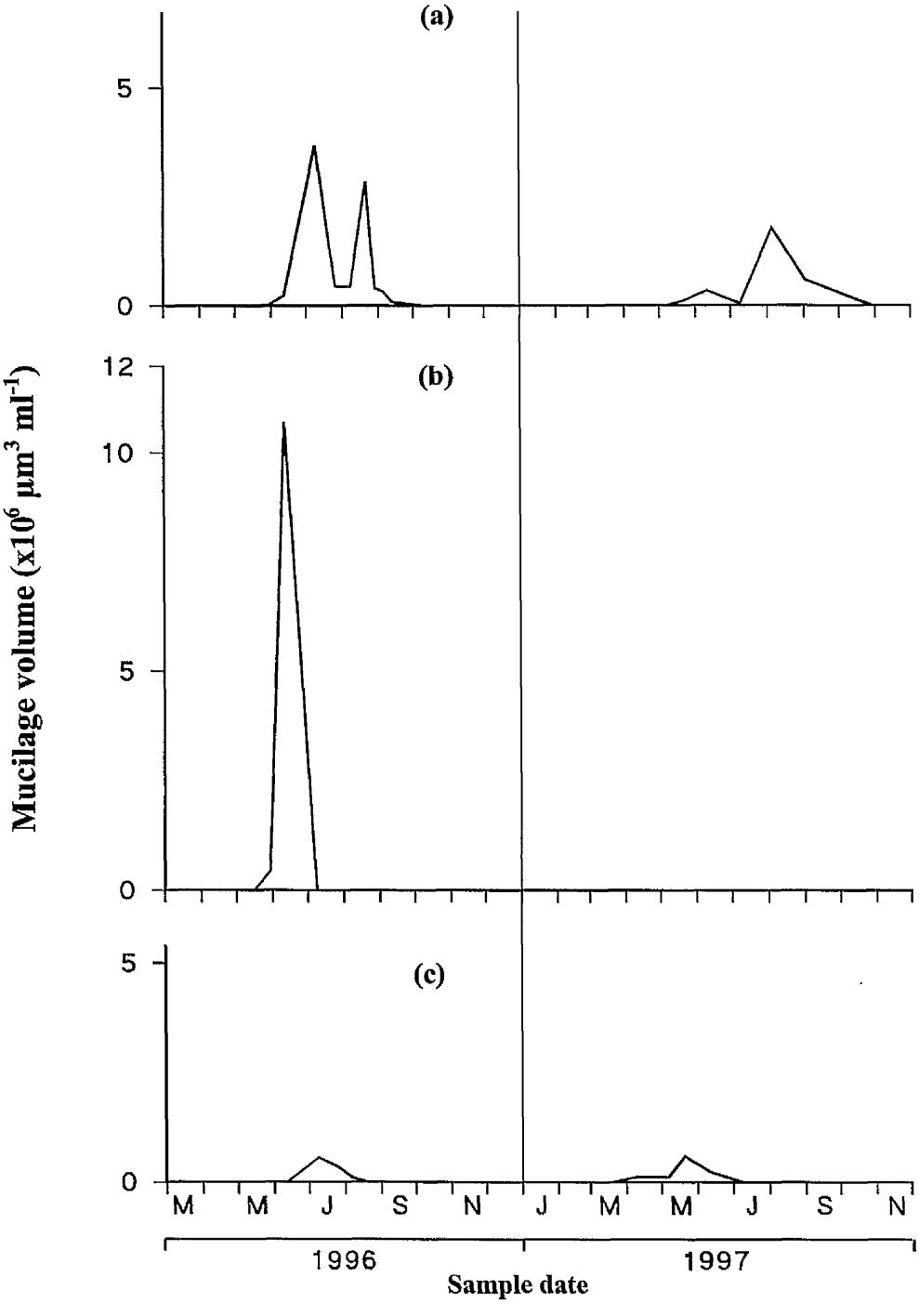


Fig. 3.17 Seasonal variation in cell-associated mucilage volume of (a) *Anabaena flos-aquae,* (b) *Anabaena spiroides* and (c) *Anabaena circinalis* in Rostherne Mere from October 1995 to November 1997.

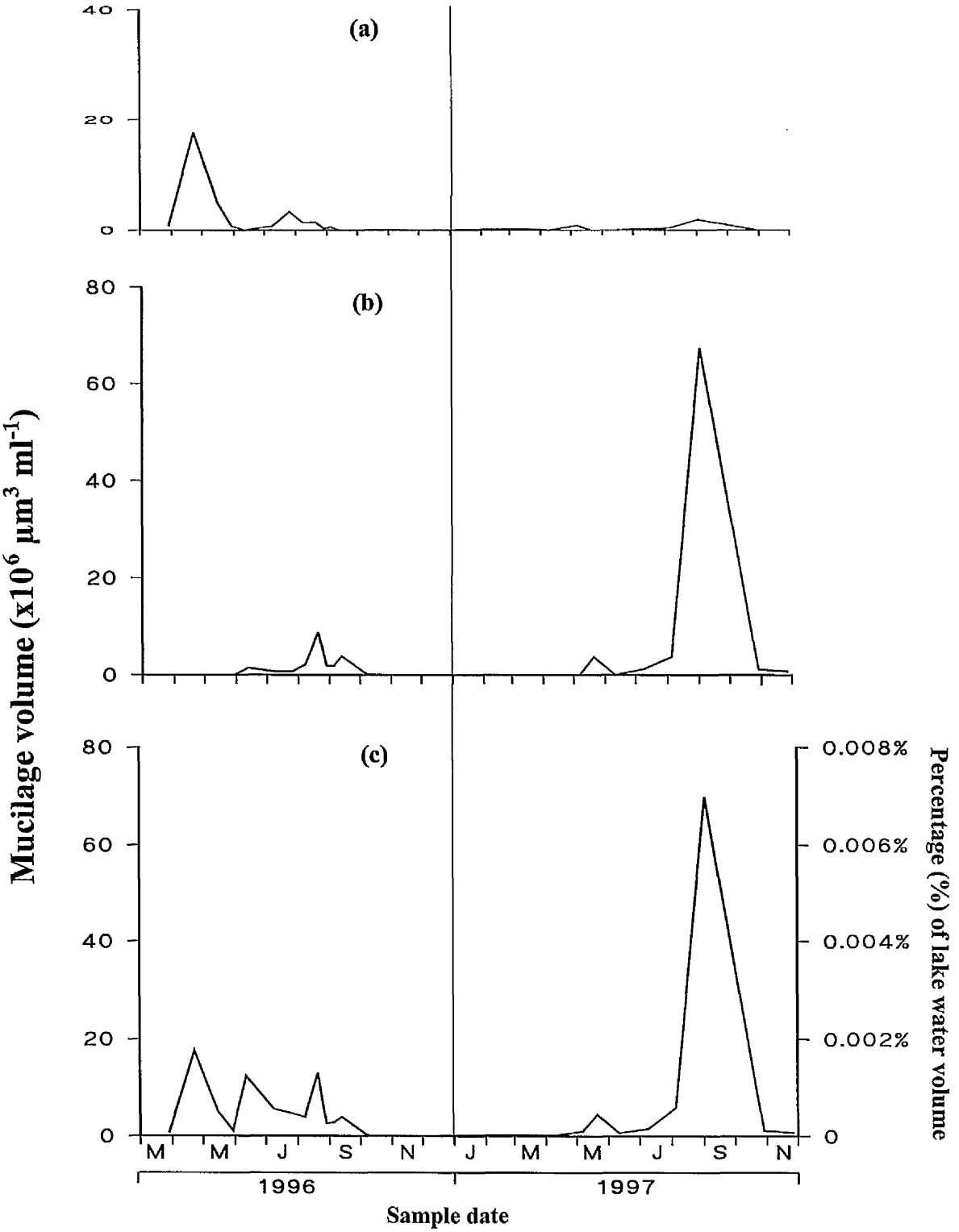


Fig. 3.18 Sasonal variation in cell-associated mucilage volume of (a) *Eudorina elegans,* (b) *Microcystis aeruginosa* and (c) total of the main mucilage-producing species in Rostherne Mere from October 1995 to November 1997. The percentage of the lake water occupied by mucilage produced by the five species is also shown in (c).

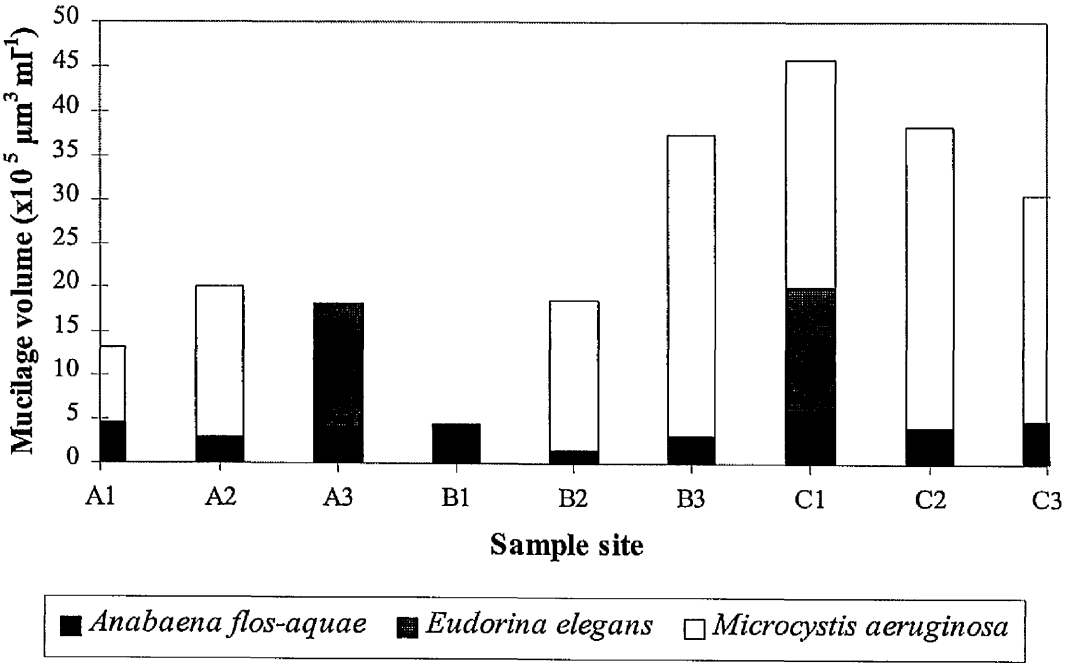


Fig. 3.19 Variation in mucilage volume of the three main species with associated mucilage, *Anabaena flos-aquae, Microcystis aeruginosa* and *Eudorina elegans,* collected from Rostherne Mere on 29 August 1996.

3.2.4 Quantification of soluble extracellular polysaccharides in lake water

Soluble extracellular polysaccharides (EPS) in lake water were determined using the assay described in Chapter 2. Section 2.2.3. Using this assay, the colorimetric absorbance showed a strong linear relationship (R^2>0.99) with polysaccharide concentration using pig mucin as a standard (Fig. 3.20).

Changes in mean concentration of soluble EPS, derived from sites A, B and C, are shown in Fig. 3.21. Soluble EPS concentrations showed considerable variation over the annual cycle, ranging from 2.5 mg l^{-1} to 60 mg l^{-1}. Each set of measurements included mucin standards which gave consistent readings indicating that measured differences in lake water polysaccharide concentration were genuine. Concentrations of soluble EPS were low (below 10.38 mg l^{-1}) during winter and spring, and increased markedly in the summer up to the autumn overturn, with peak values in May (37.7 mg l^{-1}), July (60.5 mg l^{-1}) and August (59.7 mg l^{-1}) 1997. However, in June the concentration decreased markedly to 8 mg l^{-1}.

The high concentration of soluble EPS in May 1997 corresponded with the algal late spring bloom (dominant species: *Asterionella formosa,* cryptophyta and *Chlamydomonas spp.*) where there were small amounts of cell-associated mucilage. The peak of chlorophyll a concentration in May 1997 was followed by the further peak in soluble EPS in July-August 1997. No increase in soluble EPS occurred at the time of the autumnal (September) bloom where large amounts of associated mucilage occurred in relation to *Microcystis aeruginosa* (Fig. 3.21).

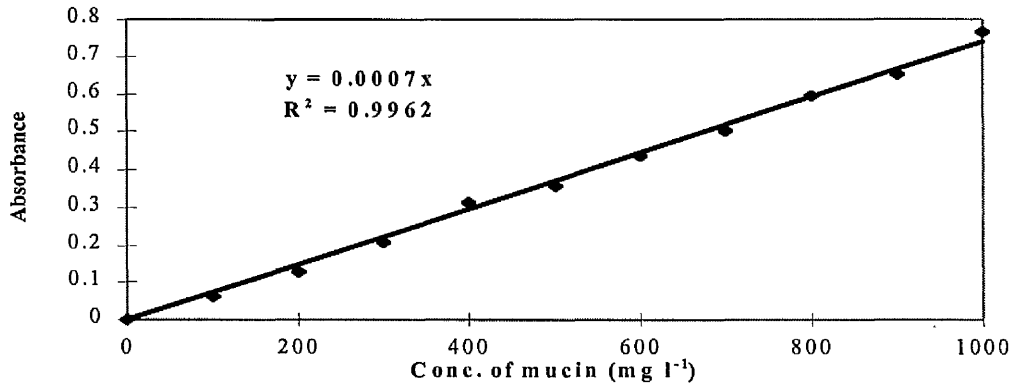


Fig. 3.20 Relationship between concentration of pig mucin (mg l^{-1}) and absorbance at a wavelength of 565nm using the method from Jugdaohsingh et al. (1998). (R^2: coefficient of determination)

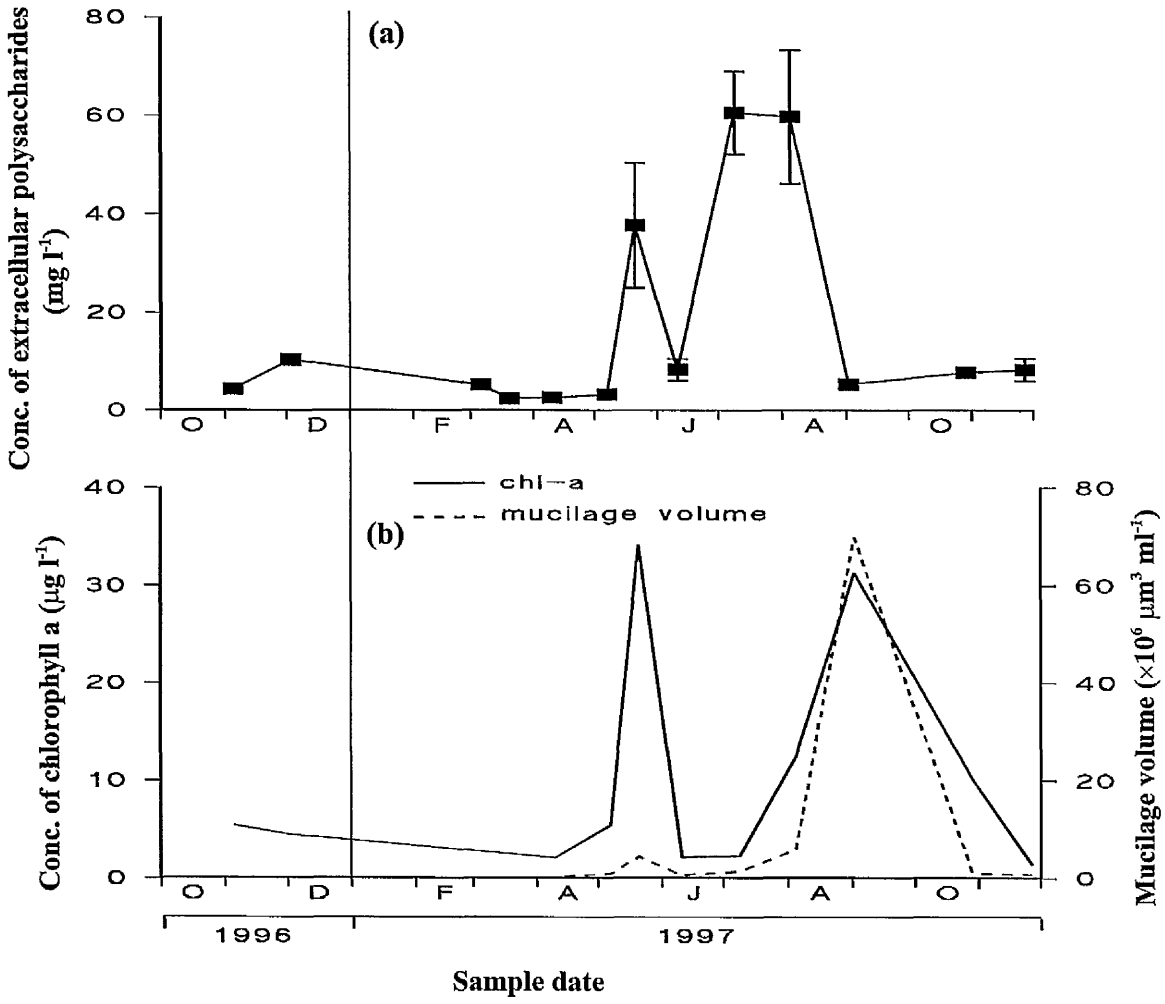


Fig. 3.21 Seasonal changes in (a) soluble extracellular polysaccharides, and (b) chlorophyll a concentration and associated mucilage volume in Rostherne Mere from November 1996 to November 1997. (Error bars represent standard deviation of one measurement from three sampling sites.)

3.3 Discussion

In this section, cell-associated and soluble extracellular polysaccharides were investigated over up to two annual cycles. Physico-chemical characteristics of the lake (particular in relation to stratification) and changes in the phytoplankton community were also examined in relation to changes in mucilage.

3.3.1 Lake stratification

The temperature profile indicated clear stratification between May and October 1996 and 1997. This is similar to the observations made during 1988 and 1989 by Clay (1992) and during 1990 and 1991 by Carvalho (1993). A thermocline (metalimnion) typically formed between 6 m and 10 m in the water column, with the hypoliminion extending from 10 m to the maximum depth of the lake (30 m).

The development of a deoxygenated hypolimnion coincided with the development of stratification in 1996 and 1997, as was also recorded by Carvalho (1993). A well developed oxycline formed between 6 m and 10 m during the summer months in 1996 and 1997 clearly correlated with the position of the thermocline (metalimnion). The oxygen distribution with depth in Rostherne Mere [Fig. 3.3(b)] corresponds to a clinograde curve and indicates a high level of lake productivity. The marked minimum in oxygen concentration seen in the metalimnion may result from oxygen consuming organisms (e.g. zooplankton) being concentrated at this depth (Hutchinson, 1957). Percentage oxygen saturation in Rostherne Mere fell below 5% at around 16 m depth in October 1996 and 1% at 17-20 m in August 1997, showing the severe loss of oxygen typical of a eutrophic lake.

The high pH seen during 1996 and 1997 coincided with a high phytoplankton biomass (R=0.52). This is due to uptake of CO_2 by phytoplankton for photosynthesis, exhausting the supply of CO_2, and causing bicarbonate ions to react with water,

yielding more CO_2 and carbonate ions. The carbonate ions react with water to produce hydroxyl ions (OH^-) and therefore pH rises (Carvalho, 1993). Eutrophic species have the ability to use both free carbon dioxide and bicarbonate ions or to use free CO_2 at very low levels (Moss, 1973). This emphasizes the importance of pH in influencing of the phytoplankton community, and high pH is often an indicator of a eutrophic lake.

3.3.2 Changes in algal biomass and species composition

Secchi depth is a measurement of the transparency of lake water. The greatest Secchi depths in May 1996 and between June and July 1997 were due to the breakdown of the spring diatoms blooms as the heavy dead diatoms rapidly sunk through the water column reducing the turbidity of the epilimnion. Algal blooms in spring and late summer corresponded with low Secchi depths (Fig. 3.4). The results demonstrated how the clarity of the lake varied with phytoplankton population (inversely correlated with R= - 0.66). The succession of Secchi depths in 1996 and 1997 was similar to that in 1990 to 1992, but the highest Secchi depth of 8 m occurred in May 1991 (Carvalho, 1993).

Rostherne Mere contained relatively few species of phytoplankton but supported a large biomass. It also displayed a phytoplankton succession similar to other eutrophic lakes (categorized by Reynolds, 1993), therefore confirming that Rostherne Mere remains a eutrophic system.

The succession of algal species found in this study was similar to that reported by other studies of Rostherne Mere since the 1970s (Reynolds, 1978b ; Booth, 1988 ; Reynolds & Bellinger, 1992 ; Clay, 1992 ; Carvalho, 1993), but with variations in some of the dominant and subdominant species. Differences in dominant species between October 1995 to November 1997 may be due to variation from one year to the next in physical (e.g. light, oxygen, and turbulence: Reynolds & Bellinger, 1992) and

chemical conditions (e.g. nutrients). For example, *Anabaena spp.* and *Aphanizomenon flos-aquae* bloomed in 1996's summer but were only present in low numbers in 1997's, while surface water temperatures and nitrate concentrations in 1997 were higher than those in 1996.

The succession of species composition of phytoplankton may be considered as five major phases: spring diatom bloom, clear water stage with abundance of chlorophyta or cryptophyta, summer cyanobacteria bloom, autumn diatom bloom and winter clear water stage. In the following discussion, particular emphasis is given to the major mucilage producers- including cyanobacteria and bacillariophyceae (diatoms).

The dominance of the diatom *Asterionella formosa* in spring and autumn may be attributed to their being cold-water organisms (with high photosynthetic efficiency at low light and low temperature) and the availability of sufficient nutrients, particularly Si (Fig. 3.22). This is derived from the hypolimnion and sediments through mixing within the lake and from the inflow water (Hutchinson, 1967 ; Reynolds, 1993). The peaks of diatom growth coincided with the onset and breakdown of stratification of the lake in 1996 and 1997. This may be because diatoms required high water turbulence to remain in the uppermost layers of lakes due to their rather heavy silica cell wall (Van Den Hoek, et al., 1997). The rapid decline of the spring and autumn bloom corresponded with the decline of silicon concentration, but there was little decline in nitrate and phosphate (Fig. 3.22). It may be that silicon was the major limiting factor in the *Asterionella* bloom, though parasitism by fungi chytrids also occurred and might be another limiting factor. Carvalho (1993) also suggested that silicon limited the spring diatom bloom in Rostherne Mere in 1990 and 1991.

Mucilage-producing *Eudorina elegans* attained its maximum biomass in April 1996, then declined quickly. This pattern is similar to that observed by Reynolds and Reynolds (1985) who found the characteristic growth of *Eudorina* was sustained only briefly, reaching a maximum at the beginning of May 1972 in Crose Mere. However, they found *Eudorina* again bloomed in June, but in this study it remained at low densities throughout the rest of the year.

Large numbers of the mucilage-producing cyanobacteria *Anabaena spp.* and *Microcystis spp.* were found in summer in Rostherne Mere during 1996 and 1997. In 1996, *Anabaena spp.* dominated in the early part of the summer, followed by *Aphanizomenon flos-aquae* and *Microcystis aeruginosa*. Although nutrient levels decreased with the lowest nitrate and phosphate concentrations occurring in August (Fig. 3.22), large numbers of *Anabaena spp.* and *Aphanizomenon flos-aquae* were present. This may be due to their ability to fix elemental nitrogen. *Microcystis* colonies are able to survive and produce high populations under conditions of low nutrient (N and P) level due to the presence of intracellular nutrient reserves and the ability of these colonies to migrate into the nutrient-rich hypolimnion (Reynolds et al. 1981). The growth of *Anabaena* and *Aphanizomenon* before *Microcystis* may be because the former can grow well at temperatures which are marginally below those preferred by *Microcystis* (Reynolds & Bellinger, 1992).

Planktonic cyanobacteria can succeed during stratified conditions due to their ability to regulate their position by gas vacuoles in the water column to depths most favourable for growth (Ganf and Oliver, 1982). Therefore, cyanobacteria could float near the water surface to obtain enough light for photosynthesis and shade out competing algae. This probably accounts for the dominance of cyanobacteria in summer, generating a high biomass which corresponded to the minimum recorded

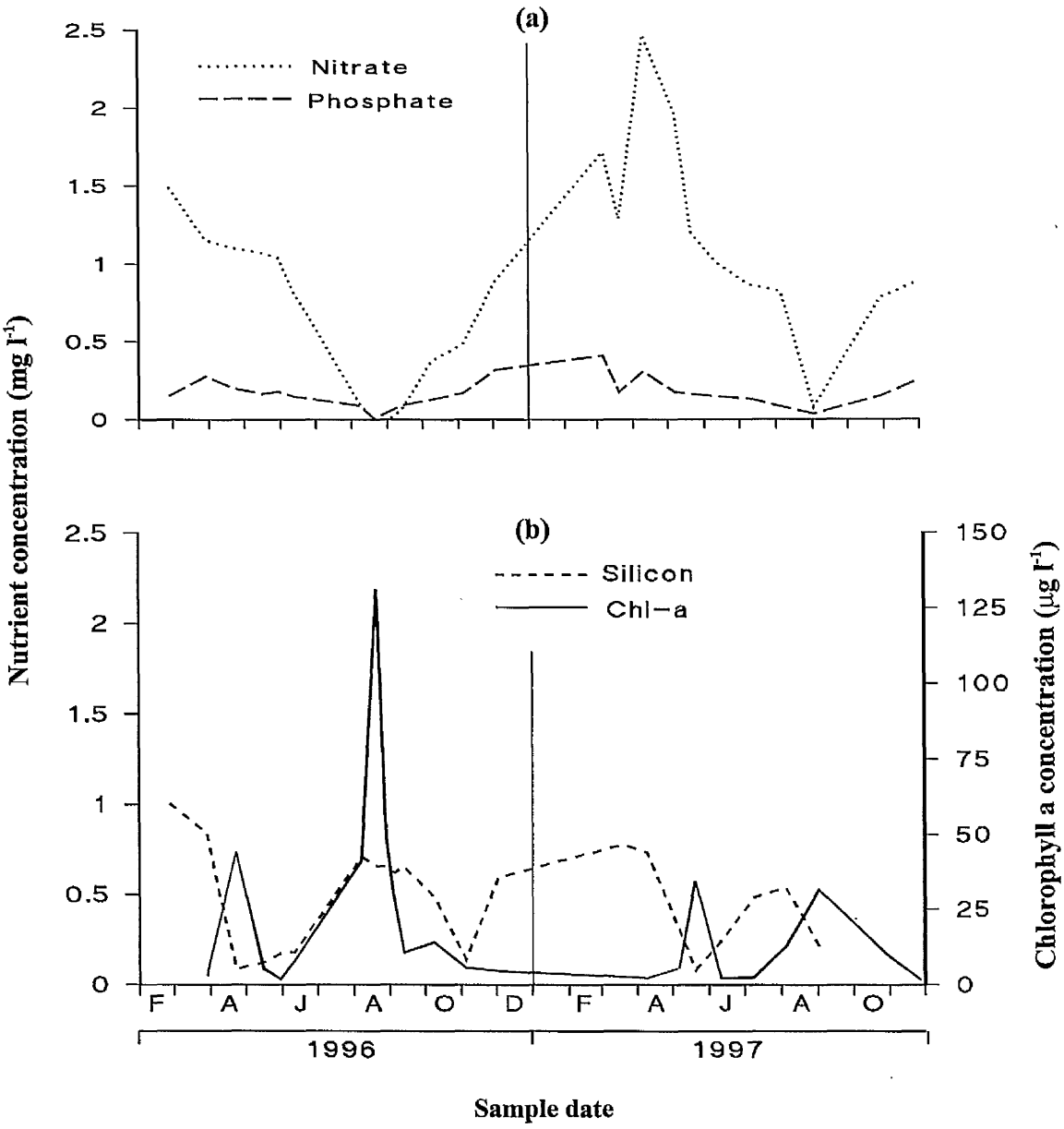


Fig. 3.22 Comparison of seasonality of concentration of (a) nitrate and phosphate, and (b) silicon and chlorophyll a in Rostherne Mere from February 1996 to November 1997. (redrawn from data provided by V. Krivtsov and E. Levado)

Secchi depth (0.8 m) in August. Failure of zooplankton to graze may be another reason for the bloom of cyanobacteria. Filter-feeding zooplankton exhibit size-selective feeding behaviour and the relatively large cyanobacteria may be thus have a selective advantage under grazing pressure (Richman et al., 1990).

However, in 1997, *Anabaena spp.* and *Aphanizomenon flos-aquae* only appeared in small numbers. Why those two genera failed to dominate in the summer of 1997 is not clear but may relate to the greater availability of nitrate. Ernst (1990) found that if nitrate is present, nitrogen fixation, an energy-consuming process, does not give *Anabaena* an ecological advantage over species with a requirement for nitrate.

Microcystis aeruginosa was found in late summer in both 1996 and 1997. At this time, CO_2 became limiting because of intensive photosynthesis. As *Microcystis* was more able to tolerate CO_2-limiting conditions compared to diatoms such as *Asterionella formosa* (Talling, 1976), this could explain the presence of *Microcystis* at this time. Paerl and Ustach (1982) suggested that cyanobacteria were able to use CO_2 at the air-water interface when blooms form. The development of *Microcystis aeruginosa* was very rapid with an increase from less than 10 colonies ml^{-1} to over 100 colonies ml^{-1} between July to September. This may be because vegetative colonies of *Microcystis* are able to sink and overwinter on the sediment, and then rapidly re-establish themselves when suitable conditions occur (Reynolds, 1979). Growth of *Microcystis* on the lake sediment and transition to a planktonic phase depends on conditions of high light penetration and oxygen depletion during the early part of the summer (Reynolds et al., 1981). *Microcystis* blooms at Rostherne Mere were preceded by a high Secchi depth and low hypolimnion saturation, in agreement with this. The decline in cyanobacteria, in particular *Microcystis,* coincided with the breakdown of stratification. This agrees with the statement made by Reynolds (1978b) that the

periodicity of *Microcystis aeruginosa* in Rostherne Mere is closely related to the annual cycle of stagnation and de-stratification.

To summarize, seasonal variations in the phytoplankton assemblage was strongly influenced by physico-chemical and biological factors in the lake during the sampling period. The success of particular phytoplankton species correlated with optimum conditions for the species' requirements.

3.3.3 Quantification of cell-associated mucilage and soluble extracellular polysaccharides

As mentioned in section 1.1.1, there are two forms of mucilage in the aquatic environment, cell-associated (insoluble) and not cell associated (soluble) mucilage. Insoluble mucilage was estimated in species with cell-associated mucilage and the soluble form was measured as soluble extracellular polysaccharides in lake water. Both were quantified in Rostherne Mere throughout the year.

3.3.3.a Variation in the volume of cell-associated (phytoplankton) mucilage in Rostherne Mere

Five of the main species found in Rostherne Mere from October 1995 to November 1997 possessed cell-associated mucilage. Four were cyanobacteria (*Anabaena spiroides, Anabaena flos-aquae, Anabaena circinalis* and *Microcystis aeruginosa*) and one was a member of the chlorophyta (*Eudorina elegans*). This indicates that cyanobacteria were the major algae accounting for much of the cell-associated mucilage production in Rostherne Mere and is in agreement with the observations made by Grossart and Simon (1993), who found that macroscopic mucilaginous aggregates ('lake snow') occurred from late August until early October in Lake Constance (Germany) and were composed predominantly of colony-forming mucilage-producing cyanobacteria. In the marine environment, bacillariophyceae

(diatoms) are the major algae contributing to mucilage in the water column (Viviani et al., 1995 ; Innamorati, 1995).

The attribution of cell-associated mucilage clearly relates to species present. This mucilage was mainly from *Eudorina elegans* in late spring 1996 and from *Anabaena spiroides* and *Microcystis aeruginosa* in summer 1996 and 1997. These mucilage levels relate both to mucilage volume per colony and algal abundance at a particular time. For example, *Anabaena spiroides* reached a maximum population level in June 1996 (filament length of 74 mm ml^{-1}), contributing a cell-associated mucilage volume of 1.1×10^7 μm^3 ml^{-1}. However, *Anabaena flos-aquae* dominated in early summer 1996 (maximum filament length of 340 mm ml^{-1}), but did not have a high overall mucilage biomass (maximum associated mucilage volume of 3.7×10^6 μm^3 ml^{-1}) because of its low mucilage volume per colony. *Microcystis aeruginosa* contributed most of the algal associated mucilage volume (> 70%) during its bloom period, due largely to the high mucilage volume per colony. Maximum levels occurred in September 1997 (chlorophyll a concentration 31 μg l^{-1}), when the *Microcystis* bloom contributed an estimated 96% of total algal associated mucilage, occupying 0.007% of lake water. Lake chlorophyll a concentrations reaching 280 μg l^{-1}, under intense bloom conditions (Schalles et al., 1998), will be equivalent to cell-associated mucilage levels of 0.06% if *Microcystis* is the main alga present.

The major abundance of the above five species with cell-associated mucilage occurred during the late spring and summer at Rostherne Mere in both 1996 and 1997, during which time the lake was stratified. The presence of mucilage in cyanobacteria is closely related to their buoyancy [Section 1.1.3.(3)], so that stratification is directly related to high mucilage levels ($2 \times 10^6 \sim 7 \times 10^7$ μm^3 ml^{-1}) at this time. Early high light

may be related to the high mucilage level in summer, as it influences the development of *Microcystis* during this time (Reynolds et al., 1981, as discussed in Section 3.3.2).

In the marine environment, the total volume of mucilage aggregates with a size > 0.5 mm was within the ranges of 11 ~ 350 mm^3 l^{-1}, occupying 0.0011% ~ 0.035% of the volume of seawater (including the volume of associated mucilage and that of the cells within the aggregates, MacIntyre et al., 1995). In Rostherne Mere, the highest volume of species with mucilage (including the volume of the cells and associated mucilage) occupied approximately 0.08% of the lake water.

High levels of cell-associated mucilage have implications for a range of freshwater activities – including cation binding, association of bacteria and fungi, and zooplankton feeding, as summaried previously (Section 1.1.3). Amemiya and Nakayama (1984) found the amounts of Na, Ca, Fe, Mn and Zn in the sheath of *Microcystis* were five or more times higher than in the cells. This shows that cell-associated mucilage has the ability to concentrate essential metals and implicates its relatively high metal-binding activity (Nakagawa et al., 1987 ; Tease & Walker, 1987 ; Weckesser et al., 1988). Therefore, the high percentage of cell-associated mucilage present in the lake may have potential for binding metals or other ions in the water. Chapter 5 will examine the capacity for metals to bind to cell-associated mucilage.

Horizontal variation

The volume of cell-associated mucilage between the site A, B, C areas was similar ($p>0.05$) except between the site A and C areas ($p<0.05$) on 29 August 1996. Since *Microcystis aeruginosa* was the major species accounting for most (at least 60%) of total mucilage volume and had higher numbers of colonies in site C than in site A, this may explain the different mucilage volumes between these two sites. That site C contained more *Microcystis aeruginosa* than site A may be due to its higher

temperature and pH, as nutrient levels (Krivtsov, 1996) were similar. The horizontal variation in the population of *Microcystis* was in agreement with George and Heaney (1978) who found that non-uniform horizontal distribution of phytoplankton resulted from the presence of buoyant cyanobacteria or *Ceratium hirundinella*. Since wind-induced water movement is important in the formation of patches of phytoplankton and horizontal patchiness disappeared at wind speed > 5 m s^{-1} (Therriault et al., 1978 ; George & Heaney, 1978 ; Reynolds, 1993), the difference in total mucilage volume between Site A and C may result from low water movement (with wind speed <5 m s^{-1}) on 29 August 1996.

3.3.3.b Variations in soluble extracellular polysaccharides in lake water

Soluble extracellular polysaccharides in lake water were quantified by the Periodic acid/Schiff method (Jugdaohsingh et al., 1998). This method is an easy and sensitive colorimetric method for measuring polysaccharides in a large number of samples and includes those present in glycoprotein (Mantle & Allen, 1978).

There was a marked seasonal variation in soluble extracellular polysaccharides (EPS) in lake water from November 1996 to November 1997. This variation was related to algal populations as it was found that the high concentration of soluble EPS in May 1997 corresponded with the late spring algal bloom. The reason may be that large numbers of algae may secrete relatively high amounts of carbohydrates (as metabolites, photosynthetic intermediates or mucilage) during active growth, as there is evidence that healthy, actively growing phytoplankton release a considerable proportion of their photoassimilated carbon into the aquatic environment (Hellebust, 1974 ; Fogg, 1983). The secretion of considerable amounts (ranging from 5% to 45% of dry extracellular products) of soluble carbohydrates by seven laboratory-cultured algae was also found in Section 4.2.2.a. The dominant species at this time in Rostherne

Mere were *Asterionella formosa,* cryptophyta and *Chlamydomonas spp.*. These species did not produce significant amounts of cell-associated mucilage, though some species of *Chlamydomonas* secreted a mucilaginous surround, but this was not observed in this study. Ramus (1972) investigated the production of both encapsulating and dissolved polysaccharides by the red alga *Porphyridium aerugineum* and suggested that a mucilaginous capsule is formed when the rate of excretion is greater than the rate of solubilization; extracellular polysaccharides will not form a capsule but dissolve into medium in the opposite situation. Therefore, these dominant species may secrete polysaccharides dissolved into surrounding water even though they do not form cell-associated mucilage. Planktonic diatoms such as *Asterionella* have rather heavy silica cell walls. In order to remain in the uppermost layers of lakes or oceans to obtain enough light for photosynthesis, diatoms develop mechanisms to decrease their sinking rate such as excluding heavy ions form cell sap and producing surface fibers (Anderson & Sweeney, 1978 ; Walsby & Xylopyta, 1977; Walsby & Reynolds, 1980). It has also been suggested that diatoms may secrete soluble mucilaginous matter which had a relatively high viscosity into the lake water to increase the viscosity of the water surrounding the cells and so decrease their sinking rate (Myklestad et al., 1972). I have also found that *Asterionella fomosa* secreted nearly 11% of soluble carbohydrates into the culture medium (Section 4.2.2.a). As secretion of extracellular carbohydrates by algae will increase when nutrients become limited (Myklestad, 1995), depletion of nutrient due to an algal bloom may also increase the amount of soluble EPS released by the dominant algae.

The late spring algal blooms in 1997 were followed by a sudden decrease and then an increase in the concentration of soluble EPS (Fig. 3.21). This could be explained by bacteria activity and/or leaching from dead cells. Kato (1994) showed

that soluble extracellular organic carbon released from photosynthetic phytoplankton was one of the most important carbon sources for the growth of planktonic bacteria and Gajewski and Chróst (1995) found high bacterial enzymatic activity during phytoplankton blooms. Lee and Rhee (1997) also found that dissolved organic carbon excreted from live phytoplankton cells is largely of low molecular weight and directly available for uptake of bacteria, thus bacteria populations and enzyme activity increased with or soon after the phytoplankton maximum (Booth, 1988 ; Jones, 1971). Therefore, high concentration of soluble EPS produced by phytoplankton blooms in May 1997 may promote the growth of bacteria which may account for the sudden decrease in soluble EPS in June 1997. The peak of soluble EPS in July-August 1997 may be mainly due to release of soluble EPS by autolysis or lysis of cells by bacteria, as the algal population at that time is low and algal cell debris were found. Lee and Rhee (1997) found that the dissolved organic carbon from dead cells consisted mainly of high-molecular-weight compounds that are not available directly to bacteria and must be hydrolysed by bacteria enzymes before uptake (Chróst et al., 1989). Those high-molecular-weight compounds may accumulate in the water column and account for these two peaks of soluble EPS.

An abundance of bacteria may also account for part of the extracellular release of soluble EPS, as Jørgensen and Jensen (1994) found that bacteria release polysaccharides. Also, Serratore et al. (1995) suggested that the significant numbers of the bacteria Vibrionaceae and Pseudomonadaceae present in seawater are likely to be actively contributing to the formation of marine mucilage. *Pseudomonas* was found by Booth (1988) as the most commonly occurring bacterial genus in Rostherne Mere. This species may release polysaccharides into the surrounding water. On the other hand, small bacteria (<0.45 μm) which would pass through the filter membrane (with the

pore size of 0.45 μm) may also account for part of the soluble EPS measured in my study.

High concentration of soluble extracellular polysaccharides at a time of low algal population may also result due to the activity of zooplankton. These organisms are known to increase to high population levels during the early summer clear water phase (Carvalho, 1993). Sala and Güde (1996) found that polysaccharide enzyme activities increased when grazing cladocerans were added to the system containing algae and bacteria, and suggested that the 'sloppy feeding' behaviour of cladocerans and defecation may increase the level of polysaccharides in the medium and influence strongly qualitatively and quantitatively the carbon supply for bacteria. Thus, this may be another reason for the high concentration of soluble EPS detected in July-August 1997 in Rostherne Mere.

There was an autumn bloom in September 1997 and the dominant species were *Microcystis aeruginosa* and *Asterionella formosa*. However, the concentration of soluble EPS was low. This may be because the extracellular polysaccharides secreted by these algae are mainly present as an insoluble form which accounted for the highest mucilage volume in Rostherne Mere, as discussed in the previous section 3.3.3.a. The low concentration of soluble EPS (less than 10 mg l^{-1}) throughout the rest of sampling period is presumably due to the low numbers of mucilage-producing species (algae and\or bacteria) present. As different algal species secrete different amounts of soluble EPS (Myklestad, 1995, also shown in Section 4.2.2.a) and appear at different periods of time (as discussed in Section 3.3.2), this may explain different amounts of soluble EPS measured through the year.

To summarize, it is suggested that the seasonal variation in concentration of soluble extracellular polysaccharides might be the result of secretion by and

decomposition of algae and bacteria, and utilization by heterotrophic bacteria. Soluble extracellular polysaccharides are the major fraction of dissolved organic carbon (DOC) in the freshwater environment which has been estimated to make up from less than 1 to 30% of the pool of DOC (Chróst et al., 1989 ; Jørgensen & Jensen, 1994). Therefore, the soluble extracellular polysaccharides recorded in this study may make at important contribution to the biogeochemical cycling of carbon in lakes.

In conclusion, this study has revealed significant seasonal differences in the amounts of cell-associated mucilage and soluble extracellular polysaccharides in a eutrophic lake. These differences mainly depend on the species composition of phytoplankton which in turn result from interactions between the physico-chemical and biological parameters in the lake. It has also been found that different species produce different amounts of cell-associated mucilage and, possibly, soluble EPS. As mucilage composition is often species-specific (Myklestad, 1995), the next chapter (Chapter 4) will examine the sugar and protein composition of associated mucilage and soluble EPS from laboratory-cultured algae and the phytoplankton from Rostherne Mere.

CHAPTER 4. CHARACTERISATION OF SURFACE SUGARS, AND ANALYSIS OF CARBOHYDRATE AND PROTEIN CONTENTS OF MUCILAGE

4.1 Introduction

Algal mucilage is composed mainly of polysaccharides (sometimes upto 90% of dry weight ; Plude et al., 1991), with a small amount of protein (Section 1.1.2). Different algae show large differences in the proportion of different sugars and protein in the mucilage (Table 1.1), the composition of which may be species-specific. In the aquatic environment, the great diversity of different algal species will therefore lead to a great diversity of mucilage composition within the water column. The early studies of Sengbusch and Müller (1983) used lectins to examine the distribution of glycoconjugates (specific sugars) at the cell surface (cell wall or mucilage) of over a hundred algae. They demonstrated species-specific diversity in the content and distribution of sugars on the cell surface. The use of lectins to examine the distribution of sugars on the cell surface appears advantageous because of their sensitive sugar-binding specificity and possibility for direct application *in situ* (López-Rodas & Costas, 1997). A detailed discussion of lectins is given in Section 1.1.2.a.

In this part of the study, the two major components of mucilage, polysaccharides and protein, were measured in cell-associated mucilage and soluble extracellular products from laboratory-cultured algae and environmental samples. The major sugars were also characterised on the associated mucilage using FITC-conjugated lectins.

4.2 Results

4.2.1 Characterisation of cell surface sugars by lectin binding

4.2.1.a Qualitative fluorescence microscopy

I. Lectin binding activities of laboratory-cultured Anabaena cylindrica

Lectin binding (using FITC-Conjugated lectins) was initially examined using the laboratory-cultured alga *Anabaena cylindrica,* as it possesses a thin layer of surface mucilage (Plate 1). The fluorescence of FITC-lectin treated preparations was derived both from the cell constituents (autofluorescence) and the FITC marker.

(A). Autofluorescence

Red autofluorescence was found in cultures of untreated (living) *Anabaena cylindrica* and those which had been fixed (killed) with 2.5% glutaraldehyde solution. The intensity of red autofluorescence was similar, regardless of treatment (Plates 6 & 7). However, no clear red fluorescence was observed in cells fixed with acetic ethanol.

Mature laboratory cultures of *Anabaena cylindrica* contain three cell types, akinetes, heterocysts and vegetative cells. Heterocysts showed the least autofluorescence of the three cell types. Akinetes and vegetative cells showed a similar intensity of autofluorescence (Plate 6).

(B). FITC-ConA lectin labeling

The effect of cell preparation was investigated to determine its effect on the fluorescence characteristics of different cell types in *Anabaena cylindrica.* In living and glutaraldehyde-fixed cells showing autofluorescence, clear green fluorescence (indicative of surface labelling by FITC-ConA) was detected in akinetes and heterocysts, but not in vegetative cells. Green labelling of vegetative cells could be

seen, however, in acetic ethanol fixed cells, where autofluorescence had been suppressed by the fixative (Plates 8-10).

(C). Sugar-binding activity on the cell surface with different FITC-conjugated lectins

Qualitative comparisons were made to determine differences in labelling using a range of FITC-lectins (ConA, PHA-E, PWM and PNA). The binding patterns of these four FITC-lectins are shown in Table 4.1. ConA, PHA-E and PWM showed clear labelling of akinetes and heterocysts, with a lower intensity of labelling of vegetative cells (Plate 10 & 11). Binding of PNA to heterocysts and vegetative cells was minimal and too weak to be seen clearly. PNA labelled akinetes but, overall labelling was much less than with the other lectins. All 4 lectins bound to the surface of the three cell types of *Anabaena cylindrica* to different degrees, but the labelling on the cell wall could not be distinguished as strong autofluorescence was observed.

Table 4.1 Binding patterns of FITC-lectins with laboratory-cultured *Anabaena cylindrica*. Different lectins were tested against the same sample (from cultures in stationary growth phase).

Cell types\FITC-lectins	ConA	PHA-E	PWM	PNA
vegetative cells	+	+	+	(+ –)
akinetes	++	++	++	+
heterocysts	++	++	++	(+ –)

+++ : highly positive staining, ++ : positive staining, + : poor staining, (+ –) : doubtful staining, and – : nondetectable staining.

Table 4.2 Binding patterns of FITC-lectins in environmental samples from Rostherne Mere. Different lectins were tested against the same sample.

Species\FITC-lectins	Con A	PHA-E	PWM	PNA
Anabaena spiroides[a]	+++(m)	+++(m)	+++(m)	++(m)
Anabaena flos-aquae[b]	++(m)	(+ –)(m)	(+ –)(m)	(+ –)(m)
Microcystis aeruginosa[c]	+++(m) ++(w)	++(m) ++(w)	++(m) ++(w)	+(m) (+ –)(w)
Eudorina elegans[d]	+++(m) +++(w)	+(m) ++(w)	+(m) ++(w)	+(m) ++(w)
Sphaerocystis spp.[d]	ND	+++(m) +++(w)	+++(m) +++(w)	+(m) –(w)
Asterionella formosa[d]	++	(+ –)	(+ –)	(+ –)
Ceratium hirundinella[c]	++	+	+	+++
Stephanodiscus spp.[d]	++	ND	+	ND

m: mucilage ; w: cell wall. ND: not done.
+++ : highly positive staining, ++ : positive staining, + : poor staining, (+ –) : doubtful staining, and – : nondetectable staining.
a-d: date of sampling ;
a: 12 June 1996 ; b: 09 July 1996 ; c: 21 August 1996 ; d: 23 April 1996.

II. Assessment of lectin binding activities on environmental phytoplankton from Rostherne Mere

Studies were carried out on phytoplankton from Rostherne Mere to determine the degree of autofluorescence and the labelling characteristics of different lectins.

(A). Autofluorescence

The extent of red autofluorescence in fresh and glutaraldehyde-fixed cells varied considerably, being relatively high in *Eudorina elegans* (Plate 14) and *Stephanodiscus sp.* (Plate 20), but low in *Asterionella formosa* (Plate 18). Cells fixed with acetic ethanol showed less (faint red or yellow) or no autofluorescence.

(B). Sugar-binding activity on the cell surface with different FITC-conjugated lectins

Qualitative results for a range of phytoplankton cells are summarized in Table 4.2.

(1) Species with cell-associated mucilage:

Clear labelling with FITC- ConA, PHA-E, PWM, and PNA occurred for the surface mucilage of cells of *Anabaena spiroides*, which showed less autofluorescence (Plate 12) compared to laboratory samples (Plates 10 & 11). The intensity of fluorescence with ConA, PHA-E and PWM was higher than with PNA. The distinct shape of the mucilage sheath surrounding *Anabaena spiroides* could be seen due to the strong green fluorescence on the outside of the autofluorescent cells. The fibrillar nature of the mucilage was also observed and can be clearly seen in Plate 12.b2.

As shown in Plate 16, green FITC-ConA labelling was found on the surface (mucilage) of *Anabaena flos-aquae*. Red autofluorescence of the cells within the mucilage sheath was also observed. Binding of PHA-E, PWM and PNA to *Anabaena flos-aquae* was too weak to be detected. The bright autofluorescence of *Anabaena spiroides* and *Anabaena flos-aquae* tended to mask the labelling of the cell wall.

Acetic ethanol fixed, FITC-ConA treated *Microcystis aeruginosa* showed a strong overall green fluorescence, with clear labelling of the associated mucilage (Plate 13.a2). Less fluorescence was observed following labelling with FITC-PHA-E, PWM, and PNA (Plate 13.b2, c2 & d2). There was strong ConA, PHA-E and PWM labelling of the cell wall. Weak autofluorescence was observed on cells treated with PHA-E, PWM and PNA, but was not observed with ConA.

ConA showed the strongest binding activity of all the lectins on both the mucilage and the cell wall of *Eudorina elegans*. PHA-E, PWM and PNA bound strongly to the cell wall and less strongly to the mucilage (Plate 15). No autofluorescence occurred in the cells of this alga.

Sphaerocystis spp. also showed clear labelling of the surface mucilage and cell wall with PHA-E and PWM (Plate 17). Red autofluorescence was not detected.

(2) Species without associated mucilage:

The diatoms *Asterionella formosa* (Plate 19) and *Stephanodiscus spp.* (Plate 21) typically showed poor labelling with PHA-E, PWM, and PNA, but strong labelling with ConA.

PNA bound strongly to *Ceratium hirundinella*, especially at the base of the flagella. Compared to PNA, *Ceratium hirundinella* showed less labelling with ConA and much less with PHA-E and PWM (Plate 22). Some organic debris were attached to the cell surface resulting in areas of strong fluorescence scattered irregularly over the cell. None of the three species without associated mucilage showed red autofluorescence following fixation in acetic ethanol.

In conclusion, Con A was shown to bind to all species examined, with other lectins showing variable binding. Mucilage contains binding sites for all four lectins, though there were differences in the labelling intensity.

Plate 6. Laboratory-cultured *Anabaena cylindrica*. Unstained living cells.
(a): Phase contrast light micrograph.
(b): Fluorescence micrograph, showing strong red autofluorescence on akinetes and vegetative cells, but less autofluorescence on heterocysts.
(A: akinetes, H: heterocysts, V: vegetative cells) (scale bar = 10 μm)

Plate 7. Laboratory-cultured *Anabaena cylindrica*. Glutaraldehyde-fixed cells.
(a): Phase contrast light micrograph.
(b): Fluorescence micrograph, showing strong red autofluorescence on akinetes and vegetative cells.
(A: akinetes, V: vegetative cells) (scale bar = 10 μm)

Plate 8. Laboratory-cultured *Anabaena cylindrica*. Unfixed cells, labelled with FITC-ConA.
(a): Phase contrast light micrograph.
(b): Fluorescence micrograph.
Note the strong green fluorescence on akinetes, but not on vegetative cells and heterocysts which showed strong red autofluorescence.
(A: akinetes, H: heterocysts, V: vegetative cells) (scale bar = 10 μm)

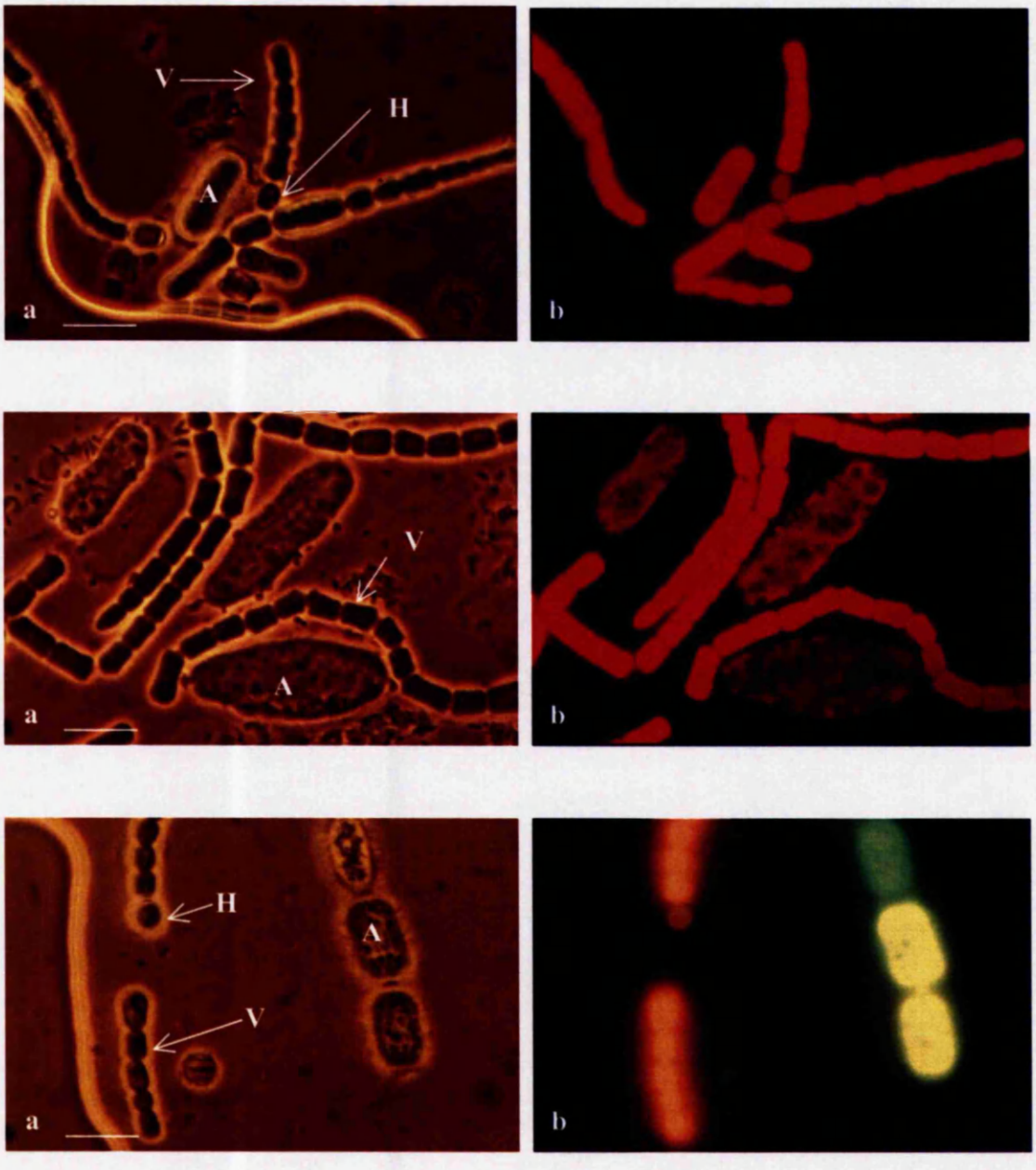

V
H
A
a
b
V
A
a
b
H
A
V
a
b

Plate 9. Laboratory-cultured *Anabaena cylindrica*. Glutaraldehyde-fixed cells, labelled with FITC-ConA.
(a): Phase contrast light micrograph.
(b): Fluorescence micrograph.
Strong green fluorescence was shown by akinetes and heterocysts, but not on vegetative cells which showed strong red autofluorescence.
(A: akinetes, H: heterocysts, V: vegetative cells) (scale bar = 10 μm)

Plate 10. Laboratory-cultured *Anabaena cylindrica*. Acetic ethanol-fixed cells, labelled with FITC-ConA.
(a): Phase contrast light micrograph.
(b): Fluorescence micrograph.
Strong green fluorescence was shown by akinetes, heterocysts and the surface mucilage of vegetative cells. Weak red autofluorescence was shown by vegetative cells.
(A: akinetes, H: heterocysts, V: vegetative cells) (scale bar = 10 μm)

Plate 11. Laboratory-cultured *Anabaena cylindrica*. Acetic ethanol-fixed cells, labelled with FITC-PHA-E.
(a): Phase contrast light micrograph.
(b): Fluorescence micrograph.
Strong green fluorescence was shown by akinetes, heterocysts and surface mucilage of vegetative cells. Vegetative cells showed weak red autofluorescence.
(A: akinetes, H: heterocysts, V: vegetative cells) (scale bar = 10 μm)

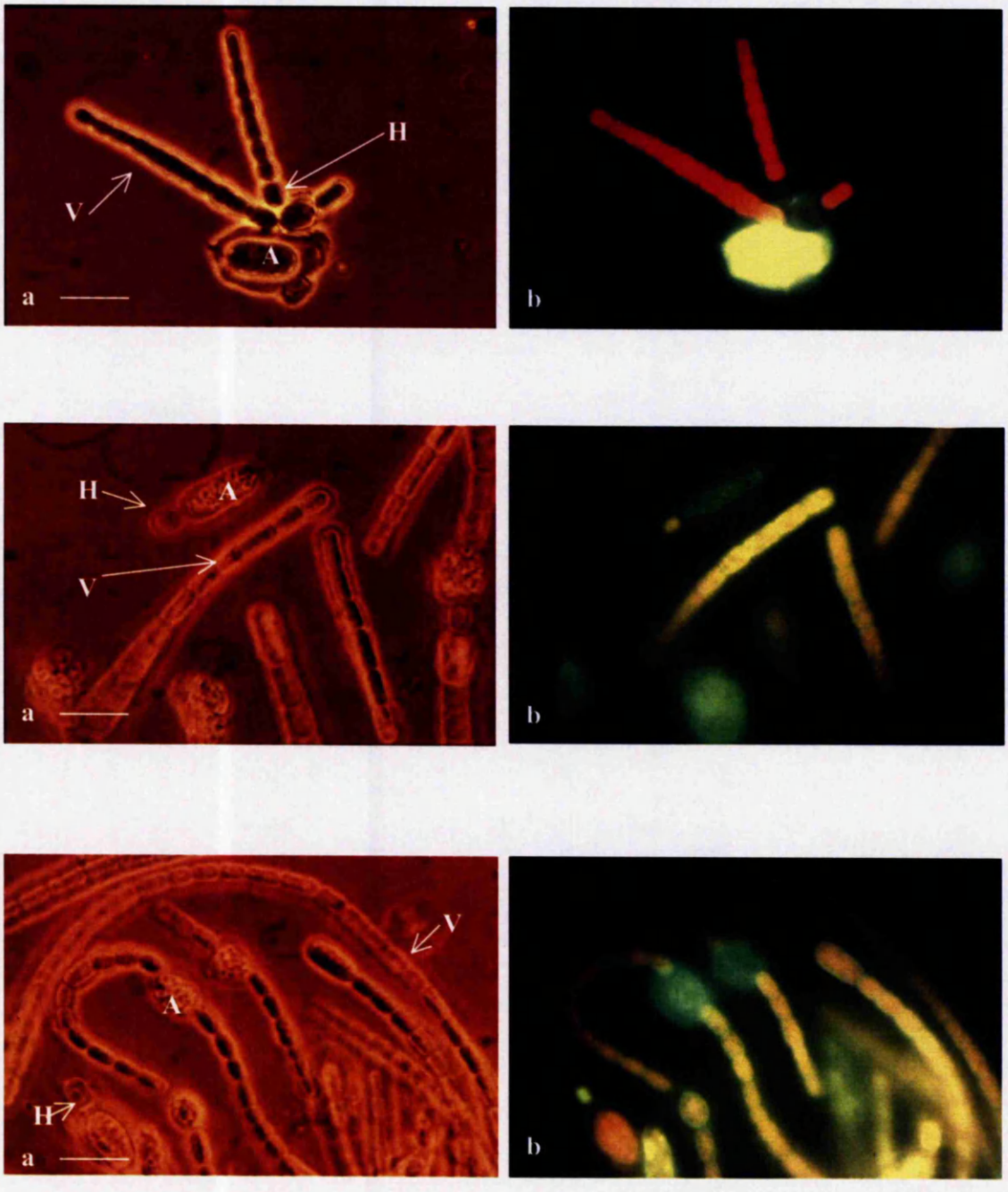

H
V
A
a
b
H
A
V
a
b
V
A
H
a
b

Plate 12. *Anabaena spiroides* collected on 12 June 1996 from Rostherne Mere. Acetic ethanol-fixed cells, labelled with FITC-conjugated (a) ConA, (b) PHA-E, (c) PWM and (d) PNA. All cells in these micrographs are in the vegetative stage.
(a1), (b1), (c1), (d1): Phase contrast light micrograph.
(a2), (b2), (c2), (d2): Fluorescence micrograph.
Strong green ConA, PHA-E and PWM fluorescence was shown on the mucilage of *Anabaena spiroides* which also showed weak red autofluorescence. PNA showed the least labelling on the mucilage among four lectins and a high level of cell autofluorescence was therefore apparent.
(scale bar = 10 μm)

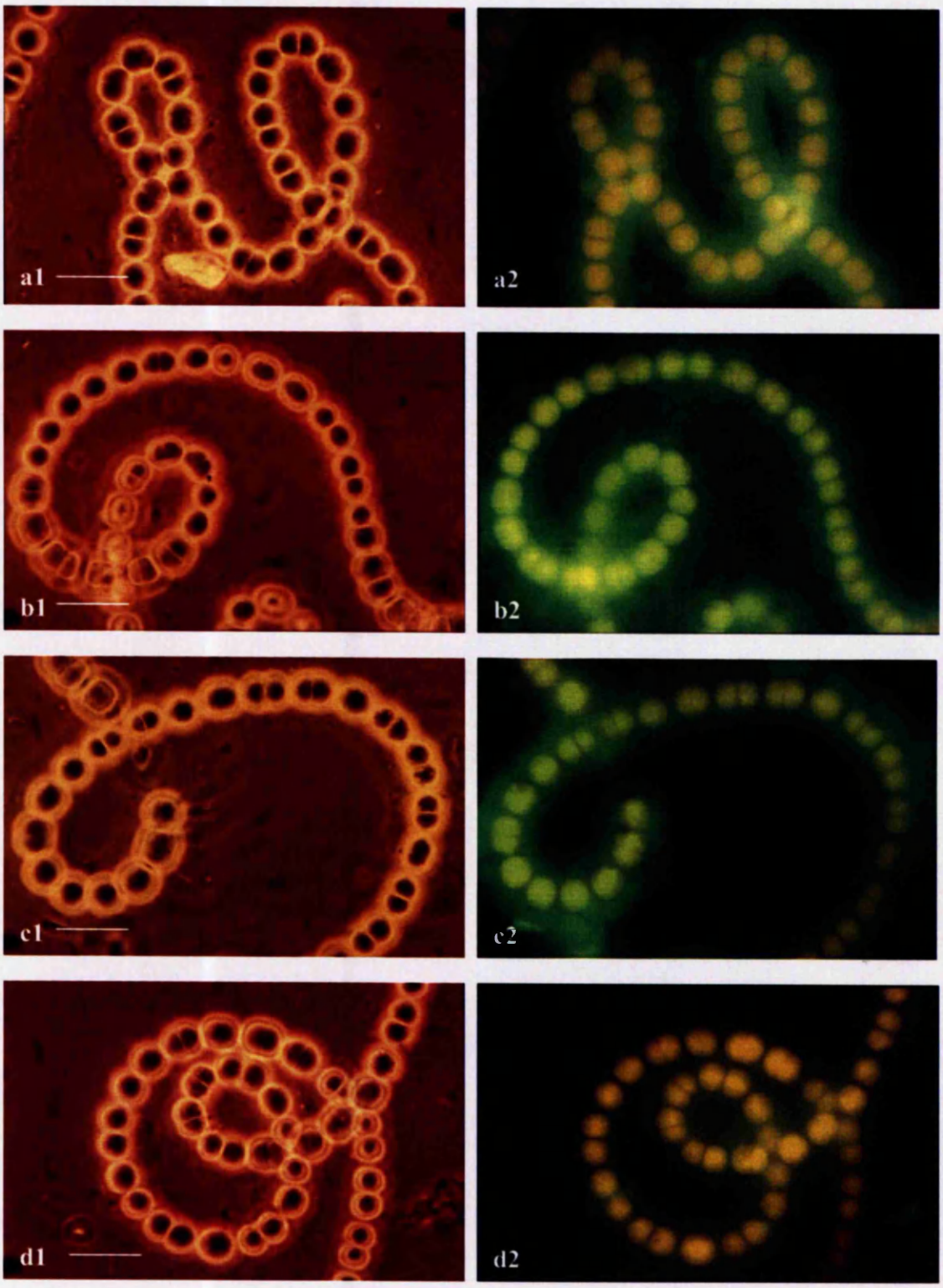

a1
a2
b1
b2
c1
c2
d1
d2

Plate 13. *Microcystis aeruginosa* collected on 21 August 1996 from Rostherne Mere. Acetic ethanol-fixed cells, labelled with FITC-conjugated (a) ConA, (b) PHA-E, (c) PWM and (d) PNA.
(a1), (b1), (c1), (d1): Phase contrast light micrograph.
(a2), (b2), (c2), (d2): Fluorescence micrograph.
Cells labelled with ConA showed strong green fluorescence on both mucilage and cell wall but no red autofluorescence. PHA-E and PWM labelled cells showed fluorescence on the mucilage and cell wall and weak autofluorescence. PNA labelled cells showed least fluorescence and relatively high autofluorescence.
(scale bar = 10 μm)

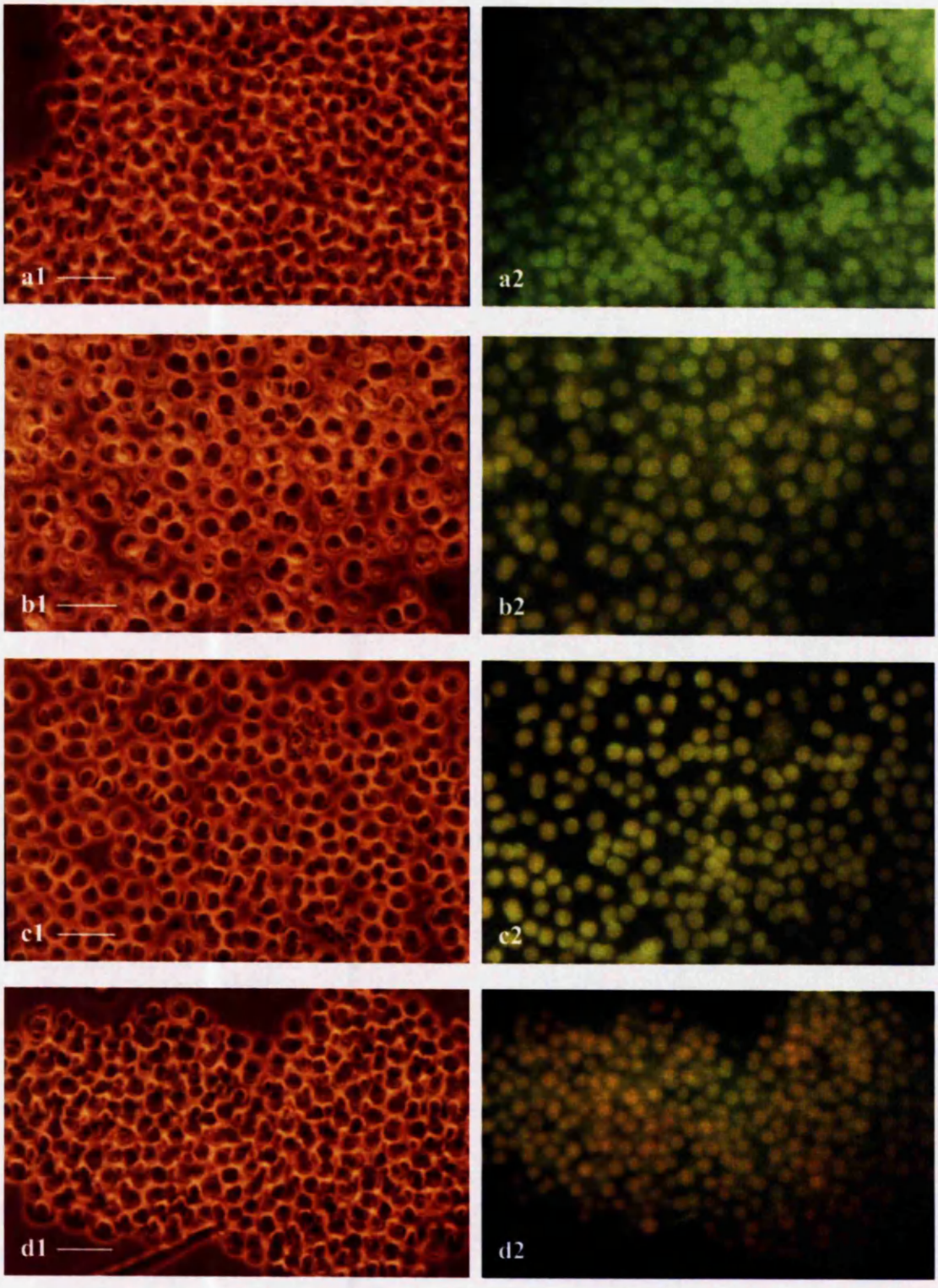

a1
a2
b1
b2
c1
c2
d1
d2

Plate 14. *Eudorina elegans* collected on 23 April 1996 from Rostherne Mere. Glutaraldehyde-fixed cells.
(a): Phase contrast light micrograph.
(b): Fluorescence micrograph.
Note the strong autofluorescence.
(scale bar = 10 μm)

Plate 15. *Eudorina elegans* collected on 23 April 1996 from Rostherne Mere. Acetic ethanol-fixed cells, labelled with FITC-conjugated (a) ConA, (b) PHA-E, (c) PWM and (d) PNA.
(a1), (b1), (c1), (d1): Phase contrast light micrograph.
(a2), (b2), (c2), (d2): Fluorescence micrograph.
ConA showed the strongest labelling on both mucilage and cell wall among four lectins. More PHA-E, PWM and PNA bound to the cell wall than to the mucilage. No red autofluorescence was detected.
(scale bar = 10 μm)

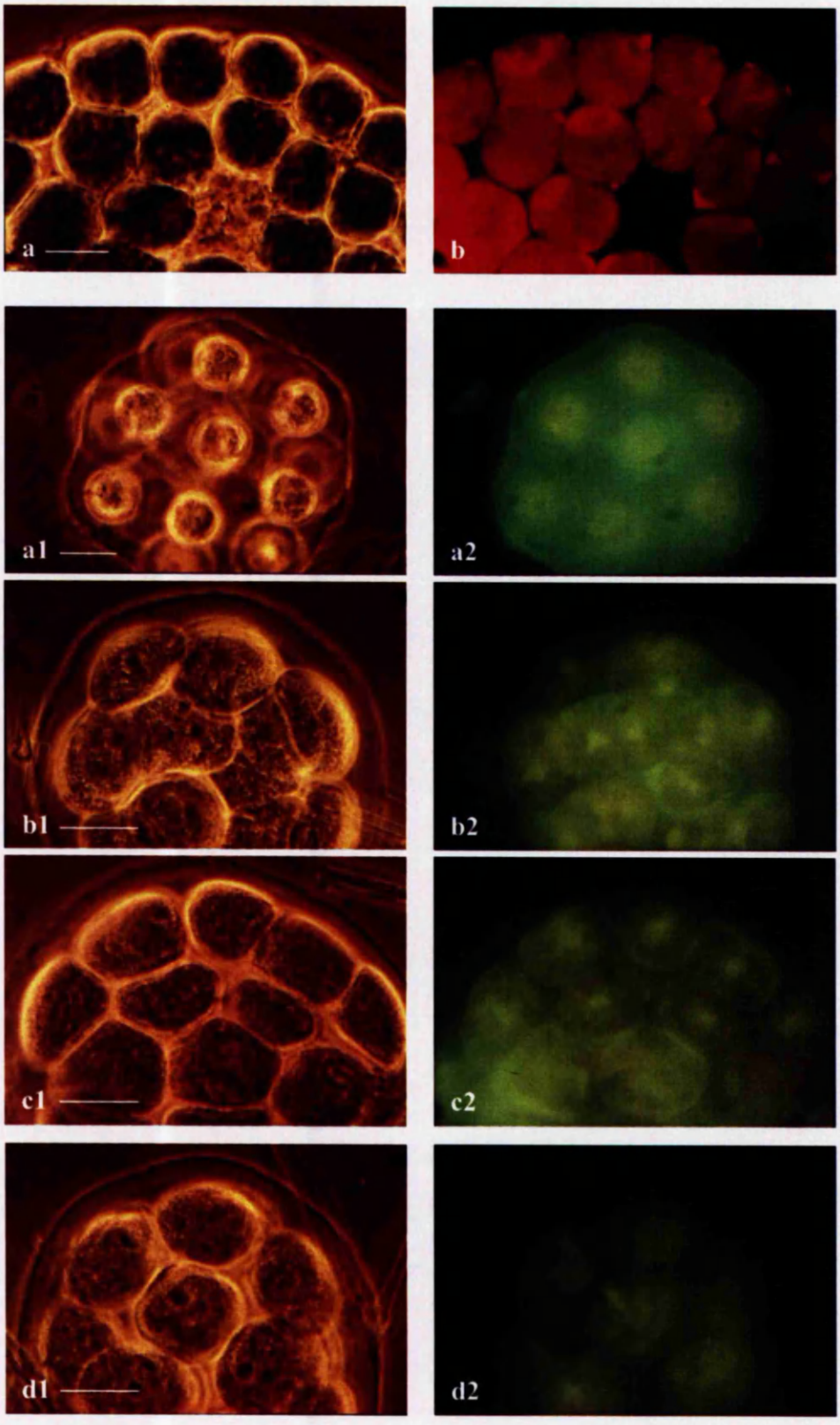

a
b
a1
a2
b1
b2
c1
c2
d1
d2

Plate 16. *Anabaena flos-aquae* collected on 09 July 1996 from Rostherne Mere. Acetic ethanol-fixed cells, labelled with FITC-ConA.
(a): Phase contrast light micrograph.
(b): Fluorescence micrograph, showing green labelling of the surface mucilage and red autofluorescence within the cells.
(scale bar = 10 μm)

Plate 17. *Sphaerocystis spp.* collected on 23 April 1996 from Rostherne Mere. Acetic ethanol-fixed cells, labelled with FITC-conjugated (a) PHA-E, (b) PWM and (c) PNA.
(a1), (b1), (c1): Phase contrast light micrograph.
(a2), (b2), (c2): Fluorescence micrograph.
Strong PHA-E and PWM labelling fluorescence was shown on both the mucilage and cell wall. PNA only bound to mucilage. No red autofluorescence was detected.
(scale bar = 10 μm)

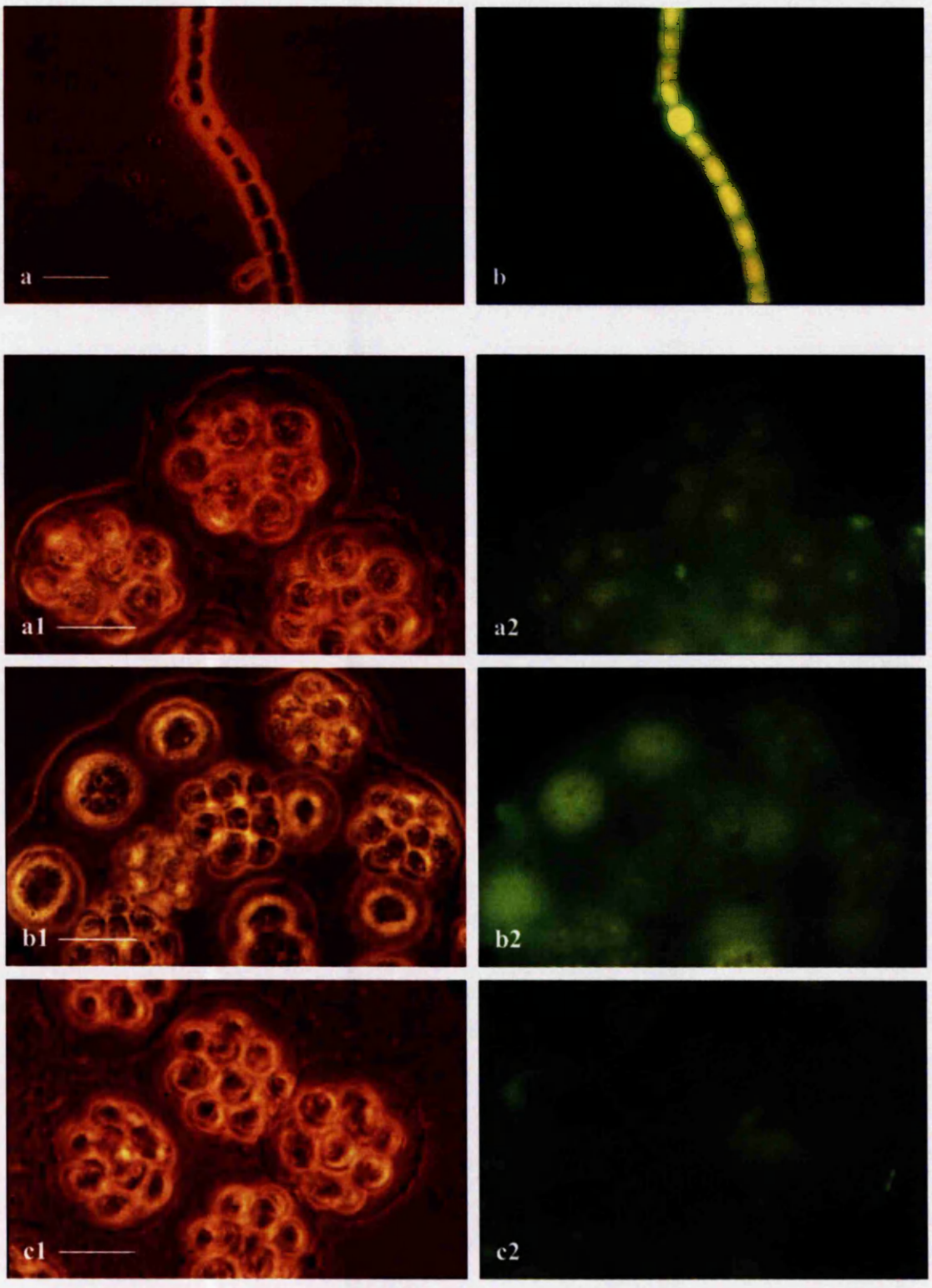

a
b
a1
a2
b1
b2
c1
c2

Plate 18. *Asterionella formosa* collected on 23 April 1996 from Rostherne Mere. Glutaraldehyde-fixed cells.
(a): Phase contrast light micrograph.
(b): Fluorescence micrograph, showing weak autofluorescence of the cells.
(scale bar = 10 μm)

Plate 19. *Asterionella formosa* collected on 23 April 1996 from Rostherne Mere. Acetic ethanol-fixed cells, labelled with FITC-ConA.
(a): Phase contrast light micrograph.
(b): Fluorescence micrograph, showing strong green labelling of the cell wall and no autofluorescence on the cells.
(scale bar = 10 μm)

Plate 20. *Stephanodiscus spp.* collected on 23 April 1996 from Rostherne Mere. Glutaraldehyde-fixed cells.
(a): Phase contrast light micrograph.
(b): Fluorescence micrograph, showing strong red autofluorescence of the cell.
(scale bar = 10 μm)

Plate 21. *Stephanodiscus spp.* collected on 23 April 1996 from Rostherne Mere. Acetic ethanol-fixed cells, labelled with FITC-conjugated (a) ConA and (b) PWM.
(a1), (b1): Phase contrast light micrograph.
(a2), (b2): Fluorescence micrograph.
ConA showed a greater intensity of labelling on the cell wall than PWM. There was no red autofluorescence.
(scale bar = 10 μm)

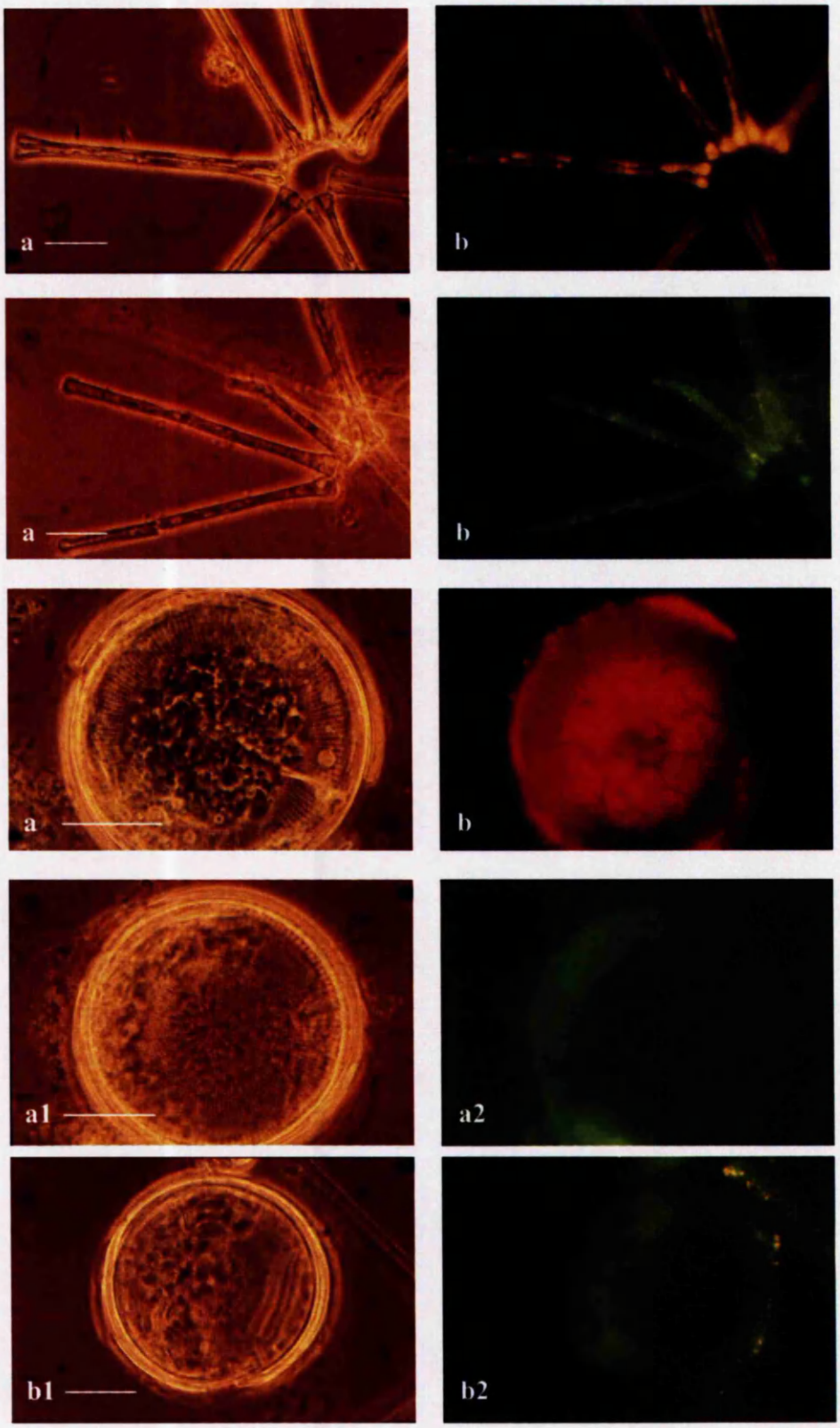
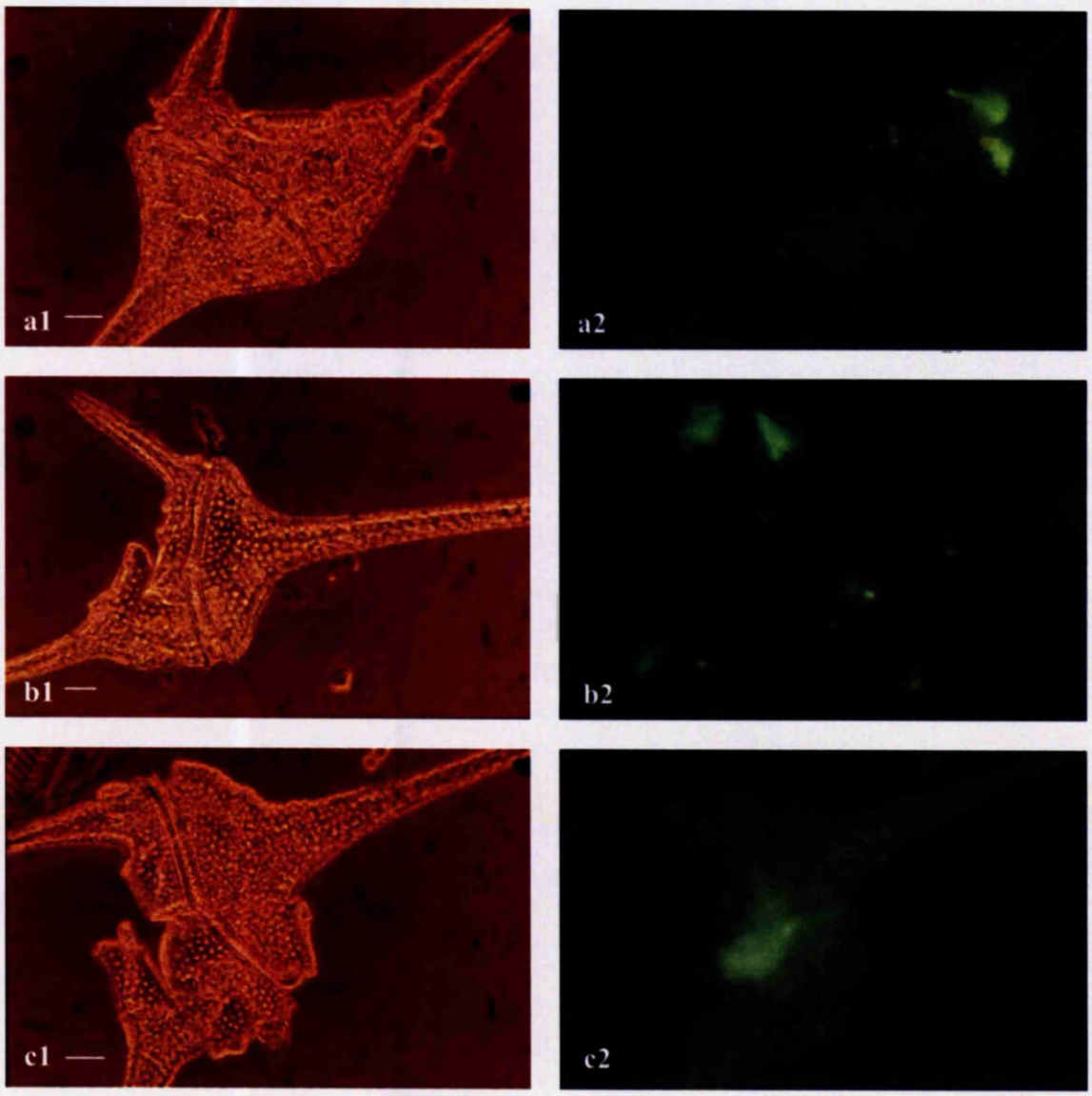

a
b
a
b
a
b
a1
a2
b1
b2

Plate 22. *Ceratium hirundinella* collected on 21 August 1996 from Rostherne Mere. Acetic ethanol-fixed cells, labelled with FITC-conjugated (a) ConA, (b) PHA-E and (c) PNA.
(a1), (b1), (c1): Phase contrast light micrograph.
(a2), (b2), (c2): Fluorescence micrograph.
PNA showed the greatest degree of binding to the cell wall, following by ConA, then by PHA-E and PWM. Bright staining on some parts of the cell wall was due to organic debris attached to the cell wall which showed strong lectin binding activity. No autofluorescence was detected.
(scale bar = 10 μm)


a1
a2
b1
b2
c1
c2

4.2.1.b Determination of lectin binding using confocal microscopy

Plate 23 shows a series of optical images (Z series) collected from different depths of the specimen (with 1 μm depth difference between each image) using a single labelled imaging technique to examine the degree of autofluorescence in environmental *Anabaena spiroides*. Images were recorded using an Argon 25mW laser which excited fluorescence at 488nm. The plate shows autofluorescence images of five optical sections out of a total of 11. Autofluorescence and FITC-ConA labeling fluorescence could be seen clearly using a double labelled imaging technique at detection wavelengths of 488nm and 512nm (Plate 24). In order to optimise optical resolution, images were recorded in black and white. By restoring the original colour, as shown in Plate 25, clear localization of green FITC-ConA labelling to mucilage was recorded. This green fluorescence also reveals the detailed shape of the mucilage sheath. The highest level of fluorescence was found in the isthmus region of the cell and at the edge of the mucilage sheath. The image obtained in Plate 26 was derived by reconstructing the series of optical images shown in Plate 25.

The fluorescence image of environmental *Anabaena flos-aquae* (Plate 27) shows clear FITC-ConA labelling of the mucilage. This could not be seen clearly using qualitative fluorescence microscopy (Plate 16, Section 4.2.1.a). No optical images were recorded from laboratory-cultured *Anabaena cylindrica* fixed in acetic ethanol and labelled with FITC-ConA because the resulting fluorescence was too weak to be recorded by a confocal microscopy.

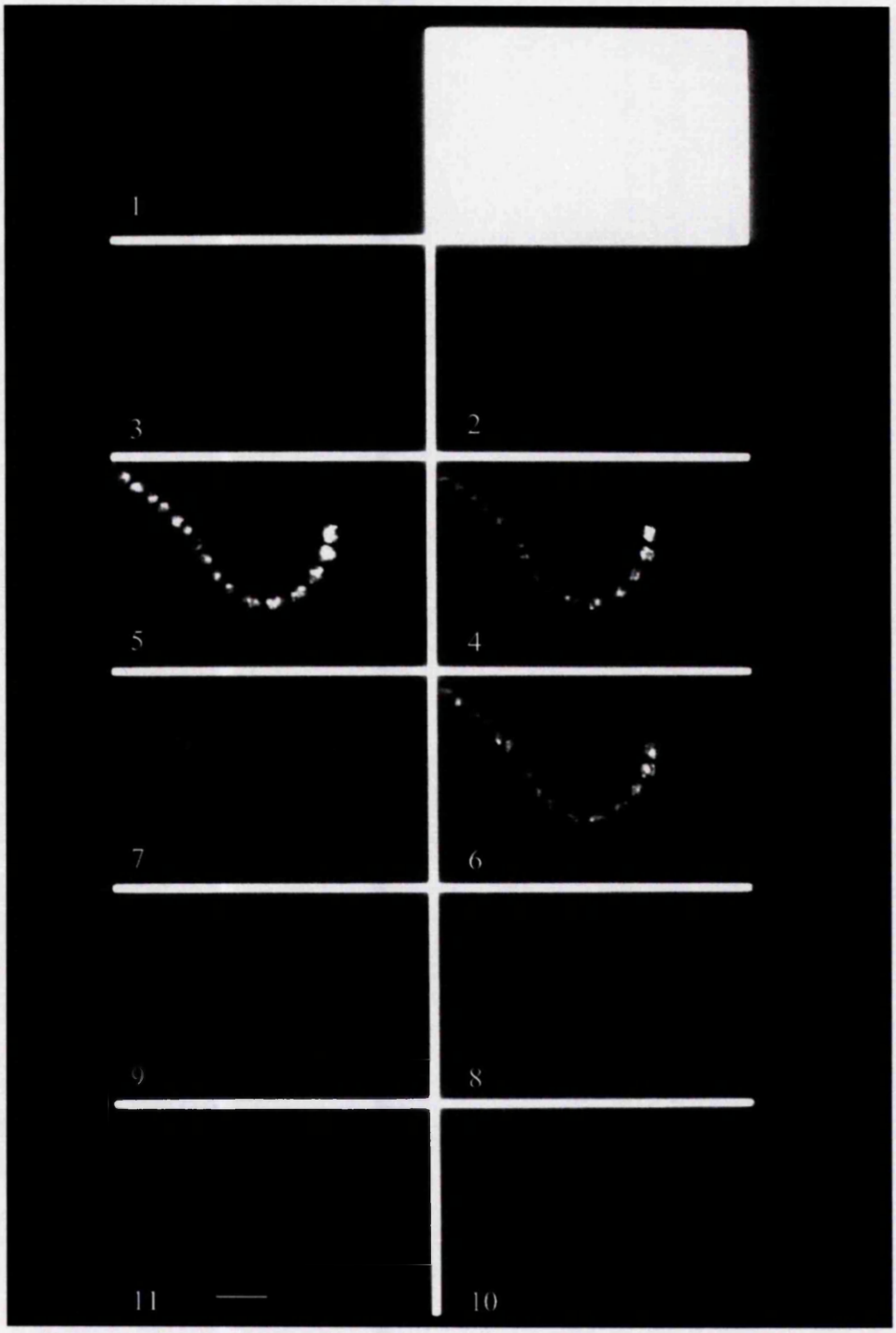


Plate 23. A series of optical images of *Anabaena spiroides* (images 3-7) collected on 12 June 1996 from Rostherne Mere using a single labelled imaging technique. (Detection wavelength: 488 nm) (Scale bar = 10 μm)

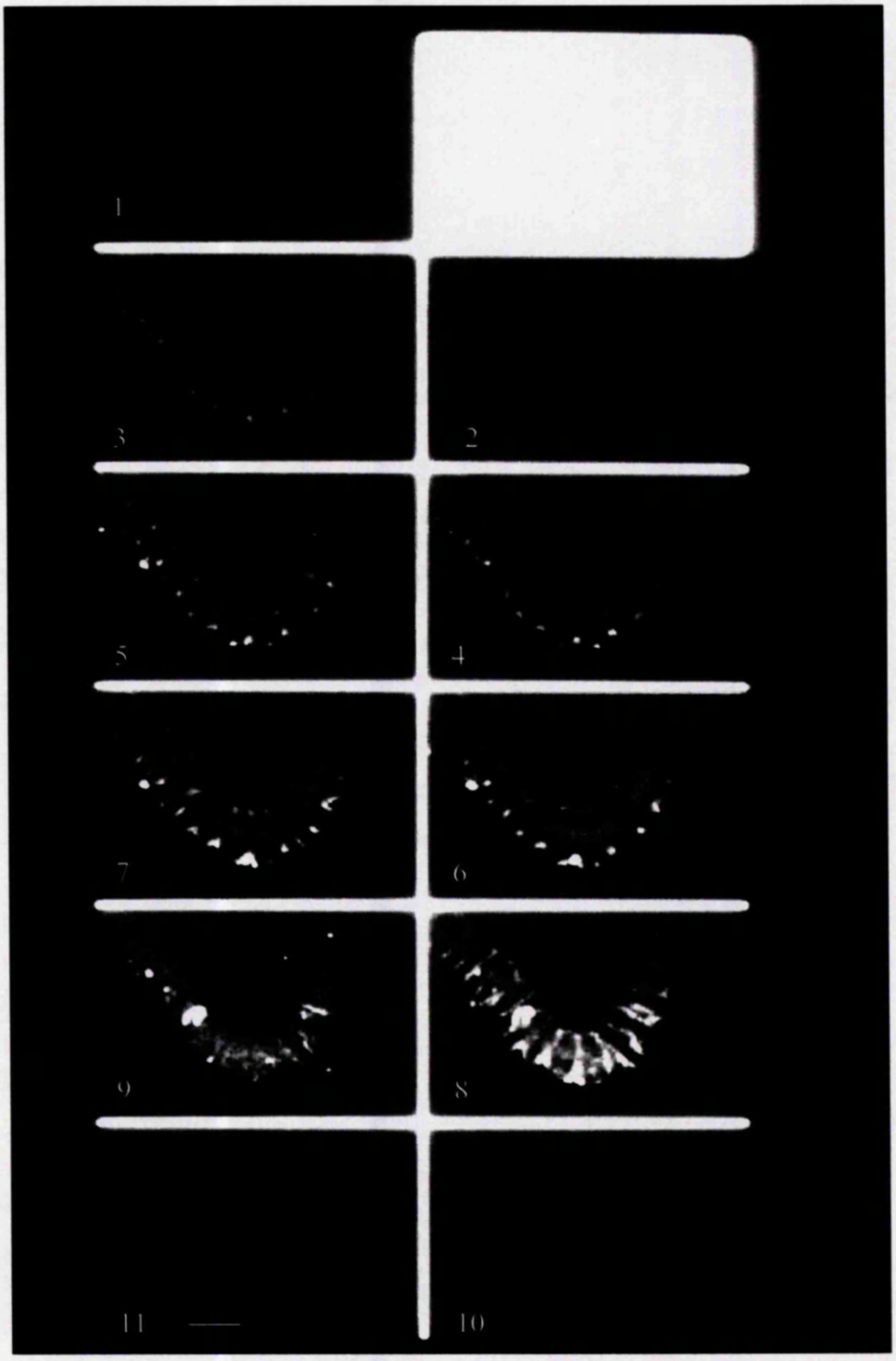


Plate 24. A series of optical images of *Anabaena spiroides* collected on 12 June 96 from Rostherne Mere using a double labelled imaging technique. (Detection wavelength: 488 nm and 512 nm) (Scale bar = 10 μm)

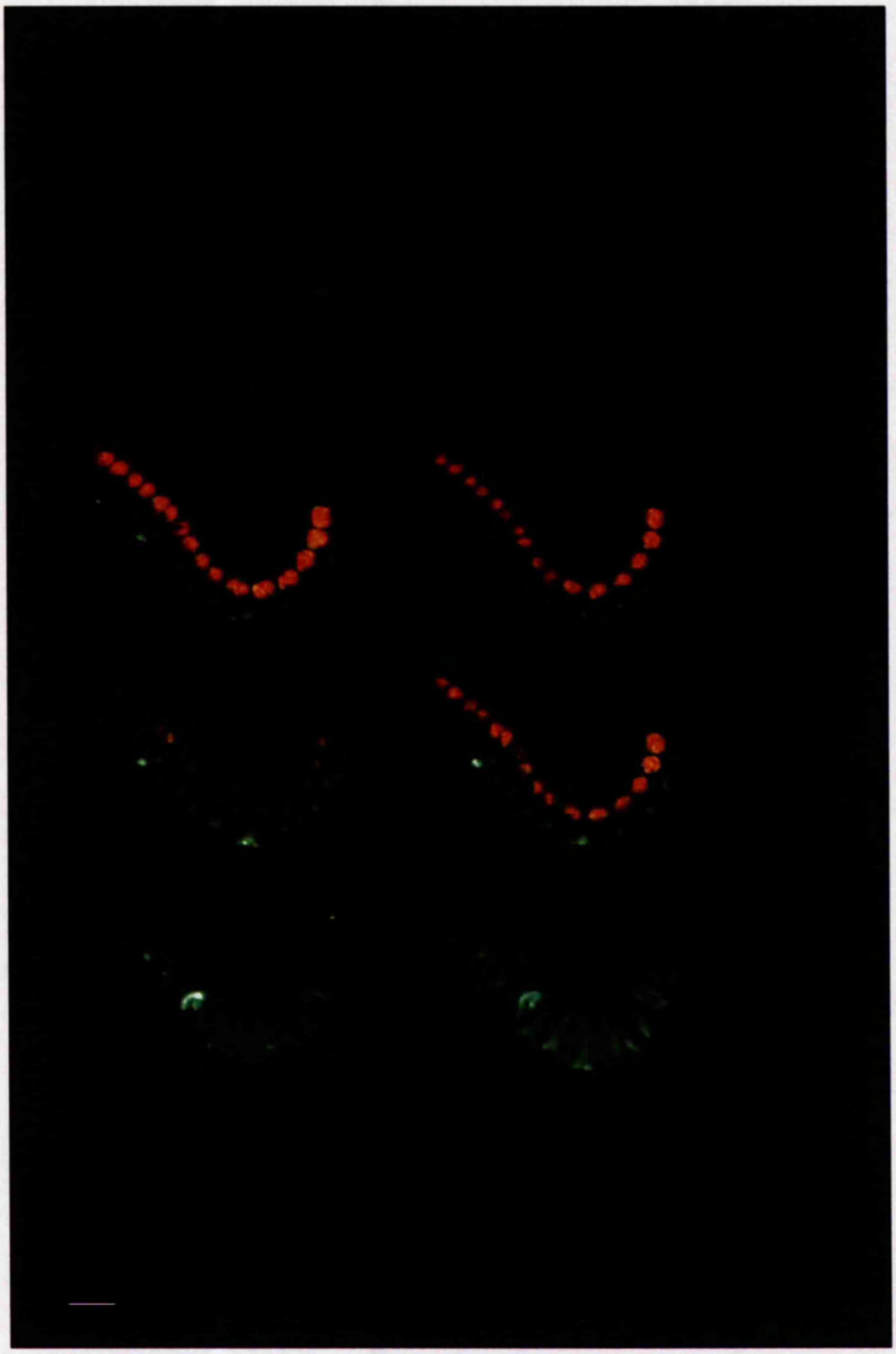


Plate 25. A series of double labelled optical images of environmental *Anabaena spiroides* recovered with original colours. (Scale bar = 10 μm)

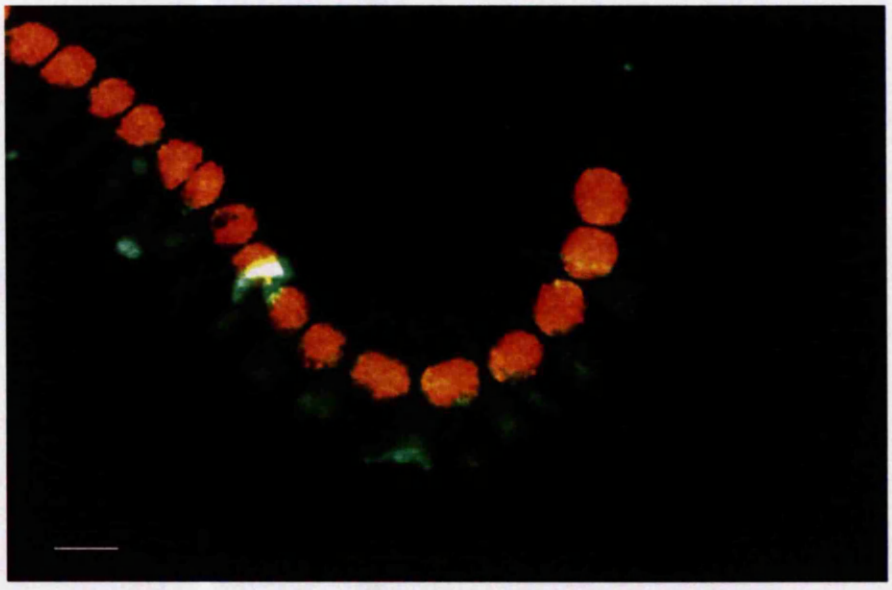


Plate 26. Reconstructed optical image of environmental *Anabaena spiroides* from a series of optical images in Plate 25. (Scale bar = 5 μm)

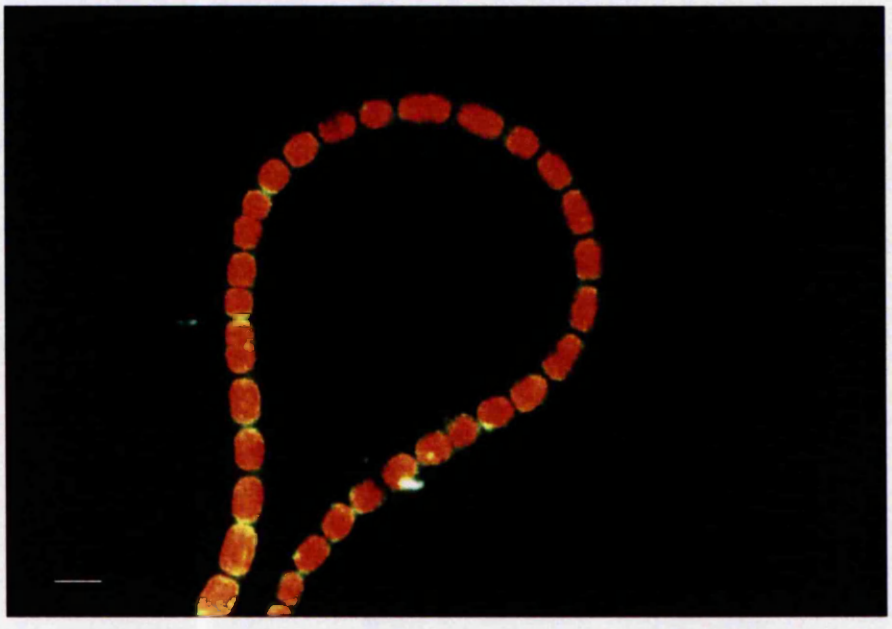


Plate 27. Reconstructed optical image of environmental *Anabaena flos-aquae* from a series of optical images using a double labelled imaging technique. (Detection wavelength: 488 nm and 512 nm) (Scale bar = 5 μm)

4.2.1.c Analysis of lectin binding to different growth phases of *Anabaena cylindrica* by quantitative fluorescence microscopy

This experiment investigated the lectin-binding activity of laboratory-cultured *Anabaena cylindrica* over a 26 day culture period.

A growth curve of *Anabaena cylindrica* is shown in Fig. 4.1 and comprises clear lag (up to 6 day post-incubation), exponential (6-23 days) and stationary (post 23 days) phases. Cells taken from the different growth phases (Day 2, lag phase ; Day 9 and Day 16, exponential phase ; and Day 26, stationary phase) were treated with four FITC-lectins to determine their binding activity. The intensity of fluorescence for vegetative cells, mucilage, akinetes and heterocysts in each of these samples is shown in Table 4.3. The measured fluorescence (total intensity) of the cell is the sum of the autofluorescence of the cell plus lectin-labelled fluorescence associated with the mucilage and/or cell wall.

In the two day cultures, lectin binding activity of all four lectins to the cells was very low with heterocysts having the highest fluorescence intensity (139). Binding to heterocysts was greater than to mucilage. No akinetes were found in these cultures. Mucilage showed a high intensity of labelling with ConA, PHA-E and PNA, but not with PWM. ConA, PHA-E and PNA labelling to mucilage was similar ($p>0.05$), but PWM showed significantly lower ($p<0.05$, with the nonparametric Mann-Whitney U-Wilcoxon Rank Sum W Test) than ConA and PNA. All four lectins bound similarly ($p>0.05$) to heterocysts.

After 9 days in culture, mucilage showed increased labelling with PHA-E, PWM and PNA, but the intensity of ConA binding remained the same as at 2 days. PHA-E and PNA bound strongly to mucilage, followed by PWM and ConA showed the least binding. Akinetes bound strongly to ConA, PHA-E and PWM and weakly to

PNA, where binding of the former three lectins was similar ($p>0.05$). Heterocysts bound strongly to ConA, PWM and PNA and weakly to PHA-E. Akinetes showed higher lectin binding than heterocysts, vegetative cells and mucilage. The intensity of ConA, PHA-E and PWM binding to akinetes was approximately twice that of heterocystis ($p<0.05$), but PNA binding was similar between these two cell types ($p>0.05$).

At 16 days incubation, ConA showed very strong overall labelling for mucilage and all cell types, but PNA-induced fluorescence was much less. Akinetes showed greater labelling with ConA and PWM than did heterocysts and mucilage, but all three cell types showed similar labelling with PHA-E and PNA ($p>0.05$).

PHA-E showed the highest binding of all the lectins to all three cell types after 26 days growth. PWM and PNA bound similarly ($p>0.05$) to mucilage, akinetes and heterocysts. All four lectins showed less labelling to mucilage and heterocysts than to akinetes. The intensity of four lectins binding to akinetes was approximately twice that to mucilage.

A comparison of the four data sets shows that cells sampled in different incubation times showed different lectin binding activities. Highest ConA labelling was found in cells after 16 days growth (late exponential growth phase) where PHA-E bound much strongly to cells from 26 days incubation (stationary phase). Cells in lag phase showed the least lectin binding activity. Akinetes typically showed higher lectin binding activity than the other cell types.

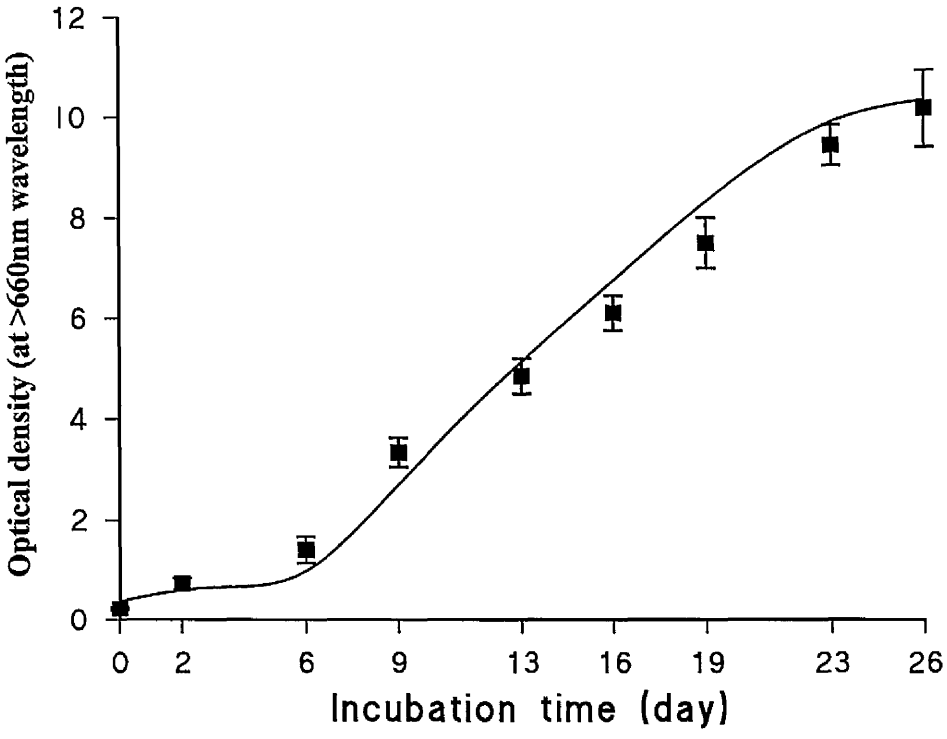


Fig. 4.1 Growth curve of *Anabaena cylindrica* grown in 100 ml BG-11 medium at 23 °C, under fluorescent light (22 μmol photon m^{-2} s^{-1}) and a 15 hour light / 9 hour dark cycle. (Error bar represents the standard deviation of triplicate samples.)

Table 4.3 FITC-lectin labelling of laboratory-cultured *Anabaena cylindrica* at different periods of incubation representing different growth phases.

	ConA	PHA-E	PWM	PNA
2 days				
Vegetative cells --cells[1]	131±3	108±2	109±5	112±32
--mucilage[2]	107±9	105±7	89±17	103±23
--mucilage[3]	56±9	54±7	38±17	52±23
Heterocysts[1]	127±10	123±0	139±22	124±28
9 days				
Vegetative cells --cells[1]	122±1	206±12	203±10	216±12
--mucilage[2]	99±1	216±10	199±14	236±13
--mucilage[3]	48±1	165±10	148±14	185±13
Akinetes[1]	609±57	550±83	520±121	233±4
Heterocysts[1]	311±3	195±11	312±64	233±6
16 days				
Vegetative cells --cells[1]	1457±106	263±3	263±14	139±3
--mucilage[2]	1708±151	282±3	275±12	144±3
--mucilage[3]	1657±151	231±3	224±12	93±3
Akinetes[1]	2160±248	283±51	391±30	130±9
Heterocysts[1]	1676±0	ND	271±52	152±2
26 days				
Vegetative cells --cells[1]	456±45	921±44	258±6	229±6
--mucilage[2]	417±43	1174±74	253±4	247±6
--mucilage[3]	366±43	1123±74	202±4	196±6
Akinetes[1]	915±128	2249±337	451±58	417±39
Heterocysts[1]	ND	610±0	347±52	240±46

Data is presented as mean ± standard error (arbitrary units of fluorescence), n>5,
where, mean = average fluorescence intensity of measured areas.
Background intensity was 51 ± 0.5 (mean ± standard error).
1: Total fluorescence, including autofluorescence and background.
2: Total fluorescence, including background (no autofluorescence).
3: Fluorescence, background subtracted.
ND: not done.
All four lectins were tested on the same algal cultures.

4.2.1.d Analysis of lectin binding to laboratory-cultured and environmental algae by quantitative fluorescence microscopy

In the previous experiment, *Anabaena cylindrica* showed different lectin-binding activities in different growth phases. In this study, the cultured cells were at the same (stationary) growth phase and environmental phytoplankton were collected during their bloom period (and on a specific date) to obtain comparable data. Lectin binding activities of the laboratory-cultured algae and the environmental phytoplankton samples were analysed by quantitative fluorescence microscopy.

I. Laboratory-cultured algae:

For species with cell-associated mucilage, the intensity of fluorescence in cells and mucilage was measured as shown in Plate 5 (Section 2.4.4) and Plates 28-29. Quantitative data using different lectin labelling to different cultured algae are presented in Table 4.4.

(A). Species with cell-associated mucilage:

The binding activity of four different lectins to *Anabaena cylindrica* decreased in the order for both cell and mucilage: PWM > PHA-E > ConA > PNA.

Where, A>B denotes significantly greater labelling of A than B ($p<0.05$)

A=B denotes no significant difference in labelling ($p>0.05$)

using the nonparametric Mann-Whitney U-Wilcoxon Rank Sum W Test.

The intensity of PWM binding to mucilage was approximately twice that of ConA.

Two distinct layers of mucilage were found surrounding *Anabaena spiroides* [Plate 28(c)]. Measurements were taken from each layer of mucilage and whole cells. The binding activities of four lectins decreased in the order:

On the cell: ConA > PHA-E > PNA > PWM,

On the inner region of mucilage: ConA > PNA > PHA-E > PWM,

On the outer region of mucilage: ConA > PHA-E > PNA > PWM.

The inner mucilage layer showed higher lectin-binding activity than the outer mucilage. The intensity of ConA binding to the two layers of mucilage was three times that of PHA-E and PNA and about five times that of PWM.

The intensity of fluorescence of whole cells and mucilage of *Microcystis aeruginosa* was: ConA > PWM > PHA-E > PNA. The highest lectin (ConA) binding to mucilage was five times that which bound least (PNA).

The intensity of fluorescence recorded by *Eudorina elegans* decreased in the order:

on the cell: PWM > PHA-E > ConA > PNA,

on mucilage: PHA-E > PWM > ConA > PNA.

The intensity of PHA-E binding to mucilage was six times that of PNA.

PNA typically showed the lowest mucilage labelling of all the lectins with the above algae, with the exception of *Anabaena spiroides* (PWM). Relative labelling with ConA, PHA-E and PWM varied with species (see above). Mucilage from *Eudorina elegans* showed significantly higher PHA-E and PWM binding activity than the other three species ($p<0.05$). ConA showed similar ($p>0.05$) binding to the mucilage of *Microcystis aeruginosa* and *Eudorina elegans* but higher ($p<0.05$) than to that of the two species of *Anabaena*. PNA bound similarly ($p>0.05$) to the mucilage of *Anabaena cylindrica* and *Eudorina elegans* but to a greater degree ($p<0.05$) than to that of *Anabaena spiroides* and *Microcystis aeruginosa.*

(B). Species without cell-associated mucilage:

The intensity of fluorescence measured in four laboratory-cultured species without cell-associated mucilage labelled with four lectins decreased in the order shown below:

Chlorella vulgaris: PWM > ConA > PHA-E > PNA.

Asterionella formosa: ConA = PHA-E > PWM > PNA.

Melosira varians: PHA-E > ConA > PNA > PWM.

Ceratium hirundinella: PHA-E = PWM > PNA > ConA.

The connection between individual *Melosira varians* cells showed a higher degree of fluorescence [Plate 29(b)] and different lectin binding activities (PHA-E > ConA > PWM > PNA) compared to the main body of the cell. The dinoflagellate *Ceratium hirundinella* only showed strong labelling on the middle part of cell wall, but not on the edge [Plate 29(c)]. Autofluorescence of these four species was too weak to be recorded with less than background intensity (51), so the measured fluorescence intensity could be accounted for in terms of lectin labelling. Only *Ceratium hirundinella* had higher cell wall lectin labelling than the other three species.

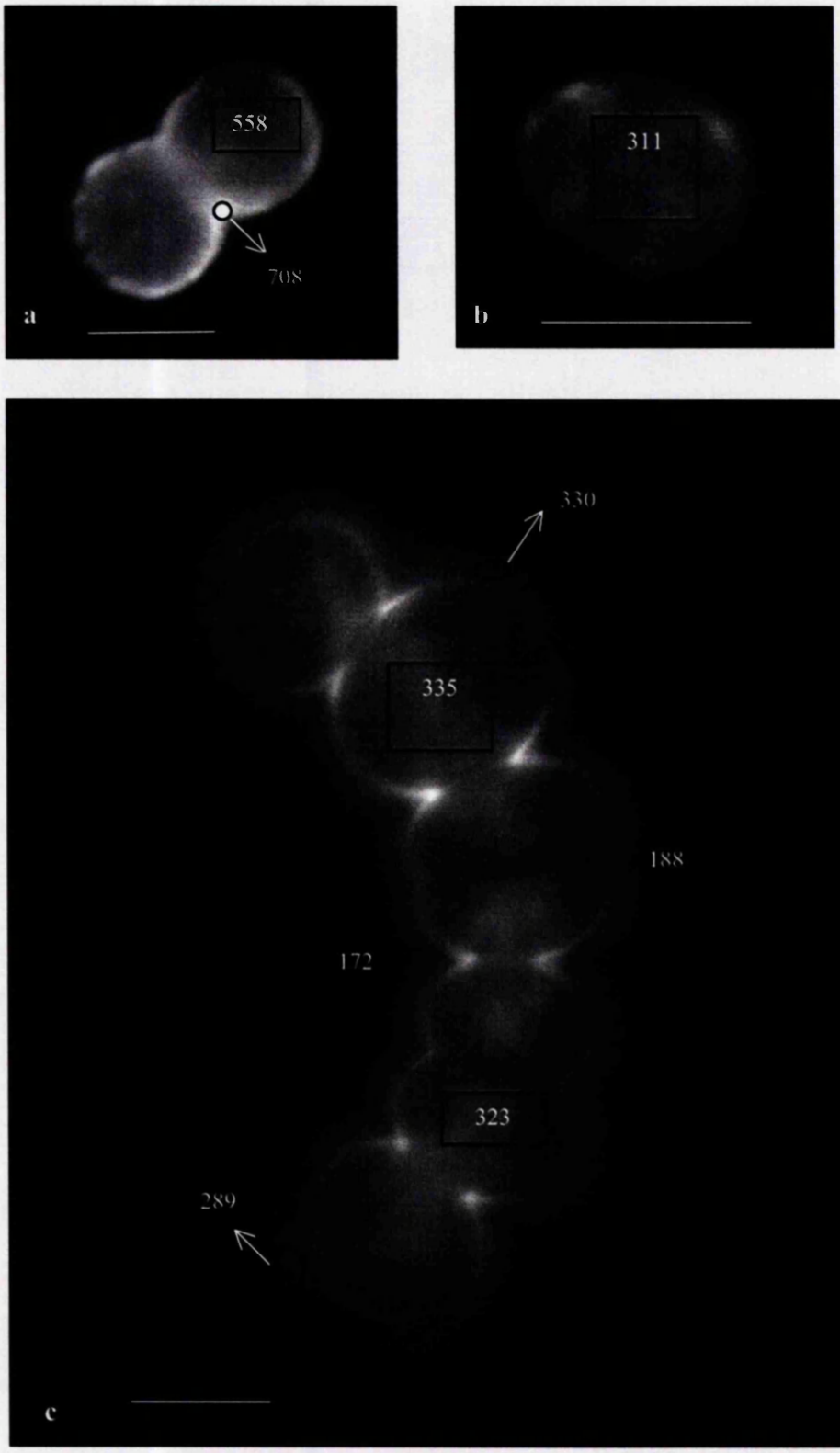


Plate 28. Fluorescent images of laboratory-cultured FITC-ConA labelled (a) *Microcystis aeruginosa,* (b) *Chlorella vulgaris* and (c) *Anabaena spiroides* taken by a photometrics slow scan CCD camera. The black box and white number show the measured area and average light intensity within the area. (Scale bar = 5 μm)

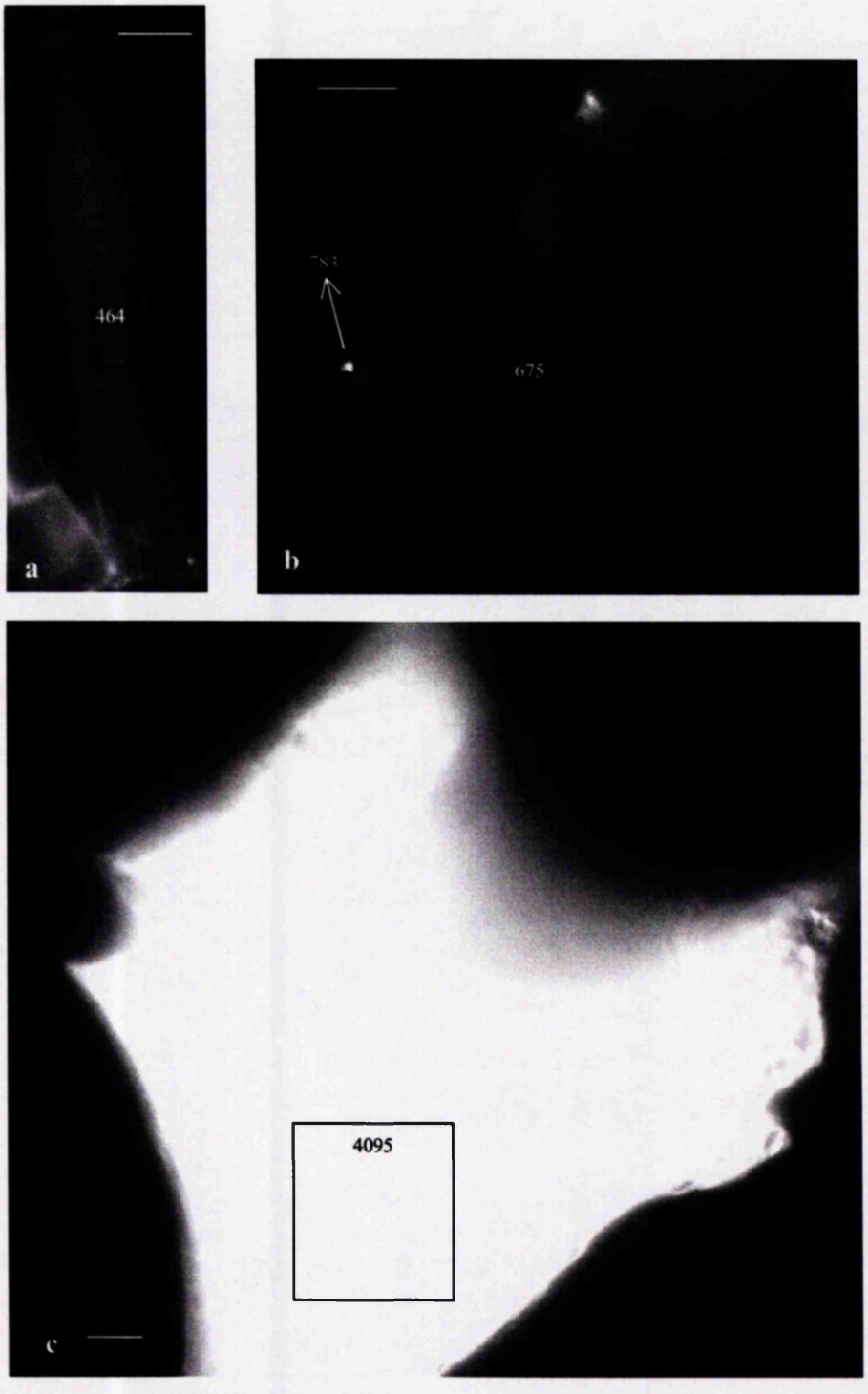


Plate 29. Fluorescent images of laboratory-cultured FITC-ConA labelled (a) *Asterionella formosa* and (b) *Melosira varians,* and FITC-PWM labelled (c) *Ceratium hirundinella* taken by a photometrics slow scan CCD camera. The black box and number show measured area and light intensity within the area. (Scale bar = 5 μm)

Table 4.4 FITC-lectin labelling of laboratory-cultured algae in stationary growth phase.

Species \ lectins	ConA	PHA-E	PWM	PNA
Anabaena cylindrica				
cells[1]	254±7	411±30	503±18	194±10
mucilage[2]	268±8	472±27	543±32	202±21
mucilage[3]	217±8	421±27	492±32	151±21
Anabaena spiroides				
cells[1]	447±32	202±9	162±7	197±8
inner mucilage[2]	421±22	153±7	113±2	179±9
inner mucilage[3]	370±22	102±7	62±2	128±9
outer mucilage[2]	259±17	131±7	88±2	111±5
outer mucilage[3]	208±17	80±7	37±2	60±5
Microcystis aeruginosa				
cells[1]	482±38	298±11	406±66	173±11
mucilage[2]	634±27	330±11	411±25	164±6
mucilage[3]	583±27	279±11	360±25	113±6
Eudorina elegans				
cells[1]	1483±130	1913±65	2357±97	405±12
mucilage[2]	617±26	1068±78	799±69	222±13
mucilage[3]	566±26	1017±78	748±69	171±13
Chlorella vulgaris				
cells[1]	238±27	120±1	312±40	84±4
cells[4]	187±28	69±1	261±40	33±4
Asterionella formosa				
cells[1]	379±20	391±20	234±6	114±3
cells[4]	328±20	340±20	183±6	63±3
Melosira varians				
cells[1]	509±51	624±76	200±17	327±25
cells[4]	458±51	573±76	149±17	276±25
connection[1]	836±119	1045±116	609±62	328±27
connection[4]	785±119	994±116	558±62	277±27
Ceratium hirundinella				
cells[1]	648±67	4024±70	4011±83	1612±162
cells[4]	597±67	3973±70	3960±83	1561±162

Data is presented as mean ± standard error (arbitrary units of fluorescence), n>5, where, mean= average fluorescence intensity of measured areas.
Background intensity was 51±0.5 (mean± standard error).
1: Total fluorescence, including autofluorescence and background.
2: Total fluorescence, including background (no autofluorescence).
3: Fluorescence, background subtracted.
4: Fluorescence, including autofluorescence but background subtracted.
Individual lectins were tested against the same culture of each alga to ensure direct comparability.

II. Environmental samples:

Lectin labelling of eight main algae *(Anabaena spiroides, Anabaena circinalis, Anabaena flos-aquae, Microcystis aeruginosa, Aphanizomonen flos-aquae, Eudorina elegans, Asterionella formosa* and *Ceratium hirundinella)* collected from Rostherne Mere was determined. Each species was collected at a particular sampling date during its bloom period. The intensity of fluorescence was measured on the cell (including autofluorescence and labelling fluorescence on mucilage and/or cell wall) and mucilage as shown in Plates 30 and 31, with data presented in Table 4.5.

Anabaena spiroides had a high level of lectin-binding fluorescence from the cell and mucilage which decreased in the order: ConA > PWM > PHA-E > PNA. Mucilage of *Anabaena spiroides* had the highest ConA labelling, nearly twice that of the least (PNA). For all four lectins, labelling of cells and mucilage in *Anabaena circinalis* and *Anabaena flos-aquae* was considerably less than *Anabaena spiroides.* The pattern of labelling on mucilage also differed, being ConA = PHA-E = PWM > PNA in *A. circinalis* and PHA-E > PWM = ConA > PNA in *A. flos-aquae.* The intensity of ConA binding to mucilage of *A. spiroides* was four times that of *A. circinalis* and six times that of *A. flos-aquae.* PNA showed the least labelling on mucilage of these three *Anabaena* species among all four lectins.

After fixation and lectin-labelling processes, *Microcystis aeruginosa* showed two kinds of colony, a densely compact colony and a more loose aggregation of cells. The lectin binding activity of *Microcystis aeruginosa* mucilage was:

Dense colony: ConA > PHA-E > PWM = PNA,

Loose colony: Con A > PNA > PHA-E = PWM.

ConA showed four times the binding activity of PWM and PNA in the mucilage of the dense colony, and two times higher than PHA-E and PWM in that of the loose colony.

The dense colony had much higher lectin binding activities than the loose colony [Plates 30(c) & (d)]. The intensity of ConA and PHA-E binding to the mucilage of the dense colony was four times that to the loose colony. PWM and PNA showed twice the intensity of binding to the mucilage of the dense colony than to the mucilage of the loose colony.

The intensity of fluorescence on the cell surface of *Aphanizomenon flos-aquae* decreased in the order: ConA > PHA-E > PWM = PNA.

Strong ConA labelling was observed on the mucilage of *Eudorina elegans.* This bright fluorescence totally masked the image of the cells [Plate 31(a)]. However, cells could be seen due to the lower degree of labelling with PHA-E, PWM and PNA [Plate 31(b)]. Lectin binding to the mucilage of *Eudorina elegans* decreased in the order: ConA > PHA-E > PWM > PNA. The intensity of ConA binding to mucilage of *Eudorina elegans* was nearly seven times that of PNA.

ConA bound strongly to the cell wall of *Asterionella formosa* with less binding of PHA-E, PWM and PNA. The former showed about twice the binding activity than the latter three lectins. The binding activity of PHA-E, PWM and PNA on the cell wall was similar ($p>0.05$).

Lectin-binding fluorescence in *Ceratium hirundinella* decreased in the order: ConA >> PHA-E > PWM > PNA. The intensity of ConA binding to *Ceratium* was six times that of PNA. The distribution of lectin binding sites on the cell wall of this species was large (50% of coefficient of variation), as shown in Plate 31(c).

In these environmental samples, lectin binding to mucilage varied considerably with species. ConA showed the highest labelling of all the species examined, except *Anabaena circinalis* and *Anabaena flos-aquae.* PNA showed the least labelling of mucilage in *Anabaena spp., Eudorina elegans* and the dense form of *Microcystis*

aeruginosa, but not loose form of this species. Greatest overall lectin-binding to mucilage occurred with *A. spiroides* and *E. elegans*. Algal species without cell-associated mucilage showed highest lectin labelling with ConA compared to the other three lectins.

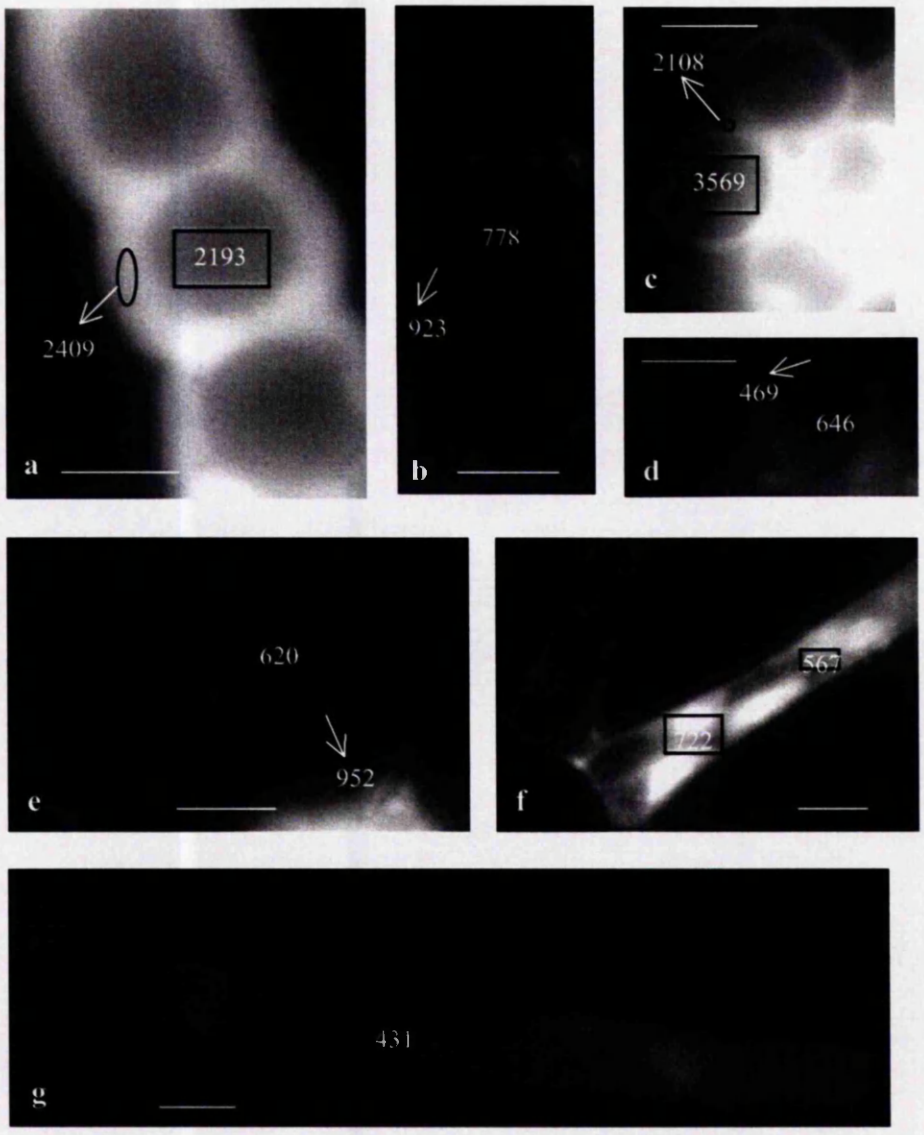


Plate 30. Fluorescence images of FITC-ConA labelled environmental phytoplankton: (a) *Anabaena spiroides*, (b) *Anabaena flos-aquae*, (c) the dense form of *Microcystis aeruginosa*, (d) the loose form of *Microcystis aeruginosa*, (e) *Anabaena circinalis*, (f) *Asterionella formosa* and (g) *Aphanizomonen flos-aquae* taken by a photometrics slow scan CCD camera. The black box and white number show the measured area and average light intensity within the area. (Scale bar = 5 μm)

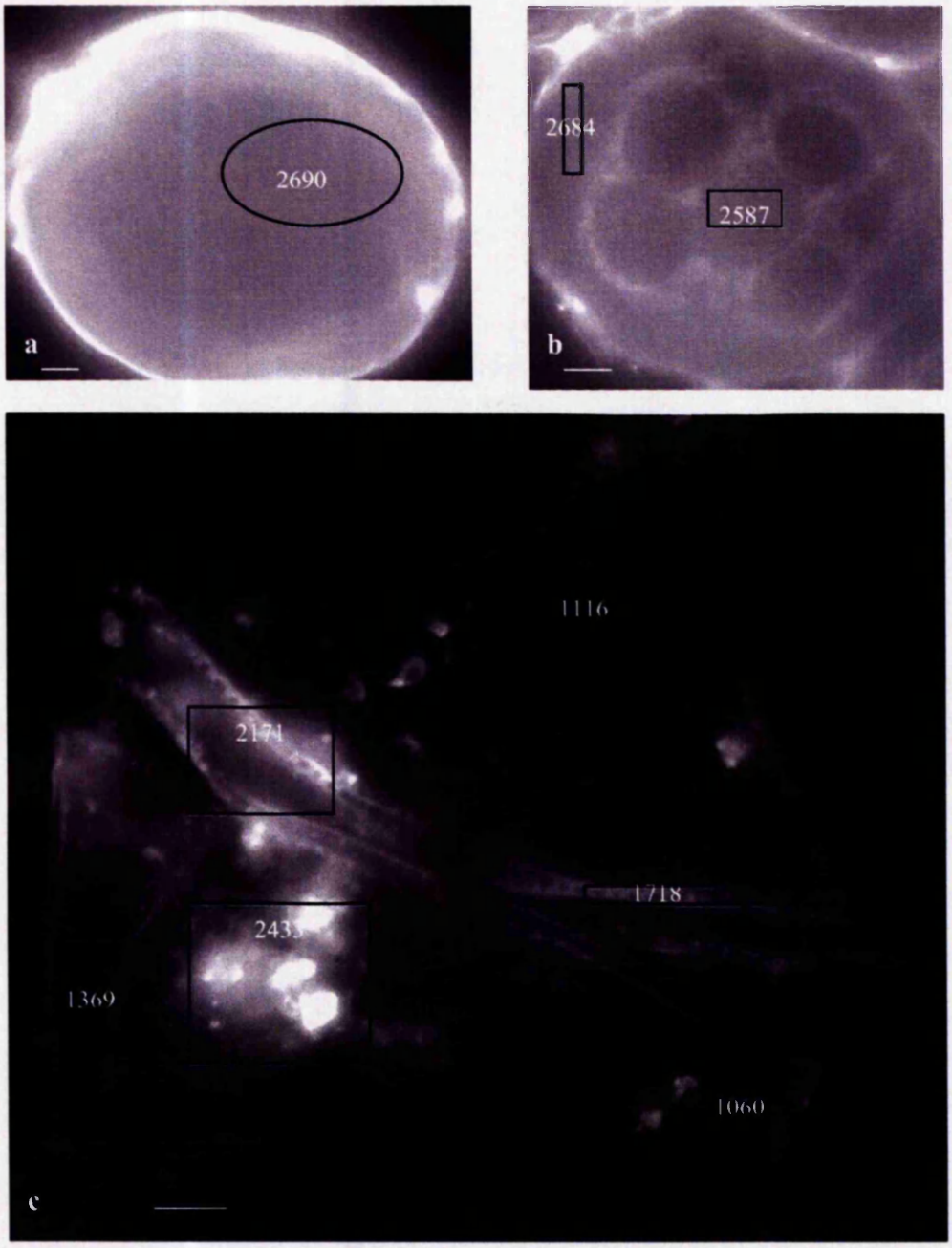


Plate 31. Fluorescent images of environmental *Eudorina elegans* labelled (a) with FITC-ConA and (b) with FITC-PHA-E, and (c) *Ceratium hirundinella* labelled with FITC-ConA taken by a photometrics slow scan CCD camera. The black box and white number show the measured area and average light intensity within the area. (Scale bar = 5 μm)

Table 4.5 FITC-lectin labelling of environmental phytoplankton from Rostherne Mere.

Species \ lectins	ConA	PHA-E	PWM	PNA
Anabaena spiroides[(a)]				
cells[1]	2831±101	2151±23	2651±80	1976±35
mucilage[2]	3884±102	2424±59	2925±61	2057±42
mucilage[3]	3833±102	2373±59	2874±61	2006±42
Anabaena circinalis[(b)]				
cells[1]	843±82	919±86	1100±27	770±49
mucilage[2]	1005±124	1192±114	1029±59	887±35
mucilage[3]	954±124	1141±114	978±59	836±35
Anabaena flos-aquae[(b)]				
cells[1]	616±16	1511±115	801±37	295±7
mucilage[2]	632±29	1390±116	730±36	340±7
mucilage[3]	581±29	1339±116	679±36	289±7
Microcystis aeruginosa[(c)]				
(dense colony) cells[1]	2400±264	791±33	636±47	614±33
mucilage[2]	2335±210	838±28	620±25	553±29
mucilage[3]	2284±210	787±28	569±25	502±29
(loose colony) cells[1]	658±21	266±17	232±14	292±13
mucilage[2]	592±15.5	237±14	234±19	289±14
mucilage[3]	541±16	186±14	183±19	238±14
Aphanizomenon flos-aquae[(c)]				
cells[1]	429±16	353±11	242±3	243±5
cells[4]	378±16	302±11	191±3	192±5
Eudorina elegans[(d)]				
cells[1]	3019±168	2380±87	2181±102	548±35
mucilage[2]	3019±168	2467±106	1386±83	494±25
mucilage[3]	2968±168	2416±106	1335±83	443±25
Asterionella formosa[(d)]				
cells[1]	485±42	251±13	259±11	217±12
cells[4]	434±42	200±13	208±11	166±12
Ceratium hirundinella[(c)]				
cells[1]	1199±144	491±35	284±21	229±5
cells[4]	1148±144	440±35	233±21	178±5

Data is presented as mean ± standard error (arbitrary units of fluorescence), n>5, where, mean= average fluorescence intensity of measured areas.
Background intensity was 51±0.5 (mean± standard error).
1: Total fluorescence, including autofluorescence and background.
2: Total fluorescence, including background (no autofluorescence).
3: Fluorescence, background subtracted.
4: Fluorescence, including autofluorescence but background subtracted.
a-d: the date of the sample collected.
a: 12 June 1996 ; b: 09 July 1996 ; c: 21 August 1996 ; d: 23 April 1996.
Individual lectins were tested against the same sample of each alga to ensure direct comparability.

4.2.2 Carbohydrate and protein analysis of extracted cell-associated mucilage and soluble extracellular products

The carbohydrate and protein content of both cell-associated mucilage (derived from laboratory-cultured algae and environmental phytoplankton) and extracellular soluble products (in both culture medium and lake water) were determined as described previously (see Section 2.5).

4.2.2.a. Carbohydrate analysis

The carbohydrate content of extracted cell-associated mucilage and extracellular products from both laboratory-cultured algae and environmental samples was determined using the phenol sulphuric acid method (Dubois et al., 1956 ; Kochert, 1978 ; Section 2.5.1).

The relationship between the concentration of glucose (x) and absorbance at 485nm of wavelength (y) using the phenol sulphuric acid method (Fig. 4.2) shows a strong correlation (R^2=0.97).

I. Laboratory-cultured algae:

Anabaena cylindrica, Anabaena spiroides, Microcystis aeruginosa and *Eudorina elegans* were extracted for their cell-associated mucilage (Section 2.5). Mucilage produced by *Anabaena spiroides* (69%) and *Eudorina elegans* (65%) had nearly twice the sugar content of that extracted from *Microcystis aeruginosa* (36%), while the least amount of carbohydrate (17%) occurred in mucilage from *Anabaena cylindrica* [Fig. 4.4(a)].

The carbohydrate content of soluble extracellular products was analysed in the culture medium of algae which had been cultured for about 24 days and were in stationary growth phase. The material released from *Eudorina elegans* showed the

highest carbohydrate content (45.1%), followed by *Anabaena spiroides* (31.2%) (Table 4.6). A carbohydrate content of less than 11% occurred in the extracellular products produced by the other five species, with the lowest from *Microcystis aeruginosa* (4.5%) and *Melosira varians* (5.3%). The carbohydrate content of cell-associated mucilage was higher than that of the extracellular products.

II. Environmental samples:

The carbohydrate content of phytoplankton-associated mucilage [Fig. 4.4(b)] and soluble extracellular products (Table 4.7) was determined on different sampling dates. As shown in Fig. 4.4(b), the highest carbohydrate content was present in the mucilage extracted from phytoplankton collected on 21 May (43%) and 05 August 1997 (40%) and the least (11%) was on 07 May 1997.

There was a significantly lower level of sugar in soluble extracellular products (Table 4.7) compared to extracted mucilage. The carbohydrate content of soluble extracellular products ranged from 1.4% to 2.4%, with the highest percentage in the sample collected in July 1997. The carbohydrate content in extracellular products thus showed little variation between samples from different sampling dates.

4.2.2.b Protein analysis

The protein content of extracted cell-associated mucilage and soluble extracellular products from laboratory-cultured algae and environmental samples was determined using the bicinchoninic acid (BCA) protein assay (Section 2.5.2). The standard assay curve between concentration of bovine serum albumin (x) and absorbance at 562nm of wavelength (y) (Fig. 4.3) shows a strong correction of R^2=0.95.

I. Laboratory-cultured algae:

Mucilage extracted from *Eudorina elegans* had the highest protein content (8%), with that from *Anabaena cylindrica* containing the least (1.4%). The protein content of *Anabaena spiroides* and *Microcystis aeruginosa* were similar at 3% and 3.6% respectively [Fig. 4.4(a)].

The protein content of extracellular products released into the culture media were less than 1% except for that from *Chlorella vulgaris* (2%) (Table 4.6).

II. Environmental samples:

The protein content of cell-associated mucilage extracted from environmental phytoplankton on different sampling dates ranged from 4% to 25%. The highest protein content was found in mucilage from April (22%), July (23%) and September (25%) and the least (4.2%) on the 21 May [Fig. 4.4(b)].

A low level of protein was found in soluble extracellular products from lake water, ranging from 0.13% to 0.48% and with the highest in those from 21 May (Table 4.7).

4.2.2.c Comparison of carbohydrate and protein content in cell-associated mucilage and soluble extracellular products

Carbohydrate and protein content in cell-associated mucilage and soluble extracellular products was compared from the results shown in the previous Sections 4.2.2.a and 4.2.2.b.

I. Cell-associated mucilage:

The ratio of carbohydrate and protein content was calculated in extracted cell-associated mucilage from laboratory-cultured algae and environmental phytoplankton (Fig. 4.4). Of the laboratory-cultured species with associated mucilage, *Anabaena spiroides* contained the highest ratio of carbohydrate to protein (22.7) and *Eudorina*

elegans the least (8.1) [Fig. 4.4(a)]. The highest ratio of carbohydrate to protein (10.3) in mucilage extracted from environmental phytoplankton was found in samples collected on 21 May. Mucilage from April (0.8), July (0.8) and September (0.9) all had a carbohydrate/protein ratio of less than 1, indicating higher protein than carbohydrate content in the mucilage at these times [Fig. 4.4(b)].

II. Soluble extracellular products:

Extracellular products released into the culture media had much higher carbohydrate than protein. The highest ratio of carbohydrate to protein was found in extracellular products from *Anabaena spiroides* (124.7) and *Eudorina elegans* (124.2) (Table 4.6).

The total dry weight of soluble material in lake water is shown in Table 4.7. The amount of carbohydrate or protein per unit volume of lake water could be calculated by multiplying the total dry weight of soluble material by the percentage of carbohydrate or protein of dry weight. The results are shown in Table 4.7. The highest amount of carbohydrate in lake water occurred in July (10.21 mg l^{-1}) and August (12.47 mg l^{-1}). However, the highest amount of protein in lake water was found on 29 October (1.51 mg l^{-1}), following samples taken on 05 August (1.35 mg l^{-1}) and on 21 May (1.32 mg l^{-1}).

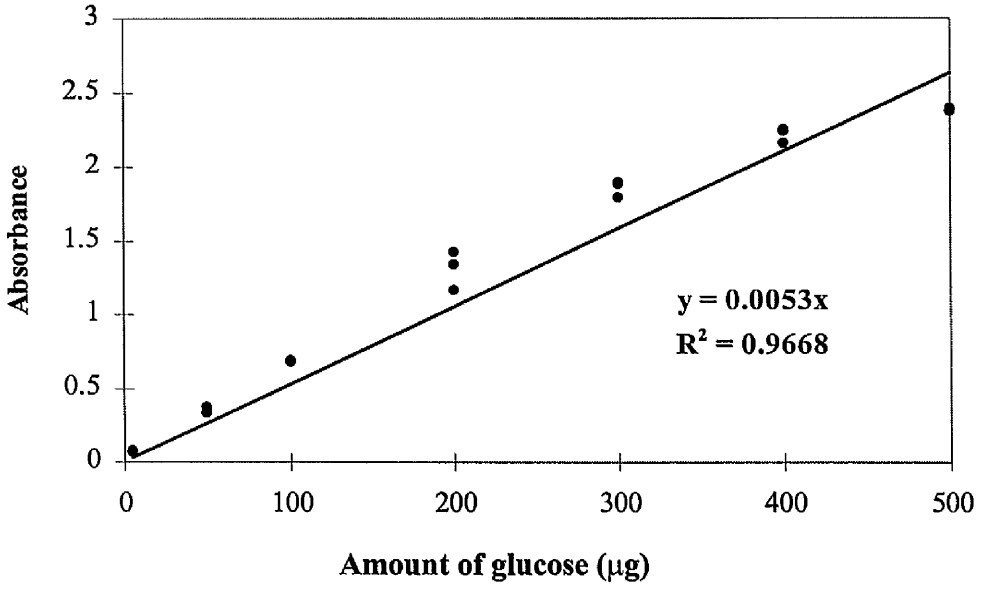


Fig. 4.2 Standard curve produced by the carbohydrate assay using the phenol sulphuric acid method with glucose as the reference sugar.

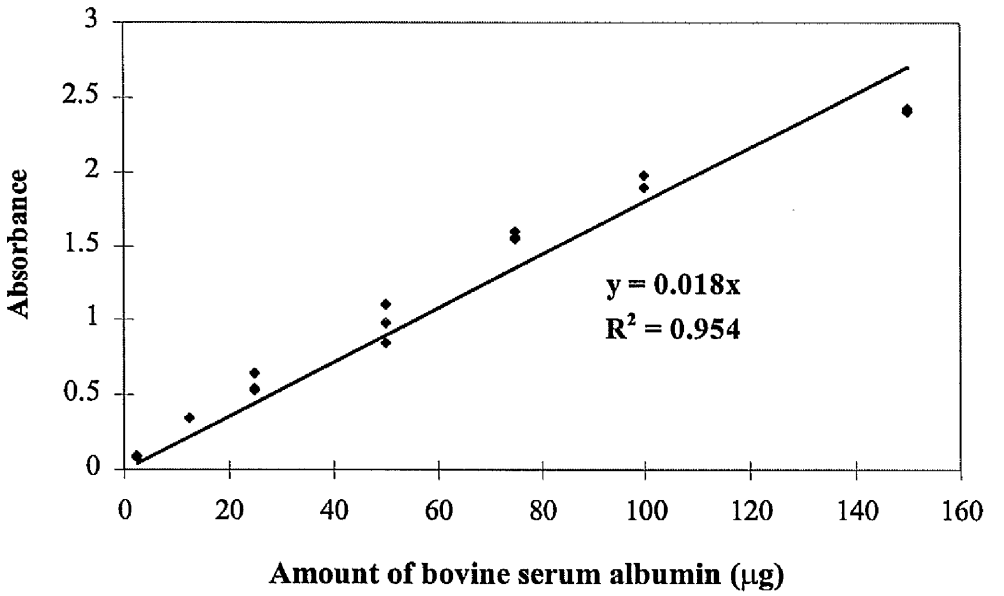


Fig. 4.3 Standard curve produced by the protein assay using the bicinchoninic acid assay (BCA).

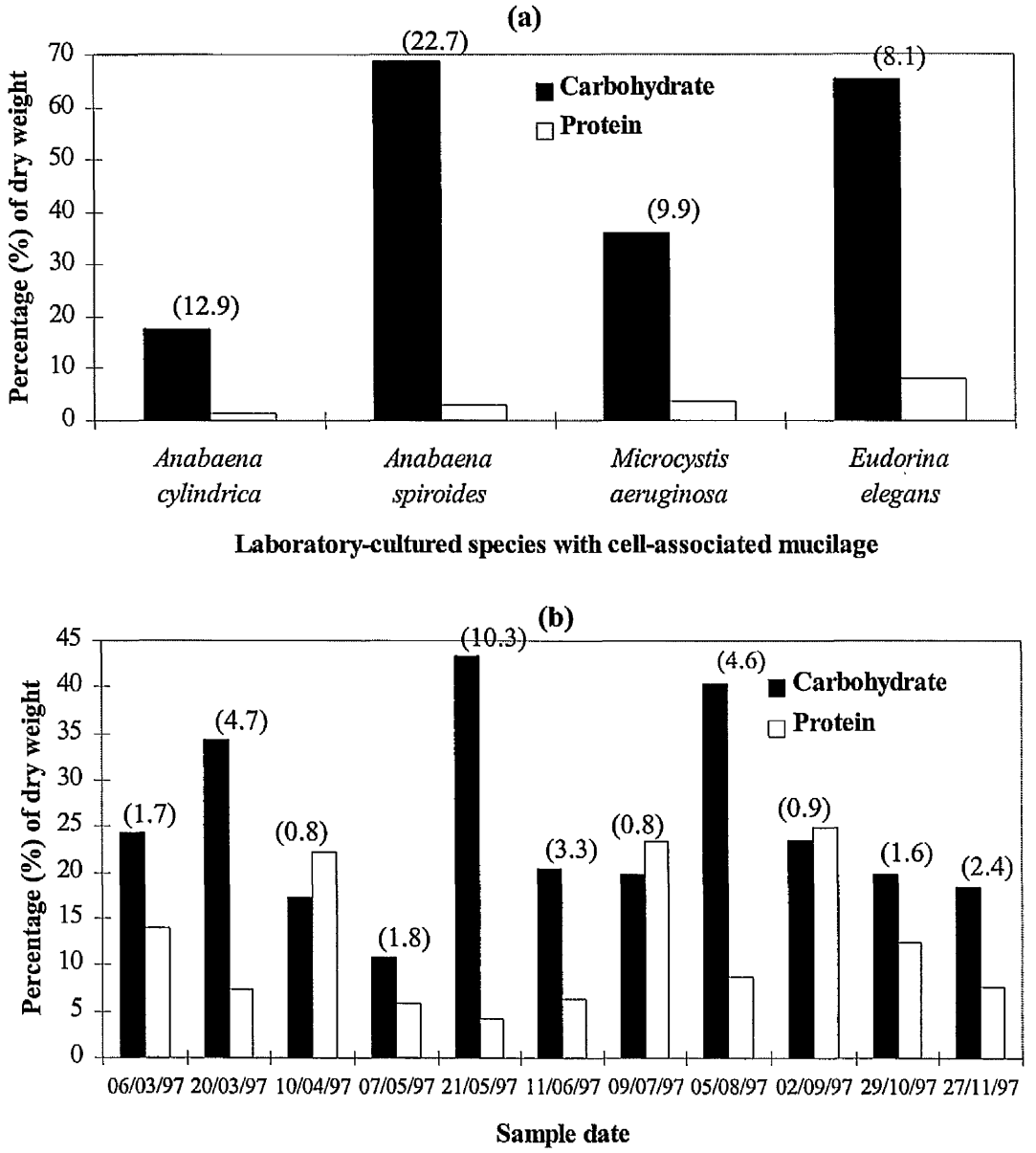


Fig. 4.4 Percentage of carbohydrate (sugar) and protein in extracted cell-associated mucilage from (a) laboratory-cultured algae and (b) environmental phytoplankton from Rostherne Mere on different sampling dates. (The number shown in parentheses is the ratio of carbohydrate to protein.)

Table 4.6 Carbohydrate and protein content of soluble extracellular products in the culture medium of laboratory-cultured algae.

Algae	Carbohydrate %	Protein %	Carbohydrate /Protein Ratio
Anabaena cylindrica	9.1	0.29	31.6
Anabaena spiroides	31.2	0.25	124.7
Microcystis aeruginosa	4.5	0.81	5.6
Eudorina elegans	45.1	0.36	124.2
Chlorella vulgaris	7.8	2.00	3.9
Asterionella formosa	10.6	0.98	10.8
Melosira varians	5.3	0.64	8.4

Carbohydrate analysis: the phenol sulphuric acid method.
Protein analysis: BCA protein assay.

Table 4.7 Carbohydrate and protein content of soluble extracellular products (EP) in lake water from Rostherne Mere.

	Carbohydrate %	Protein %	dry weight of EP(mg l^{-1})	Carbohydrate (mg l^{-1})	Protein (mg l^{-1})
06/03/97	1.79	0.27	151	2.70	0.41
20/03/97	1.95	0.14	214	4.17	0.29
10/04/97	1.41	0.13	182	2.57	0.24
07/05/97	2.03	0.24	150	3.05	0.36
21/05/97	1.78	0.48	276	4.91	1.32
11/06/97	2.02	0.26	270	5.45	0.70
09/07/97	2.42	0.23	422	10.21	0.97
05/08/97	1.94	0.21	643	12.47	1.35
02/09/97	2.15	0.16	205	4.40	0.33
29/10/97	1.62	0.30	504	8.16	1.51
27/11/97	2.12	0.17	378	8.01	0.64

Carbohydrate analysis: the phenol sulphuric acid method.
Protein analysis: BCA protein assay.

4.3 Discussion

In this study, algal surface sugars were characterised by a number of lectins with different sugar-binding specificity using three types of microscopy to identify and quantify the lectin-binding activity of laboratory-cultured algae and environmental phytoplankton from Rostherne Mere. The carbohydrate and protein composition of cell-associated mucilage and soluble extracellular products was also determined in laboratory-cultured algae and environmental samples.

4.3.1 Characterisation of cell surface sugars by lectin binding

4.3.1.a Interference from autofluorescence

Phytoplankton contain fluorescent photosynthetic pigments which exhibit autofluorescence under a fluorescence microscope. The colour of the autofluorescence displayed by phytoplankton depends on their pigment composition, with chlorophyll-a having a red fluorescence at a wavelength of 680-690nm and phycoerythrins an orange fluorescence at a wavelength of 580-630nm (Hofstraat et al., 1994). In laboratory-cultured *Anabaena cylindrica*, the strong red autofluorescence is consistent with the presence of chlorophyll-a in vegetative cells and akinetes, but not in heterocysts where weak fluorescence was observed. Heterocysts are distinguishable from vegetative cells by their thick walls, relatively weak pigmentation due to low amounts of chlorophyll-a and refractile polar granules (Rippka et al., 1979). The red autofluorescence of environmental algae such as *Eudorina elegans* and *Stephanodiscus spp.* is also due to the presence of chlorophyll-a. Weak red autofluorescence in *Asterionella formosa* indicates a relatively low concentration of chlorophyll-a.

Comparison of the autofluorescence displayed by *Anabaena cylindrica* and environmental phytoplankton cells after different treatments showed that

glutaraldehyde fixation did not decrease autofluorescence, while acetic ethanol fixation did. These results were the same as obtained by Hofstraat et al. (1994).

In this study, algal surface sugars were characterised by lectins conjugated with fluorescein isothiocyanate (FITC) to visualize the lectin binding activity. The reason for using FITC as a marker was because it has a yellow-green colour which can not be confused with the red autofluorescence. Other similar markers such as TRIC and Texas Red have the disadvantage of producing a red fluorescence (Brooks et al., 1997).

FITC-ConA binding activity was initially tested on cells of *Anabaena cylindrica* prepared in three ways (living, glutaraldehyde-fixed and acetic ethanol-fixed). Detection of the binding activity of akinetes and heterocysts was not influenced by autofluorescence owing to the bright stain. However the weak green stain on living and glutaraldehyde-fixed vegetative cells was masked by the strong red autofluorescenc. Therefore, cells fixed with acetic ethanol were used as this fixation suppressed autofluorescence.

4.3.1.b Assessment of lectin labelling on *Anabaena cylindrica* in different growth phases

There was a great diversity in lectin binding activity with no distinct patterns in the binding activity of different lectins to the surface of *Anabaena cylindrica* after different incubation times. This suggests that the sugar composition of mucilage varies with the phase of growth. These results are in agreement with those of Waite at al. (1995) who pointed out that surface sugar composition of marine diatoms differ with physiological state of cells using FITC-ConA labelling and suggested that biological mediation of cell-surface sugars-containing compounds during different growth phases and growth conditions (e.g. N or Si limitation) occurred. Panoff et al. (1988) also found mucilage of *Synechocystis* strain PCC 6803 and strain PCC 6714 extracted from

young and old cultures had different sugar and protein composition. In contrast, López-Rodas and Costas (1997) found lectin binding remained constant for each clone of *Microcystis spp.* under different conditions (e.g. growth medium, temperature, irradiance, cell cycle and culture age).

The highest ConA labelling on the mucilage and cell wall was found after 16 days incubation (the late exponential growth phase), suggesting that the cell surface was rich in mannose, glucose and N-acetyl glucosamine at this time. Of the three different cell types of *Anabaena cylindrica,* akinetes showed the highest lectin binding activity. This may be because akinetes may contain much more lectin binding receptors than mucilage and cell wall of vegetative cells and heterocysts.

4.3.1.c Qualitative and Quantitative assessment of lectin binding activity on laboratory-cultured and environmental algae

Lectin binding activity on different algae was initially assessed using conventional fluorescence microscopy of different algal species. Bright lectin labelling fluorescence was found on species with a high level of mucilage (e.g. environmental *Anabaena spiroides* and *Eudorina elegans*) but not on those with a low level of mucilage (e.g. laboratory-cultured *Anabaena cylindrica* and environmental *Anabaena flos-aquae*). This suggests that mucilage may contain large numbers of lectin binding sites.

Confocal microscopy was used to increase the image resolution. The confocal microscope has the advantage of reducing blurring of the image from light scattering, increasing effective resolution and improving signal-to-noise ratio (Inoué, 1995). Clear localization of ConA labelling fluorescence on the mucilage of *Anabaena spiroides* was seen using a double labelled imaging technique which captured autofluorescence and labelling fluorescence. Both the strong labelling in the isthmus region of the cell

and the shape of mucilage visualized by labelling fluorescence suggested that mucilage may be secreted from the isthmus region. The results agree with those of Hoiczyk and Baumeister (1998) who found that the junctional pores were the sites of mucilage secretion on cyanobacteria. Bright fluorescence on the edge of the mucilage suggested that region was rich in mannose, glucose and N-acetyl glucosamine.

Clear green fluorescence in the mucilage secreted by *Anabaena flos-aquae* labelled with FITC-ConA (Plate 30) demonstrated the higher resolution of confocal microscopy compared with the blurred image (Plate 16) obtained from a conventional fluorescent microscope. However, use of this technique with FITC-ConA labelled *Anabaena cylindrica* only resulted in weak fluorescence and no optical images was obtained. This is because the confocal microscope captures the small amount of focussed fluorescence and eliminates the large amount of unfocussed light. Thus confocal microscopy can only be used when a high intensity of fluorescence is present.

Five different scores (+++, ++, +, + –, and –, see Table 4.1) were used to determine the relative amount of lectin-induced fluorescence. The results showed differences in lectin binding activity between different lectins and algal species but these differences could not be clearly distinguished. Therefore, quantitative measurements of bound lectin fluorescence were also made using a IP Lab Spectrum software which can measure light intensity of images captured by a CCD camera. In most cases, quantitative data were in agreement with the qualitative results.

The binding of particular sugars by lectins on the surface of cells is quite complicated as it involves terminal and sub-terminal monosaccharides on the cell surface (Brooks et al., 1997). The distinct lectin binding patterns on akinetes, heterocysts, and vegetative cells suggested that there were different amounts of sugars present on the surface of different cell types. The low PNA binding to these three cell

types indicated that there were small amounts of galactose and N-acetyl galactosamine present on the termini or sub-termini of polysaccharides on the cell surface. Autofluorescence plus fluorescence labelling on mucilage masked any of the lectin binding to the cell wall. Sengbusch and Müller (1983) examined FITC-ConA and PNA binding to *A. cylindrica* strain G1403-2 and showed that there was no binding to the cell surface. These results were thus different from those obtained here and may be due to different strains of algae and/or the low concentration of lectins used by Sengbusch and Müller (1983) (50 $\mu g\ ml^{-1}$, compared to 1 $mg\ ml^{-1}$ in this study), plus a shorter incubation time (30 min, compared to 1 hour in this study).

Laboratory-cultured *Anabaena spiroides* is characterised by two distinct layers of mucilage with a higher degree of labelling on the inner mucilage layer than on the outer, suggesting that the inner layer of mucilage may have a denser composition containing more lectin binding sites. However, environmental *Anabaena spiroides* only possessed one layer of mucilage but this showed much higher level of fluorescence. These differences in the structure and sugar content of mucilage may be due to differences in growth conditions (in the environment compared to the laboratory) and the strain of algae. A much higher lectin binding activity of mucilage of environmental samples compared to cultured cells was also observed in *Eudorina elegans* and *Microcystis aeruginosa*. This agrees with the results of Waite et al. (1995), as mentioned in Section 4.3.1.b, and of Tease and Walker (1987) who found mucilage extracted from *Gloeothece* ATCC 27152 grown in the presence and absence of nitrate showed qualitative and quantitative differences in sugar and protein composition. The cell wall of laboratory-cultured and environmental *Asterionella formosa* and *Ceratium hirundinella* also showed a great diversity in lectin binding patterns. This could also be explained by differences in the strains of algae and growth conditions.

The highest lectin binding activity was found on the mucilage of both cultured and environmental *Eudorina elegans* and environmental *Anabaena spiroides,* suggesting it had higher level of target sugars and denser mucilage structure with more lectin binding sites than that of the other species. PNA bound less strongly than the other lectins to mucilage from both laboratory-cultured and environmental algae (except for cultured *Anabaena spiroides* and the loose form of *Microcystis aeruginosa* from Rostherne Mere), indicating a low level of galactose and N-acetyl galactosamine. In the case of environmental samples, mucilage from *Anabaena spiroides, Microcystis aeruginosa* and *Eudorina elegans* had the highest ConA labelling, indicating large amounts of mannose, glucose and N-acetyl glucosamine on the surface of the mucilage structure. Overall lectin binding on mucilage from environmental samples was greater than that in cultured algae, suggesting that the former produce mucilage containing higher levels of the target sugars than the latter. Lectin binding to the cell wall of *Microcystis aeruginosa* and *Eudorina elegans* could be seen in the qualitative studies, but this binding intensity could not be quantified as measurements made on the cell also including autofluorescence and labelling fluorescence resulting from the presence of mucilage.

There were significant differences in lectin binding to the cell wall of *Chlorella vulgaris, Asterionella formosa, Stephanodiscus spp., Melosira varians* and *Ceratium hirundinella,* indicating differences in sugar composition of the cell wall. The cell junction of *Melosira varians* had higher ConA, PHA-E and PWM labelling than the other part of cell wall, suggesting differences in cell wall structure between these two areas. However, the absence of lectin binding to the cell surface does not mean a total absence of specific sugars or groups of sugars in the mucilage. Surek and Sengbusch (1981) found L-fucose was present in the hydrolysed mucilage of *Cosmocladium*

saxonicum but no lectin of *Ulex europaeus,* which has L-fucose specificity, bound to the mucilage. They therefore concluded that this sugar residue is not exposed at the surface but is present within the mucilage matrix.

To summarize, there was a great diversity of lectin binding activity on the cell wall and the associated mucilage secreted by different species. Such diversity was also observed by Sengbusch and Müller (1983). Moreover, the same species subject to different growth conditions (laboratory-cultured and collected from the natural environment) also displayed different lectin binding patterns, demonstrating considerable intra-specific variation.

4.3.2 Carbohydrate and protein analysis of extracted cell-associated mucilage and soluble extracellular products

4.3.2.a Analysis methods

Determination of sugars, methylated sugars and polysaccharides by the phenol sulphuric acid method is considered a simple, rapid, inexpensive and highly sensitive method giving reproducible results (Dubois et al., 1956 ; Kochert, 1978 ; Decho, 1990). It can detect most sugars (e.g. hexoses, disaccharides, oligosaccharides, polysaccharides and uronic acid) possessing a free or potentially free reducing group, with the exception of glucosamine and galactosamine (Kochert, 1978). This method is not affected by hexosamines, amino acids or true proteins, so it can determine the monosaccharide content of glycoprotein or the hexuronic acid content of mucopolysaccharides without removal of these other residues (Lee & Montgomery, 1961).

The bicinchoninic acid (BCA) protein assay is a simple, stable and highly sensitive method for colorimetric detection and quantitation of total protein. This assay is similar to the Lowry method (Lowry et al., 1951) which has been used widely for

protein quantification in biological samples. Both methods rely on the biuret reaction for the generation of a coloured complex between peptide bonds and copper atoms when protein is placed in an alkaline environment containing Cu^{+2}. The difference between them is that the Lowry method employs Folin-Ciocalteu reagent to enhance the colour response of the biuret reaction, while the BCA assay utilizes the sodium salt of bicinchoninic acid, a highly specific chromophore for Cu^{+1}. Owing to the high specificity and stability of the bicinchoninate chromophore and great tolerance to many detergents and substances known to interfere with the Lowry method, BCA assay is considered as an excellent protein assay, and thus was used in this study. (Smith et al., 1985 ; Brown et al., 1989 ; Pierce Scientific Ltd., 1996).

4.3.2.b Variation of carbohydrate and protein content in cell-associated mucilage

Cell-associated mucilage from laboratory-cultured algae and environmental phytoplankton showed large differences in the carbohydrate and protein content, reflecting a great diversity in mucilage composition between species. The result was similar to that obtained in the previous lectin-binding studies which also showed great differences in the sugar and glycoprotein composition of mucilage.

The carbohydrate (17%) and protein content (1%) of cell-associated mucilage from laboratory-cultured *Anabaena cylindrica* in this study was much lower than determined by Dunn and Wolk (1970) where the sheath material contained 66% carbohydrate and 5% protein. Further more, the carbohydrate (36%) and protein content (4%) in associated mucilage of laboratory-cultured *Microcystis aeruginosa* in this study was different from those analysed by Nakagawa et al. (1987) who found 67% of carbohydrate and 13% of protein in associated mucilage from *Microcystis aeruginosa* K-3A and Plude et al. (1991) who found 72-90% of carbohydrate and <1% of protein from *Microcystis flos-aquae* C3-40. Although the carbohydrate content in

associated mucilage of *Microcystis aeruginosa* was similar to that analysed by Amemiya and Nakayama (1984) who found 35-47% from environmental mixed *Microcystis spp.*, the protein content was different (mucilage of the latter contained 18-25% protein). These differences may be because of the use of different strains and my method of isolation using mild mechanical forces which may have not removed all the mucilage from the cell. However, this method has the advantage of not breaking the cells and so causing contamination. Mucilage of laboratory-cultured *Anabaena spiroides* and *Eudorina elegans* showed higher carbohydrate and protein content than that of cultured *A. cylindrica* and *M. aeruginosa.* This is generally consistent with the earlier lectin-binding studies (Section 4.2.1.d.I).

The high variation in the carbohydrate and protein content of cell-associated mucilage found in the environmental phytoplankton may be due to seasonal variation in phytoplankton species composition. *Eudorina elegans* may account for most of extracted associated mucilage in spring and *Anabaena flos-aquae* and *Microcystis aeruginosa* in summer. Environmental samples collected from July and September containing large amounts of *Microcystis aeruginosa* or *Anabaena flos-aquae* had the highest protein fraction (23-25%) in their mucilage. These results were similar to those of Amemiya and Nakayama (1984) who found 18-25% of protein in the mucilage of environmental *Microcystis spp.*. However, the carbohydrate content of mucilage from these two sampling dates (20-24%) was less than that obtained by Amemiya and Nakayama (1984) (35-47%).

Plude et al. (1991) compared the chemical composition and morphology of *Microcystis aeruginosa* and *Microcystis flos-aquae.* They suggested that morphological differences in mucilage between these two species may be due to differences in the sugar composition of polysaccharides, and their protein content.

They also suggested that the protein content correlated with slime firmness. Therefore, the ratio of carbohydrate to protein could be used to explain the interspecific differences in the morphology of mucilage. Laboratory-cultured *Eudorina elegans* secreted mucilage with a distinct outer margin which could be seen without staining. The firm mucilage correlated with a low ratio of carbohydrate to protein (high protein content). Mucilage of cultured *Anabaena spiroides* had the highest ratio of carbohydrate to protein which may result in its dispersed appearance and a diffuse outer margin, which could only be seen with negative staining (e.g. indian ink).

4.3.2.c Variation of carbohydrate and protein content in soluble extracellular products

Actively-metabolising phytoplankton cells may liberate considerable amounts of extracellular products into the surrounding medium. These substances often play important roles in algal growth and physiology, as well as in aquatic food chains (Hellebust, 1974 ; Fogg, 1983). The extracellular products liberated by algae include carbohydrates, nitrogenous substances, organic acids (e.g. glycolic acid), lipids, phenolic substance, organic phosphates, volatile substance, enzymes, vitamins, sex factors, growth inhibitors and stimulators, and toxins (Fogg, 1966 ; Hellebust, 1974 ; Agrawal & Sharma, 1996). Soluble mucilage mainly containing polysaccharides and proteins is part of these extracellular products. In this study, the carbohydrate and protein of soluble extracellular products in culture media and Rostherne Mere lake water were analysed.

Great differences in the carbohydrate content of soluble extracellular products from cultured media were found. This may be due to differences in amounts of carbohydrate secretion between algal species (reviewed by Hellebust, 1974). For example, *Phaeocystis poucheti* liberated approximately 16-64% of photoassimilated

carbohydrates into the cultured medium (Guillard & Hellebust, 1974) and *Anabaena flos-aquae,* 28% (Moore & Tischer, 1965). Species with high amounts of carbohydrate in their cell-associated mucilage showed relatively high concentrations of carbohydrate in their extracellular products, suggesting that part of associated mucilage was dispersed into the culture medium. The carbohydrate content of dry material from lake water during 1997 was low and similar (ranging from 1.4% to 2.5%). This may be due to the utilization of carbohydrate by bacteria and, possibly, zooplankton. Low levels (less than 2%) of protein were found in the soluble extracellular products from both the culture media and Rostherne Mere lake water, indicating that only a small amount of protein was secreted by cells into surrounding water.

The high ratio of carbohydrate to protein in extracellular products from cultured media and lake water indicates that cells secrete more polysaccharides than protein into the surrounding water. Both the carbohydrate and protein content in the soluble extracellular products present in the lake water was low (less than 14 mg l^{-1}, 3% of total dry material) with only small differences between each sampling date. Since samples of extracellular products in lake water were obtained by freeze-drying filtered lake water, it contained not only extracellular products secreted by aquatic organisms but also soluble ions and debris. By calculating the results of the concentration of major ions (Ca, Na, Mg, S, K, Si, P, Al, Mn, Fe) in filtered lake water (Levado, 1997), the total major ions in freeze-dried samples is estimated to be at least 27% of total dry weight of soluble material in August lake water and up to 88% in September lake water. This indicated a relatively large proportion of ions collected in the samples of soluble extracellular products from lake water, resulting in low amounts of carbohydrate and protein detected in lake water and suppressing the true variation between different sampling dates.

CHAPTER 5. METAL-BINDING ACTIVITIES OF ALGAL SURFACES

5.1 Introduction

Algal surfaces function both as cationic and as anionic exchange sites for adsorption of metal ions (Weber, 1972 ; Ting et al., 1989 ; discussed in Section 1.2.3). Since there is wide structural and chemical variation in the composition of the algal cell wall and associated mucilage (see Section 1.2.3 and Section 1.1.2 respectively), metal-binding activity will vary with species. The aims of this part of study were: (1) to examine the metal-binding (adsorption) capacity of different algae in relation to the presence of mucilage, type of cell wall and surface area and, (2) to determine the metal-binding capacity of mixed phytoplankton samples and relate this to species composition. Copper was chosen to investigate metal-binding activity due to its high efficiency in binding to algal surfaces (Huntsman & Sunda, 1980 ; Zhang & Majidi, 1994 ; Kiefer et al., 1997).

5.2 Results

5.2.1 Assessment of the growth of Anabaena cylindrica *in modified BG-11 medium*

In studying the behaviour of trace metals in phytoplankton cultures, it is very important to consider the growth medium since this can complex with the added metal and so change its bioavailability and behaviour. Citric acid, ferric ammonium citrate, EDTA and trace metals were omitted from BG-11 medium (Appendix 1) to reduce the possibility of metal chelation and interaction between metals. The aim of this experiment was therefore to investigate whether *Anabaena cylindrica* could grow well in this modified medium.

Anabaena cylindrica was grown in modified BG-11 medium and normal BG-11 medium respectively for 17 days. Growth curves of *Anabaena cylindrica* in modified BG-11 medium and in BG-11 medium (Fig. 5.1) show a similar pattern of development, with a three day lag phase in each case. After this lag phase, there was a marked increase in the optical density of *Anabaena cylindrica* grown in BG-11 medium, but only a gradual rise when *Anabaena cylindrica* was grown in modified BG-11 medium. The growth of *Anabaena cylindrica* in modified BG-11 medium was significantly lower ($p<0.05$) after 3 days incubation.

Growth of *A. cylindrica* in modified BG-11 medium was also more variabe (average coefficient of variance: 14.5%) between replicate cultures than that in normal BG-11 medium (average coefficient of variance: 6.4%).

No difference in the occurrence of different cell types was observed between the two growth media during the lag phase, but thereafter, qualitative observation indicated that more akinetes were present in modified BG-11 medium compared to BG-11 medium. Moreover, smaller filaments were observed in modified BG-11 medium at the end of the experiment and akinetes formed the major cell type.

As it was found that *A. cylindrica* remained viable in modified BG-11 medium (but did not grow as well as in normal medium), the algae were cultured in normal medium then transferred to modified medium during exposure to copper in the subsequent experiment.

5.2.2 Assessment of copper uptake by Anabaena cylindrica *in modified BG-11 medium*

Anabaena cylindrica was initially cultured in normal BG-11 medium for 10 days (to the exponential growth phase) and then transferred to modified BG-11 for 18 hours before adding copper. This was to stabilize cells within the modified medium.

Rapid accumulation of copper by *Anabaena cylindrica* was observed (Fig. 5.2). Within 15 min (which was the minimum sampling time due to the necessity to centrifuge the sample) of adding copper to the culture, the concentration of copper in *Anabaena cylindrica* had increased from 200 μg g^{-1} to 1100 μg g^{-1}. The copper concentration in the alga reached a peak of 2900 μg g^{-1} after a 1 hour exposure to added copper. Further accumulation did not occur with increased exposure time. There was slight decline between 2 hours and 32 hours exposure. The copper concentration of *A. cylindrica* in controls remained relatively stable between 80 μg g^{-1} and 200 μg g^{-1}. Accumulation of copper in copper-treated *A. cylindrica* was significantly higher than in the control ($p<0.05$).

The concentration of copper in the surrounding medium showed a sharp and significant ($p<0.05$) decreased, falling to approximately 63 μg l^{-1} 1 hour after commencement of the experiment (Fig. 5.3). A similar concentration was recorded in the control (copper, no algae) and there was no significant difference ($p>0.05$) between test and control after 2 hours exposure time. The pH remained near-neutral (7.1 to 7.7) throughout the experiment.

Calculation of the total amount of copper removal from culture medium was considered in relation to total amount retained by algae. The amount of copper removed (37 μg) from the surrounding medium at 1 hour exposure time was similar to the amount detected in the pellet (32 μg). However, after 1 hour exposure time, about 40 ~ 50 μg of copper was removed from the medium, but the copper concentration in the pellet fell to between 12 ~ 24 μg.

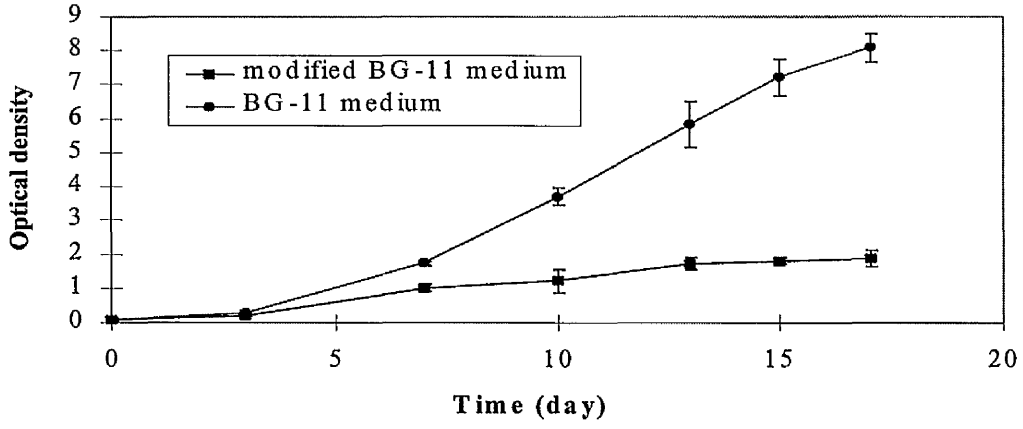


Fig. 5.1 Growth curve of *Anabaena cylindrica* in BG-11 medium and in modified BG-11 medium. (Error bar represents the standard deviation of triplicate samples.)

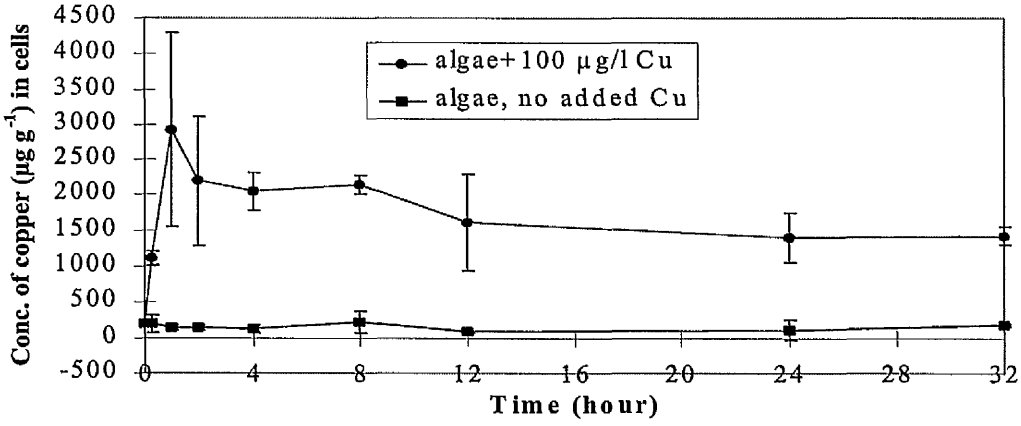


Fig. 5.2 Copper accumulation in *A. cylindrica* after different exposure times. (Error bar represents the standard deviation of triplicate samples.)

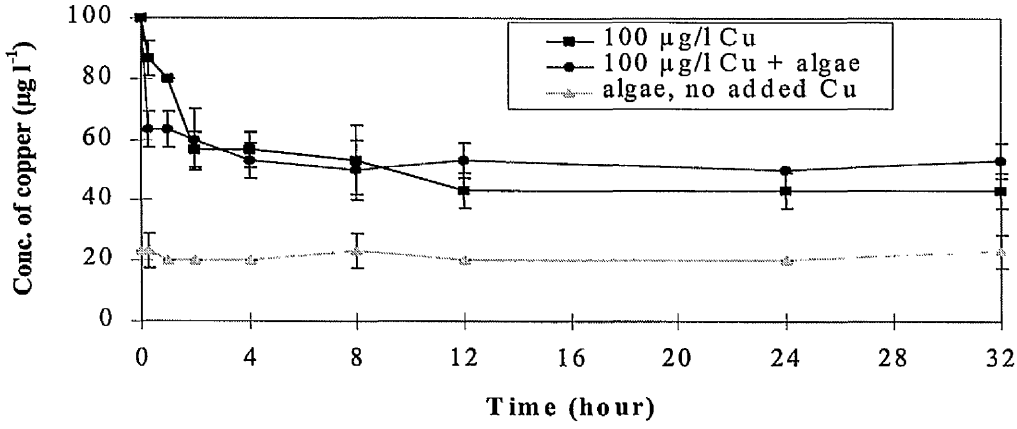


Fig 5.3 Copper concentration remaining in the modified BG-11 medium after different exposure times. (Error bar represents the standard deviation of triplicate samples.)

5.2.3 *Copper adsorption onto* Anabaena cylindrica *in distilled water*

After the initial studies, it was considered that even the modified BG-11 medium, plus the use of glass containers, might reduce the degree of copper binding by complexing and adsorption respectively. Thus in this experiment, distilled water and polycarbonate tubes were therefore used. The period of exposure to copper was reduced to 4 hours since the previous experiment revealed that adsorption was completed by this time.

The adsorption of copper onto cells of *Anabaena cylindrica* was studied in relation to four main parameters:

(a) Changes in the surrounding medium. As shown in Fig. 5.4, there was a dramatic fall in the copper concentration in distilled water containing 100 $\mu g\ l^{-1}$ added copper in the presence of *Anabaena cylindrica* after 30 minutes. By 1 hour, approximately 80% of the copper had disappeared from the distilled water. The amount of copper remaining showed a slight rise ($p<0.05$) between 1 and 2 hours. Copper concentration in the control (no algae) remained stable at 100 $\mu g\ l^{-1}$ throughout the experiment. No (<10 $\mu g\ l^{-1}$) copper was detected in the distilled water. There was a significant difference ($p<0.05$) in the amount of copper remaining in the surrounding medium between the experimental (with algae) and control (no algae) group.

(b) Changes in intact cells. From Fig. 5.5, it can be seen that copper accumulation in cells exposed to copper increased dramatically after 1 hour of incubation ($p<0.05$), but decreased slightly thereafter.

(c) Release of copper from the cell surface following treatment with EDTA. The concentration of copper released into the water from cells reached a peak of 97 $\mu g\ l^{-1}$ when cells had been previously exposed to added copper for 2 hours (Fig. 5.6). The background level of Cu in 10 mM EDTA solution was 10 $\mu g\ l^{-1}$.

(d) Copper remaining in cells after treatment with EDTA. Analysis of copper in *A. cylindrica* exposed respectively to 100 μg l^{-1} Cu and to distilled water only (control, without Cu) showed that there was no difference of copper concentration between cells following the two treatments after 30 min, 1 hour, and 2 hours exposure time ($p>0.05$). However there was a marked increase ($p<0.05$) of copper concentration in cells exposed to added copper after four hours (Fig. 5.7).

5.2.4 Comparison of copper adsorption by living and dead (glutaraldehyde-fixed) cells of Anabaena cylindrica

In this experiment, adsorption of copper by cells of *Anabaena cylindrica,* fixed with glutaraldehyde solution, was compared to living cells over a short (4 hours) incubation time.

Both living and dead (glutaraldehyde-fixed) cells showed adsorption of copper from aqueous solution. The concentration of copper in the aqueous medium (Fig. 5.8) showed a rapid reduction (about 50%) in the presence of algae ($p<0.05$), with no significant difference ($p>0.05$) between living and dead (glutaraldehyde-fixed) cells. Addition of 10 mM EDTA to algae (Fig. 5.9) resulted in a corresponding and equal release of copper by living and dead cells ($p>0.05$). The time of maximum copper adsorption (60 min, see Fig. 5.8) corresponded to the time of maximum copper release (Fig. 5.9) from the algal cells. The amount of copper released from the cell surface was approximately 70% of that removed from the surrounding medium.

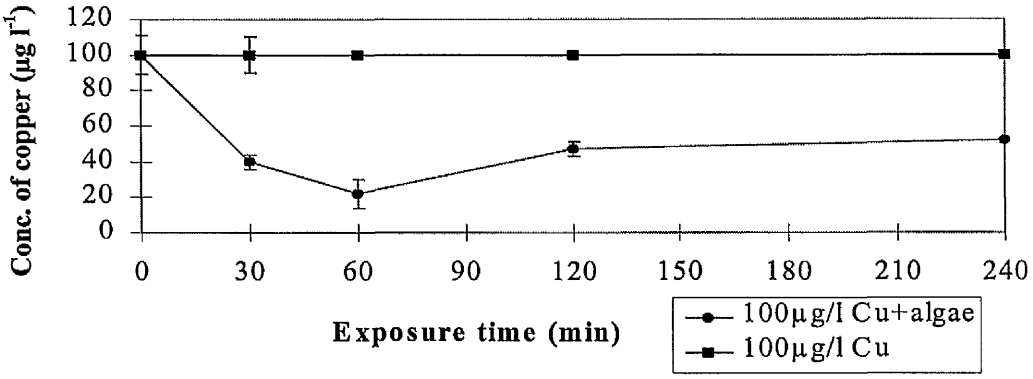


Fig. 5.4 Copper concentration remaining in distilled water after exposure to 100 µg l^{-1} added copper for up to 240 min. (Error bar represents the standard deviation of replicate samples: 100 µg l^{-1} Cu+algae, n=6 ; 100 µg l^{-1} Cu, n=3.)

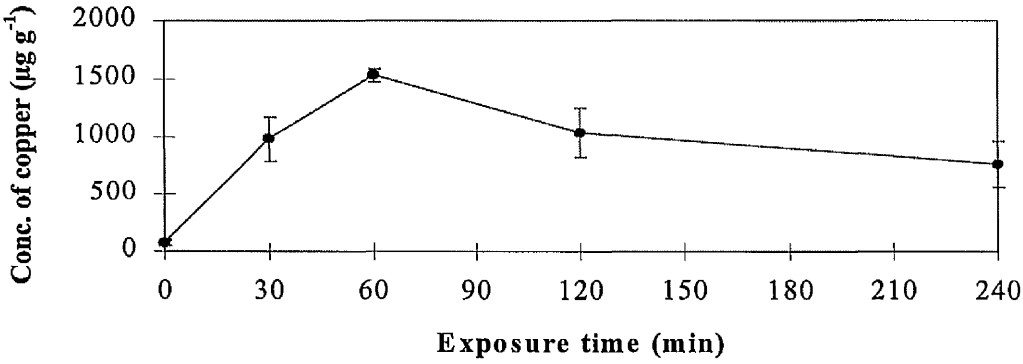


Fig. 5.5 Copper accumulation in *A. cylindrica* exposed to 100 µg l^{-1} added copper. (Error bar represents the standard deviation of triplicate samples.)

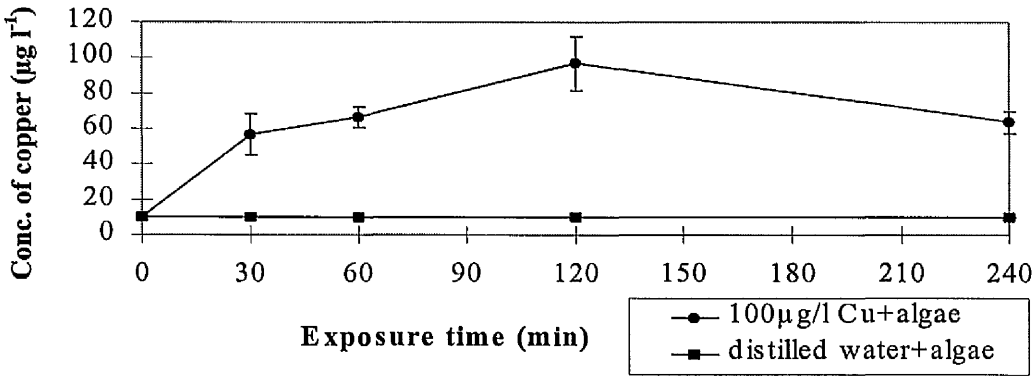


Fig. 5.6 Copper concentration released into the surrounding medium following treatment of *A. cylindrica* with 10mM EDTA solution for 1 min. Data are shown for cells treated with copper, and for control medium without copper (no added copper). (Error bar represents the standard deviation of triplicate samples.)

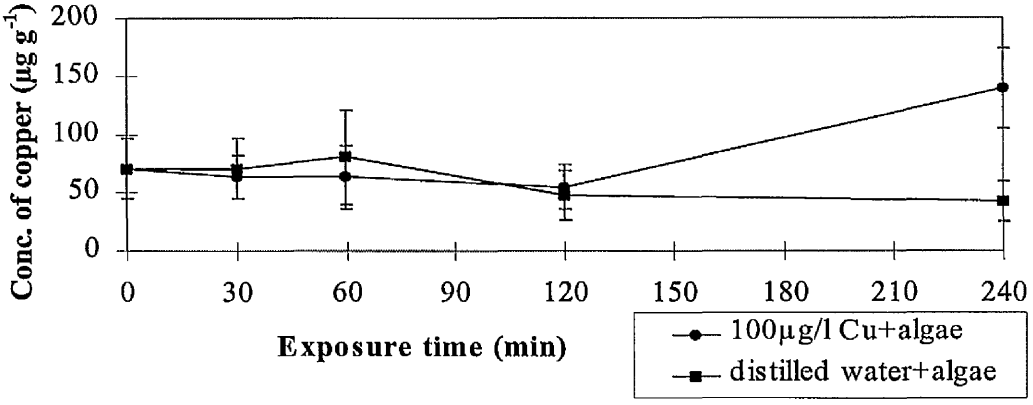


Fig. 5.7 Copper remaining in *A. cylindrica* after treatment with 10mM EDTA solution. Cells had previously been exposed to 100 µg l^{-1} added copper for up to 240 min. (Error bar represents the standard deviation of triplicate samples.)

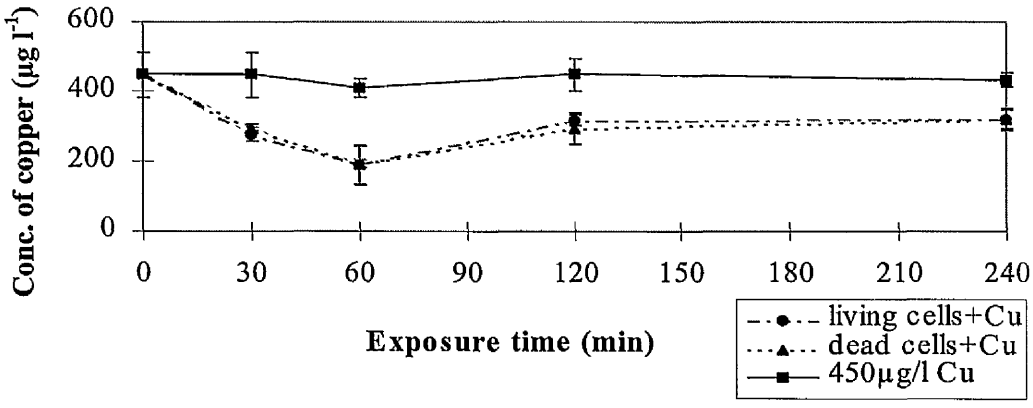


Fig. 5.8 Copper concentration remaining in distilled water after exposure to 450 µg l^{-1} added copper for up to 240 min. (Error bar represents the standard deviation in triplicates.)

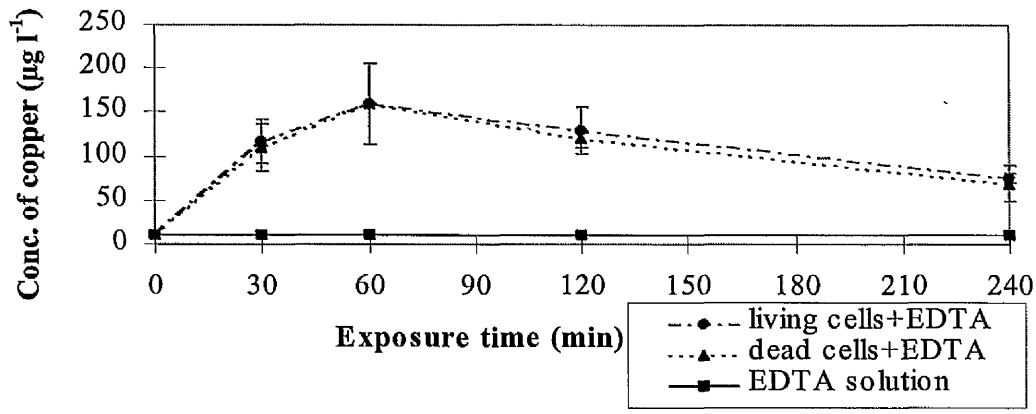


Fig. 5.9 Copper concentration released into the surrounding medium following treatment of *A. cylindrica* with 10mM EDTA solution. Data are shown for cells treated with copper. (Error bar represents the standard deviation of triplicate samples.)

5.2.5 Copper biosorption by four laboratory-cultured algae

Anabaena cylindrica, Anabaena spiroides, Eudorina elegans and Chlorella vulgaris were selected for comparative studies on copper uptake since they differed in terms of cell/colony volume, surface area and mucilage volume. Biosorption of copper by these algae was studied in relation to uptake per unit mass, uptake per cell, uptake per unit surface area and uptake per unit mucilage volume:

(a) Copper uptake per unit dry weight:

The uptake of copper by *Chlorella vulgaris* reached a maximum value of 1500 $\mu g\ g^{-1}$ within 45 min, after which it remained constant for the remaining 240 min [Fig. 5.10(b)]. In contrast, the uptake of copper by *Anabaena cylindrica, Anabaena spiroides* and *Eudorina elegans* (all mucilage-producing species) reached a maximum after 75 min, with maximum concentrations of over 2000 $\mu g\ g^{-1}$ in *Anabaena spiroides* [Fig. 5.10(a)]. There was no significant difference ($p>0.05$) of copper concentration per unit dry weight between the four algae.

(b) Copper uptake per cell:

Copper uptake per cell by *Anabaena spiroides* and *Eudorina elegans* was much higher ($7 - 9 \times 10^{-7}$ $\mu g\ cell^{-1}$), compared to *Anabaena cylindrica* and *Chlorella vulgaris* ($2 - 4 \times 10^{-8}$ $\mu g\ cell^{-1}$). The maximum copper concentration per algal cell decreased in the order *Anabaena spiroides* (9.5×10^{-7} $\mu g\ cell^{-1}$)> *Eudorina elegans* (7×10^{-7} $\mu g\ cell^{-1}$) >> *Anabaena cylindrica* (4×10^{-8} $\mu g\ cell^{-1}$) > *Chlorella vulgaris* (2×10^{-8} $\mu g\ cell^{-1}$) (Fig. 5.11). Significant differences ($p<0.05$) of copper concentration per algal cell occurred between all four species.

(c) Copper uptake per unit surface area:

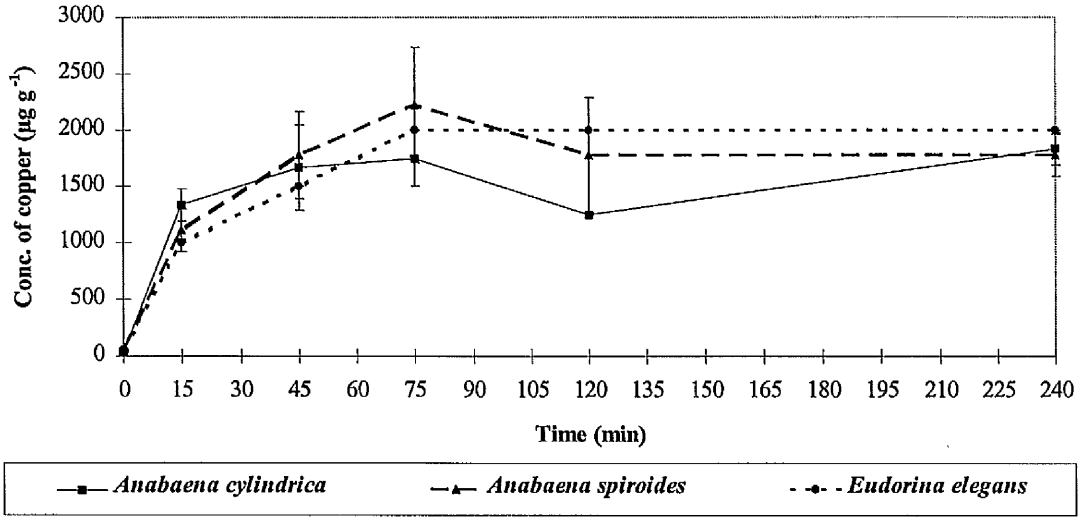
Uptake of copper per unit surface area was greatest in *Anabaena spiroides,* reaching a maximum value (after 75 min exposure to copper) of 0.16 $\mu g\ cm^{-2}$. The

maximum concentration of copper per cm^2 surface area decreased in the order *Anabaena spiroides* (0.16 $\mu g\ cm^{-2}$) >> *Anabaena cylindrica* (0.03 $\mu g\ cm^{-2}$) = *Eudorina elegans* (0.03 $\mu g\ cm^{-2}$) > *Chlorella vulgaris* (0.01 $\mu g\ cm^{-2}$) (Fig. 5.12). Differences in copper adsorption per unit surface area were significant between all species ($p<0.05$) except between *Anabaena cylindrica* and *Eudorina elegans* ($p>0.05$).

(d) Copper uptake per unit mucilage volume:

Of the three laboratory-cultured algae with cell-associated mucilage, *Anabaena cylindrica* had the highest copper concentration per cm^3 mucilage volume ($p<0.05$) with a maximum of 180 μg Cu cm^{-3}. There was no significant difference ($p>0.05$) in copper concentration per cm^3 mucilage volume between *Anabaena spiroides* and *Eudorina elegans* which had maximum adsorption values of 28 $\mu g\ cm^{-3}$ and 21 $\mu g\ cm^{-3}$ respectively (Fig. 5.13).

(a) mucilage-producing species



(b) non-mucilage-producing species -- *Chlorella vulgaris*

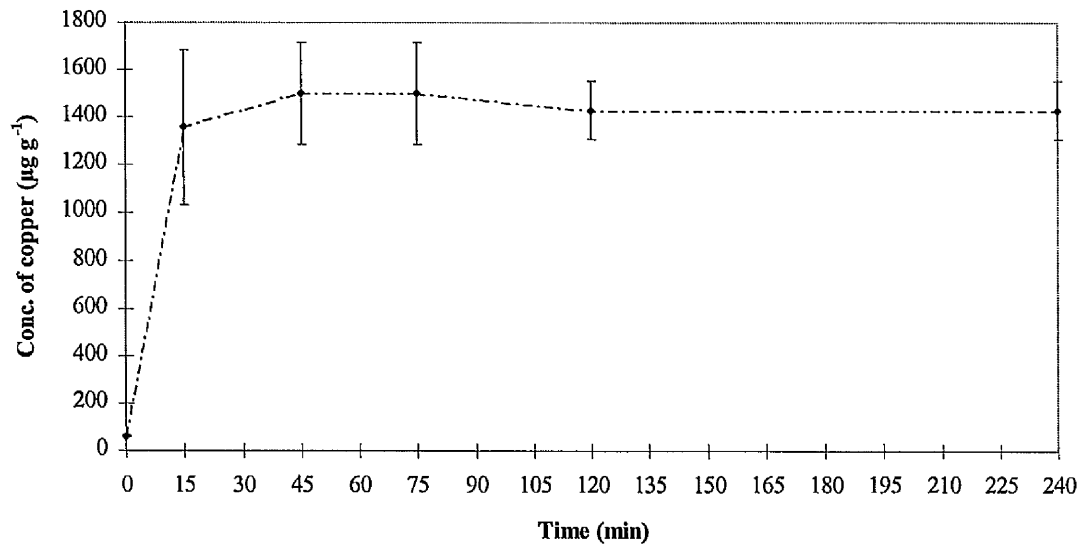


Fig. 5.10 Copper concentration per gram dry weight in (a) three mucilage-producing species and (b) one non-mucilage-producing species after exposure to 250 µg l^{-1} added copper for up to 4 hours. (Error bar represents the standard deviation of triplicate samples.)

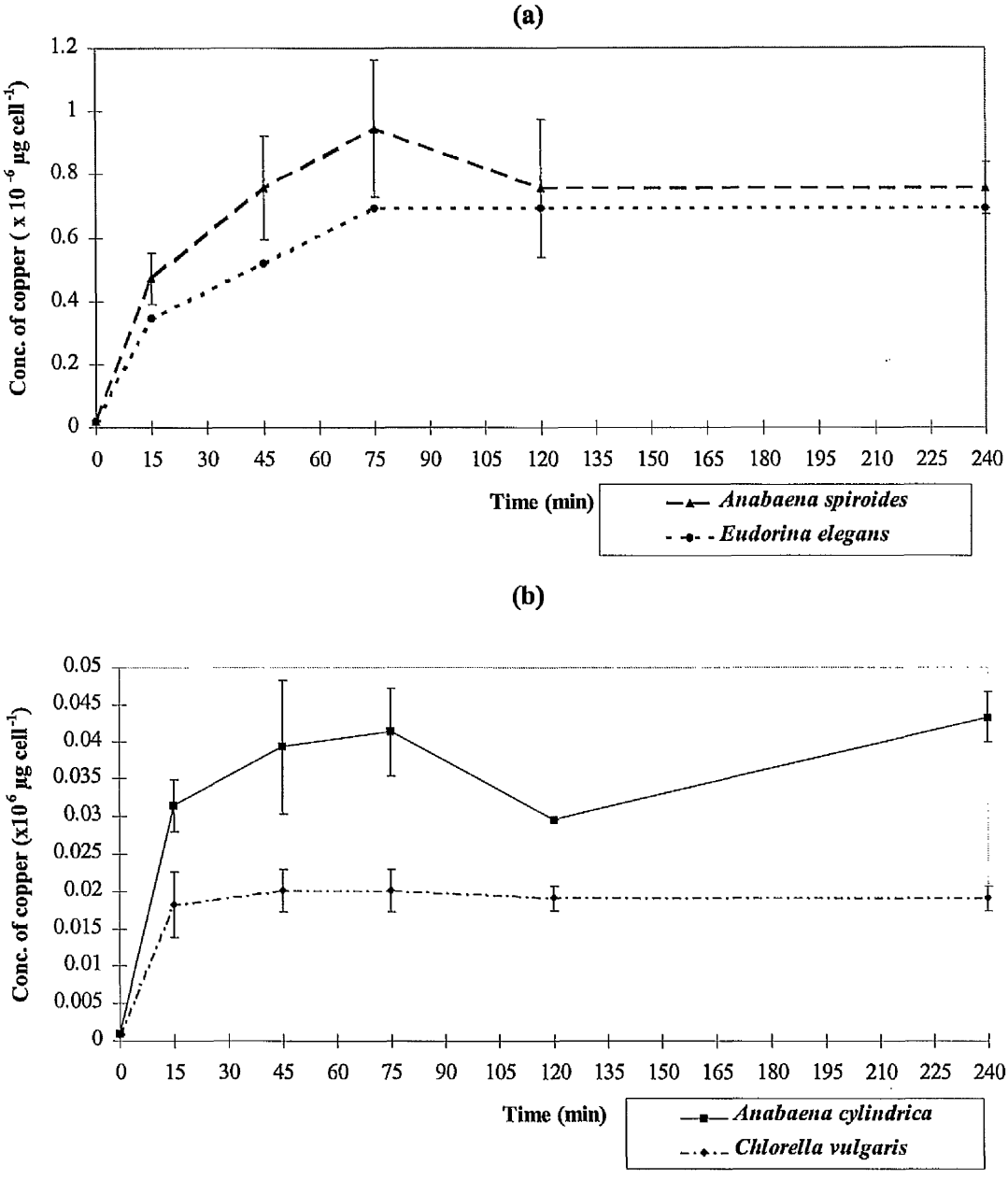


Fig. 5.11 Copper concentration per algal cell in (a) *Anabaena spiroides* and *Eudorina elegans,* and (b) *Anabaena cylindrica* and *Chlorella vulgaris* after exposure to 250 µg l^{-1} added copper for up to 4 hours. (Error bar represents the standard deviation of triplicate samples.)

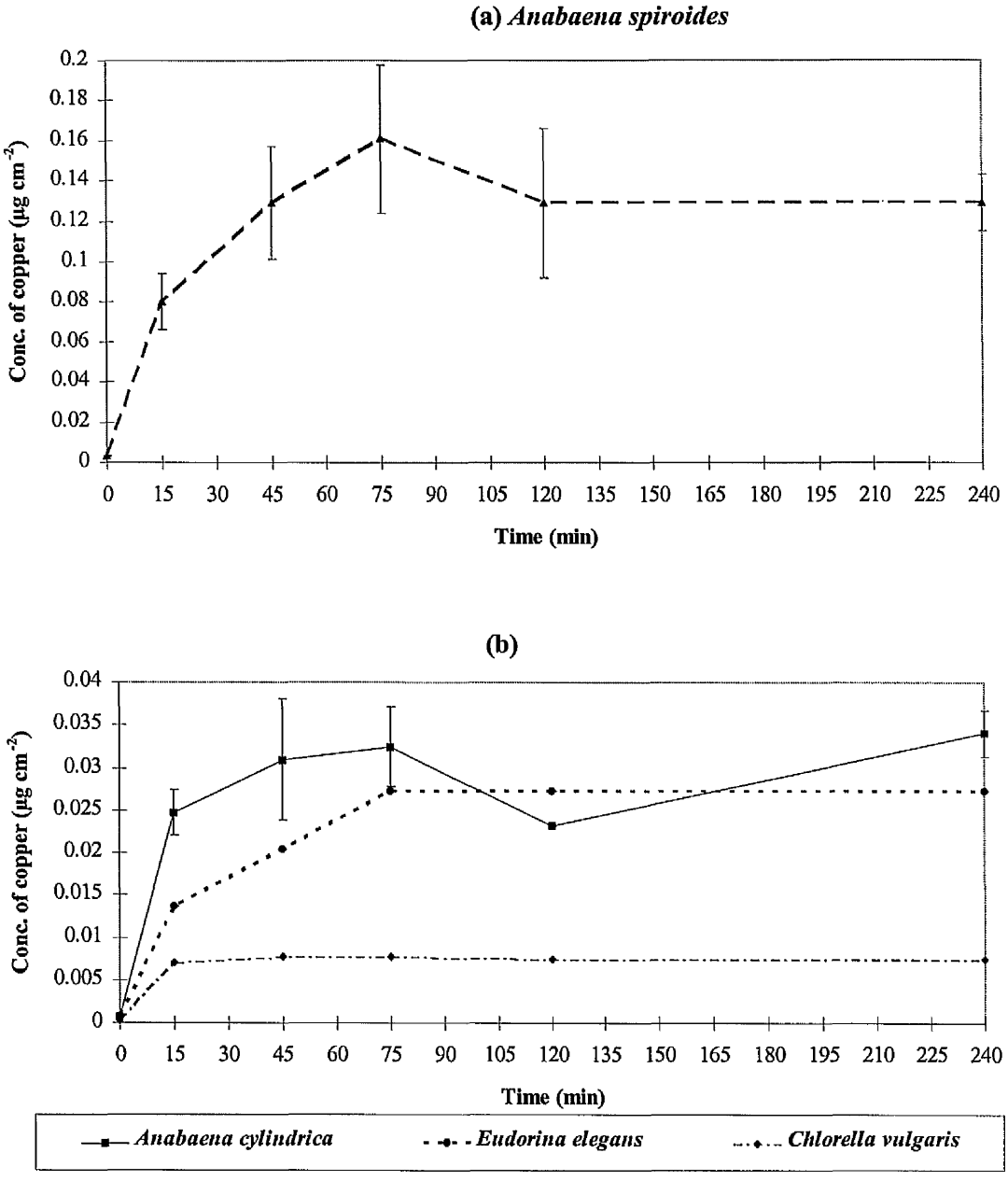


Fig. 5.12 Copper concentration per cm^2 surface area in (a) *Anabaena spiroides* and (b) *Anabaena cylindrica, Eudorina elegans,* and *Chlorella vulgaris* after exposure to 250 $\mu g\ l^{-1}$ added copper for up to 4 hours. (Error bar represents the standard deviation of triplicate samples.)

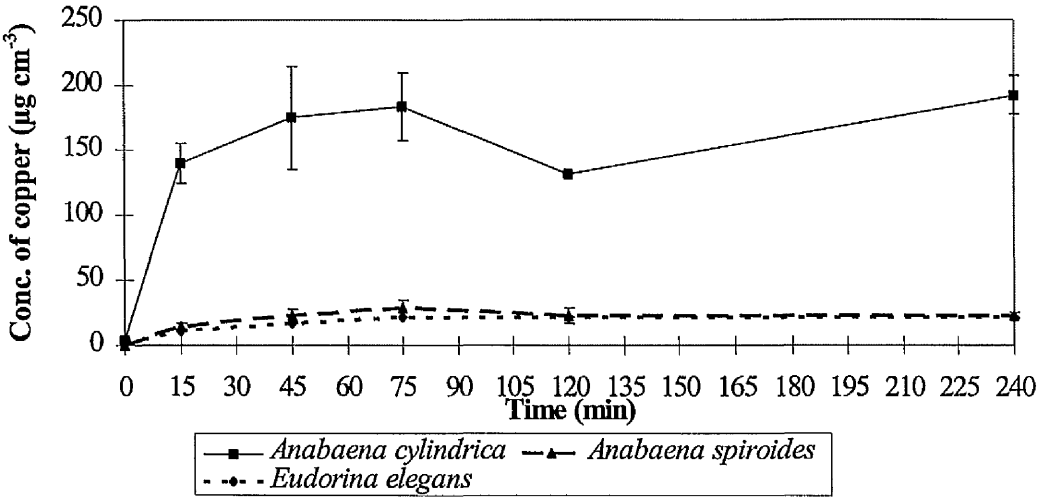


Fig. 5.13 Copper concentration per cm^3 mucilage volume in three laboratory-cultured mucilage-producing algae after exposure to 250 µg l^{-1} added copper for up to 4 hours. (Error bar represents the standard deviation of triplicate samples.)

5.2.6 Determination of copper biosorption in living and dead (freeze-dried) laboratory-cultured algae using adsorption mathematical models

The biosorption of copper(II) to eight laboratory-cultured algae was studied using two adsorption models, the Freundlich and the Langmuir model, as discussed in Section 1.2.3.b. These models allowed the degree of copper adsorption to be compared for living and dead (freeze-dried) cells.

Freeze-dried algae were examined under a Leitz Dialux 20 Phase-contrast light microscope to check that the cells had remained intact. Two species of the original eight species, *Anabaena cylindrica* and *Anabaena spiroides* were discarded because the cells had disintegrated.

(a) Freundlich model:

To obtain adsorption data for the Freundlich model, it was necessary to determine biosorption over a wide range of copper concentrations (1-1000 mg l^{-1}) [Fig. 5.14(a)]. These data were then converted to a log plot (Freundlich plot), as shown in Fig. 5.14(b). Log plots are shown for cyanobacteria (Fig. 5.14), chlorophyta [Fig. 5.15(a)], bacillariophyceae [Fig. 5.15(b)], and dinophyceae [Fig. 5.15(c)] living cells. In all cases (eight living and six freeze-dried algae), the experimental data closely fit the Freundlich model ($x/m=K_fC^{1/n}$, $\log(x/m)=(1/n)\log C + \log K_f$) over the whole range of copper concentrations ($p<0.01$ for Student's t-test of the goodness of fit of the regression line). There was a strong positive correlation (R^2, coefficient of determination, was over 90%) between the copper concentration remaining in solution at equilibrium (C) and the amount of copper adsorbed per unit weight of biomass (x/m), both in log scale (Fig. 5.14- Fig. 5.15 and Table 5.1).

The Freundlich adsorption constants K_f and 1/n (indicators of adsorption capacity and adsorption intensity respectively) calculated from the Freundlich equation and regression plots are shown in Table 5.1.

(1) Freundlich K_f values:

Adsorption capacity (K_f) decreased in the order:

Living cells:

Anabaena cylindrica > *Anabaena spiroides* > *Microcystis aeruginosa* > *Eudorina elegans* > *Chlorella vulgaris* > *Ceratium hirundinella* ≥ *Melosira varians* > *Asterionella formosa.*

Species with associated mucilage showed higher adsorption capacity (the former four species) than those without mucilage (the latter four species).

Freeze-dried cells:

Ceratium hirundinella > *Chlorella vulgaris* > *Melosira varians* > *Microcystis aeruginosa* > *Eudorina elegans* > *Asterionella formosa.*

Adsorption capacity (K_f) differed between living cells and dead (freeze-dried) cells (Table 5.1). Living mucilage-producing species (*Microcystis aeruginosa* and *Eudorina elegans*) had a higher adsorption capacity than freeze-dried cells, but living non-mucilage-producing species (*Ceratium hirundinella, Chlorella vulgaris, Melosira varians,* and *Asterionella formosa*) had lower adsorption capacity than freeze-dried cells. The K_f values ranged from 0.36 to 12.62 on living cells and from 1.21 to 5.75 on freeze-dried cells. The adsorption capacity (K_f) of living *Anabaena cylindrica* for copper was 35-fold greater than that of living *Asterionella formosa*, while that of freeze-dried *Ceratium hirundinella* was 4.8-fold greater than that of freeze-dried *Asterionella formosa.*

(2) Freundlich 1/n values:

The adsorption intensity (1/n, the slope of the regression line) ranged from 0.35 (*A. cylindrica*) to 1.1 (*Asterionella formosa*) for living cells and from 0.16 (*Melosira varians*) to 0.68 (*Microcystis aeruginosa*) for freeze-dried cells. The adsorption intensity was greater in freeze-dried than in living *Microcystis, Eudorina, Chlorella,* and *Ceratium,* but bacillariophyceae (diatoms).

Comparison of metal binding per unit surface area

As surface area and dry weight of cells are major factors influencing adsorption (Weber, 1972), the ratios of surface area and dry weight of the eight laboratory-cultured algae were determined. The ratio of surface area and dry weight ranged from 3.61 to 73.48 and decreased in the order: *Microcystis aeruginosa* > *Chlorella vulgaris* > *Anabaena cylindrica* > *Anabaena spiroides* > *Ceratium hirundinella* > *Eudorina elegans* > *Melosira varians* > *Asterionella formosa* (Table 5.2). Bacillariophyceae showed the lowest surface area and dry weight ratio. For species with cell-associated mucilage, the mucilage volume was also determined and decreased in the order *Eudorina elegans* > *Anabaena spiroides* > *Microcystis aeruginosa* > *Anabaena cylindrica.*

The Freundlich K_f values represent the predicted amount of metal bound to algal cells at an equilibrium metal concentration of 1 mg l^{-1}. Therefore, the predicted bound metal per unit algal surface area could be obtained by dividing the K_f value with the ratio of surface area and dry weight and the predicted amount of copper per unit mucilage volume by dividing K_f value by the ratio of mucilage volume to dry weight. The results are shown in Table 5.3.

The predicted copper concentration per unit surface area ranged from 0.09 to 0.6 $\mu g\ cm^{-2}$ for living cells and decreased in the order:

Melosira varians > *Anabaena cylindrica* > *Eudorina elegans* > *Anabaena spiroides* > *Microcystis aeruginosa* = *Chlorella vulgaris* > *Asterionella formosa* > *Ceratium hirundinella.*

Species with associated mucilage showed higher copper per unit surface area than those without mucilage except for *Melosira varians.*

Of four living mucilage-producing species, *Anabaena cylindrica* had the highest predicted copper concentration per unit mucilage volume following by *Anabaena spiroides* and *Microcystis aeruginosa,* and *Eudorina elegans* the least.

(b) Langmuir model:

Two Langmuir plots, derived from biosorption data over a wide range of copper concentrations [Fig 5.14.(a)], are showed in Fig 5.16 for living *Anabaena cylindrica.* Neither data from living *Anabaena cylindrica* nor that from the other seven living and six freeze-dried algae fitted the Langmuir model over the whole range of exposure concentrations (1-1000 mg l^{-1}).

Table 5.1 Comparison of the Freundlich adsorption constants obtained for laboratory-cultured algae exposed to copper.

Species	cell stage	$\log K_f \pm SE$	$(1/n) \pm SE$	K_f	R^2
Anabaena cylindrica	living	1.1±0.052	0.35±0.029	12.62	0.97
Anabaena spiroides	living	0.941±0.07	0.355±0.04	8.73	0.94
Microcystis aeruginosa	living	0.914±0.115	0.503±0.064	8.21	0.93
	freeze-dried	0.39±0.126	0.68±0.071	2.47	0.95
Eudorina elegans	living	0.598±0.063	0.449±0.035	3.96	0.97
	freeze-dried	0.33±0.129	0.55±0.073	2.13	0.92
Chlorella vulgaris	living	0.56±0.027	0.445±0.015	3.63	0.99
	freeze-dried	0.63±0.137	0.53±0.077	4.26	0.9
Asterionella formosa	living	-0.444±0.12	1.1±0.067	0.36	0.98
	freeze-dried	0.083±0.119	0.53±0.067	1.21	0.93
Melosira varians	living	0.359±0.106	0.424±0.059	2.29	0.91
	freeze-dried	0.48±0.035	0.16±0.019	3.03	0.93
Ceratium hirundinella	living	0.362±0.036	0.367±0.02	2.3	0.99
	freeze-dried	0.76±0.142	0.63±0.08	5.75	0.93

K_f and 1/n are Freundlich constants derived from the intercept and slope of the regression line. The value of K_f and 1/n can be considered as indicators of adsorption capacity and adsorption intensity, respectively. R^2 (coefficient of determination) represents the strength of the relation between the copper concentration remaining in solution at equilibrium (C) and the amount of copper adsorbed per unit weight of biomass (x/m) both following log transformation. (SE = standard error)

Table 5.2 Dry weight (DW), surface area (SA) and mucilage volume (MV) in 5 ml of laboratory-cultured algae (living cells) used in the calculation of copper biosorption from the Freundlich model.

Species	DW±SE (mg)	SA±SE (cm^2)	MV±SE (mm^3)	SA/DW (cm^2 mg^{-1})
*Anabaena cylindrica**	0.9±0	27.92±0.15	2.22±0.01	31.03
*Anabaena spiroides**	1.27±0.07	36.8±1.43	3.7±0.14	28.98
*Microcystis aeruginosa**	0.567±0.03	41.66±1.17	2.41±0.06	73.48
*Eudorina elegans**	1.6±0	18.66±1.17	13.88±0.87	11.66
Chlorella vulgaris	0.8±0.06	25.52±0.43		31.89
Asterionella formosa	0.267±0.03	0.96±0.03		3.61
Melosira varians	1.43±0.12	5.45±0.64		3.81
Ceratium hirundinella	0.04±0.01	1.03±0.03		25.78

SE refers to the standard error. * denotes species with cell-associated mucilage.

Table 5.3 Comparison of the predicted amount of copper removed by living algal cells per unit surface area (SA)* and per unit mucilage volume (MV) at an equilibrium concentration of 1 mg l^{-1}.

Species	μgCu cm^{-2}(SA)	μgCu mm^{-3} (MV)
Anabaena cylindrica	0.41	5.12
Anabaena spiroides	0.3	3
Microcystis aeruginosa	0.11	1.93
Eudorina elegans	0.34	0.46
Chlorella vulgaris	0.11	
Asterionella formosa	0.1	
Melosira varians	0.6	
Ceratium hirundinella	0.09	

* values for *Anabaena cylindrica, Anabaena spiroides, Eudorina elegans* and *Melosira varians* are examined per unit surface area of the colony.

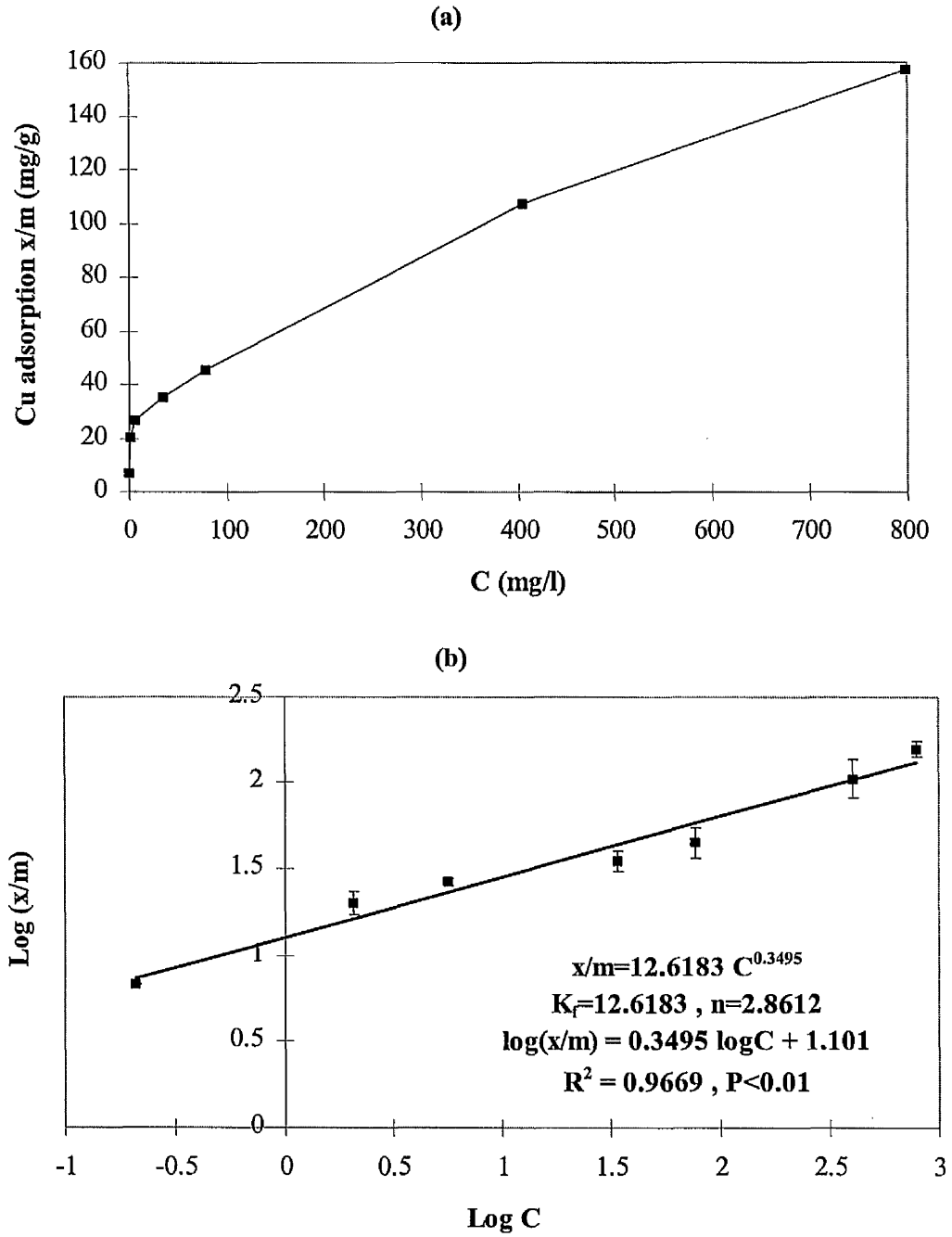


Fig. 5.14 Copper adsorption by living *Anabaena cylindrica* after exposure of the same biomass to between 1 and 1000 mg l^{-1} of copper for 1 hour at 25 °C. (a) adsorption of copper by cells at different copper concentrations and (b) Freundlich (log) plot. Where, C: copper concentration remaining in the medium; x/m: amount of copper adsorbed per unit dry weight; K_f and n: Freundlich constants; R^2: coefficient of determination. (Error bars represent the standard deviation of triplicate samples.)

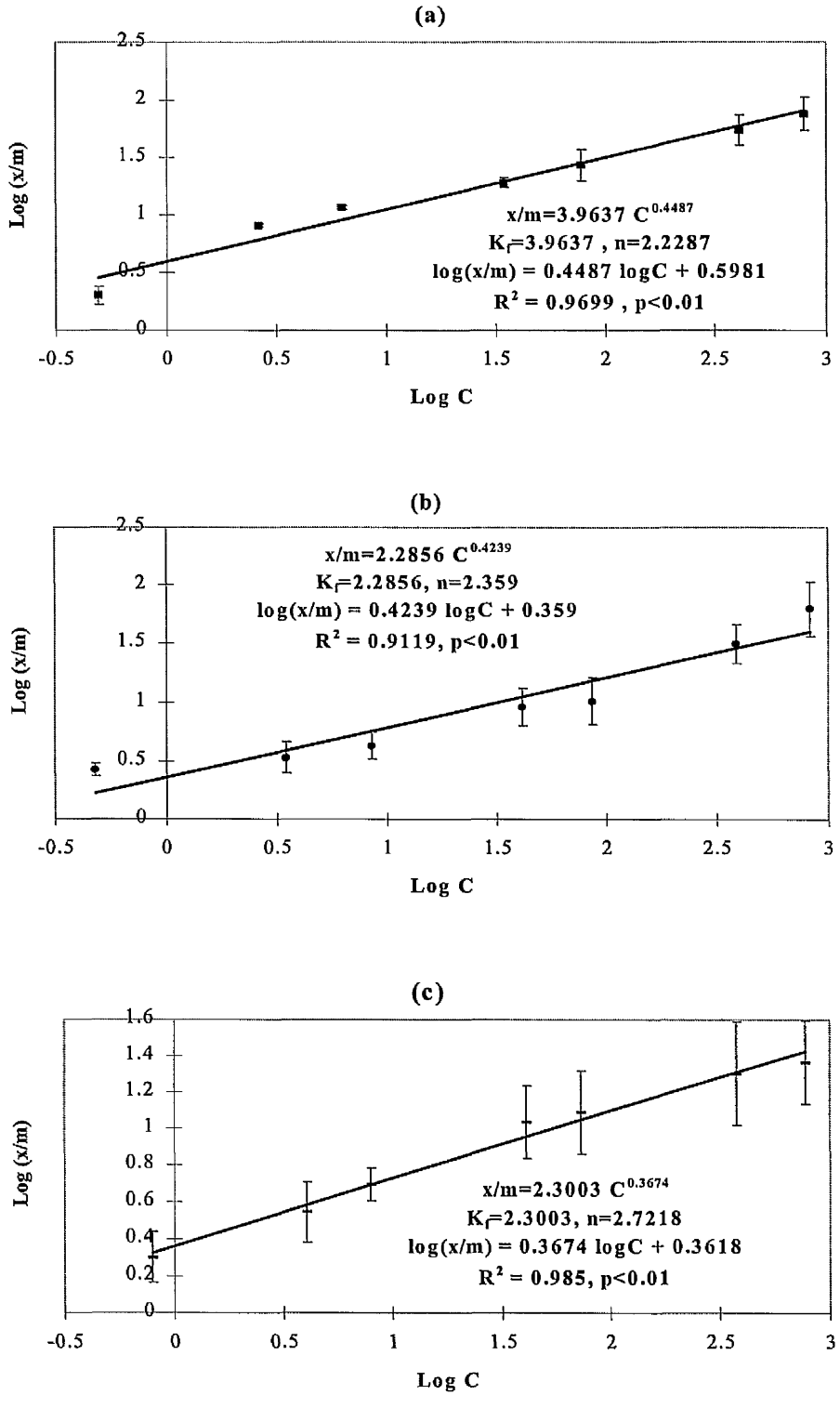


Fig. 5.15 Freundlich plots of copper biosorption by living (a) Chlorophyta *Eudorina elegans,* (b) Bacillariophyceae *Melosira varians* and (c) Dinophyceae *Ceratium hirundinella.* (Error bars represent the standard deviation of triplicate samples.)

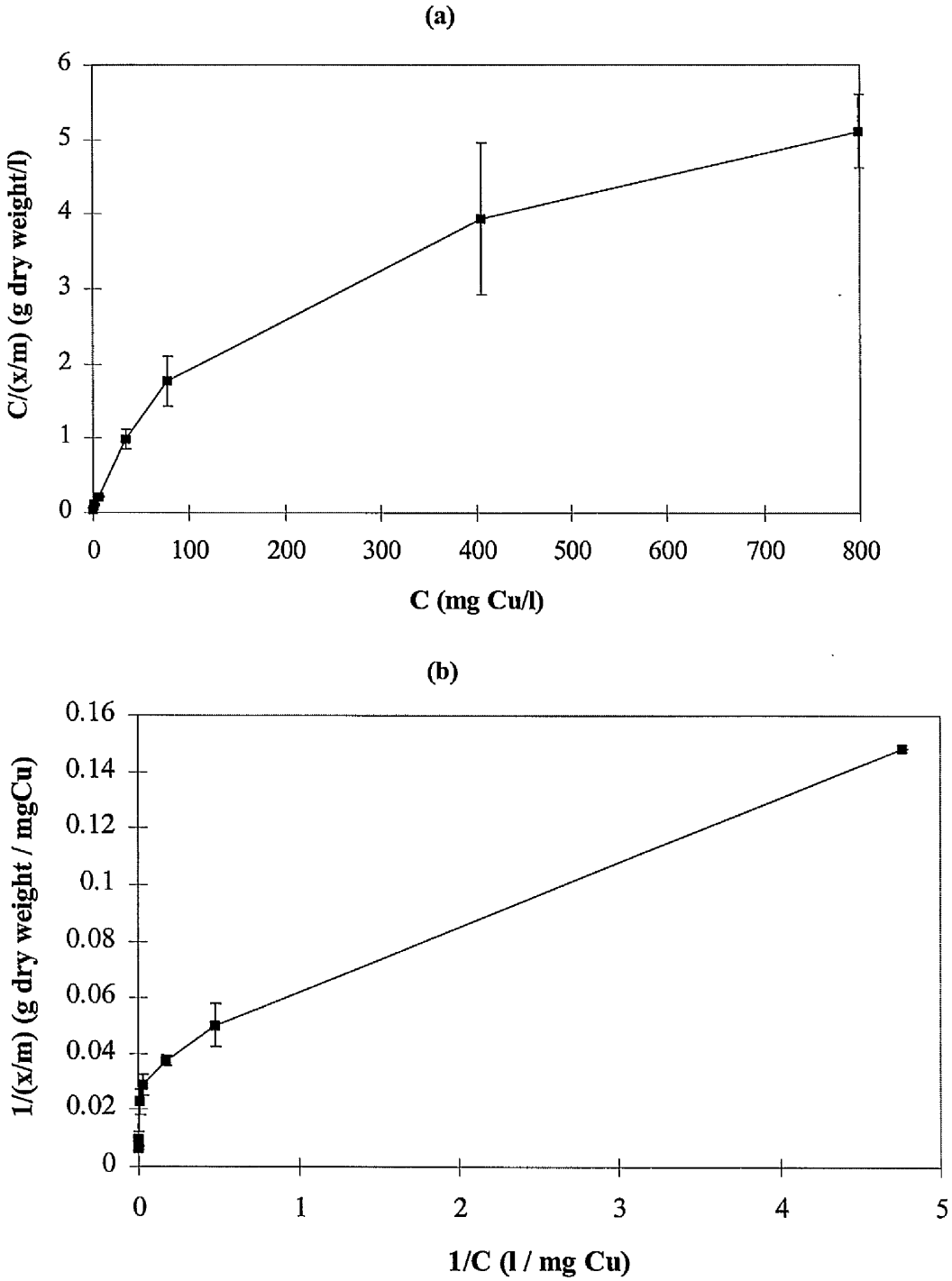


Fig. 5.16 Langmuir plots of copper biosorption by living *Anabaena cylindrica* using two versions of the Langmuir equation. (Error bars represent the standard deviation of triplicate samples.)

5.2.7 Determination of copper binding capacity of phytoplankton samples from Rostherne Mere

5.2.7.a Preliminary assessment of copper binding by mixed phytoplankton cells from Rostherne Mere

A preliminary experiment on the binding of copper by mixed phytoplankton cells was carried out using samples collected on 07 May and 11 June 1997. Copper binding activity was assessed in cells from both trawl-net samples and integrated lake water samples. The major objective of this work was to investigate the time course of copper uptake.

Rapid copper uptake (Fig. 5.17) was observed in the May sample within 20 minutes of exposure, with 20% removal of copper by concentrated phytoplankton cells (the trawl-net samples) and 8.8% by integrated phytoplankton samples. Significant differences ($p<0.05$) in the concentration of copper remaining in the lake water were found between filtered lake water and integrated samples, and between filtered lake water and trawl-net samples, within 30 min of exposure time. In the May samples, bacillariophyceae were the most abundant algae with *Asterionella formosa* (92000 cells ml^{-1} in trawel-net samples and 930 cells ml^{-1} in integrated samples) the dominant species.

In the June samples, copper removal reached a maximum value of 33% at 10 min after copper addition for trawl-net samples, while integrated samples showed a maximum copper removal (34.7%) at 40 min (Fig. 5.18). There were significant differences ($p<0.05$) in the copper concentration remaining in lakewater between filtered lake water and integrated samples, and between filtered lake water and trawl-net samples. With regard to species composition of integrated and trawl-net samples from June 1997, cyanobacteria (in praticular *Anabaena spp.* with 3500 colonies ml^{-1})

was the dominant algal group in trawl-net samples and chlorophyta (in particular *Ankyra spp.* with 900 cells ml^{-1}) in integrated samples.

With an initial level of 200 $\mu g\ l^{-1}$, maximum copper biosorption values for May were 7.5 mg g^{-1} (integrated sample) and 1.35 mg g^{-1} (net sample) and for June, 11.3 mg g^{-1} (integrated sample) and 1.6 mg g^{-1} (net sample).

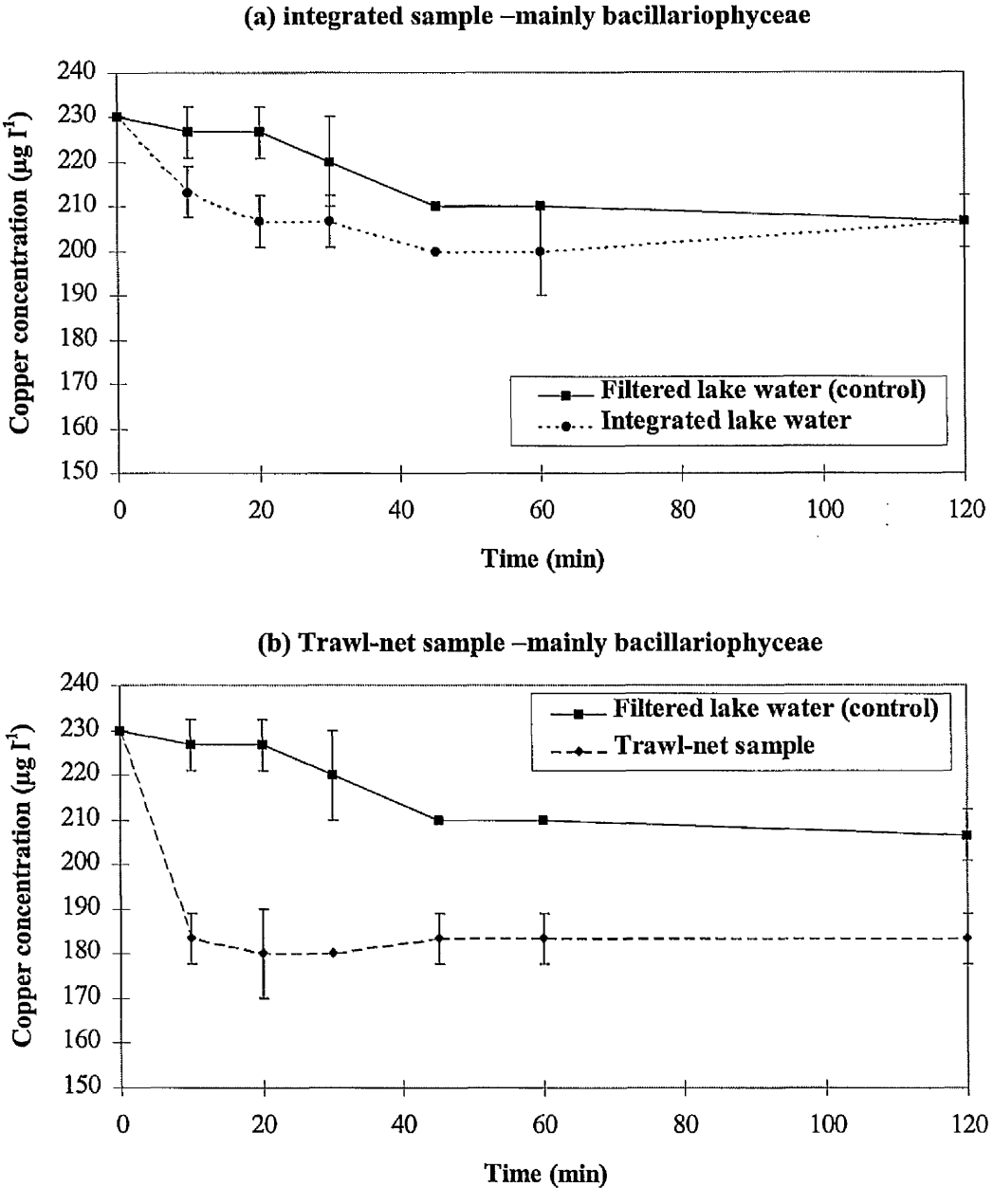


Fig. 5.17 Copper concentration remaining in lake water from Rostherne Mere on 07 May 1997 after the addition of 200 µg l^{-1} of copper. (Error bars represent the standard deviation of triplicate samples.)

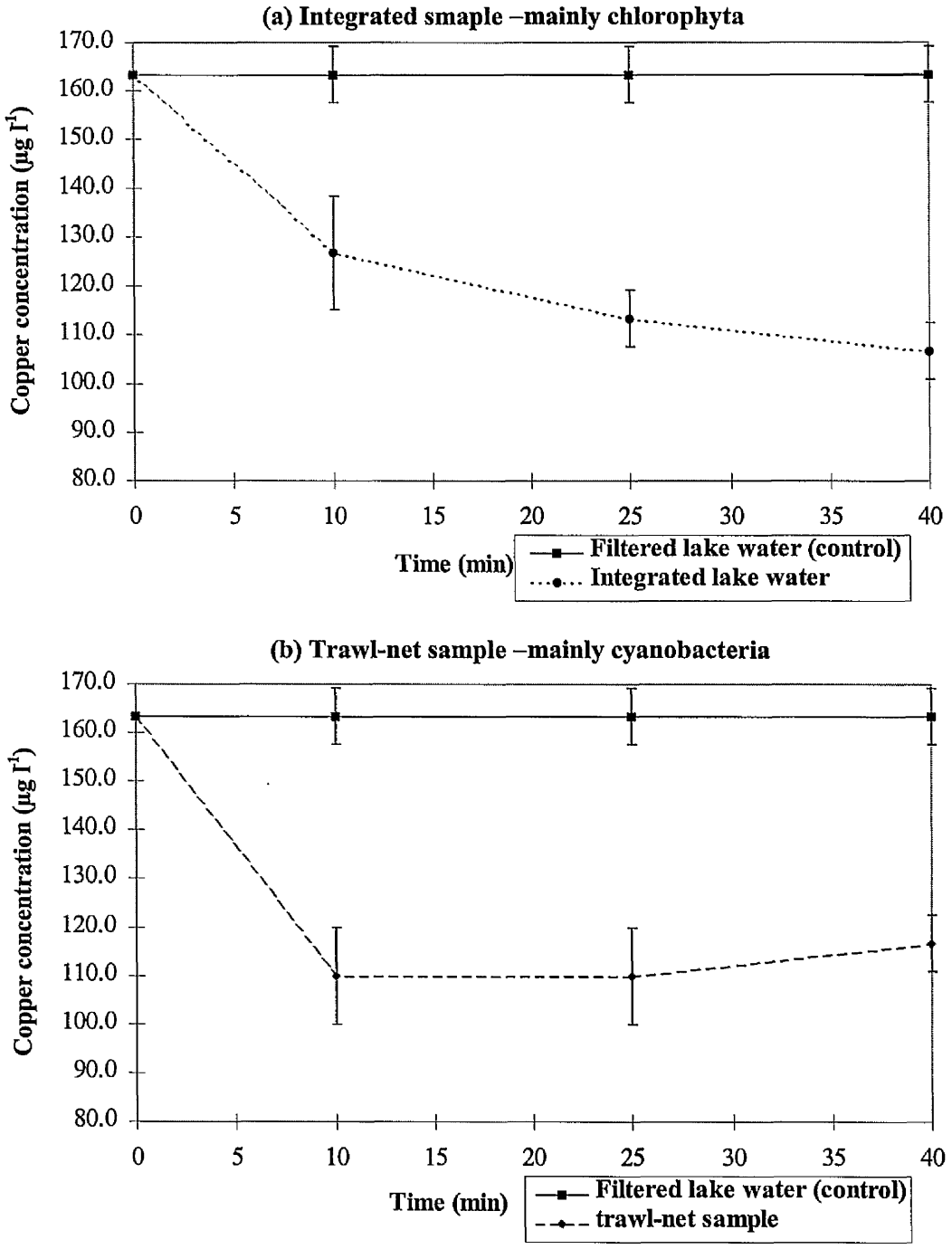


Fig. 5.18 Copper concentration remaining in lake water from Rostherne Mere on 11 June 1997 after the addition of 200 µg l^{-1} of copper. (Error bars represent the standard deviation of triplicate samples.)

5.2.7.b Assessment of copper biosorption in living and dead (freeze-dried) phytoplankton samples from Rostherne Mere using adsorption mathematical models

Copper adsorption by living and dead (freeze-dried) phytoplankton cells was assessed using the Freundlich and the Langmuir adsorption models as described previously. Experiments were carried out on fresh samples collected from Rostherne Mere on 09 July, 05 August, 02 September and 27 November 1997 and on freeze-dried samples from 09 July and 02 September 1997.

(a) Freundlich models:

The adsorption of copper by trawl-net phytoplankton cells was determined over a wide range of copper concentrations (1-1000 mg l^{-1}) [Fig 5.19(a)] and an example of a typical plot is shown in Fig 5.19(b). Freundlich plots for both living and freeze-dried mixed phytoplankton preparations showed a good fit to experimental data for all the samples with $p<0.01$ and $R^2 > 0.92$. Freundlich constants for all samples are given in Table 5.4 and Table 5.5.

(1) living phytoplankton cells:

Freundlich K_f values, an indicator of adsorption capacity, ranged from 2.29 to 7.36 for living phytoplankton cells from 4 sampling dates. Samples from September showed the highest adsorption capacity (K_f), following by those from November and August, and then those from July. However, samples from July showed the highest adsorption intensity ($1/n = 0.89$), following by those from November, September and August (Table 5.4).

(2) Comparison of living and freeze-dried phytoplankton cells:

Adsorption capacity (K_f) and adsorption intensity (1/n) of living and freeze-dried cells from two sampling dates (July and September) were compared and are shown in Table 5.5. The adsorption capacity of phytoplankton cells decreased after

freeze-drying, but adsorption intensity decreased in the July sample and increased in September.

(b) Langmuir model:

Biosorption data over a wide range of copper concentrations were plotted according to the Langmuir model. Fig. 5.20 shows an example of two Langmuir plots for living mixed phytoplankton cells collected during August. In neither case did the data fit the Langmuir model over the whole range of copper concentration (1-1000 mg l^{-1}), nor did the data from living phytoplankton cells in July, September and November.

(c) Species composition in samples from different sampling dates:

There were large differences in species composition between different sampling dates (Fig. 5.21). In July, Chlorophyta were the dominant algae with *Eudorina elegans* (1200 colonies ml^{-1}) and *Staurastrum planctonicum* (900 cells ml^{-1}) and there were also considerable numbers of *Microcystis aeruginosa* (400 colonies ml^{-1}), *Oscillastoria spp.* (300 filaments ml^{-1}) and *Ceratium hirundinella* (500 cells ml^{-1}) as the main species present. Bacillariophyceae became abundant in August and *Asterionella formosa* (30400 cells ml^{-1}) and *Melosira spp.* (7200 filaments ml^{-1}) were dominant. September had a large amount of *Ceratium hirundinella* (Dinophyceae) at 13800 cells ml^{-1}. In November, *Microcystis aeruginosa* (4200 colonies ml^{-1}) and *Staurastrum planctonicum* (1500 cells ml^{-1}) were the major algae.

Table 5.4 Comparison of the Freundlich adsorption constants obtained for copper from living environmental phytoplankton samples from Rostherne Mere on different sampling dates.

Sampling date	dominant algae*	$\log K_f \pm SE$	$(1/n) \pm SE$	K_f	R^2
09 July 1997	Chlorophyta	0.36±0.077	0.89±0.042	2.29	0.99
05 August 1997	Bacillariophy-ceae	0.38±0.074	0.54±0.042	2.37	0.97
02 September 1997	*Ceratium hirundinella*	0.87±0.128	0.59±0.075	7.36	0.92
27 November 1997	Cyanobacteria	0.51±0.087	0.67±0.05	3.2	0.97

K_f and 1/n are Freundlich constants derived from the intercept and slope of the regression line. The value of K_f and 1/n can be considered as indicators of adsorption capacity and adsorption intensity, respectively. R^2 (coefficient of determination) represents the strength of the relation between the copper concentration remaining in solution at equilibrium (C) and the amount of copper adsorbed per unit weight of biomass (x/m) both following log transformation. (SE = standard error)

* The major algal counts are shown in Fig. 5.21.

Table 5.5 Comparison of the Freundlich adsorption constants (K_f and 1/n) obtained for copper from living and freeze-dried environmental phytoplankton samples from Rostherne Mere on 09 July and 02 September 1997.

	cell stage	09 July	02 September
K_f	living cells	2.29	7.36
	freeze-dried cells	1.86	5.41
1/n	living cells	0.89	0.59
	freeze-dried cells	0.51	0.76

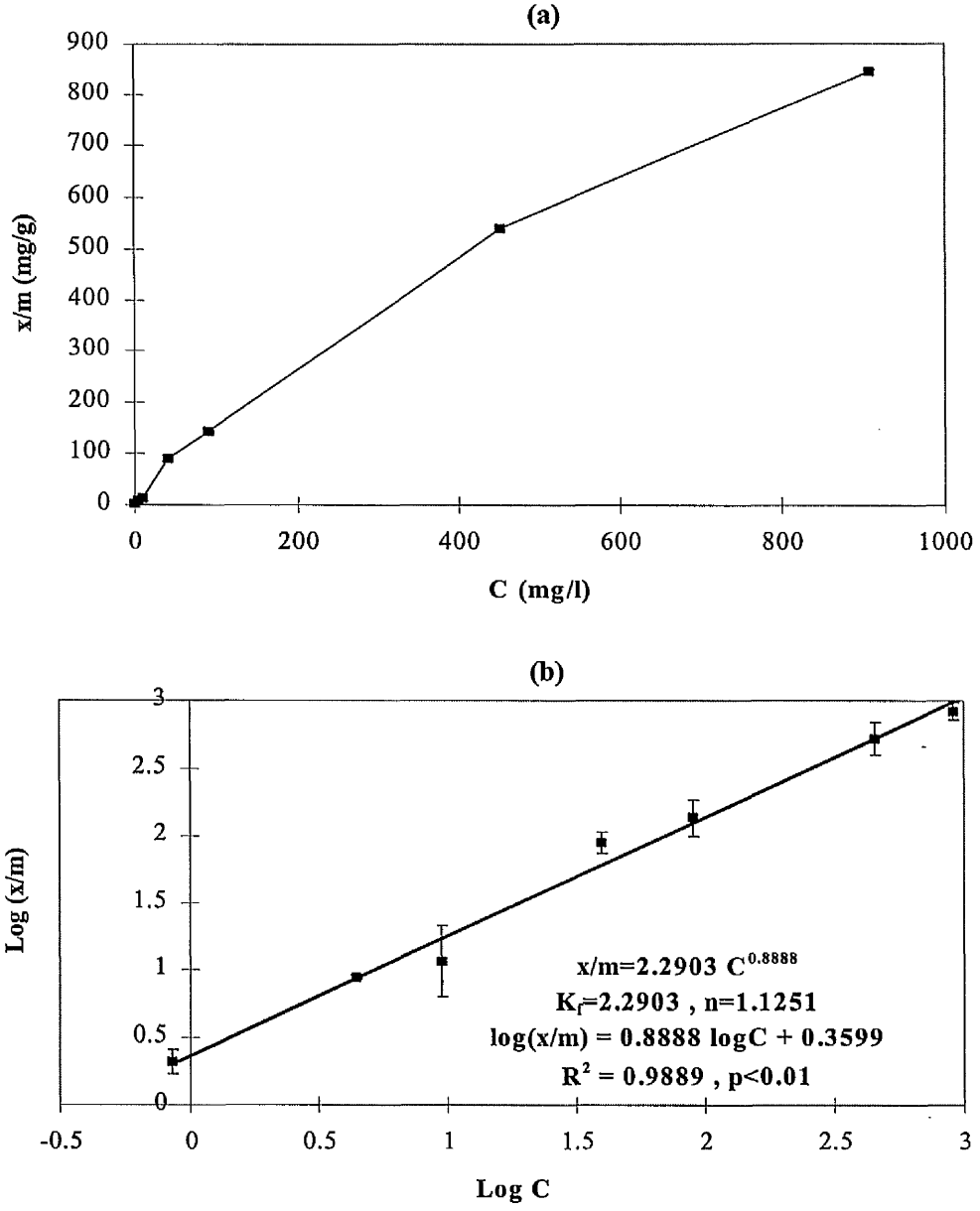


Fig. 5.19 Copper adsorption by living mixed phytoplankton cells from Rostherne Mere on 09 July 1997 after exposure of the same biomass to a range of copper concentraton for 1 hour at 25 °C. (a) adsorption of copper by cells in different copper concentrations. (b) Freundlich (log) plot. Where, C: copper concentration remaining in the medium; x/m: amount of copper adsorbed per unit dry weight; K_f and n: Freundlich constants; R^2: coefficient of determination. (Error bars represent the standard deviation of triplicate samples.)

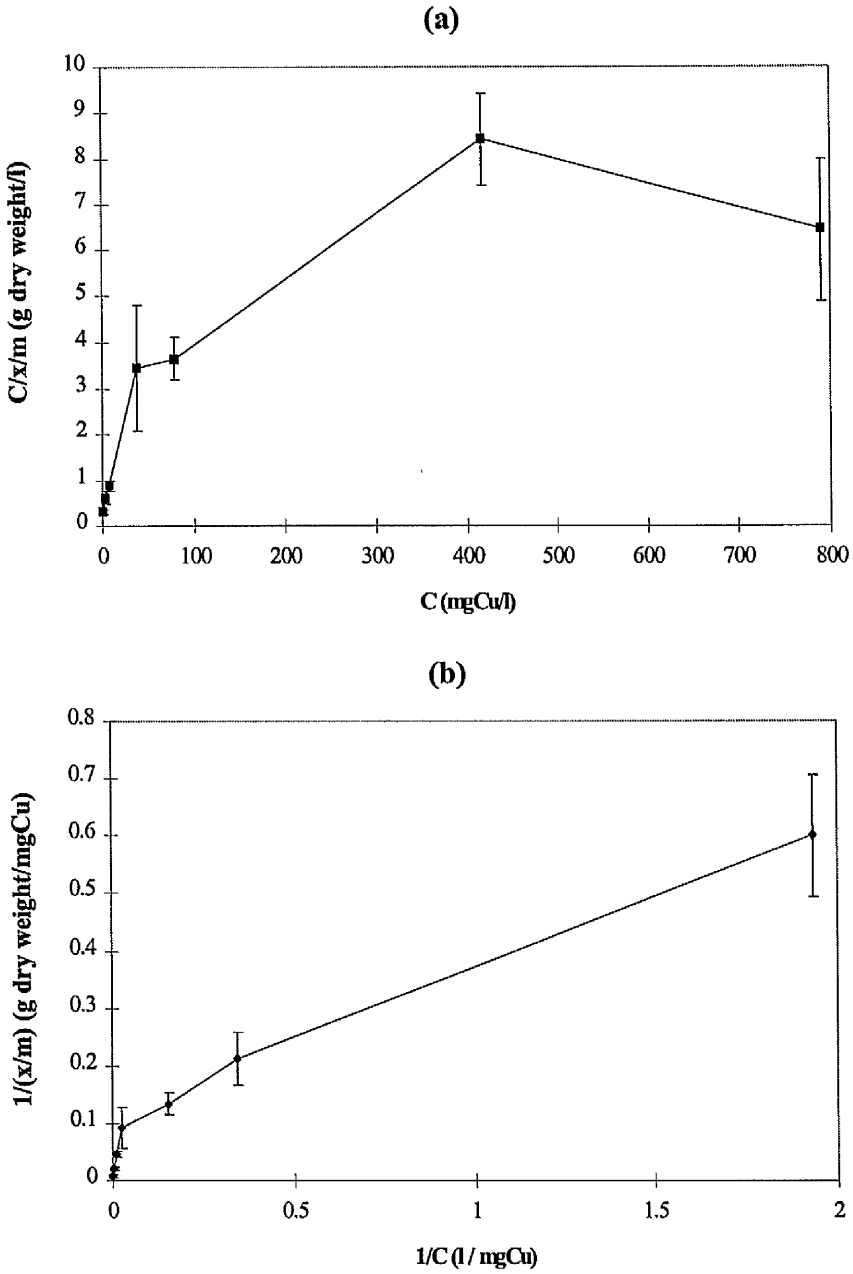


Fig. 5.20 Langmuir plots of copper biosorption by living phytoplankton cells from Rostherne Mere on 05 August 1997 using the two versions of the Langmuir equation. (Error bars represent the standard deviation of triplicate samples.)

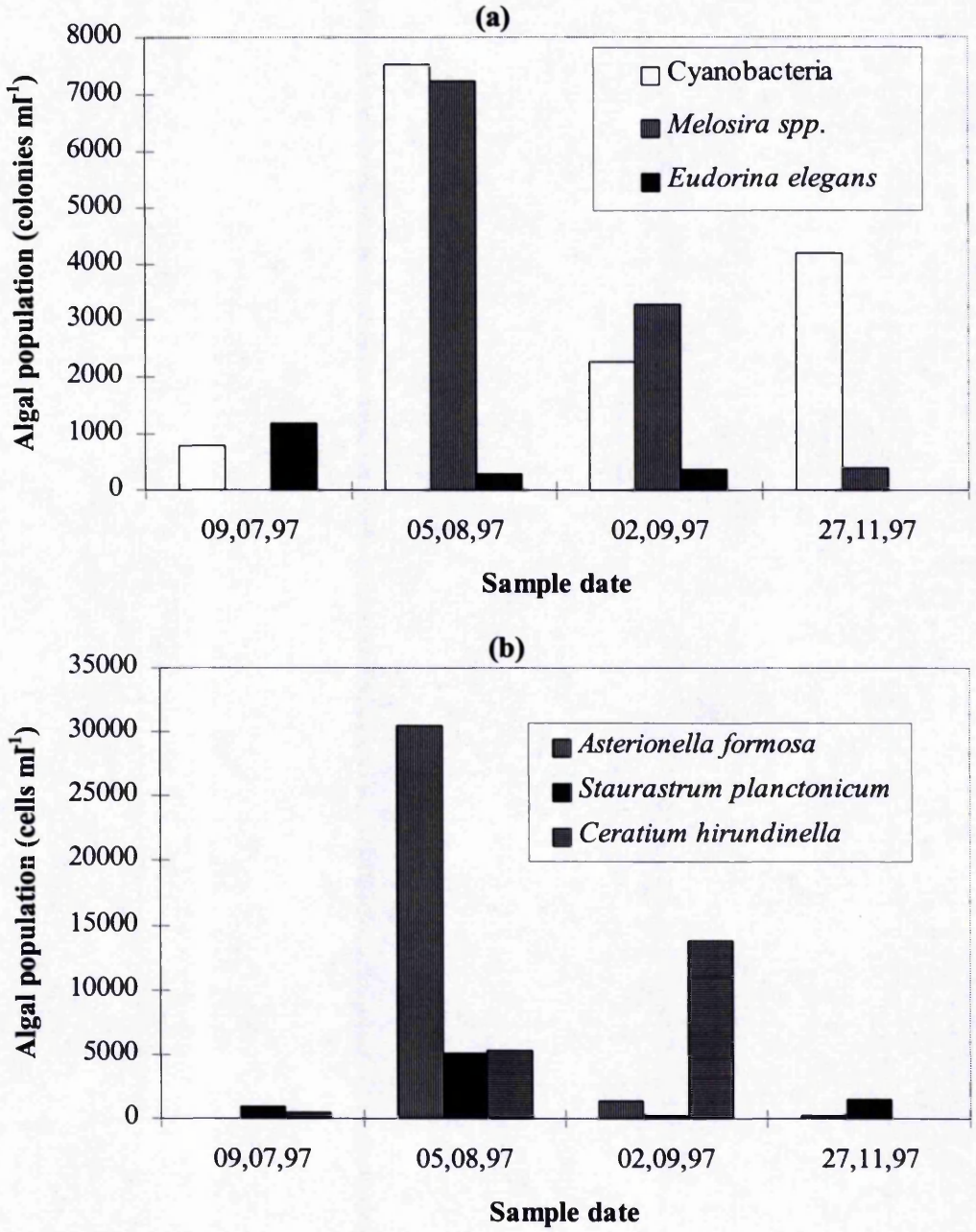


Fig. 5.21 Major algae: (a) cyanobacteria, *Melosira spp.* and *Eudorina elegans,* and (b) *Asterionella formosa, Staurastrum planctonicum* and *Ceratium hirundinella,* in mixed phytoplankton samples collected by a phytoplankton net from Rostherne Mere on 09 July, 05 August, 02 September, and 27 November 1997.

5.2.8 Examination of copper binding to cell-associated mucilage using X-Ray microanalysis

Anabaena spiroides was selected as this alga produces large amounts of mucilage to determine whether copper was directly bound to surface mucilage.

Scanning electron micrographs of freeze-dried *Anabaena spiroides* treated with 50 mg l^{-1} of copper for 30 min, washed and deposited on membranes (see Section 2.6.8) are shown in Plate 32. Owing to dehydration, the mucilage was reduced to strands of fibrillar material attaching the cells to the filter membrane. In Plate 32, the marked areas on mucilage, cells and the filter membrane illustrate regions analysed by X-ray microanalysis (XRMA) for the determination of copper. The copper concentrations measured in each marked area are also listed in Plate 32.

X-ray emission spectra from mucilage, cells (including associated mucilage) and the filter membranes (a control to ascertain if there any copper was retained on the filter membranes) are shown in Fig. 5.22. A significant copper peak appeared in the X-ray spectra from the associated mucilage and cells, but not from the membrane support. Spectra from cells also included peaks of silicon, phosphorus and sulphur, but those from mucilage and filter membranes did not. The mean mass fraction of copper in mucilage was 0.24% dry weight with 24% coefficient of variation (CV). The mean copper mass fraction in cells was 0.96% dry weight with a 13% CV. No copper peak was detected on the filter membranes.

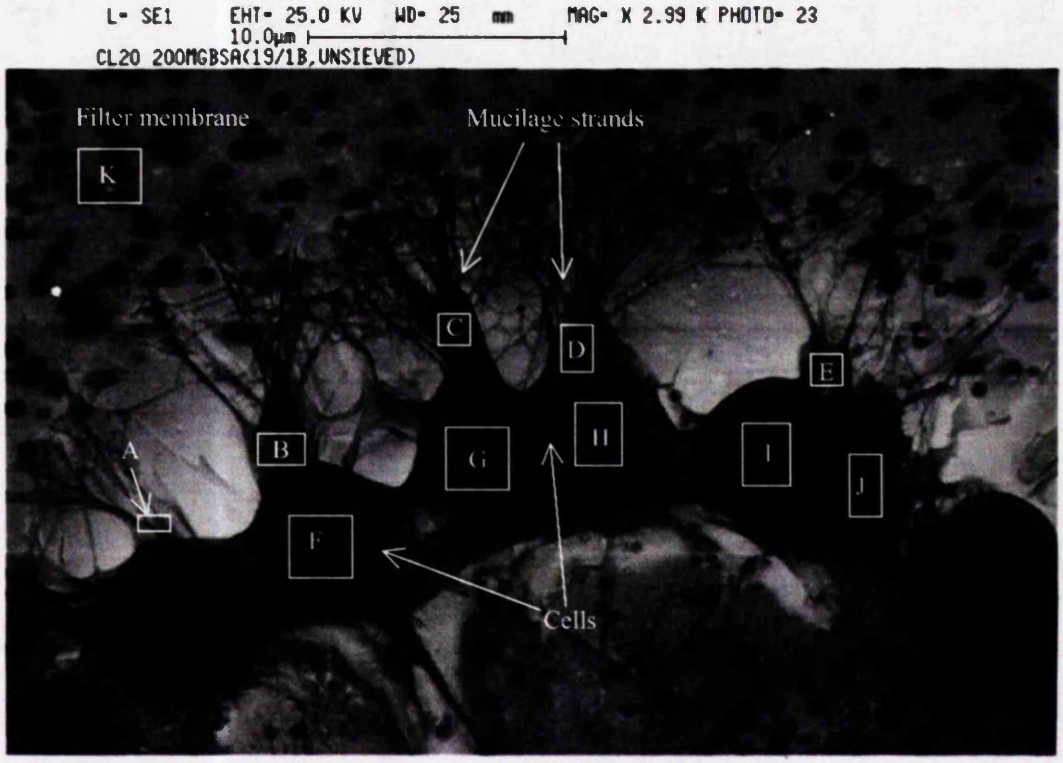


Plate 32. Scanning Electron Microscopical (SEM) view of freeze-dried *Anabaena spiroides* exposed to 50 mg l^{-1} of copper for 30 min. White boxes show measured areas. Copper concentration within each area was shown below:

Position (on mucilage strands)	A	B	C	D	E
Cu conc. (% dry weight)	0.31	0.26	0.2	0.17	0.28
Position (on the cell)	F	G	H	I	J
Cu conc. (% dry weight)	0.87	0.95	0.94	0.86	1.16
Position (on filter membrane)	K				
Cu conc. (% dry weight)	ND				

ND: not detectable.

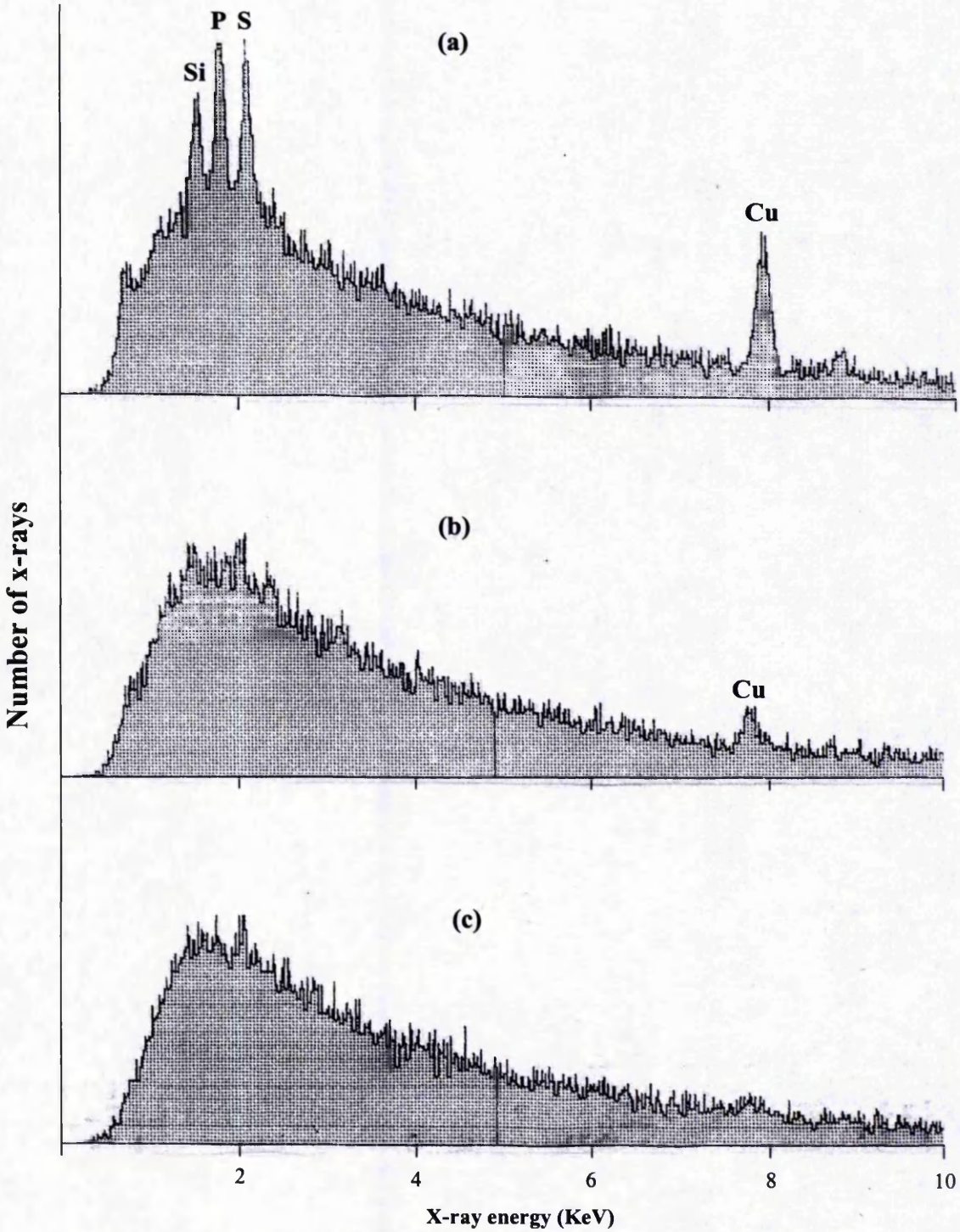


Fig. 5.22 X-Ray emission spectra from (a) the cell and (b) associated mucilage of freeze-dried *Anabaena spiroides*, plus (c) the filter membrane (control).

5.3 Discussion

Metal binding (adsorption) to algal surfaces is a result of physical and chemical processes at the surface and is a passive process which is independent of cell metabolism. These adsorptive processes are invariably followed by slower cellular absorption, which is a metabolism-dependent process. In this study, investigation of adsorption was initially carried out with laboratory-cultured *Anabaena cylindrica.* Adsorption capacity was also compared between different cultured algal species, and lake phytoplankton, using time course experiments and adsorption mathematical models.

5.3.1 Assessment of biosorption of copper by laboratory-cultured Anabaena cylindrica

5.3.1.a Effect of the growth medium in copper uptake experiments

The type of growth medium used in metal uptake experiments is important, since it may influence cation speciation or precipitation (Section 1.2.1.c) and so thus influence metal ion bioavailability. For marine phytoplankton, the culture medium "Aquil" has been developed for trace metal studies, but no equivalent medium has been developed for freshwater algae (Morel et al., 1979). My trace metal studies of *Anabaena cylindrica* involved the use of modified BG-11 medium which was derived from BG-11 medium with the omission of citric acid, ferric ammonium citrate, EDTA and trace metals. The first three of these are chelators, complexing with and precipitating metal ions. Complexing often reduces the bioavailablity of a metal (Rai et al., 1981) and precipitation will reduce the metal concentration in the water. Both processes will therefore influence the dose-response relationship in exposure experiments using algae. The omission of trace metals in the modified BG-11 medium

was carried out to avoid competition with the test cation for binding sites on the algal surface (Ting, et al. 1991). An example of such competition was reported by Nakajima et al.(1981) who found that the uptake of cadmium by *Chlorella* cells was reduced in the presence of uranyl and cupric ions.

The effect of the modified BG-11 medium on the growth of *Anabaena cylindrica* was investigated over 17 days. The growth inhibition of this alga observed in modified BG-11 medium after a three day lag phase may be due to a number of reasons, including pH changes due to the absence of buffering, nutrient limitation or accumulation of metabolic products. Rippka et al. (1979) noted that the development of akinetes occurs as cultures approach stationary phase where there is nutrient limitation and the accumulation of large amounts of metabolic products. The development of numerous akinetes in modified BG-11 medium was in marked contrast to BG-11 medium, and is consistent with growth limitation, due to, in this case, trace metals. However, no relative inhibition of growth in normal nor modified BG-11 medium occurred during the first 3 days and morphology of cells of *A. cylindirca* was similar between two culture media.

As there were no changes in cell morphology of *Anabaena cylindrica* over the initial 3 days of culture in modified BG-11 medium, copper accumulation was investigated within this period. Rapid uptake of copper occurred after a 1 hour exposure time, followed a slow loss of up to 50% of the accumulated copper. The results suggest copper adsorption onto the cell surface as a result of binding to the mucilage and/or the cell wall within 1 hour, followed by the subsequent release of copper complexes from the cell surface back to the medium. Loss of copper may also result from the operation of a detoxification mechanism by the cells. For example, *Anabaena cylindrica* may secrete extracellular polypeptides to detoxify copper (Fogg

and Westlake, 1955). A similar suggestion was also proposed by Butler et al. (1980) where an organo-copper complex was formed on contacting the cell wall of *Chlorella vulgaris,* which was then released into the medium. Moffett and Brand (1996) also suggested that marine cyanobacteria could produce extracellular copper chelators in response to copper stress, to create conditions more favourable for growth. Significant accumulation ($p<0.05$) of copper in *Anabaena cylindrica* shows that this species has the ability to concentrate copper from the surrounding medium. This ability has also been noted for the other algal species (Rai et al., 1981 ; Gowrinathan & Rao, 1990).

A smaller amount of copper (~25 μg) was detected in algal pellets, compared to the loss of copper (~50 μg) from the medium after 2 hours exposure time, suggesting that approximately 25 μg of copper was adsorbed on the surface of glass flasks and/or precipitated out of the medium (Erickson, 1972 ; Morel et al., 1979).

5.3.1.b Assessment of copper adsorption onto laboratory-cultured *Anabaena cylindrica* in distilled water

In this part of the study, copper adsorption was examined over a short period (4 hours) because metal adsorption by algae always occurs over a short exposure time (Greene & Darnal, 1990). Passive adsorption can be demonstrated in three ways:- rapid initial uptake by cells, cation release from cell surfaces by chelating agents (e.g. EDTA) and cation uptake by inactivated cells (e.g. fixed or freeze-dried cells). The use of distilled water and polycarbonate tubes instead of modified BG-11 medium and glass flasks eliminated the problems mentioned in the previous experiment.

The high degree (80% after 1h) of copper removal in the experiment using distilled water compared to only 37% in the previous experiment may be due to the difference in pH (pH ≅ 5 in this experiment and pH ≅ 7 in the previous experiment), as binding of metals by algae is pH-dependent. Harris and Ramelow (1990) found that the

binding efficiency of copper to dry algal biomass increased rapidly with pH in the range 3-5, with a maximum value at pH 5. They suggested that the decrease in binding observed after pH 5 may be due to decreased solubility of metal ions. Likewise, Zhou et al. (1998) found that uptake of copper by macroalgae *Laminaria japonica* and *Sargassum kjellmanianum* increased with increasing pH from 3 to 5, but decreased at pH above 5.

Analysis of cells after EDTA treatment which is known (Gowrinathan and Rao, 1990) to only remove copper from the cell surface showed that copper was not taken up into cells within the initial 2h period. The results indicate that copper (which disappeared from the aqueous solution) was adsorbed onto the surface of cells during this period. The marked increase of copper in cells after 4 hours exposure suggests that the metal had subsequently entered the cell.

Release of copper from the cell surface by EDTA involved the release of at least 70% of the bound cation. The lowest release rate was found on cells exposed to copper for 1 hour, when there was maximal removal, indicating strong copper binding ligands on the cell surfaces over that exposure time. The high copper adsorption within 1 hour should result in a maximal release of copper from this time. However, more copper was released from the cell after 2 hours exposure time than originally bound (>100%), suggesting removal of copper present within the cell.

The close similarity in the adsorption of copper binding (both total amount and with time) between living and dead (glutaraldehyde-fixed) cells indicates that metabolic activity is not involved in the initial (2 hours) uptake of copper. This is consistent with a simple physical adsorption of cupric ions to the cell surface. The similarity of copper binding activity on the surface of living and glutaraldehyde-fixed cells also suggests that there may be no change in the nature of cell surfaces after

glutaraldehyde treatment. This result is in agreement with other studies. He and Tebo (1998) proposed that although glutaraldehyde treatment might alter the surface properties of spores of the marine bacterium *Bacillus sp.* strain SG by reducing the numbers of charged groups on the surface, this change is likely to be small since the initial (≤4h) Cu(II) adsorption capacity was nearly the same in the treated and untreated spores. Parker et al. (1998) found heat-killed, formaldehyde-treated, and air-dried *Microcystis aeruginosa* sorbed nearly as much (or in some cases, slightly more) copper as did viable cells.

In contrast to my studies with copper, formalin-treated *Chaetoceros costatum* cells showed a two-fold greater degree of mercury adsorption than live cells, and heat-killed cells showed three fold U^{6+} adsorption than live cells (Glooschenko, 1969 ; Darnall et al., 1986). It is likely that cells subjected to different treatments may cause changes in the surface chemistry although intermetallic differences are also likely to be a factor.

5.3.2 Comparison of copper biosorption by laboratory-cultured algae

5.3.2.a Assessment of copper biosorption by four laboratory-cultured algae in the time course study

Between 76% and 80% of the copper sorption onto *Chlorella vulgaris* and *Anabaena cylindrica* took place within 15 min following exposure to added metal, while the figure for *Anabaena spiroides* and *Eudorina elegans* was 50%. Rapid copper uptake by these four species agrees with the findings of Harris and Ramelow (1990) who showed a 90% of the copper uptake by freeze-dried *Chlorella* cells occurred within 15 min, and of Zhou et al. (1998) who found 71% of copper was removed by *Sargassum platensis* within 12 min. *Chlorella vulgaris* showed the largest amount of copper after 45 min, but maximum amounts were not observed in *Anabaena*

cylindrica, Anabaena spiroides and *Eudorina elegans* until 75 min. These results indicated that *Chlorella vulgaris* had the highest rate of copper uptake which may be due to its relatively high surface area. It may be also because the cell wall of *Chlorella vulgaris* contained more high affinity copper binding sites than the mucilage which surrounds the other three species. Since metal ions bind first to surface groups with the highest affinity and subsequently to groups with lower affinity (Xue et al., 1988) at low concentration of metal and short exposure times, the more binding sites with high affinity, the faster and greater amount of metals adsorbed. The cell wall of *Chlorella vulgaris* had relatively a high level of PWM lectin labelling, showing it contained relatively large amounts of N-acetyl glucosamine (Section 4.3.1.d). As Kiefer et al. (1997) pointed out that high affinity copper binding ligands are likely to contain N (or S)-donor atoms which form stronger complexes with copper and present higher efficiency in binding copper at low copper concentration than carboxylic groups, the high level of N-acetyl glucosamine on cell wall of *Chlorella vulgaris* may account for its high copper binding rate.

Anabaena cylindrica, Anabaena spiroides, and *Eudorina elegans* contained more copper per cell and per unit surface area than *Chlorella vulgaris,* suggesting that cells with associated mucilage may have more copper binding sites than the cell wall. The mucilage of *Anabaena cylindrica, Anabaena spiroides* and *Eudorina elegans* showed higher lectin (ConA, PHA and PNA) binding activity than the cell wall of *Chlorella vulgaris,* indicating the former contained more surface sugars (mannose, glucose, galactose and N-acetyl galactosamine) than the latter (Section 4.3.1.d). Since these surface sugars may contain copper binding sites (e.g. carboxylic group and amino groups), the higher surface sugars detected on mucilage may explain the higher copper binding activity than the cell wall. However, mucilage from these three species showed

different lectin binding patterns, suggesting the differences in surface sugar composition between them. Since Fehrmann and Pohl (1993) suggested that the amount of Cd adsorbed differed between algal species, due to differences between the ratio of high affinity (e.g. amino acids, proteins) and low affinity binding sites (e.g. negatively charged carboxylic and hydroxy-carboxylic groups), this may explain the differences in copper binding activity of mucilage from these three species shown here. *Anabaena spiroides* had the highest copper concentration both per cell and per unit surface area. This may be because *Anabaena spiroides* has a relatively large amount of mucilage and the lowest surface area. However, among the three species with cell-associated mucilage, *Anabaena cylindrica* showed the highest copper concentration per unit mucilage volume. Although *Anabaena cylindrica* had a low level of mucilage volume, it may account for high cation binding activity per unit amount of mucilage. *Anabaena spiroides* and *Eudorina elegans* had a similar copper concentration per unit mucilage volume, but the former showed much higher copper per unit surface area than the latter. This difference may be because mucilage of *Anabaena spiroides* was more diffuse and may contain more copper binding sites than the dense mucilage secreted by *Eudorina elegans*. However, the diffuse form of mucilage produced by *Anabaena spiroides* makes an estimate of its true surface area difficult. Mucilage of *Eudorina elegans* showed much higher lectin binding activity than that of *Anabaena spiroides,* but the former showed less copper binding activity than the latter, suggesting lectin binding sites may not all present as functional groups for metal-binding.

The metal-binding activity on the surface of these four laboratory-cultured algae could not be distinguished in terms of uptake per mass, but there were clear differences in uptake per cell, uptake per surface area and uptake per mucilage volume.

This indicates the importance in surface area and the nature of algal surface on defining the metal-binding activity of cell surface.

5.3.2.b Determination of copper biosorption on living and dead (freeze-dried) laboratory-cultured algae using adsorption mathematical models

Freundlich and Langmuir mathematical models have been used to describe the uptake of heavy metal ions by microorganisms (Section 1.2.3.a) and were used to compare metal binding capacity between different algal species in this study. The Freundlich model effectively ($R^2 \geq 0.9$) described the sorption of copper by eight living and six freeze-dried laboratory-cultured algae well over a broad concentration range (1-1000 mg l^{-1}), however, the Langmuir model did not fit the full range of copper binding data. This may be because the Langmuir model is based on a monolayer of uniform functional groups which is not fulfilled by biological surfaces, while the Freundlich model relates to heterogeneous binding sites (Xue et al., 1988 ; Wehrheim and Wettern, 1994a). The results obtained here agree with those of Xue et al. (1988) who found metal (Cu and Cd) binding to *Chlamydomonas* surfaces did not fit the Langmuir model over a wide range of metal concentrations, suggesting complex metal binding sites on the algal surfaces and also those of Zhou et al. (1998) who obtained similar data. Metal-binding by soil bacteria and soil fungi also fits the Freundlich model (Mullen et al. 1989 & 1992).

Although in this study, the experiment data only fit the Freundlich model, there are some studies showing a good fit of metal adsorption to both the Langmuir and Freundlich models. Wehrheim and Wettern (1994a) found both the Langmuir and Freundlich models were suitable for describing the short-term adsorption of cadmium, copper and lead by cell walls and cadmium and copper adsorption by whole cells of *Chlorella fusca,* while de Rome and Gadd (1987) showed copper adsorption by the

fungi *Cladosporium resinae* and *Penicillium italicum* obeyed the Freundlich and Langmuir isotherms for single-layer adsorption. This may be due to differences in the fitting range of copper concentrations, with 1-1000 mg l^{-1} in my study compared to 0.3-25 mg l^{-1} in the studies by Wehrheim and Wettern (1994a) and de Rome and Gadd (1987). Weber (1972) noted that in specific ranges of metal concentration, data may fit both the Freundlich and the Langmuir models.

The Freundlich constant K_f represents the predicted amount of metal sorbed in milligram per gram of a sorbent at an equilibrium concentration of 1 mg l^{-1} and is an indicator of adsorption capacity. Therefore, the higher K_f values obtained for the four living species having cell-associated mucilage than the species without cell-associated mucilage indicate that algae with mucilage have a higher adsorption capacity than those without. *Chlorella vulgaris*, which does not secrete cell-associated mucilage, had a higher ratio of surface area and dry weight than species with cell-associated mucilage except for *Microcystis aeruginosa*. However, it did not show higher adsorption capacity, suggesting that mucilage may have more copper-binding sites than the cell wall. These results support the previous experiment showing species with cell-associated mucilage had a higher copper content per cell and per unit surface area than those without mucilage. It also agrees with the results obtained by Su et al. (1995) who found higher K_f values for activated sludge enriched with extracellular-polymer-producing bacteria, *Zoogloea ramigera*. They suggested that the extracellular polymers provided more sorption sites available for metal uptake. Scott et al. (1988) also found capsular bacteria removed more cadmium than non-capsular species.

Among the four species with cell-associated mucilage, *Microcystis aeruginosa* had the highest surface area/dry weight ratio, but as it did not show a high adsorption capacity, it is likely to have fewer metal binding sites than the other species. *Eudorina*

elegans had the highest mucilage volume but the least adsorption capacity of four species with associated mucilage, suggesting that there is no apparent relationship between copper-binding activity and mucilage level. After freeze-drying, the adsorption capacity (K_f) of mucilage decreased, but that of cell wall increased. This may be due to loss of many of the metal binding sites on mucilage after freeze-drying. It also indicated that changes in cell surface chemistry occur following freeze-drying.

A wide range of K_f values for copper sorption by the eight cultured algae (0.36 to 12.62) indicate a great diversity in surface composition. This result supports previous lectin-binding experiments which reveal large differences in surface sugars (Section 4.3.1.d). Zhou et al. (1998) found macroalgae had K_f values of 24.9 to 34.3, while microalgae only had K_f values of 0.2 to 3.2 for copper and cadmium biosorption. Much lower K_f values were found by Wehrheim and Wettern (1994a) with values of only 0.27 by intact cells of *Chlorella fusca* and 0.039 by isolated cell wall for copper adsorption. Mullen et al. (1989) found K_f values from 2.2 to 4.2 for sorption of copper and cadmium by four bacteria. There were only 0.74 and 0.87 of K_f values for copper sorption by two fungal species *Mucor rouxii* and *Aspergillus niger* respectively (Mullen et al., 1992). These results indicate that fungi showed less adsorption capacity than bacteria, but algae showed a wider range of adsorption capacity.

The predicted amount of copper removed by *Anabaena cylindrica, Anabaena spiroides, Eudorina elegans* and *Chlorella vulgaris* per unit surface area at an equilibrium concentration of 1 mg l^{-1} was much higher than the actual amount of copper removed per unit surface area by cells exposed to 250 μg l^{-1} copper in the previous experiment. This may be because the cells were exposed to higher concentration of copper than in the earlier experiment, resulting in higher copper adsorption on the cell surface. *Anabaena cylindrica* showed the highest copper

removal per unit surface area among these four species in this experiment, but *Anabaena spiroides* showed the highest removal in the previous experiment. Since there were differences in the ratio of high affinity and low affinity binding sites between different algal surfaces as discussed above, copper binding activity may vary with copper concentration. *Melosira varians* showed the highest copper removal per unit surface area at an equilibrium concentration of 1 mg l^{-1} (low copper concentration), suggesting it had a higher affinity for copper binding sites than the other species. The three cyanobacteria (*Anabaena cylindrica, Anabaena spiroides* and *Microcystis aeruginosa*) showed a higher copper concentration per unit mucilage volume than *Eudorina elegans* which is a member of chlorophyta, suggesting that mucilage produced by cyanobacteria may have a higher copper binding capacity than chlorophyta. As the mucilage of *Anabaena cylindrica, Anabaena spiroides* and *Microcystis aeruginosa* is in a diffuse form, there may be more copper binding sites present on the surface compared to the mucilage secreted by *Eudorina elegans*.

The slope of the isotherm (1/n) is an indicator of adsorption intensity which presents the affinity series for metal binding by an adsorbent at higher concentrations than at the 1 mg l^{-1} equilibrium concentration. Therefore differences in the isotherm slopes indicate different metal sorption efficiencies at different concentrations (Mullen et al., 1992). The 1/n values were low, ranging from 0.16 to 1.1. This suggests that the eight cultured algae had high metal binding affinity at low metal loading status but had less binding sites with low affinity which only bind metal at high concentrations. Low 1/n values also found by Zhou et al. (1998) (0.22 for macroalgae with copper binding and 0.86~1.31 for microalgae with cadmium binding).

5.3.3 Determination of copper binding capacity on phytoplankton samples from Rostherne Mere

5.3.3.a Preliminary assessment of copper binding by mixed phytoplankton cells from Rostherne Mere

The rapid (within 10-20 min) removal of copper by phytoplankton cells collected from Rostherne Mere in May and June 1997 was similar to the results (15 min) for laboratory-cultured algae (Section 5.2.5). Since the integrated phytoplankton samples represented the normal cell concentration in the upper 5 metres of the lake water column, the relatively high copper removal (9% in May and 35% in June) indicates the importance of phytoplankton in regulating metal ions in the lake. The differences in copper removal by samples collected from different dates may result from differences in algal species composition, as different algae showed different metal-binding activity (discussed in Section 5.3.2).

Phytoplankton cells from June showed higher copper removal per unit dry weight than those from May. Cyanobacteria and chlorophyta were the dominant species in the trawl-net sample and the integrated samples from June and bacillariophyceae in May, and it is likely that the former algae showed higher copper binding activity, as was observed in laboratory-cultured algae. Therefore, copper removal by the natural phytoplankton community may be related to the dominant species present in the lake.

5.3.3.b Assessment of copper biosorption on living and dead (freeze-dried) phytoplankton samples from Rostherne Mere using adsorption mathematical models

The experimental data closely fit the Freundlich model but not the Langmuir model over the range of copper concentrations (1-1000 mg l^{-1}) in all cases. This suggested that, in common with laboratory-cultured algae (Section 5.3.2.b), metal

uptake occurred as monolayer adsorption, with heterogeneous binding sites on the algal surfaces.

Freeze-dried phytoplakton showed different adsorption capacities and intensity of binding compared to living cells, as found in laboratory-cultured algae (Section 5.2.6). This indicates changes in surface chemistry after freeze-drying (as discussed in Section 5.3.2.b).

5.3.4 Examination of copper binding to cell-associated mucilage using X-ray microanalysis

X-ray microanalysis has been widely used in determining the elemental composition of organisms, such as bacteria (Booth, 1988) and algae (Bistricki & Munawar, 1982 ; Clay, 1992 ; El-bestawy et al., 1996 ; Sigee & Holland, 1997 ; Sigee et al., 1998). This technique can measure the elemental composition of a particular area and was used here to examine the copper level in the cell and associated mucilage of *Anabaena spiroides*. There were considerable amounts of copper (0.24% dry weight) detected in the mucilage strands, indicating the presence of copper in the mucilage. Cells (including mucilage) contained more copper than mucilage strands, suggesting that the former may have more metal binding sites than the latter. However, the X-ray emission spectra taken from the cells of *Anabaena spiroides* could not give direct information on the ultrastructural location of particular elements which may be present in an intracellular compartment, within the cell wall, or in surface mucilage (Clay et al., 1991).

CHAPTER 6. CONCLUSIONS AND FUTURE WORK

Three inter-related aspects of phytoplankton mucilage were investigated in this study.

1. Quantification of cell-associated and soluble mucilage in Rostherne Mere

- Monthly monitoring of Rostherne Mere has shown clear seasonal changes in the phytoplankton community, which was closely correlated with the physico-chemical (temperature, oxygen, pH, conductivity) and biological (Secchi depth and phytoplankton) parameters within the lake.
- Seasonal changes in the total volume of associated mucilage reflected the succession of mucilaginous algal species in Rostherne Mere. Colonial cyanobacteria were found to be the major contributors to cell-associated mucilage, particularly *Microcystis aeruginosa.*
- Over most of the annual cycle, cell-associated mucilage occupied between 0.0001 - 0.0018% of lake water volume. A maximum value of 0.007% was observed in September 1997, corresponding to a peak of *Microcystis* population and a total chlorophyll a concentration of 31μg l^{-1}. Analysis of the lake on one occasion (August 1996) demonstrated patchiness in algal distribution, resulting in a horizontal variation of cell-associated mucilage of 0.00005-0.0005% of lake water volume.
- Over most of the annual cycle (September to May), the concentration of soluble extracellular polysaccharides was about 10 mg l^{-1}. Two major peaks in summer were observed, corresponding to the May bacillariophyceae bloom (reaching 37.7 mg l^{-1}) and the end of the clear water phase (July-August, reaching 60 mg l^{-1}). The second phase did not appear to relate directly to changes in algal population, and may result from bacterial activity, algal lysis or zooplankton activity. Although *Microcystis* is

mucilaginous, no peak in soluble EPS appeared to be derived from this alga. Future work should include an examination of bacterial populations and quantification of the amount of mucilage associated with this group of microorganisms. As soluble EPS is now thought to form a major component of the total amount of dissolved carbon in lakes (discussed in Section 3.3.3.b), the further study of the soluble EPS is important if one is to understand the carbon cycle in freshwaters.

2. Characterisation of surface sugars, and analysis of carbohydrate and protein content of mucilage

- Changes in lectin binding activity indicate a diversity of molecular structure on algal cell surfaces (mucilage and/or cell wall). The diversity of surface mucilage composition was also demonstrated by quantification of the sugar and protein content of extracted mucilage. The surface mucilage composition was found to vary with growth conditions in that different lectin-binding activities were found between laboratory cultures and envionmental samples from Rostherne Mere, and between cultured *Anabaena cylindrica* in different growth phases. Carbohydrates were demonstrated to be the major component of surface mucilage with small amounts of protein. This was also shown by the relatively high lectin-binding intensity of mucilage. Thus, laboratory-cultured *Eudorina elegans* had the highest lectin-binding intensity of mucilage among cultured algae due to its highest carbohydrate (65%) and protein (8%) content. Lectin binding to surface mucilage identified particular sugars present as termini and subtermini on the polysaccharide chains that are a big component of mucilage. A low level of galactose and N-acetyl galactosamine (detected by PNA) was found on surface mucilage of most of algal species with relatively high amounts of mannose, glucose and N-acetyl glucosamine (detected by ConA, PHA-E and PWM) and which varied with algal species. Further

quantification of sugar composition (e.g. using gas-liquid chromatography) may help understand the detailed structure of algal mucilage. The protein component of the glycoprotein also requires investigation, including the amounts of metal binding amino acids such as cysteine which are known to be present.

- Soluble extracellular products secreted by cultured algae showed large differences in sugar and protein composition. Relatively low levels of sugar and protein content in lake water suggested high levels of consumption of organic matter by heterogeneous bacteria in the aquatic environment and hence a short half-life in the environment. Nevertheless, concentrations of soluble EPS in eutrophic lakes may exceed that of humic substances (Boult, pers. comm.). They are therefore likely to have an important influence on the biogeochemical cycling of metals.

3. Metal-binding activity of algal surfaces

- A high level of copper adsorption was initially seen in laboratory cultures of *Anabaena cylindrica,* consistent with a high copper-binding activity at the cell surface. The binding of copper by adsorption rather than uptake into the cell is indicated by the rapid release by EDTA and similar binding activities of living and dead cells.
- Copper binding activity between different algal species in pure cultures was examined by time course studies and using adsorption mathematical models. Both approaches showed clear differences between algal species. These differences appeared to relate particularly to the surface area/dry weight ratio and to surface chemistry. The diversity in algal surface sugars shown in the lectin-binding experiments may explain the differences in copper binding activity. Heterogeneous algal surfaces containing metal-binding sites with different metal binding affinity were demonstrated by being able to fit adsorption experimental data to the

Freundlich model but not to the Langmuir model. Mucilage showed higher copper binding activity (higher copper per unit surface area and higher K_f values) than the cell wall, suggesting mucilage may have more copper binding sites than the cell wall. Since the lectin binding experiments showed that mucilage had more surface sugars than the cell wall, these sugars may act as metal binding sites. That the adsorption capacity did not simply relate to the amount of mucilage was shown by the adsorption behavour of *Eudorina elegans*.

- Copper binding activity on algal surfaces was further investigated in mixed phytoplankton from Rostherne Mere. Different phytoplankton samples showed significant differences in their adsorption capacity. These differences may relate to different species composition between samples. As mucilage generally has a high ability to bind metals, the large number of algal species with cell-associated mucilage that appear during the late spring and summer in eutrophic lakes such as Rostherne Mere may play an important role in metal removal from lake water and the transfer of metal ions to higher trophic levels (e.g. zooplankton or benthic animals) in the aquatic environment.
- All eight cultured algae and environmental mixed phytoplankton samples showed considerably higher adsorption capacity (shown by high K_f) than fungi (Mullen et al., 1992) and had more high affinity copper binding sites (shown by low 1/n). Algae are thus potential biosorbents for removing metals from the surrounding water, especially species producing cell-associated mucilage.
- The presence of copper in cell-associated mucilage was also demonstrated using X-ray microanalysis. Considerable amounts of copper were found on mucilage strands, with a mean concentration of 0.24% dry weight after 30 min exposure to 50 mg l^{-1} copper solution.

- This study examined cation adsorption using a single metal, copper. Future work should include an examination of other metals. It could be particularly interesting to examine the binding of, respectively, class A, borderline and class B metals. Class A metals such as the geochemically important metal Al have a affinity to oxygen-containing ligands such as present in carboxylic groups and may thus preferentially bind to the polysaccharide component of soluble EPS and insolube mucilage. In constrast, class B and borderline metals, such as Zn and Cd (and Cu) preferentially bind to the N or S-containing ligands that occur on the protein component of the glycoprotein molecule.
- This and other work suggests that soluble EPS and cell-associated mucilage is an important influence on the behavour of trace metals in freshwaters, both because of the amount present in the natural environment and the ability to strongly bind large amounts of metal. It is, however, necessary to establish the half-life of the metal EPS/mucilage association which will be determined by the rate of breakdown of the mucopolysaccharides. If the turnover of the mucopolysaccharide is very rapid, it is possible that the bulk of metal is rapidly incorporated into other sectors of the environment with a longer half-life, in particular humic substances.

REFERENCES

Adamson, A.W. (1967). *Physical Chemistry of Surfaces.* Second edition, Interseience Publishers, Inc., New York.

Adhikary, S.P. ; Weckesser, J. ; Jürgens, U.J. ; Golecki, J.R. & Borowiak, D. (1986). Isolation and chemical characterization of the sheath from the Cyanobacterium *Chroococcus minutus* SAG B.41.79. *Journal of General Microbiology,* **132,** 2595-2599.

Agrawal, S.C. & Sharma, U.K. (1996). Chemical and biological properties of culture filtrates of *Westiellopsis prolifica* and *Chaetophora attenuata. Israel Journal of Plant Science,* **44,** 43-48.

Albergoni, V. & Piccinni, E. (1981). Biological response to trace metals and their biochemical effects. In: *Trace Element Speciation in Surface Water and its Ecological Implications.* (Leppard, G.G. ed.), 159-175. Plenum Press, New York.

Amemiya, Y. & Nakayama, O. (1984). The chemical composition and metal adsorption capacity of the sheath materials isolated from *Microcystis,* Cyanobacteria. *Japanese Journal of Limnology,* **45,** 187-193.

Anderson, L.W.J. & Sweeney, B.M. (1978). Role of inorganic ions in controlling sedimentation rate of a marine centric diatom *Ditylum brightwelli. Journal of Phycology,* **14,** 204-214.

Aspinall, G.O. & Stephen, A.M (1973). Polysaccharide methodology and plant polysaccharides. In: *Carbohydrates.* (Aspinall, G.O. ed.), 285-317. University Park Press, Baltimore.

Bailey-Watts, A.E. (1978). A nine-year study of the phytoplankton of the eutrophic and non-stratifying Loch Leven (Kinross, Scotland). *Journal of Ecology,* **66,** 741-771.

Barondes, S. (1988). Bifunctional properties of lectins: lectins redefined. *Trends in Biochemical Sciences,* **13,** 480-482.

Belcher, J.H. & Storey, J.E. (1968). The phytoplankton of Rostherne and Mere Meres, Cheshire. *Naturalist,* 57-61.

Bellinger, E.G. (1974). A note on the use of algal sizes in estimates of population standing crops. *British Phycological Journal,* **9,** 157-161.

Bellinger, E.G. (1992). *A Key to Common Algae.* Fourth edition, The Institution of Water and Environmental Management, London.

Bistricki, T. & Munawar, M. (1982). Application of X-ray microanalysis for the detection of heavy metals in Great Lakes algae. *Canadian Journal Fisheries and Aquatic Sciences,* **39,** 506-509.

Bold, H.C. ; Alexopoulus, C.S. & Delevoryas, T. (1980). *Morphology of Plants and Fungi.* Fourth edition, Harper and Row Inc., New York.

Boney, A.D. (1981). Mucilage: the ubiquitous algal attribute. *British Phycological Journal,* **16,** 115-132.

Booth, K.N. (1988). *The Occurrence and Elemental Composition of Phytoplankton and Bacteria in Rostherne Mere, Cheshire.* PhD Thesis, University of Manchester, U.K..

Boyd, W.C. & Shapleigh, E. (1954). Specific precipitating activity of plant agglutinins (lectins). *Science,* **119,** 419.

Brinkhurst, R.O. & Walsh, B. (1967). Rostherne Mere, England, a further example of guanotrophy. *Journal of the Fisheries Research Board of Canada,* **24,** 1299-1313.

Brook, A.J. & Williamson, D.B. (1988). The survival of desmids on the drying mud of a small lake. In: *Algae and the Aquatic Environment.* (Round, F.E. ed.), 185-196. Biopress Ltd., Bristol.

Brooks, S.A. ; Leathem, A.J.C. & Schumacher, U. (1997). *Lectin Histochemistry: A Concise Practical Handbook.* BIOS Scientific Publishers Limited, U.K..

Brown, R.E.; Jarvis, K.L. & Hyland, K.J. (1989). Protein measurement using bicinchoninic acid: elimination of interfering substances. *Analytical Biochemistry,* **180,** 136-139.

Burkett, R.D. (1975). Uptake and release of methylmercury-203 by *Cladophora glomerata. Journal of Phycology,* **11,** 55-59.

Butler, M. ; Haskew, A.E.J. & Young, M.M. (1980). Copper tolerance in the green alga, *Chlorella vulgaris. Plant, Cell and Environment,* **3,** 119-126.

Calvo, S. ; Barone, R. & Naselli Flores, L. (1995). Observation on mucus aggregates along Sicilian coasts during 1991-1992. *The Science of the Total Environment,* **165,** 23-31.

Canter, H.M. & Lund, J.W.G. (1951). Studies on plankton parasites III. Examples of the interaction between parasitism and other factors determining the growth of diatoms. *Annals of Botany,* **15,** 359-371.

Canter, H.M. & Lund, J.W.G. (1969). The parasitism of planktonic desmids by fungi. *Öesterreichische Botanische Zeitschrift,* **116,** 351-377.

Canter, H.M. (1979). Fungal and protozoan parasites and their importance in the ecology of the phytoplankton. *Annual Report Freshwater Biological Association,* 43-50.

Canter-Lund, H. & Lund, J.W.G. (1995). *Freshwater Algae: Their Microscopic World Explored.* Biopress Ltd, Bristol.

Carney, H.J. & Elser, J.J. (1990). Strength of zooplankton-phytoplankton coupling in relation to lake trophic state. In: *Large Lakes: Ecological Structure and Function.* (Tilzer, M.M. & Serruya, C. ed.), 615-632. Springer-Verlag Press, Berlin.

Carrillo, P. ; Reche, I. ; Sanchez-Castillo, P. & Cruz-Pizarro, L. (1995). Direct and indirect effects of grazing on the phytoplankton seasonal succession in an oligotrophic lake. *Journal of Plankton Research,* **17,** 1363-1379.

Carvalho, L. R. (1993). *Experimental Limnology on Four Cheshire Meres.* Ph.D Thesis, University of Liverpool, U.K..

Chang, T.-P. (1980). Mucilage sheath as a barrier to carbon uptake in a cyanophyte, *Oscillatoria rubescens* D.C. *Archiv Für Hydrobiologie,* **88,** 128-133.

Cho, D.-Y. ; Lee, S.-T. ; Park, S.-W. & Chung, A.-S. (1994). Studies on the biosorption of heavy metals onto *Chlorella vulgaris. Jouranl of Environmental Science and Health,* **A29,** 389-409.

Chowdhury, T.K. (1974). ConA: an introduction. In: *Concanavalin A, Advances in Experimental Medicine and Biology, Vol.55.* (Chowdhury, T.K. & Weiss, A.K. ed.), 1-12. Plenum Press, New York.

Chróst, R.J. ; Münster, U. ; Rai, H. ; Albrecht, D. ; Witzel, P.K. & Overbeck, J. (1989). Photosynthetic production and exoenzymatic degradation of organic matter in the eutrophotic zone of a eutrophic lake. *Journal of Plankton Research,* **11,** 223-242.

Clay, S. ; Sigee, D.C. ; Bellinger, E. (1991). X-ray microanalytical studies of freshwater biota: changes in the elemental composition of *Anabaena spiroides* during blooms of 1988 and 1989. *Scanning Microscopy,* **5,** 207-217.

Clay, S.M. (1992). *An X-ray Microanalytical Study of The Elemental Composition of Phytoplankton at Rostherne Mere, Cheshire.* Ph.D. Thesis, University of Manchester, U.K..

Costas, E. & López Rodas, V. (1994). Identification of marine dinoflagellates using fluorescent lectins. *Journal of Phycology,* **30,** 987-990.

Coward, T.A.(1914). Faunal survey of Rostherne Mere. II. vertebrata. *Memoirs and Proceedings of the Manchester Literary and Philosophical Society,* **58,** 1-37.

Crist, R.H. ; Oberholser, K. ; Shank, N. & Nguyen, M. (1981). Nature of bonding between metallic ions and algal cell walls. *Environmental Science and Technology,* **15,** 1212-1217.

Crist, R.H.; Oberholser, K.; McGarrity, J.; Crist, D.R.; Johnson, J.K. & Brittsan, J.M. (1992). Interaction of metals and protons with algae. 3. marine algae, with emphasis on lead and aluminum. *Environmental Science and Technology,* **26,** 496-502.

Daft, M.J. & Steward, W.D.P. (1971). Bacterial pathogens of freshwater blue-green algae. *New Phytologist,* **70,** 819-829.

Darnall, D.W. ; Greene, B. ; Hosea, M. ; Mcpherson, R.A. ; Henzl, M. & Alexander, M.D. (1986). Recovery of heavy metals by immobilized algae. In: *Trace Metal Removal From Aqueous Solution.* (Thompson, R. ed.), 1-24. The Royal Society of Chemistry Burlington House, London.

de Rome, L. & Gadd, G.M. (1987). Copper adsorption by *Rhizopus arrhizus, Cladosporium resinae* and *Penicillium italicum. Applied Microbiology and Biotechnology,* **26,** 84-90.

Decho, A.W. & Herndl, G.J. (1995). Microbial activities and the transformation of organic matter within mucilaginous material. *The Science of the Total Environment,* **165,** 33-42.

Decho, A.W. (1990). Microbial exopolymer secretions in ocean environments: their role(s) in food webs and marine processes. *Oceanography and Marine Biology Annual Review,* **28,** 73-153.

Degobbis, D. ; Fonda-Umani, S. ; Franco, P. ; Malej, A. ; Precali, R. & Smodlaka, N. (1995). Changes in the northern Adriatic ecosystem and the hypertrophic appearance of gelatinous aggregates. *The Science of the Total Environment,* **165,** 43-58.

Desikachary, T.V. (1959). *Cyanophyta.* Indian Council of Agricultural Research, New Delhi.

Dillon, P.J. ; Yan, N.D. & Harvey, H.H. (1984). Acidic deposition: effects on aquatic ecosystems. *CRC Critical Reviews on Environmental Controls,* **13,** 167-194.

Domozych, C.R.; Plante, K.; Blais, P.; Paliulis, L. & Domozych, D.S. (1993). Mucilage processing and secretion in the green alga *Closterium.* I. Cytology and biochemistry. *Journal of Phycology,* **29,** 650-659.

Domozych, D.S. & Domozych, C.R. (1993). Mucilage processing and secretion in the green alga *Closterium.* II. Ultrastructure and immunocytochemistry. *Journal of Phycology,* **29,** 659-667.

Doyle, R.J. (1994). Introduction to lectins and their interactions with microorganisms. In: *Lectin-Microorganism Interactions.* (Doyle R.J. & Slifkin, M. ed.), 1-66. Marcel Dekker, Inc., New York.

Drews G. (1973). Fine structure and chemical composition of the cell envelopes. In: *The Biology of Blue-Green Algae.* (Carr, N.G. & Whitton, B.A. ed.), 99-116. Blackwell Scientific Publication, Oxford.

Drews, G. & Weckesser, J. (1982). Function, structure and composition of cell walls and external layers. In: *The Biology of Cyanobacteria.* (Carr, N.G. & Whitton, B.A. ed.), 333-357. Blackwell Scientific Publications, Oxford.

Driessche, E.V. (1988). Structure and function of leguminosae lectins. In: *Advances in Lectin Research, Vol.1.* (Franz, H. ed.), 73-134. Springer-Verlag Press, New York.

Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry,* **28,** 350-356.

Dunn, J.H. & Wolk, C.P. (1970). Composition of the cellular envelopes of *Anabaena cylindrica. Journal of Bacteriology,* **103,** 153-158.

El-Bestawy, E. ; Bellinger, E.G. & Sigee, D.C. (1996). Elemental composition of phytoplankton in a subtropical lake: X-ray microanalytical studies on the dominant algae *Spirulina platensis* (Cyanophyta) and *Cyclotella meneghiniana* (Bacillariophyceae). *European Journal of Phycology,* **31,** 157-166.

English Nature (1995). *Rostherne Mere-- National Nature Reserve.* English Nature Press, Cheshire.

Erickson, S.J. (1972). Toxicity of copper to *Thalassiosira pseudonana* in unenriched inshore seawater. *Journal of Phycology,* **8,** 318-323.

Ernst, A. (1990). Cyanobacteria in large lakes: a case study in lake Constance. In: *Large Lakes: Ecologycial Structure and Function.* (Tilzer M.M. & Serruya, C. ed.), 428-439. Springer-Verlag Press, Berlin.

Fay, P. (1983). *The Blue-Greens.* The Camelot Press Ltd., Southampton.

Fehrmann, C. & Pohl, P. (1993). Cadmium adsorption by the non-living biomass of microalgae grown in axenic mass culture. *Journal of Applied Phycology,* **5,** 555-562.

Ferguson, J. & Bubela, B. (1974). The concentration of Cu(II), Pb(II) and Zn(II) from aqaueous solutions by particulate algal matter. *Chemical Geology,* **13,** 163-186.

Fergusson, J.E. (1990). *The Heavy Elements: Chemistry, Environmental Impact and Health effects.* Pergamon press, Oxford.

Fisher, M.M. ; Wilcox, L.W. (1996). Desmid-bacterial associations in *Sphagnum*-dominated Wisconsin peatlands. *Journal of Phycology,* **32,** 543-549.

Fogg, C.E. & Westlake, D.F. (1955). The importance of extracellular products of algae in freahwater. *Proceeding of the International Association of Theoretical and Applied Limnology,* **12,** 219-232.

Fogg, G.E. (1965). *Algal Cultures and Phytoplankton Ecology.* University of London, The Athlone press, London.

Fogg, G.E. (1966). The extracellular products of algae. *Oceanography and Marine Biology Annual Review,* **4,** 195-212.

Fogg, G.E. (1983). The ecological significance of extracellular products of phytoplankton photosynthesis. *Botanica Marina,* **XXVI,** 3-14.

Förstner, U. & Wittmann, G.T.W. (1981). *Metal Pollution in the Aquatic Environment.* Springer-Verlag press, Berlin.

Förstner, U. (1979). Metal concentrations in river, lake, and ocean waters. In: *Metal Pollution in the Aquatic Environment.* (Förstner, U. & Wittmann, G.T.W. ed.), 71-109. Springer-Verlag press, Berlin.

Fourest, E. & Volesky, B. (1996). Contribution of sulfonate groups and alginate to heavy metal biosorption by the dry biomass of *Sargassum fluitans. Environmental Science and Technology,* **30,** 277-282.

Frank, H. ; Lefort, M. & Martin, H.H. (1962). Elektronenoptische und chemische untersuchungen an zellwänden der blaualge *Phormidium uncinatum. Zeitschrift fuer Naturforschung,* **17,** 262-268.

Freundlich, H. (1926). *Colloid and Capillary Chemistry.* Methuen and Co., Ltd., London.

Fuhs, G.W. (1973). Cytochemical examination of blue-green algae. In: *The Biology of Blue-Green Algae.* (Carr, N.G. & Whitton, B.A. ed.), 117-144. Blackwell Scientific Publication, Oxford.

Gadd, G.M. (1988). Accumulation of metals by microorganisms and algae. In: *Biotechnology, Vol.6B. Special Microbial Processes.* (Rehm, H.-J. & Reed, G. ed.), 401-433. VCH Verlagsgesellschaft mbH, Germany.

Gajewski, A.J. & Chróst, R.J. (1995). Production and enzymatic decomposition of organic matter by microplankton in a eutrophic lake. *Journal of Plankton Research,* **17,** 709-728.

Ganf, G.G. & Oliver, R.L. (1982). Vertical separation of light and available nutrients as a factor causing replacement of green aglae by blue-green algae in the plankton of a stratified lake. *Journal of Ecology,* **70,** 829-844.

Geitler, L. (1948). Symbiosen zwischen Chrysomonaden und knospenden Bakterienartigen Organismen sowie Beobachtungen über Organisationseigentumlichkeiten der Chrysomonaden. *Öesterreichische Botanische Zeitschrift,* **95,** 300-324.

George, D.G. & Heaney, S.I. (1978). Factors influencing the spatial distribution of phytoplankton in a small productive lake. *Journal of Ecology,* **66,** 133-155.

Gibson, J.F. ; Poole, R.K. ; Hughes, M.N. & Rees, J.F. (1984). Effect of nitrosyl complexes of ruthenium on *E. coli* K12 and yeast and the uptake of these complexes by marine bacteria. In: *Role of Microorganisms on the Behaviour of Radionuclides in Aquatic and Terrestrial Systems and Their Transfer to Man.* (Gelderand, E.B. & Kirchmann, R. ed.), 82-91. International Union of Radioecologists, Brussels.

Giesy, J.P. (1981). Biological control of trace metal equilibria in surface waters. In: *Trace Element speciation in Surface Water and Its Ecological Implications.* (Leppard, G.G. ed.), 195-210. Plenum Press, New York.

Glooschenko, W.A. (1969). Accumulation of ^{203}Hg by the marine diatom *Chaetoceros costatum. Journal of Phycology*, **5**, 224-226.

Goldstein, I.J. (1972). Use of concanavalin A for structural studies. In: *Method in Carbohydrate Chemistry: General Carbohydrate Method. Vol.VI.* (Wistler, R.L. ; Smith, R.J. ; BeMiller, J.N. & Wolfrom, M.L. ed.), 106-119. Academic Press, London.

Goldstein, I.J. (1974). Studies on the combining sites of concanavalin A. In: *Advances in Experimental Medicine and Biology, Vol.55.* (Chowdhury, T.K. & Weiss, A.K. ed.), 35-53. Plenum Press, New York.

Goldstein, I.J. ; Hughes, R.C. ; Monsigny, M. ; Osawa, T. & Sharon, N. (1980). What should be called a lectin. *Nature*, **285**, 66.

Goldstein, I.J. & Poretz, R.D. (1986). Isolation and chemical properties of lectins. In: *The Lectins: Properties, Functions and Applications in Biology and Medicine.* (Liener, I.E. ; Sharon, N. & Goldstein, I.J. ed.), 33-220. Academic Press Inc., New York.

Gonzalez-Davila, M. ; Santana-Casiano, J.M. ; Perez-Peña, J. & Millero, F.J. (1995). Binding of Cu(II) to the surface and exudates of the alga *Dunaliella tertiolecta* in seawater. *Environmental Science and Technology,* **29,** 289-301.

Gorham, E. (1957). The ionic composition of some lowland lake waters from Cheshire, England. *Limnology and Oceanography,* **2,** 22-27.

Gowrinathan, K.P. & Rao, V.N.R. (1990). Detoxification of copper by *Nitzschia obtusa* Wm. Sm., a pennate diatom. *Bulletin of Environmental Contamination and Toxicology*, **45**, 612-618.

Greene, B. & Darnal, D.W. (1990). Microbial oxygenic photoautotrophs (Cyanobacteria and algae) for metal-ion binding. In: *Microbial Mineral Recovery.* (Ehrlich, M. & Brierley, C.L. ed.), 277-302. McGraw-Hill, Inc., New York.

Griffeths, B.M. (1925). Studeis in the phytoplankton of the lowland waters of Great Britain. No. III. The phytoplankton of Shropshire, Cheshire, and Staffordshire. *Journal of Linnean Society Botany,* **47,** 75-91.

Grimshaw, H.M. & Hudson, M.J. (1970). Some mineral nutrient studies of a lowland mere in Cheshire, England. *Hydrobiologia,* **36,** 329-341.

Grossart, H.-P. & Simon, M. (1993). Limnetic macroscopic organic aggregates (lake snow): occurrence, characteristics, and microbial dynamics in Lake Constance. *Limnology and Oceangraphy,* **38,** 532-546.

Grossart, H.-P. ; Simon, M. & Logan, B.E. (1997). Formation of macroscopic organic aggregates (lake snow) in a large lake: the significance of transparent exopolymer particles, phytoplankton, and zooplankton. *Limnology and Oceangraphy,* **42,** 1651-1659.

Guillard, R.R.L. & Hellebust, J.A. (1971). Growth and the production of extracellular substances by two strains of *Phaeocystis poucheti. Journal of Phycology,* **7,** 330-338.

Guy, R.D. & Chakrabarti, C.L. (1975). Analytical techniques for speciation of trace metals. *Proceedings. International Conference on Heavy Metal Environment,* Toronto, 275-294.

Hamburger, B. (1958). Bakterien symbiose bei *Volvox aureas* Ehrenb. *Archiv fuer Mikrobiologie,* **29,** 291-310.

Harris, P.O. & Ramelow, G.J. (1990). Binding of metal ions by particulate biomass derived from *Chlorella vulgaris* and *Scenedesmus quadricauda. Environmental Science and Technology,* **24,** 220-228.

He, L.M. & Tebo, B.M. (1998). Surface charge properties of and Cu(II) adsorption by spores of the marine *Bacillus sp.* Strain SG-1. *Applied and Environmental Microbiology,* **64,** 1123-1129.

Hellebust, J.A. (1974). Extracellular Products. In: *Algal Physiology and Biochemistry.* (Stewart, W.D.P. ed.), 838-863. Blackwell Scientific Publications Ltd., Oxford.

Höcht, H. ; Martin, H.H. & Kandler, O. (1965). Zur kenntnis der chemischen zusammensetzung der zellwand der blaualgen. *Zeitschrift Für Pflanzenphysiologie,* **53,** 39-57.

Hofstraat, J.W. ; van Zeijl, W.J.M. ; de Vreeze, M.E.J. ; Peeters, J.C.H. ; Peperzak, L. ; Colijn, F. & Rademaker, T.W.M. (1994). Phytoplankton monitoring by flow cytometry. *Journal of Plankton Research,* **16,** 1197-1224.

Hoiczyk, E. & Baumeister, W. (1998). The juctional pore complex, a prokaryotic secretion organelle, is the molecular motor underlying gliding motility in cyanobacteria. *Current Biology,* **8,** 1161-1168.

Holan, Z.R. ; Volesky, B. & Prasetyo, I. (1993). Biosorption of cadmium by biomass of marine algae. *Biotechnology and Bioengineering,* **41,** 819-825.

Hori, K. ; Ogata, T. ; Kamiya, H. & Mimuro, M. (1996). Lectin-like compounds and lectin receptors in marine microalgae: hemagglutination and reactivity with purified lectins. *Journal of phycology,* **32,** 783-790.

Horikoshi, T. ; Nakajima, A. & Sakaguchi, T. (1979). Uptake of uranium by *Chlorella regularis. Agricultural and Biological Chemistry,* **43,** 617-623.

Horne, A.J. & Goldman, C.R. (1994). *Limnology.* Second edition. McGraw-Hill, Inc., New York.

Horne, A.J. (1975). The ecology of Clear Lake phytoplankton. *Special Report of the Clear Lake Algal Research Unit,* Lakeport, Calif. 116.

Hughes, M.N. & Poole, R. K. (1989). *Metals and Micro-organisms.* Chapman and Hall Ltd. press, London.

Hunter, S.H. (1972). Inorganic nutrition. *Annual Review of Microbiology,* **26,** 313.

Huntsman, S.A. & Sunda, W.G. (1980). The role of trace metals in regulating phytoplankton growth with emphasis on Fe, Mn and Cu. In: *The Physiological Ecology of Phytoplankton.* (Morris, I. ed.), 285-328. Blackwell Scientific publication, Oxford.

Hutchins, D.A. ; Rueter, J.G. & Fish, W. (1991). Siderophore production and nitrogen fixation are mutually exclusive strategies in *Anabaena* 7120. *Limnology and Oceanography,* **36,** 1-12.

Hutchinson, G.E. (1957). *A Treatise on Limnology. Vol.1, Geography, Physics, and Chemistry.* John Wiley & sons, Inc., New York.

Hutchinson, G.E. (1967). *A Treatise on Limnology. Vol.2, Introduction to Lake Biology and the Limnoplankton.* John Wiley & sons, Inc., New York.

Hutton, M. (1987). Group report: cadmium. In: *Lead, Mercury, Cadmium and Arsenic in the Environment.* (Hutchinson, T.C. & Meema, K.M. ed.), **Scope 31,** 35-41, Wiley, Chichester.

Hynes, H.B.N. (1964). Report on the streams flowing into Rostherne Mere.

Ibelings, B.W. (1996). Changes in photosynthesis in response to combined irradiance and temperature stress in cyanobacterial surface waterblooms. *Journal of Phycology,* **32,** 549-557.

Ibelings, B.W. ; Kroon, B.M.A. & Mur, L.R. (1994). Acclimation of photosystem II in a cyanobacterium and a eukaryotic green alga to high and fluctuating photosynthetic photon flux densities, simulating light regimes induced by mixing in lakes. *New Phytologist,* **128,** 407-424.

Innamorati, M. (1995). Hyperproduction of mucilages by micro and macro algae in the Tyrrhenian Sea. *The Science of the Total Environment,* **165,** 65-81.

Inoué, S. (1995). Foundations of confocal scanned imaging in light microscopy. In: *Handbook of Biological Confocal Microscopy.* (Pawley, J.B. ed.), 1-17. Plenum Press, New York.

Jackson, G.A. & Morgan, J.J. (1978). Trace metal-chelator interactions and phytoplankton growth in seawater media: theoretical analysis and comparison with reported observations. *Limnology and Oceanography,* **23,** 268-282.

Jackson, T.A. (1979). Relationships between the properties of heavy metals and their biogeochemical behaviour in lakes and river-lake systems. In: *International Conference on Management and Control of Heavy Metals in the Environment.* 457-460. CEP Consultants Ltd., Edinburgh.

James, M.R. ; Burns, C.W. & Forsyth, D.J. (1995). Pelagic ciliated protozoa in two monomictic, southern temperate lakes of contrasting trophic state: seasonal distribution and abundance. *Journal of Plankton Research,* **17,** 1479-1500.

Johnson, G.D. & Araujo, G.M. de C.N. (1981). A simple method of reducing the fading of immunofluorescence during microscopy. *Journal of Immunological Methods,* **43,** 349-350.

Jones, J.G. (1971). Studies on freshwater bacteria: factors which influence the polulation and its activity. *Journal of Ecology,* **59,** 593-613.

Jørgensen, N.O.G. & Jensen, R.E. (1994). Microbial fluxes of free monosaccharides and total carbohydrates in freshwater determined by PAD-HPLC. *FEMS Microbiology Ecology,* **14,** 79-94.

Jugdaohsingh, R.; Campbell, M.M. ; Thompson, P.H. ; McCrohan, C.R. ; White, K.N. & Powell, J.J. (1998). Mucus secretion by the freshwater snail *Lymnaea stagnalis* limits aluminum concentrations of the the aqueous environment. *Environmental Science and Technology,* **32,** 2591-2595.

Jürgens, U.J. & Weckesser, J. (1985). The fine structure and chemical composition of the cell wall and sheath layers of cyanobacteria. *Annales de L'institut Pasteur: Microbiology,* **136A,** 41-44.

Kaplan, D. ; Christiaen, D. & Arad, S. (1987). Binding of heavy metals by algal polysaccharides. In: *Algal Biotechnology.* (Stakler, T. et al. ed.), 179-187. Elsevier Applied Science, London.

Kato, K. (1994). Planktonic bacterial DNA and RNA synthesis from algal extracellular products in a eutrophic lake. *FEMS Microbiology Ecology,* **15,** 291-298.

Khailov, K.M. (1964). The formation of organometallic complexes with the participation of extracellular metabolites of marine algae. *Doklady Akademii Nauk SSSR., Biological Science,* **155,** 237.

Khummongkol, D. ; Canterford, G.S. & Fryer, C. (1982). Accumulation of heavy metals in unicellular algae. *Biotechnology and Bioengineering,* **14,** 2643-2660.

Kiefer, E. ; Sigg, L. & Schosseler, P.(1997). Chemical and spectroscopic characterization of algae surfaces. *Environmental Science and Technology,* **31,** 759-764.

Kilham, S.S. (1990). Relationship of phytoplankton and nutrients to stoichiometric measures. In: *Large Lakes: Ecological Structure and Function.* (Tilzer, M.M. & Serruya, C. ed.), 403-413. Springer-Verlag Press, Berlin.

Kim, Y.H. ; Yoo, Y.J. & Lee, H.Y. (1995). Characteristics of lead adsorption by *Undaria pinnatifida. Biotechnology Letters,* **17,** 345-350.

Knauer, K. ; Behra, R. ; Sigg, L. (1997). Adsorption and uptake of copper by the green alga *Scenedesmus subspicatus* (Chlorophyta). *Journal of Phycology,* **33,** 596-601.

Kochert, G. (1978). Carbohydrate determination by the phenol-sulphuric acid method. In: *Handbook of Phycological Methods: Physiological and Biochemical Methods.* (Hellebust, J.A. & Craigie, J.S. ed.), 95-97. Cambridge Univeristy Press, Cambridge.

Kocourek, J. (1986). Historical background. In: *The Lectin: Properties, Functions, and Applications in Biology and Medicine.* (Liener, I.E. ; Sharon, N. & Goldstein I.J. ed.), 1-32. Academic Press, Orlando.

Kocourek, J. ; Hořejši, V. (1983). A note on the recent discussion on definition of the term "lectin". In: *Lectins: biology, biochemistry, clinical biochemistry, Vol.3,* (Bog-Hansen T.C. & Spengler, G.A. ed.), 3-6. Walter de Gruyter, Berlin.

Kosakowska, A. ; Falkowski, L. & Lewankowska, J. (1988). Effect of amino acids on the toxicity of heavy metals to phytoplankton. *Bulletin of Environmental contamination and Toxicology,* **40,** 532-538.

Krivtsov, V. (1996). Unpublished data.

Lamont, H.C. (1969). Shear-oriented microfibrils in the mucilaginous investments of two motile Oscillatoriacean blue-green algae. *Journal of Bacteriology,* **97,** 350-361.

Lancelot, C. (1995). The mucilage phenomenon in the continental coastal waters of the North Sea. *The science of the Total Environment,* **165,** 83-102.

Lange, W. (1974). Chelating agents and blue-green algae. *Canadian Journal of Microbiology,* **20,** 1311.

Lange, W. (1976). Speculations on a possible essential function of the gelatinous sheath of blue-green algae. *Canadian Journal of Microbiology,* **22,** 1181-1185.

Langmuir, I. (1918). The adsorption of gases on plane surfaces of glass, mica, and platinum. *Journal of American Chemical Society,* **40,** 1361.

Lapedes, D.N. (1974). *Dictionary of Scientific and Technical terms.* McGraw-Hill, New York.

Leak, L.V. (1967). Fine structure of the mucilaginous sheath of *Anabaena sp.*. *Journal of Ultrastructure Research,* **21,** 61-74.

Lee, D.-Y. & Rhee, G.-Y. (1997). Kinetics of cell death in the cyanobacterium *Anabaena flos-aquae* and the production of dissolved organic carbon. *Journal of Phycology,* **33,** 991-998.

Lee, R.E. (1997). *Phycology.* Second edition. Cambridge University Press, Cambridge.

Lee, Y.C. & Montgomery, R. (1961). Determination of Hexosamines. *Archives of Biochemistry and Biophysics,* **93,** 292-296.

Leppard, G.G. (1995). The characterization of algal and microbial mucilages and their aggregates in aquatic ecosystems. *The Science of the Total Environment,* **165,** 103-131.

Leppard, G.G. ; Massalski, A. & Lean, D.R.S. (1977). Electron-opaque microscopic fibrils in lakes: their demonstration, their biological derivation and their potential significance in the redistribution of cations. *Protoplasma,* **92,** 289-309.

Levado, E. (1997). Unpublished data.

Levene, C. ; Gilboa-Garber, N. & Garber, N.C. (1994). Lectin-blood group interactions. In: *Lectin-Microorganism Interactions.* (Doyle, R.J. & Slifkin, M. ed.), 327-393. Marcel Dekker, Inc., New York.

Lewis, J.G. ; Stanley, N.F. & Guist, G.G. (1990). Commercial production and applications of algal hydrocolloids. In: *Algae and human affairs.* (Lembi, C.A. & Waaland, J.R. ed.), 205-236. Cambridge University Press, Cambridge.

Lind, E.M. (1945). The phytoplankton of some Cheshire Meres. *Memoirs and Proceedings of the Manchester Literary & Philosophical Society,* **86,** 83-105.

Lindley, J. (1845). *School Botany.* Bradbury, Evans & Co., London.

Lis, H. & Sharon, N. (1986). Application of lectins. In: *The Lectins: Properties, Functions, and Applications in Biology and Medicine.* (Liener, I.E. ; Sharon, N. & Goldstein, I.J. ed.), 293-370. Academic Press Inc., New York.

Livingston, D. (1979). *Algal Remains in Recent Lake Sediments.* PHD thesis. University of Leicester, U.K..

López-Rodas, V. & Costas, E. (1997). Characterization of morphospecies and strains of *Microcystis* (Cyanobacteria) from natural populations and laboratory clones using cell probes (lectins and antibodies). *Journal of Phycology,* **33,** 446-454.

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. & Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *The Journal of Biological Chemistry,* **193,** 265-275.

Lund, J.W.G.; Kipling, C. & Le Cren, D.L. (1958). The inverted microscope method of estimating algal numbers and the statistical basis of estimations by counting. *Hydrobiologia,* **2,** 143-170.

MacIntyre, S. ; Alldredge, A.L. & Gotschalk, C.C. (1995). Accumulation of marine snow at density discontinuities in the water column. *Limnology and Oceanography,* **40,** 449-468.

Maki, J.S. & Mitchell, R. (1986). The function of lectins in interactions among marine bacteria, invertebrates and algae. In: *Microbial Lectins and Agglutinins Properties and Biological Activity.* (Mirelman, D. ed.), 409-425. A wiley-Interscience Publication, New York.

Mann, A.W. & Lintern, M. (1983). Heavy metal dispersion patterns from tailings dumps Northampton district Western Australia. *Environmental Pollution,* **B6,** 33-49.

Mantle, M. & Allen, A. (1978). A colorimetric assay for glycoprotein based on the Periodic Acid/Schiff stain. *Biochemical Society Transactions,* **6,** 607-609.

Martin, T.C. & Wyatt, J.T. (1974). Extracellular investments in blue-green algae with particular emphasis on the genus *Nostoc. Journal of Phycology,* **10,** 204-210.

Mcknight, D.M. & Morel, F.M.M. (1979). Release of weak and strong copper-complexing agents by algae. *Limnology and Oceanography,* **24,** 823-837.

McKnight, D.M. & Morel, F.M.M. (1980). Copper complexation by siderophores from filamentous blue-green algae. *Limnology and Oceanography,* **25,** 62.

Mingazzini, M & Thake, B. (1995). Summary and conclusions of the workshop on marine mucilages in the Adriatic Sea and elsewhere. *The Science of the Total Environment,* **165,** 9-14.

Moffett, J.W. & Brand, L.E. (1996). Production of strong, extracellular Cu chelators by marine cyanobacteria in response to Cu stress. *Limnology and Oceanography,* **41,** 388-395.

Moore, B.G. & Tischer, R.G. (1965). Biosynthesis of extracellular polysaccharides by the blue-green alga *Anabaena flos-aquae. Canadian Journal of Microbiology,* **11,** 877-885.

Morel, F.M.M. ; Rueter, J.G. ; Anderson, D.M. & Guillard, R.R.L. (1979). Aquil: a chemically defined phytoplankton culture medium for trace metal studies. *Journal of Phycology,* **15,** 135-141.

Morgan, W.T.J. & Watkins, W.M. (1959). The inhibition of the haemagglutinins in plant seeds by human blood group substances and simple sugars. *British Journal of Experimental Pathology,* **34,** 94-103.

Morris, I. (1967). *An Introduction to the algae.* Hutchinson & Co. (Publishers) Ltd., London.

Moss, B. (1973). The influence of environmental factors on the distribution of freshwater algae: an experimental study. *Journal of Ecology,* **61,** 157-177.

Mullen, M.D. ; Wolf, D.C. : Beveridge, T.J. & Bailey, G.W. (1992). Sorption of heavy metals by the soil fungi *Aspergillus niger* and *Mucor rouxii. Soil Biology and Biochemistry,* **24,** 129-135.

Mullen, M.D. ; Wolf, D.C. ; Ferris, F.G. ; Beveridge, T.J. ; Flemming, C.A. & Bailey, G.W. (1989). Bacterial sorption of heavy metals. *Applied and Environmental Microbiology,* **55,** 3143-3149.

Murphy, T.P.; Lean, D.R.S. & Nalewajko, C. (1976). Blue-green algae: their excretion of iron-selective chelators enables them to dominate other algae. *Science,* **192,** 900-902.

Myklestad, S. ; Haug, A. & Larsen, B. (1972). Production of carbohydrates by the marine diatom *Chaetoceros affinis* var. *Willei* (Gran) Hustedt. II. preliminary investigation of the extracellular polysaccharide. *Journal of Experimental Marine Biology and Ecology,* **9,** 137-144.

Myklestad, S.M. (1995). Release of extracellular products by phytoplankton with special emphasis on polysaccharides. *The Science of the Total Environment,* **165,** 155-164.

Nakagawa, M. ; Takamura, Y. & Yagi, O. (1987). Isolation and characterization of the slime from a Cyanobacterium, *Microcystis aeruginosa* K-3A. *Agricultural and Biological Chemistry,* **51,** 329-337.

Nakajima, A., Horikoshi, T. & Sakaguchi, T. (1981). Study on the accumulation of heavy elements in biological systems, XVII. Selective accumulation of heavy metal ions by *Chlorella regularis. European Journal of Applied Microbiology and Biotechnology,* **12,** 76-83.

Naumann, E. (1925). Die Gallertbildungen des pflanzlichen Limnoplanktons. Eine morphologisch-okologische Ubersicht. *Acta Universitatis Lundensis,* **N.F. Avd. II., 21, Nr. 5,** 26.

Nelms, R. (1984). *Palaeolimnological Studies of Rostherne Mere (Cheshire) and Ellesmere (Shropshire).* Ph.D Thesis, Liverpool Polytechnic, U.K..

Nieboer, E. & Richardson, D.H.S. (1980). The replacement of the nondescript term 'heavy metals' by a biologically and chemically significant classification of metal ions. *Environmental pollution,* **Series B1,** 3-26.

O'Colla P.S. (1962). Mucilage. In: *Physiology and Biochemistry of Algae.* (Lewin R.A. edition), 315-335. Academic Press, New York.

Özer, D. ; Aksu, Z. ; Kutsal, T. & Caglar, A. (1994). Adsorption isotherms of lead(II) and chromium(VI) on *Cladophora crispata. Environmental Technology,* **15,** 439-448.

Paerl, H.W. & Ustach, J.F. (1982). Blue-green algal scums: an explanation for their occurrence during freshwater blooms. *Limnology and Oceangraphy,* **27,** 212-217.

Paerl, H.W. (1984). Transfer of N_2 and CO_2 fixation products from *Anabaena oscillarioides* to associated bacteria during inorganic carbon sufficiency and deficiency. *Journal of Phycology,* **20,** 600-608.

Paerl, H.W. (1992). Epi- and endobiotic interactions of cyanobacteria. In: *Algae and Symbioses- Plants, Animals, Fungi, Viruses interactions explored.* (Reisser, W. ed.), 537-565. Biopress Ltd., Bristol.

Panoff, J.-M. ; Priem, B. ; Morvan, H. & Joset, F. (1988). Sulphated exopolysaccharides produced by two unicellular strains of Cyanobacteria, *Synechocystis* PCC 6803 and 6714. *Archives of Microbiology,* **150,** 558-563.

Parker, D.L. ; Rai, L.C. ; Mallick, N. ; Rai, P.K. & Kumar, H.D. (1998). Effects of cellular metabolism and viability on metal ion accumulation by cultured biomass from a bloom of the Cyanobacterium *Microcystis aeruginosa. Applied and Environmental Microbiology,* **64,** 1545-1547.

Passow, H. ; Rothstein, A. & Clarkson, T.W. (1961). The general pharmacology of heavy metals. *Pharmacological Reviews,* **13,** 185-223.

Paulsen, B.S. & Vieira, A.A.H. (1994). Structure of the capsular and extracellular polysaccharides produced by the Desmid *Spondylosium panduriforme* (Chlorophyta). *Journal of Phycology,* **30,** 638-641.

Paxton, J. (1849). *A Pocket Botanical Dictionary.* Bradbury & Evans, London.

Pearsall, W.H. (1923). The phytoplankton of Rostherne Mere. *Manchester Memoirs,* **67,** 45-55.

Percival, E. & McDowell, R.H. (1981). Algae walls: composition and biosynthesis. In: *Plant Carbohydrates 2.--Extracellular carbohydrates.* (Tanner, W. and Loewus, F.A. ed.), Encyclopedia of Plant Physiology New Series, **13B,** 277-316. Springer-Verlag Press, Berlin.

Pierce Scientific Ltd. (1996). *Instructions for BCA Protein Assay.*

Plude, J.L. ; Parker, D.L. ; Schommer, O.J. ; Timmerman, R.J. ; Hagstrom, S.A. ; Joers, J.M. & Hnasko, R. (1991). Chemical characterization of polysaccharide from the slime layer of the Cyanobacterium *Microcystis flos-aquae* C3-40. *Applied and Environmental Microbiology,* **57,** 1696-1700.

Post, A.F. ; Dubinsky, Z. ; Wyman, K. & Falkowski, P.G. (1984). Kinetics of light-intensity adaptation in a marine planktonic diatom. *Marine Biology,* **83,** 231-238.

Pourriot, R. (1977). Food and feeding habits of Rotifera. *Ergebnisse der Limnologie,* **8,** 243-260.

Prescott, G.W. (1969). *The algae: A Review.* Thomas Nelson and Sons Ltd., London.

Pritzer, M. ; Weckesser, J. & Jürgens, U.J. (1989). Sheath and outer membrane components from the Cyanobacterium *Fischerella sp.* PCC 7414. *Archives of Microbiology,* **153,** 7-11.

Rai, L.C. ; Gaur, J.P. & Kumar, H.D. (1981). Phycology and heavy-metal pollution. *Biological Review,* **56,** 99-151.

Ramamoorthy, S. ; Springthorpe, S. & Kushner, D.J. (1977). Competition for mercury between river sediment and bacteria. *Bulletin of Environmental Contamination and Toxicology,* **17,** 505-511.

Ramus, J. (1972). The production of extracellular polysaccharide by the unicellular red alga *Porphyridium aerugineum. Journal of Phycology,* **8,** 97-111.

Reeke Jr., G.N. ; Becker, J.W. ; Cunningham, B.A. ; Gunther, G.R. ; Wang, J.L. & Edelman, G.M. (1974). Relationships between the structure and activities of concanavalin A. *Annals New York Academy of Sciences,* **234,** 369-382.

Reinertsen, H. ; Jensen, A. ; Langeland, A. & Olsen, Y. (1986). Algal competition for phosphorus: the influence of zooplankton and fish. *Canadian Journal of Fisheries and Aquatic Sciences,* **43,** 1135-1141.

Reynolds, C.S. & Bellinger, E.G. (1992). Patterns of abundance and dominance of the phytoplnakton of Rosterne Mere, England: evidence from an 18-year data set. *Aquatic Sciences,* **54,** 10-36.

Reynolds, C.S. & Reynolds, J.B. (1985). The atypical seasonality of phytoplankton in Crose Mere, 1972: an independent test of the hypothesis that variability in the physical environment regulates community dynamics and structure. *British Phycological Journal,* **20,** 227-242.

Reynolds, C.S. (1978a). The plankton of the north-west Midland meres. *Occasional Papers of the Caradoc and Severn Valley Field Club,* **2.** 36+xxiii pp.

Reynolds, C.S. (1978b). Notes on the phytoplankton periodicity of Rostherne Mere, Cheshire, 1967-1977. *British Phycology Journal,* **13,** 329-335.

Reynolds, C.S. (1979). The limnology of the eutrophic meres of the Shropshire-Cheshire plain. *Field Studies*, **5**, 93-173.

Reynolds, C.S. (1993). *The Ecology of Freshwater Phytoplankton.* Fourth edition, Cambridge University Press, Cambridge.

Reynolds, C.S. ; Jaworski, G.H.M. ; Cmiech, H.A. & Leedale, G.F. (1981). On the annual cycle of the blue-green algae *Microcystis aeruginosa* Kutz. emend. Elenkin. *Philosophical Transactions of the Royal Society of London*, **B293**, 419-477.

Rhee, G.-Y. (1978). Effect of N:P atomic ratios and nitrate limitation on algal growth, cell composition and nitrate uptake. *Limnology and Oceanography,* **23,** 10-25.

Richman, S. ; Branstrator, D.K. & Huber-Villegas, M. (1990). Impact of zooplankton grazing on phytoplankton along a trophic gradient. In: *Large Lakes-Ecological Structure and Function.* (Tilzer, M.M. & Serruya, C. ed.), 592-614. Springer-Verlag Press, Berlin.

Rinaldi, A. ; Vollenweider, R.A. ; Montanari, G. ; Ferrari, C.R. & Ghetti, A. (1995). Mucilages in Italian seas: the Adriatic and Tyrrhenian Seas, 1988-1991. *The Science of the Total Environment,* **165,** 165-183.

Rippka, R. ; Deruelles, J. ; Waterbury, J.B. ; Herdman, M. & Stanier, R.Y. (1979). Generic assignments, strain histories properties of pure cultures of Cyanobacteria. *Journal of General Microbiology*, **111**, 1-61.

Roberts, D.D. & Goldstein, I.J. (1983). Binding of adenine and cytokinins to lima bean and other legume lectins. In: *Chemical Taxonomy, Molecular Biology, and Function of Plant Lectins. Vol.138.* (Goldstein, I.J. & Etzier, M.E. ed.), 131-141. Alan R. Liss, Inc., New York.

Rogers, D.J. & Hori, K. (1993). Marine algal lectins: new developments. *Hydrobiologia,* **260,** 589-593.

Round, F.E. (1981). *The ecology of algae.* Cambridge University Press, Cambridge.

Rüdiger, H. (1981). Lectins- an introduction. In: *Lectins: Biology, Biochemistry, Clinical Biochemistry Vol.I.* (Bog-Hansen, T.C. ed.), 3-10. Walter de Gruyter, New York.

Sakshaug, E. & Olsen, Y. (1986). Nutrient status of phytoplankton blooms in Norwegian waters and algal strategies for nutrient competition. *Canadian Journal of Fisheries and Aquatic Sciences,* **43,** 389-396.

Sala, M.M. & Güde, H. (1996). Influence of algae and crustacean zooplankton on patterns of microbial hydrolytic enzyme activities: an experimental approach. *Ergebnisse der Limnologie,* **48,** 143-154.

Schalles, J.F. ; Gitelson, A.A. ; Yacobi, Y.Z. ; Kroenke, A.E. (1998). Estimation of chlorophyll a from time series measurements of high spectral resolution reflectance in an Eutrophic lake. *Journal of Phycology,* **34,** 383-390.

Schindler, D.W. (1977). Evolution of phosphorus limitation in lakes. *Science,* **196,** 260-262.

Schrader, M. ; Drews, G. ; Golecki, J.R. & Weckesser, J. (1982). Isolation and characterization of the sheath from the Cyanobacterium *Chlorogloeopsis* PCC 6912. *Journal of General Microbiology,* **128,** 267-272.

Scott, J.A. ; Sage, G.K. & Palmer, S.J. (1988). Metal immobilisation by microbial capsular coatings. *Biorecovery,* **1,** 51-58.

Sengbusch, P.V. & Müller, U. (1983). Distribution of glycoconjugates at algae cell surfaces as monitored by FITC-conjugated lectins.--Studies on selected species from *Cyanophyta, Pyrrhophyta, Raphidophyta, Euglenophyta, Chromophyta, and Chlorophyta. Protoplasma*, **114**, 103-113.

Sengbusch, P.V. ; Mix, M. ; Wachholz, I. & Manshard, E. (1982). FITC-labeled lectins and Calcofluor White ST as probes for the investigation of the molecular architecture of cell surfaces.--Studies on Conjugatophycean species. *Protoplasma*, **111**, 38-52.

Serratore, P. ; Rinaldi, A. ; Montanari, G. ; Ghetti, A. ; Ferrari, C.R. & Vollenweider, R.A. (1995). Some observations about bacterial presence in seawater related to mucilaginous aggregates in the Northwest Adriatic Sea. *The Science of the Total Environment,* **165,** 185-192.

Sigee, D.C. & Holland, R. (1997). Elemental composition, correlations, and ratios within a population of *Staurastrum planctonicum* (Zygnematales): an X-ray microanalytical study. *Journal of Phycology,* **33,** 182-190.

Sigee, D.C. ; Krivtsov, V. & Bellinger, E.G. (1998). Elemental concentrations, correlations and ratios in micropopulations of *Ceratium hirundinella* (Pyrrhophyta): an X-ray microanalytical study. *European Journal of Phycology,* **33,** 155-164.

Smith, P.K. ; Krohn, R.I. ; Hermanson, G.T. ; Mallia, A.K. ; Gartner, F.H. ; Provenzano, M.D. ; Fujimoto, E.K. ; Goeke, N.M. ; Olson, B.J. & Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry,* **150,** 76-85.

Steemann Nielsen, E. ; Kamp-Nielsen, L. & Wium-Andersen, S. (1969). The effect of deleterious concentrations of copper on the photosynthesis of *Chlorella pyrenoidosa. Physiologia Plantarum*, **22**, 1121-1133.

Stoermer, E.F. (1990). Algae and the environment: the Great Lakes case. In: *Algae and Human Affairs.* (Lembi, C.A. & Waaland, J.R. ed.), 57-86. Cambridge University Press, New York.

Stokes, P.M. (1983). Responses of freshwater algae to metals. In: *Progress in Phycological Research.* (Round, F.E. & Chapman, D.J. ed.), **2,** 89-112. Elsevier Science Publishers B.V., Amsterdam, The Netherlands.

Stumm, W. & Bilinski, H. (1972). Trace metals in natural water: difficulties of interpretation arising from our ignorance on their speciation. In: *Advances on Water Pollution Research.* (Jenkins, S.H. ed.), 39-52. Pergamon Press, New York.

Su, M.-C. ; Cha, D.K. & Anderson, P.R. (1995). Influence of selector technology on heavy metal removal by activated sludge: secondary effects of selector technology. *Water Research,* **29,** 971-976.

Sumner, J.B. & Howell, S.F. (1936). The identification of the hemagglutinin of the jack bean with *concanavalin A. Journal of Bacteriology,* **32,** 227-237.

Surek, B. & Sengbusch, P.V. (1981). The localization of galactosyl residues and lectin receptors in the mucilage and the cell walls of *Cosmocladium saxonicum* (Desmidiaceae) by means of fluorescent probes. *Protoplasma,* **108,** 149-161.

Swallow, K.C. ; Westall, J.C. ; McKnight, D.M. ; Morel, N.M. & Morel, F.M.M. (1978). Potentiometric determination of copper complexation by phytoplankton exudates. *Limnology and Oceanography,* **23,** 538.

Talling, J.F. (1976). The depletion of carbon dioxide from lake water by phytoplankton. *Journal of Ecology,* **64,** 79-121.

Tattersall, W.M. & Coward, T.A. (1914). Faunal survey of Rostherne Mere.--1. Introduction and methods. *Manchester Memoris,* **58,** 1.

Tchobanoglous, G. & Burton, F.L. (1991). *Wastewater engineering --Treatment, Disposal, and Reuse.* Third Edition. McGraw-Hill, Inc., New York.

Tease, B.E. & Walker, R.W. (1987). Comparative composition of the sheath of the Cyanobacterium *Gloeothece* ATCC 27152 cultured with and without combined nitrogen. *Journal of General Microbiology,* **133,** 3331-3339.

Therriault, J.-C. ; Lawrence, D.J. & Platt, T. (1978). Spatial variability of phytoplankton turnover in relaiton to physical processes in a coastal environment. *Limnology and Oceanography,* **23,** 900-911.

Thompson, P.-A. & Rhee, G.-Y. (1994). Phytoplankton responses to eutrophication. In: *Algae and Water Pollution.* (Rai, L.C. ; Gaur, J.P. & Soeder, C.J. ed.), 125-166. E. Schweizerbart'sche Verlagsbuchhandlung (Nagele u. Obermiller), Stuttgart, 1994.

Tilman, D. ; Kilham, S.S. & Kilham, P. (1982). Phytoplankton community ecology: the role of limiting nutrients. *Annual Review of Ecology and Systematics,* **13,** 349-372.

Ting, Y.P. ; Lawson, F. & Prince, I.G. (1989). Uptake of cadmium and zinc by the alga *Chlorella vulgaris*: part 1. individual ion species. *Biotechnology and Bioengineering,* **34,** 990-999.

Ting, Y.P. ; Lawson, F. & Prince, I.G. (1991). Uptake of cadmium and zinc by the alga *Chlorella vulgaris*: II. multi-ion situation. *Biotechnology and Bioengineering,* **37,** 445-455.

Twiss, M.R. (1996). The importance of chemical speciation: from the bulk solution to the cell surface. *Journal of Phycology,* **32,** 885-886.

Van Den Hoek, C. ; Mann, D.G. & Jahns, H.M. (1997). *Algae: An Introduction to Phycology.* Cambridge University Press, Cambridge.

Van Donk, E. & Bruning, K. (1992). Ecology of aquatic fungi in and on algae. In: *Algae and Symbioses: Plants, Animals, Fungi, Viruses interactions explored.* (Reisser, W. ed.), 567-592. Biopress limited, Bristol.

Van Donk, E. & Bruning, K. (1995). Effects of fungal parasites on planktonic algae and the role of environmental factors in the fungus-alga relationship. In: *Algae, Environment and Human Affairs.* (Wiessner, W. ; Schnepf, E. & Starr, R.C. ed.), 223-234. Biopress Ltd., Bristol.

Van Donk, E. & Ringelberg, J. (1983). The effect of fungal parasitism on the succession of diatioms in Lake Maarsseveen (The Netherlands). *Freshwater Biology,* **13,** 241-251.

Vincenzini, M. ; De Philippis, R. ; Sili, C. & Materassi, R. (1990). Studies on exopolysaccharide release by diazotrophic batch cultures of *Cyanospira capsulata. Applied Microbiology and Biotechnology,* **34,** 392-396.

Viviani, R. ; Boni, L. ; Cattani, O. ; Milandri, A. ; Pirini, M. ; Poletti, R. & Pompei, M. (1995). Fatty acids, chlorophylls and total silicon in mucilaginous aggregates collected in a coastal area of the Northern Adriatic Sea facing Emilia-Romagna in August 1988. *The Science of the Total Environment,* **165,** 193-201.

Volesky, B. & Holan, Z.R. (1995). Biosorption of heavy metals. *Biotechnology Progress,* **11,** 235-250.

Waite, A.M. ; Olson, R.J. ; Dam, H.G. & Passow, U. (1995). Sugar-containing compounds on the cell surfaces of marine diatoms measured using *concanavalin A* and flow cytometry. *Journal of phycology,* **31,** 925-933.

Walsby, A.E. & Reynolds, C.S. (1980). Sinking and floating. In: *The Physiological Ecology of Phytoplankton.* (Morris, I. ed.), 371-412. Blackwell Scientific Publications, London.

Walsby, A.E. & Xylopyta, A. (1977). The form resistance of chitan fibres attached to the cells of *Thalassiosira fluviatilis* Hustedt. *British phycological Journal,* **12,** 215-23.

Watkins, W.M. & Morgan, W.T.J. (1952). Neutralization of the anti-H agglutinin in eel serum by simple sugars. *Nature,* **169,** 825-826.

Weber, W.J. (1972). *Physicochemical Processes for Water Quality Control.* Wiley-Interscience: A division of John Wiley & sons, Inc., New York.

Weckesser, J ; Hofmann, K ; Jürgens, U.J. ; Whitton, B.A. & Raffelsberger, B. (1988). Isolation and chemical analysis of the sheaths of the filamentous Cyanobacteria *Calothrix parietina* and *C. scopulorum. Journal of General Microbiology,* **134,** 629-634.

Weckesser, J. ; Broll, C. ; Adhikary, S.P. & Jürgens, U.J. (1987). 2-O-methyl-D-xylose containing sheath in the Cyanobacterium *Gloeothece sp.* PCC 6501. *Archives of Microbiology,* **147,** 300-303.

Wehrheim, B. & Wettern, M. (1994a). Biosorption of cadmium, copper and lead by isolated mother cell walls and whole cells of *Chlorella fusca. Applied Microbiology and Biotechnology,* **41,** 725-728.

Wehrheim, B. & Wettern, M. (1994b). Influence of the external redox state on heavy metal adsorption by whole cells and isolated cell walls of *Chlorella fusca. Biotechnology Techniques,* **8,** 221-226.

Wehrheim, B. & Wettern, M. (1994c). Comparative studies of the heavy metal uptake of whole cells and different types of cell walls from Chlorella fusca. *Biotechnology Techniques,* **8,** 227-232.

Wesenberg-Lund, C. (1930). Contributions to the biology of the Rotifera. Part II. The periodicity and sexual periods. *Kongelige danske Videnskabernes Selskab Skrifter,* Naturw. Math. Afd., (ser. 9), **2,** 230.

Whitton, B.A. (1973). Freshwater plankton. In: *The Biology of Blue-Green Algae.* (Carr, N.G. & Whitton, B.A. ed.), 353-368. Blackwell Scientific Publication, Oxford.

Williams, S.L. ; Aulenbach, D.B. & Clesceri, N.L. (1974). Sources and distribution of trace metals in aquatic environments. In: *Aqueous-environmental chemistry of metals.* (Rubin, A.J. ed.), 77-127. Ann Arbor Science Publishers Inc., Ann Arbor, Michigan, U.S.A..

Wolk, C. P. (1973). Physiology and cytological chemistry of blue-green algae. *Bacteriological Reviews,* **37,** 32-101.

Wood, J.M. & Wang, H. (1983). Microbial resistance to heavy metals. *Environmental Science and Technology,* **17,** 582A-590A.

Woof, C. & Wall, T. (1984). The morphometry of Rostherne Mere, Cheshire. *Naturalist,* **Hull.109,** 143-146.

Xue, H.-B. ; Stumm, W. & Sigg, L. (1988). The binding of heavy metals to algal surfaces. *Water Research,* **22,** 917-926.

Zhang, W. & Majidi, V. (1994). Monitoring the cellular response of *Stichococcus bacillaris* to exposure of several different metals using *in vivo* ^{31}P NMR and other spectroscopic. *Environmental Science and Technology,* **28,** 1577-1581.

Zhou, J.L. ; Huang, P.L. & Lin, R.G. (1998). Sorption and desorption of Cu and Cd by macroalgae and microalgae. *Environmental Pollution,* **101,** 67-75.

APPENDIX 1. MINERAL COMPOSITION OF THE CULTURE MEDIUM

BG-11 medium

Compounds	**per liter**
$NaNO_3$	1.5g
$K_2HPO_4 . 3H_2O$	0.04g
$MgSO_4 . 7H_2O$	0.075g
$CaCl_2 . 2H_2O$	0.036g
Citric acid	0.006g
Ferric ammonium citrate	0.006g
EDTA(disodium magnesium salt)	0.001g
Na_2CO_3	0.002g
* Trace metal mix	1.0ml

*** Trace metal mix**	**g l^{-1}**
H_3BO_3	2.86
$MnCl_2 . 4H_2O$	1.81
$ZnSO_4 . 7H_2O$	0.222
$Na_2MoO_4 . 2H_2O$	0.39
$CuSO_4 . 5H_2O$	0.079
$Co(NO_3)_2 . 6H_2O$	0.0494

Make up to 1 liter with glass distilled or deionised water.
The pH after autoclaving and cooling should be 7.4 .

Modified BG-11 medium

Compounds	**per liter**
$NaNO_3$	1.5g
$K_2HPO_4 . 3H_2O$	0.04g
$MgSO_4 . 7H_2O$	0.075g
$CaCl_2 . 2H_2O$	0.036g
Na_2CO_3	0.02g

Make up to 1 liter with glass distilled or deionised water.
The pH value should be adjusted to 7.4 .

CT medium

Compounds	**per 100ml**		
$Ca(NO_3)_2.4H_2O$	15mg	*** PIV metals**	
KNO_3	10mg	FeCl3.6H2O	19.6mg
β-Na_2 glycerophosphate	5mg	$MnCl_2 . 4H_2O$	3.6mg
$MgSO_4 . 7H_2O$	4mg	$ZnSO_4 . 7H_2O$	2.2mg
Vitamin B12	0.001µg	$CoCl_2 . 6H_2O$	0.4mg
Biotin	0.001µg	$Na_2MoO_4 . 2H_2O$	0.25mg
Thiamine HCl	1µg	$Na_2EDTA . 2H_2O$	100mg
PIV metals	0.3ml	Distilled water	100ml
TAPS	40mg		
Distilled water	99.7ml		

The PH value should be adjusted to 8.2.

Bold's Basal medium

Stock solutions:

	per 400ml
1. $NaNO_3$	10g
2. $MgSO_4 . 7H_2O$	3g
3. NaCl	1g
4. K_2HPO_4	3g
5. KH_2PO_4	7g
6. $CaCl_2 . 2H_2O$	0.036g
	Per liter
8. H_3BO_3	11.42g
9. EDTA-KOH solution	
EDTA	50g
KOH	31g
10. $FeSO_4 . 7H_2O$	4.98g
H_2SO_4(conc.)	1.0ml

7. Trace elements solution:

	Per liter
$ZnSO_4 . 7H_2O$	8.82g
$MnCl_2 . 4H_2O$	1.44g
MoO_3	0.71g
$CuSO_4 . 5H_2O$	1.57g
$Co(NO_3)_2 . 6H_2O$	0.49g

Autoclave to dissolve.

11. Soil extract stock: Mix 1 part air dried, sieved soil with 2 parts distilled water. Adjust to PH 8 with NaOH or HCl and autoclave for one hour at 15 lb in^{-2} pressure. Decant or filter the supernatant.

Final solution:

Stock solution 1-6	10ml each
Stock solution 7-10	1ml each

Make up to 1 liter with distilled or deionised water plus 50ml soil extract supernatant.

Diatom Medium (DM)

Stock solutions:	**Per 200ml**
1. $Ca(NO_3).4H_2O$	4.0g
2. KH_2PO_4	2.48g
3. $MgSO_4.7H_2O$	5.0g
4.$NaHCO_3$	3.18g
5. EDTA FeNa	0.45g
EDTA Na_2	0.45g
6. H_3BO_3	0.496g
$MnCl_2.4H_2O$	0.278g
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.2g
7. Cyanocobalamin(VitaminB_{12})	0.008g
Thiamine HCl (Vitamin B_1)	0.008g
Biotin	0.008g
8. $Na_2SiO_3.9H_2O$	11.4g
Final solution:	
Stock solution 1-8	1.0 ml each

Make up to 1 liter with glass distilled water.
Adjust pH to 6.9 with HCl.

MWC (Modified WC medium)

Stock solutions:	**Per liter**
1. $CaCl_2.2H_2O$	36.80g
2. $MgSO_4.7H_2O$	37.00g
3. $NaHCO_3$	12.60g
4. $K_2HPO_4.3H_2O$	11.40g
5. $NaNO_3$	85.00g
6. $Na_2O_3Si.5H_2O$	21.20g
7. trace elements	
EDTA Na_2	4.36g
$FeCl_3.6H_2O$	3.15g
$CuSO_4.5H_2O$	0.01g
$ZnSO_4.7H_2O$	0.022g
$CoCl_2.6H_2O$	0.01g
$MnCl_2.4H_2O$	0.18g
$Na_2MoO_4.2H_2O$	0.006g
H_3BO_3	1.00g
8. Thiamine HCl	0.1g
Biotin	0.0005g
Cyanocobalamin	0.0005g
Final solution	**per liter**
Stock solution 1-8	1.0ml each
Dry buffer-TES	0.115g

Combine stock solution 1-8 and the dry buffer and make up to 1 liter with deionized water.
Autoclave at 15psi for 15 min.