Removal of cadmium from polluted water by immobilized algae

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by

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A thesis submitted for the degree of Doctor of Philosophy in the University of Durham

Department of Biological Sciences

September 1990

This thesis is entirely the result of my own work. It has not been accepted for any other degree and is not being submitted for any other degree.

ABSTRACT

A feasibility study was planned to determine the efficiency of immobilized algal cells growing in a packed bed for removing Cd from commercial effluents. To select appropriate material for an immobilized cell system, twenty five strains of algae isolated from heavy-metal contaminated environments of known water chemistry were tested for their ability to accumulate Cd. Before accumulation experiments were initiated, ion exchange resin was employed to demonstrate that EDTA in the medium did not complex Cd to a significant degree. Synechococcus D562 cells subcultured in Cd accumulated the most metal; little was bound to the cell wall. A continuous culture of steady-state Synechococcus D562 cells tolerated a lower maximum concentration of metal (3.4 mg l^{-1} Cd) than batch-cultured cells (5 mg l^{-1} Cd), indicating that metabolic status influences the toxicity of Cd.

When flasks of calcium-alginate beads were challenged with Cd, up to 60 % of the added metal was bound within 16 h; however, further incubation did not reduce the pollutant concentration. Two axenic strains which accumulated the metal to a high concentration were then immobilized and tested for their capacity to remove Cd from the circulating medium. A packed-bed reactor containing Mougeotia D536 cells proved more effective at metal removal than Synechococcus D562, but both species grew to a lower cell density at the effluent end of the column. The medium was then aerated to overcome such growth-limiting conditions, but this treatment inhibited Cd accumulation. Column-immobilized cells reduced Cd levels more effectively than inoculated, alginate beads in stationary flasks or free cells.

Energy dispersive X-ray microanalysis located Cd only in particular Synechococcus D562 polyphosphate bodies (those with a high Ca to K ratio); peaks for Zn, Pb, Fe, Mn and Ba were also detected in algae isolated from the field. Scanning proton microanalysis provided information on the distribution of macro- and micro-elements throughout the two strains of cyanobacteria and two strains of algae selected from the Durham Culture Collection and demonstrated the presence of Cd in Klebsormidium rivulare D537.

Detergent-sensitive spheroplasts of Synechococcus D562 were produced by lysozyme and protease digestion, but were not viable for growth. To observe the extracellular mucilage of this strain by EM, lysozyme digestion proved imperative for effective ruthenium red staining to convert the material into an electron opaque material. From cultures of Synechococcus D562 grown with or without Cd a 14 kD plasmid was isolated, which contained two Eco RI, two Bam HI and five Hind III restriction sites. A radiolabelled oligonucleotide probe based on part of the nucleotide sequence of a metallothionein from Synechococcus PCC 6301 did not bind to a genomic and plasmid blot of Synechococcus D562 DNA.

The putative Cd-binding peptides ($(\gamma EC)_nG$'s) that were discovered only bound significant quantities of the metal when cells were exposed to 6.17 mg l^{-1} Cd for 2 days at the end of their log-growth phase. Indigenous peptides failed to bind substantial amounts of the metal and the presence of Cd throughout growth did not influence the quantity of chelated Cd, except for Mougeotia D536. The pH of half displacement for $(\gamma EC)_nG$'s from this strain is comparable with that of other species. Reversed-phase HPLC of the peptides from Mougeotia D536 generated a thiol profile similar to that recorded for the Cd-binding peptides of Datura innoxia.

The Cd-induced ultrastructural distortions that were recorded include potential Ca / P / Cd precipitates in Mougeotia D536, the loss of polyglucoside granules from Calothrix D184 together with a relaxation of its thylakoid packing and a lack of plastoglobuli in Cd-exposed Klebsormidium D537. The space between an immobilized cell and the matrix either represents shrinkage of the matrix during dehydration or mucilage which does not bind electron dense stains.

Release of alkaline phosphatase into the medium by Synechococcus D562, provided suitable material to study the inhibitory effects of Cd upon P hydrolysis. Ultrafiltration membranes proved effective as initial step towards enzyme purification and for the determination of activity under sub-optimal pH conditions. At pH 7.0, the activity of an enzyme concentrate was inhibited when 1 and 10 mg l^{-1} Cd were added to the assay medium, but the presence of this metal in the growth medium did not reduce activity. One-dimensional SDS PAGE revealed only one protein difference between strains grown with or without Cd; a reduction in the staining intensity of a 17 kD band of Calothrix D184.

ABBREVIATIONS

^{o} C	degrees Celsius
t	tonne
g	gramme
mg	milligramme
µg	microgramme
Mr	molecular weight
kD	kilo Dalton
kb	kilobase
l	litre
ml	millilitre
v/v	volume for volume
w/v	weight for volume
ppm	parts per million
cm	centimetre
µm	micrometre
nm	nanometre
yr	year
d	day
h	hour
min	minute
s	second
M	molar
mM	millimolar
µM	micromolar
Cd	cadmium
P	phosphorus
PP	polyphosphate
MT	metallothionein
$(\gamma EC)_nG$	poly(gamma-glutamylcysteinyl)glycine
PC	phytochelatin
APA	alkaline phosphatase activity
PME	phosphomonoesterase
PDE	phosphodiesterase
PAR	photosynthetically active radiation
x	mean
sem	standard error of the mean
log	logarithmic
BSA	bovine serum albumin
BSO	buthionine sulphoximine
DMGA	dimethyl glutaric acid
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
NAG	N-acetyl glucosamine
NAM	N-acetyl muramic acid
NTA	nitrilotriacetic acid
PIPES	piperazine-N,N'-bis (2-ethane-sulphonic acid)
SDS	sodium dodecyl sulphate (= sodium lauryl sulphate)
TRIS	tris (hydroxymethyl) methylamine
EDXMA	energy dispersive X-ray microanalysis
EM	electron microscopy
FAAS	flame atomic absorption spectrophotometry
HPLC	high performance liquid chromatography
TEM	transmission electron microscopy
SEM	scanning electron microscopy
SPM	scanning proton microanalysis
EC	European Community

ACKNOWLEDGEMENTS

Research facilities in the Department of Biological Sciences were made available by Professor D. Boulter and Northern Environmental Consultants Ltd provided the finance for this venture. Drs B. A. Whitton and P. J. Say supervised the project, proffering advice culled from many years of work in the field of heavy-metal accumulation.

My sincere thanks go to Mr J. W. Simon and Mr J. Gilroy for countless hours of useful discussion, providing a whole spectrum of apparatus and readily passing on valuable experience. Notable laboratory colleagues who maintained an effective working atmosphere, include Drs G. Davies, S. L. J. Grainger, Mr J. Hutchinson, and Dr R. Islam. Dr G. R. W. Hawley's contribution is of particular note in the humour, encouragement and sense of perspective provided at timely interludes throughout the period of study.

To Dr N. Harris, Mr A. Peat and Mr N. Powell, I wish to extend my appreciation of their advice on electron microscopy, whilst Dr R. W. Banks and Mrs A. C. Richardson were always willing to indulge in extensive EDXMA discussion without notice. Professor A. J. Brook kindly organised the submission of samples to the Oxford Scanning Proton Microanalysis Unit, where scans of cyanobacteria and algae were performed by Dr G. W. Grime. Productive advice on the subject of ion exchange resin was supplied by Dr D. Parker. In addition, Dr N. J. Robinson, Mr W. Lindsay and Mr A. Gupta provided practical assistance and erudite discussion on MT genes and Cd binding peptides.

Help with DNA manipulation was advanced by Drs G. Williams and C. Scutt. The subculture and determination of the concentration of Cd accumulated by strains isolated from the field was executed by Mr J. Hutchinson, whilst FAAS problem solving counsel, was readily given by Mr R. Coult and Mrs J. Dostal. Photographs of apparatus were promptly taken by Mr P. Sidney who also parted with constructive experience on darkroom matters.

This work would never have evolved without the continuous financial and moral support from my father, mother and sister, to them I owe this investment in time and education.

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CHAPTER 1

INTRODUCTION

1.1 General introduction

The geological association of Cd with Zn was not only responsible for its discovery (in zinc carbonate deposits), but also forms the main source of extraction and environmental pollution. Because of useful electrophysical properties, this metal is extracted from mineral deposits (at a zero oxidation state) for its inclusion in many industrial commodities (Table 1.1). When these products reach the end of their life cycle and are discarded, the Cd may be released in a more concentrated form and at a higher oxidation level. As this metal is capable of replacing biologically essential ions without retaining their function and exerts such an effect at low concentrations, cell toxicity and environmental pollution quickly become a reality (Nriagu, 1980).

As a corollary of such metal redistribution and change in valency, the maximum permissible levels of ingestion set by the World Health Organisation of 400 - 500 μg Cd per person per week are being approached in some member countries (drinking water limits = 5 $\mu g\ l^{-1}$ Cd and sea food = 0.2 $\mu g\ l^{-1}$ Cd). Consequently a proposal has been formulated by the EC (to be brought into effect by 30 June 1991) which bans the use of Cd in coloured and surface coated products that come into contact with man, encourages the recycling of Cd containing articles and initiates a drive to find alternatives for Cd loaded pigments and stabilisers. It also recommends the monitoring of industrial emissions, soil Cd content and the amount of this metal employed in fertilisers (European Council Directive, 1989).

In response to the impending legislation designed to tackle the increasing burden of environmental Cd and the inevitable demand for metal-scavenging technology (either to treat aquatic habitats which are already loaded with the metal, or integrated into the industrial effluent stream of processes which cannot withstand a Cd substitute), this research project was designed to establish the viability of deploying immobilized algal cells for the removal of Cd from polluted water.

1.11 Environmental sources

A knowledge of the background levels of Cd at various locations (in conjunction with toxicity tests) provides a bench mark against which the extent of industrial pollution can be measured. One tonne of the earths crust yields about 0.5 g Cd, igneous rock containing 0.2 ppm, limestone 0.035 ppm and soil typically 0.66 ppm. This metal occurs naturally as greenockite or is associated with lead and sulphide ores, with an abundance of 1/350 th of Zn. These minerals are liable to gradual weathering and the action of sulphur bacteria, resulting in a natural, low-level release of Cd into the environment (Chadwick, 1976). Volcanic action is the major source of atmospheric discharge, occurring as a combination of particulate matter and the condensation of vaporised Cd (Mount Etna released 2.8×10^{-2} t d^{-1}). Contributions from wind blown desert dusts, sea sprays and forest fires are relatively insignificant. Apart from Carboniferous black shales, the weathering of crustal material does not normally lead to considerably elevated levels of soluble Cd (Hutton, 1982). Therefore, in most environments natural levels of this metal do not pose a toxic risk.

1.12 Industrial sources

Although currently employed in a wide variety of articles, it took 60 yr from discovery before Cd was commercially exploited, the first large scale application being for paint pigments. The evolution of new production methods prompted the incorporation of Cd in low-melting alloys, electroplating, glass making, photography, dying, calico printing and as a chemical reagent. Today, the metal finds applications in automobile sleeve bearings, aircraft and marine engines, fire detector units, sprinkler systems, photography halides, pigments, storage batteries, paints, plastics, TV tubes, nuclear reactor control rods, radiation devices and photoconductive elements (Nriagu, 1980 ; Hutton, 1982).

Less than 25 % of the Cd currently consumed by industry is recycled (Table 1.1). Because the metal is present in so many materials, the repercussion of this low recovery figure is that Cd accumulates in the environment, a process which in some regions project this metal onto the British Red List of Pollutants (Anon., 1988).

1.13 Environmental pollution

The industrial release of Cd into the environment pervades all of the main environmental compartments (Table 1.2), which if left unchecked will disrupt the species balance of the immediate habitat and migrate up the food chain until the metal is concentrated to toxic levels. Properly constructed landfill sites represent the best form of damage limitation, but metal inputs to water and the atmosphere are very difficult to contain.

The majority of the waste Cd, is a by-product of non-ferrous metal smelting. The solid wastes from iron and steel manufacture (200 t yr^{-1}) are not generally recycled and have to be disposed of in landfill sites. By the year 2000 it is estimated that about 400 t yr^{-1} of Cd will be released in the form of solid wastes and atmospheric emissions (Hutton, 1982).

Depending on it's origin, coal contains 0.02 - 2.0 μg Cd g^{-1}, which is volatilised when burnt (condensing on small fly ash particles) and is mainly deposited in landfill sites and ash ponds. Natural gas has negligible Cd content (future coal gasification will incorporate a cleaning process) and crude oil contains about 0.05 μg g^{-1}. During refuse disposal, scrap metal, plastics, stabilisers and pigments are the main sources of metal release. Waste incineration results in a relatively high level of emission : 0.7 - 4.4 g Cd t^{-1} of waste, resulting in an atmospheric burden of 1.5 g Cd t^{-1}; the Cd derived from burnt refuse is predominantly associated with submicron debris which is least effectively retained by particle control devices. It is assumed that Cd discharges from refuse disposal will remain constant over the next 10 yr due to restrictive legislation on incinerator emissions and the control of Cd deposition onto plated articles and plastics.

The production of phosphoric acid for use in fertilisers releases Cd from one of the starting materials, gypsum (calcium sulphate). This results in a total EC discharge in 1980 of 34 t to landfill and 62 t to water, although the fabrication of phosphate fertilisers is not expected to rise significantly over the next decade (Hutton, 1982).

Therefore, Cd pollution is a widespread problem, whose presence in a number of different environments requires a variety of solutions.

1.14 Implications for human health

A high priority is assigned to a particular pollution challenge when humans begin to act as the biomonitor for toxic concentrations of a particular substance; before this stage, polluted environments often remain unmonitored.

Upon exposure to this metal via contaminated food, ones place of work, drinking water, or the atmosphere (Table 1.3), Cd is absorbed across the lungs or alimentary canal and transported throughout the body as a component of blood cells (erythrocyte MT binds the metal at about 1 μg 100 ml $^{-1}$ blood). Selective accumulation then occurs in the liver which supports 40 - 80 % of the body burden (largely bound to intracellular metallothionein with a half life of 10 to 50 yr, 5 times per unit weight more than other body tissues) and the kidney renal cortex tubules (50 times per unit weight). Cell damage may arise if the bound Cd exceeds 200 $\mu g\ g^{-1}$ net weight cortex, followed by an increase in urine Cd and associated proteinuria (characterised by the excretion of low Mr proteins e.g. β_2 microglobulin). Aminoaciduria, phosphaturia and glucosuria will result if the metal exposure continues. The bone, central nervous system and circulating blood lipids can also be affected by Cd exposure, but lesions in these organs occur less frequently than in the kidneys (Lauwerys, 1979).

Both mammary and placental tissue accumulate Cd to 10 times that of the surrounding maternal cells in order to protect offspring, resulting in foetal blood with a 30 - 50 % lower Cd content (Nriagu, 1980 ; Cherian & Goyer, 1978). Individuals with moderate Fe deficiency have significantly higher Cd adsorption rates than subjects with normal iron stores (Hutton, 1987a). The amount of ingested Cd required to induce elevated urinary β_2 microglobulin excretion (an indicator of Cd toxicity) in 10 % of the population (average body weight of 70 kg) over a 50 yr period is 150 $\mu g\ d^{-1}$ (the levels of Cd adsorbed by inhabitants of the EC are 2 - 8 times lower). As long as Cd-exposed workers do not accumulate more than 15 μg Cd g^{-1} creatine, sufficient binding sites within the body remain unsaturated and kidney lesions should not develop (Hutton, 1982 ; Hutton, 1987b ; Lauwerys et al., 1979).

The symptoms of Cd toxicity may arise after an acute experience (high intake level over a short period) which is characterised by vomiting, myalgia, osteomalacia, liver, kidney and respiratory tract damage or during chronic, occupational exposure (long term challenge at lower levels) expressed as fatigue, dental caries and low blood haemoglobin levels.

The Cd-induced testicular damage observed in mammalian germinal epithelium can be prevented by an administration of Zn, oestrogen, thiol compounds, cysteine, Se, Co or British anti-Lewisite. Above about 320 mg (the sublethal dose for Cd ingestion) individuals experience shock and collapse, followed by death within 24 h to 2 weeks (Nriagu, 1980).

The ubiquitous nature of Cd-containing products and low lethal doses, coupled with the toxic effects outlined above, demand that Cd is not released back into the environment and allowed to accumulate to concentrations at which health problems arise.

Table 1.1 Industrial applications of Cd and associated methods of disposal. The waste disposal pathway is estimated to receive a total of 2 643 t Cd yr^{-1} (Nriagu, 1980 ; Hutton, 1982).

Usage	Products	Disposal of waste Cd
35 %	electroplating of steel, iron, copper and brass for corrosion resistance	10% lost to aqueous wastes, 50 % of which delivered to the sewage system and 50 % landfilled. 40 % of product Cd enters the scrap steel cycle and 60 % is landfilled.
25 %	pigments, widely employed in plastics, ceramics, paints, coated fabrics, textiles, rubber, glass, enamels and printing inks	no significant environmental pollution; all the Cd ultimately enters the waste disposal pathway.
15 %	plastics stabilisers used in non-food grade flexible PVC	landfilled or enters the sewage system.
15 %	NiCd rechargeable batteries	50 % recycled; the remainder is sent to the sewage treatment plant.
10 %	miscellaneous use in alloys, chemicals, nuclear reactor engineering and electronics industry	

Table 1.2 Concentrations of Cd recorded in a number of environmental compartments throughout the EC (Nriagu, 1980 ; Vymazal, 1987)

Compartment	Cd concentration
Lead refinery	0.5 $\mu g\ m^{-3}$
Urban atmosphere	0.002 - 0.53 $\mu g\ m^{-3}$ - deposited at 3.9 - 29.6 $g\ ha^{-1}\ yr^{-1}$
Rural atmosphere	0.001 - 0.003 $\mu g\ m^{-3}$ - deposited at 82 - 150 $g\ ha^{-1}\ yr^{-1}$
Ocean surface water	4 - 70 $ng\ l^{-1}$
Rain water	0.2 - 2.8 $\mu g\ l^{-1}$
Sediments	1 $\mu g\ g^{-1}$ - sediment / water interface important for metal mobilisation
Street dust	4.6 $\mu g\ g^{-1}$ - abrasion of motor tyres
Household dust	10 $\mu g\ g^{-1}$ - from the rubber backing of carpets
Agricultural soils	0.1 - 2.0 $\mu g\ g^{-1}$
Phosphate fertilisers	1.6 - 9.4 $g\ ha^{-1}$
Food plants	0.05 - 0.2 $\mu g\ g^{-1}$ - leafy vegetables accumulate more Cd

Table 1.3 Concentrations of Cd in a variety of substances consumed by humans and the amount of metal absorbed from these sources (Hutton, 1982 ; Friberg et al., 1971)

Source	Cd content / intake	Cd absorbed
Dietary	25 - 60 $\mu g\ d^{-1}$	1.5 – 3.6 $\mu g\ d^{-1}$
Cereals	0.02 $\mu g\ g^{-1}$	0.3 $\mu g\ d^{-1}$
Vegetables	0.09 $\mu g\ g^{-1}$	2.7 $\mu g\ d^{-1}$
Japanese rice *	1 $\mu g\ g^{-1}$	30 $\mu g\ d^{-1}$
Drinking water	< 0.5 $\mu g\ l^{-1}$	< 1 $\mu g\ d^{-1}$
Inhalation	0.5 $\mu g\ d^{-1}$	0.125 - 0.25 $\mu g\ d^{-1}$
Smoking (20 d^{-1})	2 - 4 μg	0.8 - 1.4 $\mu g\ d^{-1}$
Dust and soil	0.4 - 1 $\mu g\ g^{-1}$	20 $\mu g\ d^{-1}$

* this particular food source was heavily contaminated by Cd from industrial effluents

1.2 Metal chemistry

The toxicity of a particular metal is governed by its electron distribution, local pH, prevailing redox potential, available ligands and metal competition.

1.21 Electron distribution

Heavy metals can be classified in terms of their co-ordination chemistry, a property which is dictated by the element's electron environment (Beveridge & Doyle, 1989 ; Collins & Stotzky, 1990) :

1. Class A (hard acids) - oxygen seeking species which are biologically essential eg. K^+, Na^+, Mg^{2+} and Ca^{2+}.
2. Class B (soft acids) - bind to sulphur, are mainly non-essential and very toxic eg. Cd^{2+}, Cu^{2+}, Hg^{2+} and Pb^{2+}.
3. Borderline ions such as Fe^{2+}, Fe^{3+}, and Mn^{2+} with biological roles as well as toxic properties

The individual characteristics exhibited by an element are influenced by :

a) the charge to radius ratio : strongly polarized (high charge density) metal ions retain a potent ligand affinity, whilst the low charge-density alkali metals do not bind well.

b) the number of electrons : ions with multiple electron shells (soft cations), eg. Cd ($4d^{10}$), are more easily polarized and form stable complexes with soft, biochemically active bases such as thiol groups. Hard cations are not easily polarized, (although they have a higher affinity for oxygen) and are readily displaced from their binding site by competing soft cations.

c) Zn, Cu and Cd exhibit partly filled orbitals and bind ligands more strongly than the s-block elements.

Once bound, the biological function of a metal is related to its electron activity; Zn^{2+} is a strong Lewis acid (has at least one vacant orbital and accepts electron pairs from ligands) and enhances the reactive potential of the co-ordinated molecule via the process of ligand-to-metal sigma bonding and metal-to-peripheral pi back-bonding. These bonds either enhance hydrolysis via polarization of a peptide group or deprotonation of a water molecule, acting as an effective nucleophile towards the substrate. In contrast, mild base centres require a weak Lewis acid

(eg. Mg^{2+}) for hydrolytic and phosphate transfer enzymes (Hughes & Poole, 1989a). Since biochemical activity is ion sensitive, the intrusion of a foreign metal with a different electron environment will prohibit many biological processes. The intracellular toxicity assigned to Cd is an example of such invasion (Hughes & Poole, 1989b).

1.22 Influence of pH

The pH of an organisms environment controls the amount of metal bound and therefore regulates the damage incurred by cell metabolism :

1. Solubility : Cd toxicity declines when the pH increases, because insoluble oxides and hydroxides predominate (Hem, 1972). Non-cytotoxic $CdOH^+$ complexes are initiated at pH 7.0 - 7.5 and peak at pH 8.2 - 9.0, whilst the formation of $Cd(OH)_2$ begins at pH 9.0 and attains a maximum at pH 11.0. Soluble $Cd(OH)_3^-$ and $Cd(OH)_4^-$ forms also exist, but only at non-environmental pH values (Babich & Stotzky, 1980).
2. Cell-surface ligand competition : a high proton concentration will saturate cell-surface anionic groups, reduce the number of available metal-binding sites and lower the toxicity of Cd. The strength of this effect depends upon the pK values of the functional groups involved (Crist et al., 1981 ; Peterson et al., 1984 ; Peterson & Healey, 1985).
3. Transmembrane electrical gradient : as the local pH drops, membrane proton pumps respond to maintain an internal negative charge, eventually leading to membrane depolarisation and a change in the metal affinity of the cell surface (Campbell & Stokes, 1985).

1.23 Redox potential

As the redox potential (Eh) of an organisms habitat is lowered, metal ions undergo valence shifts with a concurrent change in toxicity. For example, Cr^{3+} is not toxic to Salmonella typhimurium but with a change in Eh the toxic and mutagenic Cr^{6+} predominates. Whereas anaerobic environments may convert SO_4^{2-} to (S^{2-}) providing appropriate conditions for heavy-metal precipitation and thus a reduction in toxicity (Babich & Stotzky, 1980).

1.24 Metal / ligand interaction

When Cd-binding ligands are present, the metal's toxicity declines as the pool of free, reactive, divalent Cd is reduced :

a) Inorganic ligands : in natural waters Cd may be bound to suspended (> 1 μm) and colloid particles (< 1 μm), consisting of individual or mixed hydroxides, oxides, silicates, sulphides and chlorides. Chloride complexes are one of the most mobile and persistent agents, thus sea water provides some capacity for metal detoxification (Babich & Stotzky, 1980 ; Ahlf, 1988a).

b) Chemical ligands : phosphate and cyanide must be present at a high concentration to complex Cd, similarly very high levels of carboxylic and hydrocarboxylic acids would be required to bind the metal (Gardiner, 1974 ; Frevert, 1987).

c) Sewage effluent load : primary mechanical settling-treatment removes metals that are insoluble or adsorbed onto sewage particles, whilst the activated sludge process traps metals in sediment flocs or adsorbs them onto bacterial extracellular polymers (Aiking et al., 1982). But any microbial oxidation of these polymers will then result in a release of the metal (Brown & Lester, 1979).

d) Organic ligands : Low Mr humic and fulvic acids are the most active fractions as they contain polyelectrolytes i.e. COOH, C = O, phenolic, aliphatic and OH groups which bind considerable amounts of metal. These ligands exist in natural waters ranging from 0.1 (non-bioproductive fresh and sea-water) to 10 mg l^{-1} (lakes rivers, estuaries and moorland water). As Ca^{2+} and Mg^{2+} are strong competitors for organic ligands, Cd is only significantly complexed by the carbon ligands of soft water. Sorption may occur via co-ordination bonding (chelation), covalent attachment (SH groups), or cation exchange (humic acid). The two amino acids which combine with metal ions most strongly are histidine and cysteine. Mid carbon carboxyl, imidazole and thiol groups have a stronger affinity for ions than the terminal COO^- and NH_3^+ of amino acids; the overall charge on a protein also influences how much metal is bound (Gurd & Wilcox, 1956). However, concentrations of amino acids as high as 100 μg l^{-1} do not significantly interact with trace metals (Stumm & Morgan, 1981 ; Gadd & Griffiths, 1978).

e) Clays and soil : exhibit a range of cation exchange capacities depending on the number and type of free anionic groups available (Babich & Stotzky, 1977a ; 1977b).

f) Synthetic chelating agents : Both EDTA (non-biodegradable) and NTA are employed in commercial detergents to prevent the decomposition of perborate - 60 d are required for the disunion of a Cd-NTA complex (Nriagu, 1980 ; Rai et al., 1981 ; Stokes, 1983).

1.25 Metal competition

The toxic effects of Cd are further diluted when ions of similar physical profiles compete for binding sites. Zn was found to reduce Cd toxicity for Euglena gracilis (Falchuck et. al., 1975a) and Coelastrum proboscideum, except that Zn did not protect the cells from Cd toxicity when grown in a light : dark cycle; whilst a low level of Cd (0.33 mg l^{-1}) actually stimulated growth (Müller & Payer, 1980).

Increasing the concentration of Ca^{2+} in the medium thirty times, provided competitive alleviation of Cd toxicity for Chlorella pyrenoidosa, as both elements have similar ionic radii (Ca^{2+} = 99 pm, Cd^{2+} = 97 pm). The presence of 6 mg l^{-1} Fe at pH 8.0 co-precipitated the added Cd as ferric oxide, thus reducing metal toxicity. Fe may also protect the mitochondrial and chloroplast cytochrome systems as well as inhibiting Cd entry into the cells. The addition of Mn at 0.5 mg l^{-1} appeared to result in a slight rise in Cd toxicity and the effect of 1 mg l^{-1} Zn, whilst toxic in itself, was additive to that of Cd (Gipps & Coller, 1982). The repression of nitrate assimilation by Cd in Anacystis nidulans is probably due to the inhibition of ATP dependent glutamine synthetase. Whilst the restoration of nitrate uptake rates by Ca^{2+} and Zn^{2+} may either be assigned to transport site competition or intracellular detoxification (Singh & Yadava, 1983 ; Singh & Yadava, 1984).

When the euryhaline alga Dunaliella salina was exposed to increasing levels of sodium, the maximal uptake of 5 mg l^{-1} Cd occurred at 0.5 to 1.0 M Na and declined at higher salt concentrations up to 4 .0 M Na (Rebhun & Ben-Amotz, 1986).

Variations in the results of ionic competition from one species to another, probably reflects differences in their mechanisms of Cd uptake and storage.

1.26 Media ligands

Ionic algal media provide few chemical groups to which divalent Cd may bind, whilst more complex bacterial substrates contain ligands that significantly reduce the toxicity of added heavy-metals. Differential pulse

polarography, electronic absorption spectroscopy and electron spin resonance were employed to observe the effects of different media upon FeEDTA and Cu^{2+} availability. Cu^{2+} appeared to be chelated by growth media at a concentration of 1 % (v/v), whilst a reactive FeEDTA complex persisted in up to 90 % (v/v) media, indicating that anionic ligands predominate (Bird et al., 1985). When bacterial growth media were exposed to 20 ppm of a metal mixture, less than 0.80 ppm of Hg^{2+}, Pb^{2+} and Cu^{2+} remained as free cations (detected with ion specific electrodes), whilst Cd^{2+} proved more resistant to chelation. Media with a casamino acid content exhibited the highest binding activity (Ramamoorthy & Kushner, 1975).

Media components which bind metals and therefore reduce the level of toxic cations are equivalent to the many different ligands present in the environment, which preclude ions from exerting a poisonous influence.

1.3 Uptake of Cd

Microorganisms display a variety of metal-binding sites which can be roughly categorised with respect to their position relative to the cell wall. Extracellular ligands provide metal precipitation points or bind individual ions without calling upon cell transport mechanisms. In contrast, Cd that is bound by intracellular groups must cross the cell wall and plasma membrane (mainly via ion transport proteins). Such a process consumes cell energy and increases the toxicity of the metal.

1.31 Adsorption of Cd by bacterial cell-walls

Due to a heterogeneity in composition, the complexing capacity of bacterial cell walls depends on the species concerned. Gram-negative bacteria have a lower surface charge density than Gram-positive cells, but possess a more intricate wall which binds a variety of metals and thus provides more protection. In general, actinomycetes are more tolerant to metals which have been added to the medium than eubacteria, among which the gram positives are more sensitive (Hughes & Poole, 1989a).

The cell wall of Bacillus subtilis binds a variety of ions (Pb (II) 0.02 to Sc (III) 10.99 mol mg dw^{-1}) and those whose atomic number is greater than 11 were visualised under the TEM (Beveridge & Murray, 1976). Lysozyme degradation of the cell wall diminished Mg^{2+} adsorption, but not that of Ca^{2+}, Fe^{3+} or Ni^{2+}, indicating that the peptidoglycan layer contributes specifically to Mg^{2+} binding. Areas of the wall also acted as nucleation sites for

the growth of small Au crystals. The teichoic acid phosphodiester groups, peptidoglycan carboxyl regions and to a lesser extent the sugar hydroxyls of the cell wall provided metal binding sites, but no simple correlation was observed between the ionic radii, heats of hydration or number of hydration shells of the metals and the amount bound.

Characterisation of the type and number of bonds that metals form at a cell surface, permits calculations of the amount of cell material necessary for efficient metal removal and provides the information required to enhance uptake activity. The carboxyl groups of glutamic acid possess a high degree of rotational freedom (a feature which permits nucleation growth) and are probably more accessible than teichoic-acid radicals (which prove to be potent Mg^{2+} chelators). Monovalent ions require one acid group and divalent cations two teichoic acid residues for ligand formation (Beveridge & Murray, 1980).

The modern molecular-imaging techniques of electron-nuclear double resonance and electron spin-echo envelope modulations were used to formulate a tentative model of Cu-binding to the cell wall histidine of Klebsiella pneumoniae. Of the two imidazole nitrogens, only the sp^2 hybridized N acts as a donor site to which the Cu^{2+} is co-ordinated bidentally, the remaining H_2O molecules occupy axial and equitorial positions (Möhl et al., 1988).

In addition to cell wall ligands, extracellular material may also display metal binding sites, for example the capsular polysaccharides of K. aerogenes complexed more of the supplemented Cu (54%) than Cd (9%) (Bitton & Freihoffer, 1978). These workers then showed that a non-capsulated, metal-sensitive strain could be afforded Cu protection by the addition of this metal chelating polysaccharide to the medium.

An example of cell-surface modification for improving the amount of metal accumulated is provided by extracellular material from Arthrobacter viscosus (Scott et al., 1988). When treated with formaldehyde, an increase in the amount of adsorbed Cd was recorded, probably due to a suppression of the positive surface charges which would repel any metallic cation.

1.32 Adsorption of Cd by algal cell-walls

Less attention has been spent on the surface complexation of metals by photosynthetic microorganisms compared with that expended upon bacteria, even though algae often predominate metal contaminated environments.

The heterogeneity of heavy-metal binding sites present on the cell walls of Chlamydomonas reinhardtii was demonstrated by Xue et al. (1988). An excessive concentration of metals in the medium reduced the rate of surface

complex formation as the high affinity binding sites became saturated first, forcing ions to bind to lower affinity groups, with each binding site contributing a different Langmuir isotherm.

An acidic heteropolysaccharide component of the extracellular sheath of Gloeothece was found to bind up to 105 μg of Cd mg^{-1} of capsule and that cells grown with $NaNO_3$ bound more Cd than N_2-fixing cultures, probably due to an increase in the number of sheath binding sites (Tease & Walker, 1987). Cu^{2+}, Zn^{2+} and Cd^{2+} bound rapidly to the sheath forming Chroococcus paris, with 90 % of the added metal complexed in 1 min. Further significant uptake occurred at a slower rate and nearly all the bound metal was found to be EDTA extractable (Les & Walker, 1983).

Commercial attention has been drawn to Chlorella vulgaris which accumulates gold (I) and (II) from aqueous solutions; this metal is then slowly reduced to gold (0) (Greene et al., 1986). Tetrachloroaurate (III) and gold (I) sodium thiomalate were rapidly adsorbed over a high pH range, whilst dicyanoaurate (I) bound more slowly and in a highly pH-dependent manner, similar to the binding-site blockage exhibited by β-mercaptoethanol, cyanide and thiourea.

Algal cells which are killed by heat treatment often demonstrate an increase in their cell wall cation exchange capacity as more binding sites become exposed during heat shock. Dead Chlorella regularis cells adsorbed more Cd than living cells, whilst Na^{+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} tended to retard Cd^{2+} uptake (Sakaguchi et al., 1979). The metabolic inhibitors sodium azide and dinitrophenol had little influence on metal adsorption, as the majority of the complexed metal was extracellular.

An energy-independent Langmuir isotherm described Cd uptake by Stichococcus bacillaris ; 80 % of the added Cd was removed from the medium (Skowronski, 1984) ; dead cells adsorbed more metal, probably due to extra binding sites being exposed during the killing process. The addition of ammonium ions reduced Cd toxicity by interfering with the surface adsorption and transport of Cd (Skowronski, 1986).

Unlike conventional ion exchange resins, the cell wall of Chlorella vulgaris (Greene et al., 1987) provides hard and soft metal binding sites with little affinity for Mg^{2+} and Ca^{2+} (and is thus insensitive to hard water). Electrostatic binding ligands that are present on cyanobacterial and algal cell walls (carboxylate, carbonyl, hydroxyl, amine, imidazole, phosphate, thiol and thioester groups) may be divided into three groups :

class 1 : metals are tightly bound at pH ≥ 5.0 but stripped out at pH ≤ 2.0 e.g. Cd^{2+}, Cu^{2+}, Al^{3+}, Zn^{2+}, Ni^{2+} and Co^{2+}, the cell wall ligand is negative but becomes more positively charged as the pH declines.

class 2 : strongly bound at pH ≤ 2.0 and weak / unbound at pH 5.0 e.g. $PtCl_4^{2-}$, CrO_4^{2-} and SeO_4^{2-} - anionic sites which possess a positive charge only at low pH.

class 3 : no discernible pH dependence eg. Ag^{+}, Hg^{2+} and $AuCl_4^{-}$ the most strongly bound of all ions, they form covalent complexes with soft ligands which contain N and S.

Silica immobilized Chlorella pyrenoidosa and Spirulina were used to extract Cr from acidic electroplating waste; using the combination of a pH gradient and 0.5 M β-mercaptoethanol, the selective cell-release of Zn (II), Cu (II), Hg (II) and $AuCl_4$ (III) was attained. Once regenerated, the cells were ready for more metal stripping. Non-living biomass may also possess reductive capacities i.e. Au (III) to Au (0) (Greene et al., 1987).

However, the cell wall of Chlamydomonas reinhardtii was found by Cain and Allen (1980) not to play a role in Cd exclusion, as mutants lacking a cell wall did not accumulate significantly less Cd than wild type cells.

The main advantage of utilising cell walls for metal sequestration is their pH or chemical recyclability and independence of cell energy.

1.33 Precipitation of Cd by algal cell-walls

Cell-wall ligands are finite in number thus limiting the amount of Cd chelated, whilst the process of precipitation produces metal aggregates with many more ions per unit surface area of the cell boundary. By diminishing the oxidation environment of the cell-wall sulphate reductase of Cyanidium caldarium, Ahlf (1988b) enhanced the quantity of Cd precipitated at the cell surface. The precipitation of this metal on the surface of cells has also been recorded in Citrobacter sp. (Macaskie et al., 1987).

1.34 Passive uptake

Few species exhibit little control over the type and quantity of ions that cross into the cytoplasm, but for algae

which do not maintain concentration differentials, metals may accumulate internally without an energy debt. For Phaeodactylum tricornutum the rapid cell-surface adsorption of Hg^{2+} and Zn^{2+} was followed by passive, diffusion-controlled transport at rates proportional to the concentration of cell-surface bound metal following a Langmuir type adsorption isotherm (Davies, 1978). For young cells, the cytoplasmic protein content is high, providing ligands to which the internalised metal bind, but as the cell ages, cytoplasmic protein levels decline and the released metal will move out of cells down the redirected concentration gradient.

1.35 Cd accumulation

Once past the cell wall and plasma membrane, Cd will prove cytotoxic unless it is bound by metal-insensitive cytoplasmic ligands. The amount of heavy metal internalised and efficiency of the intracellular binding mechanisms determine the Cd-tolerance of each strain (Table 1.4).

Although cyanobacteria and algae have proved more sensitive to the toxic effects of Cd than bacteria and fungi (Trevors et al., 1986) the concentrations of internalised Cd and associated concentration factors are not comparable between studies due to the variation in experimental conditions which influences the amount of Cd accumulated.

As most cells do not require Cd for metabolism, no specific membrane transport protein has been discovered which imports the metal. Cell access is attained via an essential ion carrier protein, which usually proves to be the Mn^{2+} import channel. Evidence for this model was provided by Hart et al. (1979) who observed that an increase in the Mn^{2+} concentration of the growth medium of Chlorella pyrenoidosa increased competition for ion-transport sites and therefore inhibited the process of Cd accumulation. This treatment restored the growth rates of Cd-exposed cultures to that of untreated cells.

It has been suggested that the Mn^{2+} active transport system in Cd-tolerant Bacillus subtilis had been altered by chromosomal mutation so that it no longer recognised Cd^{2+} as a Mn^{2+} analogue and thus lower levels of the toxic metal were accumulated (Laddaga et al., 1985 ; Hughes & Poole, 1989a).

Table 1.4 Toxic concentrations of Cd and associated growth responses for a number of cyanobacteria and algae.

Cd conc. ($mg\ l^{-1}$)	response	organism	reference
0.01	ceased growth after 20 - 30 h	Asterionella formosa	Conway & Williams (1979)
0.013	50 % reduction in growth	Anabaena flos-aquae	Rachlin et al. (1984)
0.031	reduced cell population	Scenedesmus quadricauda	Bringmann & Kühn (1980)
0.06	significant growth inhibition	Scenedesmus sp.	Klass et al. (1974)
0.08	complete growth inhibition	Selenastrum capricornutum	Bartlett et al. (1974)
0.10	58 % reduction in growth	Chlorella vulgaris	Hutchinson & Stokes (1975)
0.10	25 % reduction in growth	Haematococcus sp.	Hutchinson & Stokes (1975)
0.4	reduced biomass	Chroococcus paris	Les & Walker (1983)
1 - 10	up to 77 % reduction in growth	Chlamydomonas reinhardtii	Cain & Allen (1980)
1 - 2	inhibition of photosynthesis	Anabaena inaequalis	Stratton & Corke (1979)
2 - 4	nitrogenase activity reduced	Anabaena inaequalis	Stratton & Corke (1979)
4	50 % reduction in NO_3^- uptake	Anacystis nidulans *	Singh & Yadava (1983)
10.12	reduction in growth	Chlorella vulgaris	Den Dooren de Jong (1965)

* = Synechococcus PCC 6301

1.4 Cd removal

Industrial-scale procedures for the eradication of Cd from polluted effluents have been developed along both chemical and biological lines. The factors which influence the choice of a particular system include process cost, lifetime, post-treatment residual effluent and methods of metal disposal.

1.41 Chemical removal

The majority of current waste-Cd disposal procedures involve chemical techniques which do not always reduce metal levels to those required by law. An example of efficient Cd extraction by a chemical approach was the coagulation of the metal with a blend of sodium sulphide (Na_2S) or calcium polysulphide (CaS_4) and iron III chloride ($FeCl_3$) followed by magnetite seeding and high gradient magnetic filtration to remove nearly 100 % of the polluting Cd^{2+} (Terashima et al., 1986).

1.42 Algal removal

An illustration of the *in situ* regulation of metal pollution by biological means is provided by the treatment of Pb-contaminated water at Viburnum Mine, Missouri, U. S. A.. The effluent containing the Pb was passed through a series of shallow ponds about 1 metre deep, favouring the growth of benthic flora that trap particulate and dissolved heavy-metals. These meanders emptied into a final settling pond with baffled weirs to prevent algal overflow. This system proved to be more than 99 % efficient (Hassett et al.,1981 ; Jennett et al., 1979 ; Jennett et al., 1980).

1.5 Immobilized cells

Biocatalysts that are restrained in a matrix exhibit the following advantages : the biomass is easily recovered for recycling, a high cell density is achieved, cell / product separation can be automatic and a resistance to shear is provided. Whilst the problems that arise from restraining cells by entrapment or surface adsorption include nutrient and product diffusion-limitation, localised peripheral growth, cell leakage and decomposition of the matrix.

1.51 Matrices and applications

Because of the benefits outlined above, a number of matrices have been employed (Table 1.5) in a variety of bioprocesses (Table 1.6). Cell immobilization procedures fall into the following categories : matrix adhesion / adsorption, cell cross linking, gel entrapment / encapsulation (beads, plates or fibres) and composite matrices. One of the most popular matrix materials for algal immobilization is alginate, a linear co-polymer of D-mannuronic acid and L-guluronic acid linked by β-1,4 and α-1,4 glucosidic bonds. When two guluronic acid residues are adjacent, they form a binding pocket of four oxygen co-ordination sites (egg box model) for calcium ions,the density and proximity of these groups determines the gels strength and stability; the highest breaking strengths are obtained with alginate composed of multiple guluronic acid polymers (Cheetham et al., 1979 ; Grant et al., 1973).

The properties of a particular matrix are often species dependent, alginate extracted from Laminaria contains many L-guluronic acid blocks, which when gelled by Ca^{2+}, produces a strong matrix, whilst the carbohydrate from Macrocystis is low in L-guluronic acid units and produces weak gels. The water-soluble Na form of alginate can be complexed by Ca^{2+}, Sr^{2+} or Al^{3+} (Cheetham et al., 1979). A thin layer of highly cross-linked alginate occurs on the surface of most gels when a drop of sodium-alginate hits the surface of a $CaCl_2$ solution; the periphery of the sphere is complexed instantly, whilst the inside of the bead is much more porous, as it is formed by a slow diffusion of Ca^{2+} into the bead.

A high concentration of P in the medium competes with the bound Ca^{2+} and results in gel disintegration, this problem can be avoided by substituting Sr^{2+} for Ca^{2+}. The matrix integrity may also be reduced by alginate lyase, an enzyme found in a variety of organisms (Cheetham & Bucke, 1984). The production of gas within immobilization matrices prompted a study on the tensile strength of gels. Alginate was found to be more stress resistant than K-carrageenan and matrices which are gelled internally have different surface layer properties than the bulk phase (Krouwel et al., 1982). Externally gelled matrix fractures never extend entirely to the surface because of a denser outer layer, although the addition of cells will reduce the strength of the matrix.

A comparison of the methods used to cross link matrices revealed that internally gelled cylinders released ten times more cells into the medium during growth than externally complexed beads. However, an increase in the alginate concentration of the cylinder from 2 to 4 % (w/v) reduced cell loss by 50 % (Johansen & Flink, 1986). Regardless of a higher matrix density at their periphery, the CO_2 generated by cell metabolism split the beads in half, but only formed gas pockets in the cylinders.

Table 1.5 Examples of matrices used for cell immobilization and appropriate methods of polymerization

Matrix	polymerization process	reference
Polyacrylamide	chemical cross linking	Cheetham et al. (1979)
Alginate	ionic gelling	Klein et al. (1983)
Polyurethane foams	-	Muallem et al.,1983
k-Carrageenan	cooling plus KCl	Cheetham & Bucke (1984)
Glutaraldehyde	X-linked with 25 % gelatin on glass	Dale et al. (1985)
Formaldehyde	20 % gelatin at 40 ^{o}C, cooled, lyophilized and treated with cold formaldehyde in ethanol	Gianfreda et al. (1980)
Microporous exchange resin	-	Daugulis et al. (1985).
Dialysis tube photobioreactor	-	Vincenzini et al. (1986)
Cellulose triacetate fibres	-	Ghose & Kannan (1978)

Table 1.6 Examples of immobilized cyanobacteria and algae, the matrix employed to retain the cells and associated products

Organism	matrix	product	reference
Anacystis nidulans * & Rhodospirillum rubrum	agar	hydrogen	Weetall & Krampitz (1980)
Chlorella vulgaris & Providencia sp.	agarose	α-keto acid	Wikström et al. (1982)
Chlorella	calcium alginate	P removal	Robinson et al. (1988)
Clostridium butyricum	polyurethane	ammonia	Rao & Hall (1984)
Anabaena azollae	calcium alginate	ammonia	Brouers & Hall (1986)
Mastigocladus laminosus	polyvinyl foams	ammonia	Brouers & Hall (1986)
Scenedesmus acutus & S. obliquus	k-carrageenan	N and P from waste water	Chevalier & de la Noüe (1985)
Bacillus subtilis & Scenedesmus obliquus	calcium alginate	α-amylase	Chevalier & de la Noüe (1988)
Phormidium luridum & Anacystis nidulans *	glutaraldehyde / albumin	cryoprotection	Papageorgio & Lagoyanni (1986)
Dunaliella tertiolecta	alginate	glycerol	Grizeau & Navarro (1986)
Anabaena cylindrica	alginate	ammonia	Jeanfils & Loudeche (1986)
Anabaena variabilis	alginate	ammonia	Kerby et al. (1986)
Anabaena ATCC 27893	alginate	ammonia	Musgrave et al. (1982)
Kluveromyces marxianus	alginate	ethanol	Kierstan & Bucke (1977)
Anabaena N-7363	k-carrageenan	hydrogen alginate & agar	Karube et al. (1986)
Botryococcus braunii	alginate	hydrocarbons	Bailliez et al. (1985)

* = Synechococcus PCC 6301

1.52 Metal removal

The process of cell immobilization has been selected for the removal of heavy-metal pollutants so that the metal laden biomass may be effectively retained. Silica was chosen as an immobilization matrix for Chlorella vulgaris in favour of polyacrylamide (which was prone to fracture) for the removal of UO_2^{2+}, Hg^{2+}, Zn^{2+}, Cu^{2+} and Au^{2+} (Darnall et al., 1986). The product proved stable after 3 months storage and could be recycled thirty times (by a pH gradient or β-mercaptoethanol) without loss of efficiency or hard-ion interference.

Lewis and Kiff (1988) grew Rhizopus arrhizus biomass in polyester foam cubes, transferred them to glass columns and exposed the cells to a variety of metals. From this study, a removal hierarchy was established : $Pb^{2+} > Fe^{3+} > Cu^{2+} > Cd^{2+} > Zn^{2+} > Mn^{2+}$, whilst the addition of Ca^{2+}, Mn^{2+}, Cu^{2+} and Zn^{2+} dramatically reduced Cd^{2+} uptake. The column was regenerated with a 0.1 M HCl wash (which removed 95 % of the bound metal) and could be reused for a maximum of six cycles with retention of up to 85 % of it's activity. The initial removal of Cd was 92 %, but after 24 litres of effluent passage this figure declined to 44 %.

Uranium recovery was effectively demonstrated with polyacrylamide immobilized Streptomyces viridochromogenes and Chlorella in a 7 ml bed volume column, fluxed with sea water. In this study the alga proved more effective in stripping the metal from the challenge flow even though S. viridochromogenes bound more uranium as free cells (Nakajima et al., 1982).

The Cd-binding capacity of extracellular polysaccharides was harnessed by Scott et al. (1986) who immobilized two species of bacteria in a 1.06 x 0.055 m fluidized bed of sand . Arthrobacter viscosus (a strain that excretes polysaccharides) bound more metal than A. globiformis (little extracellular carbohydrate released), although the runs were not axenic.

Probably the most in depth study of metal accumulation by immobilized organisms was that carried out over a number of years by Macaskie and Dean, whose model organism, Citrobacter sp., was selected from a screen of 174 bacterial strains (Macaskie & Dean, 1982). The addition of glycerol 2-phosphate to the medium ensured that Cd adsorption was maximised throughout the growth cycle and not as a discrete peak at mid-exponential phase. Cells at stationary phase tolerated a higher level of Cd exposure and immobilized cells removed 65 % of the added Cd from the medium (Macaskie & Dean, 1984a). It was discovered that a cell surface phosphatase (which continues to function in resting cells) cleaves glycerol 2-phosphate and the leaving P then precipitates Cd from the medium (Macaskie & Dean, 1984b).

After the passage of 1 litre of medium containing 200 mg l^{-1} Cd, activity was lost very slowly and at 5 litres the removal efficiency was still 58 % (Macaskie & Dean, 1984b). At 20 - 30 l elution, activity was lost rapidly with the appearance of large amounts of Cd^{2+} in the effluent. Accumulated Cd was evident as a dense white precipitate in the gel which actually generated a resistance to pumping of the medium. Doubling the volume of the column failed to enhance metal uptake, but linked columns reduced the level of metal to 0.01 mg l^{-1}; still double the maximum permissible concentration for drinking water.

An increase in pregrowth temperature from 20 to 30 ° C increased the removal efficiency by 7 %, this rise in entropy probably enhanced the rate of P cleavage. The cell-bound Cd was then removed from the column with 0.5 M citrate pH 5 (Macaskie & Dean, 1984b).

Stationary phase cells immobilized in shredded cylinders of polyacrylamide (packed into 13 x 1.75 cm columns) retained their activity over several litres of eluate from 1 to 100 μM Cd and the metals in two synthetic effluents were stripped out at an efficiency of 56 - 65 % (Macaskie & Dean, 1984c). Linking three columns together resulted in 99.86% extraction of the added Pb and 99.95% of the supplemented Cd. Unlike Pb and Cd, Cu exhibited concentration sensitivity with a loss in column activity at high Cu concentrations. The solubility of the metal phosphate under test may prove to be the limiting factor in metal extraction. At higher proton concentrations, less Cd was precipitated and 10 mM β-mercaptoethanol or 100 mM Cl^- were found to reduce Cd adsorption, probably due to binding of these molecules to the SH groups at the active site of the enzyme (Macaskie & Dean, 1984a).

For Citrobacter sp. grown in carbon-limited continuous culture and immobilized in polyacrylamide gel the percentage of Cd accumulated declined as the medium flow rate increased (from 100 to 500 ml h^{-1}), fulfilling the predictions of Michaelis-Menten kinetics (an inverse log-relationship between column activity and flow rate) (Macaskie et al., 1986). As a doubling of the biomass load had no effect on metal removal, the enzyme is in excess over the substrate. No temperature effect was recorded from 20 - 40 ° C, but above 45 ° C, enzyme half lives were first order log-decaying up to 48 ° C. Phosphatase activity remained relatively constant from pH 5 - 9, but Cd uptake was dramatically reduced below pH 6.5 due to the solubility of Cd phosphate. The cyanide from electroplating wastes severely curtailed metal accumulation at 5 mM CN^- (equivalent in effect to 250 mM Cl^-) probably due to the extraction of the bound metal from the phosphatase metalloenzyme by CN^-. The addition of equimolar Ca^{2+} and 10 times excess Zn^{2+} did not affect Cd uptake. As polyacrylamide is expensive and toxic, columns of cells adsorbed to glass were tried, with metal-removal activites of up to 80 % in the first 5 litres which

then declined to 70 % after the passage of 16 litres of medium.

Resting cells pre-grown in glycerol-limiting continuous culture accumulated Cd to 40 % of the bacterial dry weight after 5 h exposure to 200 mg l^{-1} (Macaskie et al., 1987). From X-ray microanalysis data, the Cd : P ratio of the cell wall precipitates was found to be 1 : 1 and ^{31}P magnetic angle spinning NMR confirmed that the precipitate was not $Cd_2P_2O_7$ but was probably $CdHPO_4$.

When Citrobacter sp. were immobilized in 60 ml columns of shredded polyacrylamide, 96 % of the challenge Cd was precipitated. This value was enhanced to almost 100 % when three linked columns were employed. Metal-removal activity was invariable up to a total loading of 3 - 4 g Cd^{2+} per column, but after 300 h the columns became blocked by the accumulated Cd^{2+}. Use of the cheaper substrates trimethyl and triethyl phosphate produced oscillating growth indicative of a toxic challenge, therefore these P sources could not be employed to culture the required density and quality of biomass (Michel et al., 1986). Although the alkyl phosphates (10 times cheaper than glycerol 2-phosphate) reduced column activity by a factor of 2.5, Cd precipitation was recovered by the substitution of alkyl P with glycerol 2-phosphate.

When tested for their ability to remove Pb from the eluant, immobilized Citrobacter cells could not precipitate the metal (Macaskie & Dean, 1987b). Glass-helix immobilized biomass removed 87.4 % of the added uranium and polyacrylamide gel 91.6 % (turning the gel opaque with the metal precipitate). The storage of cells before immobilization for 1 week at 4 ^{o} C resulted in enhanced activity. Such a phenomenon can probably be assigned to low-temperature cell decomposition, which exposes UO_2^{2+}-binding ligands (Macaskie & Dean, 1987a). From this extensive investigation of one organism, the precipitation of metals upon a cells surface has proved to be a very effective and regenerative method for the removal of metals from effluent streams.

1.53 Gel stabilization

Under certain conditions (Eikmeier & Rehm, 1987 ; Johansen & Flink, 1986) calcium-alginate gels will dissolve and release their entrapped cells. Such incidents prompted the development of appropriate stabilization procedures. The treatment of alginate beads with $Al(NO_3)_3$ (a trivalent cross-linking agent) (Rochefort et al., 1986) or Ba^{2+} (Dainty et al., 1986) prevents Ca^{2+} displacement when the matrix is incubated in medium containing a high concentrtaion of P.

The choice of chelating ion also influences cell activity. Calcium alginate retained the photosynthetic activity

of Euglena gracilis for at least 2 years, whilst Ba^{2+} formed gels conserved cell viability for a few months and Mn^{2+} partially inhibited the energy capture process (Tamponnet et al., 1988). Micrographs of immobilized cells demonstrated the absence of a direct physical link between cells and their matrix.

Furthermore, chemical treatments (polyethyleneimine treatment, carbodiimide and periodate activation)of a 2 % w/v alginate matrix containing Saccharomyces cerevisiae cells have been devised to enhance gel stability (Birnbaum et al., 1981).

Alginate may also be gelled internally, when mixed with tri-calcium-citrate followed immediately by glucano-δ-lactone, Ca^{2+} ions are released inside the gel and bind alginate molecules without the need for ion diffusion across a cross-linked outer layer of alginate. Such a process permits the casting of solid forms with a variety of shapes, whose strength increases during fermentation (in comparison with the resilience of externally gelled beads which declines with time) and does not exhibit the dense matrix layer present at the surface of alginate spheres which have been complexed from the outside (Flink & Johansen, 1985).

1.54 Diffusion coefficients for immobilization matrices

Cell growth-rates and product removal are parameters susceptible to diffusion limitations within immobilization matrices. The rate-controlling steps in a packed-bed reactor include external substrate mass transfer, partition effects at the fluid / matrix interface, pore diffusion and cellular uptake. Externally gelled alginate is not a homogeneous material and the diffusion resistance varies tangentially (Bucholz, 1982). For media of low viscosity, the fluid flow in a packed-bed reactor can be described by an axial, dispersed, plug flow model; information often requested by process engineers for system optimisation (Ching & Ho, 1984).

Assuming negligible surface-binding, the diffusion resistance of a matrix is a product of it's pore diameter and the substrate dimensions. The molecular diameters of glucose (0.72 nm) and ethanol (0.45 nm) are about ten times smaller than the surface pores of an alginate membrane and forty times that of the internal pores. These molecules thus experience little resistance to diffusion within the gel (Hannoun & Stephanopoulos, 1986). Free diffusion was also observed for α-lactoalbumin (Mr = 1.56×10^4) and l-tryptophan (Mr = 204) but not BSA (Mr = 6.9×10^4) or the higher Mr substrates albumin, γ-globulin and fibrinogen (Tanaka et al., 1984).

An example of the effect of diffusion resistance upon immobilized-cell systems is provided by the lack of growth observed at the centre of alginate beads which had been inoculated with Chlorella emersonii cells; this

phenomenon was attributed to CO_2 diffusion limitation (raised levels of CO_2 permitted growth throughout the beads) (Day & Codd, 1985 ; Robinson et al., 1986).

Therefore the ions present in algal growth medium and divalent Cd are not diffusion restricted in alginate beads as only high molecular weight products are withheld by the matrix material.

1.6 Cd localisation

An ability to determine sites of metal accumulation within the cell, yields evidence which can be used to build a definition of the metal-uptake mechanisms involved . These details may then be employed to maximise the amount of Cd sequestered in metal scavenging systems.

1.61 Energy dispersive X-ray microanalysis

Specimens irradiated by the beam of an EM, emit X-rays of discrete energy values which are unique for each element, as their displaced electrons drop back to a lower energy state. An energy dispersive, lithium drifted silicon X-ray detector plus beryllium window attached to an EM specimen stage, permits the subcellular analysis of elements with atomic numbers greater than 11. In addition, this technique indicates the chemical form of each element and can be engaged to determine the metabolic status of individual cells. The 1 μm resolution of a TEM beam is suited to probing particular organelles, whilst a complete elemental map of the cell can be generated by SEM (albeit at a lower resolution). A number of specimen treatments have been developed, which require different quantification approaches :

a) Unstained thin sections embedded in a resin containing peripheral standard elements yield relatively good TEM resolution. But the sample elements are not effectively immobilized (Roomans ,1979).

b) Air-dried whole cells may be viewed under SEM or TEM to provide bulk tissue for elements present at low concentrations - although the cell components are not secured and it is difficult to reproduce the matrix for effective sample-peak integration (Baxter & Jensen, 1980)

c) Cryomicrotomed and cool-stage viewed specimens with peripheral standards provide effective quantification, specimen cooling and element immobilization (Gupta & Hall, 1981).

Specimens which are not protected from the damaging influence of a TEM beam suffer cell degradation. The loss of elements during X-ray exposure was shown for microdroplets of inorganic fluid (in terms of beam stability Ca, Mg and Co > P > Na > K > S >> Cl) where the onset of chemical volatilization was found to be a direct function of the current delivered per unit area (Morgan & Davies, 1982). Elements were less stable in 3 μm diameter drops than those with a diameter of 1 μm probably due to a greater diffusion volume; local specimen heating being the most likely cause of volatilization during electron irradiation.

Freeze-dried samples on an uncooled specimen stage lost most mass (assessed by monitoring the spectrum continuum (background radiation)), whilst embedded sections appeared to retain mass stability and freeze-dried / fixed samples on a cooled stage exhibited a uniform continuum (and therefore insignificant mass loss) (Hall & Gupta, 1973).

The problems associated with specimen embedding and staining were highlighted during a study of the polyphosphate bodies of Plectonema boryanum. An Os shoulder from the OsO_4 stain interfered with the P peak and a foreign Cl signal was attributed to the embedding medium (Sicko-Goad et al., 1975). In this work EDXMA demonstrated that the elemental composition of PP bodies was dependent on the strain employed and the medium chosen for growth.

Examples of the application of EDXMA to the cellular localisation of heavy metals include a 0.2 μm layer of uranium fibrils on the surface of Saccharomyces cerevisiae cells (32 % of the population), formation of dense intracellular deposits in 44 % of Pseudomonas aeruginosa organisms (Strandberg et al., 1981) and electron dense granules (300 - 1200 nm in diameter) on the coenobial wall of Scenedesmus granulatus salina and the cell wall of Siderocelis minor (Crawford & Heap, 1978). The granules contained Fe and Mn, whilst smaller particles were composed chiefly of Cd and are believed to be liberated from lake sediments in the top oxic layer as Fe and Mn hydroxides or crystalline deposits.

The influence of cell P status on metal uptake was demonstrated when P-rich Saccharomyces cerevisiae cells took up more Ca and Sr than P-deficient cells. The metals were sequestered in PP bodies and small amounts were found bound to lipids (Roomans, 1980). Thin cryosections of the cyanobacterium Anabaena cylindrica demonstrated rapid Al uptake into PP bodies and cell walls, but not in the cytoplasm. With a high concentration of P in the medium more Al was bound; however, this metal may be released during PP body metabolism (Petterson et al., 1985).

For P-starved A. variabilis cells at least two small intracellular bodies per cell were recorded, with strong K peaks and weaker Mg and Ca peaks. Cu, Pb and Zn were accumulated in PP bodies within 18 h, including a concomitant reduction in the K and an increase in the Ca peak. The cell wall proved to bind and thus detoxify the majority of the Pb that had been accumulated by the cell. Although Zn was not detected in P-starved cells, the metal was located in cells grown in complete medium after 4 h. In all cases Cd exposure did not disturb the elemental composition and no Cd was detected in the organelles, although many of the cells were lysed at 10 $\mu g\ g^{-1}$ Cd (Jensen et al., 1986).

The ultrastructural localisation of Pb in Stigeoclonium tenue revealed electron-dense precipitates on the cell wall, pinocytotic vacuoles and Pb-binding sites within the two large peripheral vacuoles. Dense metal-deposits were never observed in mitochondria, plastids or nuclei, but at 0.15 - 0.5 mg l^{-1} Pb, alterations in the fine structure of the chloroplast were noted (Pb inhibits photosystem II and redirects lipid stores from thylakoid membranes to plastoglobuli) (Silverberg, 1975).

EDXMA also proved effective in charting the Hg-induced loss of K^+ from yeast cells (Saccharomyces cerevisiae). Such a response suggests that Hg may interact with the yeast membrane thiol groups resulting in H^+ and K^+ leakage (Kuypers & Roomans, 1979).

The negatively-charged polysaccharides from the cell walls of brown algae (alginate and fucoidan) are claimed to be responsible for the uptake of certain metals by ion exchange. When exposed to 0.1 mM $CdCl_2$ Fucus vesiculosus deposited the metal in its physodes (and bound by phenol groups), middle lamellae and cell walls (complexed by polysaccharides), but no ultrastructural damage was recorded. For specimens collected near an iron works, Al, Ti, Fe, Cu and Zn X-ray peaks were discovered. The distribution of metals in laboratory cultures closely matched those of algae from the environment, except that low levels of Cd were not detected by EDXMA of field samples (Lignell et al., 1982).

Cells may therefore be divided up into elemental compartments (as metabolic and metal sequestration categories), whose composition fluctuates in response to the prevailing chemical status of the cell and environmental conditions. However, metals accumulated to low levels (for whatever reason) are not detectable by this approach.

1.62 Scanning proton microanalysis

The detection limitations of EDXMA may be overcome by the use of SPM, which fires a beam of protons at the specimen to yield element-specific X-rays. For cells of Closterium moniliferum exposed to a range of Ba concentrations, a pixel map of metal distribution demonstrated that during cell division, the crystals of Ba are distributed between the progeny (Brook et al., 1988). A useful quantitative comparison between the two microanalytical techniques has not been discovered in the literature.

1.7 Production of spheroplasts

The incentive to remove the algal cell wall (protoplast formation) or degrade its components (spheroplast production) derives from an interest in the influence of this layer upon substrate uptake and cell-cell interactions, to prepare cells for transformation or isolate shear-sensitive cell organelles. In order to generate viable cells, the manipulator must find enzymes with effective but restricted activity and an efficacious osmoticum. The range of cell-wall complexity between organisms is reflected in the diverse types of enzymes employed. TEM confirmed the loss of the peptidoglycan layer for Anabaena variablis and A. azollae that had been incubated in lysozyme, a treatment which resulted in a 50 % cell-wall-less, intensively fluorescing population (upon safranin O staining), with greater than 75 % viability (Berliner et al., 1986). Lysozyme also proved appropriate for the creation of spheroplasts from Synechococcus PCC 7942 and PCC 6301 (Delaney, 1984 ; Stone et al., 1988). N-acetylmuramidase SG was found to effectively digest layers II and IV of the cell wall of Anabaena cylindrica, to yield viable protoplasts (Yoshida & Toyama, 1987).

For algae with more complex cell-wall structures, a number of catalytic reactions are required. Protoplasts were produced in large quantities (particularly with young tissue) from the meristematic regions and adjacent stipes of the brown alga Sargassum muticum (Yendo) with 2 % cellulase and 10 % limpet acetone powder I in 0.6 M sorbitol. Cells which excluded Evans Blue Stain were assumed to be viable and complete degradation of the cell wall was demonstrated with calcofluor white (Fisher & Gibor, 1987).

Of the twelve strains incapable of producing 2^{o} carotenoids and thus lacking the enzyme-resistant sporopollenin layer, only Chlorella ellipsoidea and C. saccharophila formed osmotically-labile protoplasts (with 4 % cellulase, 2 % maceroenzyme and 1 % pectinase treatment) whose consummate lack of a cell wall was

demonstrated by calcofluor staining and EM (Yamada & Sakaguchi, 1981).

The ten-fold difference in accumulated metal observed between Cd-resistant and Cd-sensitive protoplasts of Bacillus subtilis, indicates that the cell wall was not providing a detoxification barrier to the passage of Cd (Surowitz et al., 1984). In contrast, Cd exerted a greater toxic threat to cell-wall deficient cells of Chlamydomonas reinhardtii; this mutant strain could tolerate only half the Cd concentration in which wild-type cells survived (Collard & Matagne, 1990).

The enzymes used to produce protoplasts and the contribution that the cell wall affords to Cd tolerance is thus very dependent upon the molecular constituents of the cell wall in each species.

1.8 Tolerance mechanisms

If a cell population is to survive exposure to toxic metals either the offending ions should be excluded, or an intracellular mechanism must be in place to bind foreign metals before they disrupt cell structure and metabolism. For cells that bind heavy-metals with cytoplasmic proteins or possess cell-membrane ion-export molecules, plasmid-borne metal tolerance genes may prove to be more easily disseminated amongst metal-sensitive cells than genomic based elements. The particular detoxification processes that an organism exhibits is probably influenced by the prevailing metabolic pathways that evolution has generated.

1.81 Genomic and plasmid encoded resistance

Although plasmids have been isolated from a variety of cyanobacteria (Rebière et al., 1986 ; Potts, 1984 ; Ciferri et al., 1989) the association of heavy-metal tolerance with plasmid-encoded factors has only been probed for in bacteria. The products of such mobile genes are believed to operate mainly as part of a metal removal mechanism.

The intial association of the presence of plasmids in Staphylococcus aureus with the ability to exclude Cd (Tynecka et al., 1975) prompted a more in-depth study of this particular tolerance mechanism. The accumulation of Cd by S. aureus 17810R was found to occur via a chromosomally-determined Mn^{2+} port, in response to a respiratory-generated membrane potential (via the reversible ATPase). Internalised Cd then blocked respiration by binding to enzyme thiol groups (Tynecka et al., 1981a) and converted the ATPase from oxidative phosphorylation to

proton-pump mode, thus increasing the membrane potential and prompting further Cd^{2+} uptake (Beveridge & Doyle, 1989). The biphasic nature of accumulation suggests the presence of both a Mn^{2+} carrier with a high Cd^{2+} affinity (influenced by the membrane potential) and a low affinity, Mn^{2+} independent system.

Cd resistance in this bacterium is in fact provided by two genes resident on the penicillinase plasmid. The cadA gene product is an electroneutral, $2H^+$ driven Cd^{2+}-efflux pump capable of ejecting the metal up to an extracellular level of 100 μM Cd. Whilst the cadB protein may prove to exhibit cytosolic, metal chelating properties (Tynecka et al., 1981b ; Chopra, 1971 ; Chopra, 1975 ; Hughes & Poole, 1989a). At high Cd^{2+} concentrations this Cd^{2+} / H^+ exchange system is converted to that of a Cd^{2+} / Cd^{2+} pump, therefore increasing metal influx. When the external Cd^{2+} concentration is lowered, the antiporter seemed to revert to its normal function of exchanging H^+ for Cd^{2+}. Metal-sensitive bacteria do not exhibit a Cd^{2+} efflux pump and succumb to respiratory difficulties at low Cd concentrations (Tynecka et al., 1981a ; Silver, 1983). When exposed to Hg, this bacterium employs enzymes to convert the metal into an innocuous form rather than attempting to export it (Kondo et al., 1974). The energetics of resistance may influence the type of mechanism that is selected for a particular toxicant.

The metal-tolerance element of Alcaligenes eutrophus CH34 was found (by Tn 5 mutagenesis restriction nuclease analysis and Southern blotting) to be resident on a 9.1 kb Eco RI fragment of a 238 kb plasmid . When cloned into the broad-host range plasmid pRK290 and transferred to a plasmid free derivative of CH34, the Eco RI restrict provided the same degree of resistance as the parent plasmid (Hambuckers-Berhin & Remacle, 1987). In two other Alcaligenes strains the hybrid plasmid was expressed, but at a lower level (Nies et al., 1987). A further example of gene-encoded metal endurance is provided by the pD188 138 kb plasmid from Rhodococcus fascians which conveyed tolerance when conjugatively transferred to sensitive strains (Desomer et al., 1988).

The dissection of tolerance plasmids has been carried out for a number of plant pathogenic strains. A cosmid-library subclone of plasmid DNA from Pseudomonas syringae pv. tomato Cu^r, only hybridised to Cu^r plasmids and showed no homology with Cu^s strains (Bender & Cooksey, 1987). Whilst the 35 kb plasmid (pPT23D) conserved amongst 12 Cu^r strains of Pseudomonas syringae pv. tomato showed identical restriction patterns and the cloned Cu^r gene from one strain hybridised to the same location on the 35 kb plasmid of all other strains (Cooksey, 1987). A second plasmid pPSII (isolated from a foliar blight causative organism) conveyed resistance to a copper sensitive strain and shared 20 kb of homogeneous DNA with pPT23D (Cooksey, 1990).

Although the spatial location of metal-tolerance genes is in the process of being mapped, the protein sequence

and detoxification mechanisms of their products have yet to be elucidated.

1.82 Cd-binding peptides

If the metal exclusion / removal mechanisms available to cells fail to maintain a low level of intracellular Cd^{2+}, cytotoxic symptoms will develop unless the ion is deactivated. A second defence tactic is to bind the troublesome metal to molecules without hindering cell catalytic or structural functions. In many cases the Cd^{2+} is complexed by one or more of the following metal-inducible metallothioneins :

Class I - proteins with cysteine locations closely resembling those of equine renal MT

Class II - proteins with cysteine locations distantly related to horse MT

Class III - non-translationally synthesized polypeptides : $(\gamma EC)_nG$'s, cadystins or phytochelatins

The Cd-binding peptides,poly gamma-glutamylcysteinylglycines ($(\gamma EC)_nG$'s), were first isolated from Schizosaccharomyces pombe (Kondo et al., 1984) and later from higher plants (Grill et al., 1985). The presence of a carboximide bond (degraded by γ-glutamyl transpeptidase, but insensitive to Edman degradation or V8 protease), the absence of $(\gamma EC)_nG$ mRNA in Cd-tolerant Datura innoxia cells and retention of Cd resistance in the presence of cycloheximide (Robinson et al., 1988), indicate that the peptides are constitutive, metabolic products rather than transcriptional molecules . The synthesis of these peptides either involves the condensation of glutathione molecules with the release of glycine, or the sequential addition of glutamyl cysteine moieties to a single terminal glutathione.

Cells resistant to the toxic effects of Cd may either increase the activity of $(\gamma EC)_nG$ biosynthesis enzymes (chiefly a low Mr carrier concerned with assimilatory SO_4^{2-} reduction), enhance the S^{2-} saturation of metal / $(\gamma EC)_nG$ complexes, modify compartmentation of $(\gamma EC)_nG$s or commute the rates of $(\gamma EC)_nG$ turnover (Robinson, 1989). When potential metal binding sites on the glutathione molecule were probed with ^{13}C NMR, Cd^{2+} and Zn^{2+} were found attached to both SH and NH_2 groups, with some binding to glutamyl and glycyl carboxylic acids (at certain pH values). The sulphydryl group proved to be the main co-ordination site at pH 6.59 (Fuhr & Rabenstein, 1973 ; Perrin & Watt, 1971).

The molecular distinction between Cd-resistant and sensitive D. innoxia cells is related to their ability to form Cd complexes rather than differential rates of peptide formation (the total amounts of $(\gamma EC)_nG$ synthesized by Cd^R and Cd^S cell lines were equivalent). Tolerant strains produced longer chain, sulphide varieties of the peptide which bound 95 - 100% of the cellular Cd within 24 h (Jackson et al., 1989 ; Delhaize et al., 1989).

This requirement for inorganic sulphide has also been documented in Schizosaccharomyces pombe as peptides containing the S^{2-} complex Cd more tightly than those lacking this charged group (Murasugi et al., 1981 ; Murasugi et al.,1983). For Scenedesmus acutiformis and Chlorella fusca Cd^{2+}, Pb^{2+}, Zn^{2+}, Ag^{2+}, Cu^{2+} and Hg^{2+} induced $(\gamma EC)_nG$ synthesis, but only Cd^{2+} and Cu^{2+} were found to bind to the peptides. The addition of BSO (a glutamyl cysteinyl synthetase inhibitor) increased the toxicity of Cd to these algal cells (Robinson, 1989) and rendered Cd-tolerant tomato cells incapable of growth in the presence of the metal (Steffens et al., 1986), suggesting that resistance was due to enhanced γ-glutamyl cysteinyl synthetase activity. However,the peptides did not prove to be the main source of metal tolerance in Cd^R tobacco cells; although BSO treatment caused a significant decline in $(\gamma EC)_nG$ levels and an increase in free cytoplasmic Cd, cell growth in the presence of Cd continued. BSO exposure also failed to regulate Zn and Cu toxicity as the metals did not bind to $(\gamma EC)_nG$'s (Reese & Wagner, 1987).

Cells of Datura innoxia in plant suspension culture were also used to investigate the formation of the peptide / metal complex. When shocked with Cd, the glutathione sulphur content of D. innoxia declined, transferring completely to the $(\gamma EC)_nG$ pool, indicating a Cd-induced metabolic shift. But the $(\gamma EC)_2G$ species were not used to form $(\gamma EC)_3G$. Once co-ordinated with Cd, the peptides are probably blocked from further elongation (Berger et al., 1989). As an alternative to reverse phase HPLC, triple quadropole mass spectrometry was used to show that the accumulated Cd in resistant tomato cell suspensions was complexed by $(\gamma EC)_3$ and $_4$ G (Steffens et al., 1986).

Further evidence of the widespread distribution of these peptides emanates from a screen of seven of the ten classes of Phycophyta, which revealed evidence of phytochelatin synthesis after exposure to Cd^{2+} and also induction by Pb^{2+}, Zn^{2+}, Ag^{2+}, Cu^{2+} and Hg^{2+}. For Euglena gracilis, the $(\gamma EC)_2G$ form predominated (Gekeler et al., 1988).

Cells often rely upon more than one mode of heavy-metal detoxification, for example the exposure of E. gracilis to Cd induces the synthesis of two Cd-binding molecules (CdBP I and II). The amino acid composition of CdBP II is identical to $(\gamma EC)_3G$, whilst CdBP I resembles the type II Cu and Cd binding MTs of yeast, with low cys and high asx and glx. The important role that sulphide ions play in Cd detoxification was again demonstrated, for removal of the S^{2-} from metal complexing peptides produced a low Mr species which bound only 20 % of the Cd complexed by S^{2-} containing peptides (Shaw et al., 1988).

When the yeast, Schizosaccharomyces pombe was treated with Cd, phytochelatins n = 2 - 8 were detected; the addition of BSO abolished peptide induction, but not growth. Although Cd proved the most effective metal for prompting peptide synthesis, other ions (Cu^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}, Ag^{+}, Au^{+}, Bi^{3+}, Sb^{3+}, Sn^{2+}, Ni^{2+}, AsO_4^{3-} and SeO_3^{2-}) also resulted in $(\gamma EC)_nG$ production; alkaline and alkali earth ions however, failed to elicit a peptide response (Grill et al., 1986 ; Grill et al., 1987).

Further evidence for the role of these peptides in metal tolerance was provided by mutants of S. pombe that are unable to synthesize $(\gamma EC)_nG$. These cells were found to be hypersensitive to Cd due to the inhibition of γ-glutamyl cysteine synthetase (EC 6.3.2.2) or glutathione synthetase (EC 6.3.2.3) and other enzymes (Mutoh & Hayashi, 1988).

Employing the fission yeast, Hayashi et al. (1988) found preferential synthesis of the smaller cadystin CdBP 2 species in the early stages of induction and Cd BP 1 at the later stages. Values for their associated binding affinities indicated that the larger peptides complex Cd more firmly than the smaller molecules, coinciding with a higher internal Cd content recorded later on in growth.

Cd-binding peptides have also been detected in the field, HPLC profiles of Acer pseudoplatanus from a mining refuse area (28 g Zn kg^{-1} soil) showed typical $(\gamma EC)_nG$ profiles, but these molecules were not detected in roots from uncontaminated forest Acer. The same pattern was discovered in Silene cucubalus roots except that Acer accumulated four times more Zn and synthesized fifteen times more PCs than Silene (Grill et al., 1988). The contribution that these metal binding peptides make towards cell tolerance depends on the species concerned, in addition their sulphide component appears to exert a controlling influence. Although the storage of Cd in plant cells as phosphate or oxalate precipitates has been documented (Jackson et al., 1990), there is no evidence for such a pathway in algae. Preliminary work has been carried out on this system of metal detoxification via a number of organisms, but a picture of the kinetics and molecular architecture of intracellular peptide binding is very incomplete at the moment.

1.83 Cd-binding proteins

The selection of particular molecules for cytosolic metal-detoxification may be related to the metabolic pathways that are utilised by a species at the time of metal exposure. The physical dissimilarities between algal metal binding proteins (18 % cys residues 3, his, 2, tyr and 7 long chain aliphatics) (Kägi & Nordberg, 1979) (Table 1.7) and mammalian MTs (32% cys (binding twice the amount of Cd) with no his, tyr or aliphatic residues) may

imply that they carry out different functions. But scant evidence is available to decide whether these molecules are metal transport proteins which have been adapted for a detoxification role, or are deliberately designed as metal scavengers.

When E. gracilis was exposed to Cd, all the cytosolic metal was bound to two high Mr species - Cd-binding protein (BP)I and II. Cells incubated in Cu produced a CuBP, but a ZnBP was not found (Gingrich et al., 1986). Acid-labile sulphide was detected in CdBP II, although the inefficient induction by low levels of Cd, the kinetic lability of the bound Cd^{2+} and the thermodynamically weaker bond indicate that this protein is not as highly evolved as mammalian MT (Weber et al., 1987).

Table 1.7 Cd-binding proteins isolated from both cyanobacteria and algae. The Mr quoted are variable depending on the extraction conditions and the quantification method, † = not determined

Species	Mr (kD)	induction Cd (μM)	% Cd bound	reference
Anacystis sp.	10 -12	0.01	65	Maclean et al. (1972)
Synechococcus PCC 6301	8.1 - 10	25	†	Olafson et al. (1988)
Chlorella pyrenoidosa	12.8	4.5	84	Hart & Bertram (1980)
Chlorella pyrenoidosa	12.6	†	40 - 50	Hong-Yu & Huan-Xiao (1985)
Scenedesmus obliquus	12	†	40 - 50	Hong-Yu & Huan-Xiao (1985)
Dunaliella bioculata	10	4.4	†	Heuillet et al. (1988)
Chlorella ellipsoidea	8.5 - 9	8.9	3.2	Nagano et al. (1984)
Chlamydomonas reinhardtii *	25	49.8	67	Collard & Matagne (1986)

* for the sensitive strain only

Investigations into the structure and regulation of the Synechococcus PCC 6301 MT revealed a transcriptionally controlled protein with two aromatic residues near the centre of the molecule, making it the most

hydrophobic MT yet discovered (Olafson et al., 1979 : Olafson et al., 1980 ; Olafson, 1984). The metals Cd and Zn were found to induce MT synthesis, but Cu failed to elicit a response (Olafson, 1986). Further investigation of the molecular structure of this protein revealed a large number of variants which did not exhibit significant homology with mammalian and fungal MTs (Olafson et al., 1988).

For Pseudomonas putida 40 % of the cytoplasmic Cd was associated with PP bodies and the rest with a high-Mr Cd binding protein during a 6 h lag phase (Higham et al.,1984). At the end of the lag phase the PP bodies had been metabolized and the first low-Mr CdBP isolated. $CdBP_1$ was produced throughout exponential growth, whilst small amounts of $CdBP_2$ occurred for a short period at the end of exponential phase. Both proteins persisted during a brief stationary phase, followed by $CdBP_3$ production. Amino acid analysis revealed large amounts of cysteine, but not enough for all metals to be singly or double sulphydryl bridged, so it is proposed that His and Glu residues also play a role. Studies of this manner begin to portray the fate of cytosolic Cd^{2+} throughout a culture's lifetime rather than the static records made at a particular stage of development.

1.84 Cd-binding polysaccharides

Only cell bound and extracellular polysaccharides appear to bind metals. Such carbohydrate fractions from Chlorella stigmatophora LB993 possessed three times more uronic acid (free COO^- groups) than that of the non-metal complexing carbohydrate of C. salina (Kaplan et al.,1987). However, the 10 % sulphur component of these molecules did not play a major role in heavy-metal chelation. A significant number of uronic acid groups were also detected in the extracelluar, mucilaginous cell wall material of Klebshormidium fluitans (sic) (isolated from acidic mine-water drainage in Papua New Guinea), which bound a variety of metals at pH 3 (Strong et al., 1982).

Metal bound outside the cell probably incurs less demand upon metabolism than cytosolic detoxification. No records were found to suggest that species which excrete polysaccharides possess secondary intracellular binding mechanisms to anticipate conditions under which all the extracellular ligands have been saturated.

1.85 Polyphosphate accumulation

Cellular P stores fluctuate in response to the nutrient status of an organisms environment and it's metabolic requirements (Grillo & Gibson, 1979 ; Rigby et al., 1980 ; Lawry & Jensen, 1979 ; Nissen et al., 1987). As a Cd-complexing ligand, PP bodies exhibit three types of binding site : polyphosphate molecules with a negative surface charge, lipids (although not a major contributor) and proteins (Vymazal, 1987). These inclusion bodies may represent the final storage-site for toxic metals after peptide or protein transport within the cell. But literature concerning the fate of released metal at times of P limitation and PP body degradation has not been discovered.

1.9 Cell disorders

If tolerance mechanisms are absent (as with some sensitive cells) or the intracellular levels of Cd so high that all metal-binding ligands are saturated, the following cell chaos will ensue.

1.91 Ultrastructural damage

Cytotoxic, divalent Cd can disrupt organelles because of its avidity for free carboxyl and thiol groups. A variety of ultrastructural defects have been recorded in TEM sections of algae grown in the presence of Cd (Table 1.8).

1.92 Biochemical impairment

Because of favourable biochemical properties, SH groups are exploited throughout the cell. But their frequency ensures that Cd toxicity is expressed at many points in the metabolic pathways of an organism (Table 1.9). For Chlorella cells the observed leakage of intracellular K^+ was attributed to an attack on cell-membrane thiol groups by Cd^{2+} ions (De Fillippis, 1979). Above 0.05 mM Cd the amount of K^+ lost by Saccharomyces cerevisiae was independent of the external Cd^{2+} concentration, indicating widespread SH damage (Gadd & Mowll, 1983 ; Norris & Kelly, 1977). Ca^{2+} ions were found to shield yeast cells from the toxic effects of Cd^{2+}, whilst Mg^{2+} provided only slight protection against the deficit of K^+ (Kessels et al., 1985).

The cytotoxic effects of Cd have also been observed to block substrate supply (Passow et al., 1981). Cell-surface enzymes which split non-permeating substances (phosphoric acid, esters or sucrose) are affected, whilst inside the cell Cd is known to inhibit mitochondrial Krebs cycle enzymes and interrupt protein synthesis.

In addition to the inhibition of photosynthesis and acetylene reduction recorded by Stratton & Corke (1979) in Anabaena inaequalis, Cd exposure also resulted in some cell lysis, induced an increase in filament length and heterocyst frequency together with a loss of the cellular contents from apical cells. Other molecules which are susceptible to Cd replacement include the SH active sites of Calvin cycle enzymes, the Fe atoms in PSI / PSII cytochromes and the Mn^{2+} centre of the O_2 evolution reaction (Hart & Scaife, 1977).

In mitochondria the toxic effects depend on the type of metabolism in progress. For C-heterotrophic organisms Cd inhibits respiration (relieved by the addition of excess cysteine), whilst in C-autotrophic species Cd stimulates respiration as photophosphorylation is reduced and a demand for ATP arises (via oxidative phosphorylation) (Nriagu, 1980). If the Zn from E. coli surface alkaline phosphatase (Applebury et al., 1970) is replaced with Cd at the same ser residue, the enzyme will bind one phosphate molecule per enzyme dimer. However, it then forms a stable complex which does not yield free P.

Cytosolic Cd was also observed to influence the energy-charge values and adenylate contents of Euglena gracilis which decreased in the presence of 500 μM Cd, resulting in enhanced cell size and protein content. Photosynthesis and lactate consumption were similarly repressed, suggesting that respiration was the main point of Cd^{2+} interaction. These symptoms disappeared during the adaptation of Euglena cells to Cd (Bonaly et al., 1986).

From this catalogue of extensive cell damage (due to the prevalent number of sites for Cd attack) it is clear that the amount of cytosolic Cd^{2+} must be suppressed by intracellular ligands if a cell population is to remain viable in the presence of the metal.

1.93 Cd / DNA interactions

When Cd binds to the nucleic acids of a cell disturbances of DNA transcription, translation and replication are evident. This outcome was used to explain the loss of control over growth and division for Euglena cells exposed to cytotoxic levels of Cd (Falchuck et al., 1975b ; Bonaly et al., 1980).

Table 1.8 Cd-induced ultrastructural disorders in a selection of microorganisms and the concentration of Cd in the medium

Species	Cd conc. ($mg\ l^{-1}$)	disorder	reference
Anabaena flos-aquae	0.013 - 13	thylakoid, PP body volume, lipid inclusion, cyanophycin & cell wall changes	Rachlin et al. (1984)
Ankistrodesmus falcatus	1.0	vacuolation & cell membrane damage	Burnison et al. (1975)
Ankistrodesmus braunii	1.12	electron dense cells produced	Massalski et al. (1981)
Anabaena cylindrica	2.0	increase in % heterocysts & cell malformations	Delmotte (1980)
Chara vulgaris	0.009 - 0.023	microfibril rearrangement, lamellar wall disintegration & starch grain loss	Heumann (1987)
Chlorella pyrenoidosa	0.05	swelling & mitochondrial vacuolation	Silverberg (1976)
Euglena gracilis	56.2	mitochondrial whorls, thylakoid rupture & increase in pyrenoid / plastoglobuli number	Duret et al. (1986)
Euglena gracilis	1.12	multinucleate cells with fragmented endosomes, abnormal paramylon & inhibited cell division	Falchuck et al. (1975a)
Plectonema boryanum	100	intracellular whorls & enlarged PP bodies	Rachlin et al. (1982)
Prorocentrum micans	0.01 - 0.1	vacuolated mitochondria & diminished cristae	Soyer & Prevot (1981)
Pseudomonas putida	337	extensive blebbing of the outer membrane & Cd detected in PP bodies	Higham et al.(1986)
Skeletonema costatum	0.05	swollen granules, vacuolated cytoplasm & multivesiculated bodies	Smith et al. (1983)

Table 1.9 Influence of Cd upon enzyme activity and the inhibitory concentrations of Cd employed

Enzyme	inhibitory Cd conc. (μM)	effect	reference
malate dehydrogenase	500	50 % reduction	Ernst (1980)
nitrate reductase	2.5	50 % reduction	Ernst (1980)
alkaline phosphatase	50	strong inhibition	Hughes (1987)
carbonic anhydrase	1.35	18 % reduction	Nriagu (1980)
succinate oxidation	5	complete inhibition	Jacobs et al. (1956)
β-galactosidase	1.3 m mole g^{-1}	50% inhibition	Katayama (1986)
dehydrogenase	0.16 m mol g^{-1}	50% inhibition	Katayama (1986)
DNA polymerase I	10	complete inactivation	Mitra & Bernstein (1977)
NADPH-oxidoreductase	0.1	strong inhibition	De Filippis et al. (1981)

Utilising proton NMR, Eichhorn et al. (1970) demonstrated that Cu^{2+} binds to the N^7 of 5'AMP and 5'GMP, to N^1 of 5'CMP but does not complex 5'TMP. Bound Cu^{2+} thus disrupts nucleotide hydrogen bonds resulting in weaker DNA strands, whilst other metals eg. Mg^{2+} which attach to the backbone P groups, reduce their negative charge and strengthen the double helix. A Cu^{2+} denatured double helix can be returned to the native state upon cooling and the addition of $NaNO_3$, whereas Zn^{2+} does not bind so tightly to bases and the presence of salt is not required for recoiling.

For cultured myotubules, exposure to 100 μM Cd or 3 μM Hg, led to an accumulation of DNA strand breaks. DNA repair was partially inhibited at 100 μM Cd and nearly completely blocked at 300 μM Cd. At 0 °C, DNA strand breaks still occurred but at a slower rate (Burkart & Ogorek, 1986).

Preliminary studies by Mitra et al. (1975) suggested that E. coli cells cultured in the presence of Cd must undergo a period of DNA repair before the cells may proliferate. Further studies showed that single-strand DNA breakage (with no detectable increase in double strand breaks) was observed for cultures of E. coli grown in 3 μM Cd^{2+}, as a result, 85 - 95 % of the cells lost their colony-forming ability, whilst at 10 μM Cd^{2+} irreversible double-strand fractures of DNA may have occurred (Mitra & Bernstein, 1977).

The Cd-mediated interference of DNA translation was demonstrated for polyriboadenylic acid, which at pH 6 produced a double helical molecule in the presence of Zn^{2+}, a single coil when Ni^{2+} was added and a random coil with Cu^{2+}. The addition of Cd^{2+} to poly A at pH 7.0 converted a single helical molecule into a random coil. Cleavage of the RNA phosphodiester bond occurs because metal ions can withdraw electrons from a P group to produce a positive dipole, this species attracts a negative oxygen atom to yield pentavalent P which is then dismembered at it's weakest point. If the adjacent base to this bond is uracil there is a high tendency to cleave, if guanine, then a weak inclination. Such breakage is not found in DNA as 2' OH groups are required (Eichhorn et al., 1970 ; Hughes & Poole, 1989a).

As a result of the many industrial applications of this metal, the regulation of Cd disposal into the biosphere is difficult to enforce. To stem the accumulation of Cd in the environment (if not to protect individual habitats, at least prevent Cd from building up along the human food chain) substitutes must be introduced for metal bearing commodities, or Cd scavenging systems deployed for both industrial effluents and regions already contaminated with the metal.

AIMS

1. In response to increasing levels of Cd in the environment from industrial processes and the disposal of Cd-containing products, the main aim of this research was to identify photosynthetic microorganisms which accumulate Cd to a high concentration and to then harness this ability in a packed-bed of immobilized cells, for the removal of the metal from polluted effluents. Waterways that have been contaminated with heavy metals were used to select microorganisms which exhibit strong metal-accumulating characteristics.

2. A second objective was the cellular localization of Cd by EDXMA and SPM. Such information can be used to enhance metal-uptake by identifying the mechanisms of detoxification used by each strain and then promoting conditions which maximise metal-accumulation via particular pathways.

3. The third aim was to investigate the tolerance mechanisms that are used by strains to ensure their survival in heavy-metal contaminated environments; once identified, the production of molecules used to detoxify Cd may be manipulated to enhance the concentration of metal that cells can accumulate without suffering toxic effects.

4. A study of the cell damage which often accompanies growth in the presence of relatively high concentrations of Cd provided the fourth aim. Disorders in cyanobacterial and algal ultrastructure, enzyme activity and protein profile were investigated to determine the extent to which cell function (and in particular their metal-removal efficiency) is imparied by the presence of intracellular Cd.

CHAPTER 2

MATERIALS AND METHODS

2.1 Algal origins

Strains of algae from heavy-metal contaminated environments were screened for their ability to accumulate Cd to a high level, two strains each of cyanobacteria and algae (originating from heavy-metal sites) were chosen from the Durham University Culture Collection as suitable material for immobilization and Cd uptake. Synechococcus sp. D562 was isolated from a mine tailings pond (5.9 mg l^{-1} Zn, 0.024 mg l^{-1} Cd) in the Old Lead Belt of Missouri, USA (Whitton et al. 1981), Klebsormidium rivulare D537 originates from a stream near a zinc smelter at Viviez, France (3840 mg l^{-1} Zn, 345 mg l^{-1} Cd) (Say & Whitton, 1982), Mougeotia sp. D536 from Caplecleugh Low Level, Nenthead, England (7 mg l^{-1} Zn, 0.012 mg l^{-1} Cd) (Patterson, 1983) and Calothrix parietina D184 came from a 9 mg l^{-1} Zn laboratory tank of algae.

2.2 Media and culture techniques

2.21 Algal growth media

Growth media were freshly prepared for each experiment / subculture and used within 24 h. Gilson pipettes were employed to dispense the correct volumes of stock mineral salts (Tables 2.1, 2.2, 2.3, 2.4, 2.5 and 2.6) which were dissolved in 18 MΩ cm^{-1} resistivity water dispensed from a Milli-Q Reagent Water System (Millipore, Watford, U. K.), that was fed with distilled water. Buffering capacity was provided by 2.5 mM (0.6 g l^{-1}) HEPES (Sigma Chemical Co. Ltd, Poole, U. K.), made up in 500 ml of 18 MΩ water and adjusted to pH 7.0 with a known volume of 1.0 M NaOH. HEPES exhibits a suitable pKa of 7.55, is not toxic at low levels (Smith & Foy, 1974 ; Eley, 1988) and does not complex metals to a significant extent (Good et al., 1966). Stocks were stored in the dark at 4 ° C.

During the course of research modifications were made in the media to create comparable growth conditions. Synechococcus D562, originally maintained in ACM pH 7.6 (Table 2.1), was grown in a new PPJ medium designed to enhance doubling times. Calothrix D184 was switched from AD P (1.0) Fe (0.4) pH 7.6 to PPJ - N pH 7.0 and the DMG buffer employed in CHU 10E pH 6.6, replaced with 2.5 mM HEPES pH 7.0. The total element concentrations stated in the media tables, include contributions by the microelements and the chemicals employed throughout this investigation were AnalaR grade (BDH Ltd, Poole, England) unless otherwise stated.

2.22 Heavy-metal stocks

To ensure accurate metal additions to media, fresh Cd stocks of 1000 and 100 mg l^{-1} were formulated in 18 MΩ water from $3CdSO_4$ $8H_2O$ and autoclaved. The precision of the Gilson pipettes employed to deliver volumes of these stocks was checked periodically by determining the weight of a known volume of dispensed water.

2.23 Bacterial test-media

Cultures contaminated with bacteria were detected when grown on the following media which were formulated in 1000 ml of 18 MΩ water (except e)) and autoclaved as 500 ml volumes in 1-litre flasks :

a) Nutrient Broth	:	25 g Nutrient broth No. 2 (Oxoid Laboratories, Hampshire, England), 10 g agar (Difco Laboratories, USA)
b) SST	:	10 g glucose, 10 g tryptone (Oxoid Laboratories, Hampshire, England), 5 g yeast extract (Oxoid Laboratories, Hampshire, England), 10 g agar
c) Peptone-glucose	:	1 g glucose, 1 g peptone (Difco Laboratories, USA), 10 g agar
d) Yeast	:	5 g yeast extract, 10 g agar
e) Growth medium	:	1 g glucose, 0.1 g casamino acids (Difco Laboratories, USA), 10 g agar, in 1000 ml growth medium

Sterile Petri dishes were poured with 40 - 50 ml of media in a laminar airflow cabinet and allowed to solidify before storage in cling film at 4 ^{o} C. For bacterial tests, the plates were dried in the airflow cabinet near the flame

of a Bunsen burner to drive off condensation on the lid of the Petri dishes and samples streaked across a quartile of each plate with a flamed inoculating loop. Plates were incubated in the dark at 32 ^{o} C for at least 1 week to authenticate culture axenicity.

Table 2.1 Mineral salt composition of ACM medium pH 7.0, microelements = 1.0 ml AC (low Mn)

Compound / element	molecular / atomic weight	stock conc. ($g\ l^{-1}$)	medium conc. ($mg\ l^{-1}$)	(mM)	total element conc. ($mg\ l^{-1}$)	(mM)
$CaCl_2\ 2H_2O$	147.020	19.860	19.860	0.135		
Ca	40.080		5.414		5.414	0.135
Cl	35.453		9.578		24.072	0.679
Na_2EDTA	372.240	1.667	1.667	0.005		
Na	22.989		0.206		28.800	1.253
EDTA	326.262		1.461		1.461	0.005
$FeCl_3\ 6H_2O$	270.300	1.210	1.210	0.005		
Fe	55.847		0.250		0.250	0.005
Cl	35.453		0.476			
$K_2HPO_4\ 3H_2O$	228.230	131.030	13.103	0.057		
K	39.098		4.489		197.834	5.060
P	30.974		1.778		1.778	0.057
KNO_3	101.110	100.000	500.000	4.945		
K	39.098		193.344			
N	14.007		69.266		69.266	4.945
$MgSO_4\ 7H_2O$	246.470	50.000	250.000	1.014		
Mg	24.305		24.653		24.653	1.014
S	32.060		32.519		32.559	1.015
NaCl	58.440	46.000	23.000	0.394		
Na	22.989		9.048			
Cl	35.453		13.953			
Buffering NaOH	40.000	40.000	34.000	0.850		
Na	22.989		19.541			

Table 2.2 Mineral salt composition of 1.0 ml l^{-1} AC (low Mn) microelements

Compound / element	molecular / atomic weight	stock conc. (g l^{-1})	medium conc. (mg l^{-1})	(mM)	total element conc. (mg l^{-1})	(mM)
H_3BO_3	61.830	2.86	2.8600	0.0463		
B	10.810		0.5000		0.5000	0.0463
$MnCl_2$ $4H_2O$	197.920	0.181	0.1810	0.0009		
Mn	54.938		0.0502		0.0502	0.0009
Cl	35.453		0.0648		0.0648	0.0018
$ZnSO_4$ $7H_2O$	287.550	0.222	0.2220	0.0008		
Zn	65.380		0.0505		0.0505	0.0008
S	32.060		0.0248		0.0397	0.0012
$CuSO_4$ $5H_2O$	249.680	0.079	0.0790	0.0003		
Cu	63.546		0.0201		0.0201	0.0003
S	32.060		0.0101			
$CoSO_4$ $7H_2O$	281.100	0.042	0.0420	0.0002		
Co	58.933		0.0088		0.0088	0.0002
S	32.060		0.0048			
Na_2MoO_4 $2H_2O$	241.950	0.027	0.0270	0.0001		
Na	22.989		0.0051		0.0051	0.0001
Mo	95.940		0.0107		0.0107	0.0002

Table 2.3. Mineral salt composition of CHU10 E medium pH 7.0 ,microelements = 0.25 ml AC (low Mn)

Compound / element	molecular / atomic weight	stock conc. ($g\ l^{-1}$)	medium conc. ($mg\ l^{-1}$)	(mM)	total element conc. ($mg\ l^{-1}$)	(mM)
$Ca(NO_3)_2\ 4H_2O$	236.150	57.600	57.600	0.244		
Ca	40.080		9.776		9.776	0.244
N	14.007		6.833		6.833	0.488
Na_2EDTA	372.240	13.358	3.340	0.009		
Na	22.989		0.413		22.123	0.962
EDTA	326.262		2.927		2.927	0.009
$FeCl_3\ 6H_2O$	270.300	9.700	2.425	0.009		
Fe	55.847		0.501		0.501	0.009
Cl	35.453		0.954		0.970	0.027
$KH_2PO_4\ 3H_2O$	136.090	7.820	3.910	0.029		
K	39.098		1.123		1.123	0.029
P	30.974		0.890		0.890	0.029
$MgSO_4\ 7H_2O$	246.470	25.000	25.000	0.101		
Mg	24.305		2.465		2.465	0.101
S	32.060		3.252		3.262	0.102
$NaHCO_3$	84.010	7.925	7.925	0.094		
Na	22.989		2.169			
C	12.011		1.133		1.133	0.094
Buffering NaOH	40.000	40.000	34.000	0.850		
Na	22.989		19.541			

Table 2.4 Mineral salt composition of PPJ medium pH 7.0 microelements = 1.0 ml BG11

Compound / element	molecular / atomic weight	stock conc. ($g\ l^{-1}$)	medium conc. ($mg\ l^{-1}$)	(mM)	total element conc. ($mg\ l^{-1}$)	(mM)
$CaCl_2\ 2H_2O$	147.020	73.360	73.360	0.499		
Ca	40.080		19.999		19.999	0.499
Cl	35.453		35.381		71.359	2.013
Na_2EDTA	372.240	13.358	3.340	0.009		
Na	22.989		0.413		20.028	0.871
EDTA	326.262		2.927		2.972	0.009
$FeCl_3\ 6H_2O$	270.300	9.700	2.425	0.009		
Fe	55.847		0.501		0.501	0.009
Cl	35.453		0.954			
K_2HPO_4	228.230	131.030	13.103	0.057		
K	39.098		4.489		14.488	0.371
P	30.974		1.778		1.778	0.057
NH_4Cl	53.490	76.370	38.185	0.714		
N	14.007		9.999		10.004	0.714
Cl	35.453		25.309			
$MgSO_4\ 7H_2O$	246.470	50.706	202.82	0.8229		
Mg	24.305		20.000		20.000	0.8229
S	32.060		26.368		26.409	0.8237
KCl	74.550	19.067	19.067	0.256		
K	39.098		9.999			
Cl	35.453		9.067			
Buffering NaOH	40.000	40.000	34.000	0.850		
Na	22.989		19.541			

Table 2.5 Mineral salt composition of AD P (1.0) Fe (0.4) medium pH 7.0, microelements = 1.0 ml BG 11 stock 2

Compound / element	molecular / atomic weight	stock conc. ($g\ l^{-1}$)	medium conc. ($mg\ l^{-1}$)	(mM)	total element conc. ($mg\ l^{-1}$)	(mM)
$CaCl_2\ 2H_2O$	147.020	132.4	66.200	0.450		
Ca	40.080		18.047		18.047	0.450
Cl	35.453		31.927		182.143	5.1376
Na_2EDTA	372.240	13.358	3.340	0.009		
Na	22.989		0.413		110.505	4.807
EDTA	326.262		2.927		2.972	0.009
$FeCl_3\ 6H_2O$	270.300	9.700	2.425	0.009		
Fe	55.847		0.501		0.501	0.009
Cl	35.453		0.954			
$K_2HPO_4\ 3H_2O$	228.230	7.36	7.36	0.032		
K	39.098		1.261		14.488	0.371
P	30.974		0.999		0.999	0.032
$MgSO_4\ 7H_2O$	246.470	50.000	200.000	0.8115		
Mg	24.305		19.723		20.000	0.8229
S	32.060		26.015		26.056	0.8127
NaCl	58.440	46.000	230.000	3.936		
Na	22.989		90.477			
Cl	35.453		139.531			
KCl	74.55	1.91	19.100	0.256		
K	39.098		10.017			
Cl	35.453		9.083			
Buffering NaOH	40.000	40.000	34.000	0.850		
Na	22.989		19.541			

Table 2.6 Mineral salt composition of 1.0 ml l^{-1} BG 11 stock 2 microelements

Compound / element	molecular / atomic weight	stock conc. (g l^{-1})	medium conc. (mg l^{-1})	(mM)	total element conc. (mg l^{-1})	(mM)
H_3BO_3	61.830	2.86	2.8600	0.0463		
B	10.810		0.5000		0.5000	0.0463
$MnCl_2\ 4H_2O$	197.920	1.81	1.8100	0.0092		
Mn	54.938		0.5024		0.5024	0.0092
Cl	35.453		0.6484		0.6484	0.0183
$ZnSO_4\ 7H_2O$	287.550	0.222	0.2220	0.0008		
Zn	65.380		0.0505		0.0505	0.0008
S	32.060		0.0248		0.0406	0.0013
$Na_2MoO_4\ 2H_2O$	241.950	0.390	0.3900	0.0016		
Na	22.989		0.0741		0.0741	0.0032
Mo	95.940		0.1547		0.1547	0.0016
$CuSO_4\ 5H_2O$	249.680	0.079	0.0790	0.0003		
Cu	63.546		0.0201		0.0201	0.0003
S	32.060		0.0101			
$Co(NO_3)_2\ 6H_2O$	291.030	0.049	0.0490	0.0002		
Co	58.933		0.0099		0.0099	0.0002
N	14.007		0.0047		0.0047	0.0003
$NiSO_4\ 7H_2O$	280.760	0.048	0.0480	0.0002		
Ni	58.700		0.0100		0.0100	0.0002
S	32.060		0.0055			

2.24 Changes in the volume of media

Five flasks were filled with 50 ml of PPJ medium and weighed before and after autoclaving to ascertain deviations in fluid volume. The evaporation of medium from conical flasks incubated in a shaken water tank, was also determined over a period of 26 d.

2.25 Glassware and plastic preparation

Borosilicate glassware was utilised to reduce extraneous Cd addition / removal. Before use, glass and plastic apparatus were soaked in a phosphate free detergent - 2% Decon 90, for 12 h (Decon Laboratories Ltd, Hove), scrubbed with a nylon brush, washed in distilled water and soaked in 4% HNO_3 for at least 12 h to remove all traces of bound metal. The acid was displaced by rinsing the utensils six times in 18 MΩ water, which were subsequently dried at 105 ^{o} C for 12h (plastic and rubber items were dried at 40 ^{o} C). Equipment composed of rubber was not acid washed.

2.26 Sterile technique

Procedures which required sterile conditions were carried out in a 98 % alcohol sprayed, Microflow Pathfinder vertical laminar flow cabinet (B.S. 5295 class 1) (M. D. H. Ltd, Hampshire, U. K.). Autoclavable media and equipment were sterilised at 121 ^{o} C , 10^{5} Pascal for 20 min (fluid volumes greater than 1 litre were treated for a longer period) and equilibrated to room-temperature for 6 h. Heat labile components were filter-sterilised through a 0.22 μm nitrocellulose membrane into empty, autoclaved and cooled receptacles. Sterilised media were stored in the dark to reduce EDTA photoreduction and used within 48 h.

Cultures grown on agar-solidified media were observed under a Nikon SMZ-2 Stereoscopic Zoom Microscope housed in a horizontal laminar air-flow cabinet.

2.27 Algal subculture and experimental conditions

Stocks of Cd-resistant Synechococcus D562, Calothrix D184, Mougeotia D536 and Klebsormidium D537 were maintained in metal-amended medium, by batch subculture of log-phase algae under sterile conditions in 100 ml conical flasks holding 50 ml of media and enclosed with silicon rubber stoppers (Sanko Plastics Co., Japan). A 2.5 % inoculum of Synechococcus D562 and Mougeotia D536 was dispensed with a Gilson P 5000 Pipetman, whilst Klebsormidium D537 and Calothrix D184 were transferred as a clump on a sterile (flamed) inoculating loop. These stocks were maintained under the following growth conditions (with no agitation or CO_2 addition) :

Table 2.7 Culture conditions for the cyanobacteria and algae employed in this study

Strain	average air temperature (° C)	average incident light intensity * (μmol photon m^{-2} s^{-1})	Cd conc. (mg l^{-1})
Synechococcus D562	32	35 - 40	0, 1, 2
Calothrix D184	25	25 - 30	0, 1
Mougeotia D536	25	25 - 30	0, 0.5
Klebsormidium D537	25	25 - 30	0, 0.5

* Phillips warm white fluorescent tubes

Back up stocks were also maintained on agar at 10 ° C, 15 μmol photon m^{-2} s^{-1} and subcultured every six months. Media volumes of less than 100 ml, were incubated in a thermostatically controlled, Gallenkamp shaker tank, illuminated from below with Phillips warm white fluorescent tubes and shaken 64 times min^{-1} through a distance of 27 mm. To ensure that the flasks were exposed to an average light flux,their positions in the tank were changed each day. Growth media greater than 100 ml in volume were incubated on a rotary shaker (110 revolutions

min^{-1}) at the appropriate temperature and light levels (Appendix 1.). Cultures of Synechococcus D562 greater than 500 ml in volume were grown up in stationary flasks sparged with air.

Homogeneous inocula were obtained for all strains (except Calothrix D184 which was sterilely fragmented by passage through a 1-mm diameter hypodermic needle prior to subculture), as growth on a rotary shaker produced small filaments equally distributed throughout the media.

2.28 Cell harvesting

The filamentous green algae were collected on 0.8 μm polycarbonate, Nucleopore filters under vacuum filtration, Calothrix D184 was harvested on muslin cloth and Synechococcus D562 centrifuged in 40 ml polycarbonate Oak Ridge tubes at 30 000 x g relative centrifugal force in the SS34 rotor of a Sorvall RC-5B temperature controlled machine. For 10 litre volumes of Synechococcus D562 an MSE Continuous Action Rotor running on an MSE high speed 18 centrifuge (Measuring and Scientific Equipment Ltd, Crawley, England) was employed to harvest the cells. When sterile cell-recovery was required, all apparatus (except the muslin cloth) were autoclaved.

2.3 General procedures

2.31 Mass determination

Chemicals, centrifuge tubes and media were weighed to an accuracy of two decimal places on a Sartorius 1474 top-pan balance whilst more accurate mass determination was carried out on an Oertling R51 balance. An objects weight refers to the amount of mass present, measured under the force of the earths gravity.

2.32 Cell dry weight

At least 5 - 10 ml of Calothrix D184, Mougeotia D536 and Klebsormidium D537 culture or 50 ml of Synechococcus D562 were spun out of their salt media and resuspended in 18 MΩ water. The samples were pipetted into preweighed 20 ml snap cap vials, dried at 110 ^{o} C for 48 h, cooled in a desiccator, then reweighed and

the dry weight of algae expressed in mg l^{-1}. Weight determinations were carried out on the Oertling R51 balance and the relative humidity of the weighing chamber containing the snap cap vials, reduced by the presence of recently dried silica gel.

2.33 Cell density

25 µl of a uniform cell suspension were pipetted onto a Weber cell-counting slide, the number of fields which contained 400 cells was recorded and the culture density expressed as the number of cells ml^{-1}.

2.34 pH

The proton concentration of fluid volumes greater than 2 ml were measured with an Ingold type E 50 / SK combination electrode and EIL 7050 pH meter, 150 µl of smaller volumes were sampled and applied to the electrode of a Cardy compact pH meter (Horiba Ltd., Japan). Standard buffers of pH 4.0, 7.0 and 9.2 (BDH Ltd, Poole, U. K.) were employed at room temperature to calibrate the meters in the sample pH range.

2.35 Light

Measurements of the incident photosynthetically active radiation (400 nm - 700 nm) at the surface of culture vessels were carried out with a Macam Q101 lightmeter plus PAR sensor head (Macam Photometrics Ltd, Scotland) and recorded as µmol photon m^{-2} s^{-1}.

2.36 Total protein assay

To estimate the protein content of samples, a microtitre-well Bradford protein assay (Bradford, 1976) was developed. 10 ml of the reagent (Bio-Rad Protein Assay Dye Concentrate 500-0006) was diluted with 40 ml of 18 MΩ water, 100 µl of this stock was added to 200 µl of sample and the absorbance of the resulting blue colour recorded at 595 nm. To convert absorbance readings into protein concentrations, two stock solutions of 15 and 150

$\mu g\ ml^{-1}$ of BSA were made up in buffer (50 mM HEPES pH 7.0) and the following volumes added to 100 µl of reagent :

Table 2.8 Reagent volumes used to formulate a standard curve for the Bradford protein assay

Solution		Volumes (µl)										
15 $\mu g\ ml^{-1}$ BSA stock	:	0	10	20	40	60	80	100				
150 $\mu g\ ml^{-1}$ BSA stock	:	-	-	-	-	-	-	-	20	40	60	80
buffer	:	200	190	180	160	140	120	100	180	160	140	120
Final BSA conc. ($\mu g\ ml^{-1}$)	:	0	0.5	1	2	3	4	5	10	20	30	40

2.37 Flame atomic absorption spectrophotometry

The soluble metal component of samples was analysed with a Perkin-Elmer 5000 Atomic Absorption Spectrophotometer and Automatic Burner Control Unit using an air - C_2H_2 flame (gas box settings = 35 : 35) and deuterium-arc lamp background correction. The sensitivity of the spectrophotometer was enhanced by replacing the flow spoiler with an impact bead, and concentrations of Cd below 0.2 $mg\ l^{-1}$ were detected on a chart recorder with expansion factors of 1 to 12. The machine was calibrated with standards made from 1 000 $mg\ l^{-1}$ solutions (BDH "Spectrosol", Poole, England) and the burner position / nebulizer operation optimised with a 10 $mg\ l^{-1}$ Cd standard. The metal accumulated by algae, was determined by digesting a known dry weight of material in 3 ml of 18 MΩ water plus 1 ml of concentrated AAS grade HNO_3, heated to 120 ° C for 60 min on a Tecam DB3H heating block and analysed against matrix-matched standards. The residual organic matter not released by acid digestion was filtered off, sonicated to a fine suspension and also analysed for Cd.

2.38 Computing

The text for this thesis was processed with Microsoft Word V 5.0 running on an IBM PS / 2 model 30. The graphs were produced with Sigmaplot V 3.10 and alkaline phosphatase data-conversion carried out with Quattro Pro Spreadsheets. A database of the Durham University Culture Collection was held as Superfile records (Southdata, London) on an RM Nimbus X16 PC running MS-DOS V 3.10.

2.4 Strain selection and Cd uptake

2.41 Isolating Cd-accumulating algae from the environment.

Four heavy-metal contaminated streams were visited on 07.06.88 and the pH, temperature and conductivity of the water recorded. Two, 10 ml water samples were taken, one was syringed across a 0.2 μm filter whilst the other constituted a total sample, both were acidified with 1 μl ml^{-1} AAS grade HNO_3 and stored in teflon bottles. Large growths of algae were sampled for their Cd content and probed with EDXMA, whilst water, mud and soil specimens were collected for the isolation of Cd accumulating algae.

2.42 Small-scale screening

Sterile microtitre plates (Nunc, Denmark) were used to screen large numbers of Cd-tolerant, single-celled cyanobacteria on a small scale. The ensuing range of Cd concentrations (mg l^{-1}) were created from an autoclaved 100 mg l^{-1} stock in a total well-volume of 250 μl of sterile AC medium : 0, 5, 10, 15, 20, 25, 30, 35, 40. The total incubation volume included 80 μl of one of each of the following strains of Synechococcus : D562, D767, D768, D769, D772, D773, D797 and D799. An ACM medium blank was pipetted into the first column of wells and the algae grown up for 10 d at 32 ^{o} C in a sterile box containing filter-sterilised 18 MΩ water (to reduce evaporation). All wells were then scanned at 620 nm to determine the concentration of pigment in each culture.

2.43 Procedures employed to render Synechococcus D562 axenic

A number of physical methods were selected to remove the bacterial contaminant from cultures of Synechococcus D562, before resorting to the use of antibiotics. All culture samples were 10 d old :

i) Spray plating - A Synechococcus D562 culture was diluted in a series from 10^{-1} to 10^{-10} in 2 ml of medium (5 % (v/v) inoculation volume). Once shaken, the contents of each tube was sampled with a sterile syringe plus needle and injected into a flow of sterile air to nebulise the culture onto an open, vertical Petri dish containing solidified ACM (Fig. 2.1). Each dilution was plated out five times in this manner, all plates were incubated at 32 ^{o} C and inspected ten days later for the growth of bacteria-free colonies. Any colonies which appeared to be axenic were transferred to 2 ml of liquid medium, grown for 10 d then plated on bacterial test media. No axenic colonies were obtained on agar, even at the theoretical dilution factor of 1 cell per sample - at these cell densities Synechococcus D562 did not grow.

ii) Liquid dilution - The cell density of a culture collected on a 0.22 μm nitrocellulose filter was determined to calculate the number of dilutions required to yield one cell per test tube. Five, 15 ml test tubes containing 4.5 ml of sterile ACM were inoculated with 0.5 ml of Synechococcus and 0.5 ml transfer volumes used to form five replicates of a 10^{-1} to 10^{-10} dilution series. After 10 d growth at 32 ^{o} C samples of each dilution were plated onto solidified ACM and bacterial test media, then grown up to screen for axenic cultures. Bacteria proved capable of growing at lower cell densities than Synechococcus D562. Thus no axenic colonies were found with this technique.

iii) The bacterial contaminant of Synechococcus D562 would not form dense streak patterns on SST substrate, so an experiment was designed to use SST as a selective medium for Synechococcus D562. Solid plates of SST made with ACM medium rather than just 18 MΩ water were formed and three replicates streaked with a 10 d old culture of Synechococcus D562. Although this medium inhibited the growth of contaminating bacteria, algal growth was also repressed and no bacteria-free colonies obtained.

iv) Centrifugation - 10 ml of culture were added to six, sterile Oak Ridge type tubes which were spun at different

Fig 2.1 Apparatus used in an attempt to plate out bacteria-free colonies of Synechococcus D562

ai = air inlet f = filter s = inoculum syringe p = petri dish

n = needle a = aerosol m = solid medium

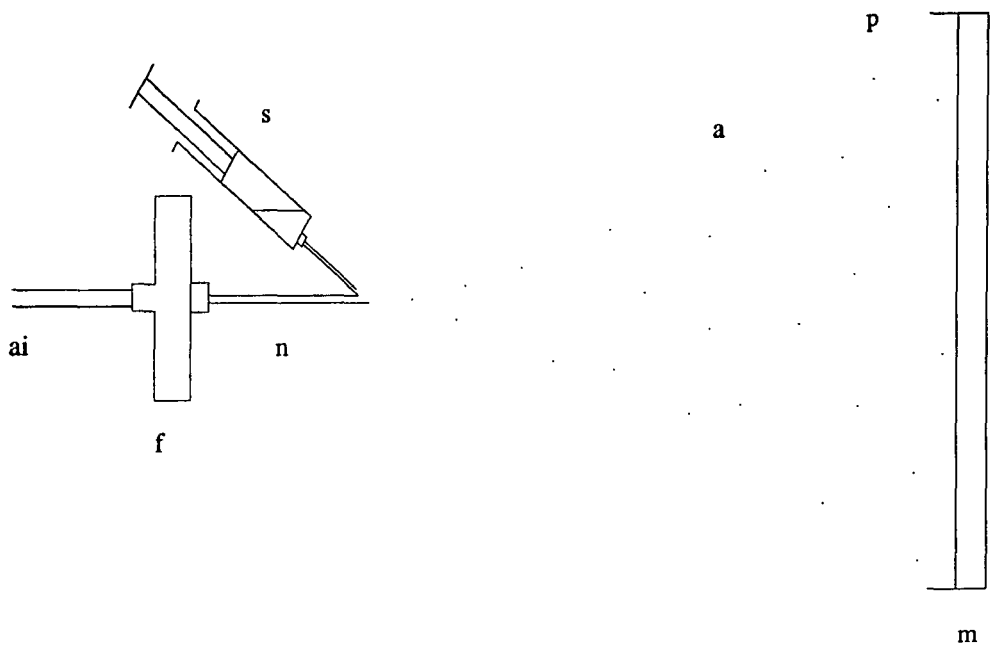
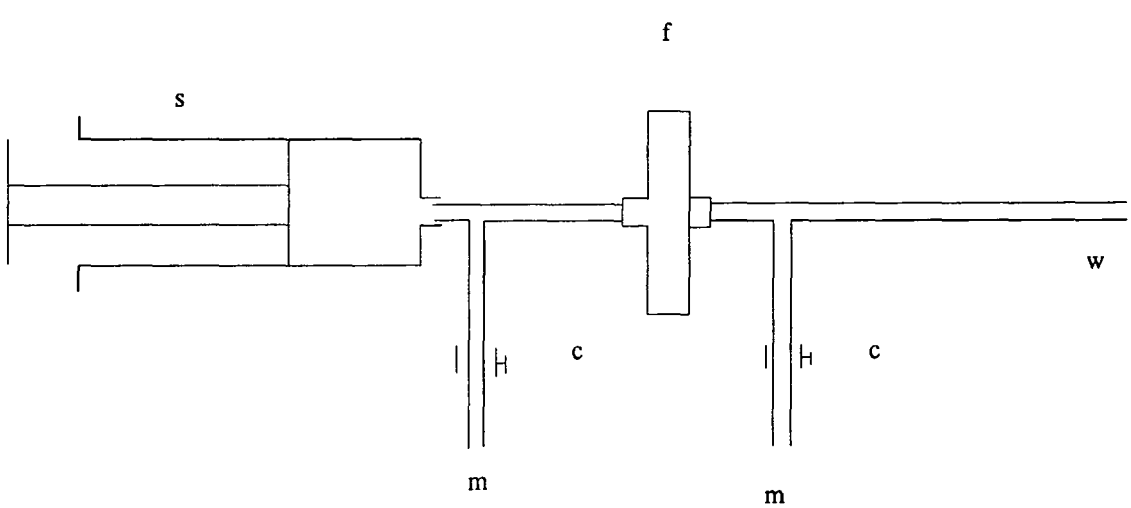


Fig 2.2 Apparatus used to filter and wash a sample of SynechococcusD562

s = inoculum syringe f = filter w = to waste

c = clamp m = sterile medium inlet



speeds (120, 480, 1070, 1910 and 2990 x g, relative centrifugal force) in a Sorvall RC 5B centrifuge and SS34 angle head rotor. One tube, the control, was not spun. The supernatant was then sampled with a flamed inoculating loop, streaked on ACM plates then poured off to allow access to the algal pellet which was streaked in the same manner. The pellet was washed three times in sterile ACM medium by resuspension / centrifugation and after each spin both the supernatant and the pellet were plated out. But none of the pellet or supernatant samples produced axenic algal cultures.

v) Microaerobic growth - the observation that cultures submerged in agar produced a brighter colony colour indicated that pour plating might prove a successful technique. Hence 50 ml of a culture was sterilely concentrated by centrifugation, the pellet resuspended in 5 ml of medium and 1 ml added to each of five sterile Petri dishes, into which was quickly poured 40 ml of cool ACM medium plus 1 % agar. The plates were gently swirled to form an even cell suspension, allowed to set, then grown up for 20 d. Following growth throughout the solidified medium, 1 cm^3 cubes of agar were sterilely cut from the middle of the plate and transferred to 100 ml flasks containing 50 ml sterile ACM and incubated for 15 d. The resulting liquid culture was sampled and streaked out on bacterial test plates. Reducing the amount of oxygen available for bacterial metabolism did not result in cell death.

vi) Diluted medium - the nutrients of the growth medium were depleted by the addition of 18 MΩ water to known volumes of medium, resulting in the following strengths : 100 %, 80 %, 75 %, 50 % and 25 % (v/v). Samples of Synechococcus D562 from each dilution were streaked on agar plates and incubated at 32 °C for 10 d. As this approach failed to yield an axenic culture of Synechococcus D562, there must have been enough carbon in the culture sample to maintain bacterial growth on a simple dilute medium.

vii) Filtration - the dimensions of the alga and contaminant were determined from EM photomicrographs and suitable filters chosen for the separation of bacteria from algae : 1.0 μm polycarbonate 1.2 μm nitrocellulose, 2.0 μm glass fibre, 3.0 μm polycarbonate, 8.0 μm nitrocellulose and polycarbonate. Each filter was checked for it's ability to retain some algal cells by placing them in a Swinnex filter holder, syringing a 5 ml sample of culture across the membrane and observing the density of retained cells by epifluorescent microscopy. Appropriate filters were then housed in a Swinnex holder and connected

to apparatus which facilitated filter backwashing (Fig. 2.2) and autoclaved. After five forward and five backward passages across the filter, the washed inoculum was spray plated onto ACM medium, whilst each filter was sterily extracted and placed on the surface of an ACM plate. This approach failed to render an algal culture without bacteria, probably due to the relatively low pore density on the filters employed.

viii) Antibiotics - 200 µl of a Synechococcus D562 culture was spread over the surface of each of six ACM / PG plates and incubated in the dark at 32 ° C for 1 h to grow up a lawn of bacteria. Three replicates of two types of Oxoid Multodisks (30-1 H and 30-12 L, see Appendix 2) (Oxoid, Basingstoke, U.K.) were sterily placed on each plate and grown up at 32 ° C without illumination for 6 d. Algal lawns were also formed on six ACM plates after two days growth, the two types of antibiotic selection discs were then applied and the plates grown in the light for a further 5 d to determine the algal toxicity of the antibiotics under test.
From initial tests 10, 50 and 100 µg ml^{-1} final concentration of the following antibiotics were chosen to render Synechococcus D562 axenic, both singly and in combination : oxytetracycline, chlortetracycline and neomycin. Antibiotic stock solutions of 1 mg ml^{-1} were made up in 18 MΩ water (oxytetracycline having been predissolved in 5 ml of methanol), 0.2 µm nitrocellulose filter sterilised and used immediately, together with a filter sterilised 5 % glucose solution (to provide organic C for bacterial growth).
The correct volume of antibiotics was added to Pyrex boiling tubes containing sterile medium together with 0.5 ml of a culture and 0.5 ml of 5 % glucose to create a final incubation volume of 5 ml. All treatments were duplicated three times and included three controls with no antibiotic. The tubes were incubated overnight in the dark at 32 ° C then spun down in a bench top centrifuge (Heraeus Christ GMBH), resuspended, spun in fresh, sterile medium three times to remove most of the antibiotics and grown up for 10 d. The resulting cultures were then streaked out on medium and bacterial test plates and cell growth assessed after 20 d for the alga and 5 d for the bacteria. Having determined the most appropriate concentrations and mixture of antibiotics, the above procedure was carried out with 70 µl of chlortetracycline plus 30 µl of an oxytetracycline / neomycin mixture and 80 µl of chlortetracycline plus 40 µl of oxytetracycline / neomycin. Cultures which had been exposed to, then freed from antibiotics were spray plated with 10^{-1} to 10^{-3} dilutions and also streaked onto ACM and bacterial test plates. Cultures from spray plates which appeared clean were selected, transferred to liquid ACM, grown up for 15 d then tested for axenicity on bacterial test plates. Apart from penicillin and streptomycin, all the antibiotics under test

inhibited the growth of bacteria but permitted the growth of Synechococcus D562 (except for sulphafurazole, furazolidone and chloramphenicol). Antibiotic-exposed cultures also required one spray plate treatment before bacteria-free cultures were obtained.

2.44 Cd complexed by the medium

The following treatments were replicated five times, with 20 ml of autoclaved media in 125 ml conical flasks. Half the contents of each flask were stored in snap cap vials and 0.3 g of Dowex 2-X8 (Cl) 18-52 mesh standard grade anion exchange resin (washed in buffered 18 MΩ water (2.5 mM HEPES pH 7.0)) added to the remaining solution and incubated on an orbital shaker at 32 ° C for 20 min. The medium was poured off from the resin, stored in vials and all samples analysed for Cd and iron by FAAS :

i) Buffered (2.5 mM HEPES, pH 7.0) 1 mg l^{-1} Cd

ii) 1 mg l^{-1} Cd and 0.5 mg l^{-1} Fe (as FeEDTA)

iii) PPJ + 1 mg l^{-1} Cd - FeEDTA

iv) PPJ + 0.5 mg l^{-1} Fe - EDTA

v) PPJ + 1 mg l^{-1} Cd

vi) 1 mg l^{-1} Cd + EDTA

vii) PPJ medium

viii) 2.9 mg l^{-1} EDTA

ix) PPJ medium used to grow Synechococcus D562 for 8 d

2.45 EDTA washing

Ten, 50 ml replicates of Synechococcus D562 were grown in PPJ medium plus 2 mg l^{-1} Cd in 250 ml flasks and grown up for 5 d in a 32 ° C shaking water bath. The cells were harvested, washed three times in PPJ medium minus Cd and FeEDTA and exposed to 25 and 0.25 mM Na_2EDTA pH 7.0 (five flasks each) for 2 h, then finally immersed in 18 MΩ water. The Cd accumulated by the cells and that of the chelator supernatant were determined.

2.46 Dead cell uptake

Five replicates of Synechococcus D562 were grown in PPJ medium, 0 mg l^{-1} Cd for 5 d, then heated at 70 ° C for 1 h, spun down, resuspended in 50 ml PPJ medium plus 2 mg l^{-1} Cd and incubated at 32 ° C for 2 h. The cells were centrifuged, washed three times in PPJ medium without Cd or FeEDTA and once in 18 MΩ water. The dry weight and concentration of Cd accumulated by the organism were then determined.

2.47 Physiological response to dilute medium

As part of the efforts to obtain an axenic strain of Synechococcus D562, 2.5 ml of a 10 d old culture were transferred to 50 ml of sterile 18 MΩ water and grown up for 6 d at 32 ° C and 85 µmol photon m^{-2} s^{-1}. Then 0.5 ml of this cell population were added to 5 ml of sterile 18 MΩ water and cultured up for 14 d. 50 µl of the resulting culture were dried on a 200 mesh Cu / Ru grid and viewed under the TEM.

2.48 Fermentation

A 2-litre LH air lift fermenter was engaged to study the growth of algae provided with a continuous supply of nutrients. Five batch runs at different light intensities were instigated to ensure that luminance was a limiting factor which could be used to control continuous-fermentation. Fifteen hundred ml of PPJ pH 7.0 (0.6 g l^{-1} HEPES) medium was autoclaved in the fermenter with a 500 ml medium overflow flask on line, to replace medium which had evaporated during autoclaving and provide an air expansion route.

The fermenter temperature of 32 ° C was maintained by a water jacket, the air inlet and off gas ports were fitted with 37 mm diameter, 0.3 µm bacterial air vents (Gelman Sciences, Northampton, U.K.) and the medium aerated through a sparger at 0.5 l min^{-1}. The air outlet was condensed to prevent filter wetting and as a buffer was incorporated in the medium, the culture pH was not monitored. Algal samples of 10 ml were taken with a sterile 20 ml syringe and hypodermic needle via a silicon septa.

Once the post autoclaved fermenter had cooled down to room temperature, it was connected up to the inoculum flask in an air-flow hood, transferred to the light bank and attached to the water jacket and air-outlet condenser

supply. The light level was adjusted and the medium aerated for at least four hours before pumping 200 ml of bubble grown inoculum into the fermenter (Fig. 2.3). Successful runs were sampled every 12 h.

The batch fermenter was then adapted (Fig. 2.4) to culture organisms continuously. Additions included a medium reservoir with injectable silicon septa, containing 15 litres of PPJ medium agitated by a magnetic stirrer bar and covered in high density, black polythene to resist FeEDTA photoreduction. A 10 ml pipette was included to check the medium-feed flow rate, which was provided by a Watson Marlow 101 U / R peristaltic pump (Watson Marlow, Cornwall, U.K.) through 1-mm diameter silicon tubing to provide a dilution rate of 0.068 h^{-1}. Grow back was prevented with a positive-pressure glass trap provided with 0.3 μm filtered air.

Fig. 2.4 2-litre air-lift fermenter run in continuous mode. Additional apparatus :

mi = medium inlet

at = anti grow-back trap

fp = flow rate pipette

pr = to product receiver vessel

ai = anti grow-back air inlet

mf = medium reservoir covered in black plastic

p = peristaltic pump

t = 1-mm diameter tubing

Fig. 2. 3 2-litre air-lift fermenter run in batch mode.

ao = air outlet

c = air outlet condenser

s = culture sampling septa

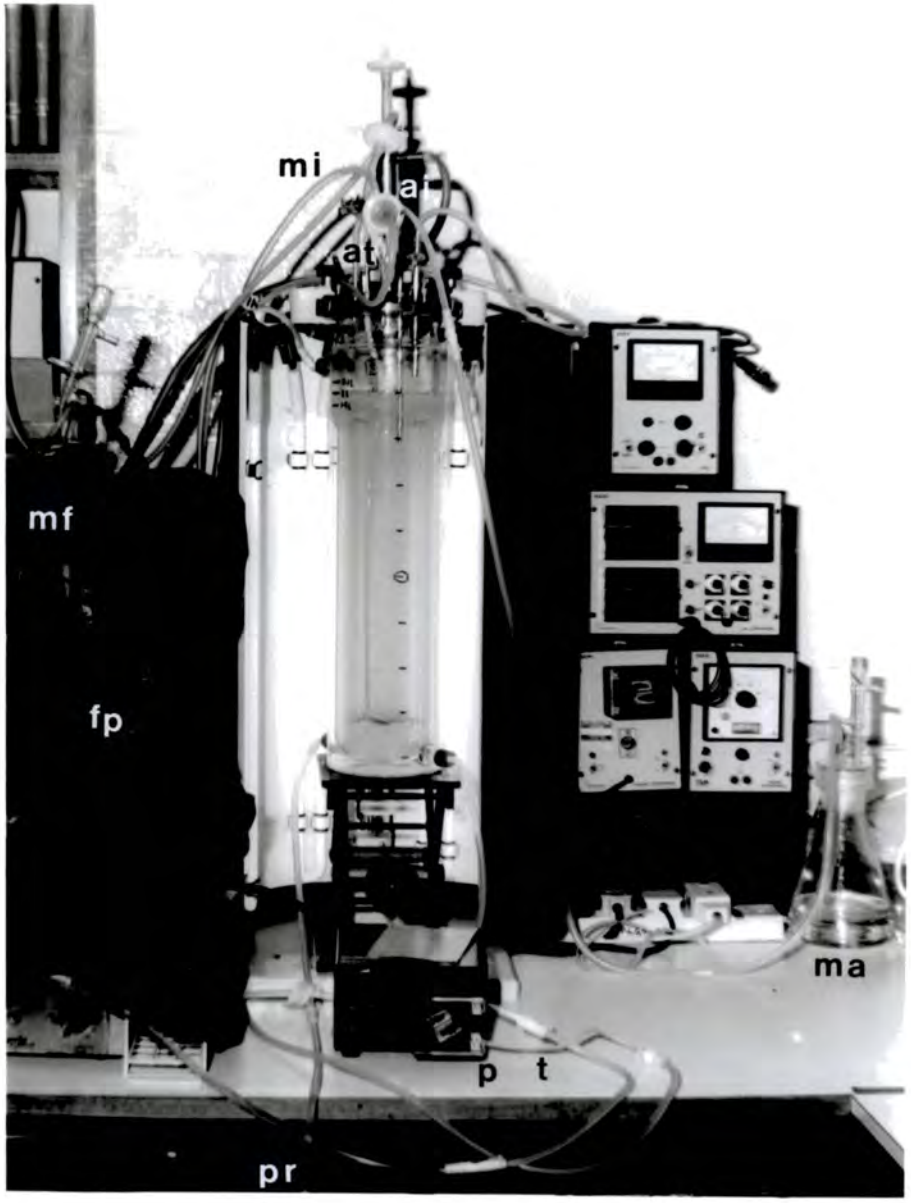
h = head plate

ai = air inlet

m = medium addition line

i = inoculum connection line

wo = 32 ^{o}C water outlet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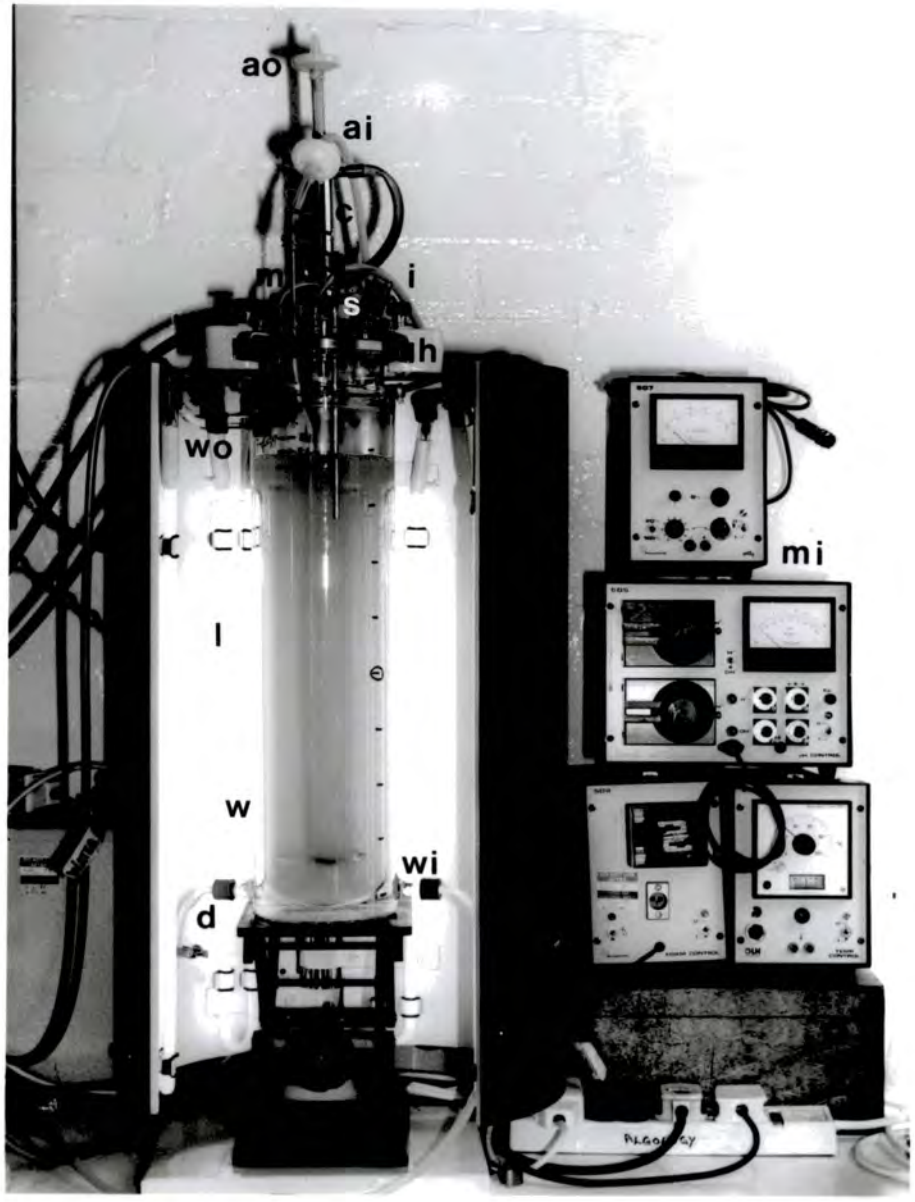
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2.5 Immobilized cells

2.51 Immobilized-cell matrices

A variety of immobilization matrices were evaluated to select the most appropriate method of cell retention :

i) A block of polyurethane foam was cut into 0.5 cm^3 cubes, ten cubes were added to each of two flasks of ACM and CHU10E media and the contents autoclaved. When cool, a 1.5 ml inoculum of Synechococcus D562 and syringe-homogenised Klebsormidium D537 were added to each flask.

ii) Low melting point Sea Plaque agarose at 3 % (w/v) was autoclaved, cooled and a 25 % (v/v) inoculum of Synechococcus D562 and Klebsormidium D537 added, poured into a sterile Petri dish and allowed to set. Small cubes (0.5 cm^3) of inoculated agarose were then cut and transferred to two flasks per species containing 50 ml of ACM .

iii) Sodium alginate was autoclaved, cooled and inoculated with Synechococcus D562 and Klebsormidium D537 to form a final alginate concentration of 4 % (w/v), pipetted into sterile 0.1M $CaCl_2$, allowed to harden in this solution for 30 min and washed in sterile medium.

iv) A 5 % (w/v) k-carrageenan solution was sterilised and inoculated with Synechococcus D562 and Klebsormidium D537, then pipetted into 100 ml of 4 % (w/v) KCl to form beads and washed with sterile medium.

v) 10 ml of media were added to 15-cm lengths of dialysis tubing, autoclaved in flasks containing 50 ml of media (the open end of the tubing was held by the flask's bung), allowed to cool and inoculated with Synechococcus D562 and Klebsormidium D537.

All immobilized cultures were maintained under appropriate growth conditions and assessed by eye for variations in growth rate until cells started to escape from the matrix.

2.52 Optimising calcium-alginate bead formation

The following sodium alginate solutions (10 ml) were formulated in 50 ml boiling tubes : 0.1, 1, 2 and 4 % (w/v) and to separate flasks, 100 ml of the appropriate $CaCl_2$ solutions added : 0.01, 0.1, 0.3 and 1.0 M. All liquids were autoclaved and the sodium-alginate sterily pipetted into each $CaCl_2$ solution. The beads thus formed were assessed in terms of their resistance to squashing, translucence and ease of formation.

2.53 Production of microbeads

To ascertain whether diffusion-resistance inhibited the growth of immobilized Synechococcus D562, a nebuliser (similar in design to that used for spray plating (Fig. 2.1)) was constructed to produce beads with diameters less than 1 mm. An inoculated 4 % (w/v) sodium-alginate solution was pumped (with a Watson Marlow 101 U/R peristaltic pump) through the nebuliser orifice into a stream of 0.2 μm nitrocellulose filter, sterilised air. The resulting microdroplets fell into a stirred 0.1 M $CaCl_2$ solution and were allowed to harden for 30 min.

2.54 Fluidised-bed reactor

The 2-litre air-lift fermenter was fitted with an overflow weir tube and connected to a 1.5-litre closed-loop reservoir of 18 MΩ water. To this reactor 500 ml of calcium-alginate beads (4 % (w/v) sodium alginate) were added, the fermenter air-flow and closed-loop pump activated and the system run for 2 weeks.

2.55 Toxicity of sodium alginate

Initial experiments showed that although the two single-celled cyanobacteria, Synechococcus D33 and Synechococcus D767 grew well in alginate beads, Synechococcus D562 productivity was inhibited. Hence a number of investigations were initiated to ascertain which step of the alginate immobilization procedure proved toxic to Synechococcus D562 :

i) 50 ml of ACM were supplemented with sodium alginate (two replicates per treatment) to create the following final alginate concentrations - 0, 0.1, 0.5, 1.0, 2.0 and 4.0 % (w/v). The flask contents were autoclaved, cooled and inoculated with 1.5 ml of Synechococcus D562. All flasks were assessed for growth after 10 d on a visual basis.

ii) Synechococcus D562 was grown in 50 ml of medium in the presence of the following final sodium concentrations : 10, 50, 100, 1000, 2000 and 5000 mg l^{-1}. (NaCl stock concentration = 46 g l^{-1})

iii) The sodium content of native alginate was determined by FAAS using 1 ml of a 4 % (w/v) alginate solution dissolved in 1 litre.

iv) Citrate was employed as a sodium-free chelator to reduce the toxicity of a 4 % (w/v) sodium alginate solution, at the following citrate concentrations : 0.5, 5, 10, 20, 40, 100 g l^{-1}.

v) Alginate solutions were supplemented with K, Mg and Zn, inoculated with Synechococcus D562, immobilized with $CaCl_2$ and cell growth assessed after 5 d :

Ionic constituents of AC medium	: 5 mM K, 1 mM Mg, 0.0008 mM Zn
Supplemented alginate	: 5 mM K, 1 mM Mg, 0.031 mM Zn
	10 mM K, 4 mM Mg, 0.031 mM Zn
	100 mM K, 25 mM Mg, 0.031 mM Zn

When the growth medium for Synechococcus D562 was changed from ACM to PPJ, Zn no longer afforded protection from the high Na concentrations, but it was discovered that K at 5 mM was effective in alleviating the Na toxicity.

2.56 Influence of aeration

Zones of reduced growth were noted for packed-bed, immobilized Synechococcus D562 and Mougeotia D536 strains - the cell density at the medium-inlet end of the columns was much higher than that at the outlet. To test whether cells at the top of the reactor experienced lower, growth-limiting gas concentrations in the medium at this point, a number of runs were aerated (by bubbling air into the medium reservoirs) to enhance CO_2 levels.

2.57 Free versus immobilized cells

A closed-loop reactor (Fig. 2.7) was autoclaved with the top bung loosened, but enclosed in aluminium foil. The 400-ml medium reservoir was weighed to determine the volume of fluid post-autoclaving and the appropriate volume of 100 mg l^{-1} Cd stock sterily added to form the desired Cd concentration . Ten litres of Synechococcus D562 were grown in a 20-litre carboy, sparged with sterile air and agitated via a magnetic stirrer, whilst 8 litres of Mougeotia D536 were generated in 16 x 500 ml of CHU 10E on a rotary shaker. After 6 d (Synechococcus D562) and 20 d (Mougeotia D536) growth, the cultures were sterily centrifuged in a continuous rotor (Synechococcus D562) or 250 ml bottles (Mougeotia D536) and resuspended in 3 ml of sterile medium.

Six ml of 500 mM K (as KCl) were injected into the matrix flask containing 590 ml of 4 % (w/v) sodium alginate pH 7.0 (Figs 2.5 and 2.6) and shaken. Two ml of the culture concentrate were injected into the alginate flask, mixed by shaking, then pumped into the magnetically stirred flask containing 1 litre of 0.1 M $CaCl_2$ pH 7.0. The resulting beads were left to stir harden for 0.5 h, washed twice in PPJ medium and transferred as a slurry to each of 4 glass columns (length = 240 mm, internal diameter = 28 mm) to form a 100 ml packed-volume of matrix per tube. Four, 100 ml clusters of beads were also added to 4 x 400 ml of PPJ medium in 1-litre flasks. In addition, 0.25 ml of free cells were added to 4 x 400 ml PPJ medium.

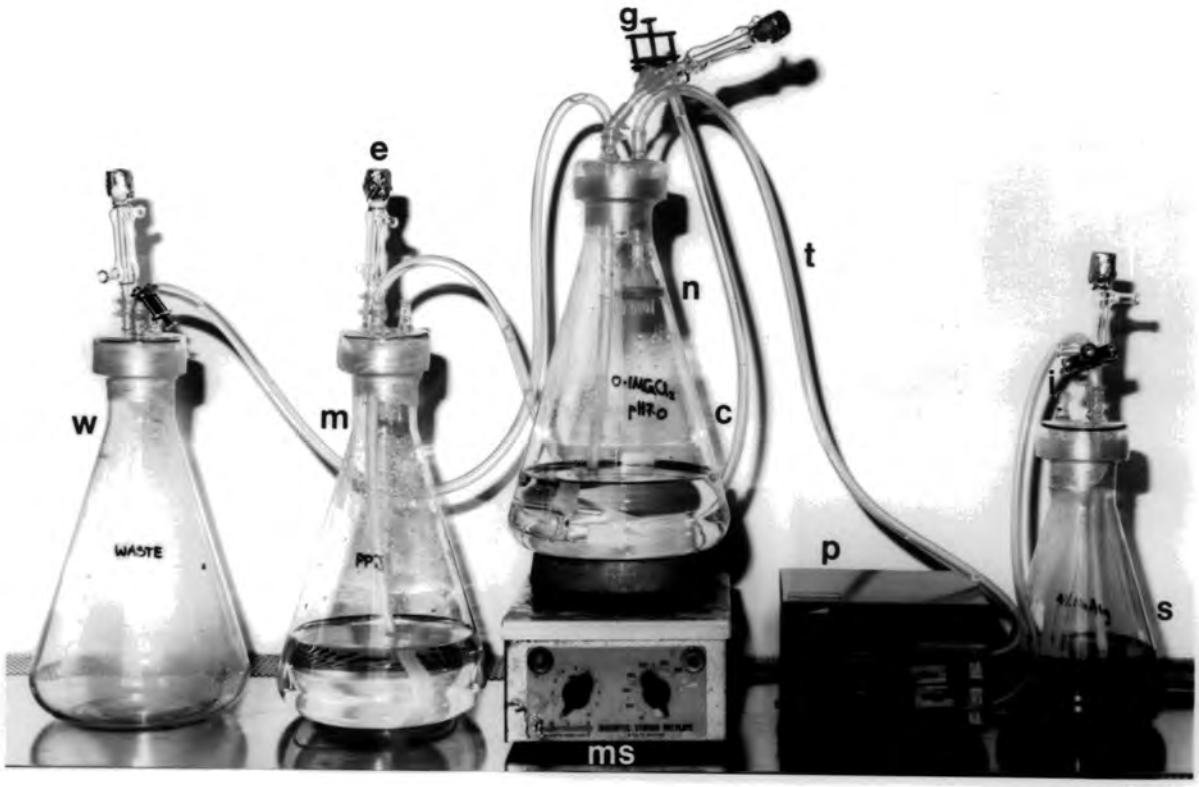
The columns were connected to a 502S Watson Marlow 4-channel peristaltic pump and run continuously on setting 50 at the appropriate temperature and light intensity. Samples (5 ml) were extracted from the immobilized cell columns each day with a sterile syringe and 0.5 mm hypodermic needle (via the silicon septa). The medium of non-immobilized cells was sterily removed by Gilson pipette. All samples were acidified with 1 µl ml^{-1} FAAS grade HNO_3, stored at 4 ^{o} C and subsequently analysed for Cd. The concentration of Cd in the medium was monitored until cells escaped from the matrix and began to grow in the medium.

Fig. 2.5 Closed-system apparatus used to form calcium-alginate beads which have been inoculated with algae

s = Na alginate reservoir
p = peristaltic pump
n = alginate injection nozzle
ms = magnetic stirrer
m = medium wash flask
t = silicon tubing
i = inoculum injection port
c = $CaCl_2$ flask
g = gate clamp
e = air exhaust port
w = waste flask

Fig. 2.6 Close up of the alginate bead-forming head with 13 injection nozzles.

s = Na alginate inlet tube
n = nozzles
sd = silicon distribution membrane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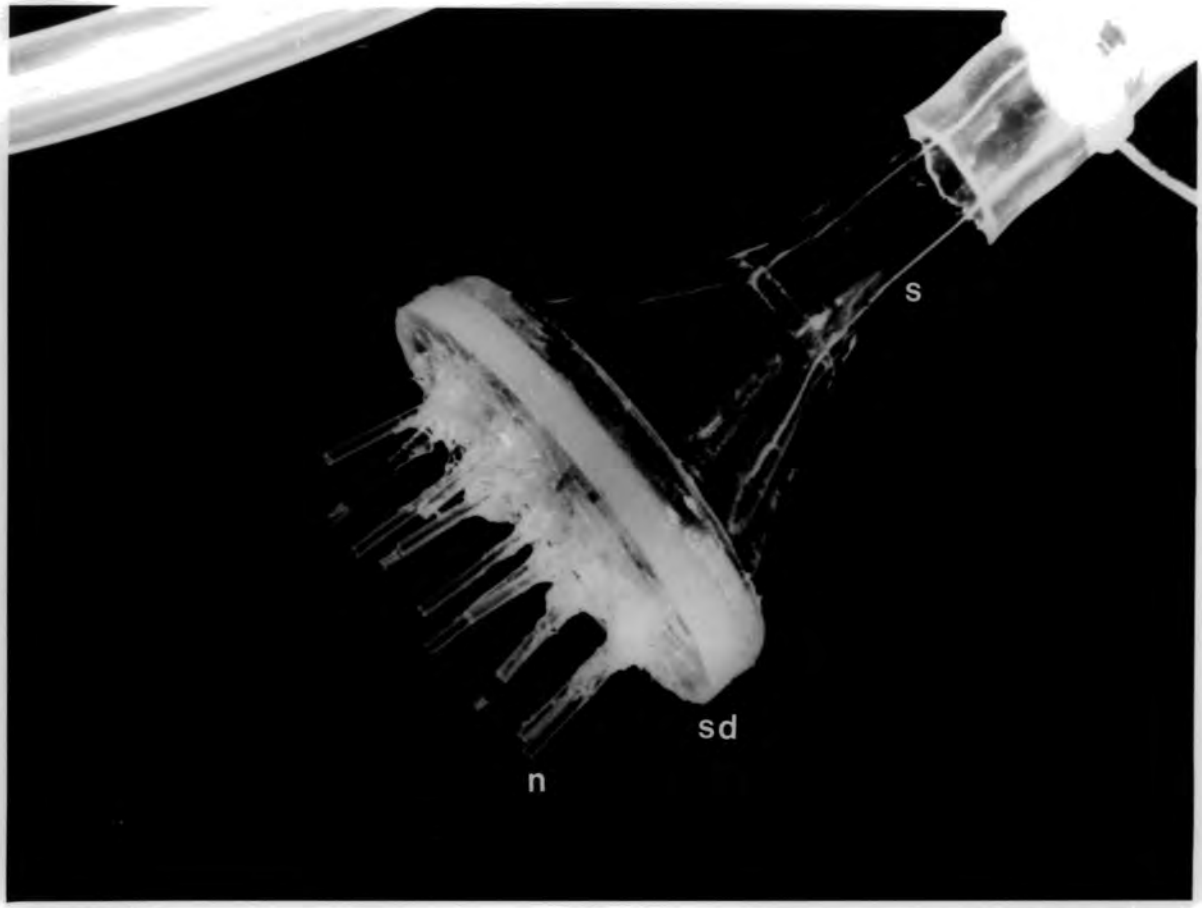
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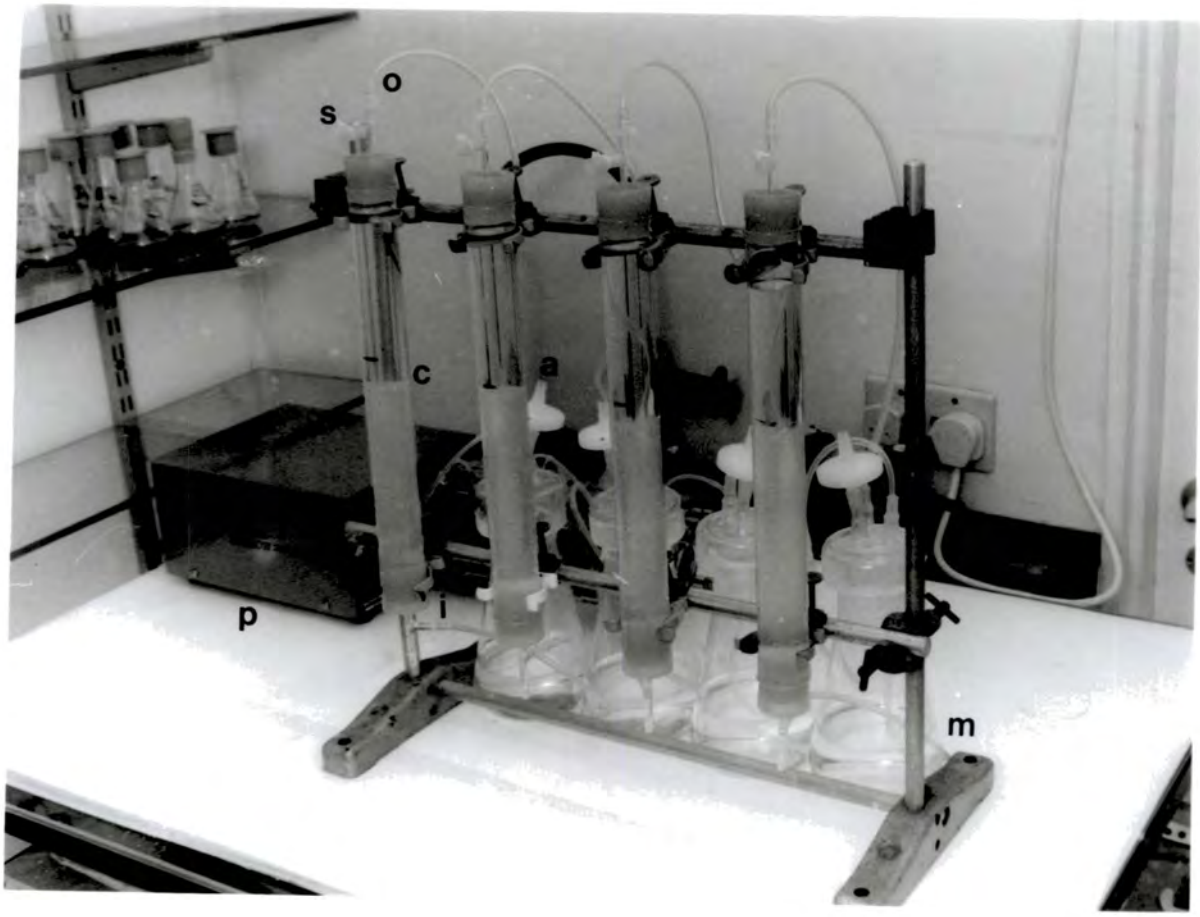
Fig. 2.7 Closed-loop, packed-bed immobilized cell columns used to accumulate Cd from the circulating medium

m = medium reservoir
p = 4 channel peristaltic pump
i = medium inlet
s = sampling septa
a = bacterial air vent
c = immobilized cell columns
o = medium outlet

Fig. 2.8 Continuous dehydration apparatus for removing the water from EM samples

e = 100 % ethanol
b = ethanol bridge
p = peristaltic pump
s = sample bottles
w = 18 MΩ water
m = magnetic stirrer
i = ethanol inlet tube
wa = to waste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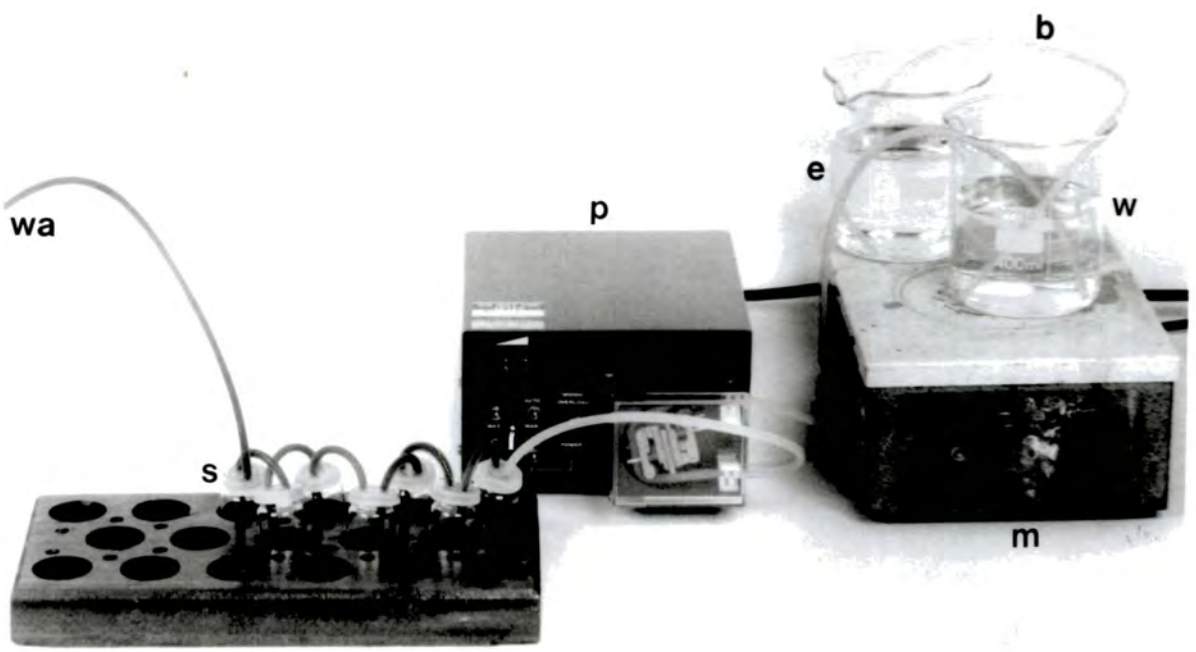
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The immobilized algae were then released from the alginate with 0.5 M sodium citrate, 0.6 g l^{-1} HEPES pH 7.0, washed three times in 18 MΩ water, resuspended and their dry weight and Cd-content determined.

2.6 Microscopy

2.61 Light Microscopy

Light and fluorescence microscopy were performed with a Nikon Fluophot type 109 microscope fitted with a Nikon M-350 automatic exposure camera and loaded with Kodacolor Gold ASA 400 film.

For Synechococcus D562 fluorescence photography the cells were vacuum filtered onto a 0.2 μm nitrocellulose membrane (prestained with a 2 g l^{-1} of irgalan black in 2 % (v/v) acetic acid solution) and washed briefly in 18 MΩ water. The filter was transferred to a refrigerated slide which had been breathed upon (to ensure filter / slide cohesion) and a drop of low-fluorescent, immersion oil added to the centre of the filter. A cover slip was then applied and finally another drop of oil.

2.62 Energy dispersive X-ray microanalysis

Bovine serum albumin was initially employed as a background matrix for standards, but did not dissolve easily and produced a strong sulphur peak upon analysis. Dextran proved to be a more suitable matrix and was used at a concentration of 5 % (v/v) in 18 MΩ water, for all EDXMA standards.

To ensure that the cytosolic Cd was immobilized, algal cells were cryomicrotomed. Ten-ml samples of Synechococcus D562 and Mougeotia D536 were spun down at a relative centrifugal force of 11 950 x g, fixed in glutaraldehyde / formaldehyde / PIPES for 2 h, (see Section 2.81) washed twice in 0.05 M PIPES pH 7.0 and resuspended in a 5 % dextran, 1 M Cd solution (as Cd $(NO_3)_2$). A small volume was then quickly frozen on the end of a copper cryostub by plunging it in liquid-nitrogen cooled Arcton 22 (ICI Mond Division, Cheshire, U.K.) and transferred to a Sorvall cryokit, attached to a Sorvall MT2-B ultramicrotome (Du Pont Instruments, Connecticut, U.S.A.). Thin frozen-sections were cut, transferred to liquid nitrogen cooled grids, dried for at least 1 h in solid CO_2 chilled Petri dishes, then analysed by EDXMA.

Due to the complexity of cryomicrotoming, a simpler technique was developed at the expense of analysing only cell inclusions in air-dried cells and not immobilizing unbound, cytosolic Cd. A small volume of culture (about 20 µl) was washed in 18 MΩ water, pipetted onto coated Cu-grids (not Cu / Rh) and allowed to dry at room temperature. Samples were then probed with a Link QX200 X-ray analyser attached to a Phillips EM 400 TEM, employing a live time of 100 s, 2000 - 2500 counts per second and spot size 2. The Be specimen holder was tilted at 12 ^{o} and the diffraction aperture was removed. Five readings were taken from each grid, the peak integrals averaged and then converted into % elemental contribution with efficiency factors and Quantem software (peak / background ratio method).

Five replicate samples of Synechococcus D562 grown in 4 mg l^{-1} Cd were taken each day for 14 d and analysed to follow Cd accumulation.

2.63 Determination of machine efficiency-factors

The response of an X-ray detector is not linear across the elemental mass-range, hence there is a need to generate figures for the detection efficiency of individual elements. A number of elements (as sulphates) were sprinkled onto formvar / carbon-coated grids and the X-rays from five small crystals were read at 100 kV with a livetime of 100 s and a specimen tilt of 12 ^{o}. Values for Si and Ni were determined from the mineral olivine, an Al figure from orthoclase, whilst P and Cl values were interpolated from a plot of % ratio of the efficiency factors with respect to S against X-ray energy (KeV) (Appendix 5). A peak stripping routine was utilised to obtain net-integral values for the buried peaks of K β and Zn l X-ray energies. The resulting spectra were averaged and the efficiency factors calculated from their net peak-integrals with respect to S.

2.64 Scanning proton microanalysis

Metal-loaded algal material was generated by growing the following strains to mid-log phase in the presence of the following Cd concentrations: Synechococcus D562 (4 mg l^{-1}), Calothrix D184 (2 mg l^{-1}), Mougeotia D536 (0.5 mg l^{-1}) and Klebsormidium D537 (0.5 mg l^{-1}). All cultures were harvested, washed with 18 MΩ water on 47-mm diameter, 0.2 and 3 µm polycarbonate filters and a sub-sample air-dried onto SPM holders. The mounted specimens were then submitted to the University of Oxford Scanning Proton Microprobe Unit for analysis.

2.7 Tolerance mechanisms

2.71 Production of Synechococcus D562 spheroplasts

Thirty ml of Synechococcus D562 cultured in 2 mg l^{-1} Cd for 8 d was spun down, sterily resuspended in a microfuge tube with 300 µl of enzyme buffer (50 mM Tris.Cl pH 8.0, 20 mM EDTA) , together with 500 µl of 1 M osmotica (sucrose, mannitol or proline) made up in the same buffer. The cell concentrate was incubated with 100 µl of 0.22 µm filter-sterilised 20 mg ml^{-1} lysozyme (Sigma L-6876 E.C. 3.2.1.179) (formulated in the above enzyme buffer) for 1 h at 37 ° C, then exposed to 100 µl of self digested (42 ° C for 2 h) 20 mg ml^{-1} protease (in the above Tris / EDTA buffer) (Sigma P-5147 type XIV) at 37 ° C for 1 h.

Enzyme-treated cells were stained with ten-times dilute, auromine O (Gurr stains, BDH, Poole, U. K.) and calcofluor white (Sigma, Poole, U. K.) to determine the extent of cell-wall removal. Spheroplasts were also washed free of all the added enzymes with sterile medium and resuspended in PPJ medium plus 0, 0.5, 1.0 and 2.0 mg l^{-1} Cd with and without 0.5 M sucrose, to assess the viability of spheroplasts and their resistance to Cd.

2.72 Plasmid extraction

4 litres of Synechococcus D562 were grown up in PPJ medium with 0 and 2 mg l^{-1} Cd (from a 5 % inoculum volume) to mid / late log-phase. The cells were then spun down in a siliconised, sterile Corex tube and resuspended in 1300 µl of enzyme buffer (50 mM Tris.Cl pH 8.0, 20 mM EDTA) plus 100 µl of a freshly 0.2 µm filtered, 20 mg ml^{-1} lysozyme, Tris / EDTA solution. These components were incubated for 1 h at 37 ° C in a shaking water bath. 100 µl of self digested (42 ° C for 2 h) 20 mg ml^{-1} protease solution (dissolved in the same enzyme buffer) was added, together with 500 µl of 3.2 % sodium-lauryl-sarcosine solution and reacted for a further hour at 37 ° C.

The resulting suspension was transferred to ice for 30 min, amended with 2000 µl of fresh, 0.2 µm filter sterilised 0.2 M NaOH / 1% SDS solution, mixed gently by inversion and retained on ice for a further 5 min.

1500 µl of 3 M Na acetate pH 4.8 was added, the sample thoroughly mixed and kept on ice for 30 min, agitating every 5 min. The cells were spun down and the clear supernatant retained. This solution was supplemented with an equal volume of distilled phenol, vortex mixed and centrifuged for 2 min. The top aqueous layer was withheld, 1000 µl of TE buffer (10 mM Tris.Cl pH 7.4, 1 mM EDTA) added to the phenol, vortex mixed,

centrifuged and the aqueous layers combined. To this aqueous phase, 1000 µl phenol plus 1000 µl isoamyl chloroform (24 : 1 chloroform : isoamyl alcohol saturated with 1 ml TE buffer) were added, vortex mixed, centrifuged and the aqueous layer extracted. 2000 µl chloroform were then added, vortex mixed, centrifuged and the aqueous layer with the extracted nucleic acids retained.

The DNA was precipitated with isopropanol at - 20 ° C for 30 min, the precipitate spun down and redissolved in 300 µl of 0.3 M Na acetate pH 4.8. Two volumes of ethanol were added and the DNA precipitated at - 20 ° C for 30 min, the pellet was washed twice in 70 % alcohol and vacuum dried, then redissolved in 100 µl sterile 18 MΩ water and stored at - 20 ° C. pUC19 was extracted via the above plasmid purification protocol (without the protease digestion-step), from a 10 ml overnight culture of E. coli DH5 grown in the presence of an antibiotic selection pressure (50 µl of 10 mg ml^{-1} ampicillin in ethanol). The plasmid was purified to ensure that the procedure employed for the extraction of plasmids from Synechococcus D562 was effective and to act as a gel electrophoresis marker.

2.73 Genomic extraction

Restriction quality, genomic DNA from Synechococcus D562 was prepared by the above plasmid extraction protocol, except that the alkali / acid precipitation step was omitted. The quantity and purity of extracted DNA was determined by the sample absorbance ratio of 260 nm : 280 nm (DNA : protein) in a quartz cuvette and Philips UV / VIS PU 8740 scanning spectrophotometer.

2.74 DNA restriction

DNA samples were digested for 2 h at 37 ° C with Eco RI, Hind III and Bam HI (Northumbria Biologicals Ltd, Cramlington, U.K.) in the following standard reaction mixture :

1 µg of ethanol precipitated sample DNA

1 µl of 0.5 U $µl^{-1}$ of RNAase

1 µl of 10 x restriction buffer

2 µl of 8 - 12 U $µl^{-1}$ of restriction enzyme

Restricted DNA was then run on 0.7 % agarose gels (containing 2.5 µl of 10 mg ml^{-1} ethidium bromide and 5 ml of 10 x TBE buffer) in a TBE electrophoresis buffer (plus 5 µl of 10 mg ml^{-1} ethidium bromide) (Sambrook et al., 1989). To estimate the size of DNA fragments, 3 µl of a commercially prepared lambda Hind III / Eco RI marker (Northumbria Biologicals Ltd, Cramlington, U.K.), were also included .

2.75 Southern blotting

The N and C terminal amino-acid sequences of Synechococcus PCC 6301 (= TX20) MT (Olafson et al., 1988) were used to design 20-mer oligonucleotide probes (Appendix 3) for hybridization to algal MT genes. Genomic and plasmid DNA from Synechococcus D562 were hydrolysed with Eco RI, Hind III and Bam HI, run together with native DNA on a maxigel and Southern blotted onto a nylon membrane. The oligonucleotides were labelled and deployed to probe the membrane-bound DNA for MT terminus sequences (Sambrook et al., 1989).

2.76 Gel permeation HPLC of Cd-binding peptides

To provide enough material for $(\gamma EC)_nG$ detection by gel chromatography, the following culture and harvesting procedures were adopted. All media were buffered to pH 7.0 and either run without metal addition or supplemented with 0.5 mg l^{-1} Cd :

After 10 d growth of Synechococcus D562 and 20 d growth of Calothrix D184, Mougeotia D536 and Klebsormidium D537, 6.17 mg l^{-1} Cd (Gekeler et al., 1988) was sterilely added and the cultures grown for a further 2 d. All cultures were flushed twice after harvesting with ice cold wash buffer (10mM Tris.Cl pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$), centrifuged in a microfuge tube, then resuspended in an equal volume of extraction buffer (wash buffer plus 50 mM β-mercaptoethanol). Samples of Mougeotia D563, Klebsormidium D537 and Calothrix D184 were sonicated on ice at 30 s intervals for 1 min (allowing the samples to cool between periods of disruption) whilst Synechococcus D562 was exposed to 5 min disruption. The material was then spun down twice to remove all traces of cell debris and 4 x 100 µl overlaid samples were injected via a Rheodyne port (Rheodyne Inc., Cotati, California, U.S.A.) into a Gilson HPLC system (302 pump and 802 C pressure regulator) (Gilson, Villiers le Bel, France).

Table 2.9 Culture conditions that were employed to screen cyanobacteria and algae for the presence of $(\gamma EC)_nG$'s

Strain	medium	conditions	total duration of growth (d)
Synechococcus D562	4 l of PPJ	bubbled	12
Calothrix D184	400 ml of PPJ - N	orbital shaker	22
Mougeotia D536	400 ml of CHU10 E	orbital shaker	22
Klebsormidium D537	400 ml of CHU10 E	orbital shaker	22

Material was transported at 0.5 ml min^{-1} in equilibration buffer (50 mM Tris.Cl pH 7.2, 150 mM NaCl), passed down a TSK guard column, then through a G 3000 SW gel permeation matrix (300 x 7.5 mm) (Anachem, Luton, U. K.) and partitioned into 1 min volumes on a fraction collector. The samples were analysed for their Cd content by FAAS, an average of three absorbance readings were taken due to the small volume of buffer in each fraction. A total protein assay was conducted with the remaining solution.

$(\gamma EC)_nG$'s were also screened for in Synechococcus D562 and Mougeotia D536 grown in the presence of 0.5 mg l^{-1} Cd without a metal shock at the end of the growth period. In addition, the presence of constitutive peptides was determined for algae grown without Cd addition, cytoplasmic material was extracted from these cells and exposed to the equivalent level of cytosolic Cd that is found in peptide binding samples. pH displacement curves at an absorbance value of 250 nm were obtained with pooled fractions of Mougeotia D536 $(\gamma EC)_nG$ in 100 mM tris pH 7.5 titrated with dilute HCl. All peptide experiments were duplicated.

2.77 Reversed-phase HPLC of Cd-binding peptides

A 500 ml culture of Mougeotia D536 grown in the presence of 0.5 mg l^{-1} Cd and shocked with 6.17 mg l^{-1} Cd for 2 d (Gekeler et al., 1988), was spun down at 30 000 x g relative centrifugal force, washed in medium three times

by centrifugation / resuspension and the cell pellet transferred to a 1.5 ml microfuge tube. Extraction buffer (10mM Tris.Cl pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 50 µM β-mercaptoethanol) was added in the ratio 1 volume of buffer : 1 volume of cells and sonicated on ice for 1 min at a power of 143 µ. To each 670 µl volume of sample, 580 µl of 1 N HCL were added to acidify the solution and the cell-debris spun down in a microcentrifuge.

The supernatant was ultrafiltrated across an Amicon 30 kD membrane (Amicon Centricon C30 and C100, Amicon, Stonehouse, U.K.) and the filtrate run on a Hi-pore RP 318, C_{18} Reversed-phase HPLC column (250 x 4.6 mm) (Bio-Rad, Watford, U.K) with a 0 - 20 % acetonitrile gradient in 0.1 % trifluoroacetic acid. Fractions were analysed for thiol groups by the addition of 150 µl of Ellman's reagent (50 mM KH_2PO_4 pH 7.6, 75 µM 5,5'-dithiobis (2-nitrobenzoic acid)) to 150 µl of sample in a microtitre plate and the absorbance of the resulting yellow colour read at 415 nm (Ellman, 1959).

2.8 Ultrastructural, enzyme and protein damage

2.81 Electron Microscopy

Samples of Synechococcus D562 were spun out of the growth medium at a relative centrifugal force of 30 000 x g for 10 min and resuspended in fixative for 12 h (2.5 % (v/v) glutaraldehyde, 1.5 % (v/v) paraformaldehyde, 0.05M PIPES pH 7.6). The cells were centrifuged out of the fixative (which was discarded), incubated in 0.05M PIPES pH 7.6 for 15 min and the resulting pellet of fixed algae microinjected (with a flame drawn, pasteur pipette) into cooling 1 % agar droplets formed in the base of a Petri dish. The encapsulated specimens were then cut down to blocks of about 1 mm^3.

The other laboratory strains (Mougeotia D536, Klebsormidium D537 and Calothrix D184) formed filamentous colonies which did not require concentration and embedding steps. All samples were then transferred to specimen bottles containing 2 ml of 18 MΩ water and stained for 1.5 h by the addition of 2 ml of 2% osmium tetroxide. The material was then washed three times in 18 MΩ water, taken through a continuous alcohol dehydration series from 0 to 100 % over 12 h (Fig. 2.8) and finally rinsed four times in dry ethanol. The inlet tube on each sample bottle was covered with a small square of plastic mesh to prevent samples moving from one bottle to the next.

The fixed cyanobacteria and algae were then transferred to a carefully mixed 1 : 1 Spurr / 100% ethanol solution (v/v) and incubated for 12 h on a rotator. The resin / alcohol mix was replaced with 100 % resin and incubated for a further 12 h (Spurr, 1969). Polymerization of the specimens was performed in a mould containing fresh resin at 70 ^{o} C for 12 h.

Ultrathin sections (80 - 100 nm) were cut from prepared block faces on an LKB microtome (4801 A Ultrotome and 4802 A control unit) (LKB, Stockholm, Sweden) with glass knifes. Algal sections were collected over freshly-filtered 18 MΩ water on uncoated Cu / Ru 200 mesh grids (Taab Laboratories, Reading, U.K.) and stained for 15 min with ethanolic uranyl acetate (freshly made and filtered through a 0.2 μm nitrocellulose membrane) by floating the samples on a droplet of the stain which had been deposited upon a sheet of parafilm.

The samples were then carefully dipped into a large beaker of distilled water, counterstained with lead citrate as above (Reynolds, 1963), given a final wash in 18 MΩ water and allowed to dry. The grids were only manipulated with clean, alcohol washed forceps and stored in plastic Petri dishes. A Phillips EM 400 TEM was used to view the sections and photographs of suitable specimens taken on Kodak EM 4489 plate film. Five grids containing at least three sections from different regions of a fixed sample were observed for each strain.

2.82 Grid coating

To optimise resolution, most sections were mounted on uncoated grids. But for air-dried samples and EDXMA standards a 0.7 % (w/v) formvar in chloroform solution was employed to form a thin film over the supports, which were then carbon coated to reduce the charge build up experienced under an electron beam.

2.83 **Electron microscopy of immobilized cells** (modification of Casson & Emery, 1986).

Calcium-alginate beads were fixed for 1 h at room temperature (4 % (v/v) glutaraldehyde in 0.1 M cacodylate buffer pH 6.5 plus 25 mM $CaCl_2$ (to maintain matrix integrity)). The beads were then washed twice in the above buffer (2 x 10 min) and secondarily fixed with 1 % (v/v) osmium tetroxide in $CaCl_2$ / cacodylate buffer. Samples

were dehydrated via a continuous alcohol series, embedded in resin, sectioned, then stained with lead citrate and uranyl acetate.

The extracellular material produced by Synechococcus D562 may account for the intercellular spaces observed in immobilized cells and could be one of the components hydrolysed during exposure to lysozyme. To visualize the effect of enzyme action, before and after incubation in 2 mg ml^{-1} lysozyme (with equivalent incubation conditions as those employed for the production of spheroplasts) a sample of cells air-dried onto an EM grid were stained for 2 min by floating their support on a drop of ruthenium red, which had been deposited on a piece of parafilm in a Petri dish. The residual metal was then carefully washed off with 18 MΩ water and the cells observed under TEM.

2.84 Alkaline phosphatase activity

The influence of Cd upon APA in Synechococcus D562 (PME and PDE) was studied using a modification of the protocol described in the Sigma Technical Bulletin 104 (Sigma, Poole, U. K.) and carried out in non-sterile Nunc InterMed 96 well microtitre plates (Nunc, Denmark). Cells (from a 2.5 % (v/v) inoculum) were grown up in 50 ml of PPJ medium at 0.5 mg l^{-1} P for 5 d, spun off from the medium and a pH response curve mapped for two groups of buffers (designated A and B) to compensate for buffer inhibition of APA (Table 2.12). For each enzyme assay the following reagents were added to the microtitre plate wells :

i) PME activity :
- 70 µl of buffer made up in sterile medium
- 140 µl of 0.25 mM PME substrate (disodium ρ-nitrophenyl phosphate) (Sigma, Poole, UK) formulated in sterile assay medium (Tables 2.10 and 2.11)
- 20 µl of alga or extracellular enzyme

ii) PDE activity :
- 90 µl of buffer made up in sterile medium
- 180 µl of 0.25 mM PDE substrate (sodium bis-ρ-nitrophenyl phosphate) (Sigma, Poole, UK) formulated in sterile assay medium (Tables 2.10 and 2.11)
- 30 µl of alga or extracellular enzyme

Reactions were run for 1 h at 32 ^{o} C then terminated with 100 µl, 4.95 M NaOH for PME and 30 µl, 0.3 M NaOH for PDE. The absorbance of the resultant yellow colour was read at 405 nm and converted into µmol ρ-nitrophenol hydrolysed mg^{-1} h^{-1} by means of the following calibration procedure. A stock ρ-nitrophenol standard of 10 µmol ml^{-1} in 0.02 N NaOH was formulated and diluted with 0.02 N NaOH to generate the ensuing ρ-nitrophenol concentrations (µM) in the microtitre wells : 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5. The range of yellow solutions produced were recorded for absorbance at 405 nm (Appendix 4).

To determine the optimum harvest time for this enzyme the release of alkaline phosphatase into the medium was related to specific points along the growth curve, 50 ml cultures (PPJ, 0.5 mg l^{-1} P medium and a 2.5 % (v/v) inoculum) were sampled each day after inoculation for 8 d.

As a preliminary step towards purification and a concentration process for pH 7.0 assays, 10 ml of extracellular enzyme (including growth medium) was centrifuged through both 30 and 100 kD ultrafiltration membranes. Both the filtrate and retentate were tested for activity. The influence of Cd upon Synechococcus D562 PME activity was determined for a range of Cd concentrations from 0.1 to 10 mg l^{-1} Cd (with 33 µl of 100, 10 and 1 mg l^{-1} Cd stocks replacing 33 µl of the buffer in a total reaction volume of 330 µl) at pH 10.3 and pH7.0. When assays were conducted at pH 7.0 a low level of activity was recorded, so the enzyme was concentrated as above before aliquoting to the microtitre plate.

2.85 SDS polyacrylamide gel electrophoresis

Cultures of Synechococcus D562, Calothrix D184, Mougeotia D536 and Klebsormidium D537 were grown in 400 ml of medium with (0.2 - 0.8 mg l^{-1}) and without Cd. The cells were harvested in mid-log phase by vacuum and muslin filtration, resuspended in buffered detergent (0.2 M Tris.Cl pH 6.8, 2 % SDS, 10 % sucrose), sonicated on ice for 30 s (algae) or 2 min (cyanobacteria) and boiled for 10 min in a water bath.

The Bradford protein assay is complexed by SDS and could not be used to determine the protein content of the samples, so their absorbance at 280 nm was read and calibrated with a standard BSA solution (Sigma, Poole, U. K.) (Appendix 7). The sample with the lowest protein content constituted the maximum loading volume (150 µl) and appropriate volumes of the other algal preparations were added to provide comparable levels of protein on the gel. Both 10 and 17 % (w/w) polyacrylamide gels were run (acryl : bisacryl = 30 : 0.8 pH 8.8) with a 5 % (w/w) pH

6.8 stacking gel. Molecular weight markers in the range 70k - 14k (Sigma Dalton Mark VII-L MWS-877L) were included in one of the outside lanes of each gel (see Appendix 9).

Table 2.10 Mineral salt composition of APA assay medium, microelements = 0.25 ml AC (high Mn)

Compound / element	molecular / atomic weight	stock conc. ($g\ l^{-1}$)	medium conc. ($mg\ l^{-1}$)	(mM)	total element conc. ($mg\ l^{-1}$)	(mM)
$CaCl_2\ 2H_2O$	147.020	35.870	35.870	0.2440		
Ca	40.080		9.778		9.778	0.2440
Cl	35.453		17.2997		19.513	0.5504
$MgSO_4\ 7H_2O$	246.470	25.000	25.000	0.1014		
Mg	24.305		2.465		2.465	0.1014
S	32.060		3.252		3.262	0.1017
$NaHCO_3$	84.010	15.850	15.850	0.1887		
Na	22.989		4.337		4.351	0.1893
C	12.011		2.266		2.266	0.1887
KCl	74.550	4.270	4.270	0.057		
K	39.098		2.239		2.239	0.0573
Cl	35.453		2.031		2.031	0.0573
Na_2EDTA	372.240	1.667	0.208	0.0006		
Na	22.989		0.013		0.013	0.0006
EDTA	326.262		0.182		0.182	0.0006
$FeCl_3\ 6H_2O$	270.300	1.210	0.151	0.0005		
Fe	55.847		0.031		0.031	0.0006
Cl	35.453		0.020		0.020	0.0006

Table 2.11 Mineral salt composition of 0.25 ml l^{-1} AC (high Mn) microelements

Compound / element	molecular / atomic weight	stock conc. (g l^{-1})	medium conc. (mg l^{-1})	(mM)	total element conc. (mg l^{-1})	(mM)
H_3BO_3	61.830	2.86	0.7150	0.0116		
B	10.810		0.1250		0.1250	0.0116
$MnCl_2$ $4H_2O$	197.920	1.810	0.4525	0.0002		
Mn	54.938		0.1256		0.1256	0.0023
Cl	35.453		0.1621		0.1621	0.0045
$ZnSO_4$ $7H_2O$	287.550	0.222	0.0555	0.0002		
Zn	65.380		0.0126		0.0126	0.0002
S	32.060		0.0062		0.0062	0.0003
$CuSO_4$ $5H_2O$	249.680	0.079	0.0198	0.00008		
Cu	63.546		0.0050		0.0050	0.00008
S	32.060		0.0025			
$CoSO_4$ $7H_2O$	281.100	0.042	0.0105	0.00004		
Co	58.933		0.0022		0.0022	0.00004
S	32.060		0.0012			
Na_2MoO_4 $2H_2O$	241.950	0.027	0.0068	0.00003		
Na	22.989		0.0013		0.0013	0.00006
Mo	95.940		0.0027		0.0027	0.00003

Table 2.12 Buffers employed to investigate the pH profile of APA in Synechococcus D562 (final assay buffer conc. = 50 mM).

Reaction pH	buffer	group	buffering range	pKa at 25 ° C
7.0	DMG-NaOH	A	3.2 - 7.6	3.66 & 6.20
7.0	HEPES-NaOH	B	6.8 - 8.2	7.50
8.0	TES-NaOH	A	6.8 - 8.2	7.50
8.0	HEPES-NaOH	B	6.8 - 8.2	7.50
9.0	AMeP-NaOH	A	9.0 - 10.5	9.69
9.0	glycine-NaOH	B	8.6 - 10.6	2.35 & 9.60
10.0	AMeP-NaOH	A	9.0 - 10.5	9.69
10.0	glycine-NaOH	B	8.6 - 10.6	2.35 & 9.60
10.3	AMeP-NaOH	A	9.0 - 10.5	9.69
10.3	glycine-NaOH	B	8.6 - 10.6	2.35 & 9.60
11.0	CAPS-NaOH	A	9.8 - 11.1	10.40
11.0	Na_2CO_3-$NaHCO_3$	B	9.2 - 10.8	10.33

RESULTS

CHAPTER 3

3. Strain selection

3.1 Introduction

In order to select strains of cyanobacteria and algae suitable for heavy-metal removal in the form of an immobilized-cell column, two criteria were used : the chosen microorganisms should accumulate Cd to a high concentration without suffering toxic symptoms and exhibit good growth rates when immobilized in calcium-alginate beads.

The following photosynthetic microorganisms were screened for their ability to accumulate Cd, having originated from aquatic sites contaminated with a high concentration of the metal.

3.2 Culture collection strains

The concentration of Cd accumulated by four clonal, axenic strains isolated from heavy-metal environments and deposited in the Durham Culture Collection was determined. Cultures of Synechococcus D562 accumulated the metal to a high concentration (see Table 3.4 for the Cd content of all four strains) in proportion to the amount of Cd present in the medium and tolerated up to 5 mg l^{-1} Cd (although cells grown at this concentration of Cd exhibited a long lag-phase, a visible culture was obtained). Mougeotia D536 displayed reduced growth at 1 mg l^{-1} Cd (observed visually), but the metal was accumulated to a relatively high concentration. The figure for accumulated Cd in Klebsormidium D537 was relatively low and this strain was found to be sensitive to 1 mg l^{-1} Cd. Calothrix D184 proved capable of growth in the presence of 2 mg l^{-1} Cd and excluded most of the metal added to the medium.

3.3 Strains recently isolated from the field

As an extension of the screening process, four aquatic sites affected by mining activity (see Table 3.1) were sampled to determine the concentration of Cd that a variety of taxa from each site had accumulated (Table 3.2) for comparison with the axenic strains listed above. The Zn and Cd measured at each site were not associated with particles greater than 0.2 μm in diameter (Table 3.1) or complexed by precipitates which form under alkaline conditions (all sites exhibited a neutral pH). These observations suggest that most of the metal is available for uptake by the resident microorganisms. The Cd content of strains sampled from these field sites was assessed (Table 3.2), whilst algae isolated from water and soil samples taken from each area , were grown in the presence of 0.25 mg l^{-1} Cd and the concentration of accumulated metal determined (Table 3.3). Due to a small data set, no correlation can be drawn between the conductivity of the water at each field site and the concentration of Cd accumulated; a reduction in the overall ionic concentration of the environment should reduce the competition for ion adsorption and transport sites and thus increase the concentration of Cd accumulated. Large, visible colonies of algae from each site did not accumulate more Cd than cultures of algae isolated from soil and water samples. Except for a strain of Stichococcus, none of the field isolates or samples proved to accumulate as much Cd as Synechococcus D562 and Mougeotia D536. Considerable time is also required to render bacteria-free cultures of the algae sampled from these heavy-metal sites. The two strains obtained from the Durham Culture Collection were therefore selected for immobilization and investigation of the molecular response to intracellular Cd; the toxic effects of this metal and resultant tolerance mechanisms were also studied for Klebsormidium D537 and Calothrix D184.

Table 3.1 Water chemistry at four heavy-metal contaminated field sites in N. E. England, sampled on 07.06.88
T = total metal, F = 0.2 μm filterable metal

Site	grid ref.	water pH	conductivity ($\mu S\ cm^{-1}$)	temperature (° C)		Zn ($mg\ l^{-1}$)	Cd ($mg\ l^{-1}$)
Bolts Burn	NY 957497	7.3	240	12	T	0.421	< 0.002
					F	0.411	< 0.002
Rampgill Level	NY 781434	7.2	718	10.2	T	7.840	0.023
					F	7.783	0.022
Caplecleugh Low Level	NY 781434	7.2	630	9.8	T	3.307	0.010
					F	3.217	0.008
Gillgill Burn	NY 795440	7.0	170	13.9	T	3.231	0.059
					F	3.201	0.057

Table 3.2 Concentration of Cd accumulated by algae which formed visible colonies at the following heavy-metal contaminated field sites

Site	taxon	width (μm)	accumulated Cd ($\mu g\ g^{-1}$)
Bolts Burn	Klebsormidium		39.48
	Mougeotia	7	138.00
Rampgill Level	Stigeoclonium		26.34
Caplecleugh Low Level	Mougeotia		59.03
	Klebsormidium		112.35
Gillgill Burn	Klebsormidium		150.00
	Spirogyra	16 - 18	36.53

Table 3.3 Concentration of Cd accumulated by clonal, non-axenic field strains grown in the presence of 0.25 mg l^{-1} Cd for 1 week at 25 ° C and 25 μ mol photon m^{-2} s^{-1}. These strains were isolated on solidified medium from water and soil samples taken from the following heavy-metal contaminated sites

Strain	site	Cd accumulated (μg g^{-1})
Anabaena sp.	Gillgill Burn	206.5
Chlorella sp.	Caplecleugh Low Level	136.6
Klebsormidium rivulare	Caplecleugh Low Level	67.4
Klebsormidium subtile	Caplecleugh Low Level	136.9
Klebsormidium subtile	Bolts Burn	9.3
Klebsormidium sp.	Caplecleugh Low Level	223.0
Lyngbya sp.	Gillgill Burn	303.0
Lyngbya sp.	Gillgill Burn	228.4
Oscillatoria sp.	Gillgill Burn	453.9
Oscillatoria sp.	Gillgill Burn	220.0
Plectonema sp.	Caplecleugh Low Level	340.2
Stichococcus bacillaris	Gillgill Burn	65.9
Stichococcus sp.	Gillgill Burn	510.7
Ulothrix sp.	Gillgill Burn	151.1

Table 3.4 Concentration of Cd accumulated by axenic strains from the Durham Culture Collection, * n = 5

Strain	Cd in the medium (mg l^{-1})	Cd accumulated (μg g^{-1})
Synechococcus D562	0	< 1
	2	1797 ± 122 *
	3	2207 ± 68 *
	4	10266 ± 1391 *
Calothrix D184	1	60
Mougeotia D536	1	800
Klebsormidium D537	1	129

CHAPTER 4

4. Accumulation of Cd

4.1 Introduction

In order to maximise the amount of metal removed by an immobilized-cell system, the concentration of divalent Cd present in the medium was determined; only free cationic Cd is believed to be capable of crossing cell walls and membranes. In addition, the partitioning of Cd within the cell and pretreatment of the inoculum were investigated so that the optimum growth conditions for Cd accumulation could be defined.

4.2 Concentration of divalent Cd in the medium

The free ionic challenge which microorganisms experience is not always related to the concentration of metal originally added to a system. Hence changes in the Cd content of the medium due to autoclaving, evaporation during incubation at 32 ^{o} C and EDTA chelation were tested, with the following results :

Loss of medium during autoclaving (5 replicates of 50 ml)	= 1.55 ± 0.023 g
	= 3.09 ± 0.045 %
Therefore increase in a 1 mg l^{-1} Cd solution	= 0.0309 mg l^{-1} Cd
Loss of medium during 32 ^{o} C incubation for 26 d (5 replicates of 50 ml)	= 0.147 ± 0.015 g d^{-1}
	= 0.30 ± 0.03 %
Therefore increase in a 1 mg l^{-1} Cd solution	= 0.003 mg l^{-1} Cd

EDTA is included in media to ensure that the added Fe (III) chloride remains in solution before being photoreduced to the more soluble Fe (II) form which is then available for cellular uptake (Finden et al., 1984). Unoccupied EDTA binding-sites (created when the accumulation of Fe by microorganisms prompts further

photoreduction of Fe (III) to Fe (II)) may chelate Cd, thereby reducing its ionic concentration and toxicity. The amount of metal bound by EDTA was effectively determined by the removal of any negatively charged metal-chelator complexes with an anion-exchange resin and determining the concentration of metal before and after incubation with the exchange material.

The majority of the Fe added to PPJ medium as a metal / chelate complex or a 0.5 mg l^{-1} Fe-EDTA solution were removed by the resin (Table 4.1). A reduction (by the ion exchange material) in the Fe concentration of medium without added EDTA suggests that in the absence of a chelating agent, negative Fe complexes are still formed. When incubated with ion-exchange resin, all solutions containing added Cd did not experience a reduction in Cd concentration, indicating that most of the metal was present in a free, unchelated, divalent form. The concentration of endogenous Cd present in PPJ medium and the EDTA solution were low. Any free EDTA made available during the uptake of Fe by Synechococcus D562 did not significantly chelate the Cd which had been added to the medium.

Table 4.1 Removal of EDTA-chelated metals from PPJ medium or 18 MΩ solutions by ion exchange resin at 25 ° C and 80 μ mol photon m^{-2} s^{-1}, n=5, † = not determined, * buffer = 2.5 mM HEPES pH 7.0
< = value below the detection limit

Treatment	Cd (mg l^{-1}) relative to ion exchange		Fe (mg l^{-1}) relative to ion exchange	
	before	after	before	after
Buffered Cd *	0.829 ± 0.010	0.811 ± 0.022	< 0.008	< 0.008
Cd + FeEDTA	0.992 ± 0.003	0.900 ± 0.0005	0.561 ± 0.007	0.030 ± 0.003
PPJ - FeEDTA + Cd	0.913 ± 0.014	0.858 ± 0.010	0.006 ± 0.002	0.011 ± 0.002
PPJ + Fe -EDTA	< 0.002	< 0.002	0.574 ± 0.004	0.132 ± 0.011
PPJ + Cd	0.958 ± 0.007	0.872 ± 0.009	0.494 ± 0.017	0.009 ± 0.003
Cd + EDTA	0.994 ± 0.011	0.964 ± 0.009	0.029 ± 0.004	0.004 ± 0.001
PPJ	0.002 ± 0.0007	0.003 ± 0.0005	0.412 ± 0.008	0.0142 ± 0.004
EDTA	0.004 ± 0.0010	0.005 ± 0.002	0.033 ± 0.003	< 0.008
PPJ + D562 growth	0.772 ± 0.026	0.615 ± 0.008	†	†

4.3 Small-scale screening

Although microtitre plates proved appropriate for culturing unicellular microorganisms, the concentration of Cd which could be tolerated in each well, was much greater than that of larger scale cultures (50 ml). In addition, the absorbance values for each culture could not be correlated with the concentration of added Cd (Table 4.2); the low absorbance readings for cells at intermediate levels of Cd indicate the variability in growth conditions between microtitre wells. When cultivated in 50 ml of medium plus metal, none of the strains grew in the presence of 1 mg l^{-1} Cd except Synechococcus D562.

Table 4.2 Microtitre-plate absorbance readings at 620 nm for a number of Synechococcus strains grown in the presence of Cd in order to select Cd-tolerant strains from low-volume cultures

Strain	Cd (mg l^{-1})				
	0	5	10	15	20
Synechococcus D562	0.150	0.091	0.124	0.077	0.078
Synechococcus D767	0.105	-0.011	-0.007	-0.013	0.001
Synechococcus D768	0.021	0.149	0.006	0.017	0.025
Synechococcus D769	0.119	0.037	0.122	0.026	0.036
Synechococcus D772	0.306	0.114	0.269	0.082	0.015
Synechococcus D773	0.168	0.136	0.019	0.012	0.030
Synechococcus D797	0.039	-0.066	0.031	-0.014	-0.066
Synechococcus D799	0.161	0.032	0.115	0.002	0.013

4.4 Physiological response to dilute medium

As part of the drive towards obtaining a bacteria-free culture of Synechococcus D562, cells were transferred to the nutrient-poor environment of 18 MΩ water. After two transfers in this medium, cells exhibited a reduction in the volume of their polyphosphate bodies and up to a doubling in cell length compared with cultures maintained in PPJ medium (Figs 4.1 and 4.2). Cells that were exposed to a third transfer in sterile 18 MΩ water did not survive, presumably because the medium carry-over effect was further diluted and the energy stores of each cell were reduced to a level which could not support growth.

4.5 Cd adsorbed by the cell wall

When Synechococcus D562 cells were exposed to 25 mM Na_2EDTA, a large quantity of Cd was released, resulting in a high figure for the potential cell-wall bound metal (Table 4.3). Cells which had been treated with this chelator did not exhibit detectable concentrations of intracellular Cd; such treatment probably permeabilises the cells resulting in a loss of most of the cytosolic metal. At the lower level of 0.25 mM Na_2EDTA, a reduced value for adsorbed Cd was obtained. Heat treated cells were found to adsorb about one-hundredth of that normally accumulated by Synechococcus D562.

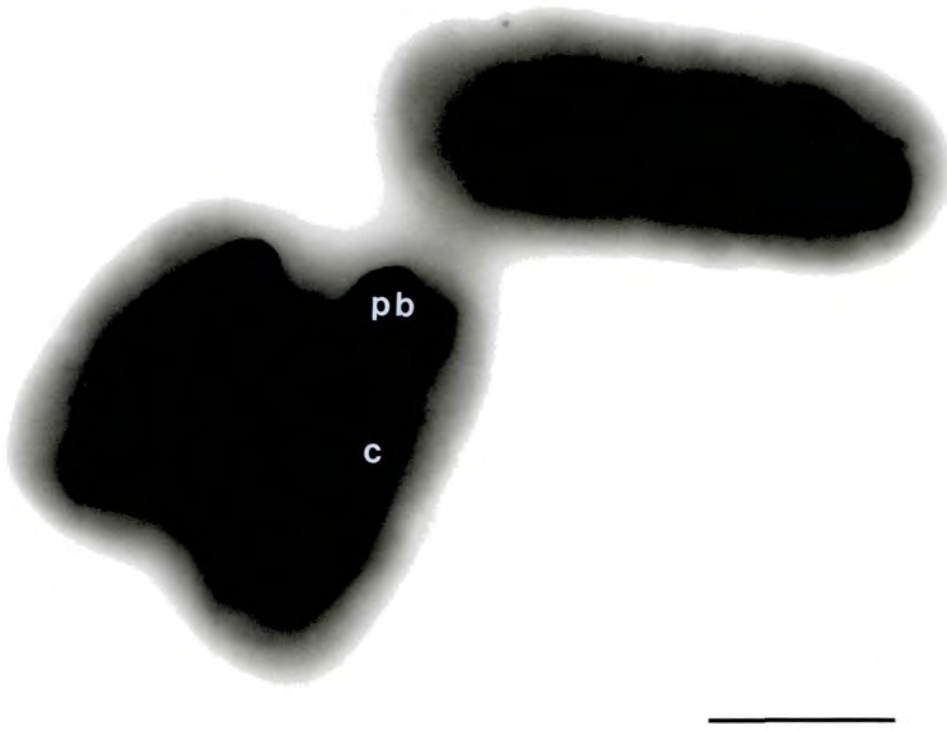
The quantity of metal accumulated by a culture of Synechococcus D562 is dependent upon the concentration of Cd present in the inoculum (Table 4.4). This strain requires only one subculture in medium without added metal to dilute out most of the Cd, a further subculture did not reduce the concentration of accumulated Cd to that of unexposed cells. Cells which had been grown with Cd, then passed through one culture cycle in the absence of the metal, did not attain the same levels of accumulated Cd when recultured in metal-amended medium, in comparison with cells which are continuously maintained on Cd.

Fig. 4.1 Electron micrograph of air-dried Synechococcus D562 cells cultured in PPJ medium at 32 ° C and 80 μmol photon m^{-2} s^{-1}. Scale bar = 1 μm

pb = PP body c = cytoplasm

Fig. 4.2 Electron micrograph of air-dried Synechococcus D562 cells transferred twice in 18 MΩ water from a PPJ medium inoculum, grown at 32 ° C and 80 μmol photon m^{-2} s^{-1}. Scale bar = 2 μm

dpb = diminished PP body c = cytoplasm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c

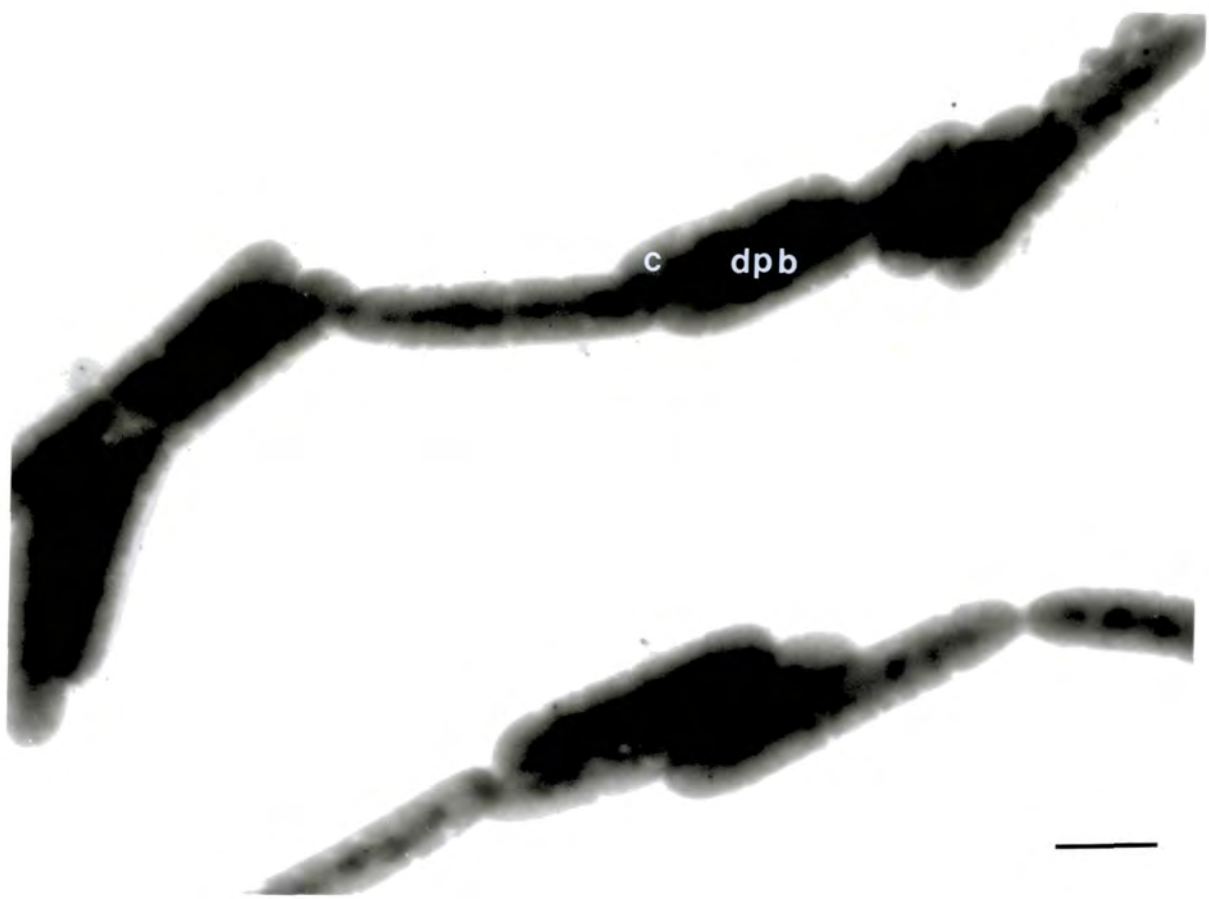

c
dpb

Table 4.3 Cd content of Na_2EDTA washed and heat treated (70 ° C for 1 h) Synechococcus D562 cells which have been exposed to 2 mg l^{-1} Cd for 2 h, n = 5, † = not determined, < = value below the detection limit

Treatment	cell-bound Cd (μg g^{-1})	internalised Cd (μg g^{-1})
25 mM EDTA wash	230.79 ± 4.15	< 1
0.25 mM EDTA wash	14.57 ± 1.05	†
Heat treated cells	11.06 ± 0.97	< 1

Table 4.4 Influence of the Cd concentration of the inoculum and culture medium upon the metal accumulated by Synechococcus D562 cells, n = 5, * = with an intermediate subculture at 0 mg l^{-1} Cd

Cd in the inoculum (mg l^{-1})	experimental Cd (mg l^{-1})	Cd accumulated (μg g^{-1})
0	0	0.21 ± 0.758
0	0.5	53.35 ± 7.08
2	0 *	3.52 ± 2.26
2	2 *	558.00 ± 21.87
2	0	4.68 ± 3.199
2	2	1342.08 ± 380.97

4.6 Batch and continuous culture

Batch culture does not provide stable conditions for the selection of metal-tolerant cells, so a culture of Synechococcus D562 (a strain amenable to culture in an air-lift fermenter) grown under photon limitation but receiving a constant supply of minerals, was exposed to the selective-agent Cd.

Batch, axenic cultures were run without Cd under different light intensities at the surface of the vessel, to ensure that photon availability was a growth-limiting parameter capable of controlling continuous culture. A rise in growth rate of the Synechococcus D562 culture was observed with increasing light intensity up to 140 μmol photon m^{-2} s^{-1}; when illuminated at 180 μmol photon m^{-2} s^{-1} the growth rate was suppressed. From the values of photon concentration used in this investigation (Fig. 4.3), 100 μmol photon m^{-2} s^{-1} was selected as an appropriate level of restraint for continuously cultured cells (Table 4.5).

A sterile, 34 d continuous culture of Synechococcus D562 was maintained with an initial Cd concentration of 2 mg l^{-1}. Both the cell density and whole cell absorbance at 626 nm remained stable during Cd addition, until a medium concentration of 3.4 mg l^{-1} Cd was attained, from that point on, both growth parameters declined rapidly (Figs 4.4 a and b). When the medium pump was activated the prevailing cell density was not perturbed, unlike some bacterial cultures.

Fig. 4.3 Influence of light intensity on the growth rate of Synechococcus D562 in 2-litre batch culture at 20 (■), 60 (▲), 100 (□), 140 (△) and 180 (◆) μmol photon m^{-2} s^{-1} and 32 °C

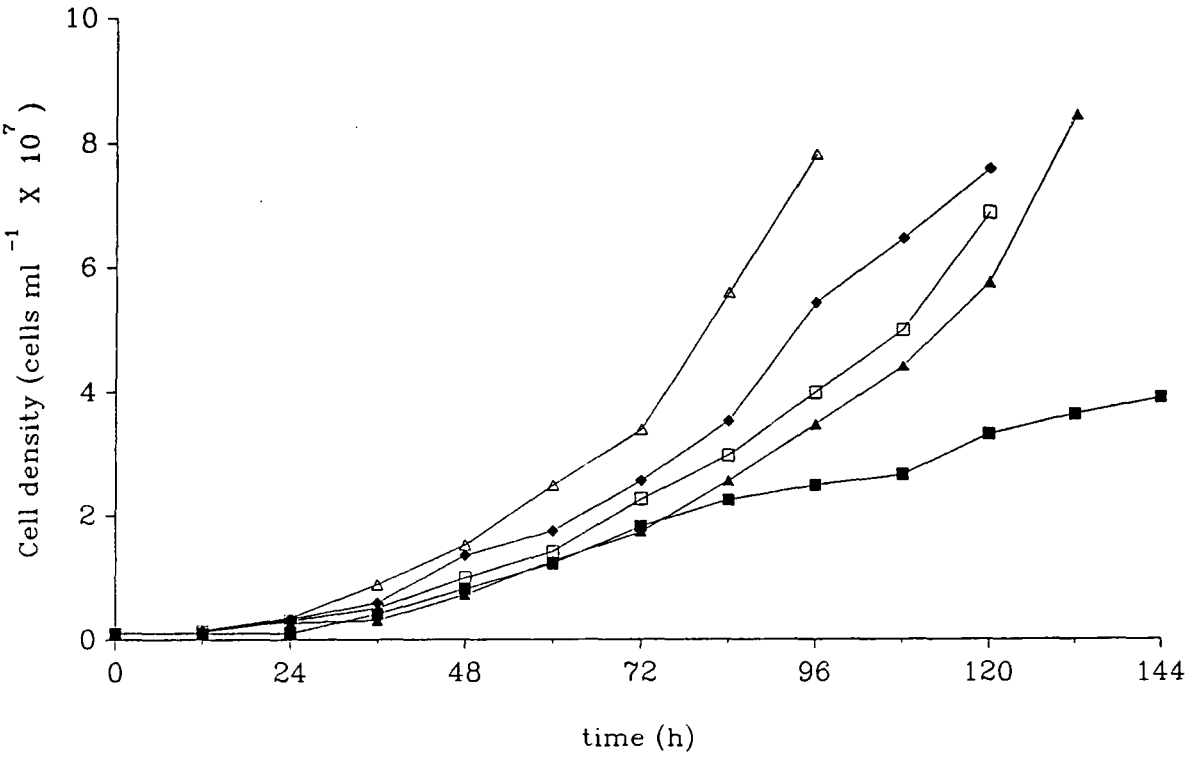
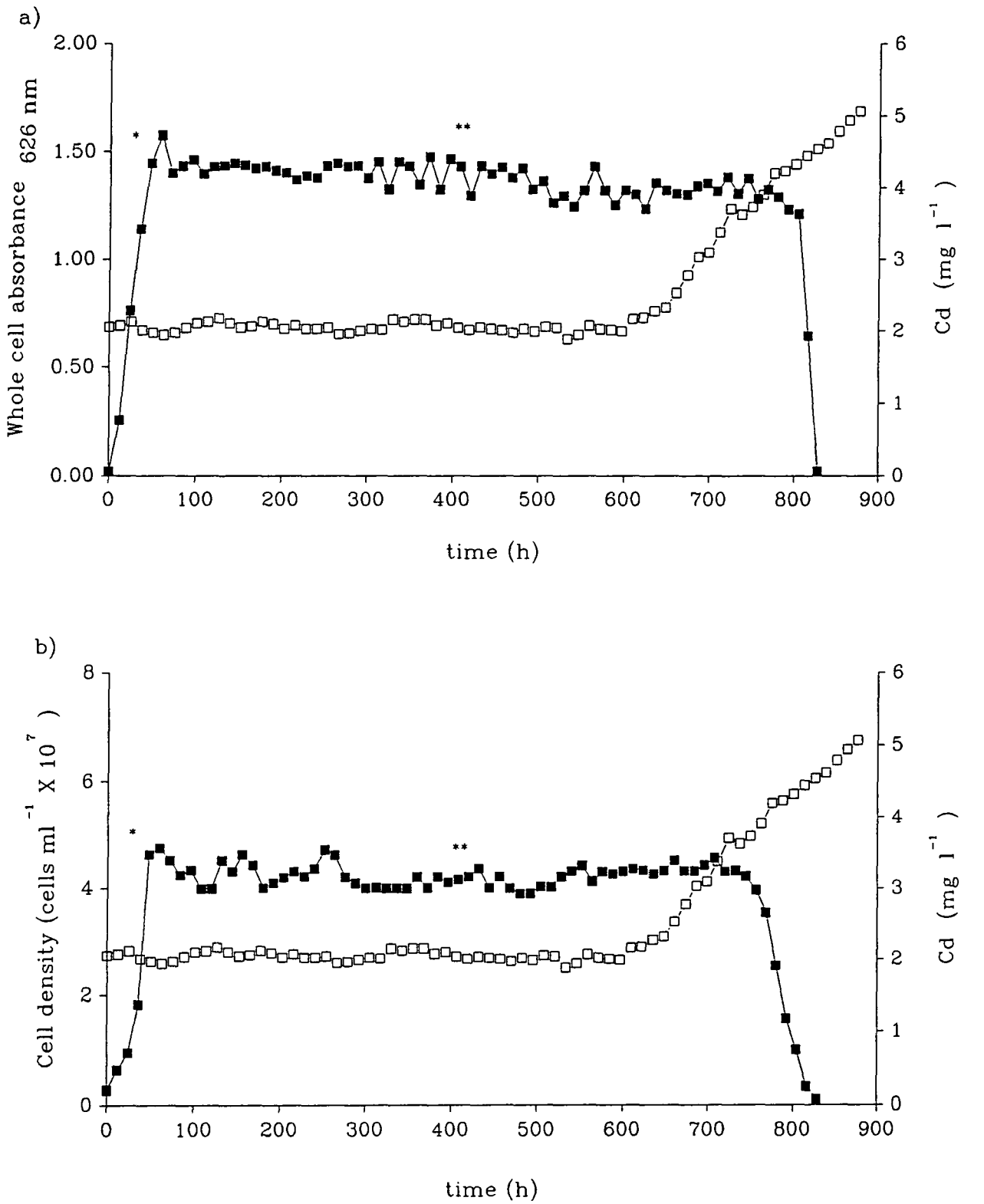


Table 4.5 Specific growth rates (calculated from the above graph) of Synechococcus D562 in 2-litre, PPJ medium batch-culture at different values of light intensity

Light intensity (μmol photon m^{-2} s^{-1})	specific growth rate (h^{-1})
20	0.0495
60	0.0644
100	0.0683
140	0.0848
180	0.0642

Fig. 4.4 Influence of Cd concentration (□) on a) whole cell absorbance at 626 nm (■), b) cell density (■) of a continuous culture of Synechococcus D562 in PPJ medium, light intensity = 100 µmol photon $m^{-2} s^{-1}$, temperature = 32 ° C, * = medium pump activated, ** = medium reservoir change over point



CHAPTER 5

5. Immobilized cells

5.1 Introduction

A system of immobilized algae was developed as a first step towards combating the release of industrial effluents into the environment which contain high concentrations of Cd. The efficiency of metal removal from the growth medium was investigated in the two axenic strains which had proved to accumulate Cd to the highest concentration. Cells were immobilized in calcium-alginate beads and contained in a packed-bed reactor.

5.2 Accumulation of Cd by the matrix

To determine the contribution that the immobilization matrix affords towards the binding of Cd, 150 alginate beads were incubated in CHU 10E pH 7.0 medium containing three concentrations of Cd. The glass surface of flasks used in this study did not bind Cd to a significant degree (Fig. 5.1a), but when calcium-alginate beads were added, a fast initial accumulation of the metal was observed followed by a slower uptake (Figs 5.1b, 5.2a and b). When FeEDTA was omitted from the medium more Cd was removed, except for flasks without beads.

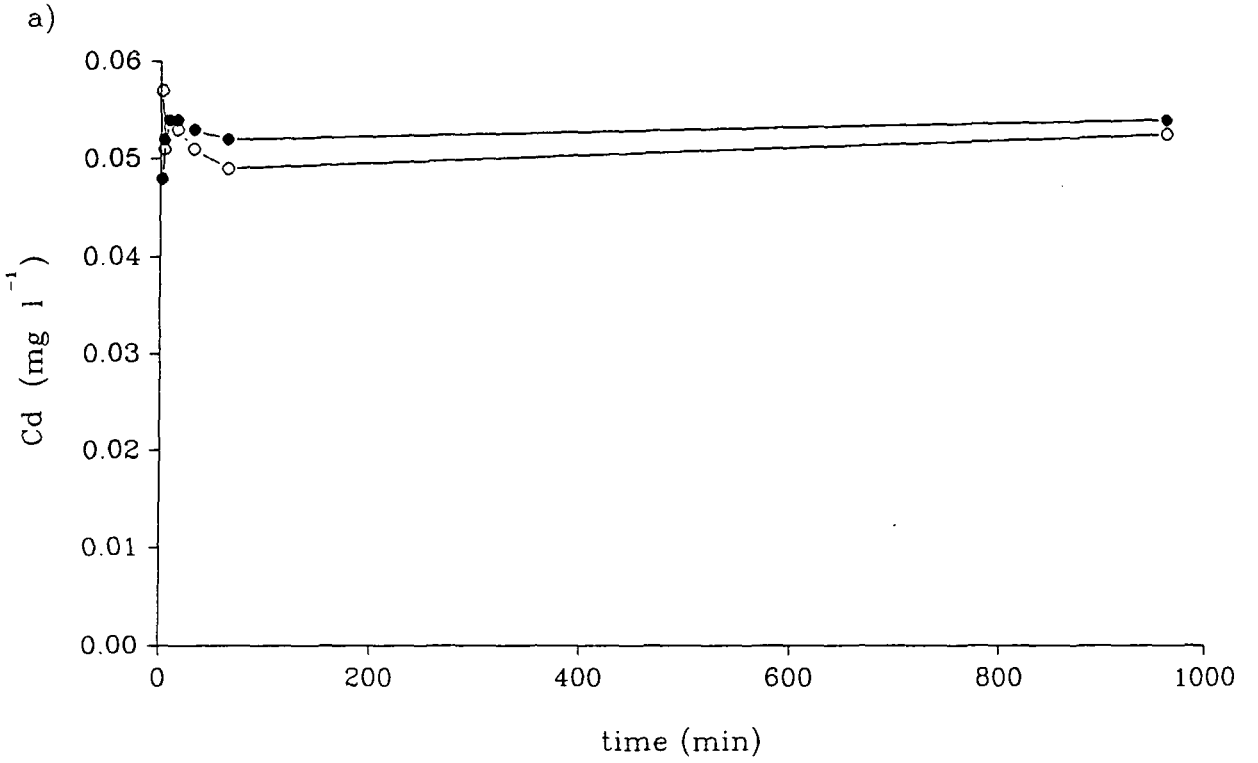
5.3 Accumulation of Cd by immobilized cells

Initial experiments showed that at a Cd concentration of 0.5 mg l^{-1} (Fig. 5.3) Synechococcus D562 cells could not perceptibly reduce the concentration of metal added to the medium. Therefore 0.05 and 0.1 mg l^{-1} Cd were chosen as appropriate concentrations of metal for investigation. Operating conditions for the immobilized-cell columns appear in Table 5.1. The observed gradation of growth up the immobilized cell column (with highest biomass at the point of medium inlet and reduced growth at the top of the bead column) prompted a column run whose medium reservoir was aerated to determine if the CO_2 partial pressure of the circulating medium was a limiting factor. The process of bubbling the medium inhibited Cd removal (Fig. 5.3) and did not encourage uniform

growth up the height of the column, so subsequent runs were not aerated. Four closed-loop reactors, alginate beads in flasks and flasks without beads were run at 0.1 mg l^{-1} Cd for 20 d as a control in order to determine the concentration of metal removed by the apparatus alone. Over this period, little Cd was removed from the medium (Fig. 5.4) except for the rapid binding of metal which occurs (Figs 5.1b and 5.2a and b) before the first sampling point on these graphs (no algae were introduced at this stage).

Synechococcus D562 cells immobilized in a packed-bed column (Figs 5.5 and 5.6), reduced the Cd concentration of the medium by 42 % (0.1 mg l^{-1} Cd) and 73 % (0.05 mg l^{-1} Cd) after 20 d, whilst immobilized Mougeotia D536 cells (Figs 5.7 and 5.8) proved more effective and reduced the Cd by 95 % (0.1 mg l^{-1} Cd) and 83 % (0.05 mg l^{-1} Cd) of the original concentration. For Mougeotia D536 cells, the packed-bed reactors removed more Cd than immobilized cells in free suspension, which in turn sequestered more Cd than free algae. Such a heirarchy of metal removal was less evident in Synechococcus D562. Cells recovered from these experiments exhibited the highest Cd concentration when grown in the packed-bed immobilized reactor (Table 5.2), whilst immobilized cells in stationary flasks accumulated less metal than free algae. Although Synechococcus D562 cells accumulated more metal than Mougeotia D536 when expressed in terms of μg of Cd g^{-1} dry weight, the total amount of Cd accumulated by Mougeotia D536 was greater than Synechococcus D562, resulting in a lower final concentration of Cd in the medium.

Fig. 5.1 Reduction in the initial Cd concentration (0.05 mg l^{-1}) of CHU 10E pH 7.0 medium at 25 ° C and 80 µmol photon m^{-2} s^{-1} in the presence (●▲) and absence (○△) of Fe EDTA, a) without alginate beads, b) with 150 alginate beads . The beads were not inoculated with cells



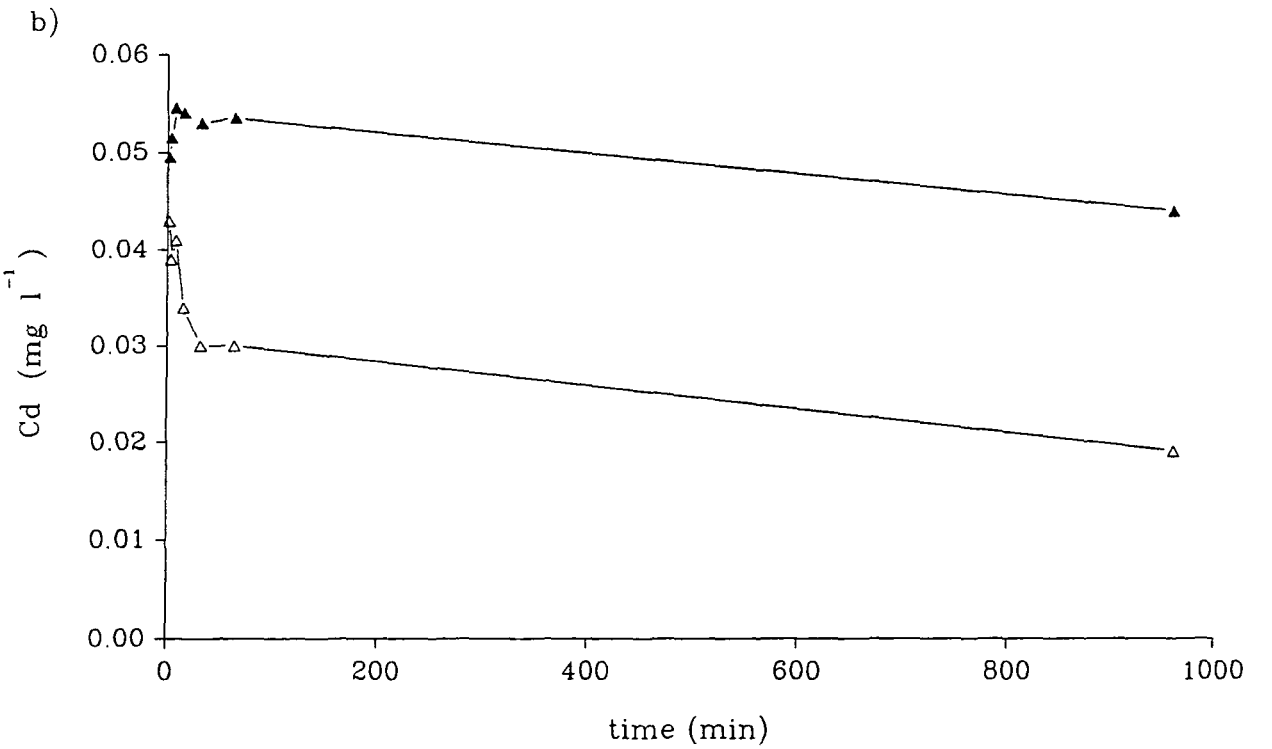
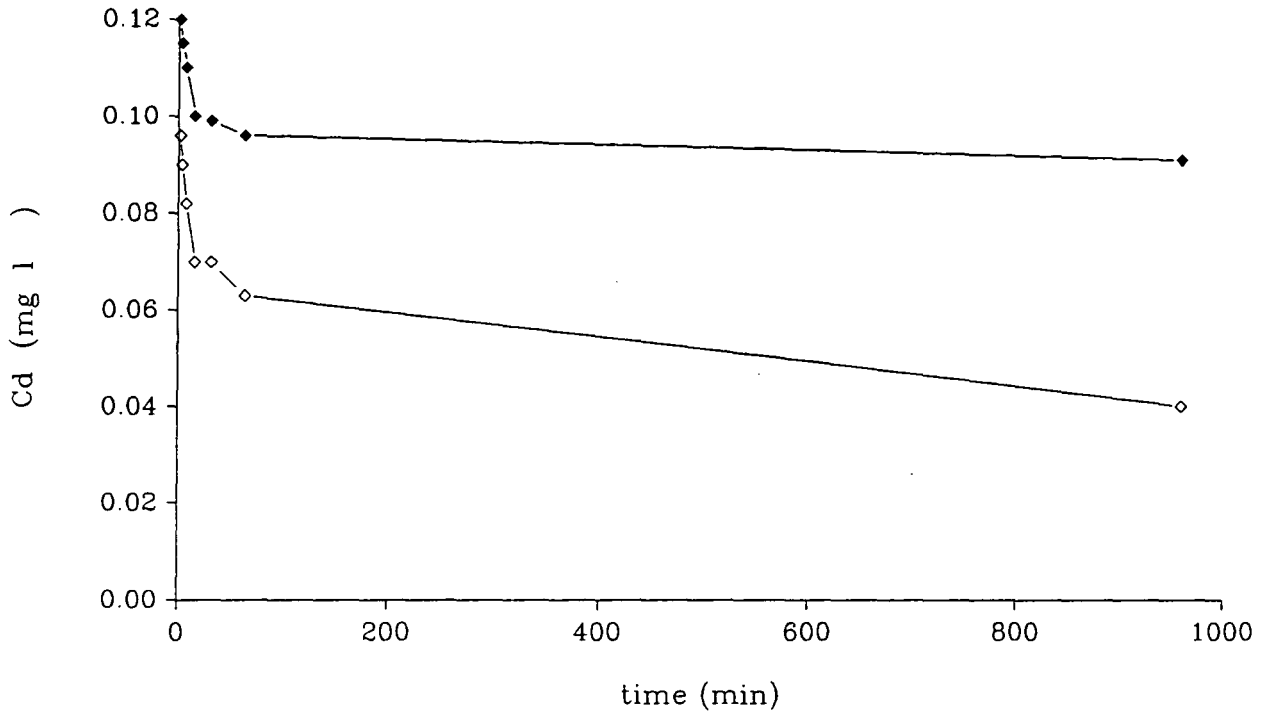


Fig. 5.2 Reduction in the initial Cd concentration of CHU 10E pH 7.0 medium at 25 ° C and 80 µmol photon m^{-2} s^{-1} in the presence (◆) and absence (◇) of Fe EDTA, a) with 150 alginate beads at 0.1 mg l^{-1} Cd, b) with 150 alginate beads at 1.0 mg l^{-1} Cd . The beads were not inoculated with cells

a)



b)

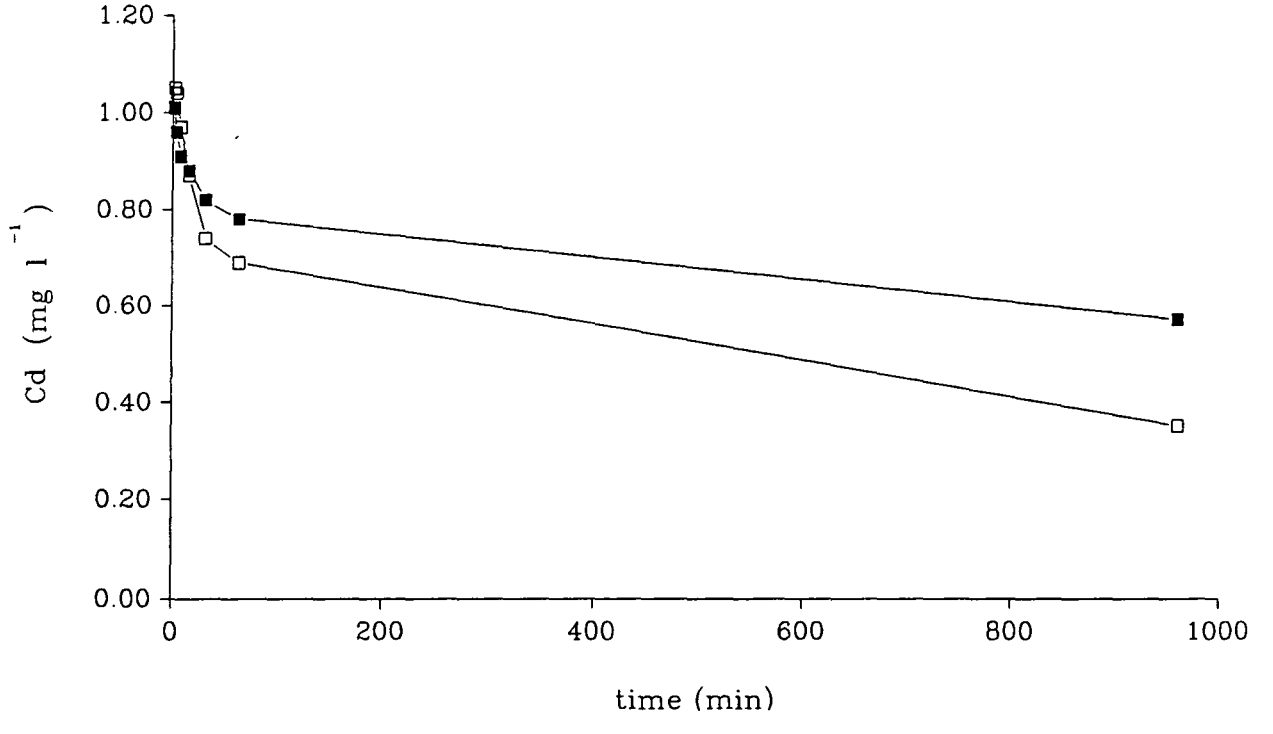


Fig. 5.3 Reduction in the Cd concentration of PPJ medium pH 7.0 at 32 ° C and 20 μ mol photon m^{-2} s^{-1} by aerated (●▲■) and non-aerated (○△□) immobilized Synechococcus D562 cells at a) 0.05 , b) 0.1 and c) 0.5 mg l^{-1} Cd

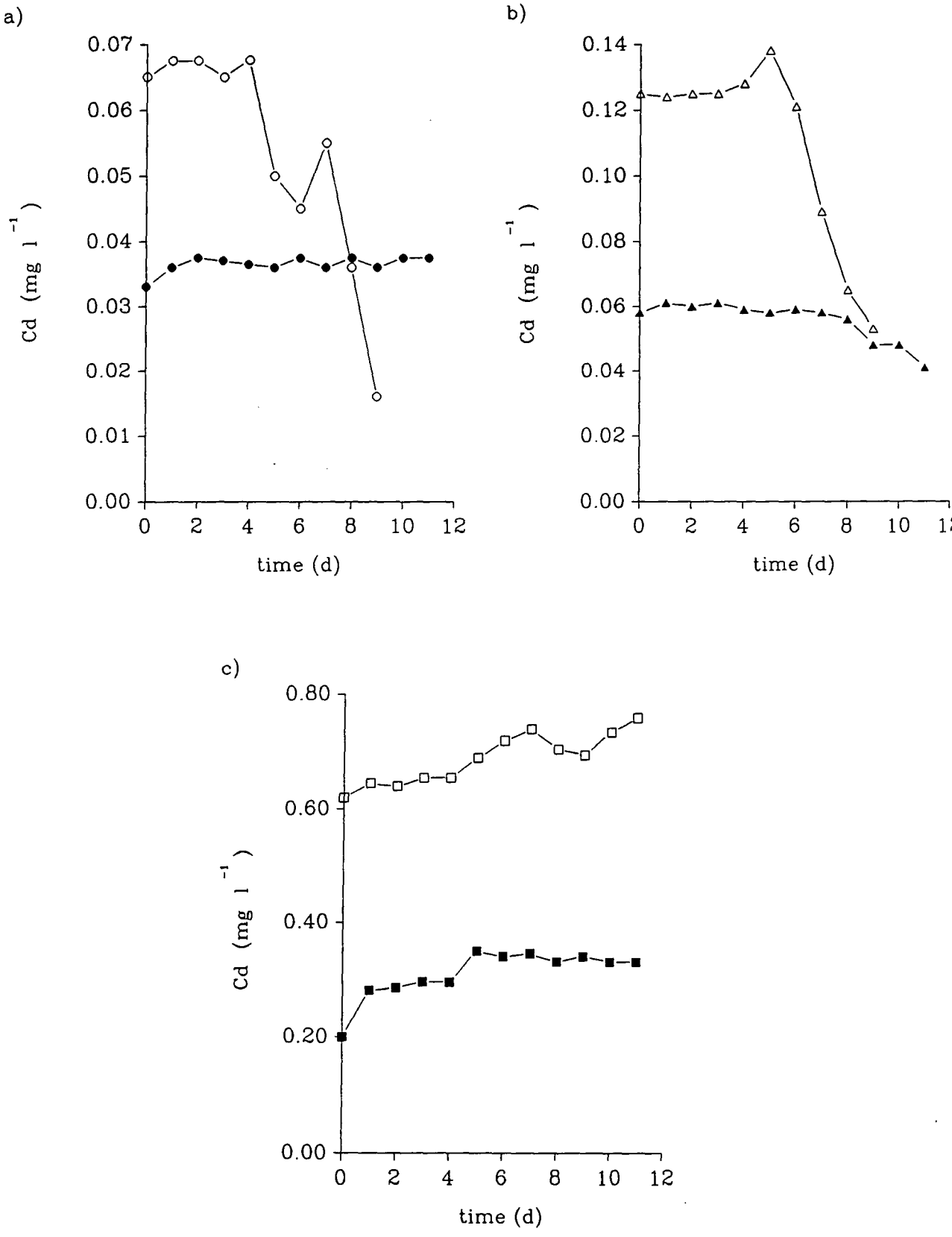


Fig. 5.4 Reduction in the initial Cd concentration (0.1 mg l^{-1}) over 20 d of PPJ medium pH 7.0 at 32 °C and 20 μ mol photon m^{-2} s^{-1} a) packed-bed alginate beads; b) beads in free suspension and c) medium without beads

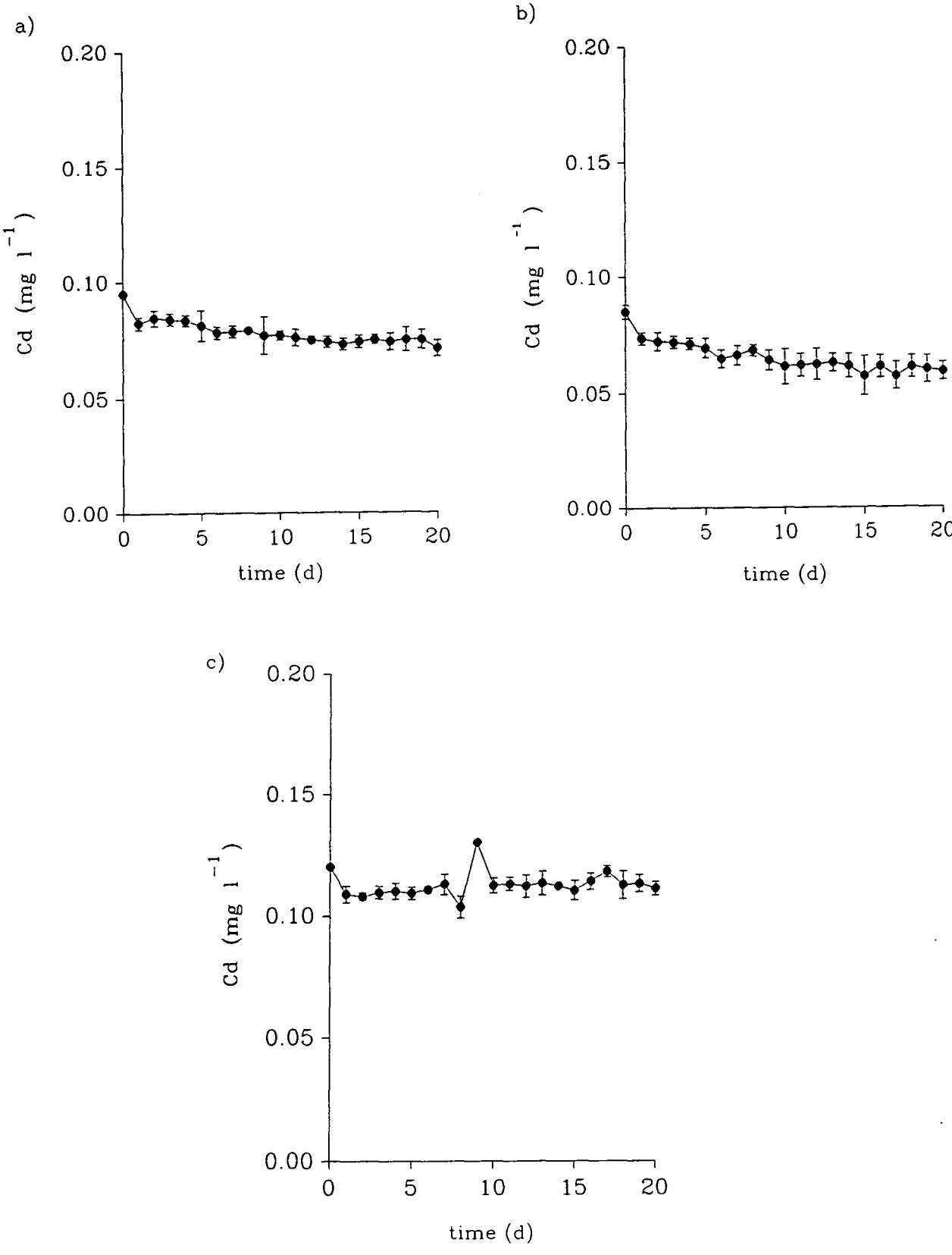


Fig. 5.5 Reduction in the initial Cd concentration (0.1 mg l^{-1}) of PPJ medium pH 7.0 at 32 °C and 20 μ mol photon m^{-2} s^{-1} by Synechococcus D562, a) packed-bed immobilized cells, b) immobilized cells in free suspension, c) free cells, error bars = sem

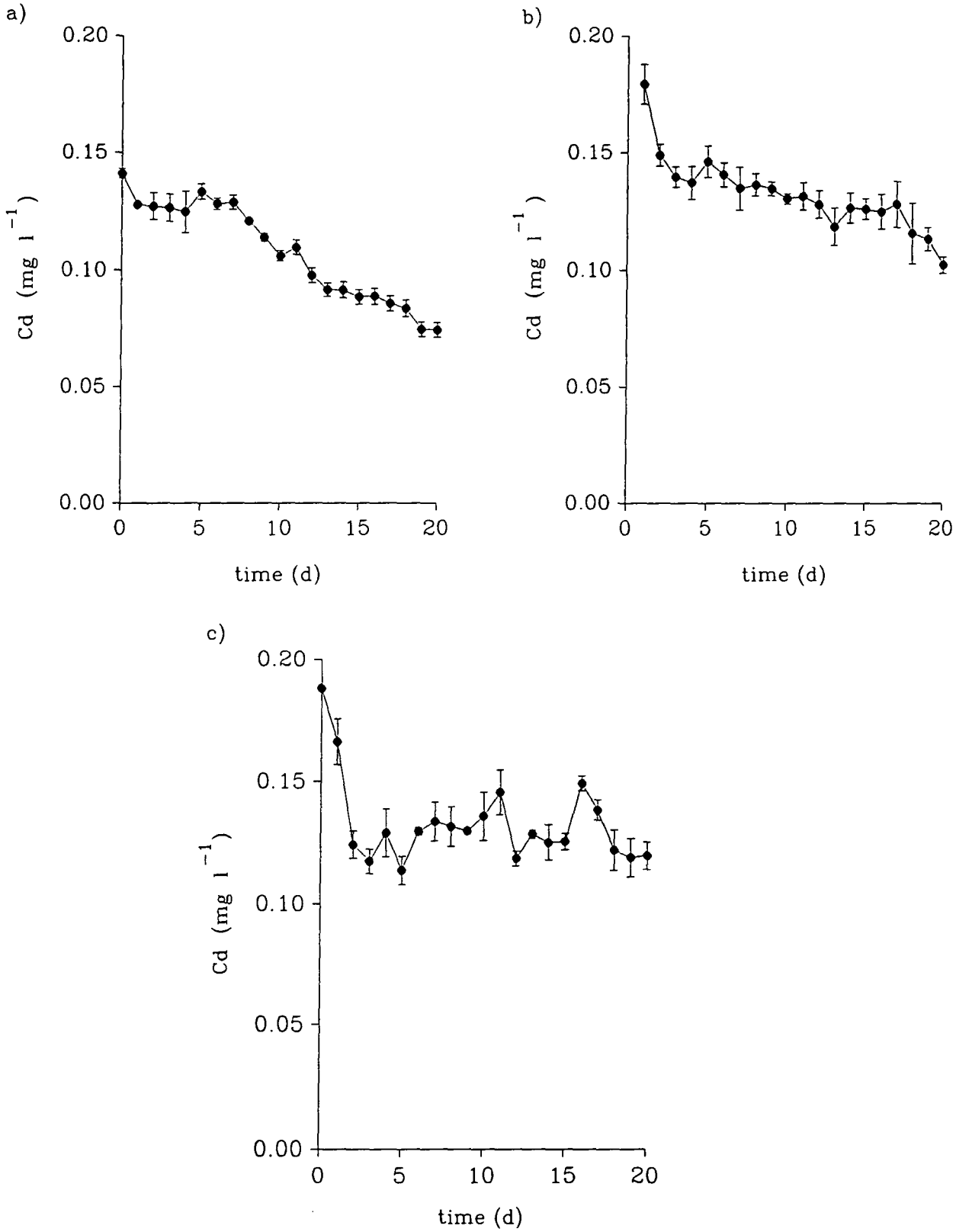


Fig. 5.6 Reduction in the initial Cd concentration (0.05 mg l^{-1}) of PPJ medium pH 7.0 at 32 °C and 20 μ mol photon m^{-2} s^{-1} by Synechococcus D562, a) packed-bed immobilized cells, b) immobilized cells in free suspension, c) free cells, error bars = sem

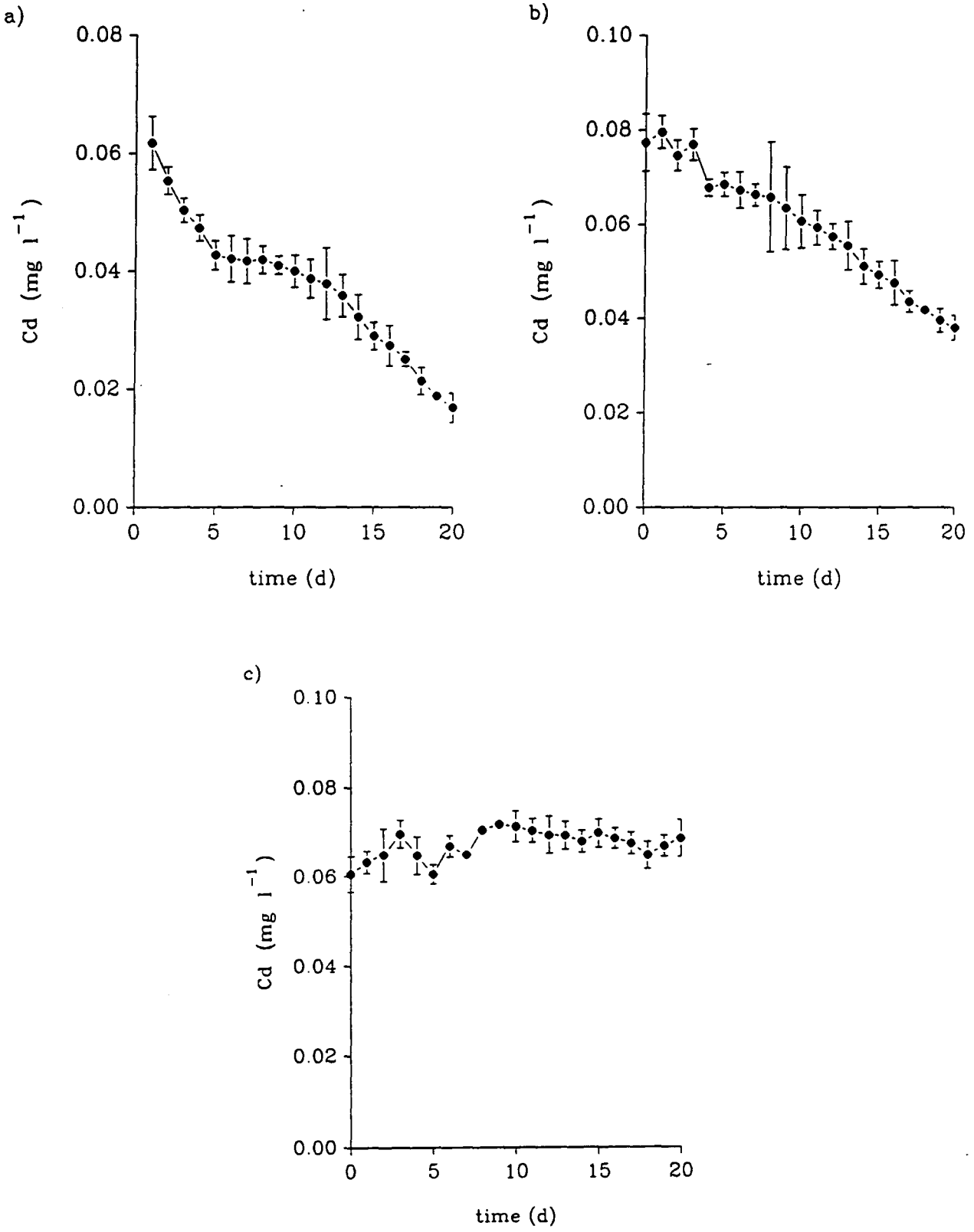


Fig. 5.7 Reduction in the initial Cd concentration (0.1 mg l^{-1}) of CHU 10E medium pH 7.0 at 25 °C and 20 μ mol photon m^{-2} s^{-1} by Mougeotia D536, a) packed-bed immobilized cells, b) immobilized cells in free suspension, error bars = sem

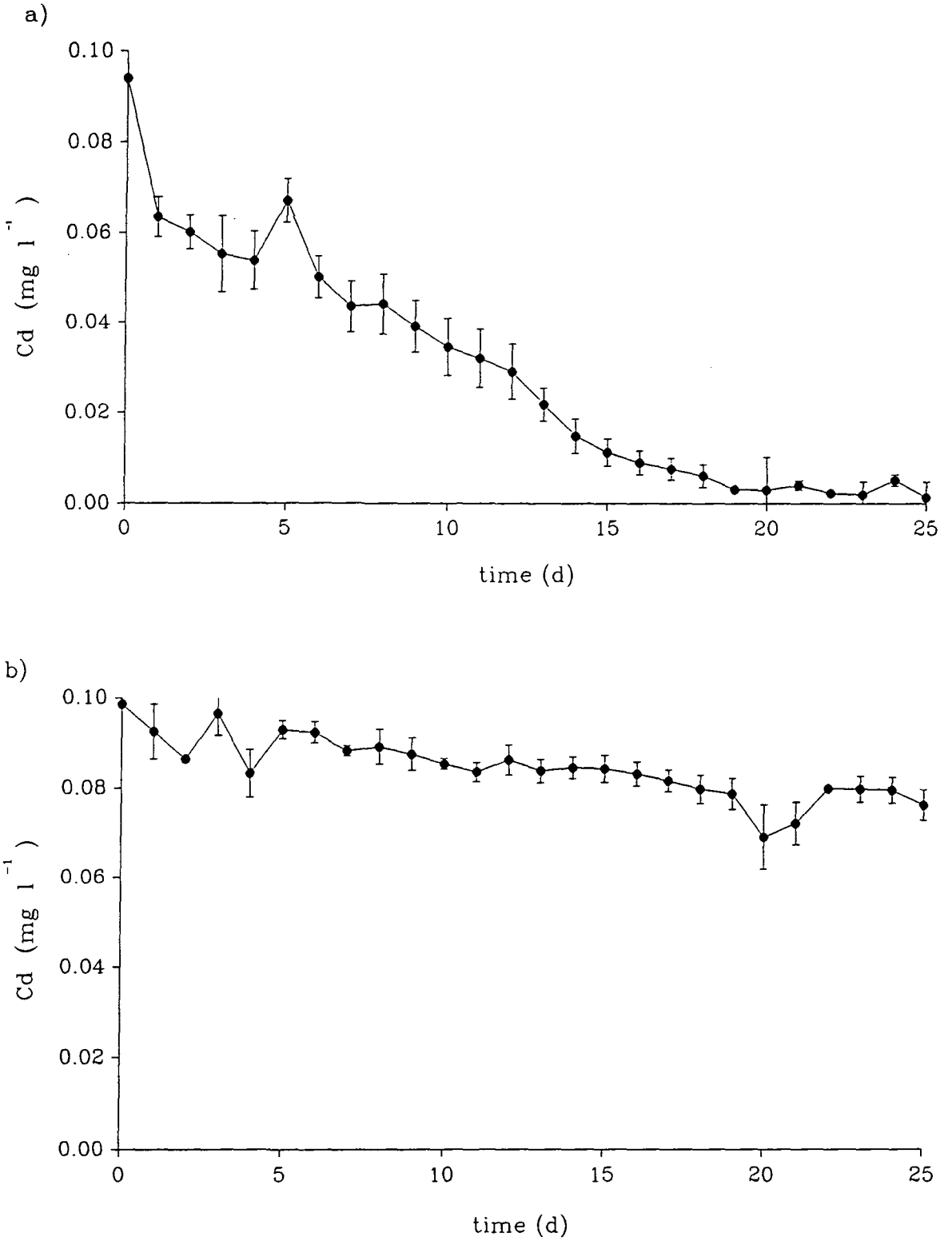


Fig. 5.8 Reduction in the initial Cd concentration (0.05 mg l^{-1}) of CHU 10E medium pH 7.0 at 25 °C and 20 μ mol photon m^{-2} s^{-1} by Mougeotia D536, a) packed-bed immobilized cells, b) immobilized cells in free suspension, c) free cells, error bars = sem

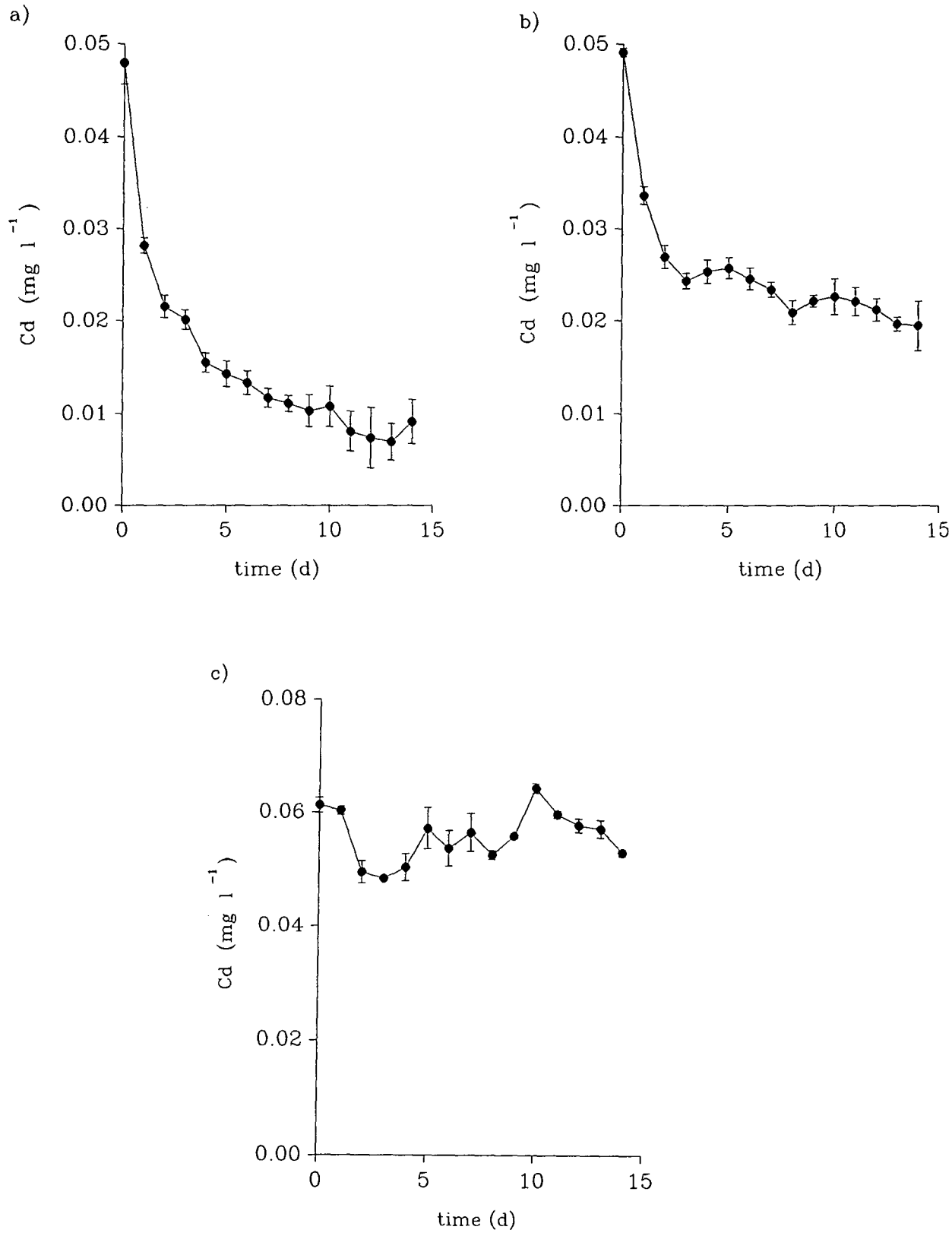


Table 5.1 Physical parameters of the immobilized cell columns used in this study, n = 4

Parameter	value ± sem
Bead diameter (mm)	3.27 ± 0.12
Gel volume per column (ml)	88.00 ± 2.52
Void volume per column (ml)	49.75 ± 2.59
Total volume per column (ml)	144.80 ± 2.34
Number of beads in a 100 ml packed bed	5418 ± 100
Flow rate with beads (ml min^{-1})	4.008 ± 0.058
Flow rate without beads (ml min^{-1})	4.013 ± 0.089

Table 5.2 Concentration of Cd accumulated by two immobilized strains and free cells exposed to 0.1 and 0.05 mg l^{-1} Cd, † = not determined, values of dry weight are from each culture vessel, n = 4

Strain	Cd (mg l^{-1})	treatment	dry weight ± sem (mg)	accumulated Cd ± sem (µg g^{-1})
Synechococcus D562	0.1	immobilized in columns	14.08 ± 0.66	1167.00 ± 36.96
	0.1	immobilized in flasks	3.69 ± 1.04	210.47 ± 8.33
	0.1	free cells	4.54 ± 0.37	519.64 ± 30.21
Synechococcus D562	0.05	immobilized in columns	14.34 ± 2.30	1430.00 ± 51.82
	0.05	immobilized in flasks	8.18 ± 2.60	70.78 ± 12.96
	0.05	free cells	2.61 ± 0.17	293.45 ± 38.29
Mougeotia D536	0.1	immobilized in columns	68.95 ± 5.11	492.94 ± 22.77.
	0.1	immobilized in flasks	51.64 ± 0.77	250.40 ± 14.00.
	0.1	free cells	†	†
Mougeotia D536	0.05	immobilized in columns	24.65 ± 0.74	312.12 ± 17.77.
	0.05	immobilized in flasks	11.75 ± 2.36	85.00 ± 21.44.
	0.05	free cells	36.77 ± 3.69	178.35 ± 4.56.

CHAPTER 6

6. Microscopy and Cd localisation

6.1 Introduction

Light microscope and EM photomicrographs of immobilized cells were taken to check that cells immobilized in calcium-alginate did not exhibit abnormal morphology, a condition which may reduce the concentration of heavy-metal accumulated. EDXMA and SPM were employed to determine the cellular sites of Cd accumulation; this information indicates part of the pathway used to detoxify intracellular Cd and may, in some circumstances, be used to increase the concentration of metal accumulated and therefore enhance the Cd-removal efficiency of immobilized cell columns.

6.2 Light microscopy

The light microscope was employed to follow the formation of spheroplasts and ascertain cell growth patterns within alginate beads. Unlike some organisms eg. Aspergillus niger and Chlorella emersonii (Eikmeier et al., 1984 ; Day & Codd, 1985), diffusion limitations did not appear to restrain cell growth in alginate beads, as colonies grew throughout the matrix (Figs 6.1 a and b).

6.3 Lysozyme hydrolysis and ruthenium red staining

As the first step in plasmid isolation, lysozyme was employed to degrade the cell wall of Synechococcus D562 cells. Although spheroplasts were not produced, the incubation of Synechococcus D562 with 2 mg ml^{-1} lysozyme resulted in cell clumping, older cells aggregating more readily than young cultures. In order to determine the site of hydrolysis, Synechococcus D562 cells which had been exposed to lysozyme were stained with Ru red and viewed under the TEM as air-dried cells. It was discovered that the extracellular mucilage of this strain could only be viewed under the TEM after enzyme treatment and Ru staining, whilst the extracellular material of undigested cells

did not bind Ru (Figs 6.2a and b). Lysozyme sensitive NAG-NAM regions must exist within the mucilage which, upon hydrolysis, provide Ru binding-sites. A requirement for the lysozyme step in the production of spheroplasts, indicates that components of the cell wall are probably also attacked in addition to the extracellular mucilage.

6.4 Immobilized cells

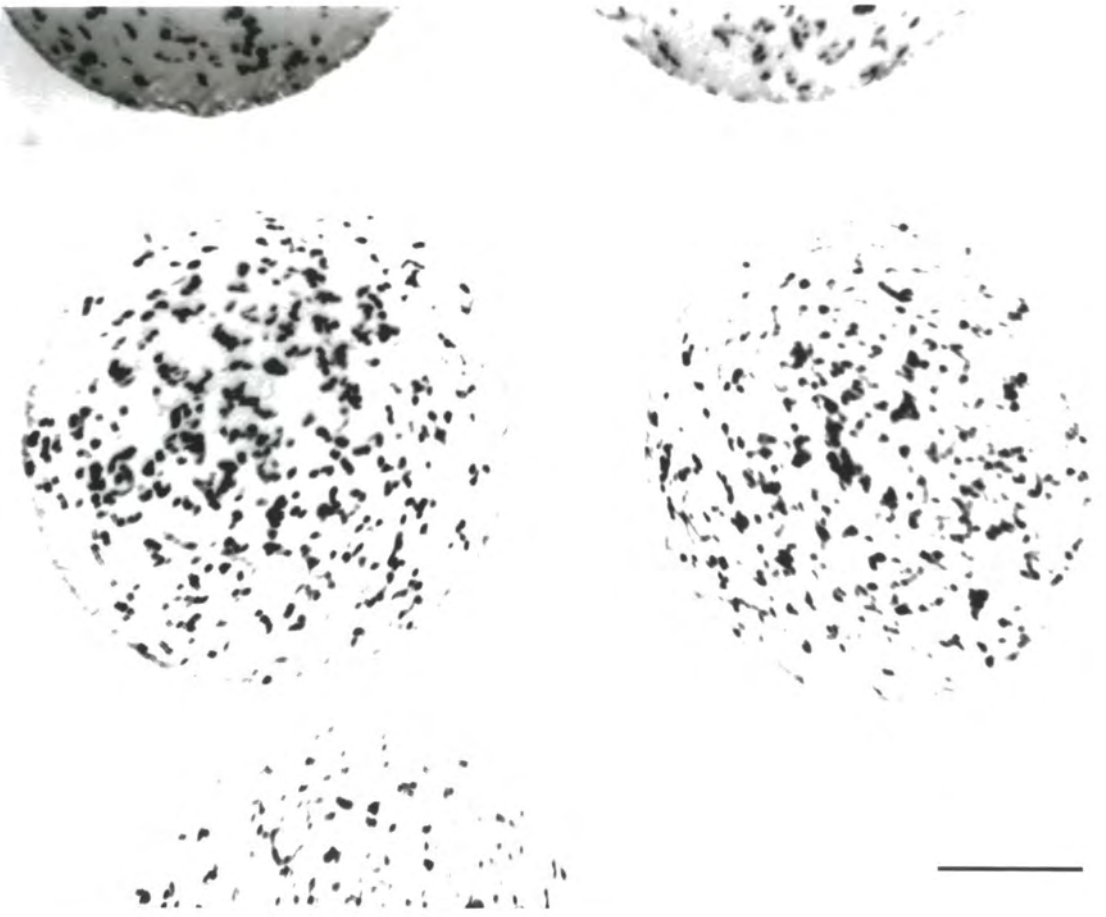
In order to ensure that the process of immobilization did not produce cell deformations, TEM sections were taken of Synechococcus D562 and Mougeotia D536 cells that had been cultured in alginate beads. No physical attachment was observed between cell and matrix after 10 d and 15 d growth, but the strains were not completely encased in alginate, even for samples sectioned immediately after immobilization (Figs 6.3a and b, 6.4a and b). Neither of these strains exhibited ultrastructural changes as a result of their immobilization.

Fig. 6.1 a) Light micrograph of Mougeotia D536 cells immobilized in calcium-alginate beads after 10 d growth. Scale bar = 1 mm

b) fluorescence micrograph of Synechococcus D562 cells immediately after immobilization in a calcium-alginate bead. Scale bar = 10 μm

s = surface of microscope slide cm = calcium alginate matrix

f = fluorescing Synechococcus D562 cells



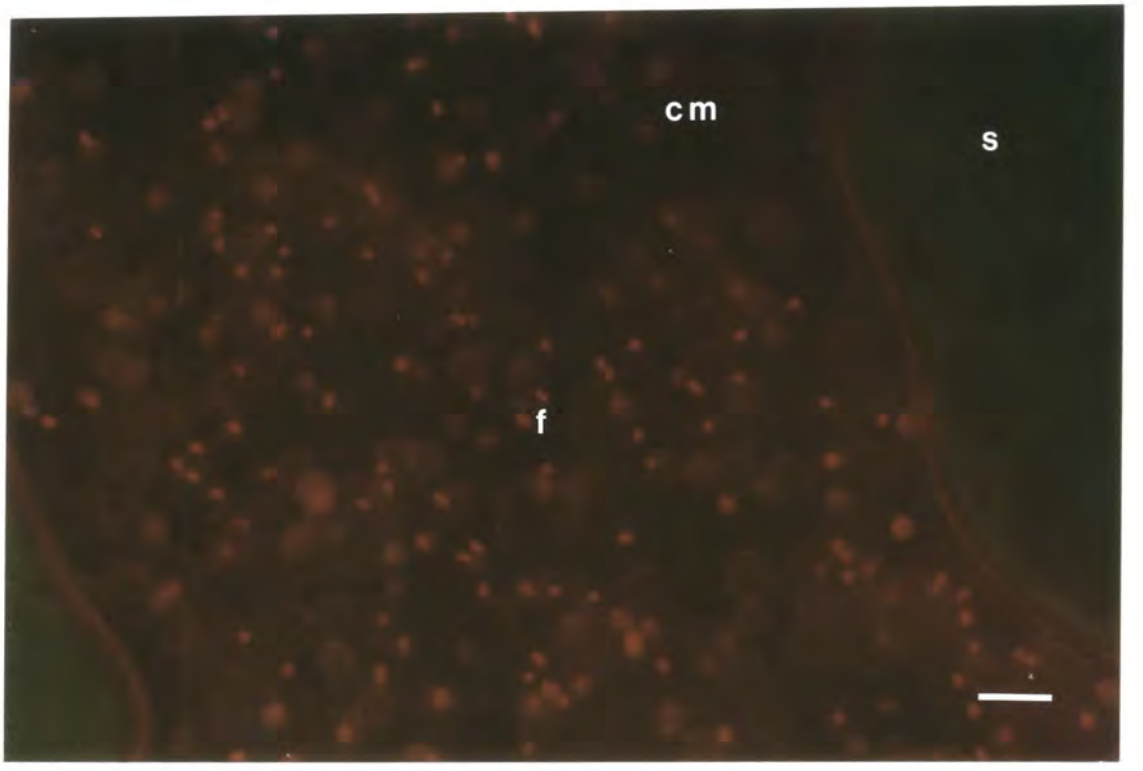

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Fig. 6.2 a) Ru red staining of a Synechococcus D562 cell without lysozyme treatment.

Scale bar = 0.5 μm

pb = PP body

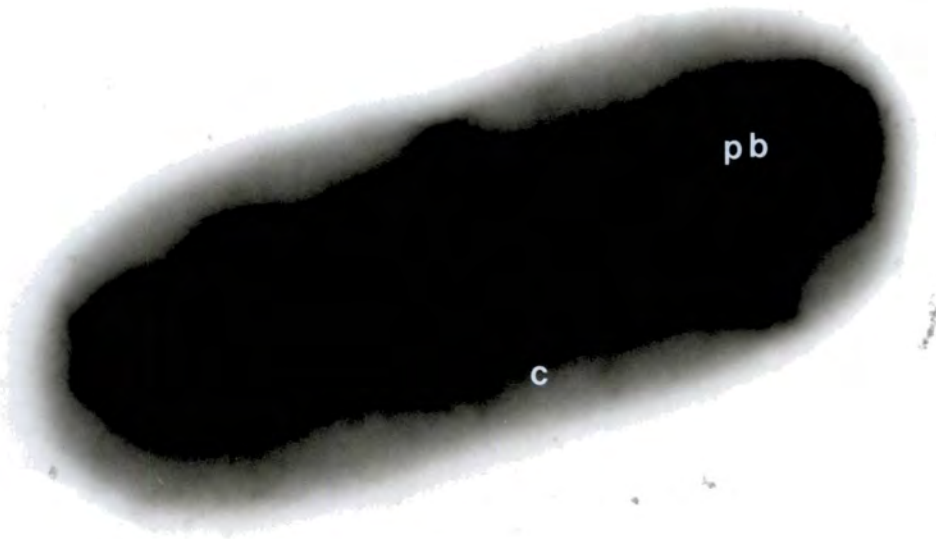
c = cytoplasm

b) A Synechococcus D562 cell incubated with 2 mg ml^{-1} lysozyme for 1 h at 37 ^{o} C then stained with Ru red. Scale bar = 0.5 μm

e = extracellular mucilage

pb = PP body

c = cytoplasm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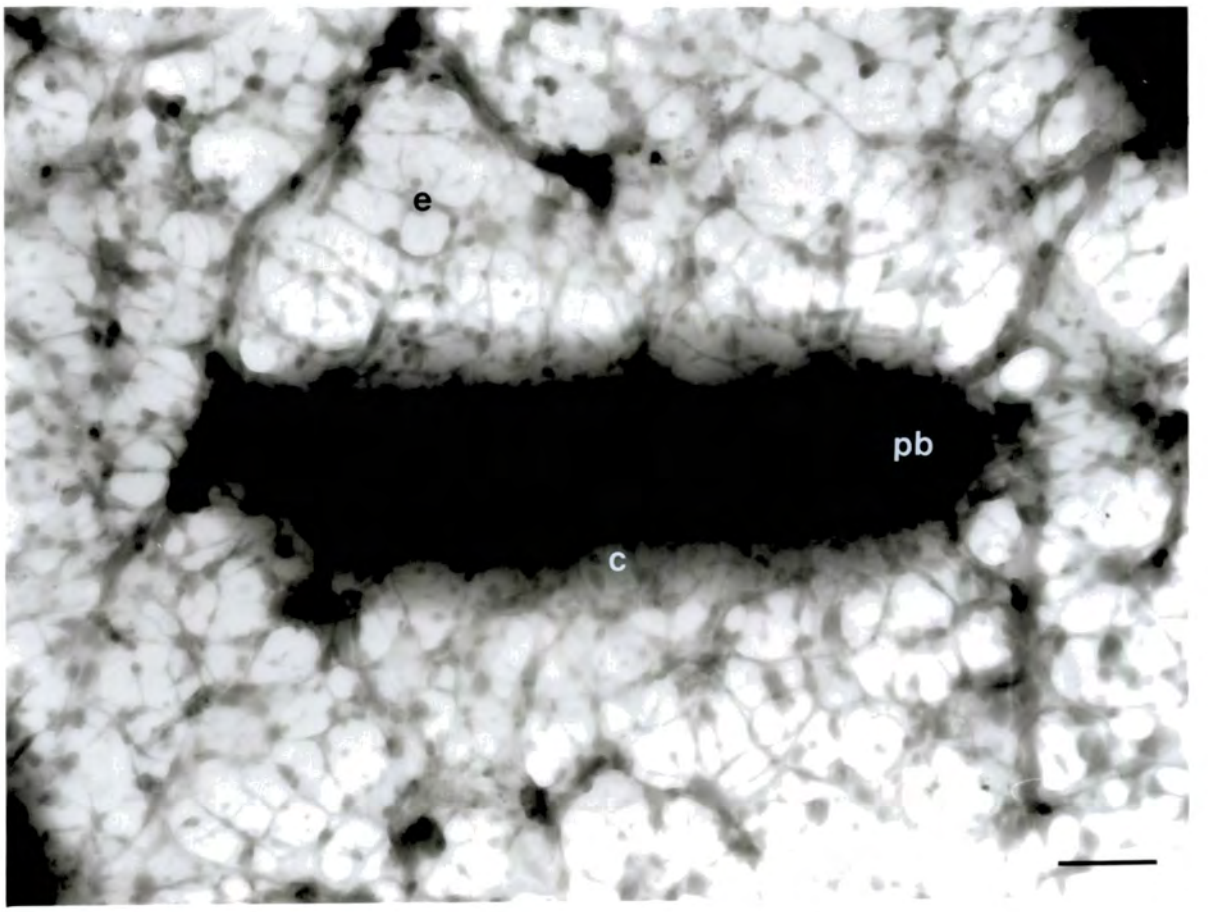
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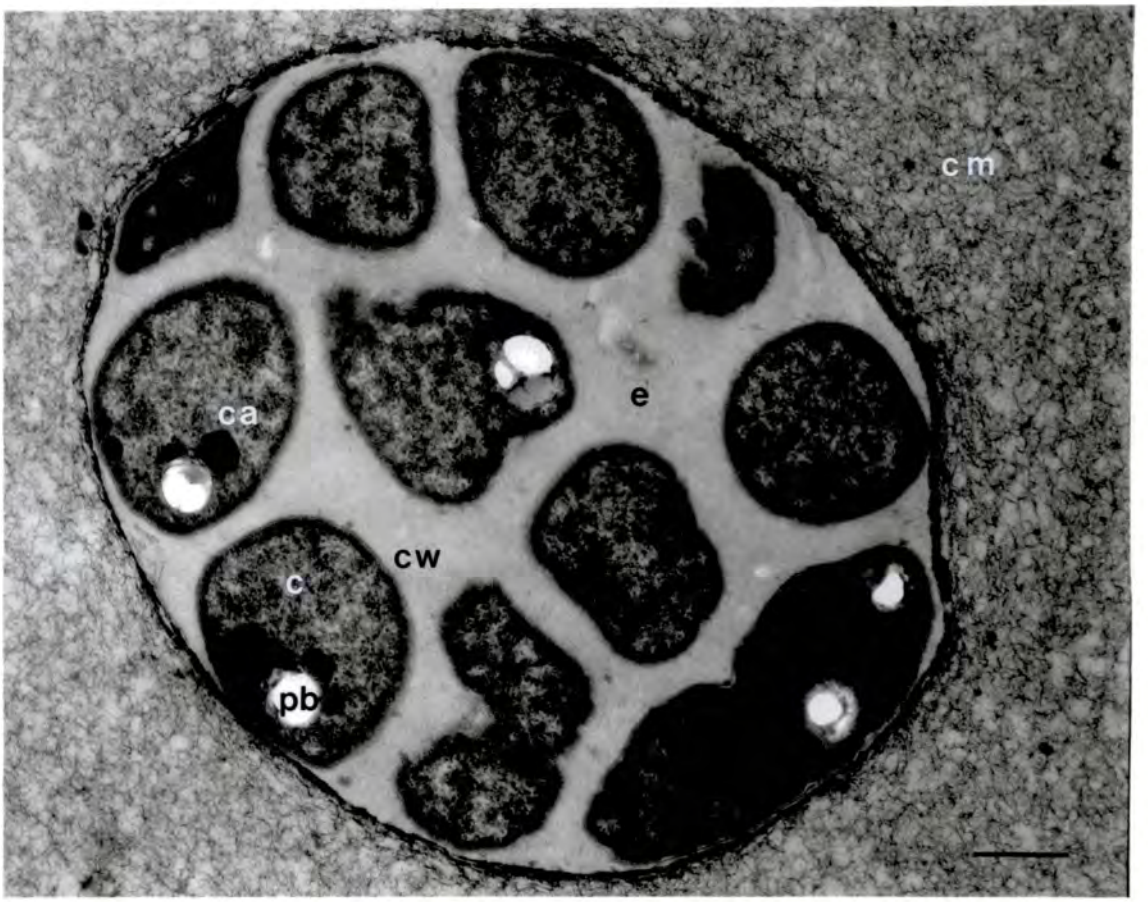
Fig. 6.3 a) Electron micrograph of Synechococcus D562 cells immobilized in 4 % (w/v) calcium alginate after 10 d growth. Scale bar = 0.5 μm

cm = calcium-alginate matrix
cw = cell wall
pb = space previously occupied by a PP body
ca = carboxysome
c = cytoplasm
e = extracellular space

b) Electron micrograph of Mougeotia D536 cells immobilized in 4 % (w/v) calcium alginate after 15 d growth. Scale bar = 5 μm

cm = calcium-alginate matrix
cw = cell wall
th = thylakoid membrane
s = starch deposit
e = extracellular space
v = vacuole
py = pyrenoid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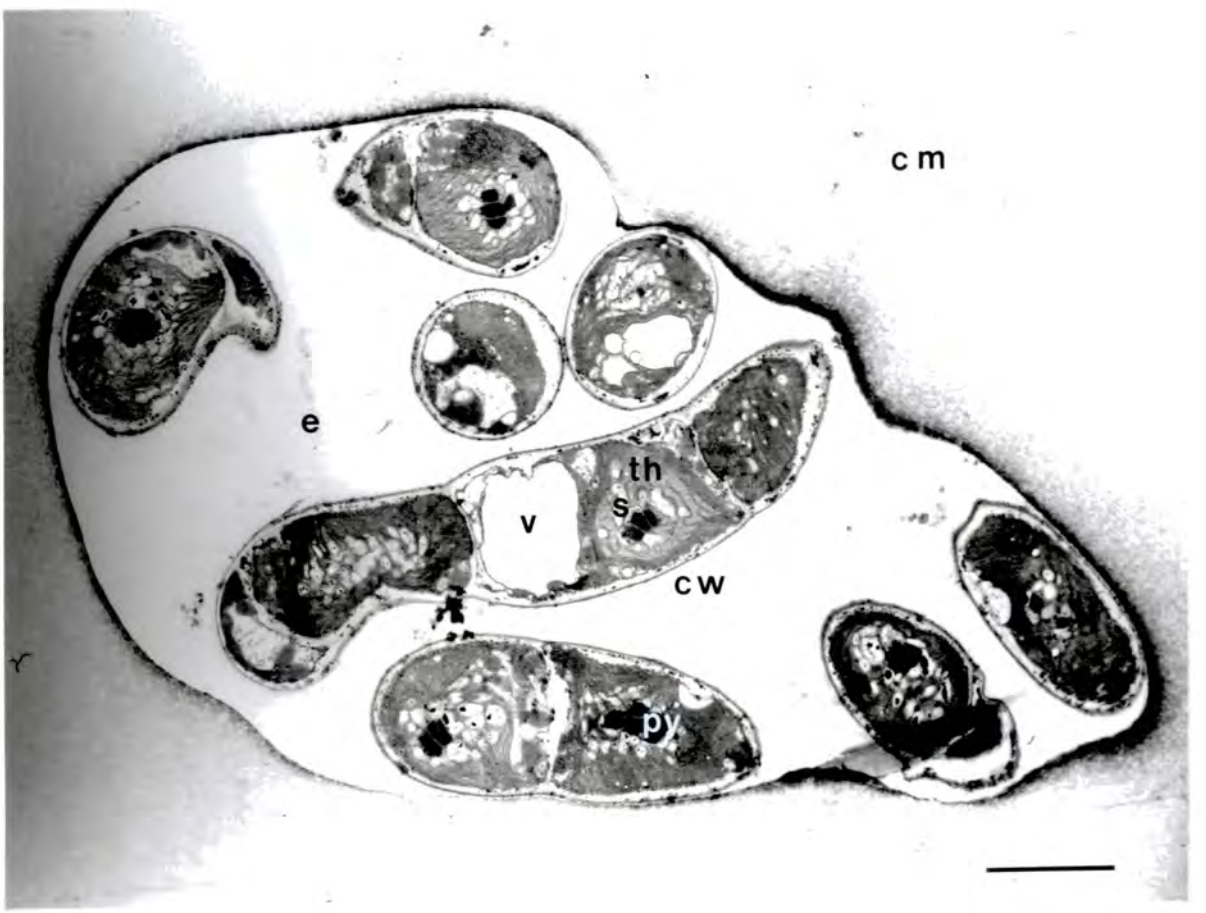
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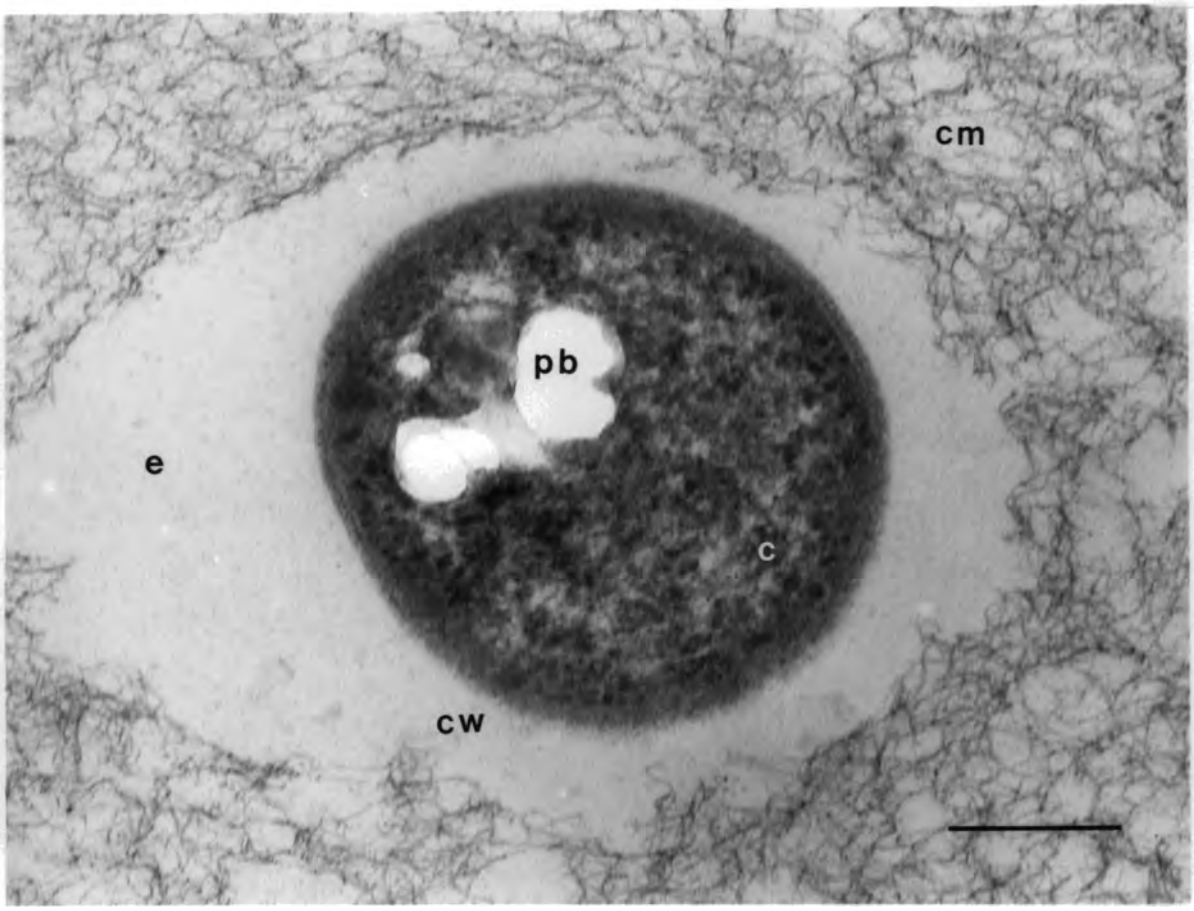
Fig. 6.4 a) Electron micrograph of a Synechococcus D562 cell immobilized in 4 % (w/v) calcium alginate and immediately processed for EM. Scale bar = 0.25 μm

cm = calcium-alginate matrix
cw = cell wall
pb = space previously occupied by a PP body
c = cytoplasm
e = extracellular space

b) Electron micrograph of Mougeotia D536 cells immobilized in 4 % (w/v) calcium alginate and immediately processed for EM. Scale bar = 1 μm

cm = calcium-alginate matrix
cw = cell wall
th = thylakoid membrane
s = starch deposit
e = extracellular space
c = cytoplasm
py = pyrenoid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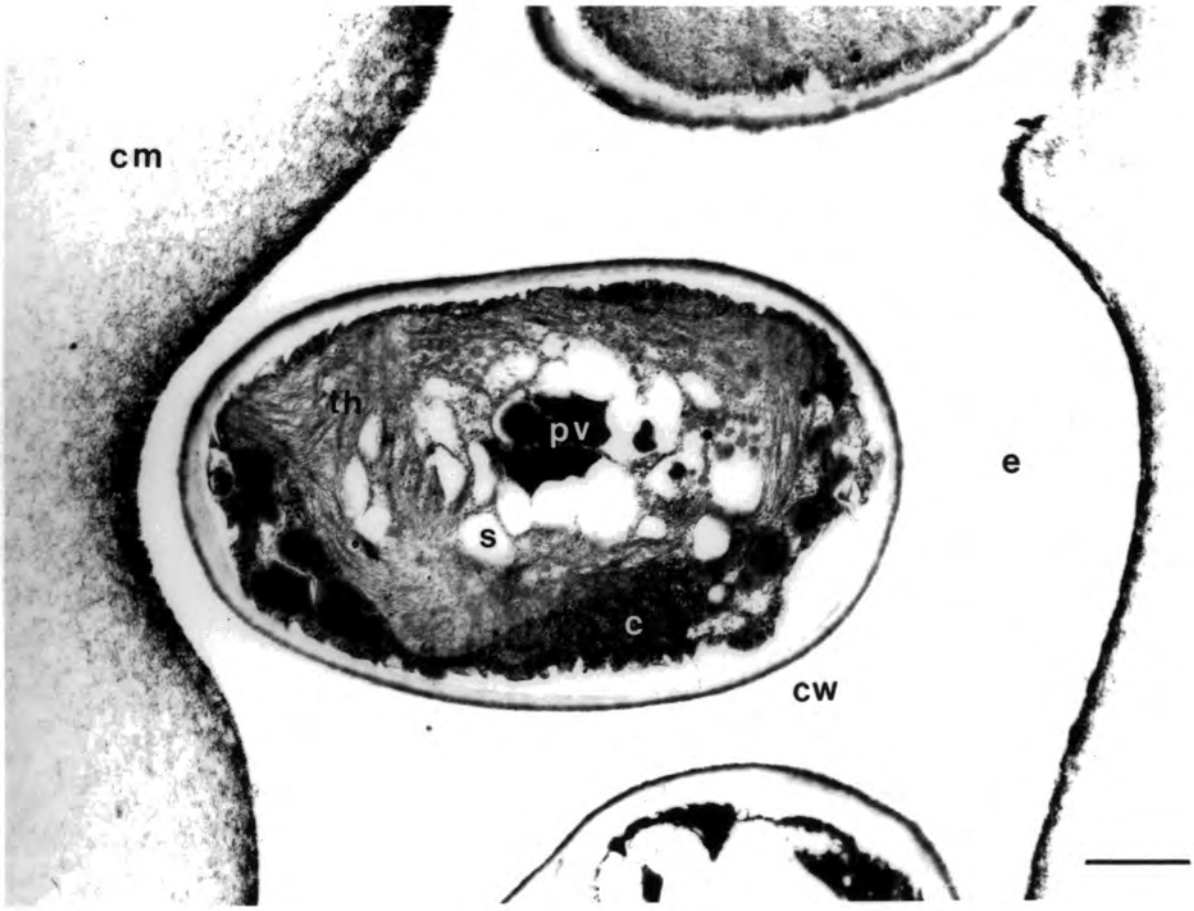
cm
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cw

6.5 Energy dispersive X-ray microanalysis

If the Cd storage sites within a cell can be defined, then the mechanism of metal accumulation used by a particular strain may be manipulated to amplify the concentration of metal stored in the cell and therefore increase the removal of Cd from the effluent stream. The search for Cd within individual cells by EDXMA and associated shifts in their elemental composition proved elusive. Background counts obtained from the formvar support film were low and no contaminating peaks occurred (Fig. 6.5a). Cryosections of algae were not successful as thin, stable sections were never cut. Dextran microdroplet standards did not yield peaks that were reproducibly proportional to the concentration of the element under study (note the maximum full scale number of counts for each scan) (Fig. 6.5b, c and d). In addition, the rate of beam-induced element loss for samples analysed at room temperature, would not be equivalent to that of the standards due to the variation in electron diffusion volume from one area of the cell to another.

Quantification of the EDXMA peaks was obtained by determining the machine efficiency-factor with respect to S, for all the elements detected (Table 6.1) and scaling individual net integrals to yield the percentage contribution that each element makes to the total scan. Cu peaks (k α = 8.041 keV and k β = 8.907 keV) emanating from the grid bars are not labelled, as the majority of the Cu counts derive from the support material and TEM column rather than the specimen. The total net-integrals of each profile is a measure of the signal strength recorded from each area that was probed. EDXMA profiles for algae that were not exposed to Cd appear in Appendix 6.

The elemental composition of PP bodies in Synechococcus D562 varied in the size of the P peak and K to Ca ratios (Figs 6.6b and c). The cytoplasm contained equivalent proportions of Mg and P but higher S values were recorded; the total net-integral for this region of the cell is inherently low as the cytoplasm is a dilute ionic matrix. PP granules which did yield Cd spectra, consistently exhibited a high Ca to K ratio but at a frequency of less than one granule in 25. The metal was not restricted to these inclusion bodies as Cd was also discovered in cytoplasmic regions (Fig. 6.6d). In addition, cellular levels of Fe appeared elevated for Cd-exposed cells compared with the metal-free, control cultures. Fe precipitates were observed on the surface of Synechococcus D562 cells only after about 5 d of growth (Fig. 6.7a). When Cd was present in the medium a relatively large concentration of this metal was observed in association with the surface Fe-granules (Fig. 6.7b). An increase in the Mn concentration of the medium

(5 mg l^{-1}) did not improve the detection of Cd in Synechococcus D562 PP bodies (Fig. 6.7c), but the appearance of Ba in the profiles may be related to a change in the concentration of Mn transported into the cells.

When daily samples of a Synechococcus D562 culture grown with 4 mg l^{-1} Cd were taken, no changes in the Cd concentration of the PP bodies or cytoplasm was observed throughout the growth curve (data not shown).

Calothrix D184 cells exhibited characteristically high concentrations of Ca in PP bodies and strong Cl peaks from cytoplasmic readings (Figs 6.8 and 6.9). Even though the Ca to K ratio was high for many PP granules of this strain (a similar metabolic state to the Cd binding PP bodies of Synechococcus D562), no Cd was detected.

Scans of Mougeotia D536 (Fig. 6.10) were delineated by a strong K signal and consistently lower Ca peaks. However, no X-ray peak for Cd was ever recorded in this strain.

Prominent Cl, K and Fe peaks were recorded in Klebsormidium D537 (Fig. 6.11) but neither Ca nor Cd spectra were generated.

When the X-ray spectra of cells grown in the presence of Cd were compared with control cultures which did not receive added metal (Appendix 6), no major Cd-induced differences in the elemental profile of each cell compartment were observed.

A number of other heavy metals were located in field material extracted from contaminated streams (Table 3.2), but X-ray peaks for Cd were not observed. As laboratory experiments involving relatively high concentrations of Cd (0.8 to 4 mg l^{-1}) failed to yield X-ray spectra for this metal, the lower concentrations of Cd normally found in the environment would have to be accumulated by field isolates to very high levels if they are to be detected by EDXMA. Zn and Fe were sequestered to a high degree in algae sampled from Rampgill Level and Caplecleugh Low Level, with K and Ca contributing many of the X-ray counts in Stigeoclonium (Figs 6.12 and 6.13); Mg peaks are also relatively high for this strain. The inclusion bodies of a moss protonema found in association with a colony of algae, proved to contain high levels of Zn and Fe (Fig. 6.14) with lower quantities of Zn recorded in a Klebsormidium strain isolated from Caplecleugh Low Level (Fig. 6.15). Cell-surface precipitates of Ba proved a significant feature of Spirogyra scans (Fig. 6.16) and indicates a high Ba load in Gillgill Burn. Cells of Klebsormidium rivulare (Fig. 6.17) from Low Gillgill Burn reflect pollution by a different metal as cell inclusions and walls containing Pb were prominent. A strain of Stichococcus isolated from Gillgill Burn accumulated large quantities of Cd when grown under laboratory conditions with 0.25 mg l^{-1} Cd (Table 3.3), but this metal was not successfully located by the EDXMA probe (Fig. 6.18).

Therefore, of all the strains scanned for accumulated Cd, only particular PP bodies (with a high Ca to K peak integral ratio) of Synechococcus D562 grown in the presence of 4 mg l^{-1} Cd produced X-ray peaks for this metal.

Table 6.1 Channel-energy widths employed to semi-quantify the elemental composition of EDXMA profiles and associated machine efficiency-factors, * efficiency-factors interpolated from the graph in Appendix 5

Element (k or l lines)	start keV	end keV	no. of channels	efficiency factor
Na k α	0.88	1.16	15	12.26
Mg k α	1.18	1.34	9	3.61
Al k α *	1.42	1.60	10	2.63
Si k α	1.54	2.08	28	2.05
P k α	1.84	2.14	16	1.27
S k α	2.16	2.38	11	1.00
k β	2.40	2.48	4	
Cl k α *	2.50	2.74	12	0.86
k β	2.76	2.92	8	
Cd l a 1	2.98	3.26	15	0.42
K k α	3.06	3.48	21	0.72
k β	3.50	3.86	9	
Ca k α	3.50	3.86	18	0.71
k β	3.88	4.18	15	
Ba l a 1	4.14	4.66	26	0.34
l b 1	4.68	5.02	17	
l b 2	5.04	5.30	13	
l g 1	5.38	5.66	14	
l g 2	5.68	6.08	20	
Mn k α	5.72	6.08	19	0.57
Fe k α	6.24	6.62	37	0.55
k β	6.64	7.52	32	
Zn k α	8.26	8.98	36	0.50
k β	9.26	9.94	34	
l a 1 & 2	0.88	1.16	15	
Pb l a 1	10.10	10.90	40	0.62
l b 1 & 2	12.06	13.12	53	
l g 1	14.50	14..98	24	

Fig. 6.5 EDXMA profiles of a grid support film and Cd standards, peak integrals not determined

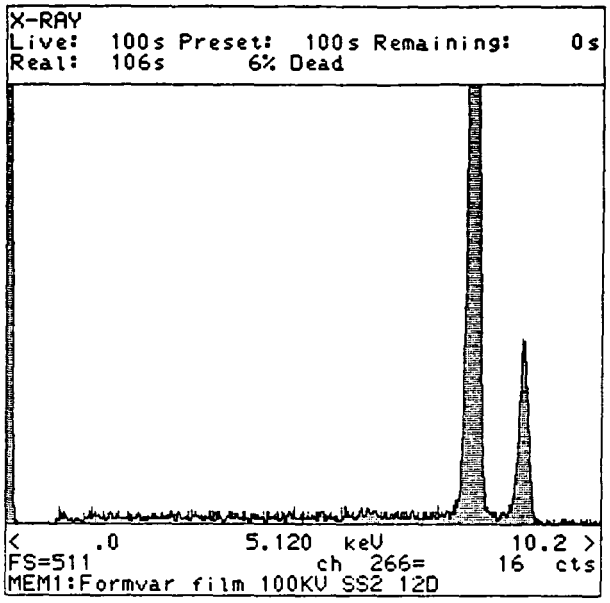
a) background scan of a 0.7 % formvar film on a Cu / Rh grid

b) 10 mM Cd standard in 5 % dextran

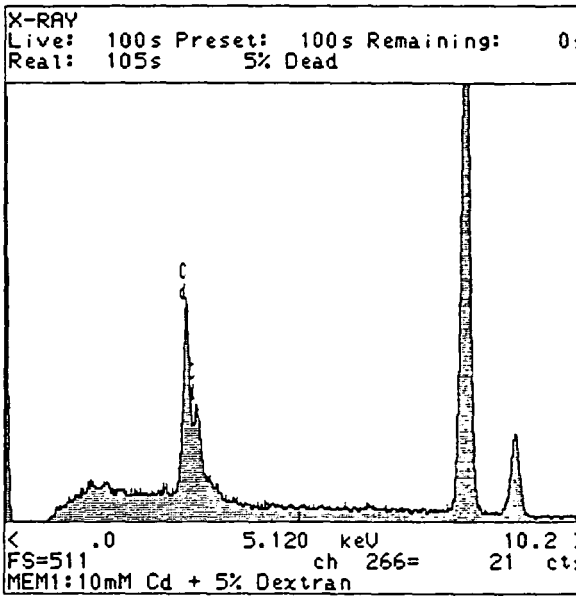
c) 100 mM Cd standard in 5 % dextran

d) 1000 mM Cd standard in 5 % dextran

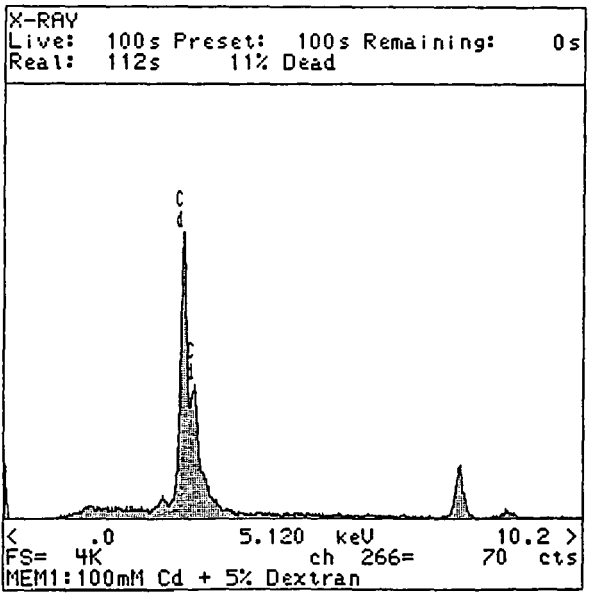
a)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 106s 6% Dead
< .0 5.120 keV 10.2 >
FS=511 ch 266= 16 cts
MEM1:Formvar film 100KV SS2 12D

b)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 105s 5% Dead
C
d
< .0 5.120 keV 10.2 >
FS=511 ch 266= 21 cts
MEM1:10mM Cd + 5% Dextran

c)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 112s 11% Dead
C
d
< .0 5.120 keV 10.2 >
FS= 4K ch 266= 70 cts
MEM1:100mM Cd + 5% Dextran

d)

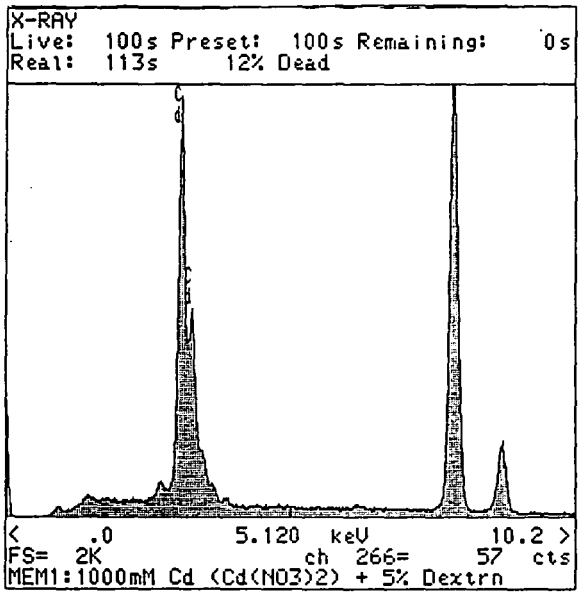

X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 113s 12% Dead
C
d
< .0 5.120 keV 10.2 >
FS= 2K ch 266= 57 cts
MEM1:1000mM Cd (Cd(NO3)2) + 5% Dextrn

Fig. 6.6 EDXMA profiles of Synechococcus D562 grown with 4 mg l^{-1} Cd

a) whole cell. Total net-integral of profile = 11239

Element	% contribution	element	% contribution
Na	43.20	Cd	0.77
Mg	8.86	K	3.93
P	32.60	Ca	2.37
S	2.83	Fe	3.78
Cl	1.66		

b) PP body, with no bound metal detected. Total net-integral of profile = 39130

Element	% contribution	element	% contribution
Na	38.91	Cl	0.19
Mg	5.23	K	5.77
Al	0.57	Ca	1.33
P	47.44	Fe	0.25
S	0.30		

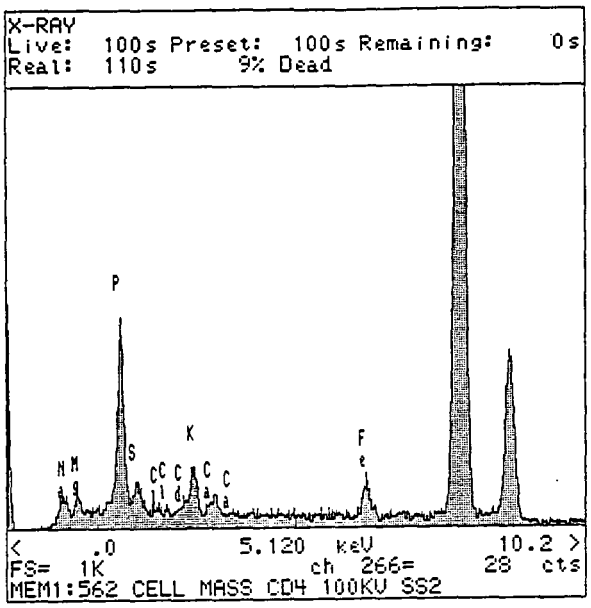
c) PP body with bound Cd. Total net-integral of profile = 97419

Element	% contribution	element	% contribution
Na	57.90	Cl	0.42
Mg	2.92	Cd	0.50
Al	0.20	K	2.38
P	27.78	Ca	6.58
S	0.97	Fe	0.34

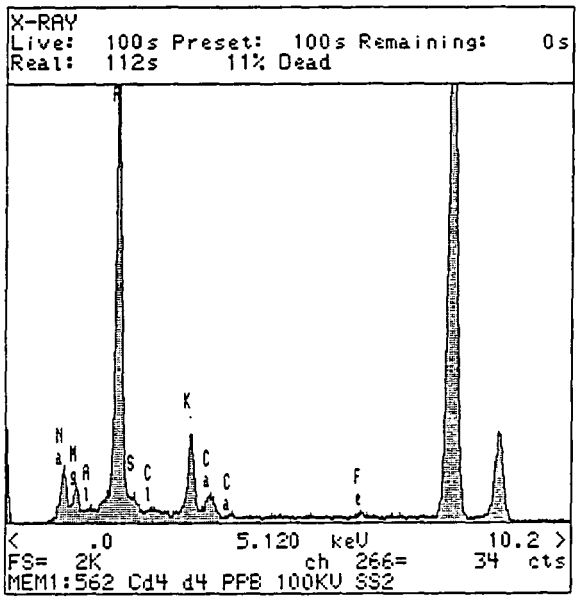
d) cytoplasm. Total net-integral of profile = 20171

Element	% contribution	element	% contribution
Na	60.00	Cl	0.34
Mg	2.37	Cd	1.50
Al	1.17	K	3.65
P	19.39	Ca	1.91
S	3.67	Fe	6.00

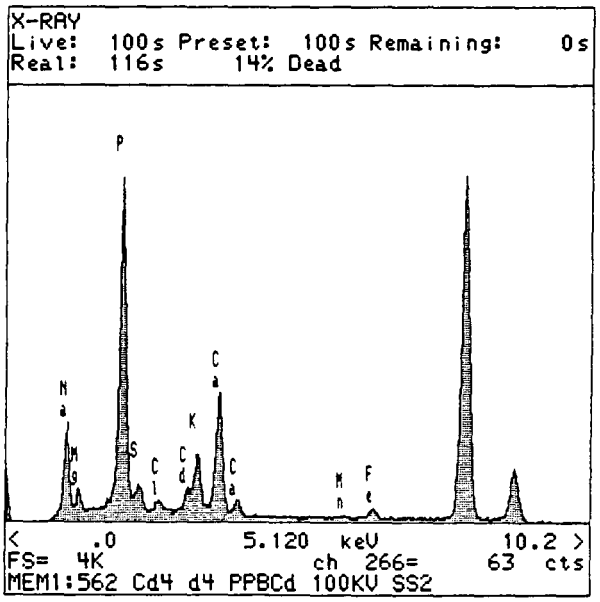
a)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 110s 9% Dead
P
S
K
F
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 28 cts
MEM1:562 CELL MASS CD4 100KV SS2

b)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 112s 11% Dead
K
F
< .0 5.120 keV 10.2 >
FS= 2K ch 266= 34 cts
MEM1:562 Cd4 d4 PPB 100KV SS2

c)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 116s 14% Dead
P
S
K
F
< .0 5.120 keV 10.2 >
FS= 4K ch 266= 63 cts
MEM1:562 Cd4 d4 PPBCd 100KV SS2

d)

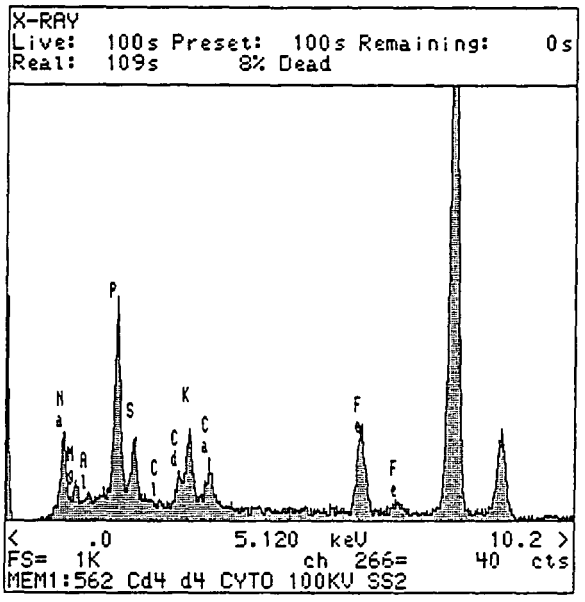

X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 109s 8% Dead
P
S
K
F
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 40 cts
MEM1:562 Cd4 d4 CYTO 100KV SS2

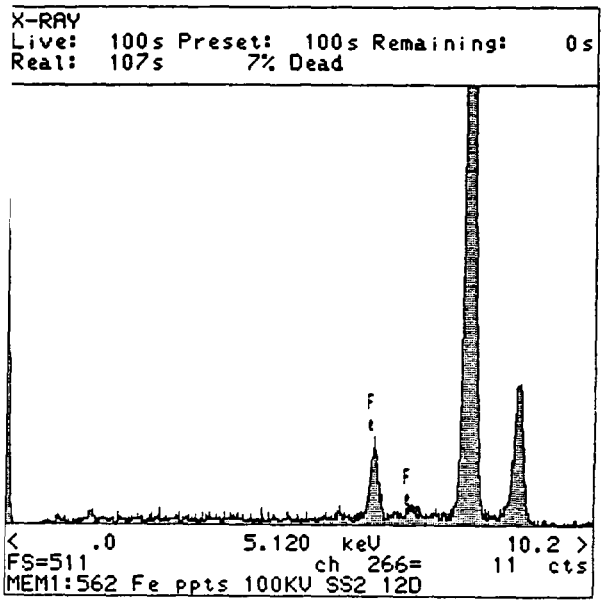
Fig. 6.7 Further EDXMA profiles of Synechococcus D562, peak integrals not determined

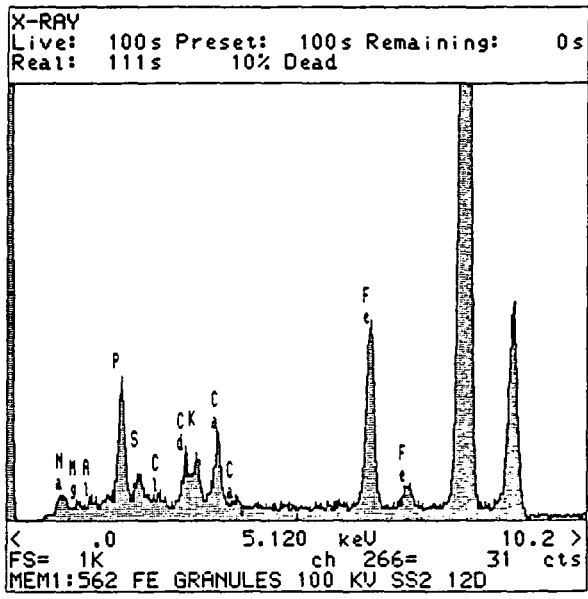
a) Precipitates of Fe recorded on the surface of Synechococcus D562 cells grown in 0 mg l^{-1} Cd

b) Precipitates of Fe recorded on the surface of Synechococcus D562 cells grown in 4 mg l^{-1} Cd

c) PP body of Synechococcus D562 grown in the presence of 4 mg l^{-1} Cd and 5 mg l^{-1} Mn

b)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 107s 7% Dead
Fe
Fe
< .0 5.120 keV 10.2 >
FS=511 ch 266= 11 cts
MEM1:562 Fe ppts 100KV SS2 12D


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 111s 10% Dead
Na
Mg
Al
P
S
Cl
Ca
Fe
Fe
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 31 cts
MEM1:562 FE GRANULES 100 KV SS2 12D

c)

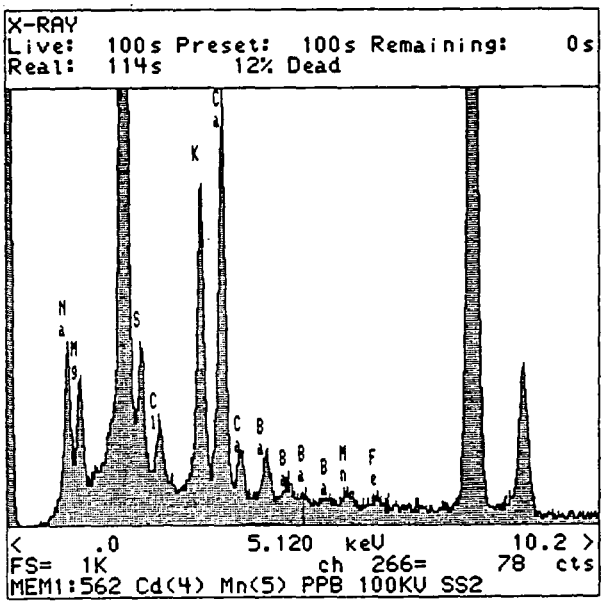

X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 114s 12% Dead
Na
Mg
Si
Cl
K
Ca
Ca
Ba
Ba
Ba
Mn
Fe
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 78 cts
MEM1:562 Cd(4) Mn(5) PPB 100KV SS2

Fig. 6.8 EDXMA profiles of Calothrix D184 grown with 1 mg l^{-1} Cd.

a) inclusion body. Total net-integral of profile = 17339

Element	% contribution	element	% contribution
Na	37.12	Cl	18.02
Mg	3.34	K	10.81
P	16.77	Ca	12.65
S	1.28		

b) inclusion body. Total net-integral of profile = 37364

Element	% contribution	element	% contribution
Mg	19.98	Cl	2.42
Al	1.15	K	23.08
P	51.18	Ca	0.59
S	1.05	Fe	0.56

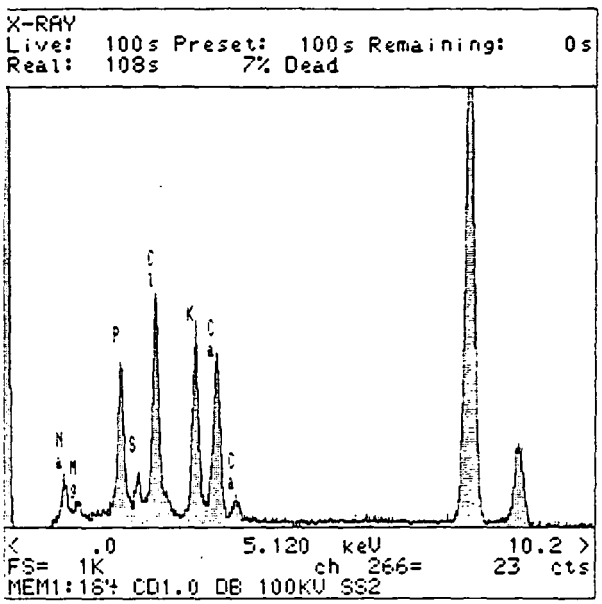
c) inclusion body. Total net-integral of profile = 87542

Element	% contribution	element	% contribution
Mg	15.79	Cl	0.74
P	53.24	K	2.74
S	0.66	Ca	26.83

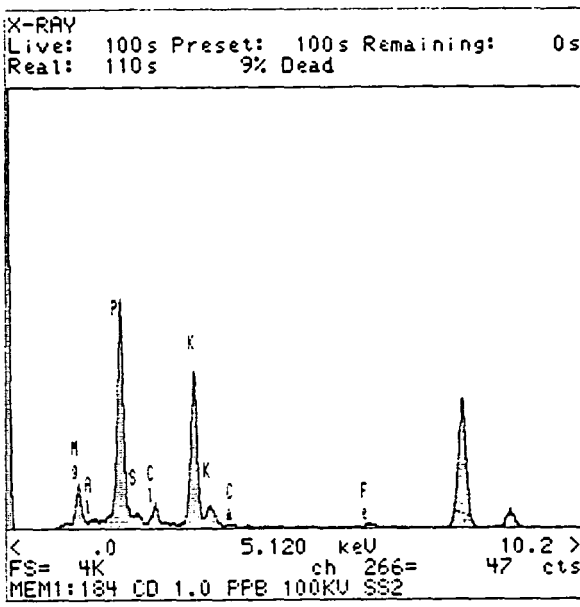
d) inclusion body. Total net-integral of profile = 25358

Element	% contribution	element	% contribution
Na	11.12	S	0.80
Mg	32.49	K	11.65
P	37.30	Ca	6.64

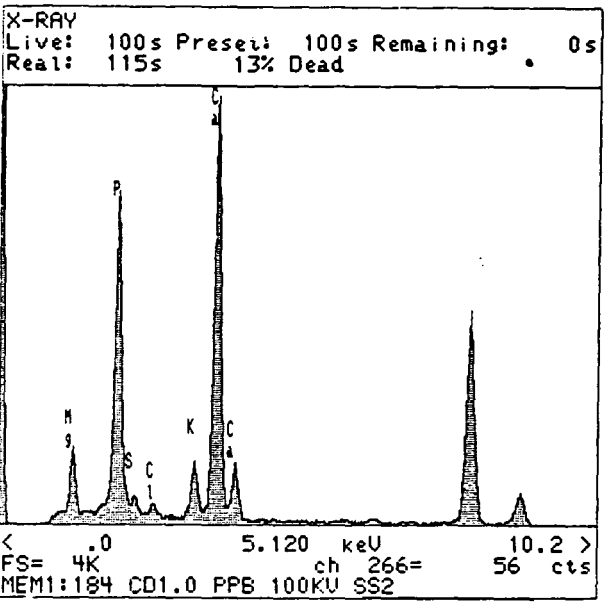
a)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 108s 7% Dead
P
Cl
K
Ca
S
N
Mg
Ca
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 23 cts
MEM1:184 CD1.0 DB 100KV SS2

b)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 110s 9% Dead
P
K
Mg
Al
S
Cl
K
Ca
F
< .0 5.120 keV 10.2 >
FS= 4K ch 266= 47 cts
MEM1:184 CD 1.0 PPB 100KV SS2

c)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 115s 13% Dead
Ca
P
Mg
S
Cl
K
Ca
< .0 5.120 keV 10.2 >
FS= 4K ch 266= 56 cts
MEM1:184 CD1.0 PPB 100KV SS2

d)

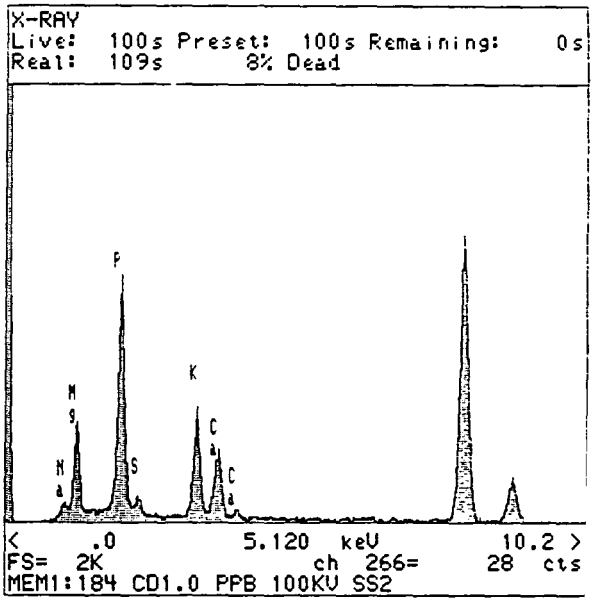

X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 109s 8% Dead
P
K
Mg
Na
S
Ca
Cl
I
< .0 5.120 keV 10.2 >
FS= 2K ch 266= 28 cts
MEM1:184 CD1.0 PPB 100KV SS2

Fig. 6.9 EDXMA profiles of Calothrix D184 grown with 1 mg l^{-1} Cd.

a). cell wall. Total net-integral of profile = 1001

Element	% contribution	element	% contribution
Mg	26.76	Cl	4.81
P	24.35	K	19.99
S	8.49	Ca	15.60

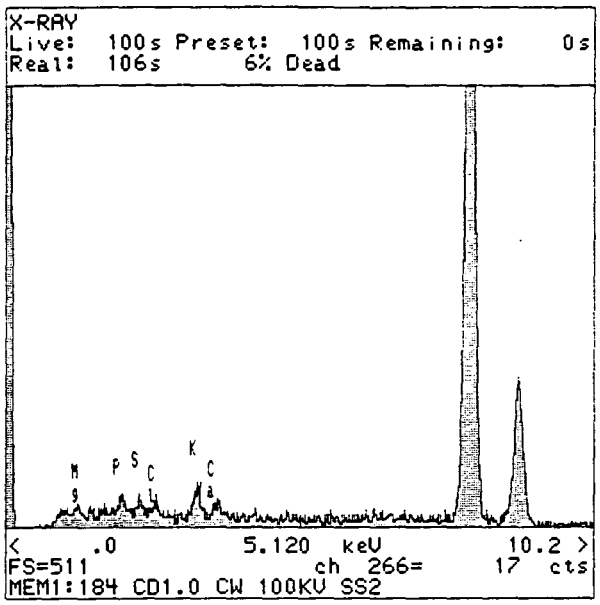
b) cytoplasm. Total net-integral of profile = 660

Element	% contribution	element	% contribution
S	11.05	Ca	21.92
Cl	43.47	Fe	2.00
K	21.57		

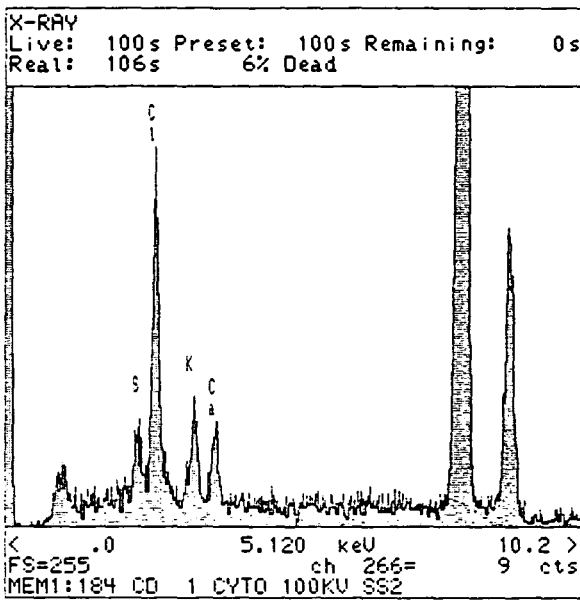
c) inclusion body. Total net-integral of profile = 660

Element	% contribution	element	% contribution
Mg	25.51	Cl	1.46
Al	43.47	K	1.61
P	58.33	Fe	0.70
S	9.40		

a)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 106s 6% Dead
Mg
P
S
Cl
K
Ca
< .0 5.120 keV 10.2 >
FS=511 ch 266= 17 cts
MEM1:184 CD1.0 CW 100KV SS2

b)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 106s 6% Dead
Cl
S
K
Ca
< .0 5.120 keV 10.2 >
FS=255 ch 266= 9 cts
MEM1:184 CD 1 CYTO 100KV SS2

c)

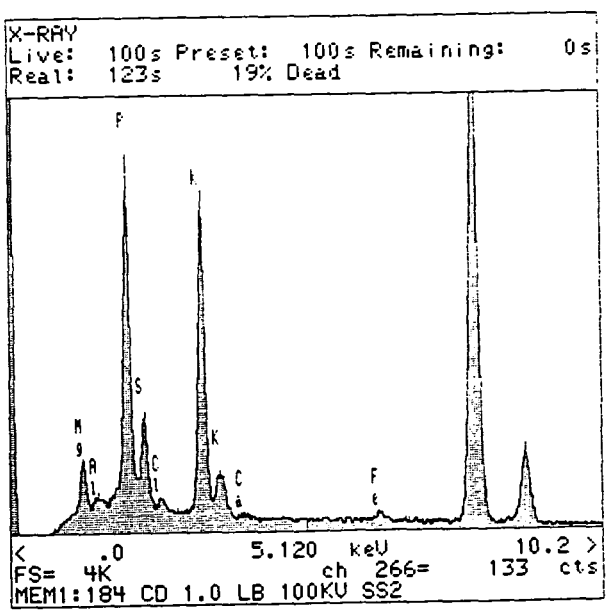

X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 123s 19% Dead
P
Cl
S
Mg
Al
K
Ca
Fe
< .0 5.120 keV 10.2 >
FS= 4K ch 266= 133 cts
MEM1:184 CD 1.0 LB 100KV SS2

Fig. 6.10 EDXMA profiles of Mougeotia D536 grown with 0.8 mg l^{-1} Cd.

a) inclusion body. Total net-integral of profile = 20036

Element	% contribution	element	% contribution
Na	3.55	Cl	0.15
Mg	10.32	K	21.70
Al	1.96	Ca	3.08
P	56.32	Fe	0.55
S	2.38		

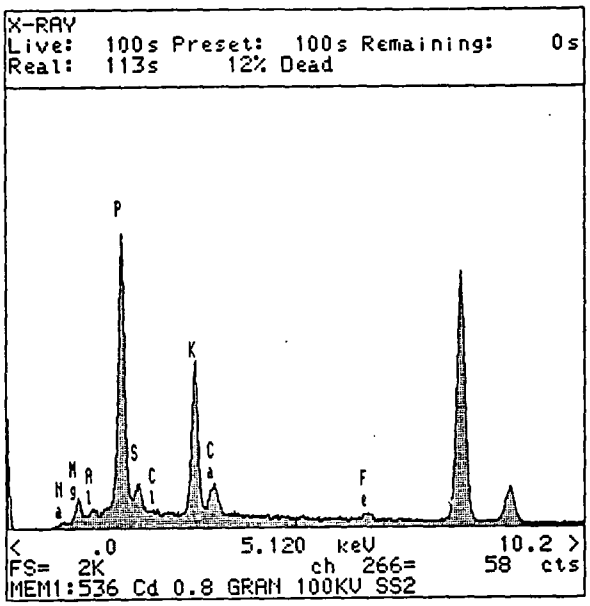
b) cell wall. Total net-integral of profile = 15026

Element	% contribution	element	% contribution
Na	11.50	S	15.13
Mg	9.30	Cl	0.37
Al	4.03	K	30.94
P	28.23	Fe	0.51

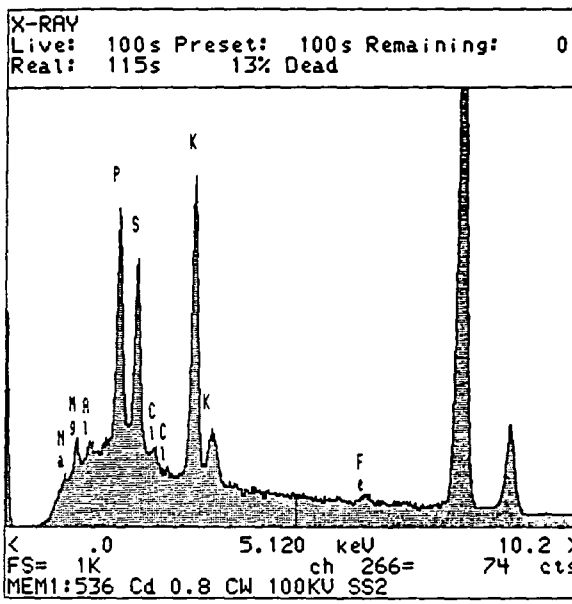
c) cytoplasm. Total net-integral of profile = 3312

Element	% contribution	element	% contribution
Na	14.81	S	14.79
Mg	10.38	Cl	2.78
P	24.81	Fe	32.43

a)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 113s 12% Dead
P
K
S
Cl
Ca
Mg
Na
Al
Fe
< .0 5.120 keV 10.2 >
FS= 2K ch 266= 58 cts
MEM1:536 Cd 0.8 GRAN 100KV SS2

b)


X-RAY
Live: 100s Preset: 100s Remaining: 0
Real: 115s 13% Dead
K
P
S
Cl
Mg
Na
Al
Fe
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 74 cts
MEM1:536 Cd 0.8 CW 100KV SS2

c)

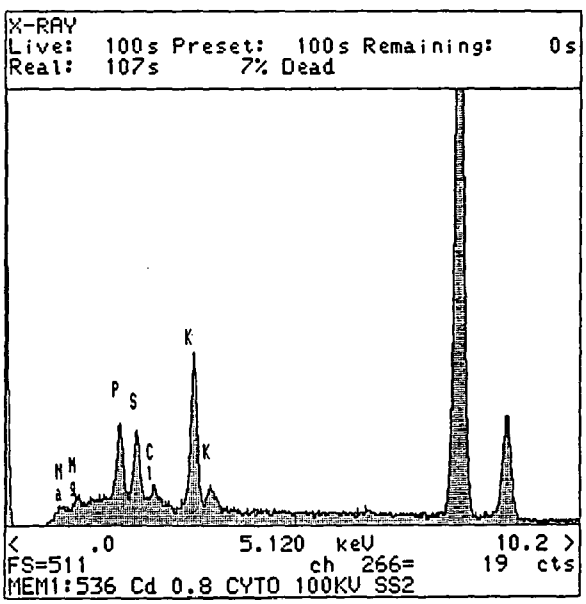

X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 107s 7% Dead
K
P
S
Cl
Mg
Na
< .0 5.120 keV 10.2 >
FS=511 ch 266= 19 cts
MEM1:536 Cd 0.8 CYTO 100KV SS2

Fig. 6.11 EDXMA profiles of Klebsormidium D537 grown with 1 mg l^{-1} Cd.

a) inclusion body. Total net-integral of profile = 46221

Element	% contribution	element	% contribution
Mg	18.15	S	5.48
Al	1.53	K	27.89
P	45.67	Fe	1.27

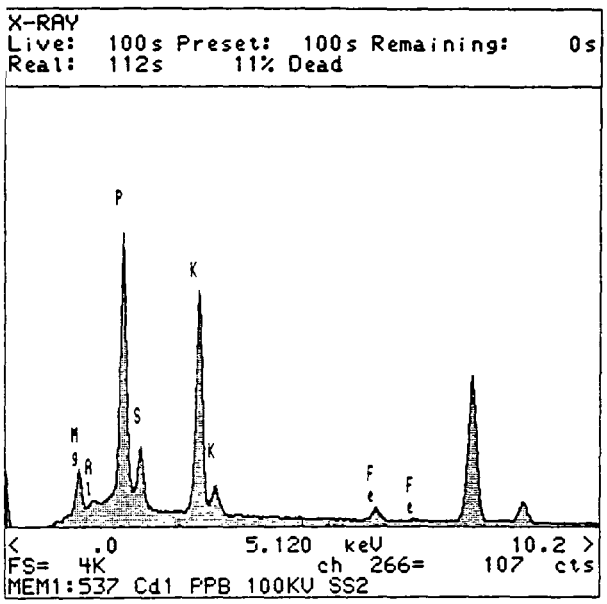
b) cell wall. Total net-integral of profile = 19812.73

Element	% contribution	element	% contribution
Mg	14.09	Cl	7.03
Al	5.81	K	29.35
P	20.76	Fe	3.11
S	19.86		

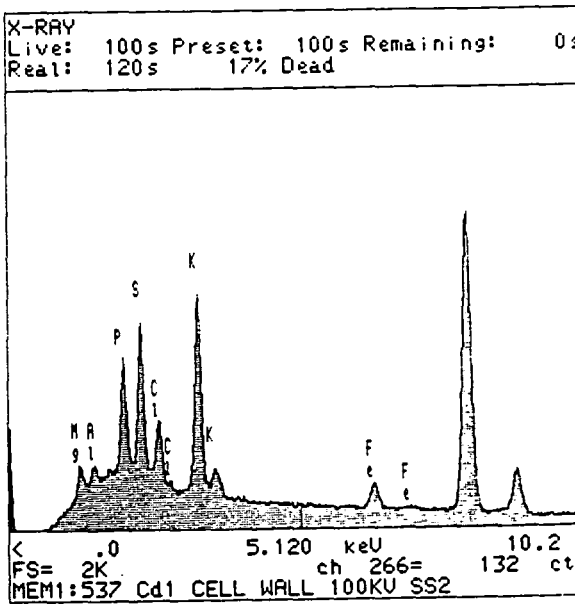
c) cytoplasm. Total net-integral of profile = 11473

Element	% contribution	element	% contribution
Mg	15.93	Cl	0.77
Al	2.27	K	35.54
P	24.79	Fe	3.08
S	17.62		

)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 112s 11% Dead
P
K
S
K
F
e
F
e
< .0 5.120 keV 10.2 >
FS= 4K ch 266= 107 cts
MEM1:537 Cd1 PPB 100KV SS2

b)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 120s 17% Dead
K
S
P
K
F
e
F
e
< .0 5.120 keV 10.2
FS= 2K ch 266= 132 ct
MEM1:537 Cd1 CELL WALL 100KV SS2

c)

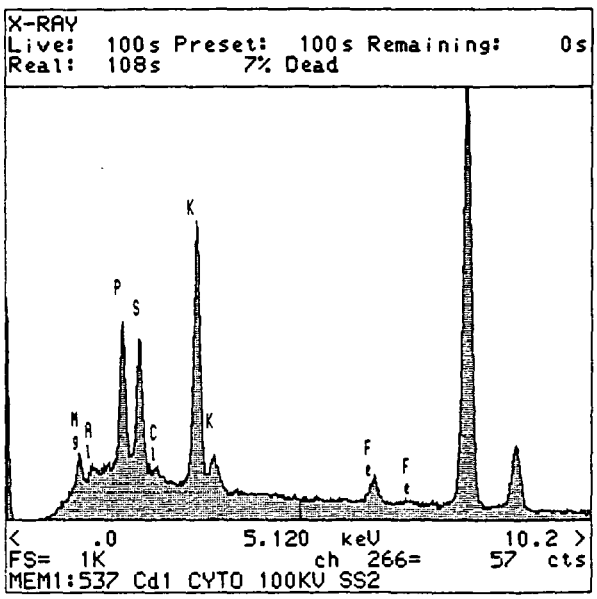

X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 108s 7% Dead
K
P
S
K
F
e
F
e
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 57 cts
MEM1:537 Cd1 CYTO 100KV SS2

Fig. 6.12 EDXMA profiles of Stigeoclonium from Rampgill Level a) (Caplecleugh High Level).

a) inclusion body. Total net-integral of profile = 18021

Element	% contribution	element	% contribution
Mg	4.60	K	31.95
Al	0.64	Ca	18.18
P	4.01	Fe	0.31
S	4.30	Zn	33.09

b) granules. Total net-integral of profile = 11649

Element	% contribution	element	% contribution
Mg	4.65	K	16.38
Al	3.59	Ca	5.42
P	3.51	Fe	33.02
S	2.35	Zn	28.60
Cl	2.48	1.05	

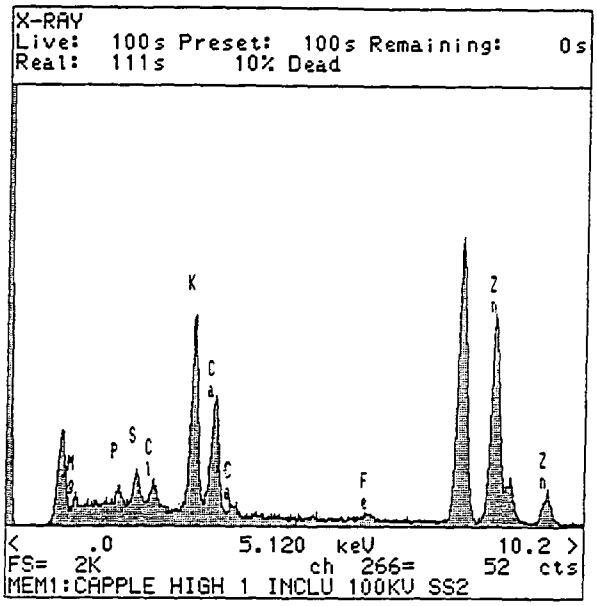
c) cytoplasm. Total net-integral of profile = 2934

Element	% contribution	element	% contribution
Na	38.87	K	29.96
Mg	13.17	Ca	8.21
S	3.14	Zn	4.19
Cl	2.46		

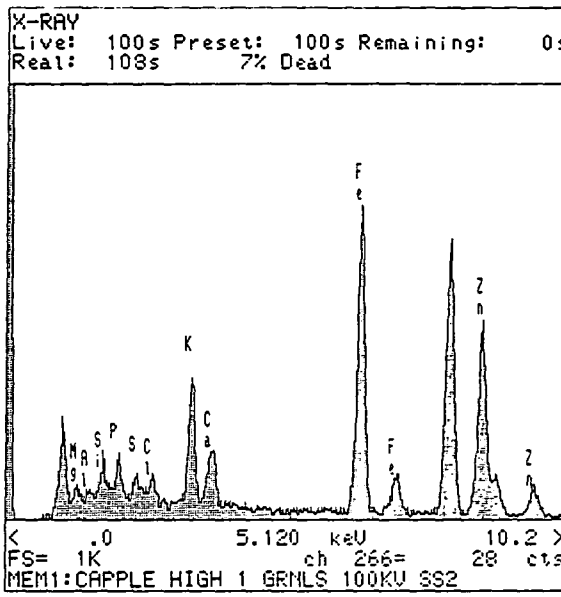
d) cell wall. Total net-integral of profile = 24430

Element	% contribution	element	% contribution
Mg	13.18	Cl	4.26
Al	4.38	K	44.04
P	1.71	Ca	14.92
S	3.84	Zn	13.65

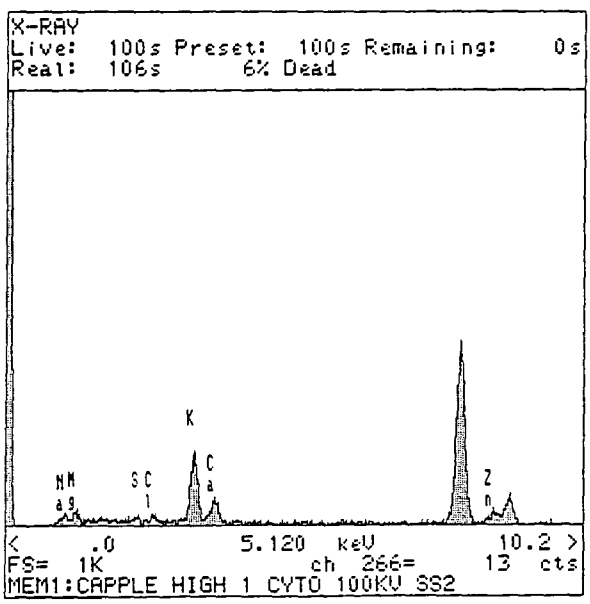
a)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 111s 10% Dead
K
Ca
Zn
Fe
< .0 5.120 keV 10.2 >
FS= 2K ch 266= 52 cts
MEM1:CAPPLE HIGH 1 INCLU 100KV SS2

b)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 108s 7% Dead
Fe
K
Ca
Zn
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 28 cts
MEM1:CAPPLE HIGH 1 GRNLS 100KV SS2

c)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 106s 6% Dead
K
Ca
Zn
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 13 cts
MEM1:CAPPLE HIGH 1 CYTO 100KV SS2

d)

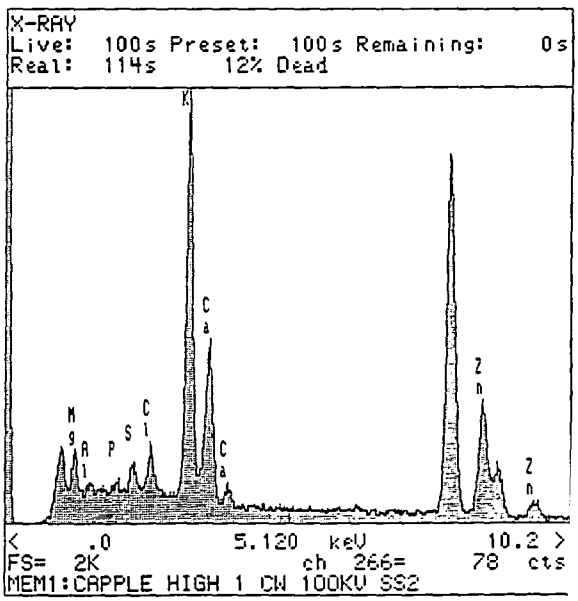

X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 114s 12% Dead
K
Ca
Zn
< .0 5.120 keV 10.2 >
FS= 2K ch 266= 78 cts
MEM1:CAPPLE HIGH 1 CW 100KV SS2

Fig. 6.13 EDXMA profiles of Stigeoclonium from Rampgill Level b) (Caplecleugh High Level).

a) inclusion body. Total net-integral of profile = 71759

Element	% contribution	element	% contribution
Mg	3.22	K	8.83
Al	3.07	Ca	14.11
P	3.56	Fe	0.79
S	7.41	Zn	59.00

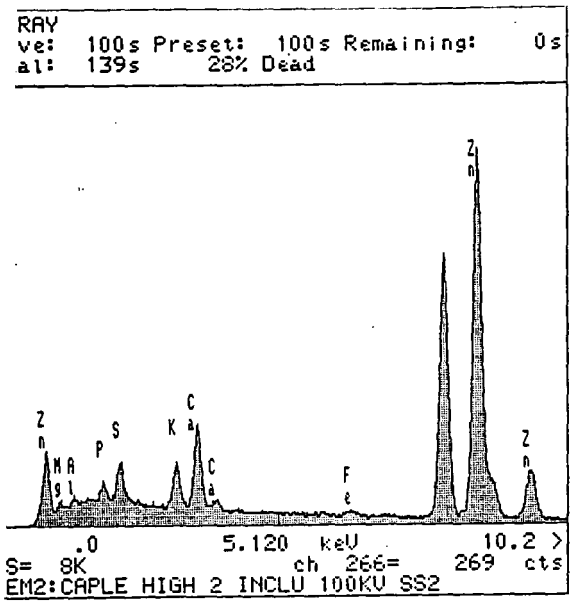
b) cell wall. Total net-integral of profile = 11641

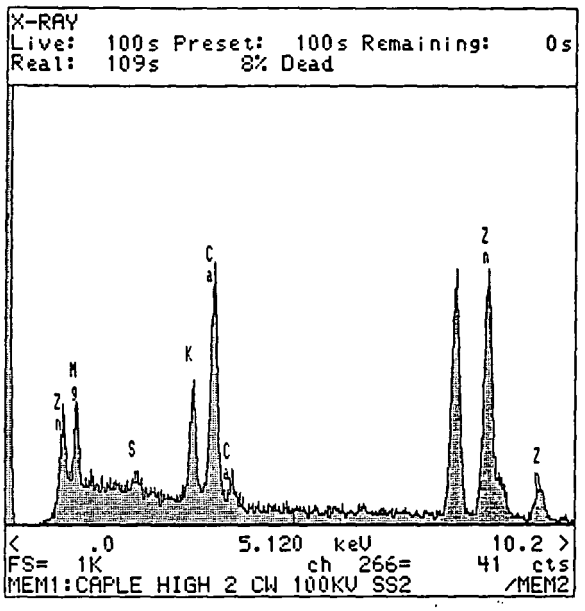
Element	% contribution	element	% contribution
Mg	24.55	Ca	28.79
S	2.43	Zn	29.32
K	14.90		

c) cytoplasm. Total net-integral of profile = 3681

Element	% contribution	element	% contribution
Mg	17.70	Ca	19.04
Al	10.65	Zn	36.73
K	15.88		

b)


RAY
ve: 100s Preset: 100s Remaining: 0s
al: 139s 28% Dead
Zn
Mg
P
S
K
Ca
Ca
Fe
Zn
Zn
.0 5.120 keV 10.2 >
S= 8K ch 266= 269 cts
EM2:CAPLE HIGH 2 INCLU 100KV SS2


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 109s 8% Dead
Zn
Mg
S
K
Ca
Ca
Zn
Zn
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 41 cts
MEM1:CAPLE HIGH 2 CW 100KV SS2 /MEM2

c)

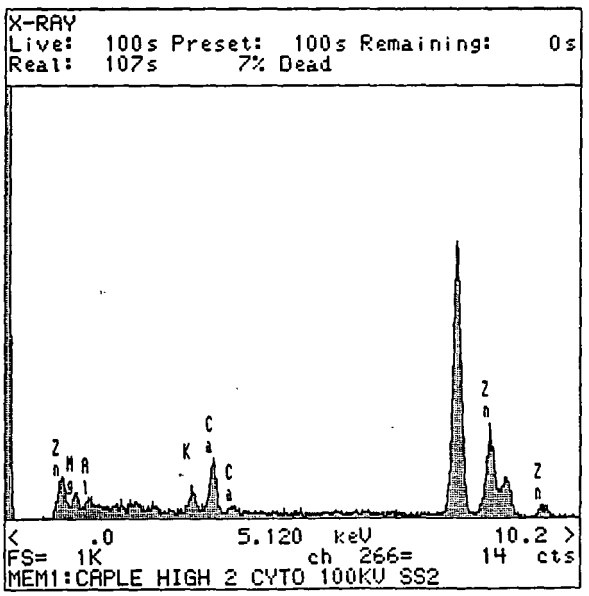

X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 107s 7% Dead
Zn
Mg
Al
K
Ca
Ca
Zn
Zn
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 14 cts
MEM1:CAPLE HIGH 2 CYTO 100KV SS2

Fig. 6.14 EDXMA scan of moss protonema from Caplecleugh Low Level.

a) inclusion body. Total net-integral of profile = 19827

Element	% contribution	element	% contribution
Mg	4.48	K	4.51
Al	6.91	Ca	14.50
S	9.47	Fe	14.72
P	20.81	Zn	15.38
S	9.22		

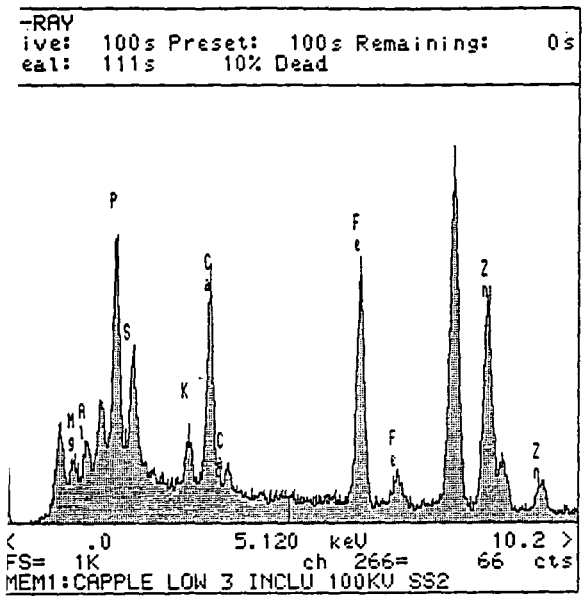
b) cytoplasm. Total net-integral of profile = 5426

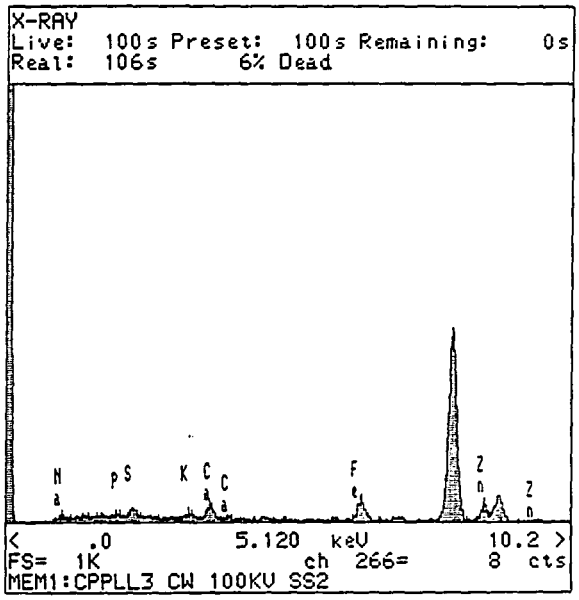
Element	% contribution	element	% contribution
Na	53.37	Ca	13.38
P	7.80	Fe	4.72
S	11.07	Zn	4.70
K	4.96		

c) cell wall. Total net-integral of profile = 879

Element	% contribution	element	% contribution
S	19.34	Fe	27.66
K	12.45	Zn	12.81
Ca	27.72		

b)


-RAY
ive: 100s Preset: 100s Remaining: 0s
eal: 111s 10% Dead
P
S
K
C
F
Z
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 66 cts
MEM1:CAPPLE LOW 3 INCLU 100KV SS2


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 106s 6% Dead
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 8 cts
MEM1:CPPLL3 CW 100KV SS2

c)

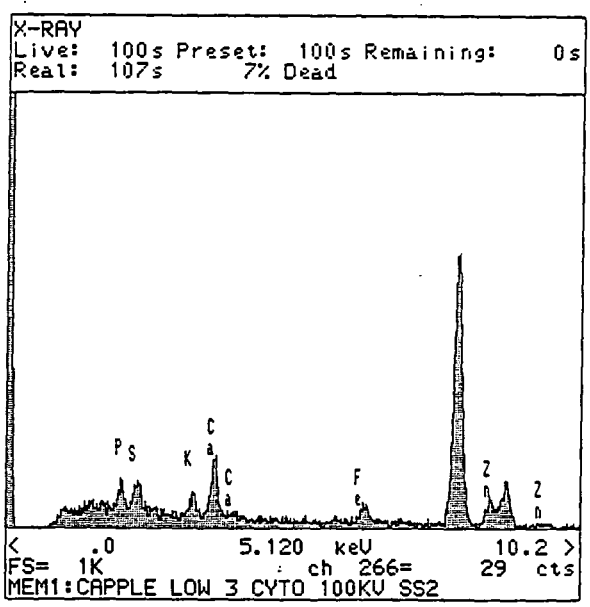

X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 107s 7% Dead
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 29 cts
MEM1:CAPPLE LOW 3 CYTO 100KV SS2

Fig. 6.15 EDXMA profiles of Klebsormidium rivulare from Caplecleugh Low Level.

a) inclusion body. Total net-integral of profile = 32588

Element	% contribution	element	% contribution
Na	25.99	K	4.51
Mg	4.81	Ca	7.78
Al	7.52	Fe	1.07
P	16.96	Zn	8.91
S	22.44		

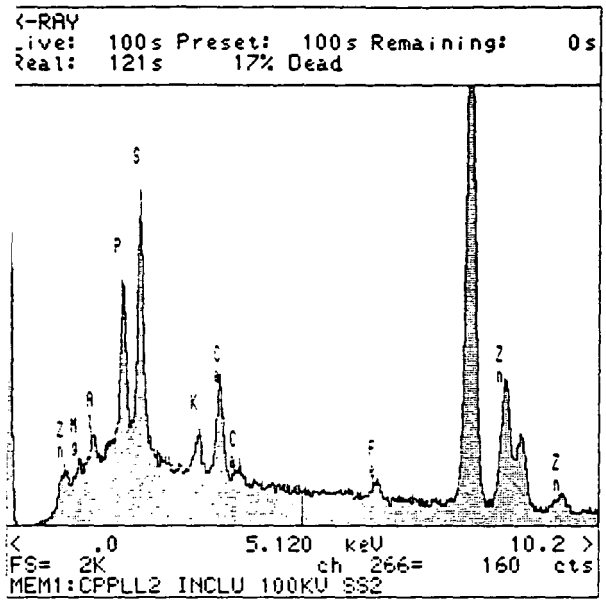
b) cell wall. Total net-integral of profile = 2743

Element	% contribution	element	% contribution
Na	34.41	K	17.56
Al	2.40	Ca	10.97
P	6.11	Fe	1.00
S	25.30	Zn	2.24

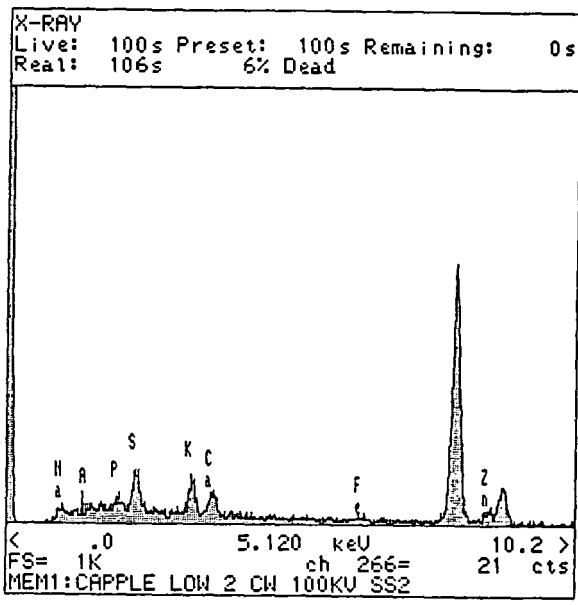
c) cytoplasm. Total net-integral of profile = 4078.81

Element	% contribution	element	% contribution
Na	49.89	K	13.45
P	7.78	Ca	8.37
S	17.21	Zn	3.29

)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 121s 17% Dead
S
P
K
Zn
< .0 5.120 keV 10.2 >
FS= 2K ch 266= 160 cts
MEM1:CPPLL2 INCLU 100KV SS2

b)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 106s 6% Dead
S
K
Ca
F
Zn
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 21 cts
MEM1:CAPPLE LOW 2 CW 100KV SS2

c)

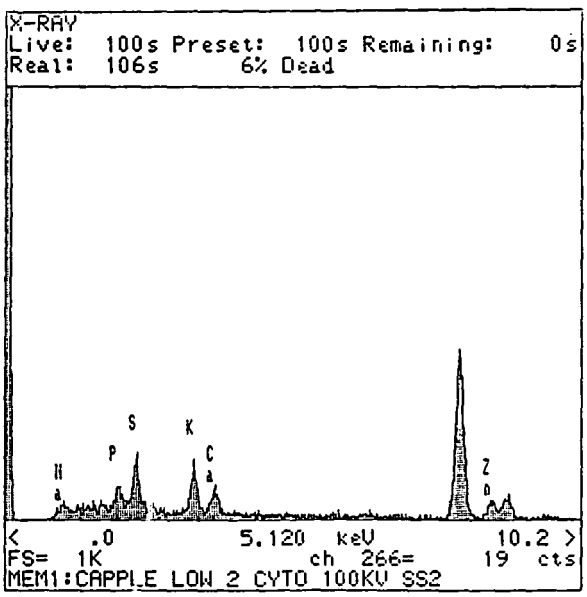

X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 106s 6% Dead
S
P
K
Ca
Zn
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 19 cts
MEM1:CAPPLE LOW 2 CYTO 100KV SS2

Fig. 6.16 EDXMA profiles of Spirogyra from Gillgill Burn.

a) photomicrograph of Spirogyra surface precipitates.

b) surface precipitates. Total net-integral of profile = 18028

Element	% contribution	element	% contribution
Na	14.35	Ca	9.48
Mg	10.42	Ba	25.10
S	37.54	Fe	1.45
K	1.66		

c) inclusion body. Total net-integral of profile = 37509

Element	% contribution	element	% contribution
Na	17.87	Ca	31.35
Mg	16.28	Ba	1.35
P	6.17	Mn	0.75
S	14.60	Fe	3.19
Cl	3.75	Zn	3.21
K	1.47		

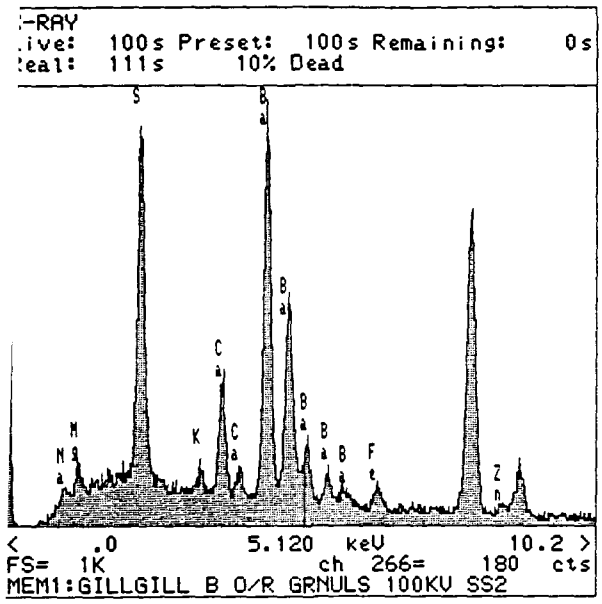
d) cell wall. Total net-integral of profile = 21139

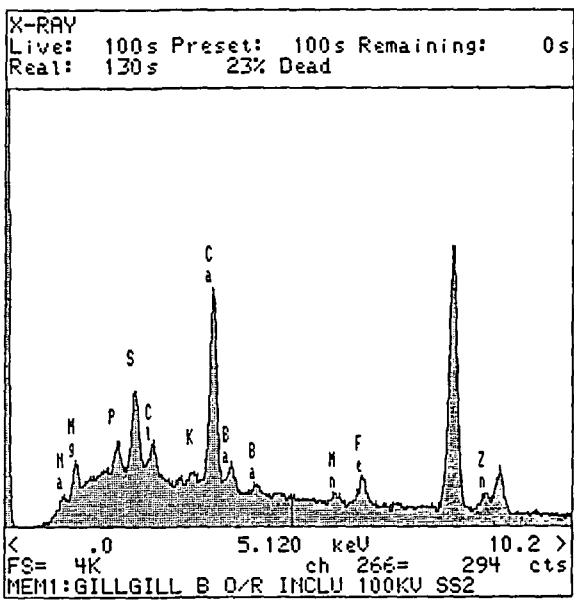
Element	% contribution	element	% contribution
Na	32.81	K	1.03
Mg	17.12	Ca	28.96
P	1.04	Fe	7.21
S	6.74	Zn	2.15
Cl	2.95		

e) cytoplasm. Total net-integral of profile = 834.68

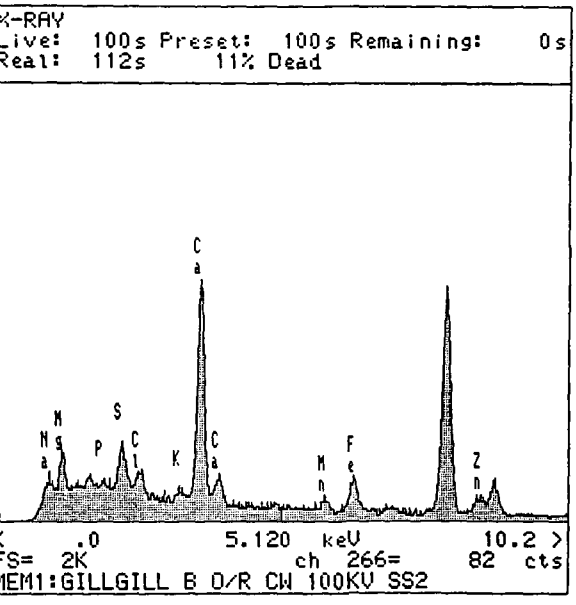
Element	% contribution	element	% contribution
S	14.31	Ca	85.69

b)


-RAY
ive: 100s Preset: 100s Remaining: 0s
eal: 111s 10% Dead
S
Ba
Ca
K
Ba
Ba
Ba
Ba
Ba
Fe
Zn
Mg
Na
.0 5.120 keV 10.2 >
FS= 1K ch 266= 180 cts
MEM1:GILLGILL B O/R GRNULS 100KV SS2


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 130s 23% Dead
Ca
S
Mg
Na
P
Cl
K
Ba
Ba
Mn
Fe
Zn
.0 5.120 keV 10.2 >
FS= 4K ch 266= 294 cts
MEM1:GILLGILL B O/R INCLU 100KV SS2

)


X-RAY
ive: 100s Preset: 100s Remaining: 0s
Real: 112s 11% Dead
Ca
Mg
Na
S
P
Cl
K
Ca
Mn
Fe
Zn
.0 5.120 keV 10.2 >
FS= 2K ch 266= 82 cts
MEM1:GILLGILL B O/R CW 100KV SS2

d)

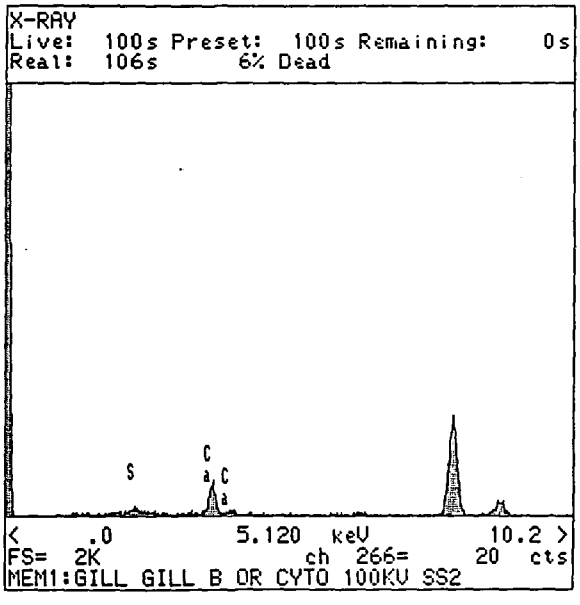

X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 106s 6% Dead
S
Ca
Ca
.0 5.120 keV 10.2 >
FS= 2K ch 266= 20 cts
MEM1:GILL GILL B OR CYTO 100KV SS2

Fig. 6.17 EDXMA profiles of Klebsormidium rivulare from Low Gillgill Burn.

a) inclusion body - high Pb. Total net-integral of profile = 81913

Element	% contribution	element	% contribution
Mg	13.41	K	29.24
Al	2.03	Mn	0.51
P	40.09	Fe	0.98
S	8.85	Pb	4.90

b) inclusion body. Total net-integral of profile = 64350

Element	% contribution	element	% contribution
Mg	3.47	K	16.56
Al	9.96	Mn	0.69
P	4.24	Fe	4.76
S	39.70	Zn	1.22
Cl	0.29	Pb	19.12

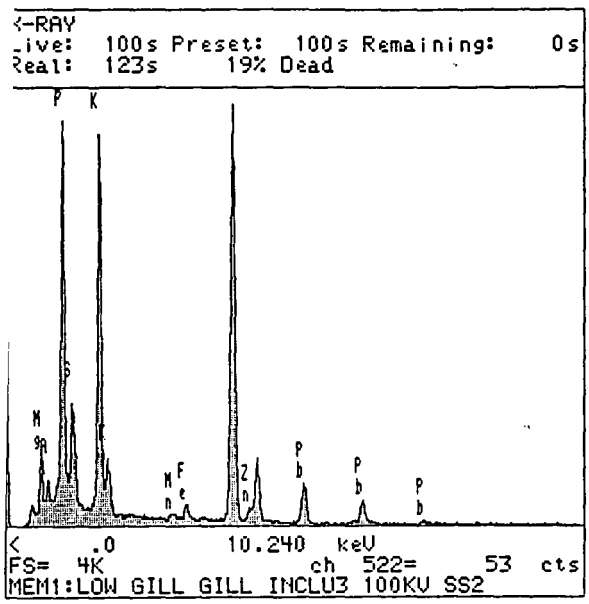
c) cell wall. Total net-integral of profile = 6975

Element	% contribution	element	% contribution
Al	10.07	K	7.19
Si	3.06	Ca	3.07
P	15.31	Fe	2.89
S	43.79	Zn	1.23
Cl	0.94	Pb	12.46

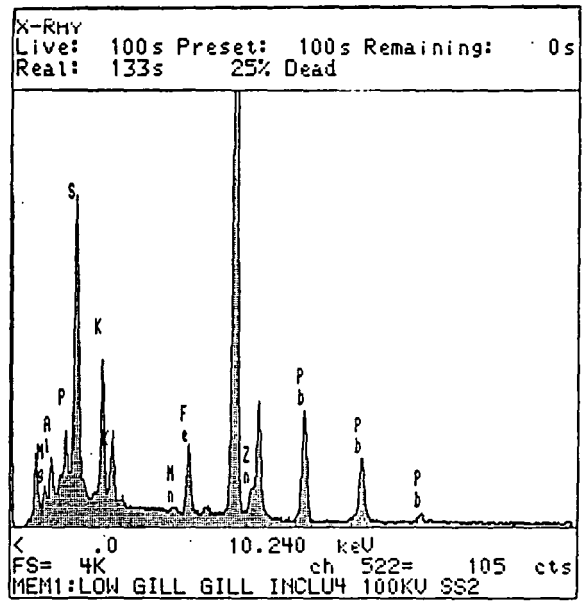
d) cytoplasm. Total net-integral of profile = 355

Element	% contribution	element	% contribution
S	36.92	K	63.08

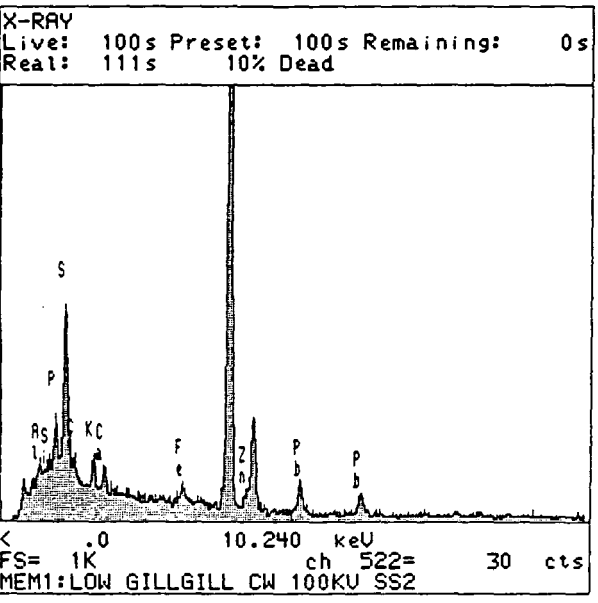
)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 123s 19% Dead
P K
< .0 10.240 keV
FS= 4K ch 522= 53 cts
MEM1:LOW GILL GILL INCLU3 100KV SS2

b)


X-RHY
Live: 100s Preset: 100s Remaining: 0s
Real: 133s 25% Dead
S K P
< .0 10.240 keV
FS= 4K ch 522= 105 cts
MEM1:LOW GILL GILL INCLU4 100KV SS2

d)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 111s 10% Dead
S P
< .0 10.240 keV
FS= 1K ch 522= 30 cts
MEM1:LOW GILLGILL CW 100KV SS2

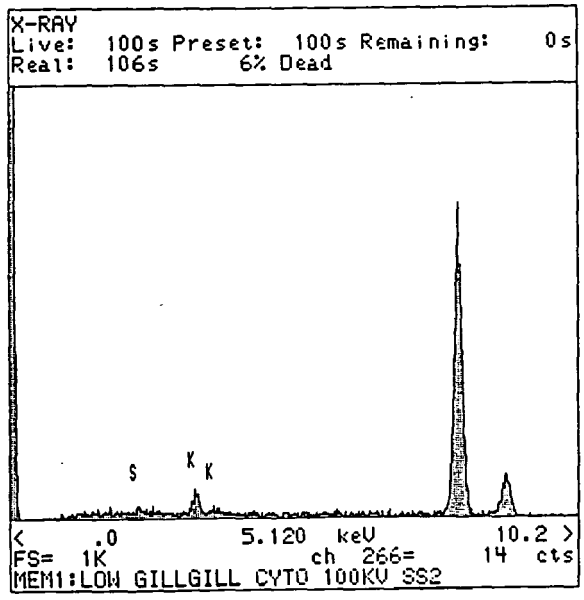

X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 106s 6% Dead
S K K
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 14 cts
MEM1:LOW GILLGILL CYTO 100KV SS2

Fig. 6.18 EDXMA scan of Stichococcus sp. D835 grown in 0.25 mg l^{-1} Cd from Gillgill Burn.

a) inclusion body. Total net-integral of profile = 11555

Element	% contribution	element	% contribution
Na	21.96	Cl	2.20
Mg	10.25	K	7.90
Al	4.00	Ca	9.83
P	31.39	Fe	1.43
S	13.65		

b) inclusion body. Total net-integral of profile = 12346

Element	% contribution	element	% contribution
Na	14.70	S	30.30
Mg	3.50	K	5.10
Al	2.05	Mn	14.31
P	2.78	Fe	14.56

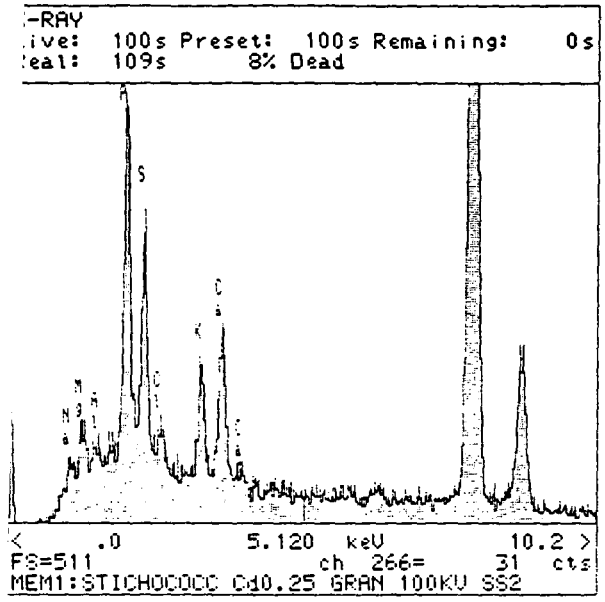
c) inclusion body. Total net-integral of profile = 28930

Element	% contribution	element	% contribution
Na	29.33	Cd	0.32
Mg	2.88	K	1.23
P	45.35	Ca	13.75
S	6.05	Fe	0.40

d) cytoplasm. Total net-integral of profile = 7284

Element	% contribution	element	% contribution
Na	20.54	S	19.07
Mg	17.40	K	6.52
Al	2.82	Ca	10.36
P	22.65	Zn	0.66

b)


-RAY
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eal: 109s 8% Dead
.0 5.120 keV 10.2
FS=511 ch 266= 31 cts
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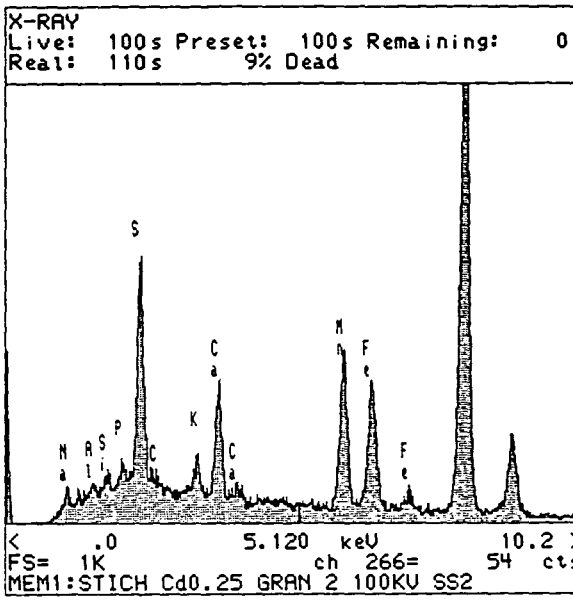
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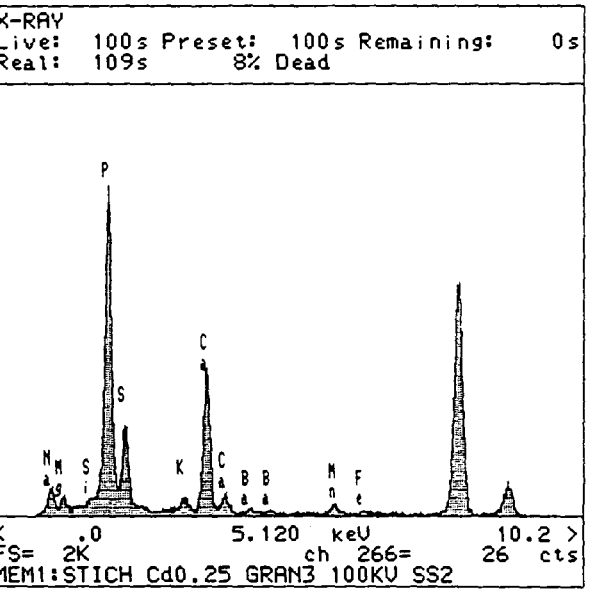
FS= 1K ch 266= 54
MEM1:STICH Cd0.25 GRAN 2 100KV SS2

)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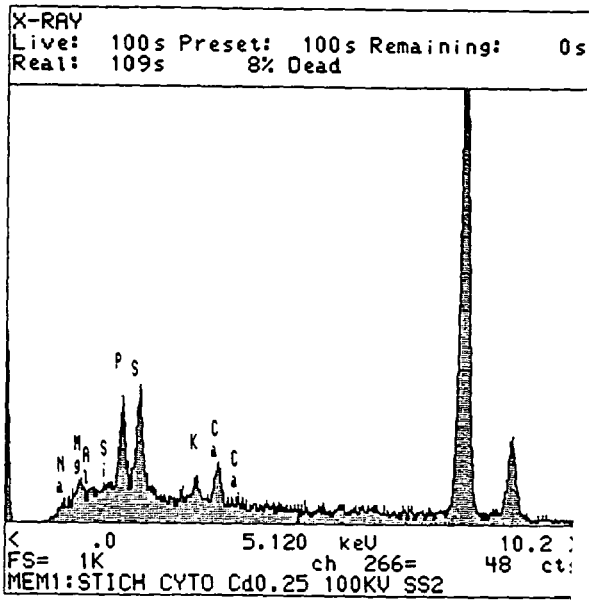
d)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MEM1:STICH CYTO Cd0.25 100KV SS2

6.6 Scanning proton microanalysis

Due to a relatively high detection limit, the technique of EDXMA localised intracellular Cd only in Synechococcus D562 grown in the presence of 4 mg l^{-1} Cd. Therefore, in an attempt to chart the cellular position of this metal and provide a map of other elements, samples of cyanobacteria and algae grown in the presence of Cd were analysed by the more sensitive technique of SPM.

As the pixel maps of each element do not discriminate between background and signal, the X-ray spectra and associated peak-integrals (not shown) were used to calculate the composition of the sample in terms of the areal density for each map (ng element cm^{-2} of algae). The figures quoted for elemental content are dependent on the estimations made during peak processing for the target thickness, density and composition. The map of captured secondary-electrons yielded information on the surface topology, akin to the scans of an SEM and the pixels representing back-scattered electrons were used for elements which cannot be detected in the form of X-ray emissions across the detector's beryllium window. The Rutherford backscattering map showed that the carbon content of algae cannot be assessed, since the specimen support material contributed a large background count.

Elemental scans of the four strains from the Durham Culture Collection revealed intracellular Cd only in Klebsormidium D537 cultured in the presence of 0.5 mg l^{-1} Cd for 15 d, but even then only at a low concentration. The predominant signals for this strain were obtained from P and K, with significant quantities of Fe, S, Si, Mg, Al and Cl detected. The trace elements Al, Cr, Cu, Mn, Ti and Zn, which were not recorded by EDXMA, also appear on SPM spectra of this strain.

Due to resolution limitations, the elemental distribution in individual cells of Synechococcus D562 grown for 5 d in the presence of 4 mg l^{-1} Cd, is not easily assessed; Al, K, Mg, P, and Si were found in high concentrations with lower levels of Ca, Cl, and S, whilst Fe was only detected at discrete positions on the scan. Small peaks for Mn, Ni, Ti and Zn, present at low concentrations within the cell were also obtained.

Although high levels of Cl, K and Si were detected in Calothrix D184, a strong signal for P was not obtained, this may be due to a reduction in the concentration of P in the medium during growth and subsequent utilisation of cellular P stores. The trace elements Al, Ba, Cr, Cu, Ni, and Zn were also detected, peaks that were not available from EDXMA scans.

For Mougeotia D536 cells cultured for 15 d in the presence of 0.5 mg l^{-1} Cd, K, Ca, Cl, and P were the main constituents, followed by S, Na, Mg and Fe.

Fig. 6.19 SPM pixel map of Synechococcus D562 cells grown at 32 ° C and 40 μ mol photon m^{-2} s^{-1} with 4 mg l^{-1} Cd for 5 d; M = maximum counts per pixel, C = % of the maximum count represented by the yellow colour, SE_M = secondary electron image, RBS3 = Rutherford backscattering map, PIX2 = X-ray data channel. Elements detected at a low concentration are not shown as pixel maps

Element	areal density (ng element cm alga $^{-2}$)	element	areal density (ng element cm alga $^{-2}$)
Al	118.87	Mn	0.77
Ca	27.09	Na	19.37
Cl	73.06	Ni	0.67
Cr	0.31	P	280.75
Cu	2.87	S	84.55
Fe	42.03	Si	153.84
K	134.46	Ti	2.73
Mg	125.43	Zn	1.87

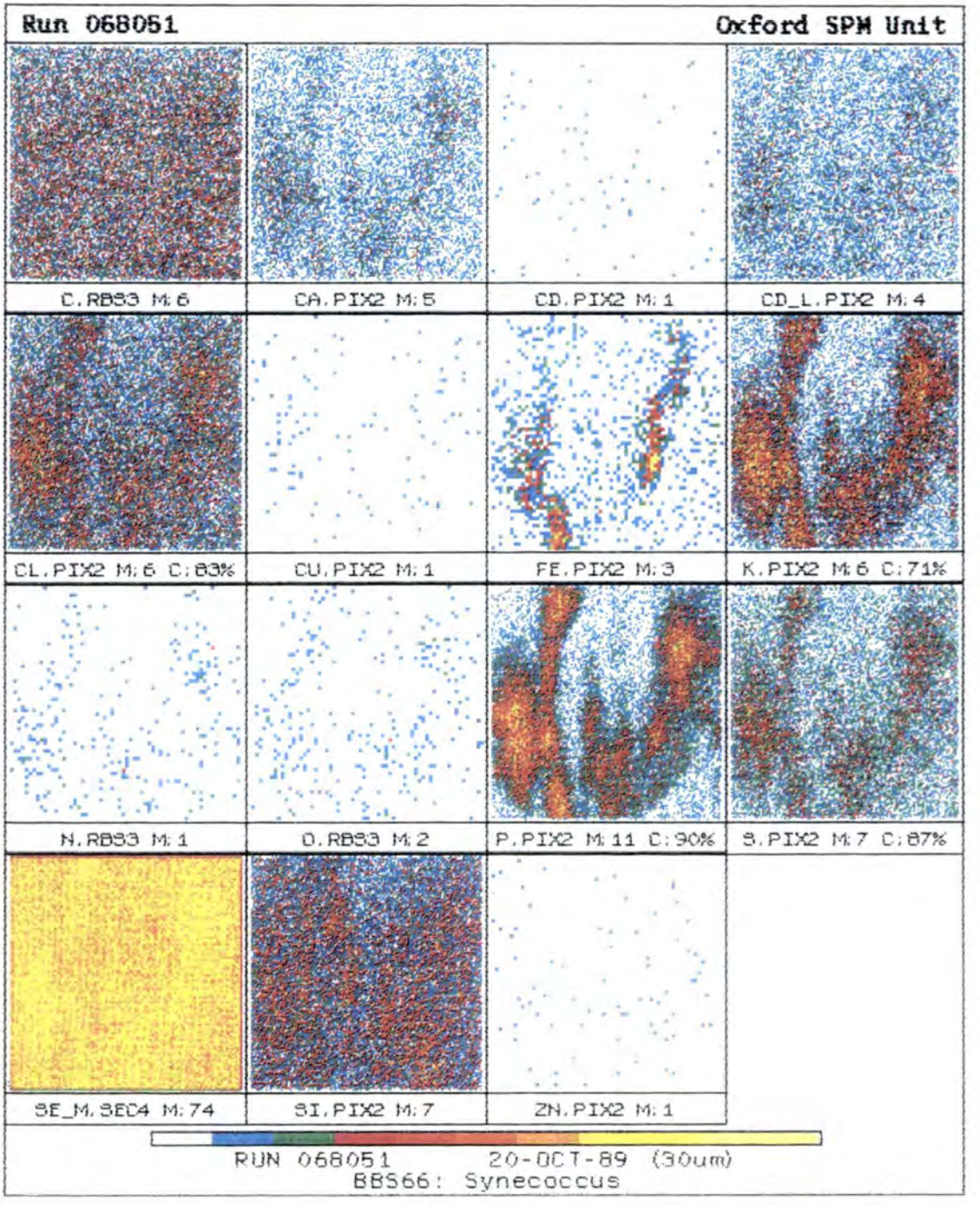
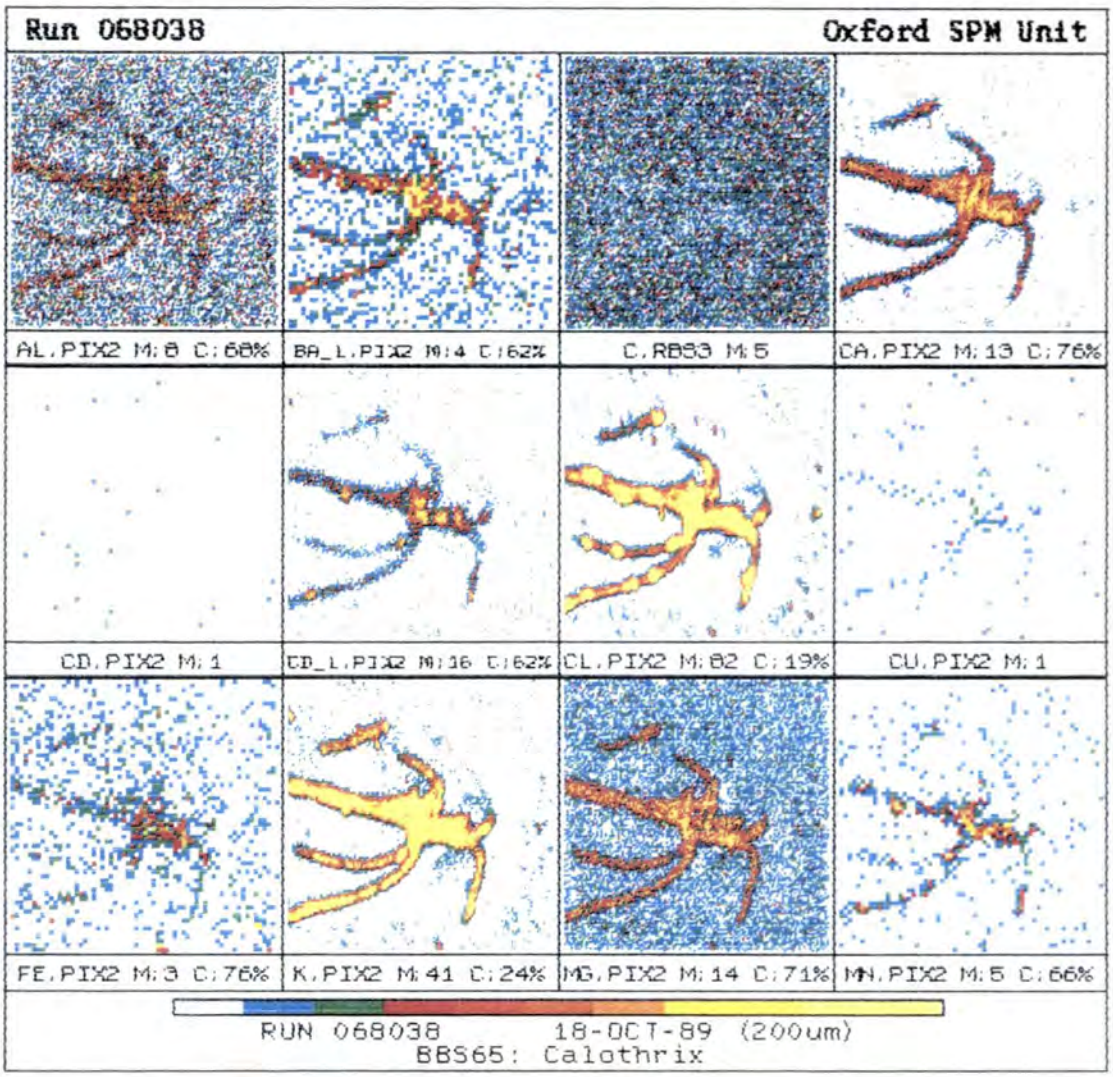

Run 068051
Oxford SPM Unit
C.RBS3 M:6
CA.PIX2 M:5
CD.PIX2 M:1
CD_L.PIX2 M:4
CL.PIX2 M:6 C:83%
CU.PIX2 M:1
FE.PIX2 M:3
K.PIX2 M:6 C:71%
N.RBS3 M:1
O.RBS3 M:2
P.PIX2 M:11 C:90%
S.PIX2 M:7 C:87%
SE_M.SEC4 M:74
SI.PIX2 M:7
ZN.PIX2 M:1
RUN 068051 20-OCT-89 (30um)
BBS66: Synecoccus

Fig. 6.20 SPM pixel map of Calothrix D184 grown at 25 °C and 25 μ mol photon m^{-2} s^{-1} for 15 d with 2 mg l^{-1} Cd

Element	areal density (ng element cm alga $^{-2}$)	element	areal density (ng element cm alga $^{-2}$)
Al	26.68	Mg	24.83
Ba	27.17	Na	720.83
Ca	961.10	Ni	5.94
Cl	5138.19	P	385.06
Cr	76.40	S	572.96
Cu	16.70	Si	1250.66
Fe	201.46	Zn	19.90
K	4195.57		


Run 068038
Oxford SPM Unit
AL.PIX2 M:8 C:68%
BA_L.PIX2 M:4 C:62%
C.RB83 M:5
CA.PIX2 M:13 C:76%
CD.PIX2 M:1
CD_L.PIX2 M:16 C:62%
CL.PIX2 M:82 C:19%
CU.PIX2 M:1
FE.PIX2 M:3 C:76%
K.PIX2 M:41 C:24%
MG.PIX2 M:14 C:71%
MN.PIX2 M:5 C:66%
RUN 068038 18-OCT-89 (200um)
BBS65: Calothrix

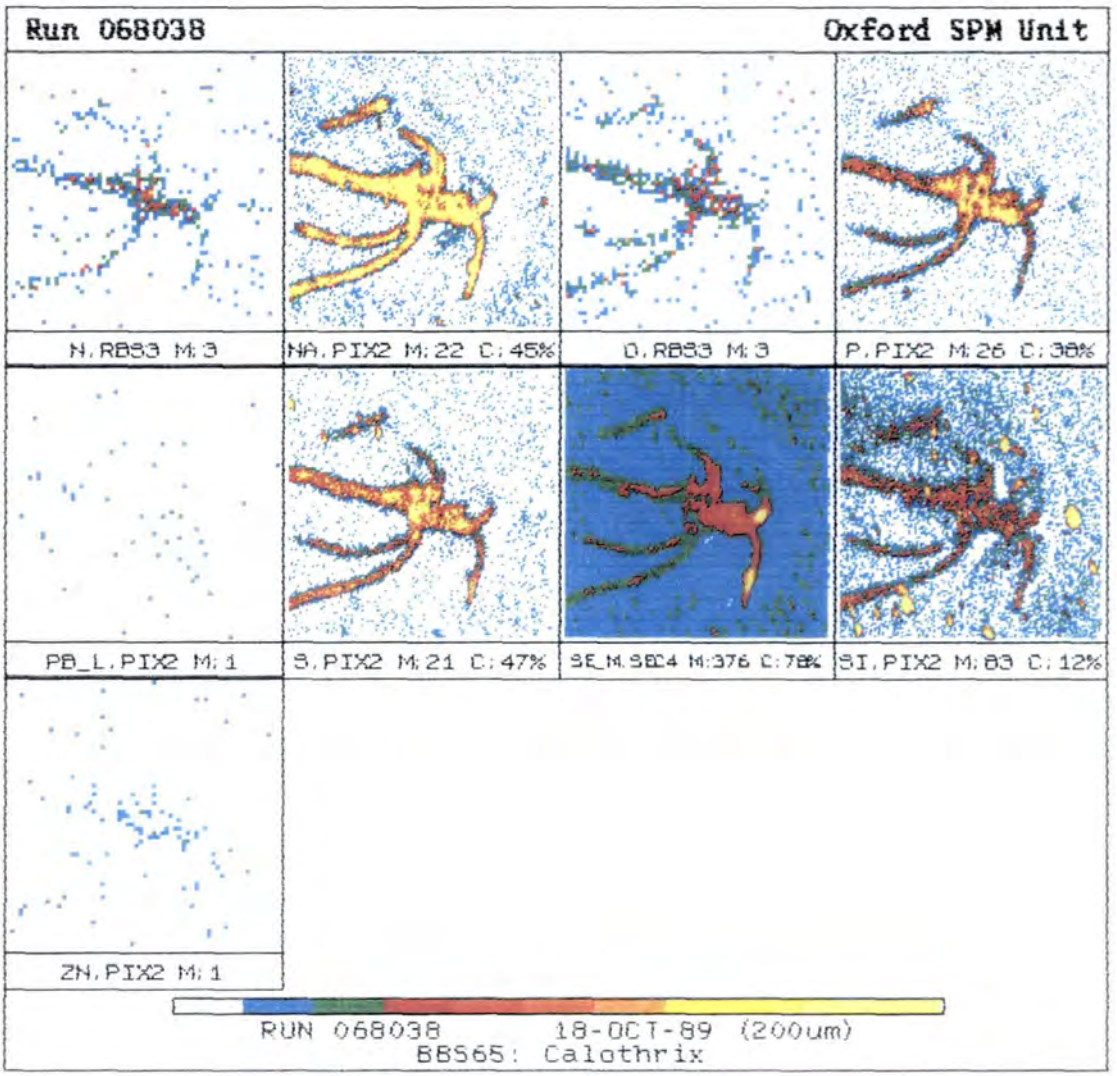
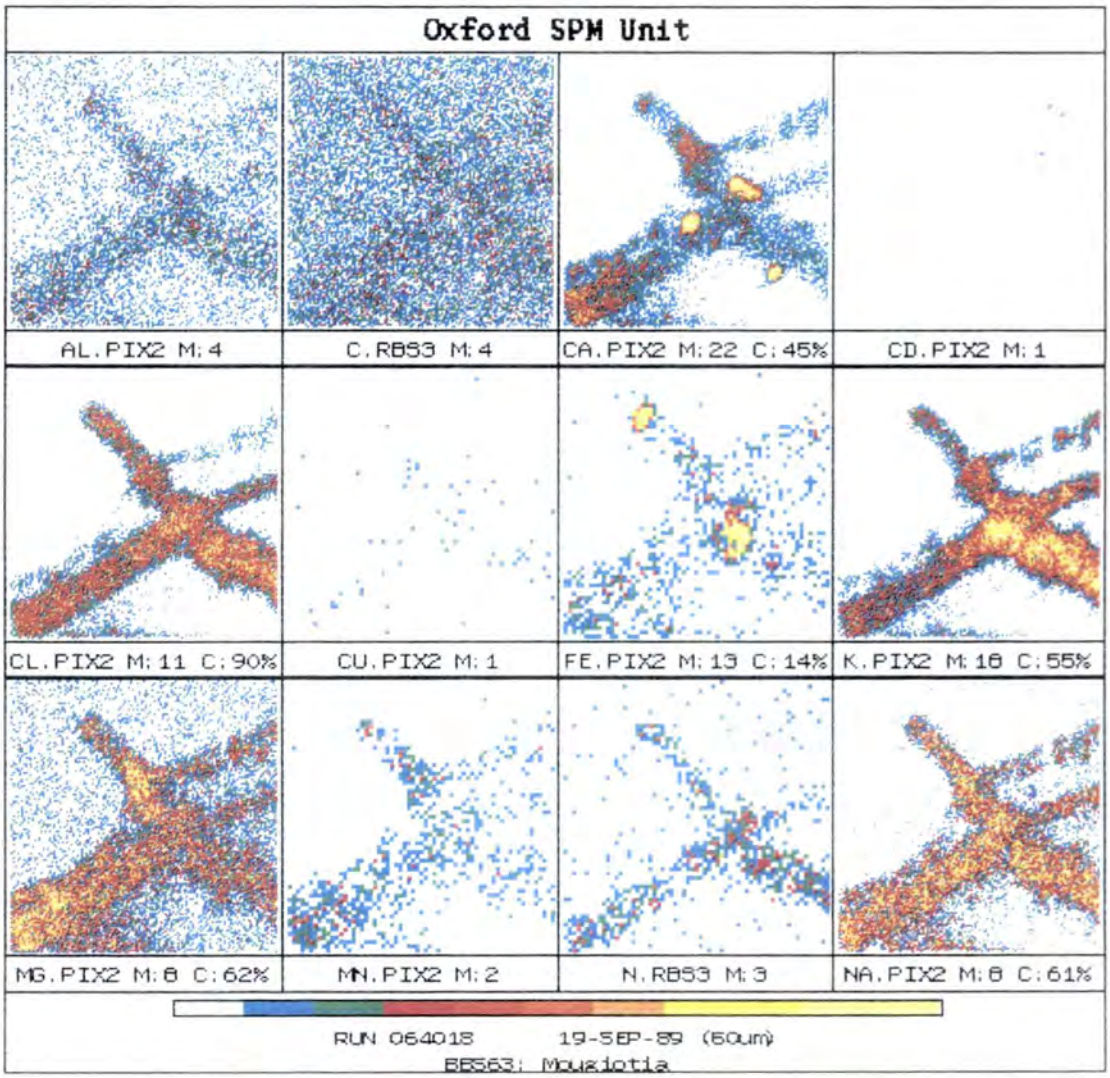

Run 068038
Oxford SPM Unit
N, RB83 M: 3
NA, PIX2 M: 22 C: 45%
O, RB83 M: 3
P, PIX2 M: 26 C: 38%
PB_L, PIX2 M: 1
S, PIX2 M: 21 C: 47%
SE_M, SEC4 M: 376 C: 78%
SI, PIX2 M: 83 C: 12%
ZN, PIX2 M: 1
RUN 068038 18-OCT-89 (200um)
BB565: Calothrix

Fig. 6.21 SPM pixel map of Mougeotia D536 grown at 25 ^{o} C and 25 μ mol photon m^{-2} s^{-1} for 15 d with 0.5 mg l^{-1} Cd

Element	areal density (ng element cm alga $^{-2}$)	element	areal density (ng element cm alga $^{-2}$)
Al	183.61	Mn	153.06
Ca	2417.869	Na	893.70
Cl	1832.89	P	2001.89
Cu	14.07	S	1537.16
Fe	448.47	Si	309.71
K	4452.24	Zn	83.85
Mg	532.97		


Oxford SPM Unit
AL.PIX2 M:4
C.RBS3 M:4
CA.PIX2 M:22 C:45%
CD.PIX2 M:1
CL.PIX2 M:11 C:90%
CU.PIX2 M:1
FE.PIX2 M:13 C:14%
K.PIX2 M:16 C:55%
MG.PIX2 M:8 C:62%
MN.PIX2 M:2
N.RBS3 M:3
NA.PIX2 M:8 C:61%
RUN 064018 19-SEP-89 (60um)
BBS63: Mouziotia

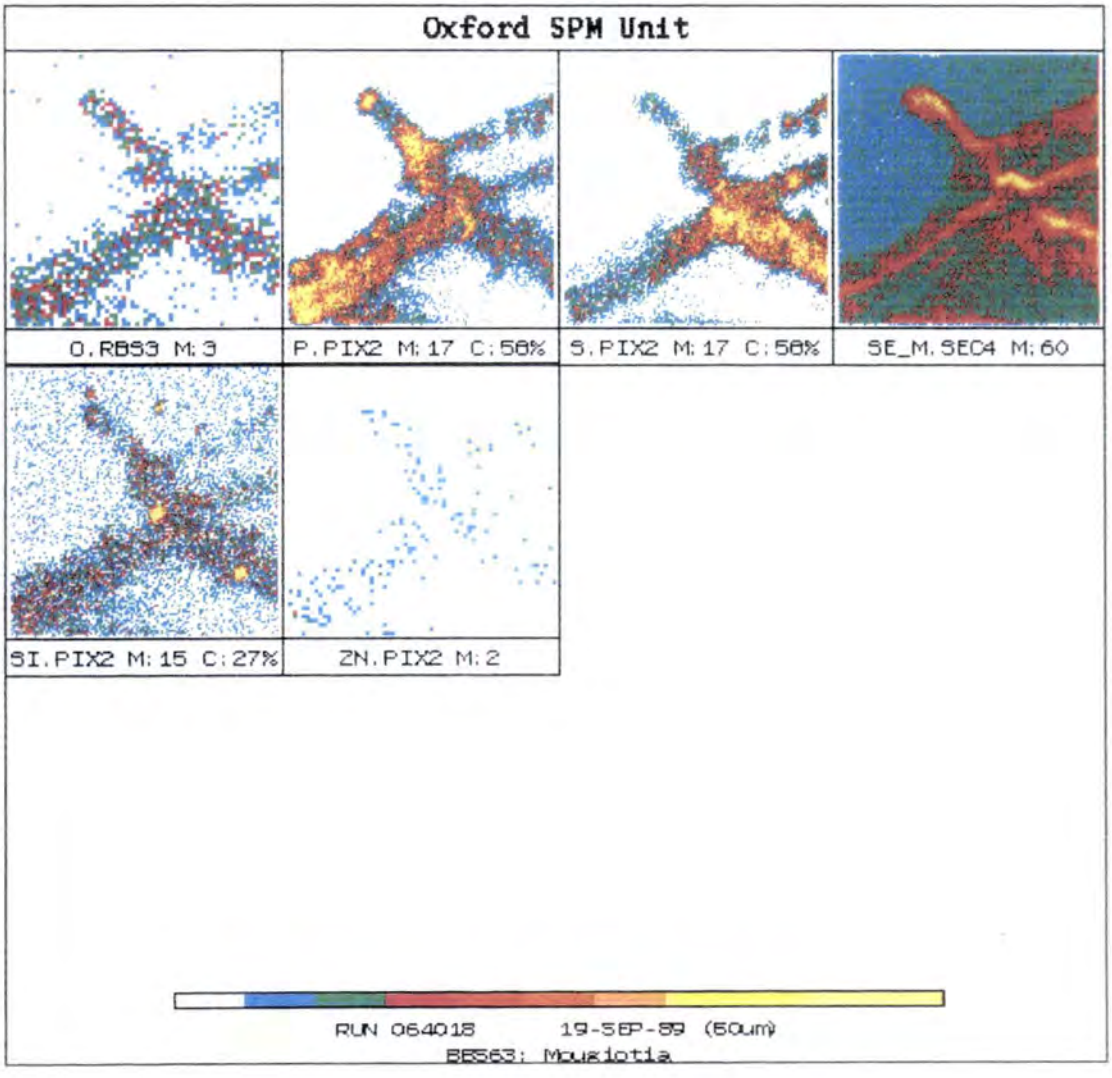
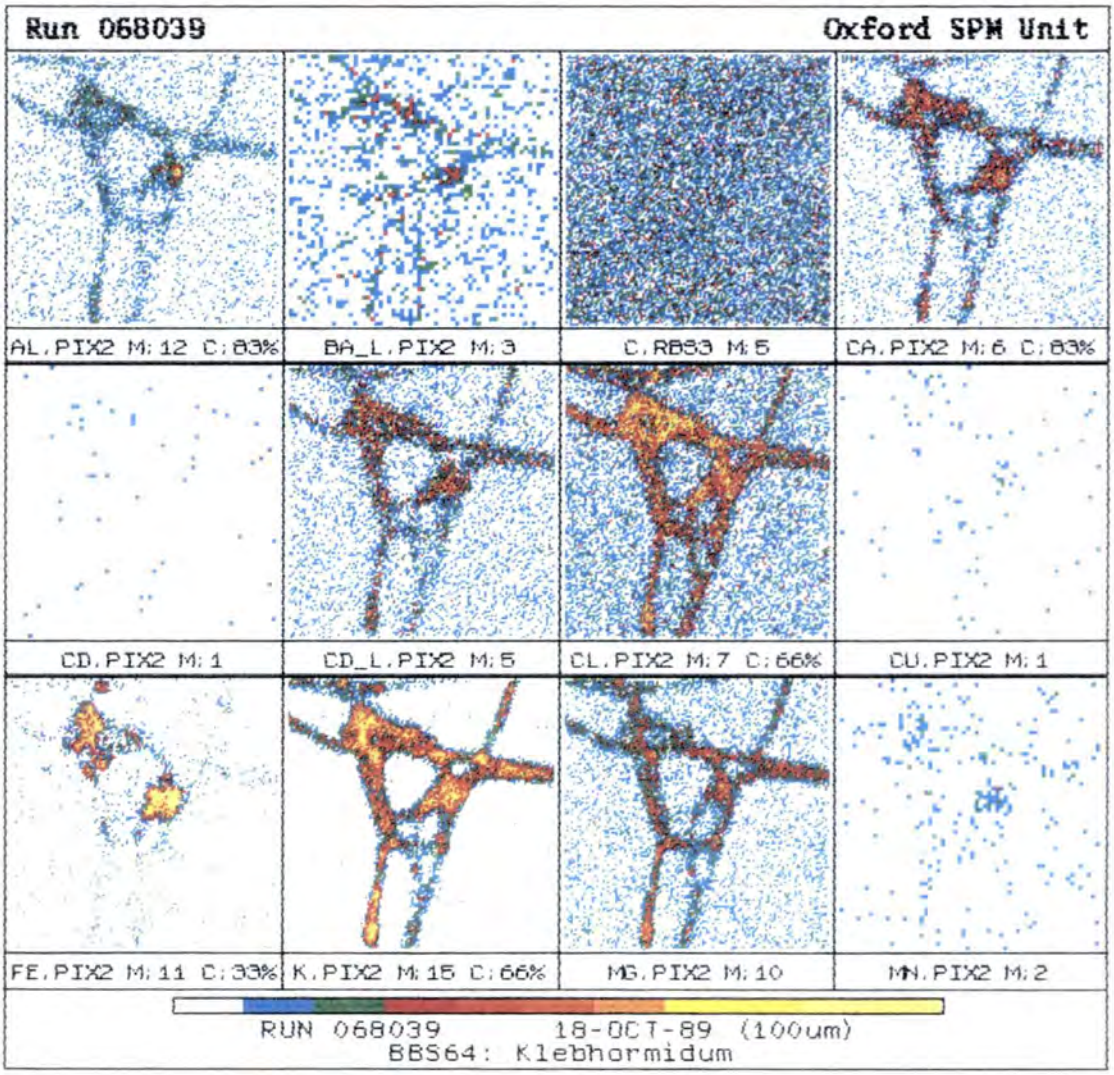
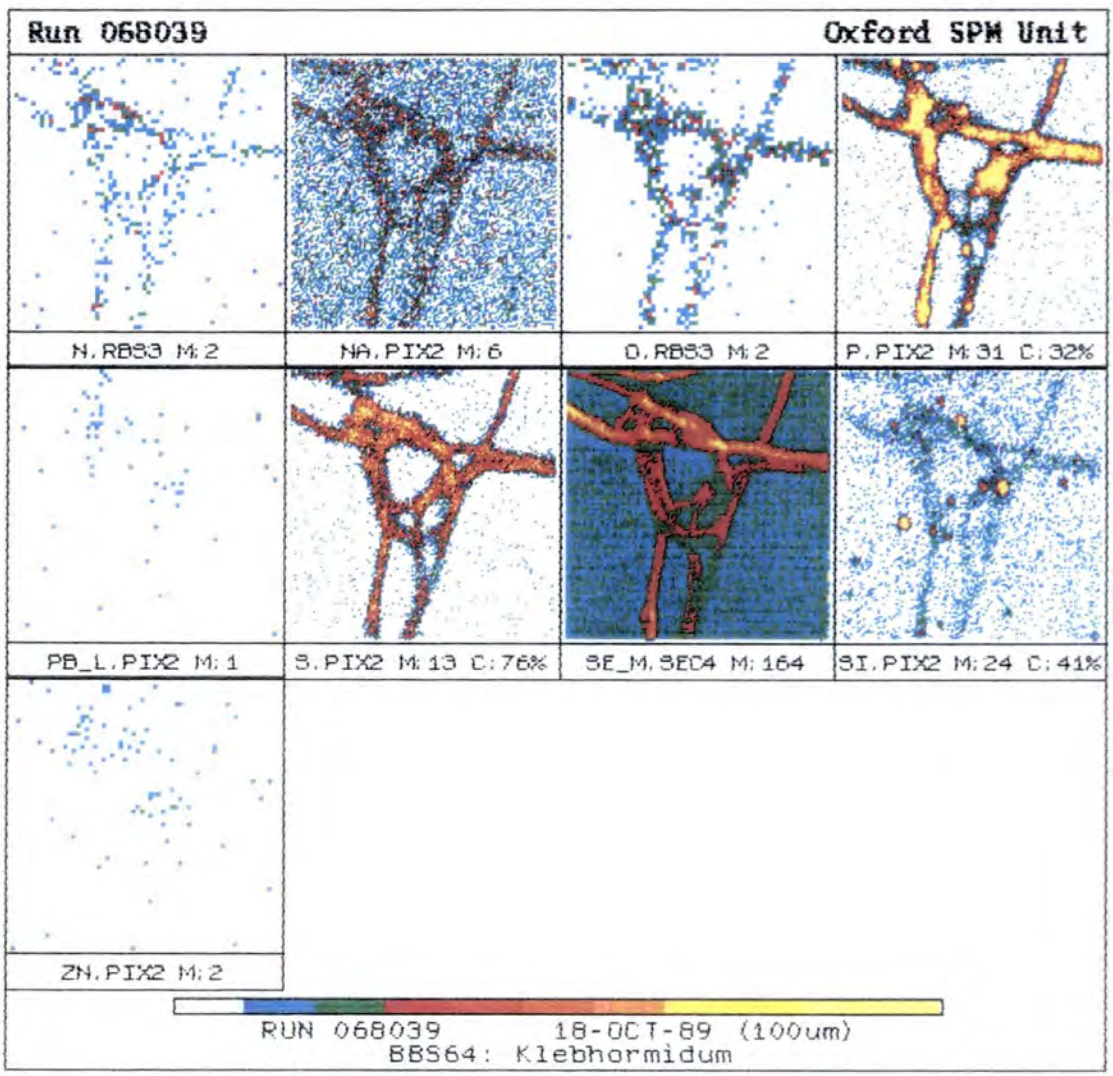

Oxford SPM Unit
O.RBS3 M:3
P.PIX2 M:17 C:58%
S.PIX2 M:17 C:58%
SE_M.SEC4 M:60
SI.PIX2 M:15 C:27%
ZN.PIX2 M:2
RUN 064018 19-SEP-89 (50um)
BB563: Mousiotia

Fig. 6.22 SPM of Klebsormidium D537 grown for 15 d with 0.5 mg l^{-1} Cd

Element	areal density (ng element cm alga $^{-2}$)	element	areal density (ng element cm alga $^{-2}$)
Al	309.78	Mg	354.68
Ca	106.94	Mn	3.84
Cd	20.40	Na	38.44
Cl	262.21	P	1115.05
Cr	5.44	S	601.29
Cu	8.07	Si	377.52
Fe	665.98	Ti	2.89
K	1023.71	Zn	15.15


Run 068039
Oxford SPM Unit
AL.PIX2 M:12 C:83%
BA_L.PIX2 M:3
C.RB83 M:5
CA.PIX2 M:6 C:83%
CD.PIX2 M:1
CD_L.PIX2 M:5
CL.PIX2 M:7 C:66%
CU.PIX2 M:1
FE.PIX2 M:11 C:33%
K.PIX2 M:15 C:66%
MG.PIX2 M:10
MN.PIX2 M:2
RUN 068039 18-OCT-89 (100um)
BBS64: Klebhormidum


Run 068039
Oxford SPM Unit
N.RBS3 M:2
NA.PIX2 M:6
O.RBS3 M:2
P.PIX2 M:31 C:32%
PB_L.PIX2 M:1
S.PIX2 M:13 C:76%
SE_M.SEC4 M:164
SI.PIX2 M:24 C:41%
ZN.PIX2 M:2
RUN 068039 18-OCT-89 (100um)
BBS64: Klebhormidum

CHAPTER 7

7 Tolerance mechanisms

7.1 Introduction

The intracellular tolerance mechanisms employed by microorganisms to ensure their survival in the presence of high concentrations of metal is of interest in this research; the biochemistry of metal storage could be manipulated to enhance the concentration of metal removed by cyanobacteria and algae that have been immobilized in a packed-bed column. The relatively high concentrations of Cd tolerated by Synechococcus D562, may possibly be attributed to the presence of a plasmid or genome encoded MT gene, whose product has the potential to reduce the cytotoxic concentration of Cd. Enhancing the number of transcripts of this gene may increase the concentration of accumulated Cd. To determine the presence of covalently-closed circles of DNA in Synechococcus D562, spheroplast production and plasmid purification protocols were developed. Potential MT encoding regions in the extracted plasmid and genomic DNA were probed for on Southern blots with a radiolabelled probe; a conserved sequence from the Synechococcus PCC 6301 MT gene (see Appendix 3). This approach assumed that any MT produced by Synechococcus D562 exhibits a similar amino acid sequence to the regions of MT DNA from Synechococcus PCC 6301 that were selected to model the probe.

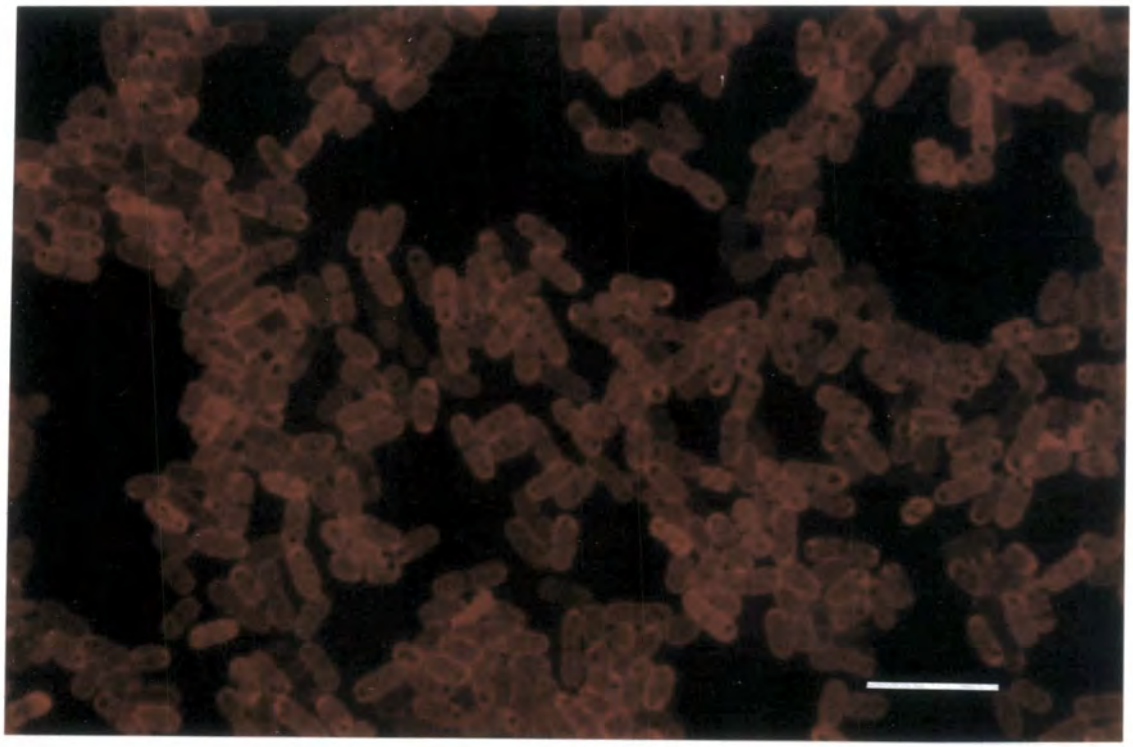
7.2 Production of Synechococcus D562 spheroplasts

The first step in DNA purification involves gentle cell lysis (frequently yielding spheroplasts or protoplasts), often with the use of enzymes that catabolise cell-wall constituents. Although lysozyme treatment resulted in some cell degradation (Fig. 7.1b) the cell wall was not sufficiently hydrolysed to allow subsequent detergent solubilisation of the plasma membrane; cells appeared intact after the addition of sodium-lauryl sarcosine. The cell clumping observed immediately after the addition of lysozyme, suggests that either the cell wall or extracellular mucilage was hydrolysed by this enzyme. The fact that Ru red (which reacts with exposed anionic acidic groups) would only stain the mucilage of enzyme treated cells, indicates that this material is one of the main sites of hydrolysis. Further

treatment with the non-specific protease from Streptomyces griseus produced a large number of spherical cells which were sodium-lauryl sarcosine sensitive and upon detergent treatment released a high percentage of their genomic and plasmid DNA (Fig. 7.2a). Protease digestion alone did not result in spherical, detergent sensitive cells (micrograph not shown).

Enzyme degradation failed to strip away all of the cell-wall material as auramine O bound to some ligands on the surface of Synechococcus D562, giving rise to orange fluorescence at the periphery of the spheroplasts (Fig. 7.2b). As this particular digestion protocol did not yield viable cells even in the presence of an osmoticum, a study of the uptake of Cd by cell-wall degraded Synechococcus D562 was not possible.

Fig. 7.1 a) Fluorescence photomicrograph of untreated Synechococcus D562 cells grown at 32 °C and 40 μ mol photon m^{-2} s^{-1}, note non-fluorescent PP bodies. Scale bar = 5 μm



b) Fluorescence photomicrograph of lysozyme treated (final concentration = 2 mg l^{-1}) Synechococcus D562 cells, note cell aggregation, but maintainence of rod-shaped conformation. Scale bar = 5 μm

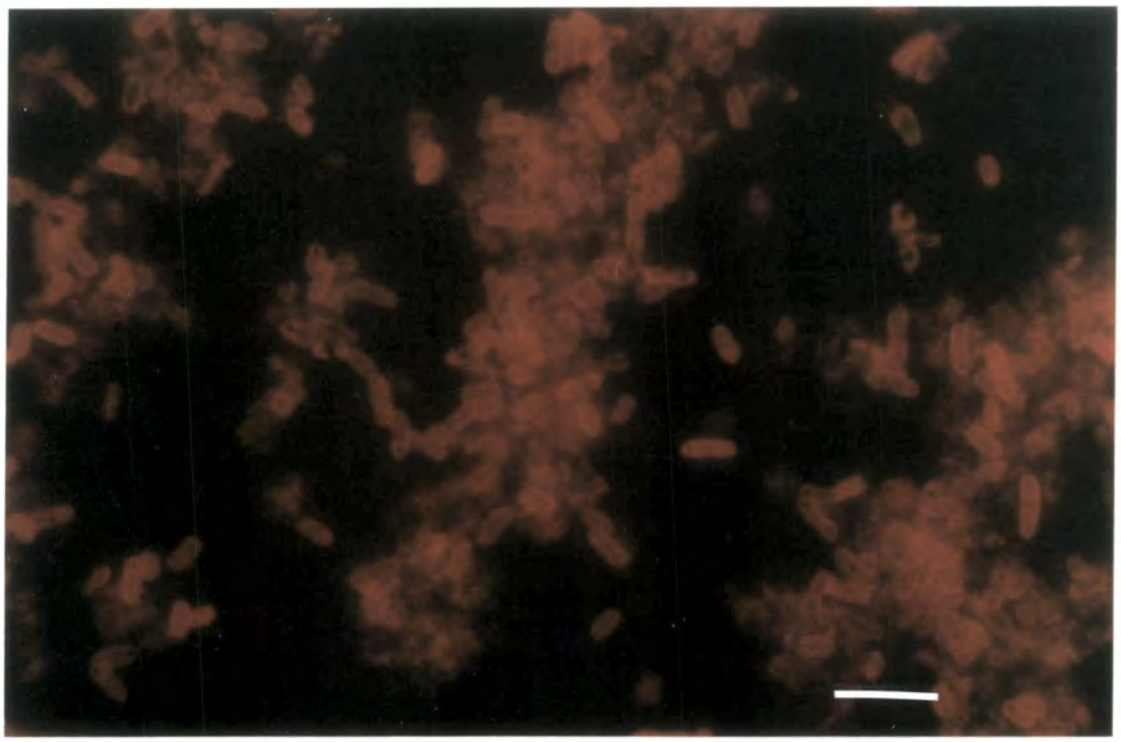
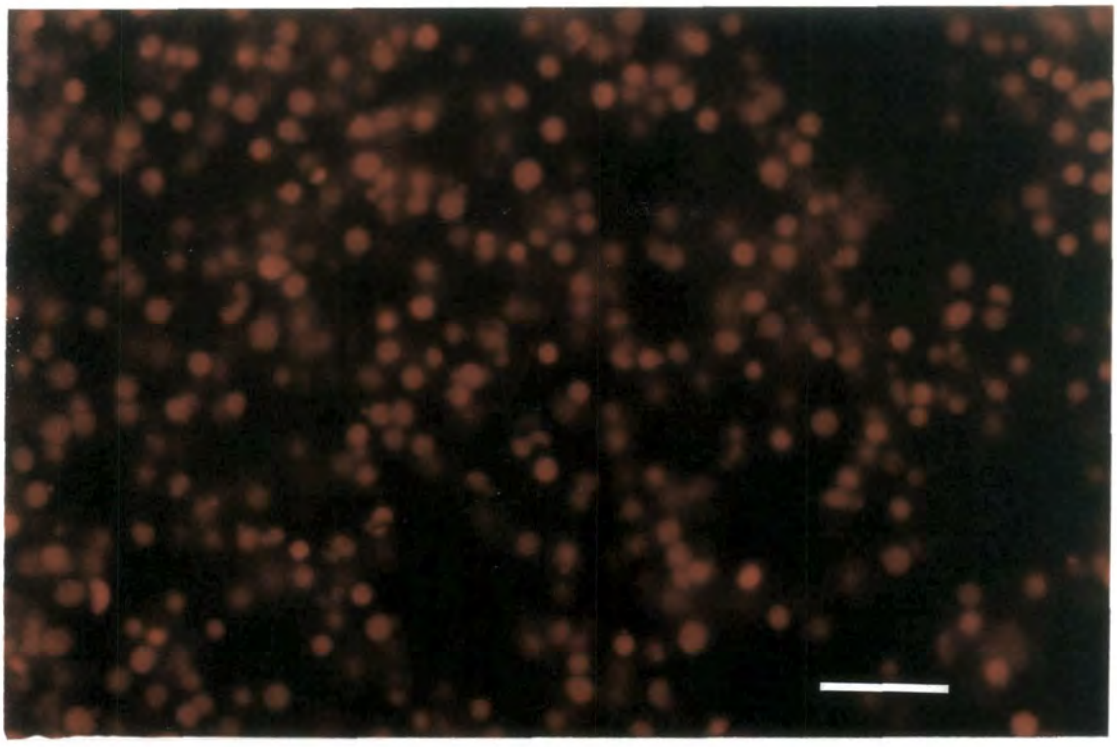
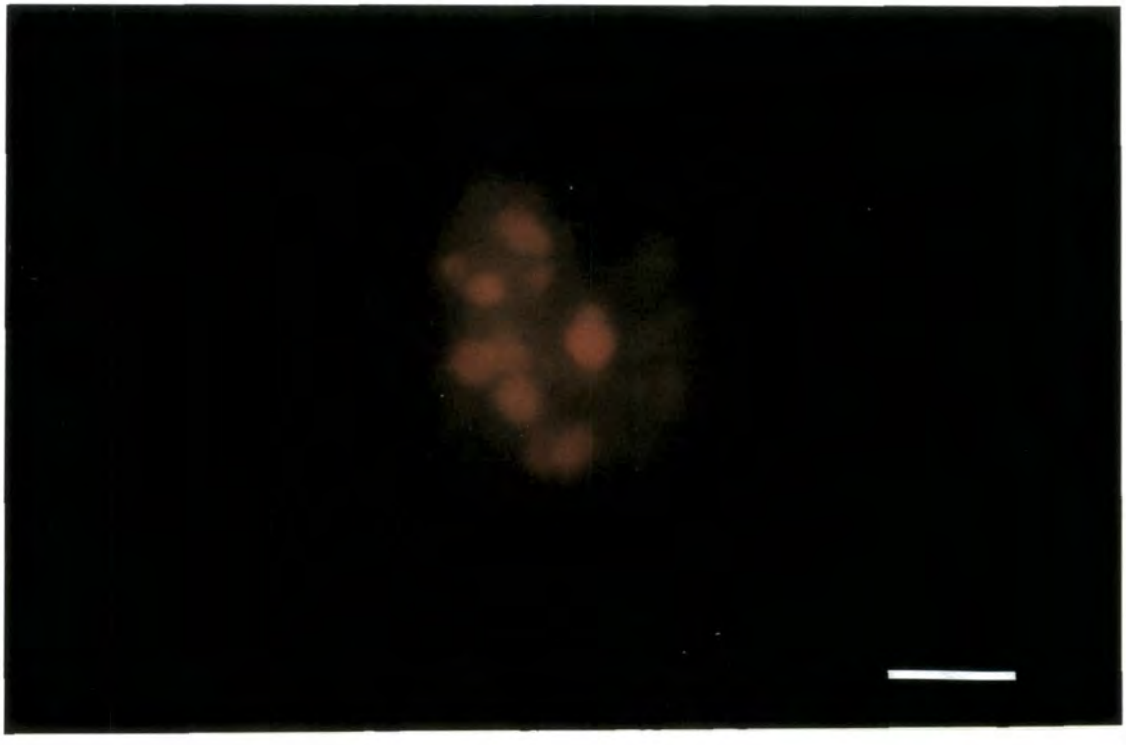


Fig. 7.2 a) Fluorescence photomicrograph of lysozyme and protease treated (final concentration = 2 mg l^{-1}) Synechococcus D562 cells, note efficient production of spherical cells. Scale bar = 5 μm



b) Fluorescence photomicrograph of Synechococcus D562 spheroplasts stained with auramine O. Scale bar = 2.5 μm



7.3 DNA extraction and restriction

Both plasmid and genomic DNA was purified from Synechococcus D562 cells to determine the presence of a Cd-binding MT. Cells cultured with or without Cd harboured a plasmid of about 14 kb in length (Figs 7.3a and b) (although Eco RI fragments are not accurately sized on 0.7 % agarose gels) with common nuclease digestion sites (Table 7.1) (see Appendix 8 for typical size marker calibration). The third band which appears between the two Eco RI fragments is the result of a partial digestion.

A Southern blot of both plasmid and genomic material did not reveal any homology between the oligonucleotide probe and the Synechococcus D562 DNA, except for the positive control at the edge of the filter (data not included). It therefore appears unlikely that an MT gene with a sequence that is complementary to primers based on the Synechococcus PCC6301 MT protein, is present either in the plasmid or genomic DNA of Synechococcus D562.

Table 7.1 Restriction fragments of the Synechococcus D562 plasmid, * the two smallest fragments were visible on the original gel photograph

Restriction enzyme	fragment sizes (bp)
Eco RI	12589, 5754
Hind III *	5039, 4120, 2035, 1504, 1360
Bam HI	7930, 5862

Fig. 7.3 Agarose-gel electrophoresis of the plasmid isolated from Synechococcus D562 which was restricted by three endonucleases, gel calibration markers are the number of base pairs in each fragment of λ DNA

a) Lane 2. Eco RI / Hind III λ markers

4. Eco RI digested pUC19

6. Eco RI digested D562 plasmid DNA from cells grown without Cd

8. Eco RI digested D562 plasmid DNA from cells grown with 2 mg l^{-1} Cd

10. Undigested pUC19

12. Eco RI / Hind III λ markers

b) Lane 2. Eco RI / Hind III λ markers

4. Hind III digested pUC19

5. Bam HI digested pUC19

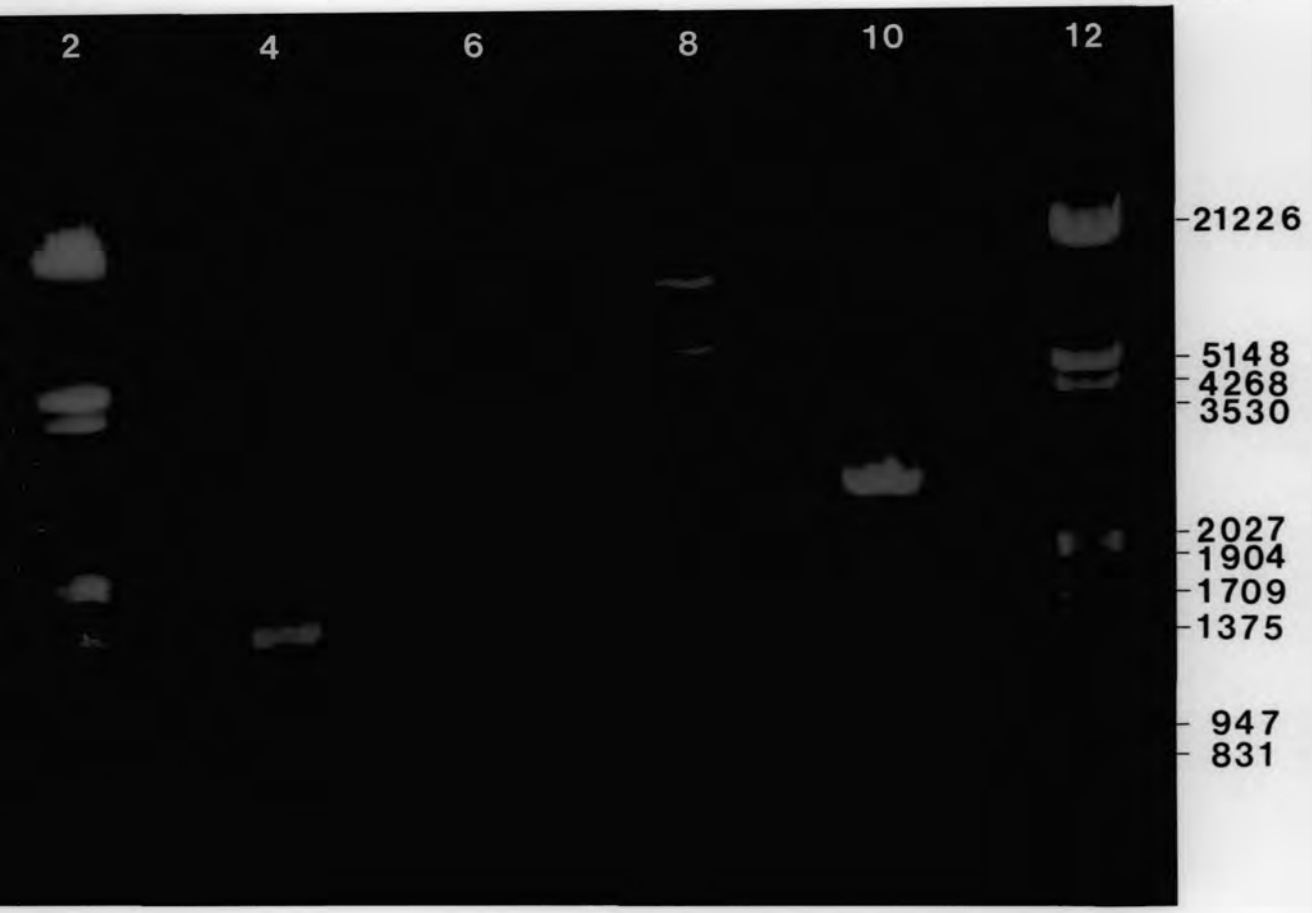
6. Hind III digested D562 plasmid DNA from cells grown without Cd

7. Bam HI digested D562 plasmid DNA from cells grown without Cd

8. Hind III digested D562 plasmid DNA from cells grown with 2 mg l^{-1} Cd

9. Bam HI digested D562 plasmid DNA from cells grown with 2 mg l^{-1} Cd

10. Eco RI / Hind III λ markers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947
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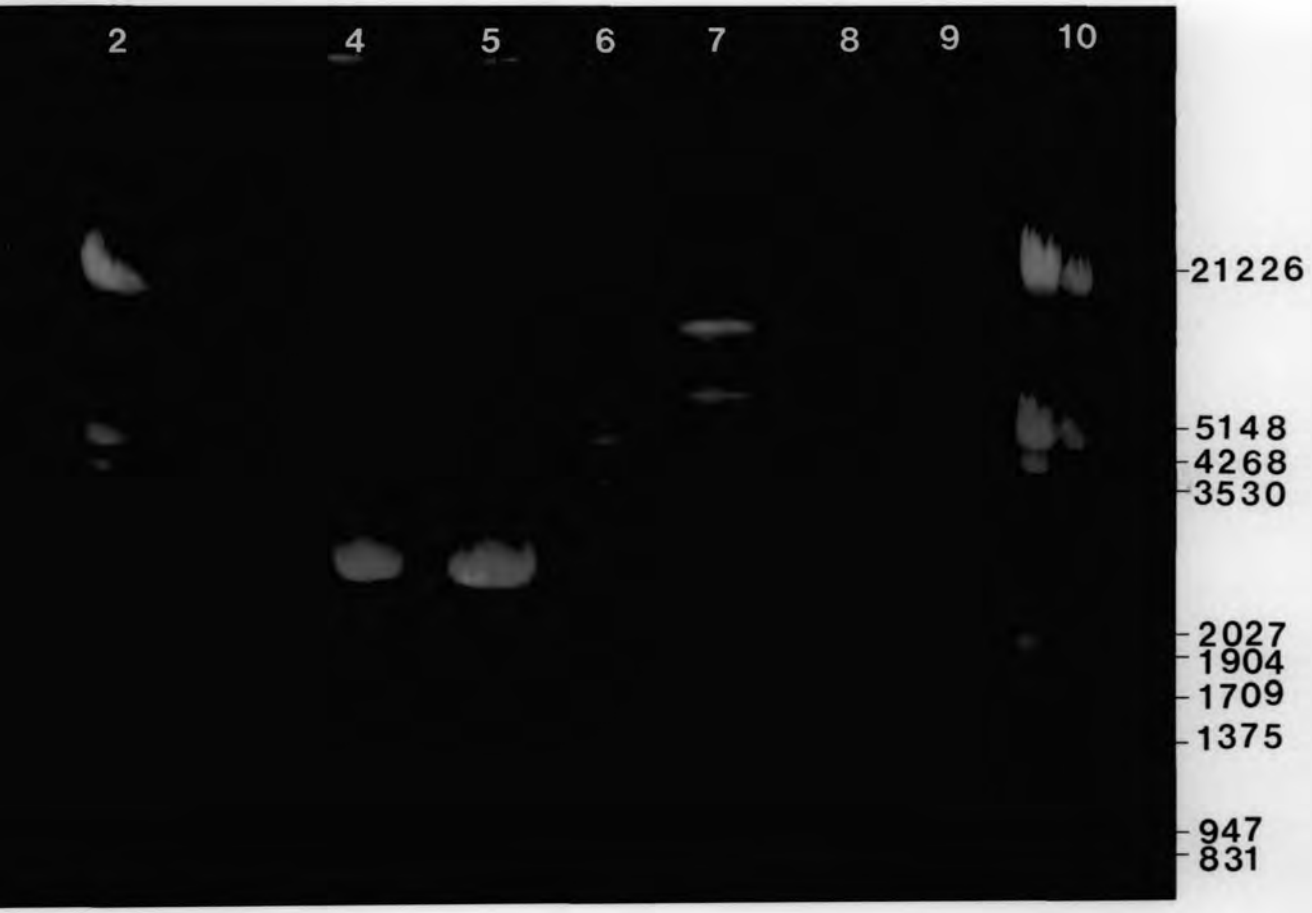
2
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21226
5148
4268
3530
2027
1904
1709
1375
947
831

7.4 Cd-binding peptides

In Cd-resistant cell lines of Datura innoxia $(\gamma EC)_nG$'s bind nearly all of the cytoplasmic metal (Jackson et al., 1987), thus permitting cultures to grow in the presence of very high concentrations of Cd (300 μM). The identification of Cd-binding peptides in cyanobacteria / algae and the extent to which they contribute towards metal detoxification, provides part of the information required to optimise another tolerance mechanism (in addition to the binding of Cd by MT) for the efficient removal of Cd from contaminated effluents.

Putative Cd-binding peptides were discovered in all of the four culture collection strains close to the same elution volume as those extracted from Datura innoxia (Fig. 7.7 c from Robinson et al., 1990), although the concentration of bound Cd varied between strains.

For Mougeotia D536, cells cultured in the presence of 0.5 mg l^{-1} Cd exhibited more Cd bound to peptides than cells which were only exposed to Cd in mid log-phase (Figs 7.5 a and b), this effect was not observed in the other strains. Without the Cd shock (6.17 mg l^{-1}), cells cultured in the presence of 0.5 mg l^{-1} Cd did not produce any Cd peaks on the gel permeation HPLC profile (Fig. 7.5 c). A cell extract exposed to equivalent concentrations of Cd as that bound by the putative $(\gamma EC)_n$g's, only produced a peak for Cd at the β-mercaptoethanol volume, no peptide / metal complex was observed (Fig. 7.5 d)

Cultures of Synechococcus D562 grown in the presence of both 0 and 0.5 mg l^{-1} Cd produced similar metal profiles (Figs 7.4 a and b). The Cd accumulated by cells that were not shocked with metal in mid log-phase was diluted to such an extent by the elution buffer that the metal profile was devoid of peaks for Cd at either the β-mercaptoethanol or peptide elution volumes (Fig. 7.4 c) For cells which had not been shocked with Cd but were exposed to the metal after extraction, no peptide-bound peak was observed, all of the added metal was collected at the same elution volume as β-mercaptoethanol (Fig. 7.4 d).

The gel permeation HPLC profile for Klebsormidium D537 cultures grown in the presence and absence of Cd did not reveal different peak integrals for the putative $(\gamma EC)_nG$-bound Cd (Fig. 7.6 a and b)

Even though Calothrix D184 did not accumulate Cd to a high concentration, $(\gamma EC)_nG$'s complexed a significant proportion of the internalised metal (Fig. 7.6 c and d).

The peak for the peptide / Cd complex did not always elute to the same position for all samples, this may represent complexes composed of different chain lengths rather than a variation in the column retention time from one strain to the next.

For eucaryotes, the main protein peak at 5.5 ml elution volume did not coincide with chelated Cd, whilst procaryotes demonstrated a broader protein distribution amongst the fractions including a peak at the same elution point as the metal binding peptides. Further evidence that the Cd-binding peak with a variable elution volume of 8 to 11.25 ml is composed of $(\gamma EC)_nG$'s emanates from the pH titration profile of Mougeotia D536 peptides (Fig. 7.7 a), which exhibits a pH of half dissociation of 5.0 in common with metal binding peptides of other species (Weber et al., 1987 and Reese et al., 1988).

The reversed-phase HPLC profile of a Mougeotia D536 cell extract exhibited peaks at the correct retention time for oxidised glutathione, $(\gamma EC)_2G$, $(\gamma EC)_3G$ and $(\gamma EC)_4G$ (Fig. 7.7 b). Such a profile provides further evidence that the Cd which elutes upstream of the β-mercaptoethanol-associated metal is complexed by $(\gamma EC)_nG$'s.

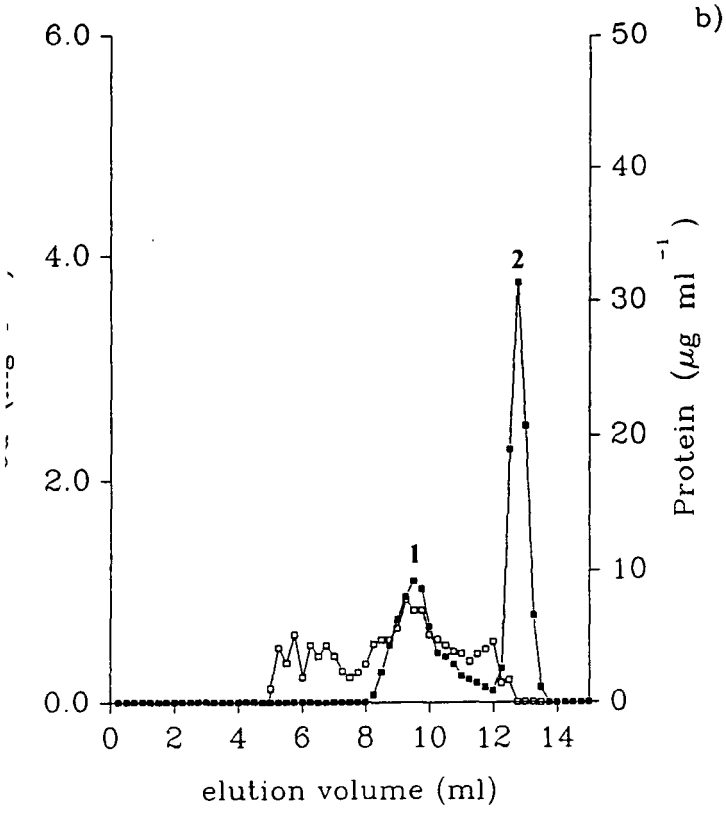
Fig. 7.4 Gel permeation HPLC profiles of Synechococcus D562 cells grown at 32 ^{o} C, 40 μ mol photon m^{-2} s^{-1} and exposed to a number of Cd treatments, elution buffer = 50 mM Tris.Cl pH 7.2, 150 mM NaCl. All plots share common axis and peak labelling ■ = Cd concentration of eluant, □ = protein concentration of eluant

a) 10 d growth without Cd, followed by exposure to 6.17 mg l^{-1} Cd for 2 d, Cd bound by putative $(\gamma EC)_nG$'s = peak 1, Cd bound by β-mercaptoethanol = peak 2

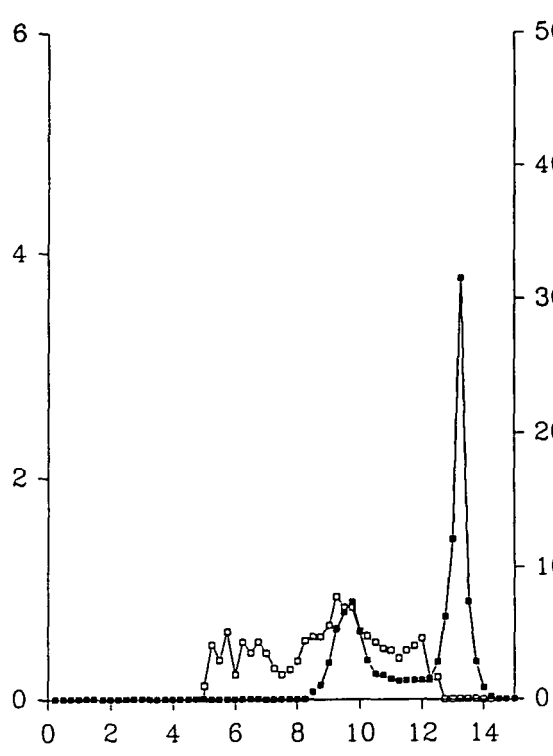
b) 10 d growth in 0.5 mg l^{-1} Cd, followed by exposure to 6.17 mg l^{-1} Cd for 2 d

c) 12 d growth solely with 0.5 mg l^{-1} Cd

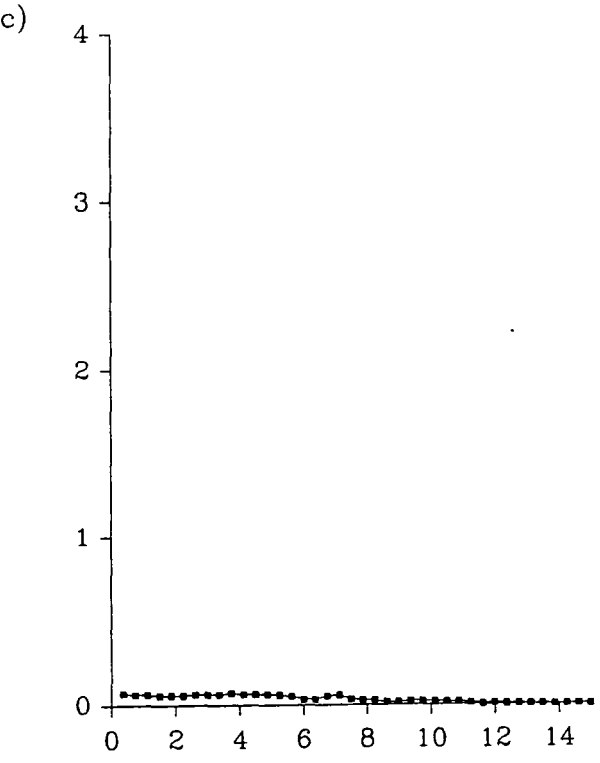
d) 10 d growth without Cd, then the cell extract was exposed to 17.1 mg l^{-1} Cd


6.0
4.0
2.0
0.0
50
40
30
20
10
0
1
2
0 2 4 6 8 10 12 14
elution volume (ml)
Protein (μg ml⁻¹)

b)


6
4
2
0
0 2 4 6 8 10 12 14

c)


4
3
2
1
0
0 2 4 6 8 10 12 14

d)

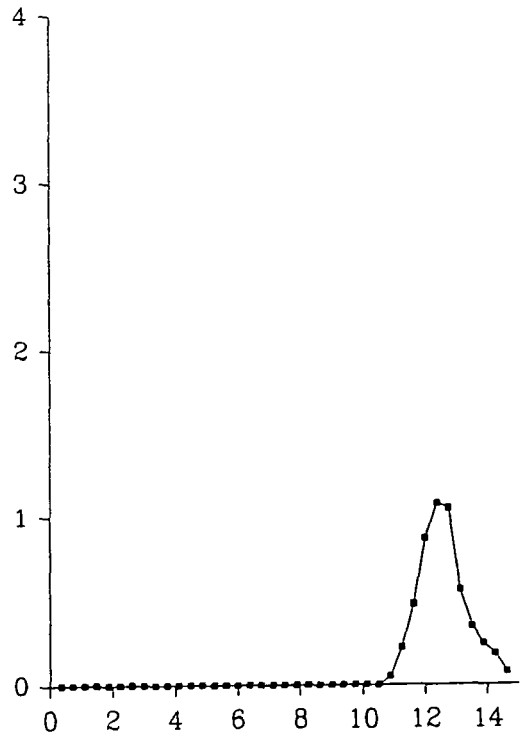

4
3
2
1
0
0 2 4 6 8 10 12 14

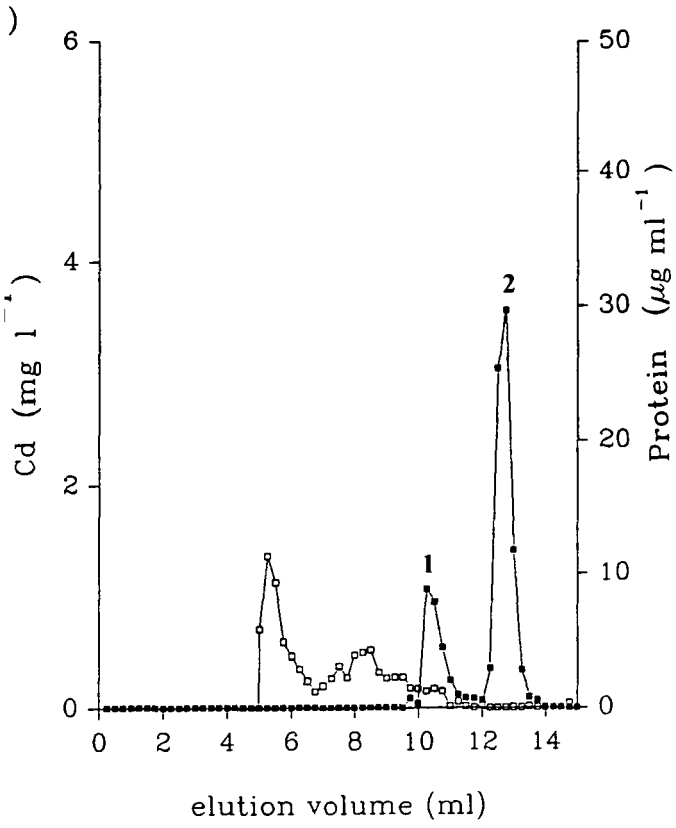
Fig. 7.5 Gel permeation HPLC profiles of Mougeotia D536 cells grown at 25 ° C, 25 μ mol photon m^{-2} s^{-1} and exposed to a number of Cd treatments, elution buffer = 50 mM Tris.Cl pH 7.2, 150 mM NaCl. All plots share common axis and peak labelling ■ = Cd concentration of eluant, □ = protein concentration of eluant

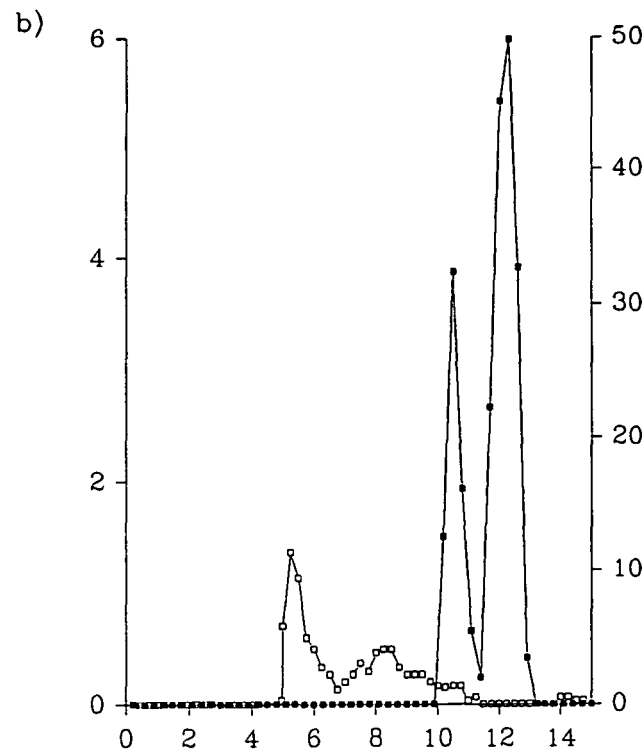
a) 20 d growth without Cd, followed by exposure to 6.17 mg l^{-1} Cd for 2 d, Cd bound by putative $(\gamma EC)_nG$'s = peak 1, Cd bound by β-mercaptoethanol = peak 2

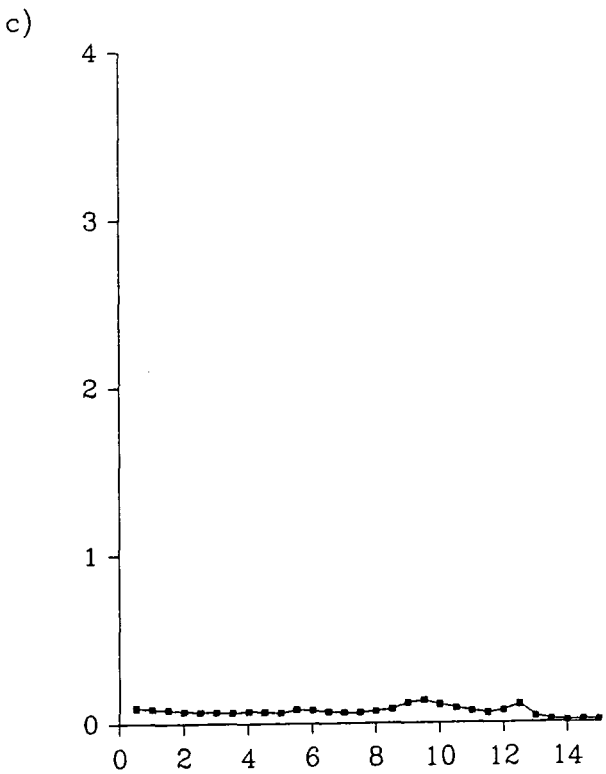
b) 20 d growth in 0.5 mg l^{-1} Cd, followed by exposure to 6.17 mg l^{-1} Cd for 2 d

c) 20 d growth solely with 0.5 mg l^{-1} Cd

d) 20 d growth without Cd, then the cell extract was exposed to 31.35 mg l^{-1} Cd


)
Cd (mg l⁻¹)
Protein (μg ml⁻¹)
1
2
elution volume (ml)


b)


c)

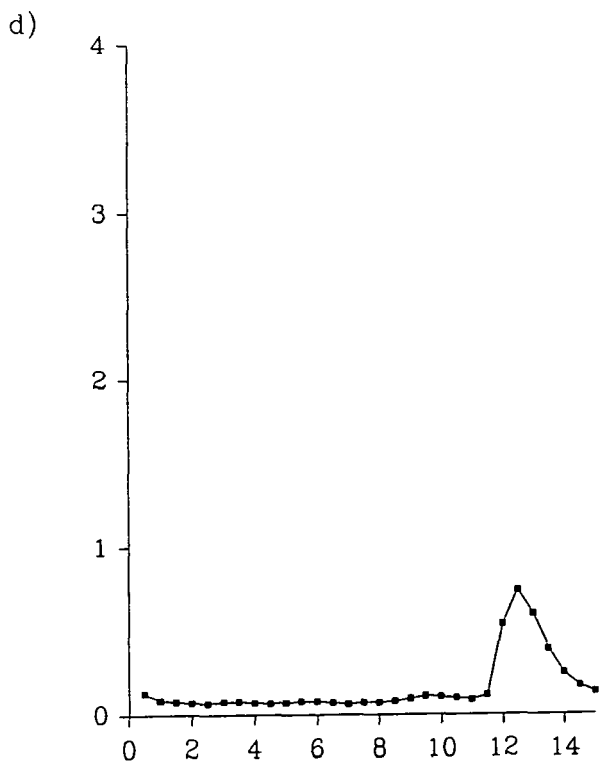

d)

Fig. 7.6 Gel permeation profiles of Klebsormidium D537 (a and b) and Calothrix D184 (c and d) grown at 25 ° C, 25 μ mol photon m^{-2} s^{-1} and exposed to a number of Cd treatments, elution buffer = 50 mM Tris.Cl pH 7.2, 150 mM NaCl. All plots share common axis and labelling ■ = Cd concentration of eluant, □ = protein concentration of eluant

a) 20 d growth without Cd, followed by exposure to 6.17 mg l^{-1} Cd for 2 d,
Cd bound by putative $(\gamma EC)_n G$'s = peak 1, Cd bound by β-mercaptoethanol = peak 2

b) 20 d growth in 0.5 mg l^{-1} Cd, followed by exposure to 6.17 mg l^{-1} Cd for 2 d

c) 20 d growth without Cd, followed by exposure to 6.17 mg l^{-1} Cd for 2 d

d) 20 d growth with 0.5 mg l^{-1} Cd, followed by exposure to 6.17 mg l^{-1} for 2 d

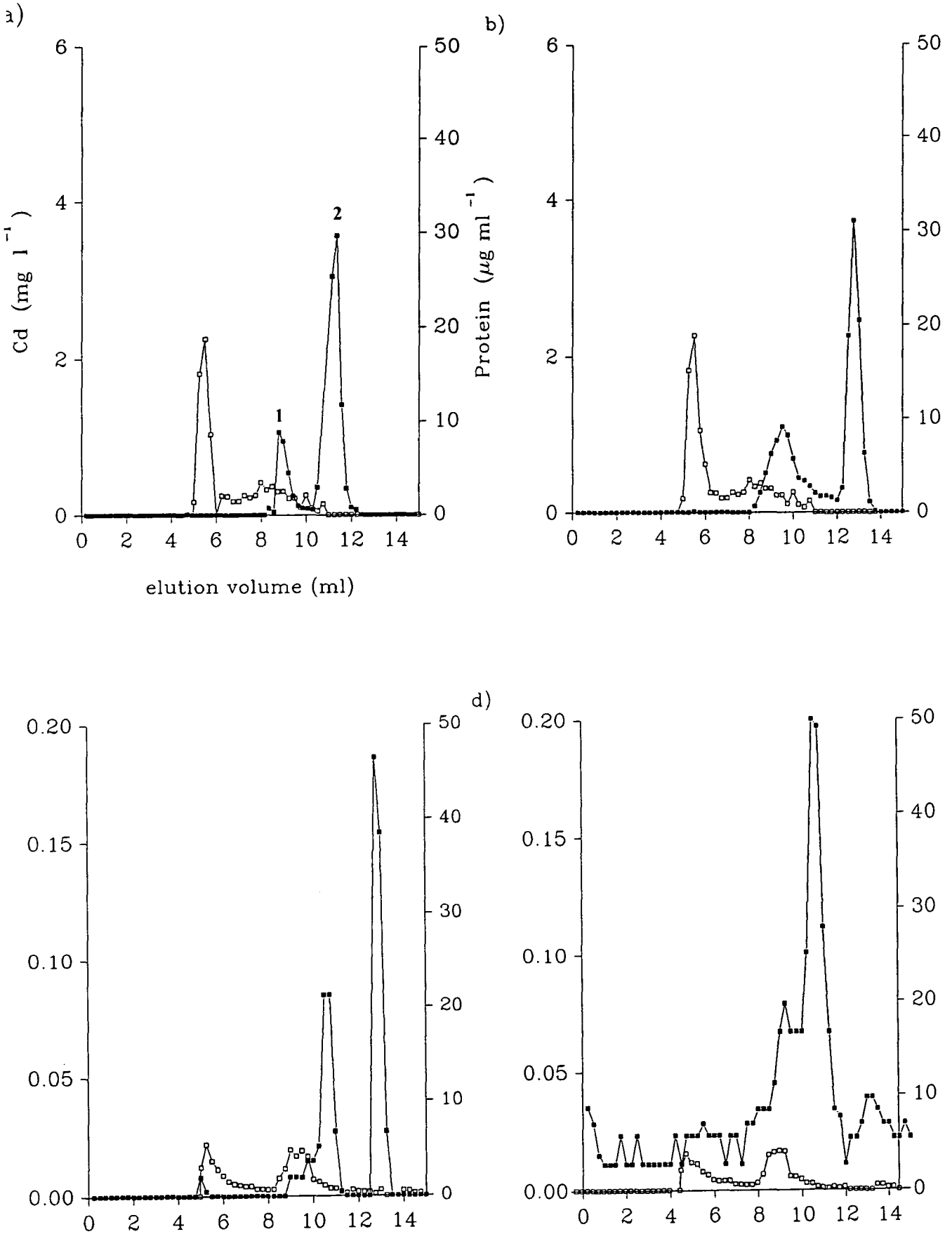
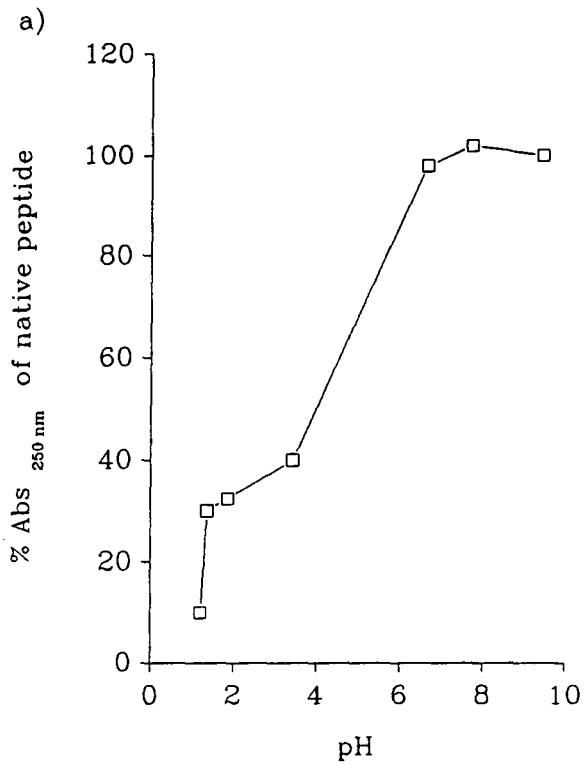

a)
b)
d)
Cd (mg l^{-1})
Protein (μg ml^{-1})
elution volume (ml)
1
2
0
2
4
6
8
10
12
14
0.00
0.05
0.10
0.15
0.20
0
10
20
30
40
50

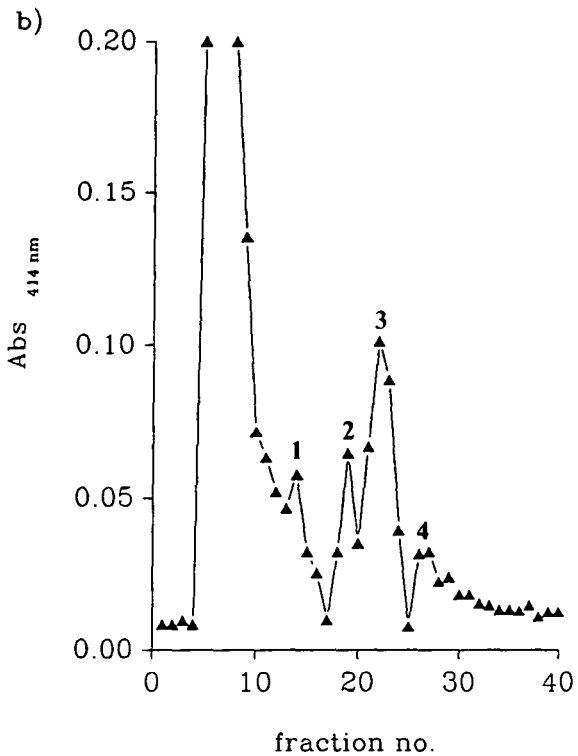
Fig. 7.7 Data used to further characterise the Cd-binding proteins from the cyanobacteria and algae used in this study and calibrate the gel permeation HPLC column

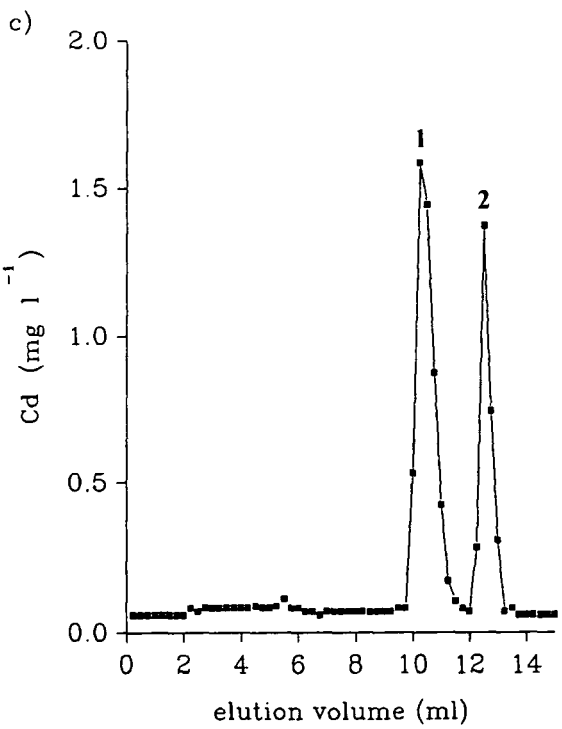
a) pH displacement-profile for Mougeotia D536 $(\gamma EC)_nG$ pooled fraction

b) reversed-phase thiol profile of Mougeotia D536 grown in the presence of 0.5 mg l^{-1} Cd followed by exposure to 6.17 mg l^{-1} Cd. The correct retention times for : oxidised glutathione = peak 1, $(\gamma EC)_2G$ = peak 2, $(\gamma EC)_3G$ = peak 3 and $(\gamma EC)_4G$ = peak 4

c) Gel permeation HPLC profile of Datura innoxia exposed to 14 mg l^{-1} Cd for 24 h at the end of log-phase, elution buffer = 50 mM Tris.Cl pH 7.2, 150 mM NaCl, ■ = Cd concentration of eluant, Cd bound by $(\gamma EC)_nG$'s = peak 1, Cd bound by β-mercaptoethanol = peak 2


a)
% Abs 250 nm of native peptide
120
100
80
60
40
20
0
0
2
4
6
8
10
pH


b)
Abs 414 nm
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
fraction no.
1
2
3
4


c)
Cd (mg l-1)
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
14
elution volume (ml)
1
2

CHAPTER 8

8 Ultrastructural, enzyme and protein damage

8.1 Introduction

The structural damage that Cd inflicts upon a cell can often be visualised by EM photomicrographs (Delmotte, 1980 ; Duret et al., 1986 ; Heumann, 1987). Cyanobacteria and algae which are susceptible to the cytotoxic action of this metal will not accumulate Cd to the same extent as strains which possess intracellular, non-exporting Cd detoxification mechanisms, therefore it is necessary to identify any ultrastructural defects caused by Cd entering the cytoplasm which may reduce the metal accumulating ability of the cells.

By replacing biologically active metal ions or affecting the synthesis and operation of cell membranes, Cd proves toxic at low concentrations. To determine which cell sites suffer ion replacement, two methods of protein analysis (enzyme activity and SDS PAGE) were selected. When faced with limiting levels of inorganic P in the environment, microorganisms synthesise enzymes to hydrolyse molecules containing organic P . The Cd substitution of Zn atoms resident in such enzymes, has been used in X-ray diffraction to obtain information on the position of the three Zn atoms at each active site (Wyckoff, 1987). It was anticipated that such a metal exchange either during enzyme synthesis or in the medium used to assay enzyme activity, may reflect the biotoxicity of Cd by a reduction in the rate of hydrolysis.

In addition, SDS PAGE was utilised to record the membrane disruption potentially caused by cytosolic Cd, a phenomena which has been documented in electron micrographs (Duret et al., 1986) and any other sub-ultrastructural toxic effects.

8.2 Ultrastructural defects

Electron micrographs were produced to search for any membrane damage or loss / induction of inclusion bodies caused by the presence of Cd in the cytoplasm. All of the strains analysed by TEM were sampled in mid-log phase, having been cultured in concentrations of Cd which inhibited growth. The micrographs presented are

representative of at least five similar sections. No obvious ultrastructural changes were observed in Synechococcus D562 cells grown for 5 d in the presence of 2 mg l^{-1} Cd (Figs 8.1 a and b). Calothrix D184 cultured for 15 d with 1 mg l^{-1} Cd exhibited suppressed polyglucoside granules and collapsed thylakoids, resulting in Cd exposed cells with a less granular appearance (Figs 8.2 a and b). When 15 d old cells of Mougeotia D536 were grown in the presence of 0.8 mg l^{-1} Cd, electron-dense material was observed around the periphery of the cytoplasm which was much less frequent in control samples (Figs 8.3 a and b). The plastoglobuli recorded in Klebsormidium D537 cells that had been cultured without added Cd, were infrequently observed in Cd-exposed cells (15 d growth in 1 mg l^{-1} Cd). These organelles were replaced by larger, more electron-opaque lipid bodies which predominated both inside and outside the chloroplasts (Figs 8.4 a and b); the relaxed thylakoid packing shown in Fig. 8.4 b was also common in samples which were grown without Cd and are probably not a result of Cd toxicity.

Fig. 8.1 a) Electron micrograph of a Synechococcus D562 cell grown in PPJ medium for 5 d at 32 ° C and 80 μmol photon m^{-2} s^{-1}, without added Cd. Scale bar = 0.5μm

cw = cell wall

co = contaminant

ca = carboxysome

c = cytoplasm

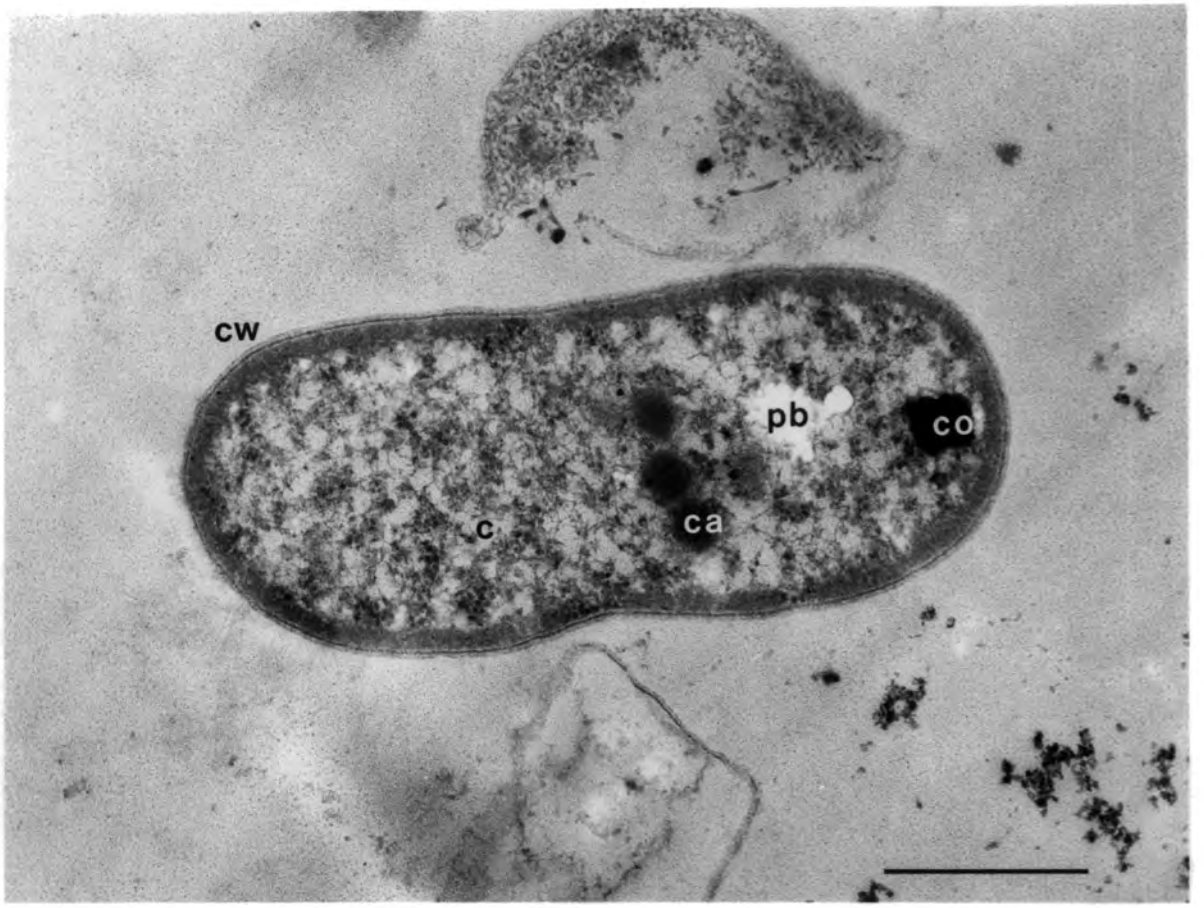
pb = space previously occupied by a PP body

b) Electron micrograph of Synechococcus D562 cells grown in PPJ medium for 5 d as above in the presence of 2 mg l^{-1} Cd. Scale bar = 0.5 μm

cw = cell wall

c = cytoplasm

ca = carboxysome


cw
pb
co
c
ca

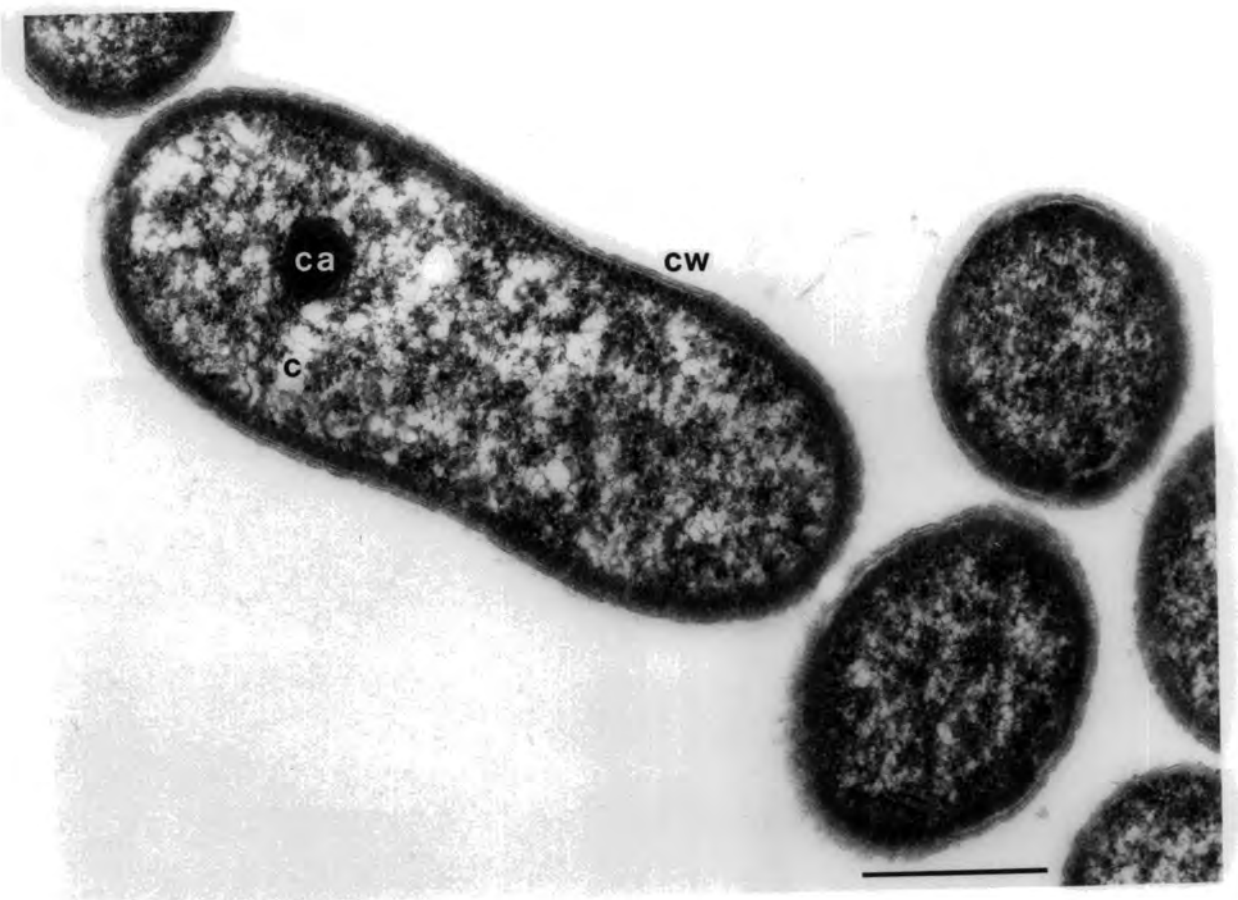
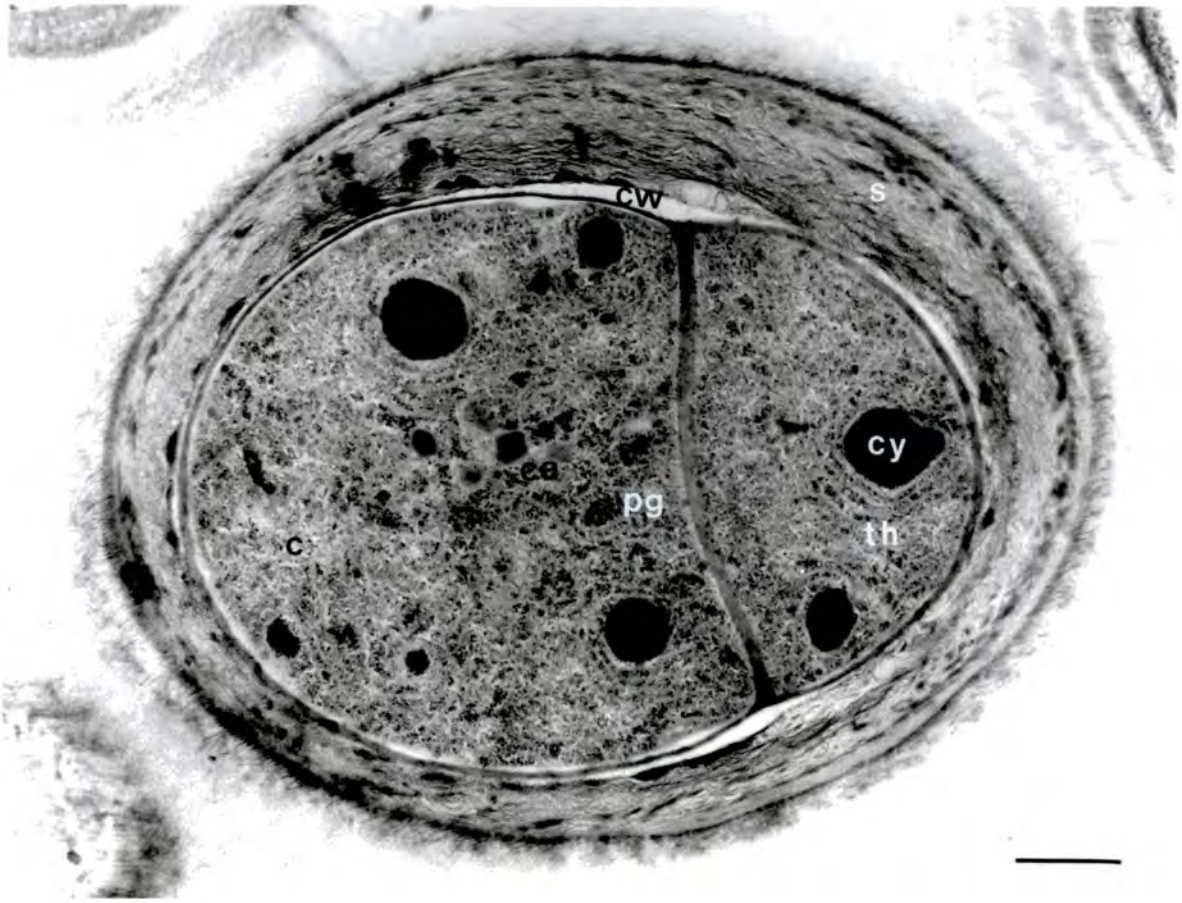

ca
cw
c

Fig. 8.2 a) Electron micrograph of a Calothrix D184 cell grown in PPJ - N medium for 15 d without added Cd at 25 °C and 80 μmol photon m^{-2} s^{-1}. Scale bar = 1 μm

cw = cell wall
pg = polyglucoside granules
ca = carboxysome
c = cytoplasm
cy = cyanophycin granule
th = thylakoid
s = sheath

b) Electron micrograph of a Calothrix D184 cell grown in PPJ - N medium for 15 d as above in the presence of 1 mg l^{-1} Cd. Scale bar = 0.5 μm

cw = cell wall
s = sheath
ca = carboxysome
c = cytoplasm
th = thylakoid membrane
nu = nucleoplasmic area
p = space previously occupied by a cyanophycin granule


cw
s
cy
ca
pg
th
c

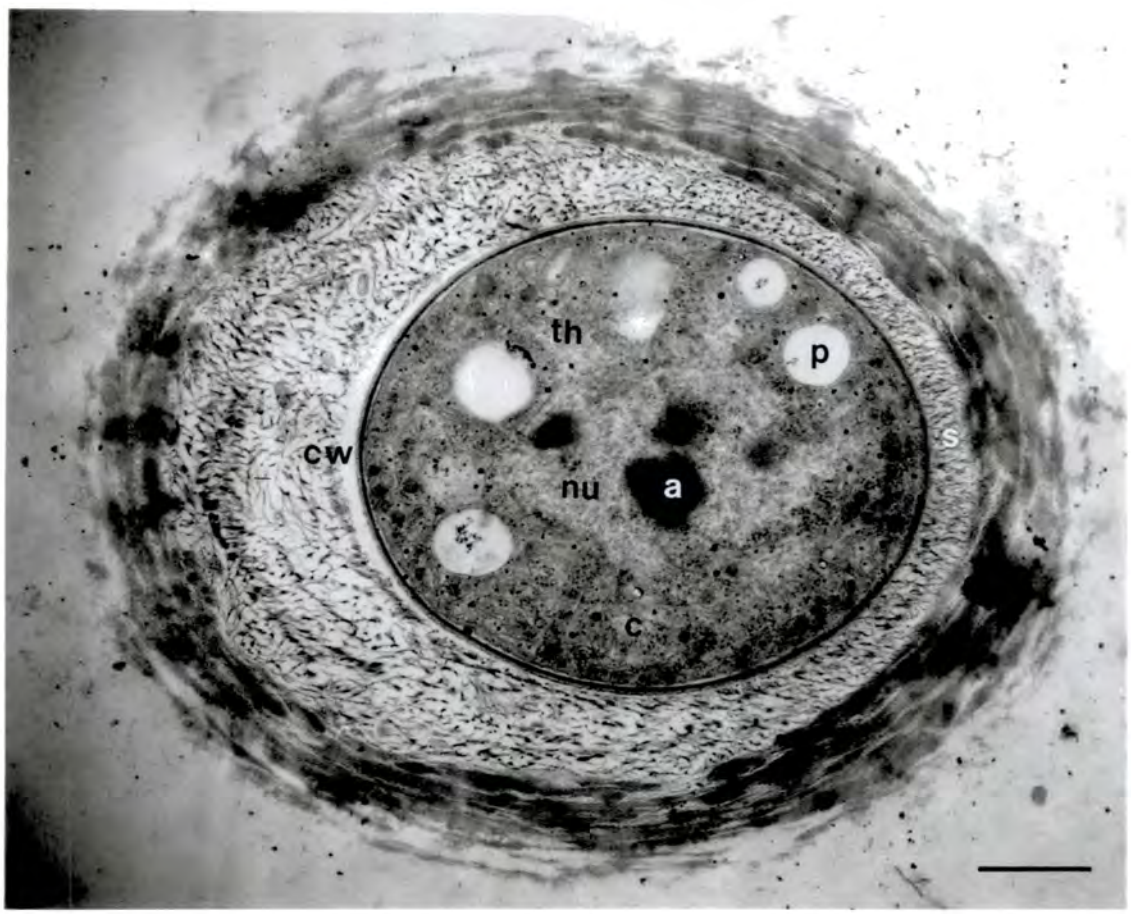

th
p
cw
s
nu
a
c

Fig. 8.3 a) Electron micrograph of a Mougeotia D536 cell grown in CHU 10E medium at 25 °C and 80 μmol photon m^{-2} s^{-1} for 15 d without added Cd. Scale bar = 0.5 μm

cw = cell wall

c = cytoplasm

th = thylakoid membrane

b) Electron micrograph of a Mougeotia D536 cell grown in CHU 10E medium for 15 d as above in the presence of 0.8 mg l^{-1} Cd. Scale bar = 1 μm

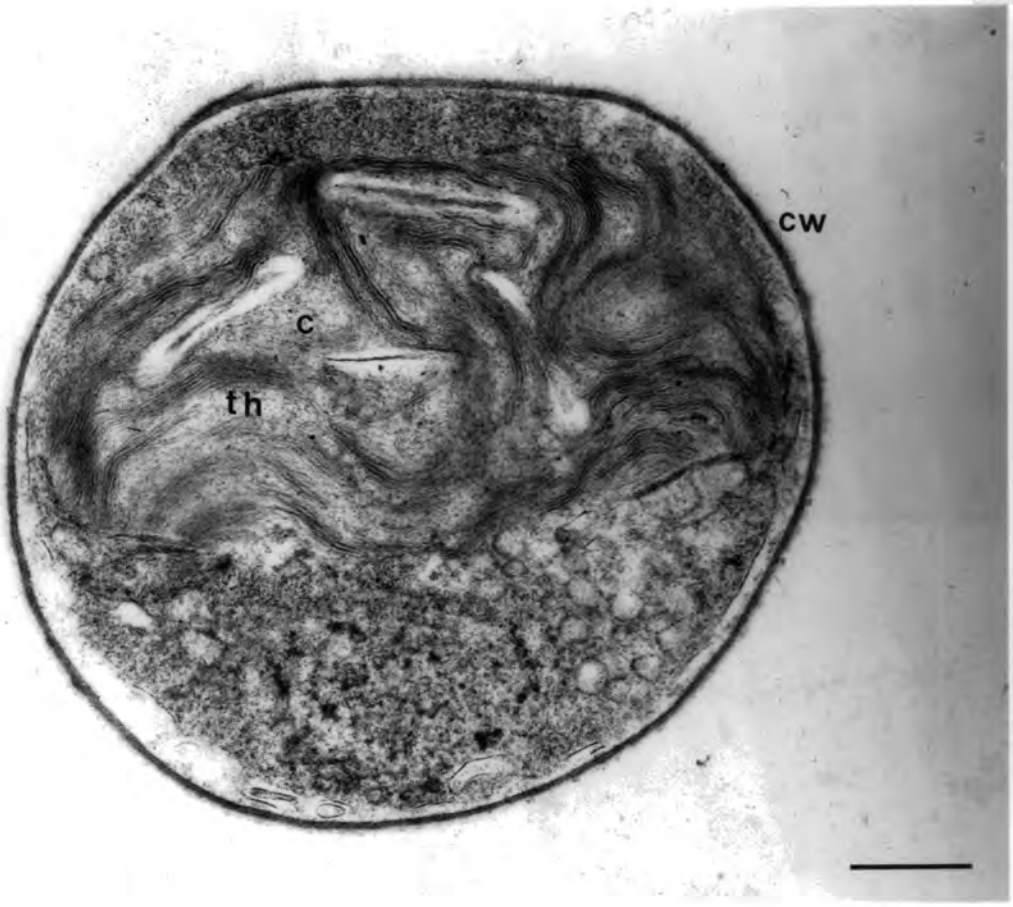
cw = cell wall

lb = lipid body

th = thylakoid membrane

c = cytoplasm

p = electron dense precipitates


cw
c
th

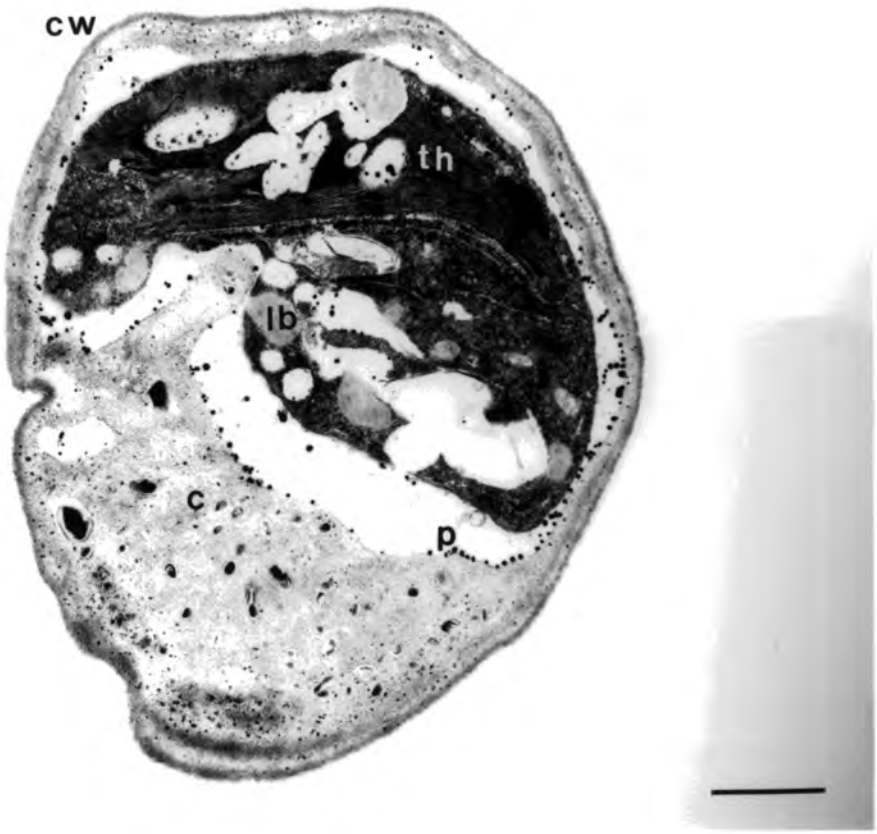
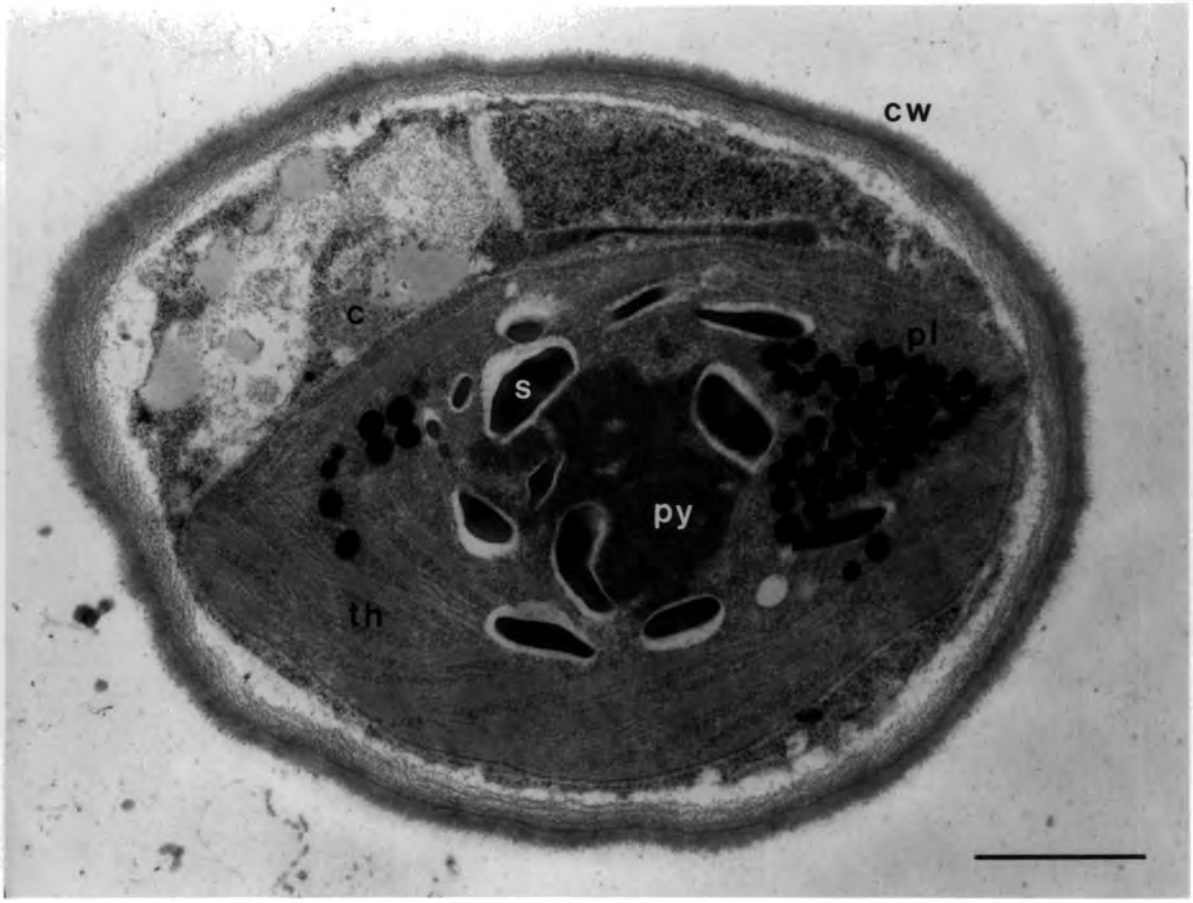

cw
th
lb
c
p

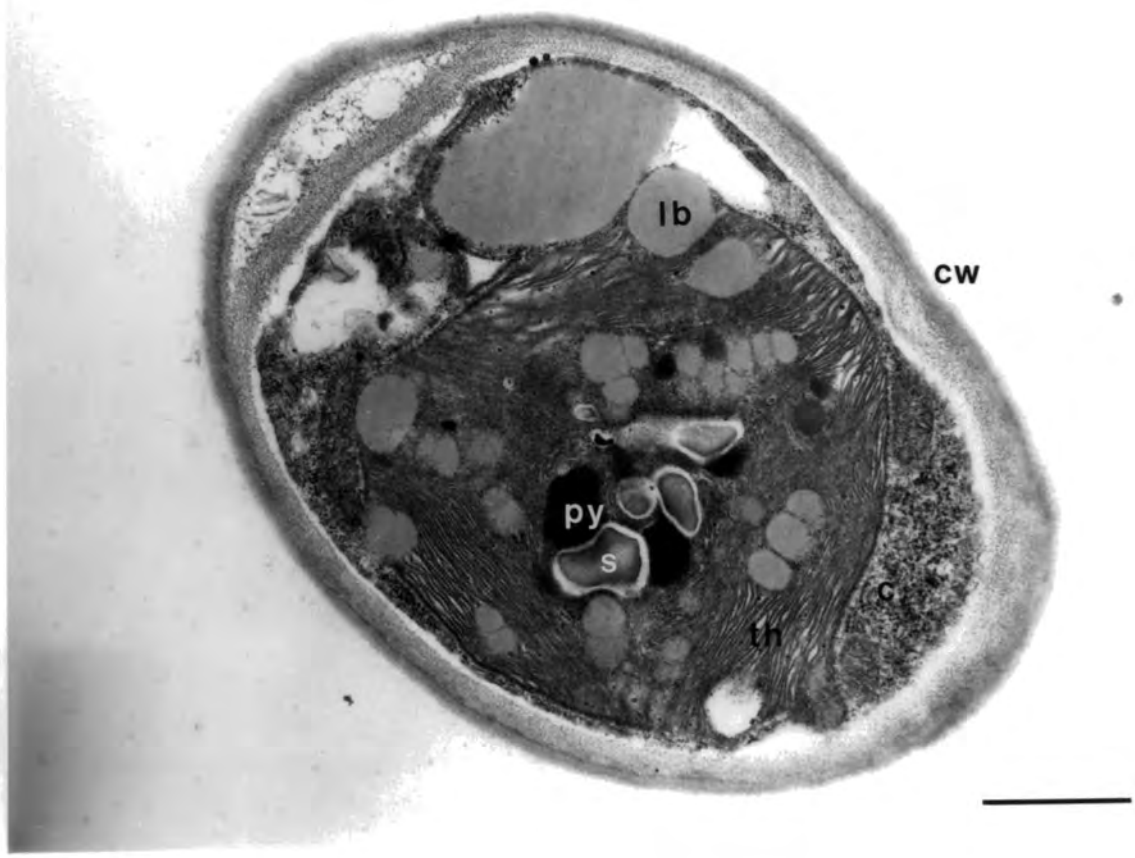
Fig. 8.4 a) Electron micrograph of a Klebsormidium D537 cell grown in CHU 10E medium for 15 d without added Cd at 25 ° C and 80 μmol photon m^{-2} s^{-1}. Scale bar = 1 μm

cw = cell wall
py = pyrenoid
s = starch deposits
c = cytoplasm
pl = plastoglobuli
th = thylakoid membranes

b) Electron micrograph of a Klebsormidium D537 cell grown in CHU 10E medium for 15 d as above in the presence of 1 mg l^{-1} Cd. Scale bar = 1 μm

cw = cell wall
py = pyrenoid
s = starch deposits
c = cytoplasm
th = thylakoid membranes
lb = lipid body


cw
c
pl
s
py
th


lb
cw
py
s
c
th

8.3 Alkaline phosphatase activity

The release of alkaline phosphatase into the medium by Synechococcus D562, provided suitable material to ascertain the inhibitory effects of Cd upon P hydrolysis. The extracellular PME activity throughout the growth curve, optimum operating pH and appropriate buffer were determined for this enzyme (Figs 8.5 and 8.6). In addition, the influence of Cd on PME activity during growth and in the assay medium was resolved (Table 8.1). Buffer group A facilitated slightly higher PME activity and the proton response curve was relatively sharp, with an optimum at pH 10.0. For all studies extracellular material was collected after five days of growth (Fig 8.6) and most assays conducted at pH 10.3 (the standard proton concentration for APA enzyme assays in this laboratory). An average value (n = 8) for the detection limit of this assay was generated with an incubation time of 1 h, dry weights of 50 - 60 mg and blank absorbance figures of 0.017 - 0.033.

Although Cd is most cytotoxic when in a divalent, cationic form at neutral pH, phosphatase activity at pH 7.0 is low. To enhance enzyme activity at this pH and as a preliminary step towards purification, ultrafiltration membranes were employed to concentrate the protein (Table 8.1). The addition of Cd to the growth and assay media resulted in slightly higher activity except when the assay pH was held at 7.0, conditions which reduced the enzyme activity by 30 % at 10 mg l^{-1} Cd. Cells grown in 4 mg l^{-1} Cd exhibit a long lag phase and reduced dry weight, producing a high value for enzyme activity which is not comparable with cultures grown at lower (non growth-supressing) Cd concentrations. Both 30 kD and 100 kD ultrafiltration membranes retained the majority of the enzyme, with a small quantity passing across the 100 kD screen, but not detectable in the filtrate.

The levels of cell-bound PME, PDE and extracellular PDE activity were below the detection limit of the assay used in this study (0.0126 µmol ρNP mg d.w. $^{-1}$ h^{-1}). However, when the samples were run through a protein concentration step, absorbance readings above the detection limit were obtained, thereby facilitating the calculation of enzyme catalysis rates (Table 8.1). A significant level of hydrolysis was exhibited by 0.2 µm-filtered culture medium, indicating that aggregations of extracellular mucilage which are witheld by the filter, do not bind all the enzyme.

Preliminary tests for PME activity in Mougeotia D536 with a 1 h incubation period, did not yield a positive result for either extracellular or cell bound activity.

Fig. 8.5 Influence of pH (buffer groups A ○ , B ●) on the extracellular PME activity of Synechococcus D562 cells grown for 5 d in PPJ medium pH 7.0, 0.5 mg l^{-1} P at 32 ° C and 80 μ mol photon m^{-2} s^{-1}

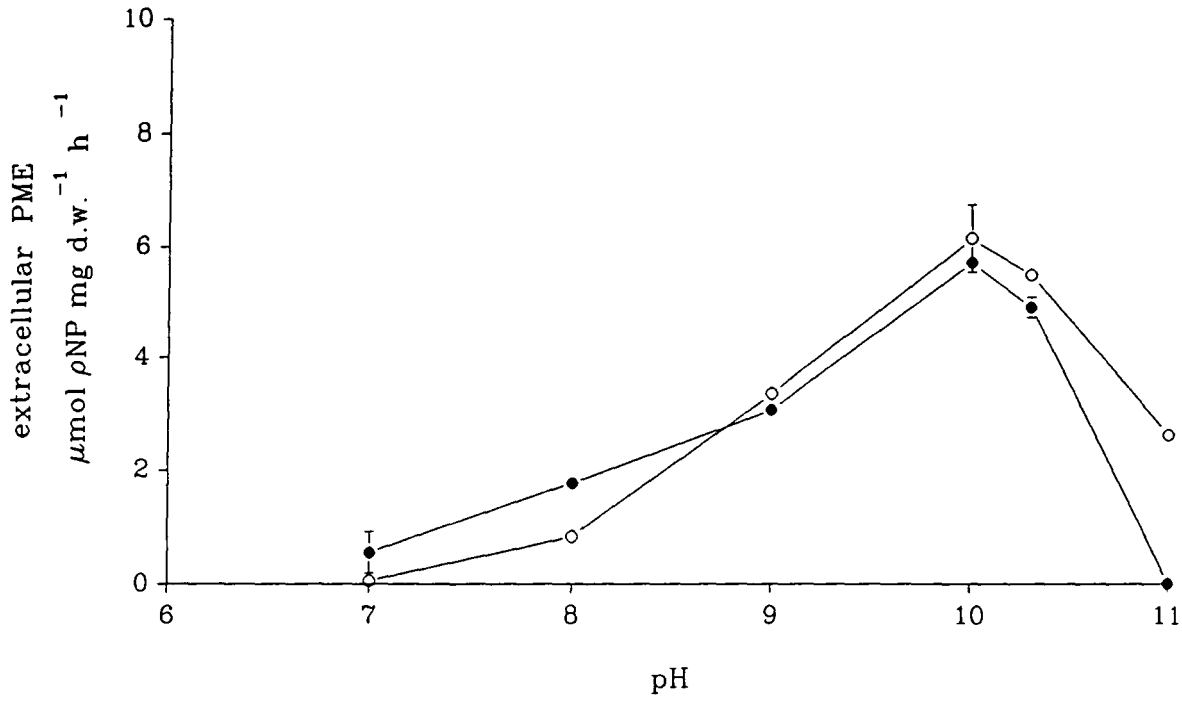


Fig. 8.6 Induction of Synechococcus D562 extracellular PME activity in batch culture, cells were grown in PPJ medium pH 7.0, 0.5 mg l^{-1} P at 32 ° C and 80 μ mol photon m^{-2} s^{-1}, the enzyme assay pH = 10.3

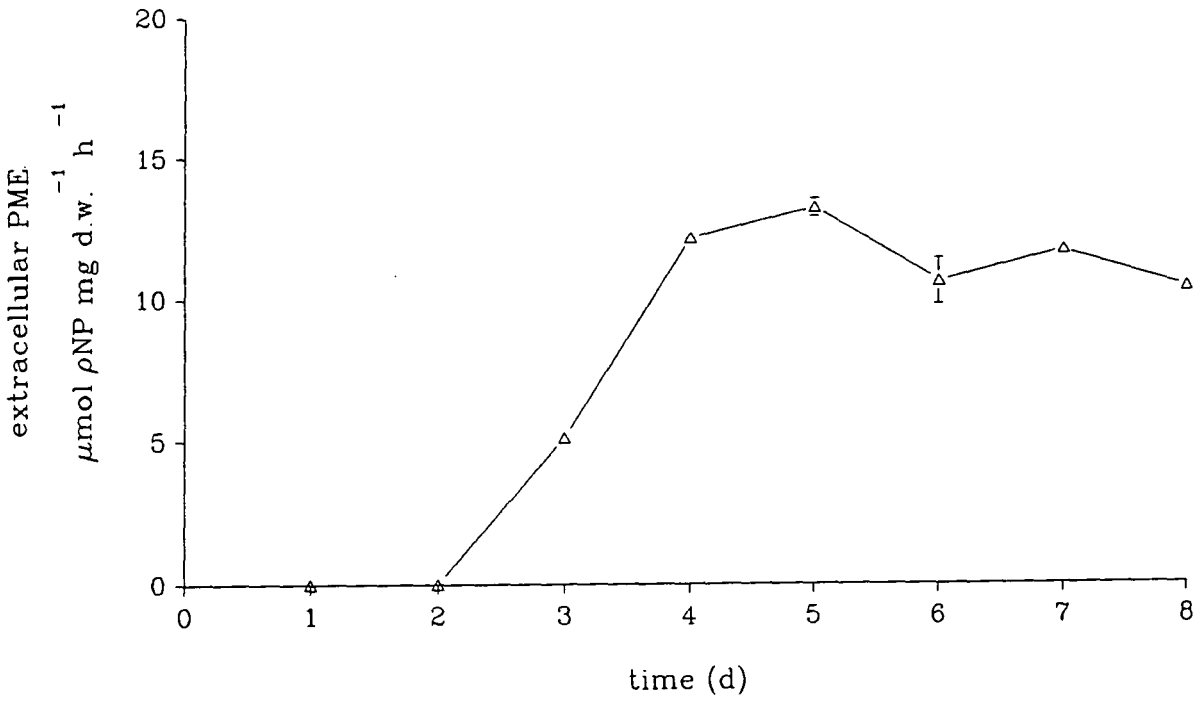


Table 8.1 Influence of Cd and filtration procedures on Synechococcus D562 extracellular APA, P = 0.5 mg l^{-1}, all assays were maintained at pH 10.3 unless stated otherwise, n = 8, < = value below the detection limit

Treatment		enzyme activity ± sem (μmol ρNP mg d.w. $^{-1}$ h^{-1})
Cd in the medium =	0 mg l^{-1}	4.795 ± 0.191
	2 mg l^{-1}	5.389 ± 0.153
	4 mg l^{-1}	13.957 ± 1.138
Cd in the assay =	0 mg l^{-1}	4.795 ± 0.191
	1 mg l^{-1}	5.068 ± 0.124
	5 mg l^{-1}	4.911 ± 0.232
	10 mg l^{-1}	5.337 ± 0.170
Cd in the assay =	0 mg l^{-1} pH 7.0	2.002 ± 0.038
	0.1 mg l^{-1} pH 7.0	1.994 ± 0.089
	1.0 mg l^{-1} pH 7.0	1.550 ± 0.251
	10 mg l^{-1} pH 7.0	1.370 ± 0.032
30 kD ultrafiltration filtrate		< 0.0126 ± 0.00123
30 kD retentate		1.166 ± 0.029
100 kD filtrate		< 0.0126 ± 0.00123
100 kD retentate		0.944 ± 0.009
Unfiltered extracellular PME		2.589 ± 0.043
Unfiltered cellular PME		< 0.0126 ± 0.00123
Unfiltered extracellular PDE		< 0.0126 ± 0.00123
Unfiltered cellular PDE		< 0.0126 ± 0.00123
30 kD cellular PME		2.269 ± 0.202
30 kD extracellular PME		2.910 ± 0.059
30 kD cellular PDE		2.826 ± 0.097
30 kD extracellular PDE		2.196 ± 0.061
0.2 μm nitrocellulose filtrate		3.058 ± 0.165

8.4 SDS polyacrylamide gel electrophoresis

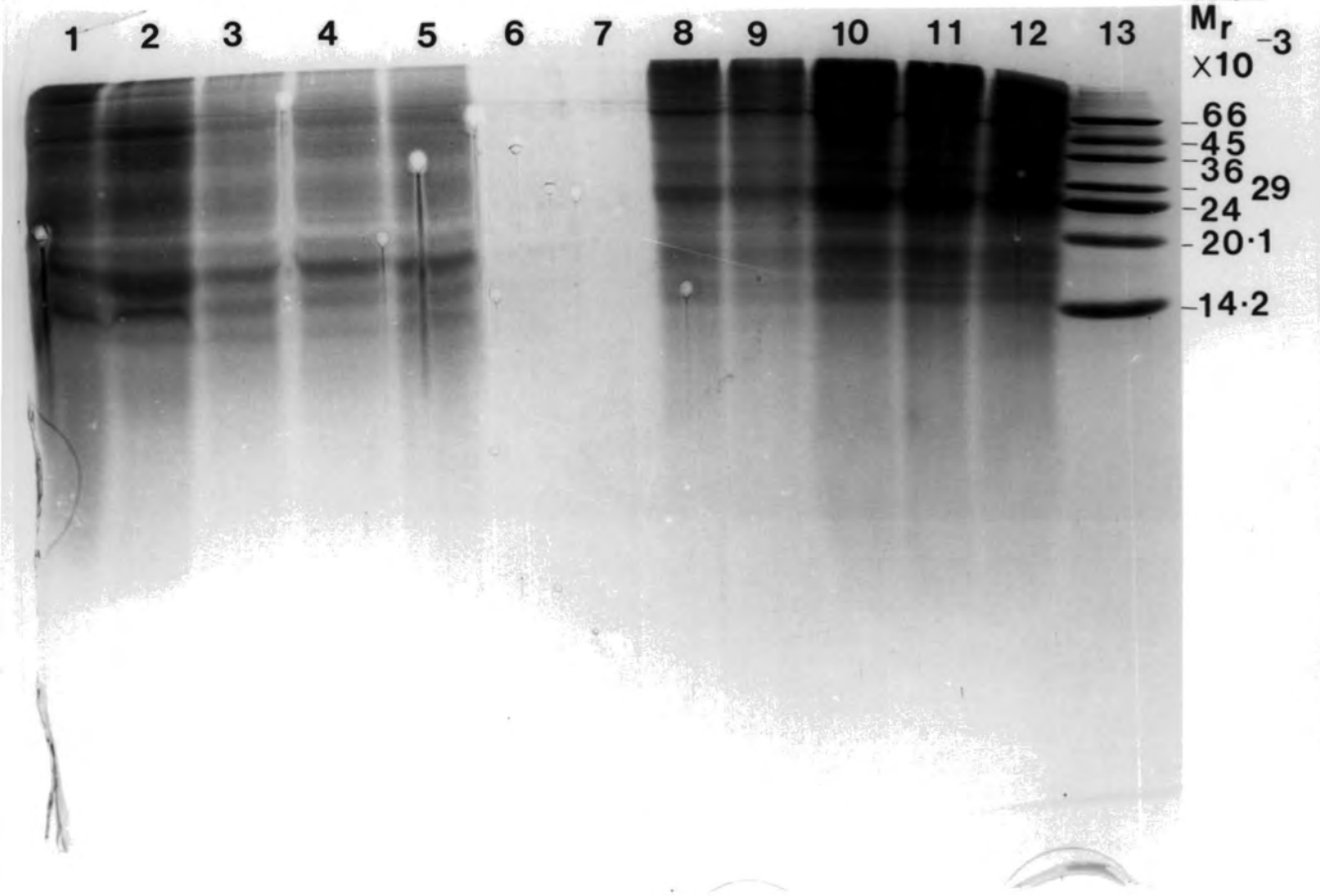
Unbound, cytoplasmic Cd may complex with free thiol and carboxyl groups, thus blocking protein subunit concatenation, or inactivate the enzymes employed in post-translational modification. Such toxic consequences should be detectable as changes in the high Mr protein-profile of cyanobacterial and algal extracts on SDS gels. Of the four strains that were examined by SDS PAGE, only one Cd-induced disruption to the protein profiles was observed. The staining intensity of a 17 kD band present in Calothrix D184 cells grown in the absence of Cd, declined as the concentration of Cd in the growth medium was increased (Figs 8.7 and 8.8). Samples of Synechococcus D562 that were exposed to increasing concentrations of Cd exhibited a general decline in their total protein content with an increase in metal, but no specific differences in the protein banding pattern was observed. The two eucaryotic strains failed to exhibit any change in their gross protein content when grown in the presence of toxic concentrations of Cd, in addition these gels revealed many proteins that were common to both strains (Figs 8.9 and 8.10) (see Appendix 9 for a description of the proteins used as Mr markers).

Fig. 8.7 17 % SDS PAGE of extracts from two algal strains (Synechococcus D562 and Mougeotia D536) grown in the presence of the following Cd concentrations (mg l^{-1}) (Mr markers are in kD) :

Lane				
	1	562 Cd (0)	8	536 Cd (0)
	2	562 Cd (0.1)	9	536 Cd (0.2)
	3	562 Cd (0.5)	10	536 Cd (0.4)
	4	562 Cd (1.0)	11	536 Cd (0.6)
	5	562 Cd (1.5)	12	536 Cd (0.8)
	6	No sample	13	Mr markers
	7	No sample		

Fig. 8.8 17 % SDS PAGE of cell extracts from two algal strains (Calothrix D184 and Klebsormidium D537) grown in the presence of the following Cd concentrations (mg l^{-1}) (Mr markers are in kD) :

Lane				
	1	Mr markers	7	No sample
	2	184 Cd (0)	8	537 Cd (0)
	3	184 Cd (0.2)	9	537 Cd (0.2)
	4	184 Cd (0.4)	10	537 Cd (0.4)
	5	184 Cd (0.6)	11	537 Cd (0.6)
	6	184 Cd (0.8)	12	537 Cd (0.8)


1 2 3 4 5 6 7 8 9 10 11 12 13
Mr -3
X10
66
45
36
29
24
20·1
14·2

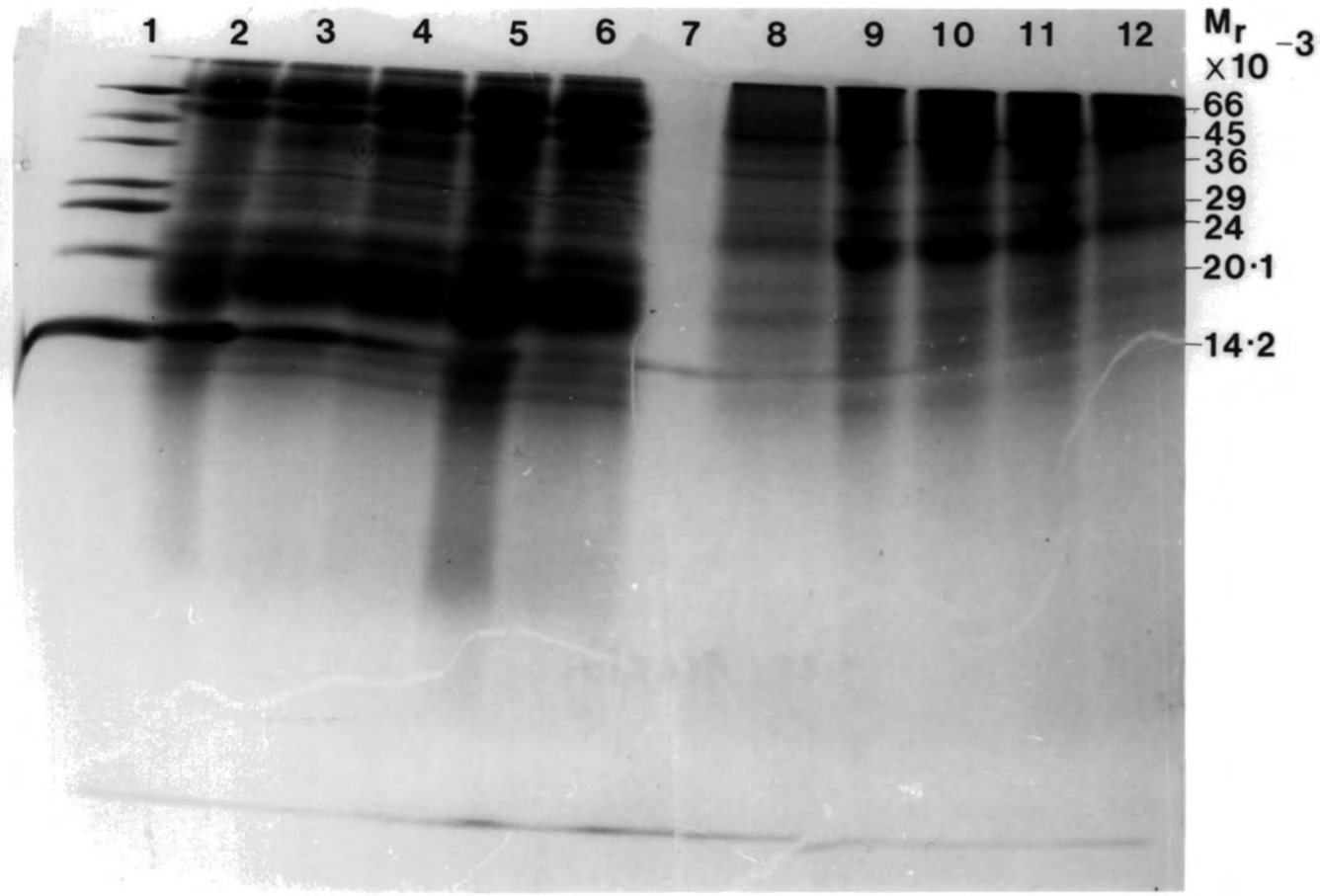
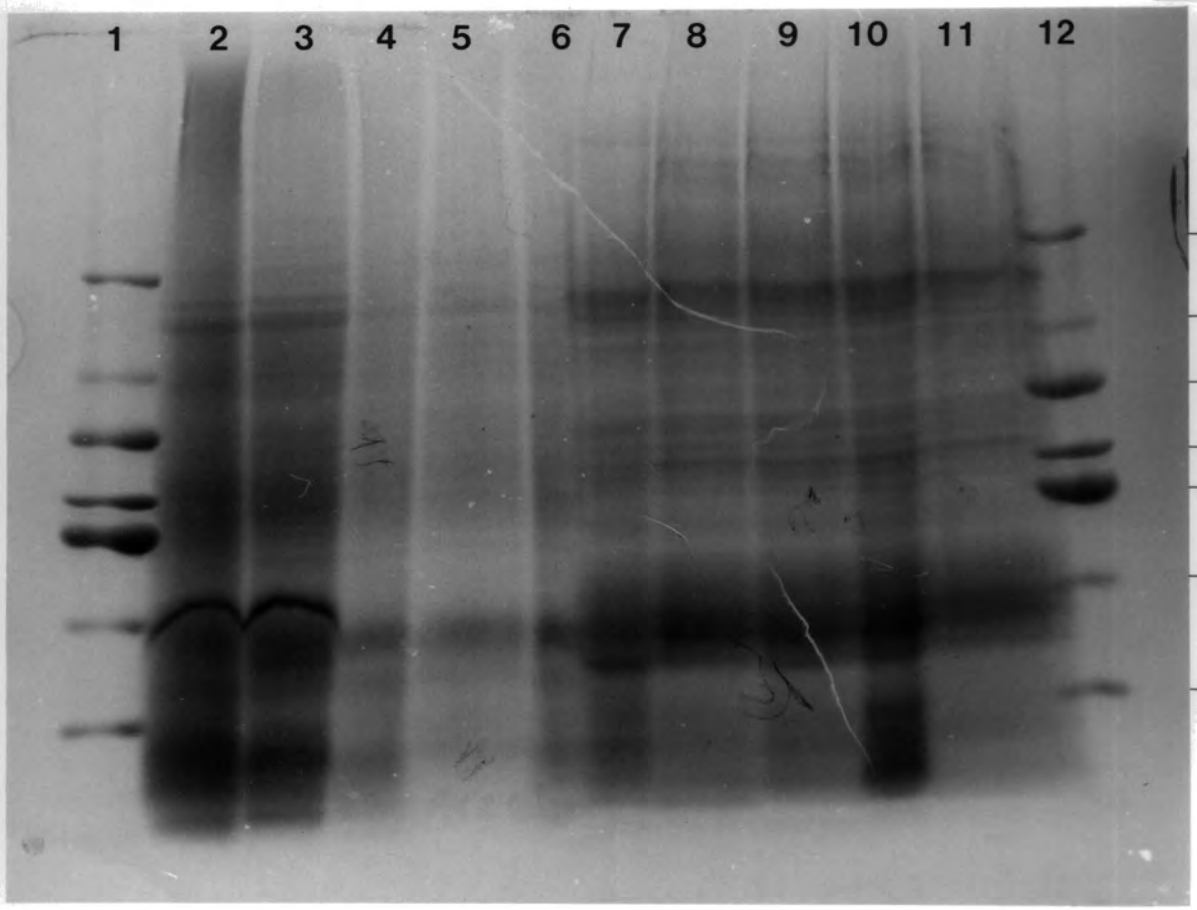

1 2 3 4 5 6 7 8 9 10 11 12
Mr -3
X10
66
45
36
29
24
20·1
14·2

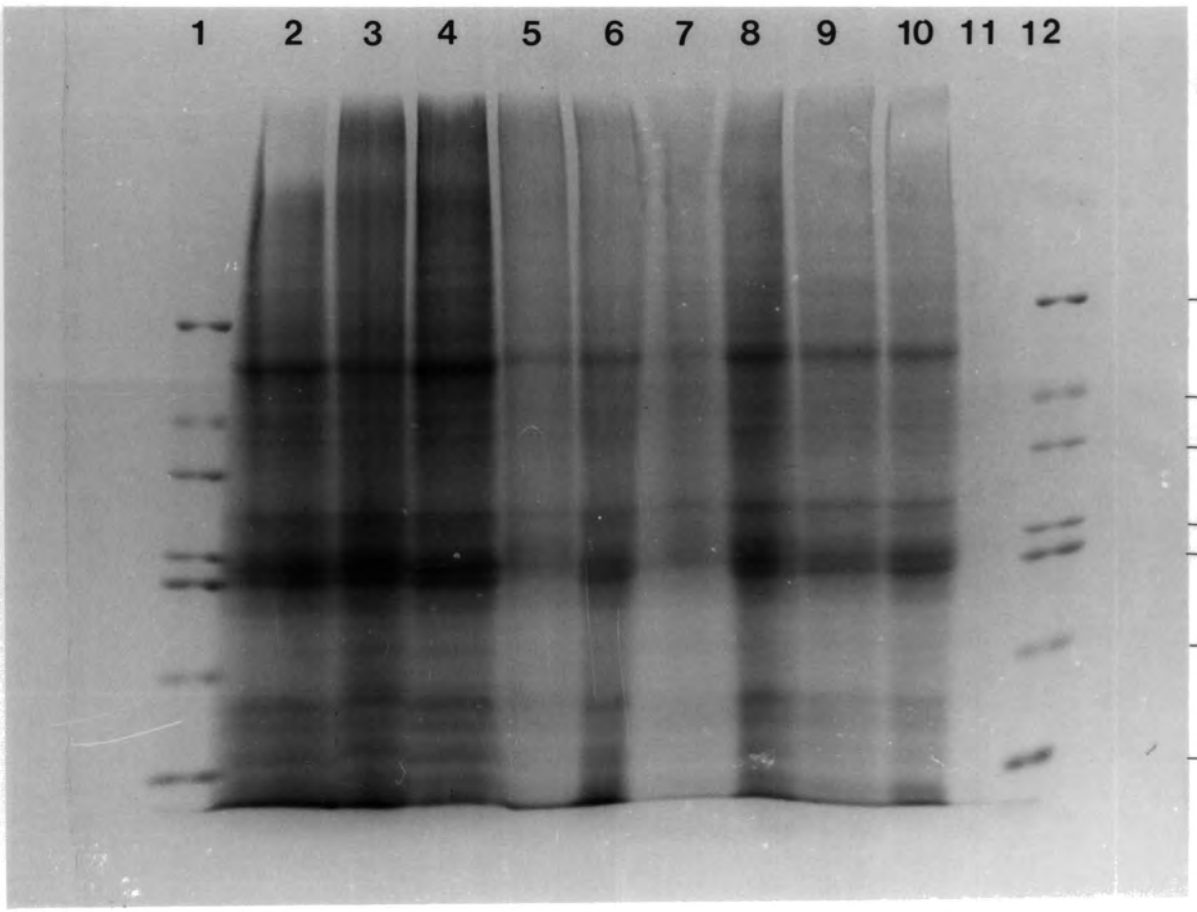
Fig. 8.9 10 % SDS PAGE of cell extracts from two algal strains (Synechococcus D562 and Calothrix D184) grown in the presence of the following Cd concentrations (mg l^{-1}) (Mr markers are in kD) :

Lane				
	1	Mr markers	7	184 Cd (0)
	2	562 Cd (0)	8	184 Cd (0.2)
	3	562 Cd (0.1)	9	184 Cd (0.4)
	4	562 Cd (0.5)	10	184 Cd (0.6)
	5	562 Cd (1.0)	11	184 Cd (0.8)
	6	562 Cd (1.5)	12	Mr markers

Fig. 8.10 10 % SDS PAGE of cell extracts from two algal strains (Mougeotia D536 and Klebsormidium D537) grown in the presence of the following Cd concentrations (mg l^{-1}) (Mr markers are in kD) :

Lane				
	1	Mr markers	7	537 Cd (0)
	2	536 Cd (0)	8	537 Cd (0.2)
	3	536 Cd (0.2)	9	537 Cd (0.4)
	4	536 Cd (0.4)	10	537 Cd (0.6)
	5	536 Cd (0.6)	11	537 Cd (0.8)
	6	536 Cd (0.8)	12	Mr markers


1
2
3
4
5
6
7
8
9
10
11
12
M_r x10^{-3}
66
45
36
29
24
20·1
14·2


1
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M_r x10^{-3}
66
45
36
29
24
20·1
14·2

CHAPTER 9

DISCUSSION

9.1 Introduction

As a result of metal smelting or the discard of Cd-containing commodities, the concentration of Cd in particular areas may increase, with consequent bioaccumulation or geochemical precipitation (Section 1.1). The additional Cd released to the environment may enter the food chain, can sometimes become concentrated during its transport up the chain and enhance the low levels of metal normally encountered by humans. A toxic threat is exerted when Cd replaces metals at the active site of metalloenzymes or disrupts cell-membrane integrity. Current EC directives demand a reduction in the use of this metal in products (European Council Directive, 1989), but until the objectives of this legislation are fully executed Cd-contaminated effluents will continue to flow, creating pools of metal concentrate in the environment; both Cd-laden products which have been abandoned and industrial waste streams require attention.

Inserting columns of immobilized cells in the path of an effluent provides one method of reducing the Cd load of industrial waste or polluted sites below proscribed limits (levels at which chemical precipitation procedures are often ineffective). In order to improve the operational efficiency of immobilized cells, additional interactions were examined between microorganisms and Cd that influence the concentration of the metal accumulated : two techniques were employed to record the distribution of Cd in microorganisms, the toxic consequences of Cd exposure was analysed and potential detoxification mechanisms were investigated.

9.2 Selection of strains for study

The selection process used to identify strains of microorganisms for immobilization was based upon the fact that organisms which exhibit tolerance to the presence of heavy metals in their environment often internalise these

metals to a high concentration as long as the most of the metal is not bound by the cell wall and the necessary ion transport-proteins are present; a relationship between the concentration of metal in the environment and that accumulated by cells has been demonstrated in a number of aquatic organisms (Kelly & Whitton, 1989). Because of the variation in cell-wall components, membrane transport proteins and detoxification mechanisms, not all microorganisms accumulate Cd to the same level for a particular environmental concentration, hence the need for a screening program. To obtain appropriate photosynthetic microorganisms for immobilization, environments which have received heavy-metal inputs from mining activity were sampled for the isolation of algae and cyanobacteria which accumulate Cd to a high concentration. In addition, the strains that are selected should be axenic so that no other organisms contribute towards Cd accumulation.

The concentration of Cd accumulated by two axenic strains from the Durham Culture Collection (Synechococcus D562 and Mougeotia D536) was considerably higher (1797 $\mu g\ g^{-1}$ and 800 $\mu g\ g^{-1}$ Cd from 2 and 1 $mg\ l^{-1}$ respectively (sections 3.2 and 3.3)) than strains that were recently isolated from the environment. However, when these concentrations are compared with values from the literature they were found to represent average examples (Cain et al., 1980 ; Sakaguchi et al., 1979 ; Khummongkol et al., 1981). Investigations were therefore focused upon these two strains. Although the process of removing bacterial contaminants from a culture can prove time-consuming, the concentration of Cd accumulated by a strain of Stichococcus isolated from Gillgill Burn (Table 3.3) suggests that this organism is worth rendering axenic.

A majority of the Cd and Zn at the five metal-polluted sites were not complexed to particles greater than 0.2 μm in diameter (Table 3.1) and were therefore probably available for microbial uptake. The concentration of Cd accumulated by the algae and cyanobacteria from these sites was species rather than concentration dependent, reflecting a variety of metal-ion transport mechanisms that exist in different strains. When cultured in the laboratory, these strains could not tolerate more than 0.25 $mg\ l^{-1}$ Cd in the medium, if the maximum concentration of metal tolerated by these strains is enhanced by repeated subculture to the same level as that tolerated Synechococcus D562 and Mougeotia D536, then the concentration of metal accumulated by the field strains is expected to rise.

Although algae sampled from the field (Table 3.2) were exposed to lower concentrations of Cd (4 to 20 times) than that of strains isolated from the same sites but cultured in the laboratory at 0.25 $mg\ l^{-1}$ Cd, they accumulated the metal to similar concentrations. At higher concentrations of Cd, these strains will either reveal a maximum threshold for internalised Cd or out-perform those from the Durham Culture Collection.

9.3 Cd accumulation

A number of factors regulate the concentration of Cd accumulated by microorganisms and the location of the metal in the cell, these include the concentration of free, divalent metal available, the type of metal-binding ligands at the cell surface and the number of metal-transport proteins present in the cell membrane. These components merit investigation if statements are to be made which relate the concentrations of metal accumulated by microorganisms to that present in the environment. Assuming that complexes of Cd (hydroxides,chlorides and chelates) are not bound to or transported across the cell wall, the concentration of divalent Cd^{2+} present in the medium should reflect the toxicity of the metal. The experimental procedures that were adopted resulted in concentrations of free Cd^{2+} which were within 4 % of the intended value (Section 4.2) this figure was judged an acceptable level of error and no additional procedures were taken to adjust the concentration of Cd in the growth media.

The accumulation of Fe by microorganisms results in free EDTA remaining in solution with a potential for chelating other metal ions in the medium and therefore reducing their toxicity. To determine the chelating power of the uncomplexed EDTA sites, ion-exchange material was used to assess the amount of Cd bound by EDTA. Results from anion-exchange resin experiments indicate that nearly all of the Fe in the medium is complexed by EDTA, whilst most of the added Cd apparently remains in solution as a divalent cation either with free EDTA, Fe-EDTA or PPJ medium.

The small reduction in Cd concentration for all treatments after ion exchange can be attributed either to Cd-anion complexes at a low level or binding of the metal to the glass flasks used to contain the solutions. Without EDTA, anionic forms of Fe appear to be present in PPJ medium but not necessarily in a form which is suitable for algal utilisation. It is important to note that the results obtained with anion-exchange resin provide a temporally static picture of what is a dynamic system, where chelated ions may also be released / adsorbed, changing the concentration of metal in the medium.

When the medium used to culture Synechococcus D562 was tested for Cd complexes, little bound metal was detected (assuming any Cd complex will have a negative charge and thus bind to the exchange resin). Such an observation supports the view that any EDTA with unbound sites does not appear to significantly chelate the Cd that is added to PPJ medium (the valency of the Cd added to CHU 10E and AD P(1.0) Fe(0.4) also needs to be tested). Because EDTA has been used to remove metals that are bound to the cell-surface and is known to increase the

toxicity of heavy metals in the environment, the low concentration of Cd / EDTA complexed by the exchange resin was not expected. Therefore, further research is required in this area, because the adsorption of chelated ions with exchange resin relies upon the assumption that Cd / EDTA complexes are not fully saturated with the metal and retain a negative charge. Cd-EDTA complexes which are neutral or positively charged are not removed from the medium by anion-exchange material and cannot be used to describe the concentration of Cd bound by EDTA. As the levels of contaminating Cd and Fe in PPJ medium are close to the detection limits of flame atomic absorption spectrophotometry, the metal added to the medium constitutes most of the total metal present. Therefore, the growth conditions employed in this study allow the full cytotoxic effects of Cd to be experienced by microorganisms.

Sterile microtitre plates were used to screen microorganisms for Cd tolerance (Section 4.3). Although the wells of these plates provided a suitable environment in which to culture at least 12 strains of photosynthetic microorganisms in each container, they have proved inappropriate for toxicity studies as many of the strains tested apparently grew in very high concentrations of Cd and no direct correlation could be drawn between the concentration of metal in the medium and the culture absorbance reading after a period of growth. This phenomenon may be attributed to the binding of Cd by the plastic wells and hence a reduction in the toxicity of the metal. The reproducibility of culturing algae on this small scale may also be unreliable.

A decline in the P status and increase in cell length of Synechococcus D562 was observed when cells were transferred to 18 MΩ water, grown up to form a visible culture and transferred a second time to fresh 18 MΩ water (forming a culture with a much reduced cell-density) (section 4.4). Such a change in morphology following nutrient stress may prove useful for obtaining bacteria-free cultures of small unicellular cyanobacteria by enhancing the difference in size between the desired strain and its contaminant. It is not known if an inoculum of cells in this condition would be capable of producing a culture in PPJ medium of comparable cell density with that of cells which have never been transferred to a nutrient-poor environment.

Before developing an immobilized cell system, the subculture conditions of Synechococcus D562 cells were investigated. A regime of fluctuating concentrations of Cd in the medium may activate Cd detoxification mechanisms which are not fully operational in cells that are continuously exposed to a high concentration of the metal. Such a hypothesis does not appear to apply to this cyanobacterial strain because the maximum concentration of Cd internalised by a population of Synechococcus D562, was recorded when the cells were subcultured continuously in the highest level of Cd which did not significantly reduce the resulting biomass (Section 4.5). Since

4.5). Since the cellular location of Cd deposition influences the rate of metal uptake (faster for cell-wall adsorption than the active transport of Cd into the cell) and the concentration of Cd that can be bound, cultures of Synechococcus D562 were analysed to determine where the accumulated Cd is partitioned. In order to assess the concentration of cell-wall complexed metal of cultures that had been grown in 2 mg l^{-1} Cd a chelating agent was utilised to bring the surface-complexed Cd into solution. Washing cells with 25 mM EDTA proved an effective treatment for the permeation of cell-wall layers and membranes resulting in the release of cytoplasmic ions, as no Cd was detected in Synechococcus D562 cells after incubation with the chelating agent. This result is analagous to a previous ion permeability study which recorded the loss of intracellular K^+ from Saccharomyces cerevisiae as a result of the damage that Cd exerted upon the cell walls (Gadd & Mowll, 1983).

The observation that Bacillus subtilis cells use Mg^{2+} to stabilise the structure of the cell wall (Beveridge & Murray, 1976) provides evidence for the hypothesis that the periphery of Synechococcus D562 contains EDTA-extractable ions which play an important role in maintaining cell-wall integrity. Although a lower concentration of EDTA was subsequently employed (0.25 mM) resulting in a lower figure for the concentration of extracted Cd, restricted cell-wall damage may still have occurred (albeit on a reduced scale to that caused by 25 mM EDTA treatment) with the release of cytoplasmic Cd, in addition to that bound by the cell wall. This approach would generate an artificially high value for surface-complexed metal and therefore only organisms with a cell wall that is resistant to the disruptive effects of EDTA treatment are suitable for investigation by this particular chelating agent.

The walls of heat-killed cells did not exhibit a high capacity for binding Cd, hence the majority of the accumulated metal must be detoxified intracellularly (Section 4.5); this result adds weight to the suspicion that data from the EDTA experiments do not reflect the concentration of Cd complexed soley by the cell wall. In fact, the process of heat treatment might be expected to increase the number of Cd-binding sites available on the cell wall as its components are broken down at elevated temperatures and reactive anionic groups exposed. Microorganisms that bind most of the accumulated metal to their cell walls are probably the best material for removing toxic heavy metals from solution; dead cultures of Paracoccus sp. and Arthrobacter globiformis bound more Cd at the cell walls than live cells (Bollag & Duszota, 1984). The metal adsorbed onto the periphery of the cell can then be easily removed by a change in the surrounding redox potential or pH (Greene et al., 1987).

9.4 Batch and continuous culture

As an alternative to the step-wise selection of metal tolerant microorganisms via batch culture (Whitton & Shehata, 1982), a continuous culture run was carried out; a technique which has been used to remove Cd from the medium rather than as a means of cell selection (Houba & Remacle, 1984). This environment was used to create a continuous selection pressure in order to obtain a population of cells which withstood the presence of more Cd in the growth medium than those cultured under batch conditions (Section 4.6). The metal-tolerant population would then have to be checked for the concentration of Cd accumulated as growth in the presence of high levels of Cd is not necessarily associated with an expansion in the concentration of internalised Cd. Light intensity demonstrated a controlling influence over the specific growth rate of Synechococcus D562 and was thus employed to govern continuous culture. The photon flux experienced by this strain at Elvins Tailings (the site of isolation) is far higher than the 180 μmol photon m^{-2} s^{-1} laboratory conditions which were found to inhibit growth (Whitton pers comm.). However, because the growth conditions *in vitro* and *in vivo* differ markedly, comparisons of the response to changes in illumination between the two environments are not valid.

A relatively constant cell-density maintained during the increase in Cd concentration of the medium, followed by a sharp decline in cell numbers at 3.4 mg l^{-1} Cd, delineates the toxicity threshold for Synechococcus D562 when grown by this continuous-selection protocol (Fig. 4.4). Hence the Cd concentration tolerated by batch cultures is higher than cells grown by continuous culture; this difference may be due to the production of extracellular mucilage (with metal-binding capabilities) towards the end of the exponential phase of growth. Alternatively, a reduction in the ionic concentration of the medium may influence the competition by other ions for the Cd transport sites, therefore reducing the concentration of Cd accumulated. As continuous culture does not provide such late-exponential conditions, the full effect of Cd toxicity is exerted throughout the duration of selection. However this approach did not prove effective in yielding a population of cells with a Cd-tolerance greater than that of batch cultures. With a change in the rate of Cd addition, light intensity and flow rate of the medium, more time would be provided for the population to adapt to the rising Cd challenge and improve the potential of this approach.

9.5 Immobilized cells

Once efficient Cd-accumulating photosynthetic microorganisms had been identified, an appropriate immobilization matrix was required to ensure that the metal-laden cells were retained and not lost to the medium flowing past the cells (a process that would release the metal back into the environment in a more concentrated form). Although immobilization has been applied to the production of a variety of cell metabolites, the use of matrices to retain metal accumulating microorganisms has been limited (Codd, 1987)

A packed-bed, closed-loop reactor was employed to accumulate the metal, as the efficiency of these immobilized strains for the removal of Cd had never been investigated and an open, single-pass system would have failed to lower the concentration of Cd in the effluent. Any swelling or contraction of the bead material throughout a run was not significant, so that the head space made available at the top of the column could have been filled with more calcium-alginate beads.

The initial adsorption of the toxicant by the alginate matrix over the first 16 h (Figs 5.1 and 5.2) may involve a cation exchange process between the Ca^{2+} ions which cross link the alginate molecules and the added Cd^{2+}; changes in the Ca^{2+} concentration of the medium in the system would provide evidence for such a theory. Alginate beads that were exposed to medium without FeEDTA removed more Cd than those incubated in the presence of the chelating agent. Although data from anion-exchange resin experiments suggest that EDTA does not chelate Cd to form a negative complex, it appears that the presence of FeEDTA inhibits the uptake of Cd by the alginate matrix. Some complicated ion-exchange process may occur in this environment which either competes for the alginate heavy-metal ligands or inhibits the Cd from binding to the matrix. Therefore the Cd-binding sites provided by the apparatus are rapidly saturated and reduce the concentration of Cd in the medium by up to 60 %. However, they do not contribute to the long-term accumulation of the metal carried out by immobilized algae or reduce the concentration of the pollutant to that obtained by columns of immobilized Mougeotia D536.

Initial experiments revealed that a Cd concentration of 0.5 mg l^{-1} was too high for the immobilized-cell system as no reduction in the pollutant concentration was recorded (Fig. 5.3). Attempts to negate the growth gradient observed along the length of each column by aeration of the medium, did not encourage a uniform cell density and actually inhibited Cd removal (Fig. 5.3); an analysis of medium sampled at the top of the column may indicate a growth-limiting concentration of a particular ion at this point due to its uptake by cells near the medium inlet of the column. Although the medium is buffered, Cd hydroxide compounds may form during aeration; such complexes

inhibit uptake of the metal and thus reduce the efficiency of the column.

Control experiments with alginate beads devoid of cells did not reduce the concentration of Cd in the circulating medium, but resulted in an initial reduction in Cd over the first 24 h (Fig. 5.4). When the beads were formed containing ~~inoculated with~~ cells, Synechococcus D562 accumulated more Cd per unit dry weight than Mougeotia D536, but did not reduce the Cd concentration in the medium to the same extent as Mougeotia D536 because the latter produced more cells per column for metal accumulation (Table 5.2). A similar distinction between the concentration of metal accumulated per unit mass and the ability to remove U from the medium was observed by Nakajima et al. (1982). Both strains removed the most Cd from the medium when grown in packed-bed immobilized columns. This is probably due to the exhausted medium surrounding each cell being replenished faster than unshaken beads or free cells, resulting in the production of more Cd-accumulating cells per bead.

Photosynthetic microorganisms that actively transport Cd across the cell membrane were immobilized in packed-bed columns in preference to strains that bind the metal at their surface, because it was believed that using cells which actively accumulated the metal would prove more effective in reducing the concentration of Cd in polluted effluents below the level of 0.005 mg l^{-1} Cd required for drinking water. For Mougeotia D536 cells immobilized in alginate beads this approach has proved correct, the Cd concentration of the medium was reduced from 0.1 to 0.002 mg l^{-1} over 25 d, lower than the figure for cells that bind the metal to the cell wall, although the experiments were run for a longer time period (Macaskie and Dean, 1984c). Immobilized Synechococcus D562 cells reduced the concentration of Cd in the medium to levels comparable with those of organisms which bind the Cd at their surface.

The majority of immobilized systems for the removal of heavy metals from effluents employ the process of cell surface / extracellular precipitation (Macaskie et al., 1986 ; Kuhn & Pfister, 1989) which often removes the metal (at an efficiency of up to 99 %) within hours rather than weeks; the bound metal is then easily recovered by a change in medium pH or redox potential and the cells are recyclable (Greene et al., 1987). Such a system is appropriate for toxic heavy-metals, as cell viability does not decline until the cell surface becomes saturated with the pollutant and exponentially growing cells, together with the constituents of the growth medium, are not usually required. However, there may be some merit in including another column containing immobilized cells that reduce the concentration of Cd further, below the designated limits that define polluted effluents. It is also recognised that industrial effluents invariably contain an assortment of compounds inhibitory to algal growth, so that the chemical approach of surface precipitation is probably more favourable than that of intracellular accumulation. Cd is not the

only heavy-metal which either acts as a pollutant or has commercial value. The technique of immobilizing live cyanobacteria or algae may have applications in accumulating high-value metals from environments which contain dilute concentrations of the element (Greene et al., 1986 ; Macaskie & Dean, 1987a ; Nakajima et al., 1982) or remove radioactive waste from contaminated aquatic sites.

Thin sections of immobilized cells exhibited normal morphology (Figs 6.3 and 6.4). The space between cells and the surrounding alginate matrix is either filled with extracellular mucilage (cells sectioned immediately after immobilization do not retain intimate contact with the matrix material), or the matrix may contract during the process of fixation / dehydration creating a gap between the cells and surrounding alginate. Incubating cell-sections on droplets of lysozyme, followed by Ru (or even perhaps Pb and U) staining would indicate whether or not the extracellular space observed between the immobilization matrix and the cells is due to the presence of mucilage.

As Synechococcus D562 is a unicellular microorganism with greater packing potential, less free-space is evident in electron micrographs of immobilized cells than that observed for immobilized filaments of Mougeotia D536, which are not amenable to close packing. Alternatively a difference in the amount of extracellular mucilage produced by each strain may account for the heterogeneous cell density.

9.6 Energy dispersive X-ray microanalysis

Information concerning the cellular location of heavy metals can also be used to identify the final storage sites for toxic ions and, once determined, contribute towards modelling the mechanisms of metal accumulation used by these cells. Ultimately this data could be employed to enhance the concentration of internalised Cd in microorganisms by redirecting the metabolic pathways associated with metal deposition. It is with this potential in mind that samples of algae and cyanobacteria that had been cultured in the presence of Cd were processed for EDXMA to probe cells for accumulated Cd and record any macroelement shifts caused by heavy-metal exposure. In addition, whilst the acid digestion and subsequent flame atomic absorption spectrophotometry of large quantities of cell material grown in the presence of Cd reflects an average figure for internalised metal throughout a cell population, the analysis of individual cells provides information on the variation of accumulated metal within a population, reflecting different metabolic states. However, EDXMA is more readily restricted by machine detection limits, due to inherently small quantities of the element in question being available for analysis.

To maximise the resolution of EDXMA so that individual cell organelles may be analysed, exploratory samples were first cryosectioned and viewed under the TEM as dried films; the process requires some skill and much time, little cell detail was revealed and good sections were infrequent. Air-dried whole cells were then probed and although fewer structural details were observed, the processing time for each sample before viewing by TEM is shorter and toxic elements which are only accumulated to a low level are present in higher concentrations than the heavy-metals in thin sections.

Initial attempts to quantify the peak integrals obtained from the analysis of individual cells, involved spraying microdroplets of elements at a known concentration in 5 % dextran (to mimic the matrix-prompted bremstrahlung background-radiation). Unfortunately the resulting peak integrals were not proportional to the quantity of element present, as little control was possible over the droplet volume (only dextran standards of equivalent circumference were analysed) and thus the amount of element deposited (Fig. 6.5). To provide some measure of the contribution that each element affords to the whole spectrum, a semi-quantitative approach was taken which applies a machine efficiency-factor to the peak integral of each element.

However, if the profiles of heavy-metal exposed cyanobacteria and algae are to be effectively compared, a fully quantitative procedure is required to convert peak integrals into concentrations. A more rewarding approach to achieving this aim might be via the production of a standard, synchronous continuous culture whose cells contain equivalent concentrations of each element. With an accurate determination of cell density and analysis of the elemental content of large volumes of culture (about 50 ml), the peak integrals of elements from single cells of this uniform population may be used to calibrate EDXMA profiles.

The only background peak recorded from the specimen support was that of Cu signals emanating from both the EM column and the specimen support. Although the use of nylon grids would negate Cu signals obtained from the specimen support and facilitate the cellular location of this metal, it is not known if components of the TEM column generate detectable Cu X-rays which would prove inseparable from those originating from the sample.

Readings from the cell wall and inclusion bodies of air-dried cells (which present a thick section to the electron beam) always included a cytoplasmic component, but the number of counts from the cytoplasm indicate that this region of the cell makes a small contribution to the overall peak integrals.

EDXMA profiles of the polyphosphate bodies observed in air-dried samples of Synechococcus D562 revealed differences in metabolic states, signified by the size of the P peak and K to Ca peak-integral ratios (Fig. 6.6). The occurence of strong Ca signals in polyphosphate bodies that had accumulated heavy-metals was also observed by

Jensen et al. (1986); the presence of a high concentration of Ca in these bodies may exert control over the capacity of individual organelles to store toxic metals. The association between polyphosphate-bound Cd and a high Ca to K ratio (recorded at a frequency of less than one granule in 25) suggests that scope exists to synchronise the metabolic profile of a cell culture in order to enhance the concentration of Cd bound by polyphosphate bodies. It is possible that the number of polyphosphate bodies which exhibit a strong Ca signal may be increased by a change in the ionic components of the medium, but further work is required with cultures grown in a variety of ion concentrations. Cd-binding organelles may represent the final storage site for this metal after transport across the cell by Cd-binding peptides or proteins (these ligands potentially contribute towards the Cd peak obtained from a cytoplasmic reading (Fig. 6.6)) and therefore warrant further study as the Cd accumulating ability of these P stores may determine the maximum concentration of cytosolic Cd that can be tolerated.

An example of the control which is possible to attain over the metal composition of a cell is the appearance of Ba peaks for cultures grown in medium with a ten fold increase in the Mn concentration of PPJ medium. Because peaks for Ba were only observed for cells grown in high Mn medium, the observed Ba may be co-transported by the Mn^{2+} cell membrane pump (although a Mn / Cd symport similar to that found in yeast (Tynecka et al., 1981a) does not appear to exist, confirmatory flame atomic absorption spectrophotometry evidence is required). As Ba was not deliberately added to PPJ medium, the element may have originated from glass flasks used to culture the cyanobacteria or impurities in the salts used to formulate stock solutions for the medium.

Although the concentration of Cd detected in the polyphosphate bodies of cells grown in high Mn medium was not amplified (in comparison with cultures from unamended PPJ medium), EDXMA profiles of Synechococcus D562 cells grown in PPJ medium with a reduced concentration of Mn are worth generating, because the concentration of accumulated Cd may be enhanced if the number of Mn^{2+} ions available for cell-membrane transport are limited and Cd^{2+} ions take their place.

Precipitates of Fe formed on the surface of Synechococcus D562 cells also co-precipitated Cd, but it is not known whether the process of Cd precipitation enhances or reduces the amount of Cd accumulated internally. Such surface precipitation was not always observed and may be related to a particular stage of growth in which Fe^{3+} becomes insoluble.

Although Calothrix D184 samples are characterised by a much higher Ca content in their polyphosphate bodies than those analysed in Synechococcus D562 (high peaks for Ca from polyphosphate bodies may be linked with the accumulation of Cd to detectable concentrations in these organelles), no Cd was observed in cells of this strain (Fig.

6.8 and 6.9). As flame atomic absorption spectrophotometry samples of Calothrix D184 proved to contain low concentrations of Cd, the absence of a Cd signal from EDXMA is not unexpected. The strong Cl signal obtained from the cytoplasm of Calothrix D184 is unique to this strain (Fig. 6.9); however, further biochemical studies are required to identify its role in cellular metabolism.

Potential Cd-induced shifts in the elemental profile of microorganisms cannot be separated from variations in the metabolic status of individual cells; many more scans are required to record the variation in elemental composition of unexposed cells before the influence of this metal upon the cell constituents can be demonstrated. Mougeotia D536 is characterised by a strong K peak in all scans, the Ca to K ratio was consistently low and once again, no peaks for Cd were ever recorded (Fig. 6.10). Although an Al peak was conspicuous in Cd-free cells and absent from formvar film spectra, flame atomic absorption spectrophotometry data are needed as further evidence for the presence of Al within these strains. Since Al was not a planned constituent of the medium, the element is probably present as an impurity in the inorganic salts used to formulate the medium (the limits for both Ba and Al in these chemicals are about 0.002 %).

Prominent Cl, K and Fe peaks were recorded in Klebsormidium D537, but no Ca or Cd (Fig. 6.11). Therefore, the Cd accumulated by laboratory strains was not detected by EDXMA except when sequestered to a high concentration, reflecting the poor detection limits of this technique. The search for Cd has also proved elusive during the analysis of thin sections (Heuillet et al., 1986); thus the limiting factor in the detection of Cd is the number of X-rays emitted from the sample (and hence the concentration of element present), rather than a specimen adsorption phenomenon which may have reduced the number of X-rays reaching the detector from thick, air-dried specimens.

After the description of a sample's elemental profile the compound nature of intracellular inclusion bodies / surface precipitates can be attempted only if two or three major peaks are present (assuming that complexes possess a stoichometric relationship). The scan of precipitates of what is presumably $BaSO_4$ on the surface of Spirogyra is an example of such extrapolation (Fig. 6.16).

Field samples also failed to yield Cd in their spectra, probably due to the relatively low concentrations of environmental Cd which these strains experience. The only EDXMA peaks obtained for Cd were from Synechococcus D562 cells cultured in the presence of 4 mg l^{-1} Cd - a metal concentration which is about 100 times higher than the Cd recorded at each field site. However, a variety of other metals were detected either as surface precipitates or stored in intracellular inclusion bodies. Zn and Fe were accumulated to high a concentration in

samples of Stigeoclonium from Rampgill Level (exhibiting the highest Zn concentration of all four sites) and Klebsormidium from Caplecleugh Low Level; although K and Ca contributed many of the X-ray counts for Stigeoclonium, Mg peaks were also relatively high for this strain (Figs 6.12 and 6.13). In addition, large peaks for Ba and Pb recorded in algae sampled from Gillgill Burn (the site with the highest Cd concentration) (Figs 6.16, and 6.17). These profiles demonstrate the application of EDXMA to environmental monitoring; the metals that have been precipitated on the surface of the algae are a record of the heavy-metal contamination that the stream has experienced as a result of mining in the area. There have apparently been no previous studies that relate the concentration of metal in microorganisms as defined by EDXMA, to the fluctuation in environmental metal.

Laboratory investigations which utilise a mixture of metals in an attempt to produce scans equivalent to those obtained with field material, may indicate that the complex chemical and microbial environments of waterways cannot be mimicked by *in vitro* models.

A quest for the cellular localisation of potentially toxic metals by EDXMA is hampered by the relatively high detection-limits of this machine. Thin sections do not contain enough of the element to yield peaks above the background radiation and the variable diffusion volume of air-dried cells (which contain Cd at detectable levels) makes quantification very difficult. Metals that are sequestered to a high concentration in intracellular bodies or precipitated on the surface of the cell provide the best material for this type of analysis.

The original reason for generating EDXMA profiles of cyanobacteria and algae that had been exposed to Cd was to gain control over the concentration of metal accumulated, but as the only strain to produce a detectable Cd peak was Synechococcus D562, efforts to control the process of Cd accumulation from a knowledge of the pathways of metal uptake should be focused on this strain

9.7 Scanning proton microanalysis

The same rationale that prompted an investigation of the cellular location of heavy metals by EDXMA applies to the decision made to employ SPM for the localization of Cd; the ultimate aim was to gain control over the maximum concentration of metal that each cell can accumulate. Due to the low number of EDXMA spectra showing Cd peaks, samples were analysed with SPM because the higher energy (3 MeV compared with 0.1 MeV) and larger particle size of the incident proton beam induces more X-rays from the specimen than the stream of electrons used in EDXMA. The scan area of 30 μm^2 represents the minimum sample dimensions that the proton

probe can discriminate, whilst EDXMA has an effective scan area of 0.1 μm^2 and facilitates the investigation of subcellular organelles. As a result, the colour scans produced by SPM cannot be used to identify individual cell components, thus limiting the resolution of this technique.

The pixel map obtained for Cd in a sample of Klebsormidium D537 (a strain which does not accumulate Cd to high concentrations) suggests that SPM has a lower detection limit for the metal (Fig. 6.22). No Cd peaks were recorded for Synechococcus D562, probably because the polyphosphate bodies which accumulated the metal to a high concentration (organelles with a high Ca to K ratio) were not scanned (Fig. 6.19). The quantities of Mn and Ni (metals not detected by EDXMA in this study) observed in all four strains, provide further evidence that the technique of SPM is suitable for the detection of elements which are accumulated to low concentrations.

A strong Cl signal from Calothrix D184 cells recorded by EDXMA (Fig. 6.20) is substantiated by SPM scans; a high Cl concentration was also recorded in Mougeotia D536 (Fig. 6.21) but no attempt has been made to assign an explanation to these high Cl profiles. Although Cd was not successfully localized by SPM, additional Synechoccus D562 scans might generate positive results for Cd; the heterogeneous distribution of Cd amongst polyphosphate bodies of different cells has been demonstrated by EDXMA and only a few SPM scans of Synechoccus D562 were made. Frequent records of Al peaks (probably present as impurities in the stock solutions used to prepare the media) for all strains suggest that the Al detected by EDXMA is not an artefact and reinforces the view that all observations should be supported by evidence from a variety of sources. An attempt to compare the data obtained by these two scanning techniques (in terms of % composition of equivalent elements) would prove erroneous due to the difference in probe resolution, sampling time and mode of analysis.

Therefore, although this approach can be used to detect the microelements accumulated by algae, substantial sample degradation must occur due to the long scanning times (up to 20 min) and little subcellular information is generated. With an increase in detection limits, EDXMA should prove to be the method of choice for the examination of cell components in the future.

Whilst the original aim of manipulating the concentration of Cd accumulated by these strains via information obtained on the cellular partitioning of heavy-metals (from EDXMA and SPM techniques) has not been achieved, some guidelines for probing heavy-metal contaminated cultures have been delineated.

9.8 Screening for a MT gene

In addition to the accumulation of Cd by polyphosphate bodies, the ubiquitous cysteine-rich MT proteins isolated from many different species have also proved to be effective ligands for reducing the toxicity of free, divalent intracellular Cd (Kägi & Nordberg, 1979). If MT were discovered to bind a significant proportion of the cytosolic Cd accumulated by Synechococcus D562, an increase in the transcription rate of this protein (either invoked by promoter control or multiple gene inserts) may increase the Cd accumulating capacity of this organism.

The extent to which enhanced levels of MT within a cell increase the concentration of Cd accumulated, depends on the function of the protein; more metal may be detoxified if the MT acts as a metal storage site rather than a transport molecule. With this target in view, plasmid and genomic DNA extracted from Synechococcus D562 was probed for the occurrence of DNA sequence homologous to that from Synechococcus PCC 6301 MT, currently the only strain of cyanobacteria shown to produce a MT (Olafson et al., 1988).

As a first step in DNA isolation somewhat destructive treatments were employed to degrade the cell wall, for although a high yield of spheroplasts was attained, medium inoculated with these emasculated cells did not produce viable cultures. The sequential requirement of lysozyme followed by protease may indicate that the extracellular mucilage protects the cells from protease action and must be partially degraded by the NAG-NAM specific enzyme before the cell wall can be attacked (Figs 7.1 and 7.2). An enzyme with a more restricted action than that of the general protease (isolated from Streptomyces griseus) that was employed in this investigation, may result in viable spheroplasts; a knowledge of the composition of the cell wall would help to identify the types of catalytic mechanism required. Once intact spheroplasts have been generated, the constituents of the medium must be reorganised to inhibit cell wall-growth before Cd uptake studies can be recorded. Differences in cell-wall composition between cyanobacteria are highlighted by the fact that some strains of Synechococcus form spherical cells after incubation with lysozyme and no further treatment (Delaney, 1984).

As an extension of the cell disruption observed during the plasmid extraction protocol for Synechococcus D562, the effect of lysozyme digestion upon the cells was investigated. The heavy-metal stains of Os, U and Pb employed in TEM sections did not stain the native mucilage and neither did Ru (Figs 6.2 and 8.1). However, the action of lysozyme on the mucilage of cells, exposed ligands (probably free acidic-groups) which bound Ru ions, revealing an extensive extracellular matrix. The EM image of this stained carbohydrate may appear distorted as a result of lysozyme digestion and the process of drying the cells on grids. Either the action of protease is dampened

by untreated mucilage, or sections of the cell wall also possess NAG-NAM groups which must be split before protease can act; for a single step digestion of Synechococcus D562 cells with this second enzyme did not produce spherical shaped units. Results from the EDTA experiments and a lack of binding by Ru to an undigested matrix, suggest that this mucilage does not bind Cd.

The large volume of culture required for Synechococcus D562 plasmid extraction indicates that the DNA is not present at a high copy number (only 10 ml of an E. coli pUC19 culture was required to produce a high concentration plasmid sample, compared with 4 litres of cyanobacteria). Although the phenol / chloroform extraction procedure proved adequate for the isolation of DNA, pulsed gel alternating field electrophoresis of whole cells may prove a faster, more productive method for generating restriction quality, plasmid DNA. Plasmid number and size varies from one species of photosynthetic microorganism to another (Cifferi et al., 1989), but few genes have been assigned to these regions of DNA and no reference to plasmid encoded, metal-tolerance factors have been found in cyanobacteria. Strains of Synechococcus D562 grown in the presence and absence of Cd proved to contain plasmids of similar size and restriction sites, showing that Cd does not operate as a selective pressure for these circular strands of DNA in the same manner as antibiotics (Fig. 7.3).

A blot of genomic and plasmid DNA with probes designed to hybridize with the MT from Synechococcus PCC 6301 did not bind the labelled oligonucleotides. This result is not conclusive as the binding stringency of the probe may have been too high if sequence heterogeneity exists between the probe and target DNA which does not favour an oligonucleotide match under these strict binding conditions.

An alternative approach would be to use the MT probe sequences as primers for a polymerase chain reaction to amplify any MT-like genes present in Synechococcus D562. A positive result in this area would then facilitate investigations of the influence of Cd upon the regulation of gene transcription and identification of metal regulatory elements.

If this strain does not prove to contain a MT then the process of Cd detoxification may be restricted to intracellular transport via Cd-binding peptides and storage in polyphosphate bodies with a relatively high Ca content. A search of the available literature reveals information on detoxification pathways in bacteria but no in-depth studies on cyanobacteria or algae.

9.9 Cd-binding peptides

Another mechanism for the detoxification of cytosolic Cd that has been identified in a variety of microorganisms and higher plants is the utilization of poly(γ-glutamylcysteinyl)glycine peptides for the binding of intracellular Cd (Reese et al., 1988 ; Robinson, 1989 ; Rauser, 1990). As the detection of such peptides in the cyanobacteria and algae used in this study would provide another cell process for manipulation to raise the efficiency of metal accumulation, samples of these strains exposed to the appropriate Cd concentrations for eliciting peptide-bound Cd were analysed.

If the concentration of toxic metal bound by these molecules is to be enhanced, then metabolic rather than genomic (appropriate for manipulating the concentration of Cd bound by MT) perturbation will have to be designed to increase the amount of ligand synthesised, as $(\gamma EC)_nG$'s are not transcriptional products. From experiments carried out on the four laboratory strains, putative $(\gamma EC)_nG$'s were found to bind intracellular Cd, but only when a high concentration of Cd was added to the medium at the end of the log-phase of growth (Figs 7.4, 7.5 and 7.6). Mougeotia D536 appears to bind more Cd with $(\gamma EC)_nG$'s than Synechoccus D562 when grown in 0.5 mg l^{-1} (Fig 7.5); this may reflect a higher utilization of the S-transport molecules for metal detoxification.

As the HPLC profiles depict only one point of the growth curve and one level of added Cd, little can be inferred about the role of $(\gamma EC)_nG$'s in heavy metal detoxification from the data produced by this study. In order to record these potential Cd-induced fluctuations in peptide production more easily, glutathione labelled with radioactive carbon could be employed as an effective tracer compound for use in the rather more dynamic technique of nuclear magnetic resonance. Complete reducing conditions cannot be guaranteed during the extraction of algal cells, therefore the ratio of Cd bound to the putative peptides and that complexed by β-mercaptoethanol does not necessarily reflect the concentration of metal bound *in vivo* by $(\gamma EC)_nG$'s. In addition, the process of extraction may also disrupt regions of the cell which contain the metal, resulting in free Cd which may then bind to $(\gamma EC)_nG$'s which were not originally intended for Cd detoxification. If the predominant role of these peptides is that of S transport (Robinson et al., 1990) the extent to which the S metabolism of the cell is disrupted by high levels of free cytosolic Cd, has yet to be elucidated. The common protein profiles that were recorded show peaks at the $(\gamma EC)_nG$ retention time for the cytosolic fractions of the procaryotes (broad distribution of protein) but not eucaryotes (most protein concentrated at the void volume), indicating that additional Cd-binding proteins may also be present in the cyanobacteria.

Cyanobacteria and algae grown in the presence of Cd at a low concentration do not yield putative $(\gamma EC)_nG$ peaks; an acetone precipitation step could be used to overcome the detection limits of Cd analysis to search for low-level indigenous peptides. The requirement for the high concentration of Cd required to visualise the ligand, may be due to the induction of peptide synthesis or a redirection of their metabolic direction from S transport to Cd-binding.

As endogenous peptides were not found to bind significant amounts of the metal, the process of Cd sequestration by peptide ligands must be under some form of inducible control. Cells which have undergone this treatment, partition about 20 - 40 % of the cytosolic Cd into one HPLC peak with the residual either weakly bound to intracellular components or as the free divalent ion. But cells which were not shocked with Cd failed to produce peaks for the metal-bound peptide. Therefore Cd-binding peptides may act as a secondary level of defence in these strains after the usual detoxification mechanisms have been saturated, because only a high concentration of Cd in the medium elicits peaks for $(\gamma EC)_nG$'s.

Alternatively Cd-binding peptides may contribute a transitory role in metal detoxification by providing ligands for the safe transport of the pollutant from the cell wall to polyphosphate bodies (or other sites of deposition eg. lipid bodies) where they are bound in a more permanent manner.

The data collected for these strains of algae should be augmented by other studies to certify that the peaks obtained on gel permeation HPLC profiles represent Cd bound by $(\gamma EC)_nG$'s : characterisation of the amino acid content of the metal-binding peaks, determination of the distribution of peptide chain lengths and the number of peptides chelating each Cd^{2+} ion.

If a MT is shown to bind cytosolic Cd in Synechococcus D562, then three mechanisms of detoxification (by polyphosphate, protein and peptide ligands) will have been demonstrated. Therefore, the spatial and temporal contribution that each mechanism affords to the complexation of Cd is the next step in building a model of the complete molecular response exhibited by this strain to a Cd challenge.

9.10 Ultrastructural, enzyme and protein damage

For cells which do not possess the heavy-metal detoxification mechanisms outlined above, the presence of Cd in the cytosol can result in wide-ranging cell damage (Tables 1.8 and 1.9); consequently, the maximum

concentration of Cd that a cell population can accumulate is reduced. In order to determine the extent to which Cd limits the efficiency of metal uptake in strains of cyanobacteria and algae, investigations were launched into the ultrastructural and protein defects which arise from growth in the presence of toxic concentrations of Cd.

The affinity exhibited by this metal for thiol groups can lead to the collapse of membranes which contain these molecules (Duret et al, 1986) or the production of intracellular inclusion bodies which store the toxic metal (Heuillet et al, 1986). Such ultrastructural changes are often documented by TEM sections of microorganisms cultured with heavy-metals (Table 1.8). Although Calothrix D184 did not accumulate Cd to a high concentration, cells cultured in 1 mg l^{-1} Cd suppressed polyglucoside granule formation and showed disordered thylakoid membranes (Fig. 8.2). In conjunction with the low levels of Cd-binding peptide discovered in this strain and the small quantity of accumulated Cd, these observations suggest that Calothrix D184 relies upon an exclusion process to combat the toxic effects of the metal and does not possess extensive intracellular defence-mechanisms. Therefore if the metal is transported into the cell, a low concentration of cytoplasmic Cd will elicit a toxic, ultrastructural response.

Mougeotia D536 cells grown in the presence of 0.8 mg l^{-1} Cd displayed small Ca / P precipitates (determined by EDXMA) (Heuillet et al., 1986) around the periphery of the cytoplasm (Fig. 8.3). These granules may contain Cd below the detection limit of EDXMA and could represent a final detoxification site which is serviced by Cd-transporting $(\gamma EC)_nG$'s.

When cultured in the presence of 1.0 mg l^{-1} Cd cells of Klebsormidium D537 did not exhibit any of the heavy-metal disruption associated with Cd bound to the thiol groups of membranes (Vymazal, 1987), but the heavily-stained plastoglobuli observed in control cultures without added Cd were absent from Cd-exposed samples (Fig. 8.4a and b) and appeared to be replaced by more opaque lipid-bodies. This result is in contrast to the heavy-metal response exhibited by Stigeoclonium tenue; an increase in plastoglobuli number was recorded for cells that had been exposed to 0.5 mg l^{-1} Pb (Silverberg, 1975). The explanation given for this ultrastructural change is that the damage inflicted upon the thylakoid membranes resulted in an excess of cytoplasmic lipids, which were therefore accumulated as plastoglobuli. In contrast, the apparent conversion of plastoglobuli into larger lipid bodies in Klebsormidium D537 may have occurred to provide Cd-binding sites in the chloroplast by changing the molecular configuration of the plastoglobuli lipids. The two different mechanisms outlined above for the detoxification of Cd require further explanation as to why a common ligand (components of plastoglobuli) does not provide the same role (binding cytosolic Cd).

The lack of ultrastructural disorders in Synechococcus D562 (Figs 8.1a and b) grown in the presence of a

relatively high level of Cd (4 mg l^{-1}) presumably indicates an ability to reduce the free divalent cytosolic metal content to non-toxic concentrations, although the limited number of organelles visible in this strain diminishes the possibility of observing Cd-induced ultrastructural defects by EM. A reduction in the dry weight of cultures that were grown in the presence of high concentrations of Cd for EM investigation indicates that a toxic effect is being exerted by the metal, but it appears that only healthy cells are available for ultrastructural studies in the exponential phase of growth. Therefore, TEM sections of cell populations at different stages of growth (especially within the first few hours following Cd addition) may reveal more severe ultrastructural damage to particular cells.

The reason for taking electron micrographs of Cd-exposed cells was to determine the extent of metal-induced ultrastructural disorders, as this may influence the maximum concentration of Cd that can be accumulated by each strain. The Cd that was employed for EM investigation was deliberately high to maximise the probability of recording defects in cell ultrastructure, however the changes in cell organisation that were observed as a result of Cd exposure cannot be related to a ceiling for the amount of Cd accumulated because only viable cells were sampled and it is difficult to relate ultrastructural defects to restrictions on metal accumulation efficiency without more analysis. But since the concentration of Cd employed in ultrastructural studies was an order of magnitude higher than that used for the immobilized cell columns, the cytotoxic effects exerted by this metal are probably not a limiting factor in the amount of Cd accumulated by packed-bed columns.

The enzyme inhibitory properties of Cd were demonstrated with the extracellular alkaline phosphatase from 5 d old Synechococcus D562 (Table 8.1). At concentrations of 1 and 10 mg l^{-1}, Cd reduced the phosphatase activity of ultrafiltered enzyme at an assay pH of 7.0, but had no effect in assays run at pH 10.3, probably because most of the Cd was present as hydroxide complexes rather than in a toxic, divalent form. Cultures grown in the presence of Cd also failed to exhibit alkaline phosphatase inhibition; this indicates that the bound Zn atoms responsible for the correct active-site electron environment, are firmly attached to the protein before export from the cytoplasm and not easily replaced by competing cations. Even if Cd can enter the active-site domain of this extracellular enzyme, no toxic effects were recorded.

The high activity of Synechococcus D562 material grown in 4 mg l^{-1} Cd, is probably a result of an extended growth period and concurrent accumulation of extracellular enzyme. Therefore, no comparisons of enzyme activity can be made between this and lower concentrations of Cd because the cultures were sampled at different time intervals. The loss of medium due to evaporation over the long period of incubation required for cells growing in the presence of 4 mg l^{-1} Cd may also concentrate the enzyme and therefore contribute to the high rate of catalysis.

The retention of enzyme activity after the passage of growth medium across a 100 kD membrane, indicates that Synechococcus D562 excretes a high Mr form of the protein which is not associated with the extracellular mucilage normally retained by 0.2 μm nitrocellulose filters. This enzyme is therefore suitable for further purification because of its lack of association with other cell components. Ultrafiltration also proved effective in enhancing the hydrolytic capability of samples with inherently low cell-bound alkaline phosphatase activity i.e. cell-bound PME and PDE. Protein concentration by the use of Mr cut-off membranes also has applications in reducing the extensive incubation times required by samples which exhibit a low enzyme activity (eg. Mougeotia D536). Other enzymes whose active-site metal is more readily replaced by Cd^{2+} may prove to be a better indicator of Cd toxicity in both growth and assay media.

If intracellular protein damage is caused by cytosolic Cd, then one-dimensional SDS PAGE is not discriminatory enough to reveal its toxic effects (Figs 8.7, 8.8, 8.9 and 8.10). Although common banding patterns were observed between cyanobacteria and algae, no low Mr Cd-response proteins or dissociated subunits were stained and the loss of protein bands as a result of transcriptional interference was not observed. The protein resolution of these gels may be enhanced either by running samples in a second dimension, or by fractionating the cells before electrophoresis to reduce the density of the main protein bands. This step may increase the sensitivity of the technique for Cd-inducible / sensitive proteins which are not present in high concentrations within the cell. Alternatively, silver staining would be appropriate for proteins at a low concentration in cell sub-fractions.

The main aim of this research was to determine the feasibility of employing immobilized, growing cyanobacteria and algae which accumulate Cd to high concentrations for the removal of the metal from polluted effluents. The two strains of algae selected for such a system cannot be recommended in isolation, as the rate of Cd removal was slow in comparison with immobilized microorganisms that precipitate toxic metals at the surface of the cell. In addition, metal-laden effluents that contain algicidal compounds (as do many complex industrial wastes) cannot be treated by a live cell system. However, immobilized, growing cells may prove effective as a final step in the process of dealing with Cd, by reducing the levels of this heavy metal below legal limits following an intial treatment with cell-surface ligands that bind the pollutant.

The use of EDXMA and SPM for the cellular localisation of toxic heavy-metals is only applicable to cells that accumulate the metal to a high concentration, particularly at the surface of the cell or when combined with intracellular inclusion bodies. But with an improvement in the detection limits and accurate methods of quantifying profiles, this technique will prove valuable for formulating heavy-metal detoxification mechanisms.

TEM sections of Cd-exposed algae will only demonstrate ultrastructural damage if affected cells with few tolerance mechanisms are sampled,. In order to avoid the analysis of cells that have been selected for heavy-metal tolerance by growth in metal-amended media, the culture should be sampled after the addition of Cd and throughout the growth phase.

The other areas of investigation covered by this thesis require further work if the mechanisms of heavy-metal tolerance utilised by the Durham Culture Collection strains are to be identified : appropriate conditions for the continuous selection of Cd-tolerant algae have yet to be defined, whilst the sequencing of Cd-induced shock proteins from isoelectrofocusing / SDS PAGE gels and changes in the metabolism of $(\gamma EC)_nG$'s during a variety of heavy-metal exposure regimes would yield useful information.

SUMMARY

1. The first step towards realising the main aim of this research (employing growing, immobilized cyanobacteria and algae for the removal of Cd from polluted effluents), was to screen microorganisms from heavy-metal environments that accumulated Cd to a high concentration. Strains of cyanobacteria and algae from the Durham Culture Collection and heavy-metal contaminated sites were investigated for this property. Two strains, Synechococcus D562 and Mougeotia D536 proved to be the best accumulators of the metal and were therefore used throughout this research. The toxic effects of the Cd added to the growth medium of Calothrix D184 and Klebsormidium D537 were also investigated.

2. In order to determine the toxicity of Cd which has been added to media, a number of factors which influence Cd activity were investigated. The process of autoclaving media, evaporation during incubation and the presence of EDTA increased the original target values for the Cd concentration that cells were exposed to by only 4 %. When Synechococcus D562 cells were washed with 25 mM EDTA, all internalised Cd was removed; a lower concentration of 0.25 mM EDTA was then used to determine the amount of metal adsorbed by the cell wall. Although less Cd was extracted by this treatment, the metal that was chelated was not necessarily derived exclusively from the cell wall. Dead cells of Synechococcus D562 (killed by heat treatment) did not exhibit many Cd-binding ligands on their surface, therefore the majority of the Cd accumulated by this strain is to be found inside the cell.

3. As an alternative approach to the step-wise selection of Cd-tolerant cells by batch culture, the process of continuous culture was employed in an attempt to obtain cells which tolerated high concentrations of Cd with enhanced levels of accumulated metal. Using batch cultures of Synechococcus D562, a light intensity of 100 μmol photon m^{-2} s^{-1} was chosen to control a continuous cell-culture in the presence of 2 mg l^{-1} Cd. A rise in the Cd concentration of the medium feed up to 3.4 mg l^{-1} Cd did not inhibit growth, but above this Cd concentration a rapid decline in whole cell absorbance and cell density were observed. In comparison, batch cultured cells tolerated 5 mg l^{-1} Cd (albeit with a long lag-phase).

4. After an intial investigation of a number of immobilization matrices, calcium alginate was selected as the most appropriate material for the retention of cells that accumulate Cd. The Cd-binding sites of calcium alginate became saturated within 16 h, complexing about 60 % of the added Cd when FeEDTA was omitted and 10 to 35 % in the presence of FeEDTA, depending upon the concentration of Cd employed. Synechococcus D562 and Mougeotia D536 were immobilized in calcium alginate beads and operated in packed-bed columns to remove Cd from the medium. Aeration of the system did not relieve the limited growth conditions observed at the top of the column and actually inhibited Cd removal, whilst 0.5 mg l^{-1} Cd proved the upper limit of added metal for this system. The additional biomass generated by immobilized Mougeotia D536 extracted more Cd from the medium stream than Synechococcus D562. Immobilized stationary cells and free cells did not remove Cd as efficiently as packed-bed columns.

5. The localization of Cd within cyanobacterial and algal cells was studied in order to generate information about the mechanisms of intracellular accumulation that are adopted by these strains, with the ultimate aim of devising procedures to enhance the concentration of Cd accumulated. Initial attempts to analyse frozen, algal sections with EDXMA proved technically intractable. Therefore the simpler procedure of probing air-dried cells was adopted. A high Cd detection limit for this machine became apparent, as only Synechococcus D562 cells (with a high Ca content to their PP bodies) displayed Cd peaks above the background radiation. Evidence that the manipulation of the metal content of a cell is possible by a change in the ionic composition of the growth medium was provided by Ba peaks, which were only observed in cells that had been cultured in medium with a high Mn concentration. None of the other strains of algae that were tested produced scans containing X-ray counts from Cd. Algae collected from field sites contaminated with mined metal residues, accumulated Ba as surface granules and Pb, Zn, Fe, Mn and Ba in intracellular inclusion bodies.

6. In order to overcome the resolution problems associated with EDXMA, samples were subjected to SPM. Peaks for Cd were recorded only in Klebsormidium D537, a species which does not accumulate the metal to high concentrations. The other strains that were subjected to this technique failed to yield X-ray signals for Cd even though these microorganisms internalise Cd to higher concentrations, however, other elements which are account for minor cell components (and not detected by EDXMA) were observed by this technique.

7. The degradation of the cell wall of Synechococcus D562 was carried out to investigate the role played by the cell wall in Cd detoxification and as a preliminary step towards DNA extraction. Both the cell wall and extracellular mucilage of Synechococcus D562 contained lysozyme digestion sites, which when stained with Ru red and viewed under the TEM could be visualized as a tortuous mesh of fibrils surrounding air-dried cells. Subsequent attack by the enzyme protease resulted in detergent-sensitive spheroplasts which were suitable for DNA extraction, but not viable for growth.

8. The presence of Cd-detoxifying MT proteins in cyanobacteria and algae was investigated because of the potential for increasing the amount of MT produced by the cell to enhance the concentration of accumulated Cd. In order to ascertain whether or not the MT gene is present and localize its position, the DNA of Synechococcus D562 was analysed. A 14 kb plasmid was isolated from this strain when grown in the presence and absence of Cd. The plasmids isolated from both cell lines exhibited identical restriction sites for three nucleases, but a radiolabelled probe modelled upon conservative regions of the MT from Synechococcus PCC 6301 did not bind to Southern blots of Synechococcus D562 genomic or plasmid DNA, indicating that the MT gene may not be present in this cyanobacterium.

9. Another detoxification mechanism was observed in all four strains from the Durham Culture Collection. Putative Cd-binding peptides (poly(γ-glutamylcysteinyl)glycine) were detected when cultures were exposed to Cd at 6.12 mg l^{-1} for 2 d at the late log-phase of growth. Indigenous poly(γ-glutamylcysteinyl)glycines did not yield a peak at the correct elution volume when Cd was added to a cytoplasmic extract and no metal-binding peptides were observed for cells grown in the presence of 0.5 mg l^{-1} Cd but not shocked with the metal at the end of their exponential growth-phase. The half dissociation pH of 5.0 (the pH at which half the original metal is bound by the ligand) for Mougeotia D536 Cd-binding peptides is in the same range as that for peptides in other species and a reversed-phase profile of the putative apopeptides (cell extracts treated with acid) produced thiol peaks similar to the well characterised poly(γ-glutamylcysteinyl)glycines from Datura innoxia.

10. An analysis of the ultrastructural damage caused by the presence of cytosolic Cd was intiated, to assess a potential reduction in the Cd accumulating ability of the strains used in this study. TEM ultra-thin sections of Calothrix D184 cells exposed to Cd revealed a loss of polyglucoside granules and collapse of the ordered

structure of the thylakoid membranes. Metal-treated Mougeotia D536 cultures exhibited heavily stained precipitates around the periphery of the cytoplasm which may bind Cd. Cell sections of Klebsormidium D537 grown in the presence of Cd exhibited an inhibition of plastoglobuli formation, the released lipids may then be stored in larger, more opaque lipid bodies whilst Synechococcus D562 failed to reveal any ultrastructural disorders when exposed to the heavy metal.

11. A second indicator of cell toxicty, that of enzyme inhibition, was used to determine the influnce of Cd upon the rate of enzyme catalysis. No cell-bound alkaline phosphatase activity was recorded in Synechococcus D562, nearly all of the activity was extracellular. The enzyme was not inhibited by a range of Cd concentrations either in the assay or growth media at pH 10.3. However, when the protein was concentrated and run at pH 7.0, the presence of Cd in the assay medium reduced activity. No enzyme inhibition was recorded for cells grown in the presence of Cd. The use of 30 kD ultrafiltration membranes proved effective in concentrating the enzyme and may be appropriate as part of an enzyme purification procedure.

12. A further technique that was used for the assessment of Cd-induced damage involved the analysis of cell proteins by one dimensional SDS PAGE. The protein bands observed on the gels did not reveal any differences between cells exposed to Cd and those grown without the metal, except for a reduction in the staining intensity of a 17 kD band in Calothrix D184, although the procedure did yield common banding patterns between cyanobacteria and algae.

13. Although Cd reduced the cell density of the four strains used in this study, no severe ultrastructural or biochemical defects were recorded; it is therefore assumed that the heavy-metal accumulating capacities of the cells that survive Cd exposure are not significantly damaged by the presence of Cd in the growth medium. Microorganisms which generate a high concentration of biomass and thus provide more Cd-binding sites are the most suitable types of cell for Cd accumulation as immobilized cells. The localization of toxic metals in individual cells proved difficult due to the low concentration of metal accumulated and the only metal-detoxification mechanism recorded in the cyanobacteria and algae used in this study were Cd-binding peptides.

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APPENDICES

Appendix 1 : Intensity of illumination for various culture environments

Apparatus	region	incident light intensity (μmol photon m^{-2} s^{-1})
Orbital shaker	periphery	20
	centre	20
Growth tank	periphery	80
	centre	60
Fermenter	top	40
	middle	100
	bottom	100
Immobilized cell columns	top	21
	middle	20
	bottom	19

Appendix 2 : Amount of antibiotic present on each arm of the Oxoid Multodisks

Code 30-12 L	(μg)	code 30 - 1 H	(μg)
chlortetracycline	10	chloramphenicol	10
chloramphenicol	10	erythromycin	10
furazolidone	50	sulphafurazole	100
sulphafurazole	100	penicillin G	1.5 units
neomycin	10	streptomycin	10
penicillin G	1.5 units	tetracycline	10
streptomycin	10		
oxytetracycline	10		

Appendix 3 : Oligonucleotide sequence of the 20-mer primers used to probe Synechococcus D562 genomic and plasmid DNA for the MT gene, based upon the Synechococcus PCC 6301 MT (Olafson et al., 1988)

MT amino acid sequence (underlined amino acids are the primer target regions) :

TSTTLVKCACEPCLCNVDPSKAIDRNGLYYCCEACADGHTGGSKGCGHTGCNC

Amino acid sequence of target regions :
- N terminus : VAL - LYS - CYS - ALA - CYS - GLU - PRO
- C terminus : GLY - HIS - THR - GLY - CYS - ASN - CYS

Nucleotide sequence of target regions :
- N terminus 3' : CACTTCACACGTACGCTGGG
- C terminus 3' : GTAAAGGTGGGTCAAACGGG

Primers :
- N terminus primer : GTIAAYTGXGCITGXGAICC
- C terminus primer : CAYTTYCAICCIGTYTGICC

A = adenosine T = thymidine C = cytidine G = guanosine I = inosine

X = thymidine or deoxycytidine incorporated into the primer without preference

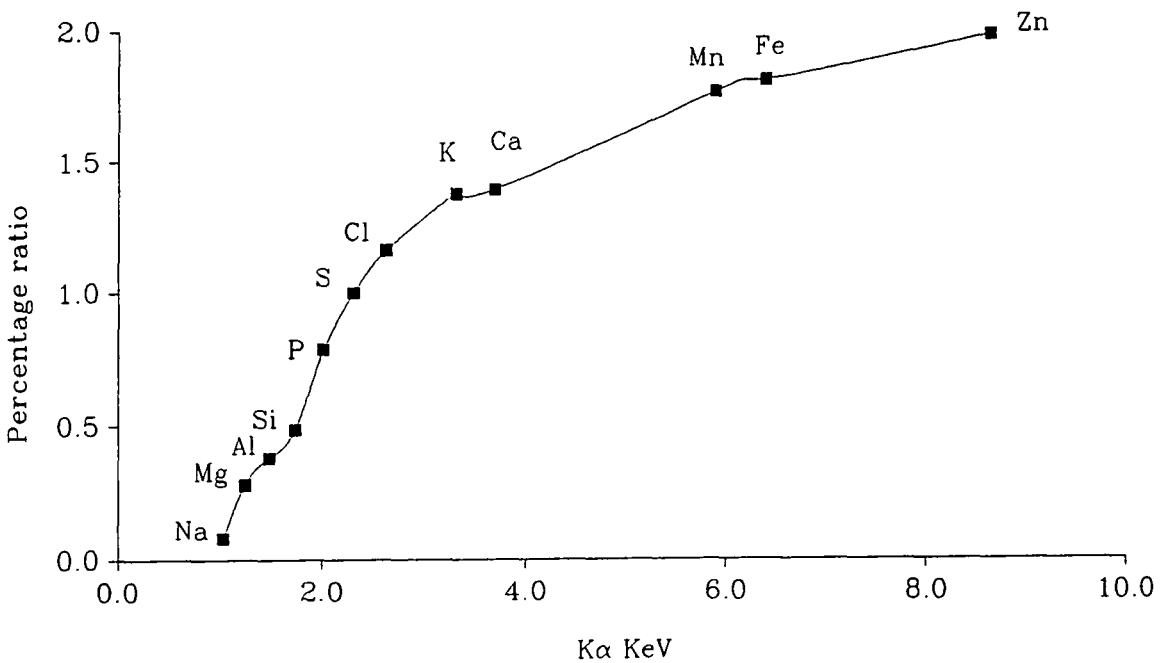
Y = adenosine or deoxyguanosine incorporated into the primer without preference

Appendix 4 : APA calibration curve of ρNP concentration versus absorbance at 405 nm, with eight well-replicates per concentration

ρNP concentration (μM)	absorbance at 405 nm ± sem	ρNP concentration (μM)	absorbance at 405 nm ± sem
100	1.581 ± 0.0036	30	0.502 ± 0.0028
90	1.451 ± 0.0063	20	0.327 ± 0.0021
80	1.296 ± 0.0091	10	0.165 ± 0.0008
70	1.148 ± 0.0040	7.5	0.130 ± 0.0008
60	0.973 ± 0.0049	5	0.081 ± 0.0008
50	0.833 ± 0.0019	2.5	0.047 ± 0.0009
40	0.652 ± 0.0027	1	0.014 ± 0.0010

Regression line : $Y = 0.01602\,X + 0.00933$ $r^2 = 0.9997$

Appendix 5 : Plot of % ratio (S = 1.0) of efficiency factors against kα X-ray energy (KeV) for EDXMA of elements at 100 kV, 100 s livetime and 12 ° specimen tilt.



Appendix 6 : EDXMA profiles of strains from the Durham Culture Collection which were not exposed to Cd

Fig. 6.1 EDXMA profile of Synechococcus D562 grown without added Cd.

a) PP body, high Ca. Total net integral of profile = 6184

Element	% contribution	element	% contribution
Mg	11.85	S	6.45
Al	0.09	K	4.06
P	63.04	Ca	14.51

b) PP body, high K. Total net-integral of profile = 60925

Element	% contribution	element	% contribution
Na	34.39	S	1.21
Mg	10.76	K	9.68
P	43.9		

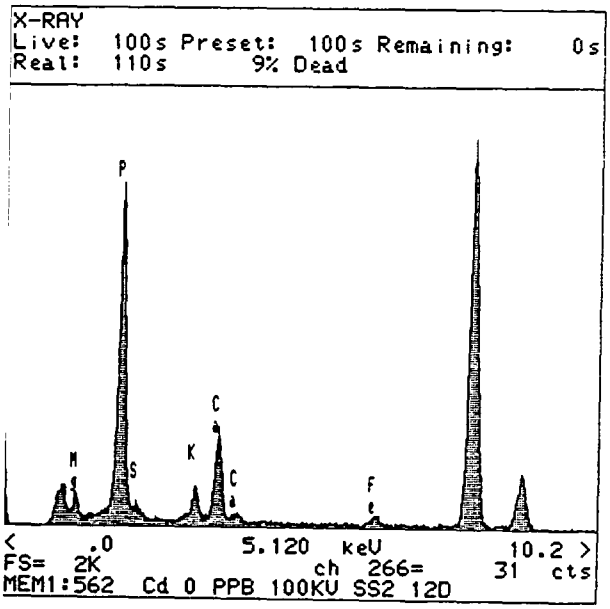
c) whole cell. Total net-integral of profile = 65042

Element	% contribution	element	% contribution
Na	40.79	P	20.18
Mg	4.64	S	5.16
Al	0.90	Cl	1.91
Si	1.49	K	24.92

d) cytoplasm. Total net-integral of profile = 1835

Element	% contribution	element	% contribution
Mg	25.98	S	11.61
Al	8.89	Ca	0.36
P	53.17		

i)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 110s 9% Dead
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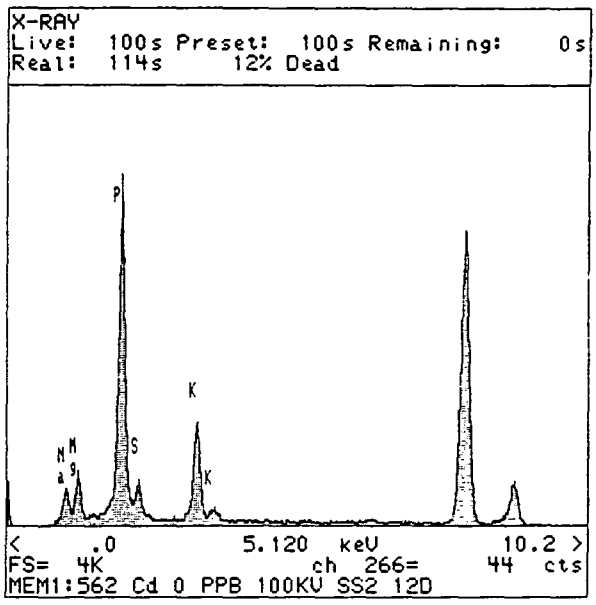
FS= 2K ch 266= 31 cts
MEM1:562 Cd 0 PPB 100KV SS2 12D

b)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FS= 4K ch 266= 44 cts
MEM1:562 Cd 0 PPB 100KV SS2 12D

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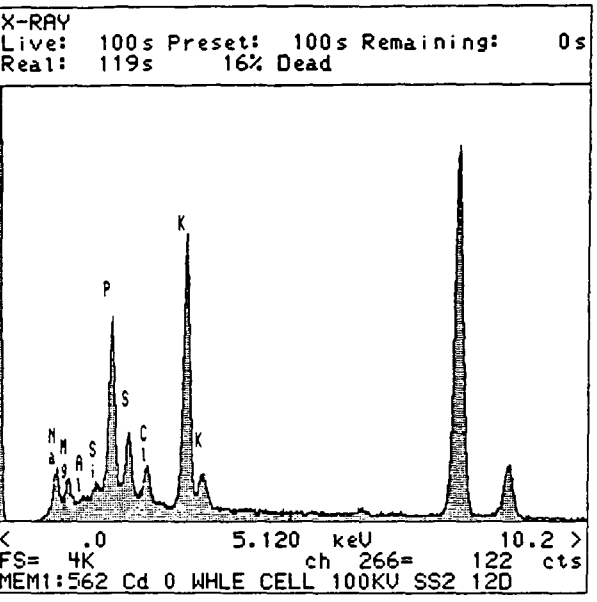
X-RAY
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FS= 4K ch 266= 122 cts
MEM1:562 Cd 0 WHLE CELL 100KV SS2 12D

d)

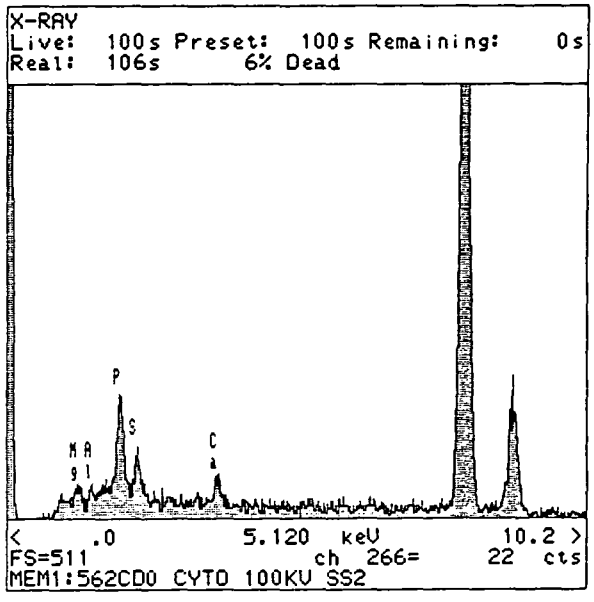

X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 106s 6% Dead
P
S
C
a
< .0 5.120 keV 10.2 >
FS=511 ch 266= 22 cts
MEM1:562CD0 CYTO 100KV SS2

Fig. 6.2 EDXMA profiles of Calothrix D184 grown without added Cd.

a) inclusion body. Total net-integral of profile = 5365

Element	% contribution	element	% contribution
Al	4.66	Cl	0.75
P	13.68	K	6.96
S	3.69	Ca	70.25

b) inclusion body. Total net-integral of profile = 13388

Element	% contribution	element	% contribution
Na	17.77	Cl	49.21
Al	2.06	K	8.51
P	0.37	Ca	21.20
S	0.96		

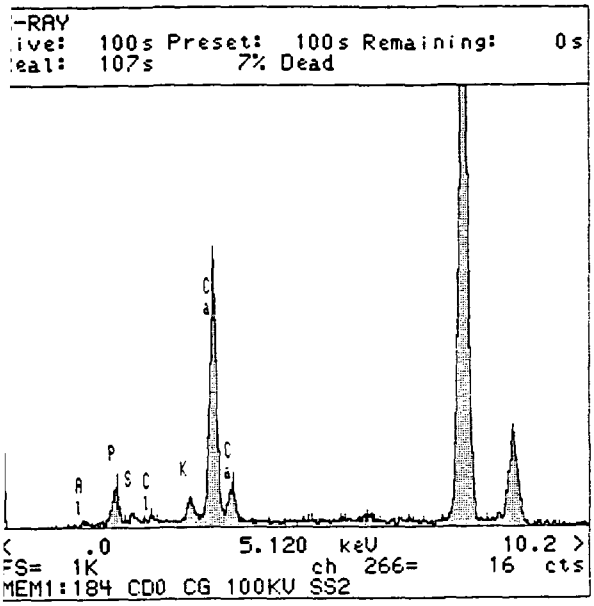
c) cell wall. Total net-integral of profile = 3150

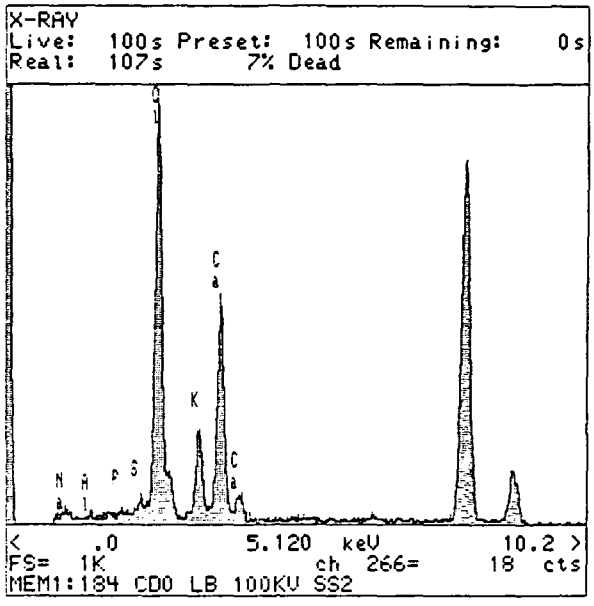
Element	% contribution	element	% contribution
Mg	11.15	Cl	9.58
Al	8.52	K	16.46
P	2.54	Ca	43.01
S	17.75		

d) cytoplasm. Total net-integral of profile = 897

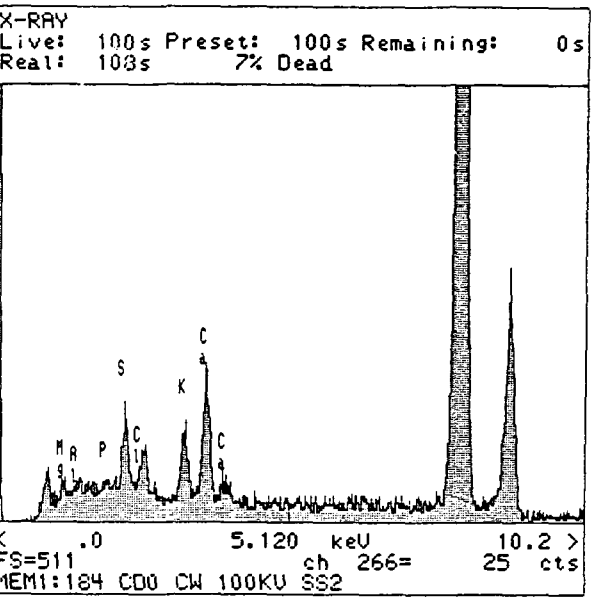
Element	% contribution	element	% contribution
S	40.35	K	19.32
Cl	11.05	Ca	29.89

b)


-RAY
ive: 100s Preset: 100s Remaining: 0s
eal: 107s 7% Dead
Al
P
S
Cl
K
Ca
Ca
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 16 cts
MEM1:184 CD0 CG 100KV SS2


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 107s 7% Dead
Na
Al
P
S
K
Ca
Ca
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 18 cts
MEM1:184 CD0 LB 100KV SS2

)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 108s 7% Dead
Mg
Al
P
S
Cl
K
Ca
Ca
< .0 5.120 keV 10.2 >
FS=511 ch 266= 25 cts
MEM1:184 CD0 CW 100KV SS2

d)

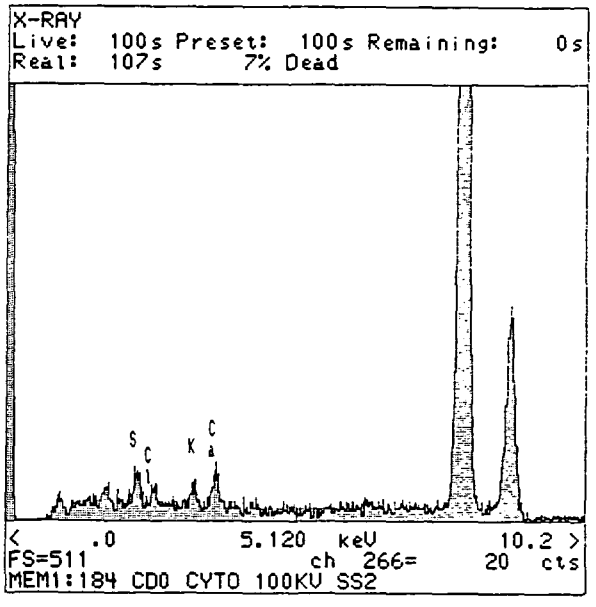

X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 107s 7% Dead
S
Cl
K
Ca
< .0 5.120 keV 10.2 >
FS=511 ch 266= 20 cts
MEM1:184 CD0 CYTO 100KV SS2

Fig. 6.3 EDXMA profiles of Mougeotia D536 grown without Cd.

a) inclusion body. Total net-integral of profile = 24047

Element	% contribution	element	% contribution
Na	41.40	Cl	1.50
Mg	1.54	K	11.72
Al	1.37	Ca	10.10
P	27.40	Fe	0.64
S	4.35		

b) inclusion body. Total net-integral of profile = 5958

Element	% contribution	element	% contribution
Al	4.10	K	53.33
P	33.59	Fe	1.51
S	7.45		

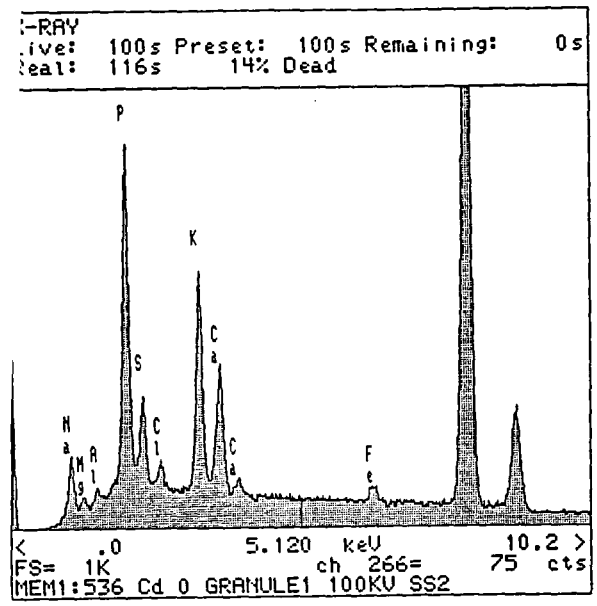
c) cell wall. Total net-integral of profile = 3123

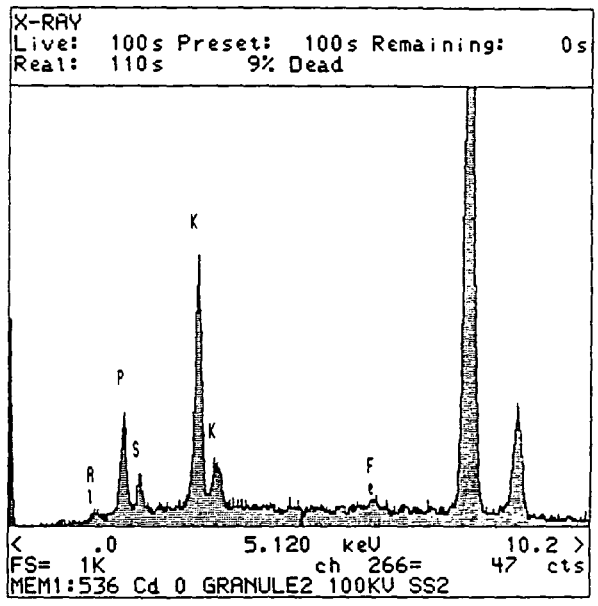
Element	% contribution	element	% contribution
Al	21.30	K	53.93
S	17.45	Fe	4.95
Cl	2.37		

d) cytoplasm. Total net-integral of profile = 1392

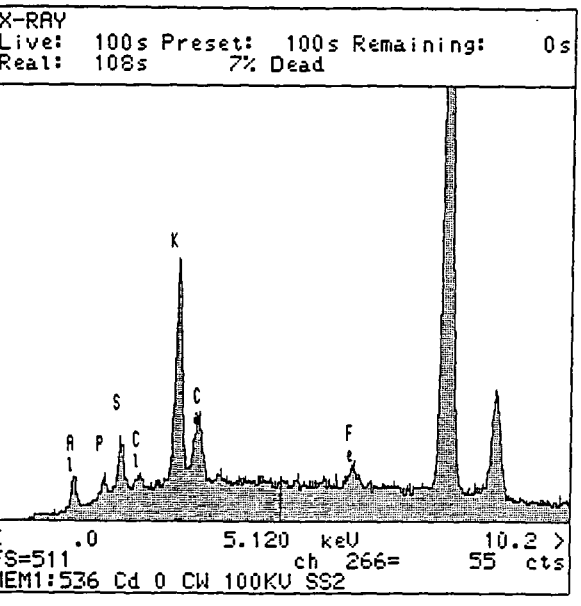
Element	% contribution	element	% contribution
Al	30.98	K	43.43
P	6.11	Ca	7.80
S	9.26	Fe	1.86
Cl	0.56		

b)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 116s 14% Dead
P
K
S
Ca
Cl
Na
Mg
Al
Ca
Fe
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 75 cts
MEM1:536 Cd 0 GRANULE1 100KV SS2


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 110s 9% Dead
K
P
S
K
Al
Fe
< .0 5.120 keV 10.2 >
FS= 1K ch 266= 47 cts
MEM1:536 Cd 0 GRANULE2 100KV SS2

c)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 108s 7% Dead
K
S
Ca
Al
P
Cl
Fe
.0 5.120 keV 10.2 >
FS=511 ch 266= 55 cts
MEM1:536 Cd 0 CW 100KV SS2

d)

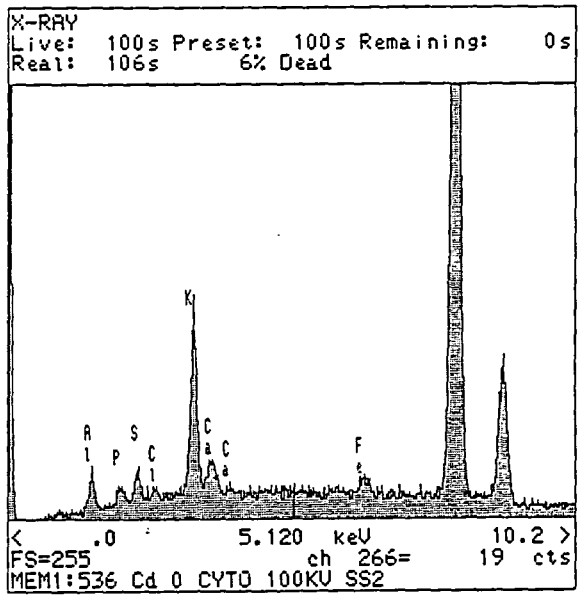

X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 106s 6% Dead
K
Al
P
S
Cl
Ca
Ca
Fe
< .0 5.120 keV 10.2 >
FS=255 ch 266= 19 cts
MEM1:536 Cd 0 CYTO 100KV SS2

Fig. 6.4 EDXMA profiles of Klebsormidium D537 grown without Cd.

a) inclusion body. Total net-integral of profile = 50870

Element	% contribution	element	% contribution
Na	8.8	S	3.97
Mg	4.32	Cl	42.41
Al	3.19	K	28.11
P	5.86	Fe	3.35

b) PP body. Total net-integral of profile = 32039

Element	% contribution	element	% contribution
Mg	19.24	Cl	0.16
P	52.91	K	23.03
S	3.79	Fe	0.87

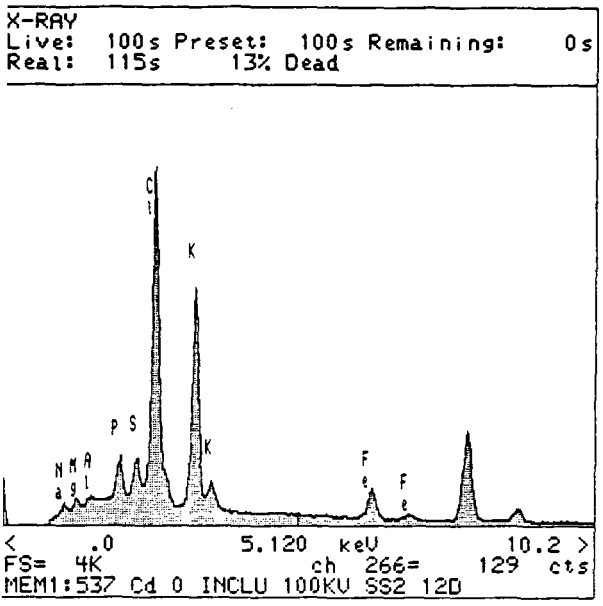
c) cell wall. Total net-integral of profile = 27016

Element	% contribution	element	% contribution
Mg	9.92	Cl	3.08
Al	1.81	K	42.99
P	26.29	Fe	0.85
S	15.06		

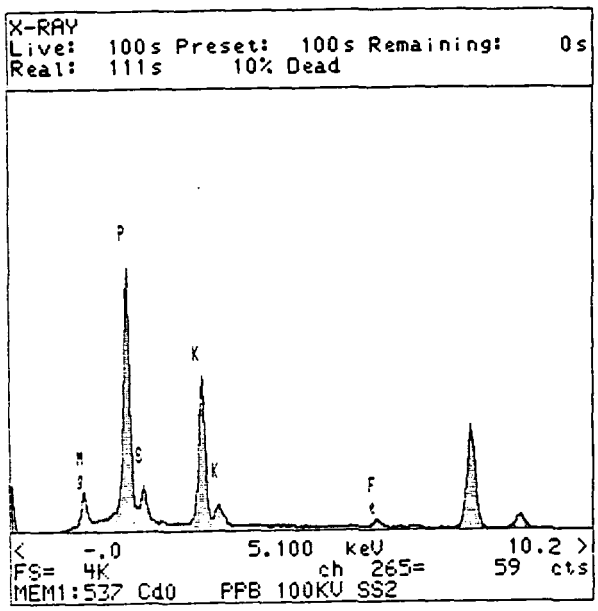
d) cytoplasm. Total net-integral of profile = 20190

Element	% contribution	element	% contribution
Na	6.19	S	4.70
Mg	5.82	Cl	42.94
Al	3.20	K	28.98
P	7.23	Fe	0.94

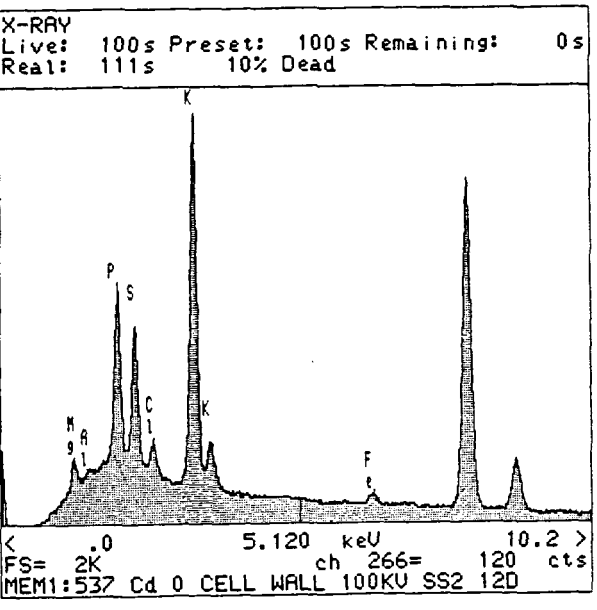
a)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 115s 13% Dead
Cl
K
P S
K
F
F
Mg
Al
< .0 5.120 keV 10.2 >
FS= 4K ch 266= 129 cts
MEM1:537 Cd 0 INCLU 100KV SS2 12D

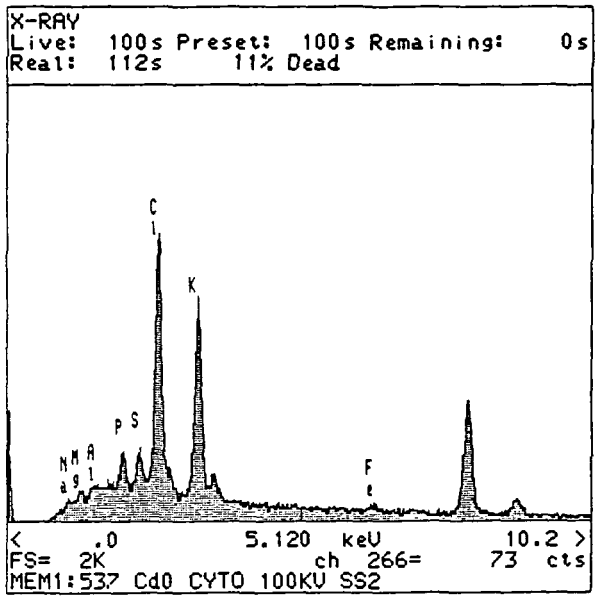
b)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 111s 10% Dead
P
K
Mg
S
K
F
< -.0 5.100 keV 10.2 >
FS= 4K ch 265= 59 cts
MEM1:537 Cd0 PPB 100KV SS2

c)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 111s 10% Dead
K
P
S
Cl
K
Mg
Al
F
< .0 5.120 keV 10.2 >
FS= 2K ch 266= 120 cts
MEM1:537 Cd 0 CELL WALL 100KV SS2 12D

d)


X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 112s 11% Dead
Cl
K
P S
Mg
Al
F
< .0 5.120 keV 10.2 >
FS= 2K ch 266= 73 cts
MEM1:537 Cd0 CYTO 100KV SS2

Appendix 7 : Ultraviolet protein assay for SDS PAGE samples, relating BSA concentration with absorbance at 280 nm

BSA concentration (mg ml^{-1})	absorbance at 280 nm	BSA concentration (mg ml^{-1})	absorbance at 280 nm
5.00	2.810	0.25	0.226
2.50	1.362	0.10	0.093
1.00	0.607	0.05	0.022
0.50	0.308	0.025	0.010

Regression line : $Y = 0.5544 X + 0.02362$ $r^2 = 0.9993$

Appendix 8 : Typical agarose-gel Mr-marker calibration relating the number of base pairs of each DNA fragment with the distance migrated, the regression line has been calculated with the $\log_{10}$ values

Base pairs	$\log_{10}$ base pairs	migration distance (mm)
21226	4.32686	10
5148 / 4973	3.71164	16.5
4268	3.63022	18
3530	3.54778	19
2027	3.30685	24.5
1904	3.27967	25.5
1709	3.23274	27
1375	3.13830	29
947	2.97635	33
831	2.91960	34

Regression line : $Y = - 17.9844 X + 84.9229$ $r^2 = - 0.9763$

Appenidx 9 : Protein composition of the standards used to calibrate SDS PAGE runs

Protein	Mr (kD)
Albumin, bovine	66
Albumin, egg	45
Glyceraldehyde 3-P dehydrogenase	36
Carbonic anhydrase	29
Trypsinogen	24
Trypsin inhibitor	20.1
α-lactalbumin	14.2