SPIRULINA AS A BIOREMEDIATION AGENT: INTERACTION WITH METALS AND INVOLVEMENT OF CARBONIC ANHYDRASE

A thesis submitted in fulfilment of the
requirements for the degree of

MASTER OF SCIENCE

in the
Department of Biochemistry and Microbiology
Rhodes University

by

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JANUARY 2000

This thesis is dedicated to

my parents

Stuart & Daphne Payne

ABSTRACT

Heavy metal contamination from mining and other industrial operations is becoming an increasing problem with regards to the depleting water resources in South Africa. This study involved the investigation of the use of an algal biomass as a possible alternative to the traditional chemical means of removing these metals.

When the toxic effects of metals were investigated, *Spirulina* was found to have a threshold level of about 30 µM for copper, zinc and lead. Copper and zinc appeared to have a direct effect on the photosynthetic pathway, thereby causing a rapid decline in cell growth. Lead on the other hand seemed to affect surface properties and hence took longer to cause deterioration in growth.

Although relatively low concentrations of metal may have a toxic effect on the cyanobacterium, *Spirulina* may have potential as a precipitation agent. The role of *Spirulina* in the precipitation of heavy metals appears to be through its ability to maintain a high pH in the surrounding medium, possibly through the enzyme carbonic anhydrase. Subsequent studies therefore focused on the assay and isolation of this enzyme.

Two different radiotracer assays, in which carbonic anhydrase converts radiolabelled bicarbonate to carbon dioxide, were investigated, but were found to have several problems. Results were insensitive and could not be reproduced. The standard Wilbur-Anderson method subsequently investigated also proved to be insensitive with a tremendous degree of variability. Although not quantitative, SDS-PAGE proved to be the most reliable method of detection, and was therefore used in subsequent procedures.

Chlamydomonas reinhardtii was the subject of initial enzyme isolation studies as these procedures are well documented. Although the published protocols proved unsuccessful, affinity chromatography of a membrane stock solution from *Chlamydomonas reinhardtii* yielded two relatively pure protein bands. These bands

were presumed to represent two subunits of carbonic anhydrase, although Western blot analysis would be required to confirm their identity. Purification of carbonic anhydrase from *Spirulina*, however, proved unsuccessful and results obtained were very inconclusive. Hence, further analysis of *Spirulina* is required.

The possibility of cloning CA from a genomic library was also considered, but suitable primers could not be designed from the aligned sequences.

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ACKNOWLEDGEMENTS

I would like to express my appreciation and gratitude to the following people for their input and support during my post-graduate studies:

- In particular I would like to thank Professor J. Duncan for his supervision of this project, and his continual guidance and encouragement.
- To my colleagues, Rob van Hille, Paula Antunes, Doug Sanyahumbi, Lynne Wallace and Denis Thompson, for their advice, encouragement and friendship throughout this project.
- To the other post-graduates and staff members of the department of Biochemistry and Microbiology for their generous support and assistance whenever necessary, particularly Clint Boyd, for his continual advice, support and friendship.
- To my colleagues, digsmates and friends Arno Venter and Carol Hartley, for all the good times we've shared, as well as for their continual encouragement, valuable input and advice.
- To Robin Cross and Shirley Pinchuck for their assistance and patience with the electron microscopy.
- To my family, for all they have sacrificed in order for me to continue this far with my studies, and for their loving support and encouragement throughout.
- To Terry Meinzer, for the important role he has played in my life over the last year, and for his moral support, encouragement, patience and understanding.
- Last, but not least, financial support for the project from the Water Research Commission and the National Research Foundation is gratefully acknowledged.

ABBREVIATIONS

AA	Atomic absorption
APS	Ammonium persulphate
BSA	Bovine serum albumin
CA	Carbonic anhydrase
Ca	Chlorophyll *a*
Cx + c	Xanthophylls + Carotenoids
CCM	Carbon concentrating mechanism
C_I	Inorganic carbon
DPM	Disintegrations per minute
EPPS	4-(2-hydroxyethyl)-1-piperazinepropanesulphonc acid
EZA	Ethoxyzolamide
HCA	Human carbonic anhydrase
IgG	Immunoglobulin G
MWCO	Molecular weight cut off
$NaC_2H_3O_2$	Sodium acetate
$NaClO_4$	Sodium perchlorate
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PVDF	Polyvinylidene difluoride
RACE	5'-rapid amplification of cDNA ends
Rubisco	Ribulose biphosphate carboxylase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
TEMED	N,N,N',N'-Tetramethylethylenediamine
TTBS	20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5
WAU	Wilbur-Anderson unit

CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

South Africa is a country rich in natural resources, with the critical exception of fresh water. This fresh water is essential to life, social development and economic progress. The country's average annual rainfall of 497 mm is well below the world average of 860 mm. Only a comparatively narrow region along the eastern and southern coastlines experience rainy conditions, while 65 % of the country receives less than 500 mm of rain annually. This is usually regarded as the minimum for successful dry-land farming. Twenty-one percent receives less than 200 mm. In addition, high evaporation rates result in the runoff-to-rainfall ratio being amongst the lowest for any populated region on earth (Department of Water Affairs, 1986; Kidd, 1997).

Coupled to the natural scarcity of water, is a simultaneous increase in human demand for water resources. This has arisen from the growth of the population and economy, as well as rising standards of living. In addition, the scarce, unutilised supplies are geographically mismatched in relation to demand. Water quality is also rapidly deteriorating due to increasing development. It has been estimated, from present growth rates of population and industry, that by the year 2020 the demand for potable water in South Africa will exceed supply. (Department of Water Affairs, 1986; Kidd, 1997).

Heavy metal ions are a major source of water contamination, and increasing levels are being released into local water supplies via effluents from industrial, military and mining sites. Metallic species released into the environment tend to persist indefinitely, eventually accumulating throughout the food chain, thus posing a serious threat to the environment, animals and humans. Not only are these effluents highly toxic but, in the case of the mining industry, valuable metals are being lost (Aderhold

et al, 1996; Bakkaloglu *et al*, 1998; Brady & Duncan, 1994a; Corder & Reeves, 1994; Ford & Mitchell, 1992; Gadd & White, 1993; Kapoor & Viraraghavan, 1995; Nourbakhsh *et al*, 1994; Puranik *et al*, 1999; Rivoallan *et al*, 1994; Roy *et al*, 1993; Srivastava & Srivastava, 1990; Volesky, 1987; Volesky & Holan, 1995; Wilde & Benemann, 1993; Wilhelmi & Duncan, 1995; Zielinski *et al*, 1998).

Removal of these metal ions and recycling of the water is consequently vital in order to avoid significant contamination of watercourses and loss of potential resources.

Traditional technologies for the removal of heavy metals from water, such as ion exchange or lime precipitation have proved ineffective and/or very expensive, especially when the metals are in the lower concentration ranges. New technologies are required that can reduce heavy metal concentrations to environmentally acceptable levels at affordable costs. Biotechnology based processes have the potential to contribute significantly to the achievement of this goal (Bakkaloglu *et al*, 1998; Çetinkaya Dönmez *et al*, 1999; Corder & Reeves, 1994; Fehrmann & Pohl, 1993; Kapoor & Viraraghavan, 1995; Kapoor & Viraraghavan, 1998; Özer *et al*, 1997; Tsezos *et al*,1996; Volesky, 1987; Wehrheim & Wettern, 1994; Wilde & Benemann, 1993).

1.2 REMOVAL OF HEAVY METALS BY BIOSORPTION

Metal deposition by microorganisms is of great importance in biogeochemical cycles, for example, microfossil and mineral formation, iron and manganese deposition, and uranium and silver mineralisation. Biotechnological approaches to the abatement of toxic metal pollution consist of selectively using and enhancing these natural processes to treat particular wastes. The processes by which microorganisms interact with toxic metals are very diverse (Figure1.1). In practice however, there are three general categories of biotechnological processes for treating liquid wastes containing heavy metals; biosorption, extracellular precipitation, and uptake by purified biopolymers and other specialist molecules derived from microbial cells. These processes are not exclusive and several physico-chemical processes may be involved.

To date, the most successful biotechnological processes utilise biosorption and bioprecipitation (Gadd & White, 1993; Gadd 1988; Volesky, 1986; Volesky 1990).

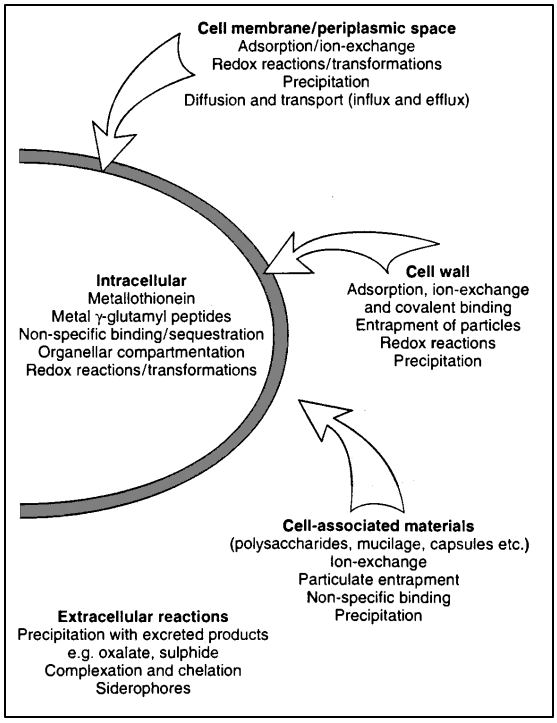


Figure 1.1: Processes contributing to microbial uptake and detoxification of toxic metals (Gadd & White, 1993)

Biosorption has been defined as the property of certain types of microbial biomass (living or dead) to bind and concentrate heavy metals (Gadd & White, 1993; Volesky, 1992). Biosorption depends not only on the chemical composition of the cell or its components such as the cell wall, but also on external physico-chemical factors and the solution chemistry of the metal. A combination of mechanisms may be involved in biosorption which include: Particulate ingestion or entrapment by flagellae or extracellular filaments, active transport of ions, ion exchange, complexation, adsorption, inorganic precipitation, co-ordination and chelation. While the first two mechanisms are associated with living cells, the latter mechanisms have been reported for living and dead microorganisms, as well as cellular debris. The sequestered metals may be found anywhere from extracellular polysaccharides to cytoplasmic granules, depending on the microbial species and/or the mechanism of metal deposition within the cell (Harris & Ramelow, 1990; Schiewer & Volesky 1996; Volesky, 1986; Volesky 1987; Volesky, 1990).

The major processes currently used to treat metal-containing solutions include the addition of chemicals for precipitation of metals and the use of ion exchange resins to bind the metals to a substrate. Other less frequently used processes include activated carbon adsorption, electrodialysis and reverse osmosis (Vilchez *et al*, 1997; Wilde & Benemann, 1993).

Biosorption is superior to precipitation in terms of ability to adjust to changes in pH and heavy metal concentrations, and superior to ion exchange in terms of sensitivity to the presence of suspended solids, organics and the presence of other heavy metals. In addition, only ion exchange can compete with biosorption in terms of residual heavy metal concentration. Overall, the available data shows that biosorption has several potential advantages over conventional methods of metal removal (Wilde & Benemann, 1993). These include:

- the use of naturally abundant, renewable biomaterials that can be cheaply produced
- the ability to treat large volumes of waste-water due to rapid kinetics
- high selectivity in terms of removal and recovery of specific heavy metals
- the ability to handle multiple heavy metals and mixed waste
- high affinity, reducing residual metals to below 1ppb in many cases
- less need for additional expensive process reagents which typically cause disposal and space problems
- operation over a wide range of physico-chemical conditions including temperature, pH and presence of other ions (including Ca^{2+} and Mg^{2+})
- relatively low capital investment and low operating costs
- greatly improved recovery of bound heavy metals from biomass
- greatly reduced volume of hazardous waste produced

The initial concept that very cheap biosorbents could be used as non-specific treatment agents has hence been revised as it has been shown that biosorbents not only have very high uptake capacities, but also that uptake can be metal selective.

Consequently, biosorbents are now being considered as replacements for ion exchangers or other metal extraction and concentration operations in 'upstream' metal recovery. In addition, if a cheap biosorbent is used it is not necessary to desorb the metal as the biomass can be incinerated yielding a high concentration of metal in the ash (Volesky, 1987).

Volesky (1987) has considered at least four broad areas of application for new biosorbent materials:

- detoxification of metal-bearing waste-waters
- decontamination of radioactive waste-waters
- recovery of metals from ore processing solutions
- concentration/recovery of strategic/rare metals from sea water

1.2.1 MECHANISMS OF MICROBIAL METAL ACCUMULATION

Due to the complex structure of a microorganism, uptake capacities and mechanisms may vary widely. Although both living and dead cells are capable of metal accumulation, there may be considerable differences in the mechanisms involved in either case (Gadd, 1988; Gadd, 1990; Veglio & Beolchini, 1997).

Microbial metal uptake is often divided into two main phases. An initial, rapid phase, which can also occur in dead cells, is metabolism-independent binding or adsorption to cell walls or other external surfaces. The second, slower phase is metabolism-dependent transport across the cell membrane. This only occurs in living cells, and may be accompanied by toxic symptoms. In some cases intracellular uptake is due to increased membrane permeability arising from toxic interactions. With some metals such as lead, uranium and thorium, most accumulation in microbial biomass is surface-based with little, or no intracellular uptake, unless by diffusion. Once inside the cells, metal ions may be preferentially located within specific organelles and/or bound to proteins such as metallothionein. In growing cultures, metabolism-independent and –dependent phases of metal uptake can be affected by changes in the

medium composition and excretion of metabolites that can act as metal chelators. Thus in a given microbial system, several mechanisms of uptake may operate simultaneously and/or sequentially (Gadd, 1988; Gadd, 1990; Garnham *et al*, 1992; Veglio & Beolchini, 1997; Vilchez *et al*, 1997).

1.2.1.1 METABOLISM -INDEPENDENT BIOSORPTION

The term 'adsorption' is often used to describe metabolism-independent uptake or binding of heavy metals to microbial cell walls and other extracellular surfaces that occur in living or dead cells (Gadd, 1988). These mechanisms vary, and any one or a combination may be operational, including; ion exchange, physical adsorption, complexation and microprecipitation. Metabolism-independent biosorption is relatively rapid, and can be reversible. In the presence of such a mechanism, which fortunately is the most common, biomass has all the characteristics of an ion exchange resin or an activated carbon, implying many advantages in the industrial application of biosorption (Veglio & Beolchini, 1997; Volesky, 1987).

Cell walls of microbial biomass, mainly composed of polysaccharides, proteins and lipids, offer particularly abundant metal-binding functional groups. These include carboxylate, hydroxyl, sulphate, phosphate and amino groups, which can all be active to various degrees in immobilising the metal (Kuyucak & Volesky, 1988; Veglio & Beolchini, 1997; Volesky, 1987).

Phenomena associated with the presence of van der Waals' forces are included in the category of physical adsorption (Veglio & Beolchini, 1997). Kuyucak and Volesky (1988) hypothesised that uranium, cadmium, zinc, copper and cobalt biosorption by the dead biomass of algae, fungi and yeast takes place through electrostatic interactions between ions in solution and cell walls. Electrostatic interactions have been demonstrated to be responsible for copper biosorption by bacterium *Zoogloea ramigera* and the alga *Chlorella vulgaris* (Aksu *et al*, 1992), for chromium biosorption by fungi *Ganoderma lucidum* and *Aspergillus niger* (Venkobachar, 1990), as well as for cadmium biosorption by marine algae (Holan *et al*, 1993). Physical

adsorption is furthermore responsible for copper, nickel, zinc, cadmium and lead biosorption by *Rhizopus arrhizus* (Fourest & Roux, 1992; Zhou & Kiff, 1991).

Cell walls of both prokaryotes and eukaryotes contain polysaccharides as basic building blocks. The ion exchange properties of natural polysaccharides have been studied in detail and it is well established that bivalent metal ions exchange with counter ions of polysaccharides such as alginic acid. Ion exchange is probably involved to a large degree in metal sequestering by algal biomass (Tsezos & Volesky, 1981; Veglio & Beolchini, 1997; Volesky, 1987).

Metal removal from solution may also take place through complex formation on the cell surface after interaction between the metal and active groups. The metal ions may bind to single ligands, or through chelation (Cabral, 1992; Veglio & Beolchini, 1997). Complexation was found to be the only mechanism responsible for calcium, magnesium, cadmium, zinc, copper and mercury accumulation by *Pseudomonas syringae* (Cabral, 1992).

1.2.1.2 METABOLISM -DEPENDENT BIOSORPTION

Metabolism-dependent uptake of metal ions is usually a slower process than 'adsorption', although larger quantities of metal may be accumulated by this mechanism in some organisms, such as yeasts. Low temperatures, the absence of an energy source, metabolic inhibitors, and uncouplers inhibit rates of uptake. Rates of intracellular uptake may also be influenced by the physiological state of the cells and the nature and composition of the growth medium (Gadd, 1988; Gadd 1990).

Many metals, such as copper, iron, zinc and cobalt, are essential for growth and metabolism, and organisms possess transport systems of varying specificity for their accumulation from the external environment. Non-essential metals may also be taken in via such systems. The metal transport system may become confused by the presence of metal ions of the same charge and ionic radius. For example, several bacteria, algae and fungi, appear to transport cadmium via a manganese system (Gadd, 1990; Veglio & Beolchini, 1997).

Most mechanisms of metal transport appear to rely on the electrochemical proton gradient ($\Delta\mu_{H^+}$) across the cell membrane. This has a chemical component, the pH gradient (Δ pH), and an electrical component, the membrane potential ($\Delta\Psi$), each of which can drive transport of ionised solutes across membranes. The membrane potential appears to be responsible for electrophoretic mono- and divalent cation transport in fungi, although other gradients, such as K^+, may also be involved (Gadd, 1990).

There are several examples where energy-dependent uptake may not be as significant a component of total uptake as general adsorption. This is particularly true for filamentous fungi and those organisms possessing extracellular polysaccharide, slime or mucilage, where high biosorptive capacities mask low rates of intracellular uptake (Gadd, 1988; Gadd, 1990).

There are some examples where intracellular uptake is not linked with metabolism, for instance, intracellular accumulation of uranium in *Synechococcus elongatus* and *Pseudomonas aeruginosa*, which leads to the formation of dense internal deposits (Horikoshi *et al*, 1979; Strandberg *et al*, 1981). In a more general sense, toxicity is obviously a consideration in heavy metal uptake by living cells. Wherever this leads to permeabilisation of cell membranes, a variety of intracellular binding sites will be exposed that are not available in intact cells (Gadd, 1988).

Metal resistance may often be associated with decreased uptake and/or impermeability. In addition those external factors which reduce uptake often result in reduced toxicity (Gadd, 1990) In contrast, there is an example of a Mn^{2+} resistant strain of *Saccharomyces cerevisiae* which accumulates considerably more Mn^{2+} than the sensitive parental strain, probably by more efficient internal sequestration (Bianchi *et al*, 1981).

1.2.1.3 EXTRACELLULAR PRECIPITATION

Precipitation of metal may take place both in solution, and on the cell surface. Furthermore, it may be dependent on the cells metabolism if, in the presence of toxic metals, the microorganism produces compounds which favour the precipitation process (Veglio & Beolchini, 1997). Scott and Palmer (1990) found that cadmium elimination from solution by some *Arthrobacter* and *Pseudomonas* species was determined by detoxification systems that precipitate cadmium on the cell surface. On the other hand, precipitation may not be dependent on the cells' metabolism, occurring after a chemical interaction between the metal and the cell surface. This phenomenon is the terminal step of uranium biosorption by *Rhizopus arrhizus* (Tsezos & Volesky, 1982) in which the formation of a uranium-chitin complex is followed by the complex hydrolysis and the precipitation of the hydrolysis product (uranylhydroxide) in the cell wall.

Cyanobacteria have been implicated in the precipitation of strontium calcite from groundwater discharge and celestite and strontianite. In general, mineral deposition by algae is most commonly silicate or carbonate in nature, a consequence of the formation of tests and frustules by diatoms and coccolithophorids (Lawrence *et al*, 1998). However, Mann and Fyfe (1984, 1985) have demonstrated the formation of uranium oxides in association with the cell wall of *Ankistrodesmus* species and other algae. Mann and Fyfe (1988) made similar observations for *Euglena* species.

Hydrogen sulphide production by sulphate-reducing bacteria can lead to precipitation of metal sulphides within and on cell surfaces (Gadd, 1988, Gadd, 1990; Gadd & White, 1993). Cadmium-adapted *Klebsiella aerogenes* contained substantial amounts of cadmium (2.4% of dry weight), which was proportional to the inorganic sulphide content of the cells. Large numbers of electron-dense cadmium sulphide granules occurred on outer surfaces of the cells (Aiking *et al*, 1984). Such sulphide precipitation can also occur in algae, yeasts and fungi. Metal precipitation as phosphates and oxalates has also been shown (Aiking *et al*, 1984; Gadd, 1988).

1.2.2 EVALUATION OF BIOSORBENTS

In order to evaluate biosorbent materials it is necessary to examine both metal uptake by the biosorbent as well as desorption from it. Data on the equilibria of these processes can be obtained from experimental biosorption isotherms similar to those used for the evaluation of the adsorption performance of activated carbons. In addition to equilibrium studies, the kinetics of the biosorption has to be determined in order to calculate the rate of metal uptake and release. Rapid uptake would provide a short contact time between solution and biosorbent. Both equilibrium and kinetic characterisation are crucial for quantitative assessment of the performance of the biosorbent as well as for process design (Volesky, 1987; Volesky & Holan, 1995).

The biosorption potential of microbial biomass depends on many aspects of its chemical composition, and metal uptake may vary widely between different microbial genera and even different strains within a species. The performance of a biosorbent may be affected by the nutrient status of the organism, age of the cells, availability of micronutrients and environmental conditions during the biosorption process, such as pH, temperature and the presence of certain co-ions. The efficiency of the biosorbent is also greatly influenced by the solution chemistry of the metal (Volesky, 1987; Volesky & Holan, 1995; Wilde & Benemann, 1993).

In the light of what has been discussed it is possible to compile the following list of criteria to which an 'ideal' biosorbent should conform:

- the uptake and release of the metal should be efficient and rapid
- the active biosorbent agent should be produced at low cost and should be reliable
- removal of the biosorbent from solution should be cheap, efficient and rapid
- the biosorbent should be metal-selective in order to separate single metals from a solution containing various metallic species
- separation of metal from the biosorbent should be metal-selective, economically feasible and loss of the solvent should be minimal

Research emphasis has largely been placed on the identification of biosorbents likely to be the most cost effective. A wide variety of novel biomaterials have been investigated for the potential use in metal bioremediation. These include, sawdust preparations from pine and cedar for nickel removal (Chatterjee *et al*, 1996), sphagnum moss peat for the removal of hexavalent chromium (Sharma & Forster, 1993), and crab shell particles for lead removal (M.Y Lee *et al*, 1998). Apple residues have also been investigated for the removal of copper, lead and cadmium (S.H Lee *et al*, 1998), and rice hulls, an unusable by-product from commercial rice harvesting, were suggested by Roy and associates (1993). Krishnan and colleagues (1987) even investigated the use of human hair cuttings for the adsorption of cadmium, arsenic and mercury.

Biosorption studies have, however, typically dealt with microbial biomass such as bacteria, yeast, fungi and algae. The biosorbents investigated are often waste products of industrial fermentation processes, which provide large quantities of biomass at a low cost (Bakkaloglu *et al*, 1998). The present research group has focused considerable attention on the biosorption of metals by a commercial strain of *Saccharomyces cerevisiae* and waste yeast obtained from the breweries post fermentation (Brady & Duncan, 1994a, 1994b, 1994c; Brady *et al*, 1994a, 1994b; Duncan *et al*, 1997; Wilhelmi & Duncan, 1995). Marine algal biomass types available in abundance in the oceans have shown high metal removal capacities (Holan & Volesky, 1994; Bakkaloglu *et al*, 1998). Microorganisms may also be selected from their natural environments (usually metal contaminated sites) for their specific metal removal capabilities, which may allow high and selective recovery of target metals (Volesky & Holan, 1995).

Azolla filiculoides, is a free-floating and fast growing water fern that is capable of colonising nitrogen deficient waters. Not only is it an environmental waste product, but it also presents a problem in freshwater as it forms complete coverage on ponds and dams in some areas, hence exerting a negative effect on the oxygen content of the water and harming the aquatic ecology. In considering a solution to this environmental problem, Zhao and Duncan (1997a; 1997b; 1998a; 1998b) have

investigated *A. filiculoides* as a possible low cost biosorbent for the removal of hexavalent chromium, nickel and zinc from contaminated wastewater.

Metal uptake by many different biosorbents has been reported extensively, however, comparison of results between different laboratories is difficult because of the many variables involved and the inconsistency of interpretations. The superiority of microalgae over yeast, bacterial and fungal biomass for bioremoval has not been established, as there are few comparative or comparable studies. Although considerable research has been conducted on bacteria, and fungi (Avery & Tobin, 1993; Bakkaloglu *et al*, 1998; Beveridge, 1986; Brady & Tobin, 1995; Fourest & Roux, 1992; Kapoor & Viraraghavan, 1995; Kapoor & Viraraghavan, 1998; Nourbakhsh *et al*, 1994; Özer *et al*, 1997; Puranik *et al*, 1999; Tsezos *et al*, 1996), there is a very large data base on heavy metal accumulation by microalgae. As this research project deals with the blue-green alga, *Spirulina*, the following section will be a review restricted to the bioremoval of metals using algae. Both unicellular microalgae and cyanobacteria (formerly known as the blue-green algae) will be considered.

1.3 ALGAL ACCUMULATION OF HEAVY METALS

Many authors (de Wet *et al*, 1990; Gale & Wixon, 1978; Mann & Fyfe, 1988) have noted that plant life in the vicinity of mining and industrial operations accumulate heavy metals. Algae and other aquatic plants have for many years helped to prevent the ubiquitous exposure of natural ecosystems to excessive toxins by binding, localising and sedimenting heavy metals (Whitton & Say, 1975).

Microalgae demonstrate the presence of very high affinity metal binding sites as they are capable of accumulating metal ions from very dilute (parts per trillion) solutions. They are able to sequester heavy metal ions by the same adsorption and absorption mechanisms as other microbial biomass, in addition to the formation of polychelatins which they synthesise in response to toxic heavy metal stress (Gekeler *et al*,1988; Wilde & Benemann, 1993).

Microalgae are among the most prolific producers of plant biomass, having photosynthetic efficiencies broadly similar to those of higher plants. In addition, they do not require organic substrates or electron acceptors, and hence the large scale culture of microalgae is theoretically, simpler and cheaper than that of bacteria and fungi (Çetinkaya Dönmez *et al*, 1999; Kerby & Stewart, 1988; Wilde & Benemann, 1993).

Microalgae can also be cultivated in open ponds or in large scale laboratory cultures, providing a reliable and consistent supply of biomass for biosorption studies and eventual scale up work (Çetinkaya Dönmez *et al*, 1999; Wilde & Benemann, 1993). However, the mass culture of algae in outdoor pond systems requires extensive land use, depends on sunlight and climate, has large requirements for nutrients and water, and is threatened by contamination and invasion by weeds (Borowitzka & Borowitzka, 1990; Kerby & Stewart, 1988). Species best suited to open pond cultivation are those which have rapid growth rates or those which can withstand environmental extremes such as high pH, high salinity or high temperatures. Species of *Chlorella*, *Scenedesmus*, *Spirulina* and *Dunaliella* are examples presently cultivated in open pond systems (Borowitzka & Borowitzka, 1990; Kerby & Stewart, 1988).

Thousands of algal species have been identified during the last two centuries, but very few have been investigated for their biosorption potential. In the few limited studies where species have been compared, results have often revealed major differences in metal binding efficiency between species, and even between strains of a single species, for any given metal and/or set of physico-chemical conditions. It is also apparent that algae and other microbes are much more efficient at removing some metal ions than others. For example, >95% Cd^{2+} removal is commonly reported, while Cr^{6+} is typically removed at low percentages and is considered one of the most difficult metals to remove using biomass. The medium the algae is grown in, and the age or growth phase of the culture also appear to be significant factors influencing metal binding efficiency. The presence of other ions, especially cations such as H^{+} and Ca^{2+} and other trace metals will also influence biosorption efficiency (Çetinkaya Dönmez *et al*, 1999; Stokes, 1983; Wilde & Benemann, 1993).

The vast majority of biosorption studies involving algae have been conducted using unicellular green algae, principally *Chlorella vulgaris* as it is relatively easy to grow in laboratory culture and it is readily attainable from numerous culture collections (Wilde & Benemann, 1993). *C. vulgaris* also offers a range of potential binding sites for metal ions which differ in affinity and specificity. Binding sites include sulphydral and amine groups and examples of 'soft' ligands which undergo covalent bonding to 'soft' metal ions such as Ag^{+} (Cordery *et al*, 1994). The research groups of Cordery *et al* (1994) and Harris and Ramelow (1990) have found *C. vulgaris* to have a particularly high sorptive capacity for Ag^{+} ions over a wide pH range. Nakajima *et al* (1981) have shown that *Chlorella* has the ability to accumulate heavy metal ions selectively, as uranyl ions were far more readily taken up than copper and cadmium ions. They also suggested that this selectivity is mainly due to the strength of coupling between heavy metal ions and cell components, especially proteins. Pempkowiak and Kosokowska (1998) showed that humic substances isolated from seawater decreased the accumulation of cadmium by *C. vulgaris*. Darnall and associates (1986) have shown that *C. vulgaris* is also capable of binding mercury, silver and gold (III), although the pH dependence of the binding is different to that of other metals.

A few authors have investigated biosorption from multi-component systems. Sag and colleagues (1998) demonstrated that the combined effect of Chromium (VI) and Iron (III) ions on *C. vulgaris* was antagonistic, since the initial biosorption rates and equilibrium metal removal decreased with increasing concentration of the other metal ion. In contrast, with cadmium and zinc as the two-metal system, the presence of the second metal ion does not appreciably affect the instantaneous uptake of the other (Ting *et al*, 1991). Aksu and Açikel (1999) have also shown that *C. vulgaris* biomass offers a practical approach for removing of mixtures of copper (II) and chromium (VI) from waste waters containing mainly these two components.

A recent investigation by Brady and collaborators (1994c) revealed that *Scenedesmus*, *Selenastrum* and *Chlorella* species were capable of accumulating metals such as copper, lead, and chromium with 67 to 98% efficiency. These species have also been shown to accumulate significant amounts of uranium (Muraleedharan *et al*, 1991).

Little work has been done exclusively with cyanobacteria as metal-binding biosorbents. One recent report (Ahuja *et al,* 1999) showed *Oscillatoria anguistissima* to have a very high capacity for zinc ions. Results were comparable to the commercial ion-exchange resin IRA-400C, and biosorption was rapid, pH dependent and temperature independent. Singh and associates (1992) studied the effect of population size, and complexing agents on copper uptake by free and immobilised cyanobacterium *Nostoc calcicola.* Copper uptake was found to be regulated by population size and immobilised cells had greater longevity. Almost identical patterns of copper uptake were found for free and immobilised cells, suggesting that complexing agents exhibit the same type of regulation.

Synthetic and natural complexing agents are known to regulate metal toxicity to algae and other plants. Certain specific peptides and proteins, which bind metals, could become over expressed in microorganisms exposed to heavy metals (Vilchez *et al,* 1997). For example, Humble and collaborators (1997) have shown significant binding of copper and zinc to three microcystins, which are released by cyanobacteria in response to heavy metal stress. Similar observations exist for other cyanobacteria. Mcknight and Morel (1979) found that four species of cyanobacteria excreted strong copper-complexing agents, which they later (1980) concluded to be siderophores. Cell exudates were also shown to decrease adsorption of copper, cadmium and nickel by cyanobacteria (Laube *et al,* 1980) as well as cadmium uptake by *Anacystis nidulans* (Singh and Yadava, 1985).

Chlorella and the alga *Spirulina* are both produced commercially in large quantities, primarily for use in the health food market. Due to their availability, they have frequently been used in bioremoval studies and have been shown to remove a variety of metals from solution including Cu^{2+}, Pb^{2+}, Zn^{2+} and Au^{3+} (Gadd & White, 1993; Vilchez *et al,* 1997; Wilde & Benemann, 1993). *Chlorella* and *Spirulina* are both used in AlgaSORB^{TM}, a bioremoval product developed by Darnall and collaborators (1986) and produced by Biorecovery Inc. of New Mexico.

There appear to be two principal mechanisms in the reversible surface binding of heavy metal ions to algae. The first is ion exchange where ions such as Na^{+}, Mg^{2+},

and Ca^{2+} become displaced by heavy metal ions. The second is complexation between metal ions and various functional groups that can interact with the metal ions in a co-ordinated manner (Wilde & Benemann, 1993). The relative importance of the different functional groups is dependent, at least to some degree, on the metal in question. Gardea-Torresdey and colleagues (1990) have shown that carbonyl groups on five different algal species are responsible for a great portion of Cu^{2+} and Al^{2+} binding, and that carboxyl groups play an inhibitory role in Au^{3+} binding.

Microalgae, like other microbes, require a number of metals as trace nutrients, since they are part of the active sites of essential enzymes. Thus, they have evolved highly efficient mechanisms for recovering specific metal ions, often from very low concentrations. Due to their specificity, these uptake systems can act even in the presence of other ions, which interfere with fewer specifics binding sites. Furthermore, the number of binding sites for trace metals increases in response to a limitation in these trace elements, increasing the rate of uptake into the cell (Morel *et al*, 1991).

Non-essential toxic heavy metals can also be taken up by the systems evolved for essential trace nutrients. Singh and Yadava have shown that in *Anacystis* Cd^{2+} is taken up by the Zn^{2+}transporter and in *Thalassiosira weissflogii* Cd^{2+}can actually substitute for Zn^{2+} (Price and Morel, 1990). It has also been shown that Cd^{2+} shares a common transport system with Mn^{2+} in *Chlorella pyrenoidosa* (Hart *et al*, 1979) and Ting and colleagues (1991) found that in *Chlorella* Cd^{2+} and Zn^{2+} bind to different components of the cell wall. Thus it can be seen that there is a great deal of variability in the active uptake systems.

Microalgae bioremoval technologies are still being developed and much more work is required. Some practical applications have been achieved, and the fundamentals look promising. Microalgae have the potential to remove metal ions to very low concentrations, to grow on light energy, and to accumulate large amounts of specific toxic elements. They appear to function well even in the presence of other ions, in particular Ca^{2+} and Mg^{2+}, and organics. Only future research and the discipline of the market place will determine their role in the clean up of the environment.

1.3.1 *SPIRULINA* AS A POTENTIAL BIOREMEDIATION AGENT

Spirulina is currently being investigated as a potential bioremediation agent for the removal of heavy metals from mining and other industrial effluents. An initial aim of this research was to determine the threshold levels of *Spirulina* for toxic metals, as it was an attractive possibility that *Spirulina* would be capable of binding heavy metal ions and could be used for biosorption of heavy metals form industrial waste-water. However, these studies, discussed in chapter two, have indicated that *Spirulina* has a very low capacity for binding metal ions, as well as a very low tolerance for heavy metals. *Spirulina* does however show considerable potential as a precipitation agent as it is able to maintain a high pH in the surrounding medium. This is thought to be due to the enzyme carbonic anhydrase (CA) which catalyses the interconversion of dissolved carbon dioxide and bicarbonate ions ($CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$). In the algal system extracellular bicarbonate is converted into CO_2 and hydroxide. The CO_2 is actively taken up by the algae and incorporated into the photosynthetic pathway, leaving the hydroxide in solution. The net result is an increase in pH, which leads to metal precipitation (Haglund *et al,* 1992; Raven, 1995; Shirawa *et al,* 1993). Due to the possible involvement of CA in bioremediation systems, the focus of this research became redirected towards the investigation of this enzyme. The following section is a review of the literature on CA.

1.4 CARBONIC ANHYDRASE

Carbonic anhydrase (CA; carbonate hydrolyase; carbonic dehydratase) is a zinc-containing metalloenzyme that catalyses the reversible interconversion of CO_2 and HCO_3^- with a maximum turnover number in excess of 10^6/s (Atkins *et al,* 1972a; Atkins *et al,* 1972b; Bowes, 1969; Braus-Stromeyer *et al,* 1997; Dionisio-Sese & Miyachi, 1992; Eriksson *et al,* 1996; Hiltonen *et al,* 1998; Jebanathirajah & Coleman, 1998; Johansson & Forsman, 1993; Johansson & Forsman, 1994; Karlsson, 1998; Lindskog, 1997; So & Espie, 1998; Sültemeyer, 1998; Tashian, 1989). CA is a ubiquitous enzyme that has been shown to occur in many organisms including animals, plants, eubacteria, archaebacteria and viruses. However, no fungal CA has yet been described (Eriksson *et al,* 1996; Hiltonen *et al,* 1998; Lindskog, 1997;

Sültemeyer, 1998). CA has been implicated in multiple, and diverse physiological processes, including pH homeostasis, facilitated diffusion of CO_2, interconversion of CO_2 and HCO_3^-, ion transport, photosynthesis, efficient virus replication, calcification, bone resorption, formation of aqueous humour and gastric juice, and the synthesis of urea, glucose and lipids (Braus-Stromeyer *et al*, 1997; Eriksson *et al*, 1996; So & Espie, 1998; Sültemeyer, 1998; Tashian, 1989).

The enzyme is classified into three independent CA gene families designated α, β and γ. Little sequence similarity occurs between the families indicating that the genes have evolved independently at least three times. The α-CAs are found primarily in animals, but homologues have also been identified in the bacterium *Neisseria gonorroae* and in the periplasmic space of the green algae *Chlamydomonas reinhardtii*. This is the most extensively studied CA family and includes the mammalian CA isozymes. Conversely the γ-CAs are a newly discovered gene family, with the enzyme from *Methanosarcina thermophila* being the only γ-CA isolated and characterised thus far. Related sequences have been found in several eubacteria and in Arabidopsis, but it is not yet known if they encode functional CAs. The β-CAs were first found in the stroma of higher plant chloroplasts, but have since been found in various eubacteria as well as in the mitochondria of *Chlamydomonas reinhardtii* (Eriksson *et al*, 1996; Hiltonen *et al*, 1998; Karlsson *et al*, 1998; Lindskog, 1997; Sültemeyer, 1998).

1.4.1 STRUCTURE OF CARBONIC ANHYDRASE

1.4.1.1 α-CARBONIC ANHYDRASES

There is extensive homology in the primary structures of mammalian CAs I, II and III, each having a molecular mass of approximately 29 kDa. Human CAs I and II show a 59 % identity in amino acids, while human isozymes I and III show 56 % identity (Tashian, 1989). In addition, the crystal structures have shown that the secondary and tertiary structures are also similar (Silverman, 1991).

The crystal structures of human CA I (HCA I) and II, bovine CA III, and a truncated form of murine CA V, expressed in *E. coli*, have been determined, and were seen to

be very similar. They are ellipsoidal molecules of approximately 5 x 4 x 4 nm^3, which could be considered one-domain proteins, were it not for the loosely connected amino terminal region of about 24 amino acids. A 10-stranded, twisted β-sheet is the dominating secondary structure (Figure 1.2), which divides the molecule into two halves: The upper half includes the N-terminal helical region and the active site, while the lower half contains a large hydrophobic core. The 10 β-strands are connected by hairpin loops and some helices, and are all antiparallel, with the exception of two pairs of parallel strands. A few relatively short helices are also present on the surface of the molecule (Freskgård *et al*, 1991; Hammarström *et al*, 1997; Lindskog, 1997).

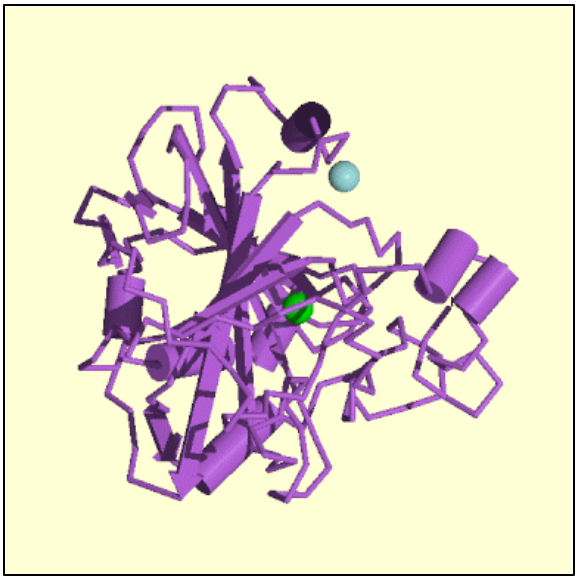


Figure 1.2: Secondary structure of HCA II. The zinc ion is shown as a green circle

The active site is located in a large, cone-shaped cavity that reaches almost to the centre of the molecule. The zinc ion is located at the base of the cleft (Figure 1.2), and is co-ordinated tetrahedrally to three imidazole rings. A water molecule, the ionisation of which is critical in the catalytic pathway, occupies the fourth co-ordination position. This water ligand is hydrogen bonded to the side chain of Thr^{199}, which in turn is hydrogen bonded to the side chain of Glu^{106}, residues conserved in all sequenced α-CAs. Additional histidyl side chains protrude into the cavity in the region in front of the metal ion, the number and position depending on the isozyme in question (Johansson & Forsman, 1994; Silverman, 1991; Wells *et al*, 1975).

In addition to the zinc ligands, 17 amino acid residues are strictly conserved in all sequenced α-CAs. Some of these are important for catalytic activity, while others are involved in stabilising the protein structure. His-107 is conserved in all known sequences of α-carbonic anhydrases, except in CA from *N. gonorrhoeae,* which has Asn in this position. A His-107→Tyr mutation is one of the causes of HCA II deficiency, and when this mutant is produced in *E.coli* it is very unstable, but fully active when stabilised by bovine serum albumin (Lindskog, 1997).

1.4.1.2 β-CARBONIC ANHYDRASES

No crystal structures of β-CAs have yet been determined (Lindskog, 1997). They do, however, differ considerably from animal α-CAs in many respects. Most striking is the entirely different primary structure, making it impossible to identify any active site residues from sequence similarity assignments (Johansson & Forsman, 1993). HCA II contains only one cysteine residue per molecule, while bovine CA contains none (Cybulsky *et al,* 1979). The four sequenced plant CAs all have a higher cysteine content, and in the pea there are five cysteine residues per subunit (Johansson & Forsman, 1993). Spinach CA has been reported to contain 7 cysteine residues per subunit (Cybulsky *et al,* 1979).

Another difference is the oligomeric structure of β-CAs. Different values for the native molecular mass of CA from the pea and spinach have been reported. Most authors have proposed CA to be a hexamer (Silverman, 1991), but others have indicated that it is in fact an octamer (Cybulsky *et al,* 1979; Johansson & Forsman, 1993). This issue will, however, probably not be resolved until the crystal structure of a β-CA has been determined. Circular dichroic spectra measurements indicate that the secondary structures also differ, in that pea CA appears to have a higher content of α-helix than the α-CAs, which are composed primarily of β-sheets (Johansson & Forsman, 1993).

In addition, evidence from X-ray absorption spectroscopy and mutagenesis suggests that the zinc ion in the spinach enzyme has a Cys-His-Cys-H_2O co-ordination sphere, in contrast to the α-CAs (Lindskog, 1997).

1.4.1.3 γ- CARBONIC ANHYDRASES

Very recently, the structure of the γ-CA from *M. thermophila* was presented by Kisker and associates (cited in Lindskog, 1997). This CA is a trimeric molecule, which is completely different from the α-CAs, emphasising its separate ancestry. Each subunit is dominated by seven turns of a left-handed β-helix, with three short strands per turn (Figure 1.3). Thus there are three nearly flat β-sheets, two with seven, and one with eight parallel strands. The β-helix has a short α-helix on top, as well as a longer, C-terminal α-helix.

Three zinc ions (Figure 1.3) are located between the subunits and are co-ordinated to His-81 and His-122 from one subunit, and His-117 from a neighbouring subunit. A putative water molecule completes a distorted, tetrahedral co-ordination geometry (Lindskog, 1997).

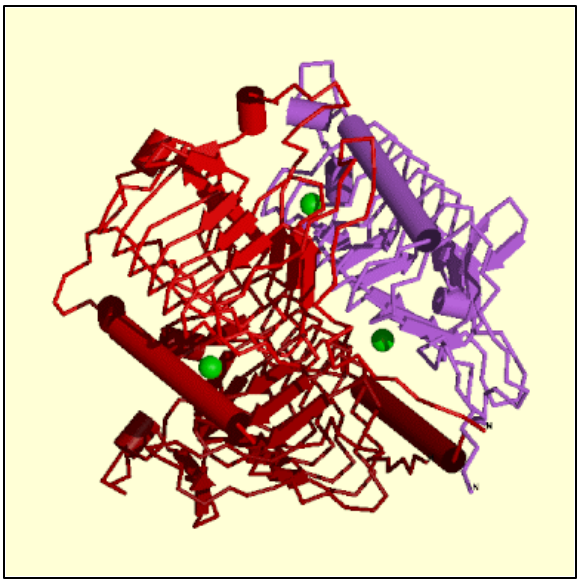


Figure 1.3: Secondary structure of γ-carbonic anhydrase from *M. thermophila*

1.4.2 CATALYTIC MECHANISM

A somewhat detailed understanding of the catalytic mechanism of HCA II has been obtained from kinetic studies, site-specific mutagenesis and X-ray crystallography. Specific details may differ for other isozymes, although they appear to have the same general mechanism (Lindskog, 1997).

The catalytic mechanism has been thoroughly investigated, and can be divided into two parts. The first part is the interconversion of CO_2 and HCO_3^- (Equation 1), which is controlled by the catalytically active zinc-bound water molecule which can ionise to a hydroxide ion. The second part is the regeneration of the active form of the enzyme, which involves the transfer of a proton from the catalytic site to the surrounding medium (Equation 2). The His-64 residue acts as an intramolecular proton-transfer group in this second reaction (Johansson & Forsman, 1993; Lindskog, 1997; Silverman, 1991).

$$[1] \qquad EZnOH^- + CO_2 + H_2O \rightleftharpoons EZnH_2O + HCO_3^-$$

$$[2] \qquad EZnH_2O + B \rightleftharpoons EZnOH^- + BH^+$$

In molecular terms this implies that a zinc-bound OH^- ion attacks a CO_2 molecule to form a metal bound HCO_3^- ion, which is then displaced by a H_2O molecule (Equation 1). The zinc bound OH^- is regenerated by a rate-limiting transfer of a water proton from the metal centre to His-64, which delivers the proton to a buffer base (Equation 2). These events are shown in greater detail in figure 1.4 (Lindskog, 1997).

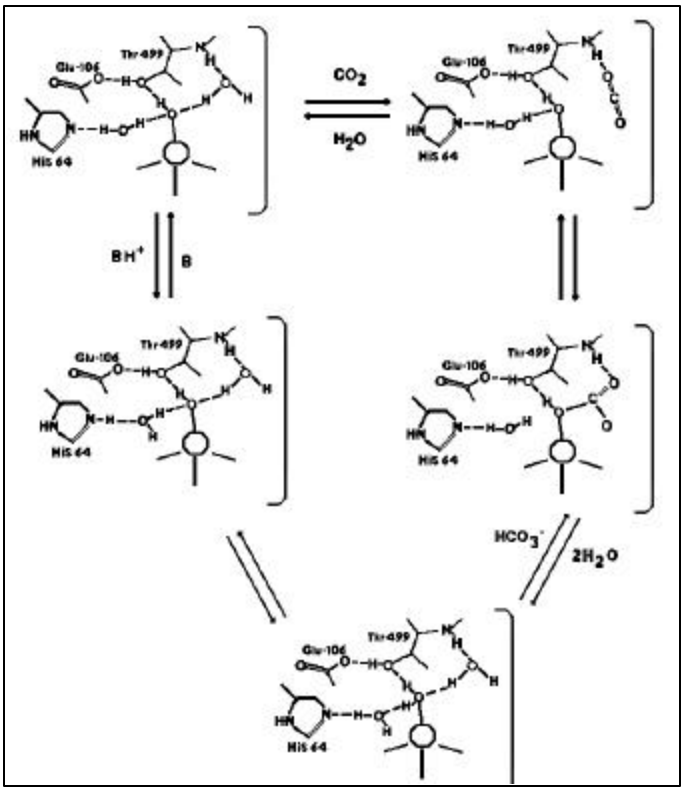


Figure 1.4: Scheme of the catalytic mechanism of HCA II (Lindskog, 1997)

1.4.2.1 ISOZYME-SPECIFIC FEATURES

Although the 'zinc hydroxide' mechanism probably applies to all α-CAs , there are certain isozyme-specific functional differences. HCA I is distinctive from HCA II in that it is six times less active on the basis of maximal turnover number. It also shows distinct buffer specificity, with much higher activities in imidazole-type buffers. This could indicate that the proton transfer system is less efficient in HCA I than HCA II, with additional pathways dominating under certain conditions (Lindskog, 1997).

HCA I has three unique amino acid residues in its active site, Val-62, His-67 and His-200. Behravan and associates (1990, 1991) have shown that His-200 plays a major role in CA I-specific kinetic properties, while Val-62 and His-67 are less important. His-200 appears to stabilise the enzyme-HCO_3^- complex and slow down the rate of HCO_3^- dissociation to the extent that this step can become rate-limiting in the presence of an imidazole-type buffer. The His-200 residue doesn't, however, effect proton transfer rates. His-64 becomes unsuitable as a proton shuttle in HCA I, due to the pK_a of 4.6. It is possible that Val-62 and His-67 are involved to some extent in this pK_a shift, although this is yet to be confirmed (Lindskog, 1997).

CA III is unique among the mammalian isozymes. It is more than 100-fold less active than CA II, it has virtually no 4-nitrophenyl acetate hydrolase activity, and it is comparatively insensitive to inhibition by sulfonamides. In addition, the pK_a of the zinc-bound water molecule is quite low at approximately 5. The active site contains a number of isozyme specific residues, for example, Lys-64 and Arg-67, which are responsible for kinetic properties. The introduction of His-64 establishes a proton shuttle pathway, greatly enhancing the maximal turnover rate. Another isozyme specific residue is Phe-198, which appears to be responsible for alterations to CO_2 hydration, 4-nitrophenyl acetate hydrolysis, and the pK_a of the zinc-bound water molecule (Lindskog, 1997).

1.4.2.2 β-CARBONIC ANHYDRASES

Although the β-CAs are evolutionary unrelated to the α-CAs, they appear to have similar catalytic capabilities. Despite structural differences, kinetic parameters indicate that pea carbonic anhydrase is equally efficient as HCA II in catalysing the hydration of CO_2, although kinetic patterns observed are far more complex than for HCA II. Results are consistent with a zinc-hydroxide mechanism, and the presence of a buffer-dependent proton-transfer step, although the mechanism for proton transfer seems to differ (Johansson & Forsman, 1993; Lindskog, 1997). Johansson & Forsman (1993) found that pea CA was dependent on a reducing agent in order to retain catalytic activity. In addition, high concentrations of buffer were required for the CA to work at a maximal rate. Catalytic activity of pea CA was also found to be dependent on the chemical nature of the buffer, with Barbital buffer giving the highest turnover rate (Johansson & Forsman, 1994).

1.4.3 INHIBITORS OF CARBONIC ANHYDRASE

1.4.3.1 SULFONAMIDES

Aromatic and certain heterocyclic sulfonamide derivatives possessing the general formula $R\text{-}SO_2NH_2$ (or $R\text{-}SO_2NH(OH)$) are powerful and specific inhibitors of most α-CAs. They bind to the metal ion as anions, $R\text{-}SO_2NH^-$ (or $R\text{-}SO_2N\text{-}OH^-$), via the nitrogen atom of the sulfonamide group (Lindskog, 1997; Moroney *et al*, 1985; Scozzafava and Supuran, 1998). The NH function of the ionised sulfonamide group replaces the zinc-bound water molecule and hydrogen bonds to the OH group of Thr-199. One of the oxygen atoms forms a hydrogen bond with the peptide NH of Thr-199, while another oxygen atom points towards the zinc ion. The affinity of the sulfonamide correlates with its acidity. While $CH_3SO_2NH_2$ (pK_a = 10.5) has a dissociation constant of 0.3 mM, the fluorinated analogue $CF_3SO_2NH_2$ has a pK_a value of 5.8 and binds to CA II with a dissociation constant of 2 nM (Lindskog, 1997).

Figure 1.5 illustrates the acetazolamide-CA II complex. The thiadiazole ring of acetazolamide comes into van der Waals contact with Val-121, Leu-198 and Thr 200.

The carbonyl oxygen of the amido group hydrogen bonds with the side chain amide of Gln-92, and the methyl group interacts with Phe-131 (Lindskog, 1997).

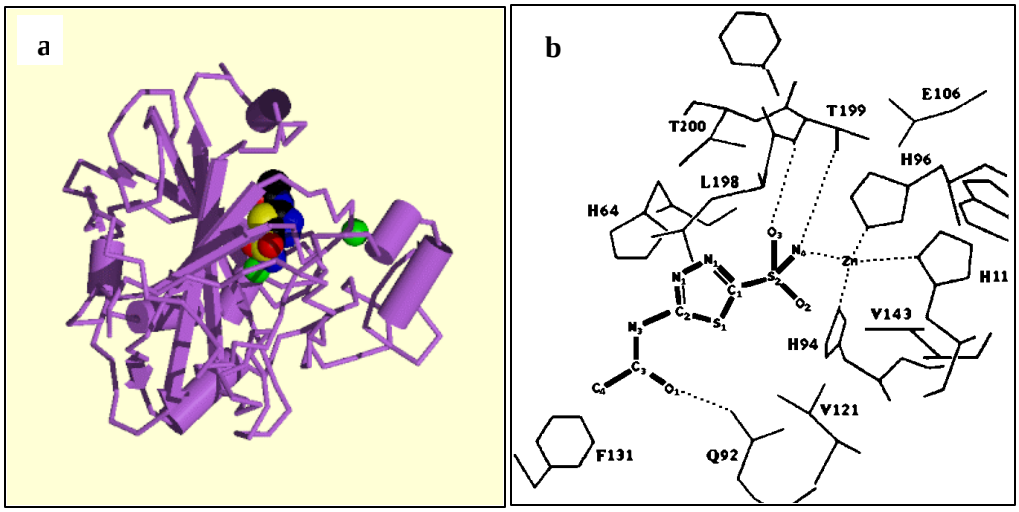


Figure 1.5: Acetazolamide-CA II complex a) Ribbon diagram b) Schematic drawing (Lindskog, 1997)

1.4.3.2 ANIONS

CA is inhibited by most monovalent anions, although the dissociation constants vary considerably from a few micromolar, in the case of CN^-, to about 1 M for F. Glu-106, Thr-199 and the zinc-bound water make up a hydrogen-bonded system, which is crucial to the active site. Glu-106 is probably ionised, and therefore, acts as an acceptor in the hydrogen bond with Thr-199. Consequently, the Thr-199 hydroxyl accepts a hydrogen atom in the bond with metal-bound solvent. Another water molecule called the 'deep water' located in the hydrophobic pocket, and hydrogen bonded to the peptide NH of Thr-199, forms a hydrogen bond with the zinc bound water molecule. These hydrogen-bond interactions appear to have a definite influence on the binding of inhibitors (Lindskog, 1997).

Inhibitors having a protonated ligand atom replace the metal-bound solvent molecule. There is no distortion of the tetrahedral co-ordination geometry, and the hydrogen bond with the OH group of Thr-199 is maintained. HSO_3^- and HS^- are examples of this group. Thiocyanate, formate and acetate are examples of inhibitors lacking a protonated ligand atom. These inhibitors do not remove the zinc-bound solvent molecule. It does, however, change position somewhat, but still maintains its

interaction with Thr-199. These anions bind close to the metal ion, displacing the deep water. In some cases they may hydrogen bond to the NH group of the Thr-199 (Lindskog, 1997).

A third group of anions co-ordinate to the metal ion and displace the zinc-bound water molecule, but do not hydrogen bond to Thr-199. This results in rather distorted tetrahedral co-ordination geometries. The halide ions Br^- and I belong to this group, as well as azide, which is a rather strong inhibitor that forms a hydrogen bond with the peptide NH of Thr-199 (Lindskog, 1997).

1.4.3.3 OTHER INHIBITORS

Some neutral, organic molecules have also been shown to inhibit mammalian CAs. One example, is phenol, a competitive inhibitor of the CA II catalysed CO_2 hydration. It is bound near the zinc ion forming a hydrogen bond with the metal-bound solvent. The phenolic hydroxyl group also forms a hydrogen bond with the peptide NH of Thr-199, displacing the deep water. The phenyl ring is located in the hydrophobic pocket, interacting with Val-121, Val-143, Leu-198 and Trp-209. Imidazole is a weak competitive inhibitor of CA I catalysed hydration of CO_2. It does not, however, significantly affect CA II (Lindskog, 1997).

A completely different kind of CA inhibitor has been found in the blood plasma of some species of mammals and fish, although not in human plasma. It is a 79 kDa glycoprotein which binds very strongly to CA II, but more weakly to other CA isozymes (Lindskog, 1997).

1.4.4 HUMAN CARBONIC ANHYDRASE ISOZYMES AND RELATED PROTEINS

Seven genetically distinct α-Ca isozymes have been identified in humans. These isozymes have different tissue distributions and intracellular locations. They also vary broadly in catalytic and inhibitory properties. Four of these seven genes, I (formerly called B), II (formerly C), III and VII, are cytosolic, and have been fully or partially

characterised for intron and exon structure (Behravan *et al*, 1990; Lindskog, 1997; Silverman, 1991; Tashian, 1989).

CA II is the most studied form, and is traditionally purified from red blood cells. However, CA II has a wide tissue distribution and is found in many different organs and cell types. The hydration of CO_2, caused by CA II, is among the most rapid of enzyme reactions at 10^6/s (Lindskog, 1997; Silverman, 1991). Deficiency of CA II has been associated with osteoporosis, renal tubular acidosis and cerebral calcification, indicating the importance of this isoenzyme in the bone, kidney and the brain (Sly & Hu, 1995).

CA I is the major non-haemoglobin protein in human red blood cells. It is also found in other tissues such as the colon, but it is not as widely distributed as CA II. It is also not as active as CA II, with a maximal CO_2 hydration turnover of 2×10^5/s. Its physiological function, other than as a back-up for CA II, is unclear (Lindskog, 1997).

CA III is a low-activity isozyme, with a maximal CO_2 hydration turnover rate of 8×10^3/s. It is found mainly in skeletal muscle, where it has been implicated in the facilitated diffusion of CO_2. It is also expressed in hepatocytes, and at low levels in certain other cells (Lindskog, 1997; Tashian, 1989). CA VII is expressed in salivary glands, and might be involved in the secretion of salivary bicarbonate (Lindskog, 1997). CA VI is secreted from the salivary glands and is believed to be involved in the pH regulation of saliva (Lindskog, 1997; Zhu & Sly, 1990).

There is a membrane-bound CA IV found in the lung and kidney (Zhu and Sly, 1990) that has catalytic properties very similar to isozyme II (Silverman, 1991). A mitochondrial enzyme, CA V, has also been characterised. CA is localised to the matrix of mitochondria from certain tissues, and is believed to be involved in ureagenesis and gluconeogenesis in the liver. Possible roles for mitochondrial CA in muscle, brain and gastric mucosa have yet to be fully explored (Lindskog, 1997; Tashian, 1989).

1.4.5 CARBONIC ANHYDRASE IN MICROALGAE AND CYANOBACTERIA

The function of CA in algae, as in higher plants, is generally considered in terms of the photosynthetic assimilation of inorganic carbon (C_i). Both cyanobacteria and green microalgae have been shown to possess an inducible inorganic carbon concentrating mechanism (CCM) that ultimately acts to raise the intracellular CO_2 concentration to a level far in excess of that present in the surrounding environment. Carbon dioxide derived from this internal pool is used by the enzyme ribulose biphosphate carboxylase (Rubisco) to initiate the first reaction in the Calvin cycle, the carboxylation of ribulose biphosphate. The C_i transport system and CA are of central importance to the functioning of the CCM. (Amaroso *et al*, 1996; Badger & Price, 1989; Badger *et al*, 1991; Badger *et al*, 1994; Beardall *et al*, 1998; Espie *et al*,1991; Furla *et al*, 1998; Funke *et al*, 1997; Kaplan *et al*,1991; Maeda *et al*; 1997; Moroney & Chen, 1998; Raven, 1995; Sültemeyer, 1998; Tsuzuki & Miyachi, 1991).

1.4.5.1 MICROALGAE

Studies of *Chlamydomonas reinhardtii* have lead to a broader understanding of the CCM in eukaryotic algae, including the role of CA. The model of the CCM, shown in figure 1.6, is therefore based on this organism (Sültemeyer, 1998). The extracellular C_i has to cross the cell wall, the plasmalemma and the membranes of the chloroplast envelope before it reaches the pyrenoid containing Rubisco (Badger & Price, 1989; Moroney & Mason, 1991).

Until recently bicarbonate was often assumed to be the carbon species actively transported across the plasmalemma or chloroplast envelope (Moroney & Mason, 1991). However, Sültemeyer and associates (1989) have proposed an active CO_2 uptake system, and Amoroso *et al*, (1996) have revealed that plastids are able to actively transport CO_2 and HCO_3^-. Marcus and associates (1984) have also provided evidence that CO_2 rather that HCO_3^- is the C_i species actively translocated across the plasmalemma. On the other hand, active CO_2 transport may only occur at the chloroplast level, thus creating a CO_2 sink so that CO_2 entry into the cell may occur

by passive diffusion (Moroney & Tolbert, 1985; Moroney & Mason, 1991; Ratatore & Colman, 1991; Sültemeyer, 1998).

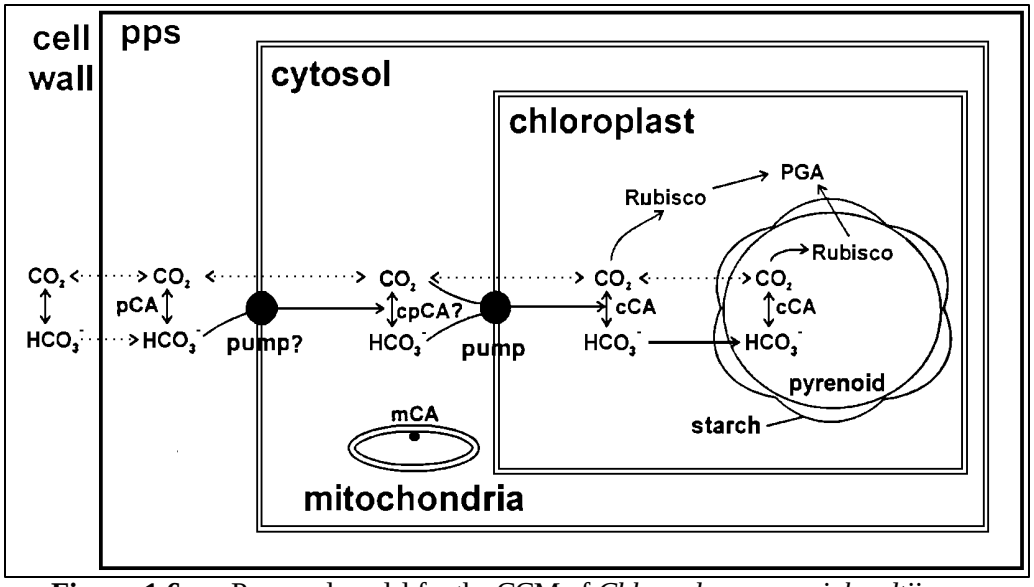


Figure 1.6: Proposed model for the CCM of *Chlamydomonas reinhardtii* as a model organism for microalgae (Sültemeyer, 1998)

A key component of any CCM is the location of CA isozymes (Badger *et al*, 1998; Moroney & Mason, 1991). Although the presence of both external and internal forms of CA have been known for a long time (Coleman *et al*, 1984; Husic *et al*, 1989; Husic, 1991; Ishida *et al*, 1993; Moroney *et al*, 1987; Sültemeyer *et al*, 1995; Yang *et al*, 1985), two distinct internal CAs have been isolated from *Chlamydomonas reinhardtii* recently. One is located in the chloroplast (Husic & Marcus, 1994; Karlsson *et al*, 1995) and the other in the mitochondria (Eriksson *et al*, 1996; Park *et al*, 1999). Figure 1.6 highlights the position of the various CAs in this alga.

In addition to the CAs from *Chlamydomonas reinhardtii* extracellular CA has been identified in *Chlorella saccharophila* (Williams & Coleman, 1995; Williams & Coleman, 1996), *Phyllariopsis purpurascens* (Flores-Moya, 1998) and *Micromonas pusilla* (Iglesias-Rodríguez *et al*, 1998). It is believed that this form of the enzyme accelerates the extracellular equilibration of CO_2 and HCO_3^- to provide CO_2 at a sufficient rate to enter the cell and serve as a substrate for photosynthetic reduction. The periplasmic enzymes are particularly important for the acquisition of inorganic

carbon at alkaline pH values where HCO_3^- is the predominant form of C_i in the medium (Husic & Marcus, 1994).

Intracellular CAs have been identified in *Coccomyxa* (Hiltonen *et al*, 1995; Palmqvist *et al*, 1995), *Chlorella vulgaris* (Villarejo *et al*, 1998), *Chlorella sorokiniana* (Satoh *et al*, 1998) and the soil alga *Porphyridium purpureum* (Dixon *et al*, 1987). External as well as internal CAs have been identified in *Dunaliella* species (Amoroso *et al*, 1996; Goyal *et al*, 1992).

1.4.5.2 CYANOBACTERIA

The involvement of CA activity in the process of C_i accumulation and utilisation in cyanobacteria is not very clear due to the lower and more variable activities. In addition, most cyanobacteria lack periplasmic CA (Badger & Price, 1989). There is however low, but variable activity associated with the cell homogenates of cyanobacteria growing at limiting levels of C_i. This CA appears to be both particulate and soluble in nature, depending on the species. Ingle and Coleman (1975) concluded that the CA in *Oscillatoria* sp. was cytoplasmic. Yagawa and associates (1984) found that 50 to 70% of CA in the homogenates of two *Anabaena variabilis strains* was particulate, but that from a third was almost entirely soluble. CA activity was found to be strongly associated with the carboxysomes in *Synechococcus* PCC7942 (Price *et al*, 1992). The amount of activity found in homogenates is also variable, ranging from 0.8 to 30 units per mg protein, depending on the species. In certain strains of *A. variabilis* and *Anacystis nidulans* it has been reported to be undetectable (Badger & Price, 1989).

The mechanisms by which C_i transport occurs in cyanobacteria is still not well understood. One of the central questions is which C_i species is used by the cell, and how is this affected by growth conditions. A number of methods have been applied in order to try and discriminate between transport of CO_2 and HCO_3^-, and a general picture has been drawn in which the C_i-transport system can utilise both CO_2 and HCO_3^- as substrates. The CO_2 transport is, however, considered to be constitutive, and the ability of HCO_3^- uptake is induced as a response to the adaptation of cells to low C_i concentrations. Regardless of the species taken up from the external medium, it is

HCO_3^- that is delivered at the inner side of the plasma membrane. The internally accumulated HCO_3^- penetrates into the carboxysomes, which contain most, if not all, of the cells Rubisco. Here specifically located CA generates CO_2 in the immediate vicinity of Rubisco (Al-Moghrabi *et al,* 1996; Badger & Andrews, 1982; Badger & Price, 1992; Badger *et al,* 1985; Bedu & Joset, 1991; Espie *et al,* 1991a; Espie *et al,* 1991b; Miller *et al,* 1990; Sültemeyer *et al,* 1995).

1.4.6 THE ROLE OF CARBONIC ANHYDRASE IN ALKALISATION

Alkalisation of the medium during photosynthesis has been reported for intact cells of Anabaena *variabilis, Chlamydomonas reinhardtii, Dunaliella parva, Chlorella vulgaris* and intact pea chloroplasts, or chloroplasts from higher plants (Shiraiwa *et al,* 1993). Haglund and associates (1992) have also reported alkalisation of the medium by two marine brown algae. Investigation by these authors led to the conclusions that extracellular CA converts HCO_3^- in the surrounding medium to CO_2, which then enters through the plasmalemma for use in photosynthesis. Hydroxyl ions remain in the medium resulting in a net increase in pH (Haglund *et al,* 1992; Shiraiwa *et al,* 1993). It is this process that is of interest in this study.

Traditionally, toxic metals have been precipitated from liquid discharges, by the addition of lime, limestone, caustic soda or sulphide. Increasing the pH of the effluent in this manner leads to the conversion of soluble metal into an insoluble form such as hydroxide (Eccles, 1999; Summers, 1992). *Spirulina* offers a potential alternative to the traditional technology, in that the required increase in pH can be achieved without the addition of any chemicals. The enzyme CA is central to the ability of *Spirulina* to achieve this increase in pH, leading to metal precipitation. For this reason, the present study involved the investigation of assay systems and isolation of CA in order to gain an improved understanding of the enzyme and the factors affecting its activity. It was also envisaged that this study would reveal an easy method of CA detection for use in screening other potential bioremediation agents.

CHAPTER TWO

THE EFFECTS OF TOXIC METALS ON *SPIRULINA*

2.1 INTRODUCTION

Spirulina is one of the few algal species that has been investigated for its potential as a bioremediation agent, although little data is available to support its use for bioremoval of heavy metals. An attractive possibility is that *Spirulina* may be capable of binding heavy metal ions as well as removing nutrients from the surrounding medium, thereby decreasing the eutrophication potential of waters which receive treated fluids. The advantage of using *Spirulina* is that it can be grown in ponds with little nutritional input or maintenance. Laboratory or small-scale experiments have also been performed on the growth of *Spirulina* on city waste-waters, cow manure and swine wastes. In addition *Spirulina* is non-pathogenic, which gives it an advantage over other forms of microbial biomass (Brady *et al*, 1994c; Ciferri, 1983; Hulse, 1982; Oswald, 1988; Shelef *et al*, 1980). The indications that *Spirulina* has potential as a bioremediation agent and the availability of the algae warranted this investigation into the use of *Spirulina* as a biosorbent.

2.1.1 CHARACTERISTICS OF *SPIRULINA*

2.1.1.1 MORPHOLOGY

Spirulina is a multicellular, filamentous, non-heterocystous cyanobacterium. The blue-green filaments are composed of cylindrical cells arranged in unbranched, helicoidal trichomes (Figure 2.1). The filaments are motile, gliding along their axis (Ciferri, 1983; Durand-Chastel, 1980; Martel *et al*, 1992; Richmond, 1986; Vonshak *et al*, 1996a).

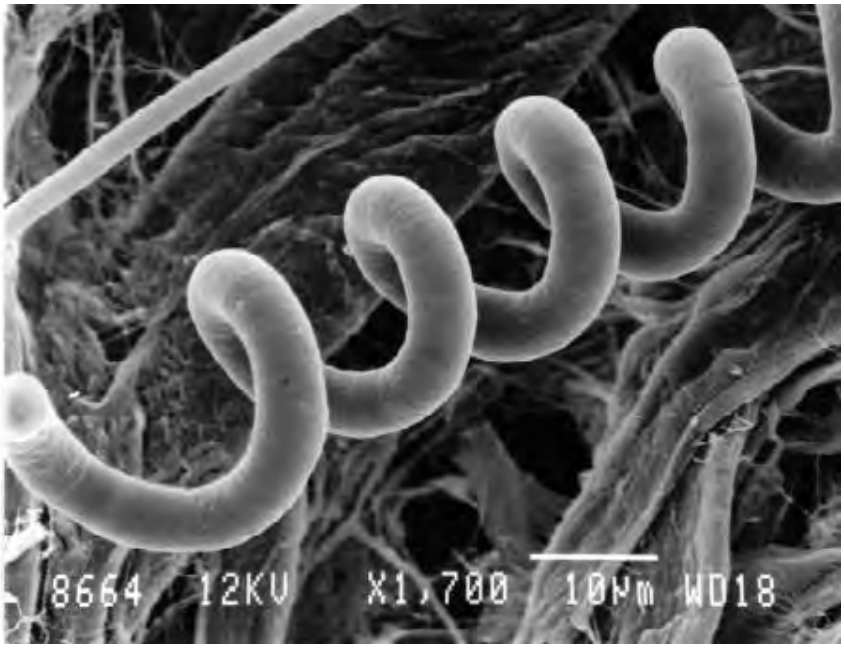


Figure 2.1: Scanning electron microscope preparation of healthy *Spirulina*

The helical shape of the trichome is characteristic of the genus, but the helical parameters, such as pitch, length and helix dimensions, vary with the species, and even within the species. The diameter of the cells ranges from 1 to 3 µm in the smaller species and from 3 to 12 µm in the larger ones. The larger species have a granular cytoplasm containing gas vacuoles and the septa are clearly visible (Ciferri, 1983; Durand-Chastel, 1980; Richmond, 1986).

The cell wall of *Spirulina* consists of four different layers. The outer layer consists of structural material analogous to the Gram negative bacterial cell wall. Underneath this is a layer of protein fibrils helically bound to the trichomes. The second layer is a peptidoglycan layer, which folds towards the inside of the filament. This second layer, together with the inner, fibril layer give rise to the septum, which separates the cells (Ciferri, 1983; Richmond, 1986).

2.1.1.2 ECOLOGY

Spirulina are obligate photoautotrophs that were first isolated by Turpin in 1827 (cited in Ciferri, 1983) from a freshwater stream. Since then species of *Spirulina* have been found in a variety of environments including soil, marshes, brackish water, seawater and freshwater. Species of *Spirulina* have been isolated from tropical waters and the North Sea, from thermal springs, salt pans, warm waters from power plants, and from fishponds. Thus the organism appears to be capable of adapting to a variety of very different habitats, and it colonises certain environments in which life for other organisms is, if not impossible, very difficult (Ciferri, 1983; Richmond, 1986; Vonshak *et al,* 1996a).

2.1.1.3 *SPIRULINA* AS A BIOSORPTION AGENT

A potential problem associated with the use of a living biomass as a biosorbent is the potential toxic effects of the heavy metals in solution (Wilde & Bennemann, 1993). Therefore, in order to determine the feasibility of *Spirulina* as a biosorption agent it is necessary to determine the effects of various metals on the growth of *Spirulina*.

Copper is one of the most common industrial metals. The use of zinc is also widespread, mainly in the manufacture of alloys, galvanising and paper production. Due to the numerous activities involving these two very common metals there are numerous industrial effluents discharging them into the environment. Although copper is an essential trace element for humans, large doses can have harmful, or even fatal effects. Wilson's disease is a result of excess copper being deposited in the brain, skin, liver and pancreas. There is also some evidence to suggest that copper is carcinogenic. Zinc is essential for enzyme activators in humans, but is also toxic at levels of 100-500 mg/day (Volesky, 1990; Volesky & Holan, 1995; Wase & Forster, 1997).

Lead is toxic to humans, aquatic fauna and livestock. It is estimated that approximately 180 000 tons of lead is mobilised by the natural weathering process each year, and approximately 2 million tons is mined yearly. Although lead pollution from mining activities presents a relatively localised problem, its magnitude is

significant and, particularly on the water pollution side, it is compounded by the presence of other heavy metals. In the aquatic environment it will exist mainly in the inorganic divalent state. It can also exist in an organic tetravalent state (such as the alkyl leads) which, in some cases is more toxic than inorganic lead. The earliest signs of lead poisoning seem to be physical symptoms such as excitement, irritability and depression. Young children are particularly affected and can suffer retardation and semi-permanent brain damage. One of the most insidious effects of inorganic lead is its ability to replace calcium in bones and remain there to form a semi-permanent reservoir for long-term release, well after the initial absorption (Volesky, 1990; Wase & Forster, 1997).

The aim of the research dealt with in this chapter was to determine the threshold levels of *Spirulina* for the toxic metals copper, zinc and lead. Electron microscopy was also used in order to determine the effects of metal accumulation on the morphology and internal organelles of the organism.

2.2 MATERIALS AND METHODS

2.2.1 BIOMASS

The *Spirulina* used in this study (Figure 2.2) was obtained from an existing culture maintained under constant environment conditions (constant illumination at 27ºC) in the Department of Biochemistry and Microbiology at Rhodes University. The biomass was cultivated in Zarrouk's medium (Appendix A), a chemically defined medium used specifically for the cultivation of *Spirulina*. In these experiments healthy *Spirulina*, resuspended in half strength Zarrouk's media, was used in batch studies. The EDTA and $FeSO_4$ were, however, excluded from the media. This was done because EDTA is a metal chelating agent and could affect the results. $FeSO_4$ tends to precipitate in the absence of EDTA and therefore was also excluded.

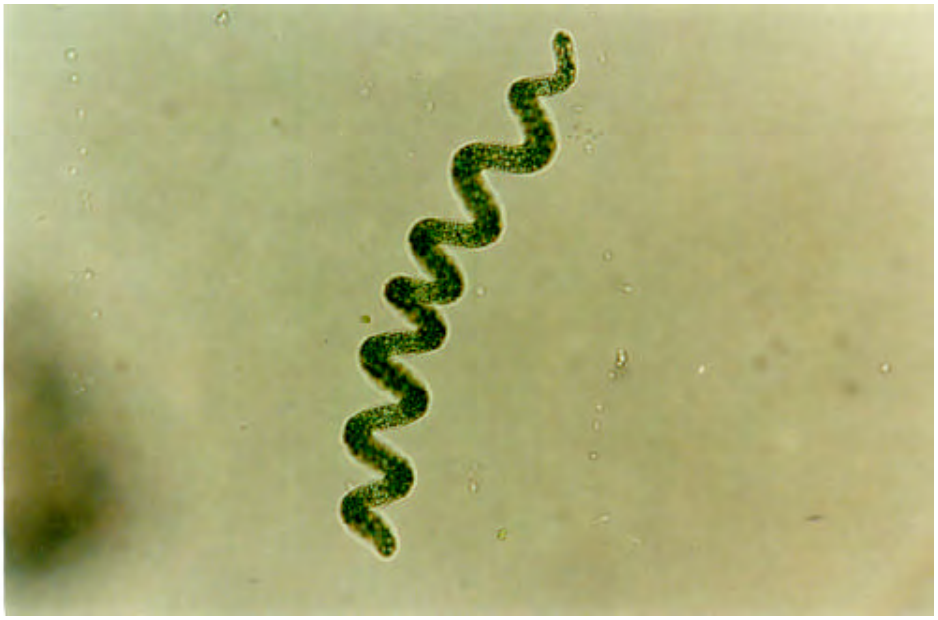


Figure 2.2: Light microscope preparation of healthy *Spirulina*

2.2.2 ATOMIC ABSORPTION SPECTROSCOPY

Metal concentrations were analysed on a GBC 909AA atomic absorption (AA) spectrophotometer linked to a GBC integrator. Absorption values were converted directly to units of µM by the integrator. Appendix B shows the specific parameters for the analysis of copper, zinc and lead.

2.2.3 METAL STOCK SOLUTIONS

Stock solutions of 2 mM $CuSO_4$, $ZnSO_4$, and $Pb(NO_3)_2$ were prepared and used to spike the algal cultures with varying concentrations of metal .

2.2.4 METAL STANDARDS

Appropriate dilutions (2, 5, 10, 20, 50 µM) of concentrated metal solutions were prepared and standard curves for copper, zinc and lead were generated using the AA spectrophotometer.

2.2.5 DETERMINATION OF ALGAL CONCENTRATION

Due to the filamentous nature of *Spirulina*, cell counts or photometric methods cannot be used to determine the strength of the culture. A novel method of determining culture strength is therefore required. Dry weight measurement is a direct method of estimating biomass production and chlorophyll determination is one of the most rapid methods for estimating quantities of living plant material (Richmond, 1986). By equating these two methods and producing a standard curve of chlorophyll *a* versus algal dry mass an estimate can be made of dry mass from chlorophyll *a* concentrations.

2.2.5.1 ALGAL DRY WEIGHT

Whatman glass microfibre filter paper discs were numbered, dried in an oven at 50°C for 3 hours and then weighed out individually. Sample volumes of 1, 2, 5, 10 and 20 ml (in triplicate) were removed from the algal culture and filtered onto the appropriately numbered discs using a bench top vacuum pump. These were then dried in the oven for a further 3 hours, re-weighed and the difference calculated.

2.2.5.2 CHLOROPHYLL EXTRACTIONS

Samples of the algal culture were taken as for the dry weight standards. The samples were filtered onto 25 mm Whatman GF/C filters using a bench top vacuum pump. The alga laden discs were then transferred into McCartney bottles containing 10ml of acetone and covered in foil to prevent light degradation of chlorophyll *a*. The discs were then crushed using a glass rod and the bottles were stored in a 4°C refrigerator overnight. The following day the samples were centrifuged, in a Heraeus Sepatech Labofuge, for 5 minutes at 5000 rpm, to remove filter paper and cell debris. The absorbance of the supernatants was then read in a UV-visible spectrophotometer at 661.6 nm, 644.8 nm and 470 nm. Chlorophyll concentrations were then calculated from the equations given in Appendix C.

Chlorophyll *a* values were plotted against algal dry mass to form a standard curve (Appendix D). For all further experiments 5 ml samples of algae were taken from experimental flasks and chlorophyll *a* concentrations determined as above. These concentrations could then be used to estimate dry mass of algae.

2.2.6 ACID DIGESTS

Acid digests were performed in order to determine the total amount of metal associated with the cells as well as the amount left in solution.

Samples of 3 ml were removed from experimental cultures and filtered using 0.45 µm cellulose acetate filters (Whatman). The filtrate was retained and the metal concentration determined using the AA spectrophotometer.

The filters with the algae were placed in Pyrex boiling tubes and 0.2 ml 55 % nitric acid was added to each. The tubes were then placed in a beaker, containing a few glass beads and a little water, over a Bunsen burner. They were boiled for approximately half an hour until the yellow cloud dissipated. To each tube 4 ml of water was then added and the metal concentration determined using the AA spectrophotometer. After each acid digest was performed the number of moles of metal in the flask was determined. The appropriate amount of metal was then added to each flask to restore it back to the starting concentration.

2.2.7 LIGHT MICROSCOPY

Samples of 25 µl, taken from the experimental flasks, were mounted on glass slides and photographed at the Electron Microscopy Unit, Rhodes University, using an Olympus BX50 camera-microscope attached to an Olympus PM-30 exposure control unit.

2.2.8 SCANNING ELECTRON MICROSCOPY (SEM)

Samples of 5 ml were taken from experimental flasks and filtered onto 25 mm Whatman CF/C filters. Specimens were incubated in cold 2.5 % gluteraldehyde in 0.1 M phosphate buffer for 12 hours. Once fixation was complete, the samples were washed twice in phosphate buffer, followed by a 30 % to 100 % ethanol dehydration sequence, each of 10 min duration. Specimens were then allowed to incubate in increasing concentrations of amyl acetate before critical point drying. Specimens were mounted on metal stubs, gold coated in a sputter chamber, and observed using a JEOL JSM-80 scanning electron microscope (Cross, 1987).

2.2.9 TRANSMISSION ELECTRON MICROSCOPY (TEM)

Samples of 1 ml were removed from experimental flasks and centrifuged at 2000g for 5 min in a Hereaus Biofuge. The pellets were then prepared for TEM. Fixation was achieved by incubation in cold buffered gluteraldehyde for 12 hours. After two washes in phosphate buffer, this was followed by secondary fixation in osmium tetroxide for 90 min. A further two washes preceded an ethanol dehydration sequence as described in the previous section. This was followed by a propylene oxide transition and a sequential embedding process, as described by Cross (1987). Trapezium shaped sections of 120 nm were cut using a RMC MT7 ultramicrotome. The sections were stained with 5 % uranyl acetate and lead citrate, followed by examination under a JEOL JEM 100 CX transmission electron microscope.

2.2.10 BATCH EXPERIMENTS

The following experiments were carried out using copper, zinc and lead and all experiments were performed in duplicate.

Healthy *Spirulina* from the existing culture was filtered through a nylon mesh and washed with distilled water to remove any traces of EDTA. The algal slurry was then resuspended in 50 % Zarrouk's mixture (Zarrouk's medium made up with no EDTA or $FeSO_4$ and diluted 50 % with water).

Twelve 500ml flasks were inoculated with 30 ml concentrated algal slurry and 270 ml 50 % Zarrouk's mixture. A chlorophyll extraction, an acid digestion and a pH reading was performed on each culture. The cultures were then allowed to grow overnight, at 120-130 rpm on an orbital shaker, under constant environment conditions.

On the following day appropriate volumes of metal stock solution were added to each flask to make the final concentrations up to 5, 10, 20, 30 and 50 µM.
Chlorophyll extractions, acid digestions and pH readings were performed again, and every second day thereafter.

2.3 RESULTS AND DISCUSSION

For all experiments algal mass (mg dry weight) in the 300 ml culture was calculated from the standard curve (Appendix D). From the acid digest results the number of moles of metal per milligram was calculated and hence the total amount of metal associated with the algae. The number of moles of metal remaining in solution was also calculated.

2.3.1 COPPER

The results for algal concentration and pH are shown in figures 2.3 and 2.4. From these results it can be seen that for the first three days all six groups exhibited essentially similar behaviour. Both the algal concentration and the pH increased steadily, after which the 20, 30 and 50 µM groups showed a decline in both pH and algal concentration. The 50 µM cultures were the first to start declining and by day eleven all of the 20, 30 and 50 µM cultures were dead. The controls and the 5 and 10 µM flasks were all still healthy, although the growth in the 10 µM cultures was starting to decline.

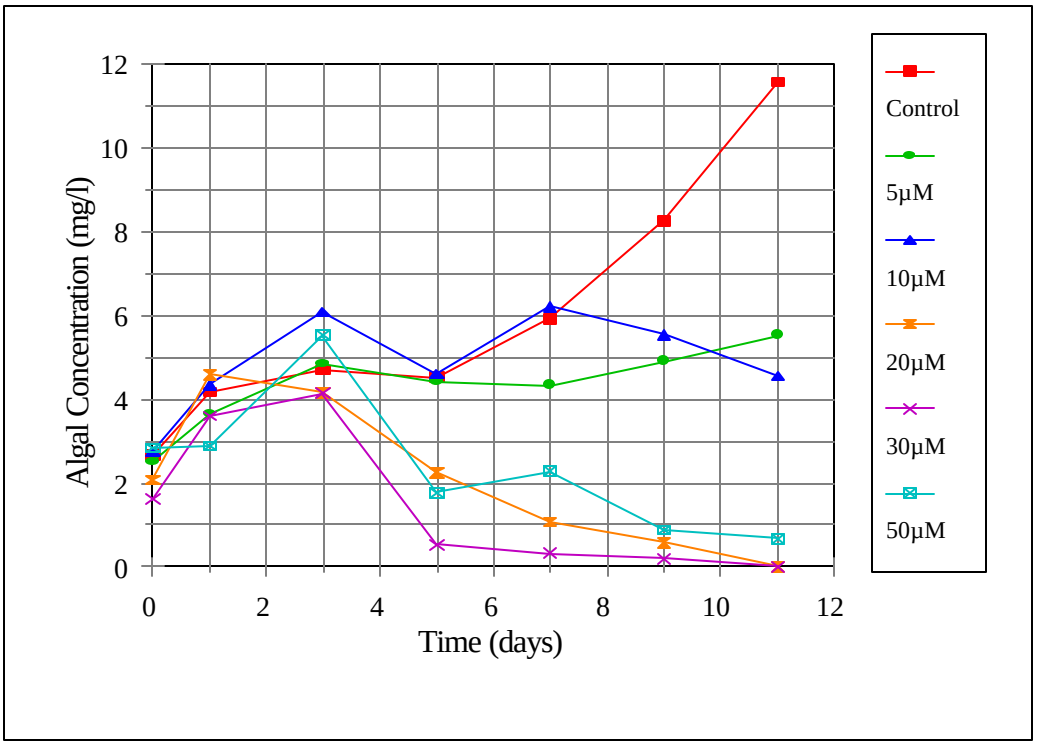


Figure 2.3: Growth curves of *Spirulina* cultures containing $CuSO_4$

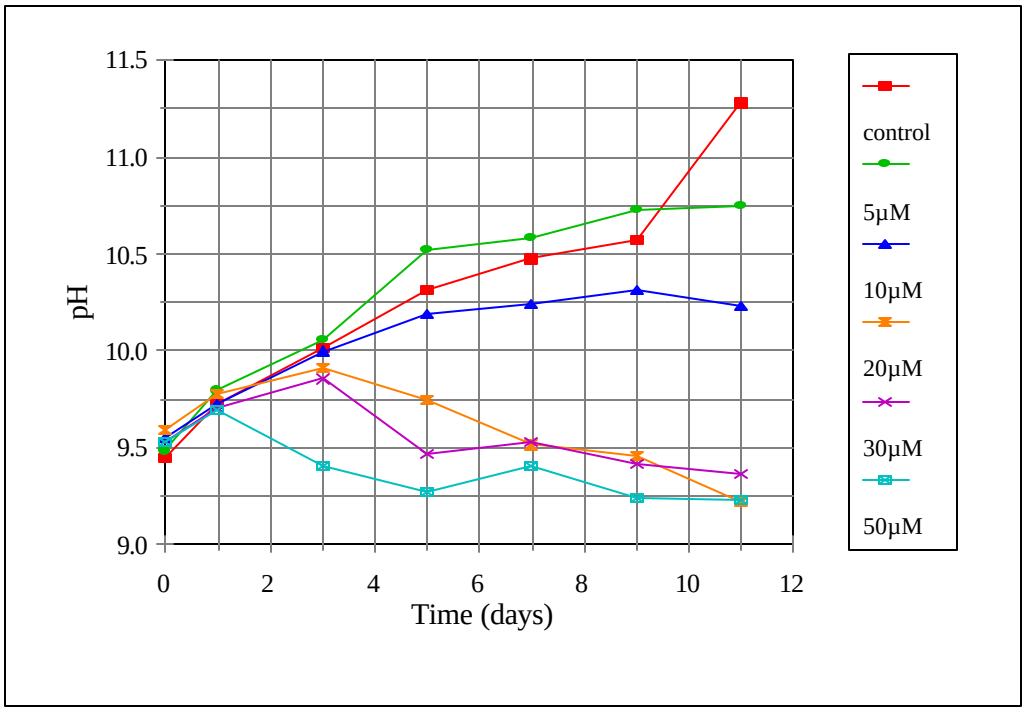


Figure 2.4: pH Profiles for copper toxicity experiments

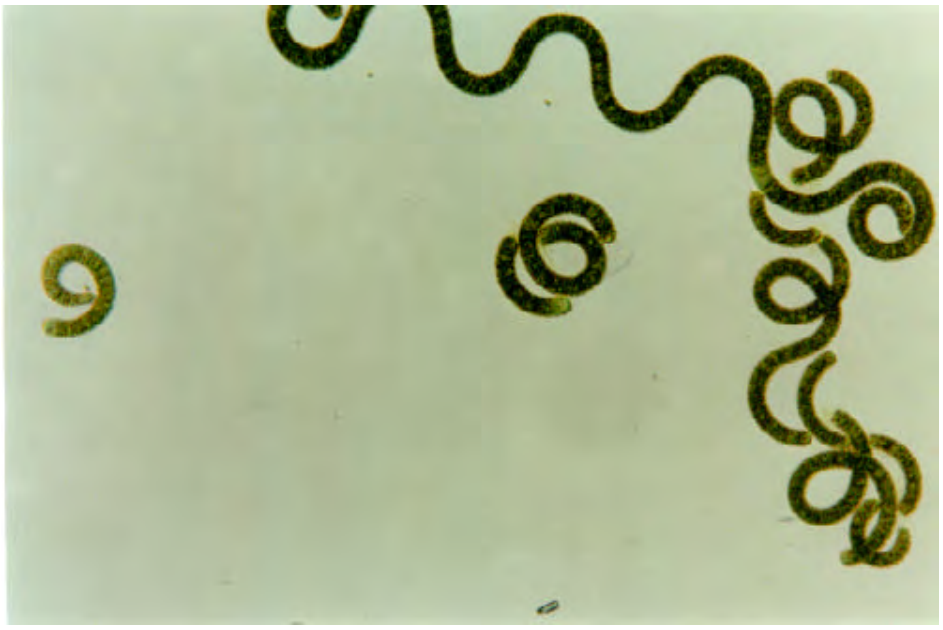


Figure 2.5: Light microscope preparation of *Spirulina* after 2 days in 50µM $CuSO_4$

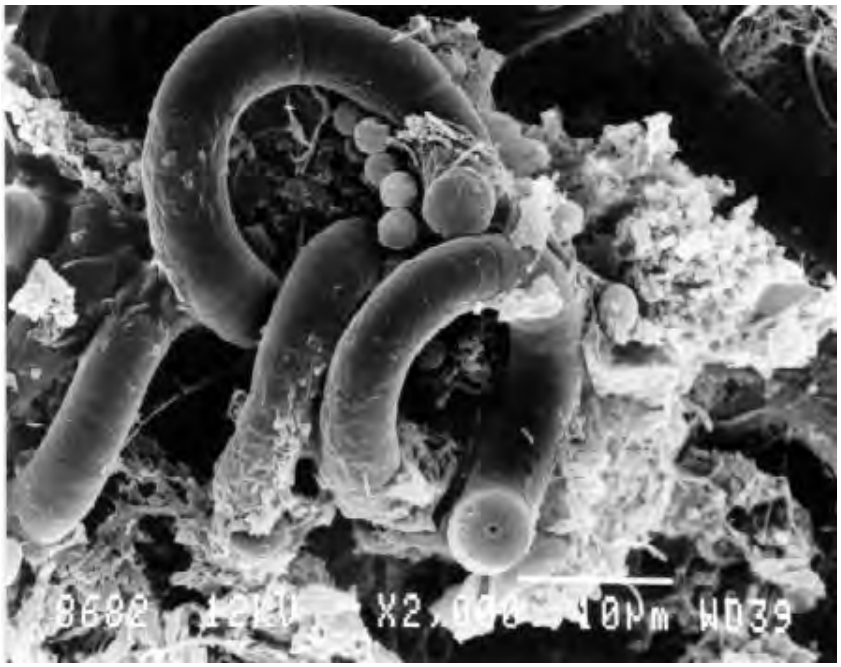


Figure 2.6: SEM preparation of *Spirulina* after 2 days in 50µM $CuSO_4$

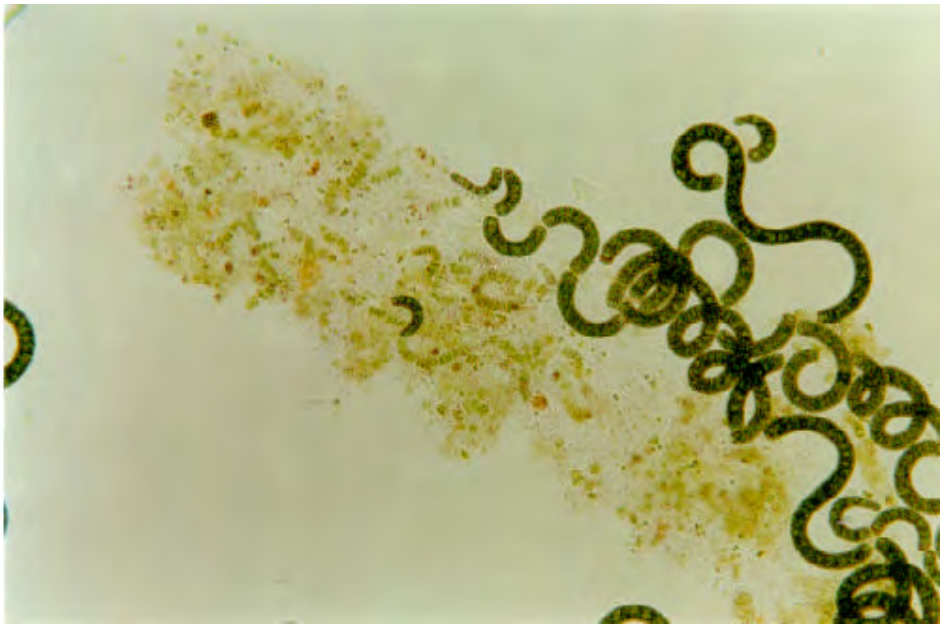


Figure 2.7: Light microscope preparation of *Spirulina* after 4 days in 50µM $CuSO_4$

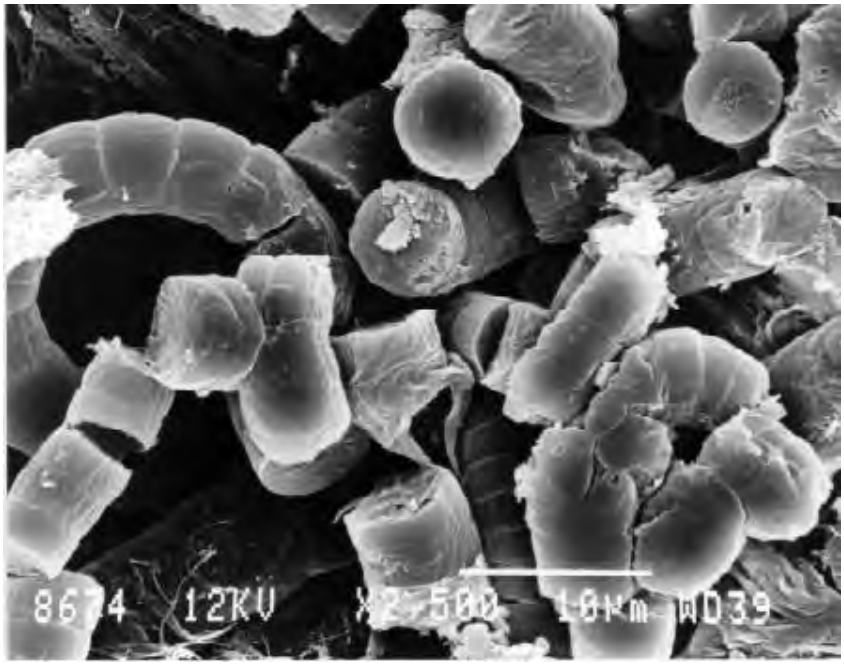


Figure 2.8: SEM preparation of *Spirulina* after 4 days in 50µM $CuSO_4$

From the photographs in figures 2.5 to 2.8 the gradual breakdown of the cells can be seen. After two days in 50 µM $CuSO_4$ (Figures 2.5 & 2.6) the filaments started to unwind and a few had started to break up. By day four (Figures 2.7 & 2.8) the filaments were almost completely broken up and a lot of cell debris was observed (Figure 2.7). The filaments appeared to lose a certain degree of cell contents during the break up process (Figure 2.8), and hence their shape became disfigured, most likely as a result of turgor pressure.

The results from the acid digests, showing how much copper was associated with the algae and how much remained in solution are shown in figures 2.9 and 2.10. Figure 2.9 shows that there is a rapid increase in Cu accumulation in the 20, 30 and 50 µM groups on day three, but if one compares figure 2.9 to the growth curve (Figure 2.3) it can be seen that day three is when the same groups started dying. Figure 2.9 is expressed in µmoles of copper per gram of algal dry mass, which is determined from the chlorophyll *a* values. As the cells started to die chlorophyll *a* values decreased, although the dead cell mass would remain, and hence the data represented the copper accumulation for a much lower concentration of algae than that which is actually present. Therefore, the graph only gives an accurate representation of the copper uptake in the 20, 30 and 50 µM groups until day three, after which the cells started to die. These results suggest that the threshold level for copper associated with algae is approximately 7 µmoles/g, because as soon as the Cu accumulated exceeded this value the cells started to die. The control, 5 and 10 µM groups never exceeded this value, and they were still healthy at the end of the experiment.

Figure 2.10 gives a more accurate representation of what actually occurred. The peaks on day one were seen due to the initial addition of metal. By day three there was a distinct drop in the copper in solution, presumably due to uptake by the algae, as any precipitation would have occurred almost immediately. On day five extra $CuSO_4$ was added to each culture to restore them to their original concentrations, hence the increase seen. After day five very little decrease was seen suggesting that once the cells start dying, no further uptake occurred. The percentage not accounted for by uptake or Cu in solution can possibly be accounted for by precipitation, or by binding to a metal-complexing agent, such as polysaccharide, excreted by the algae.

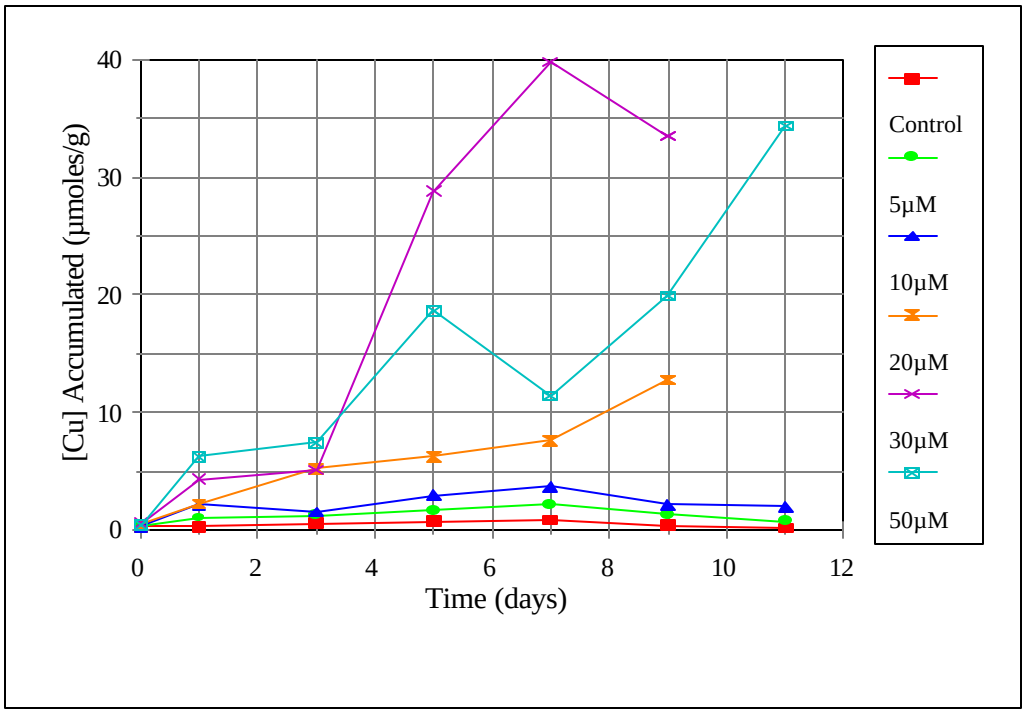


Figure 2.9: Copper accumulated by *Spirulina* over 11 days

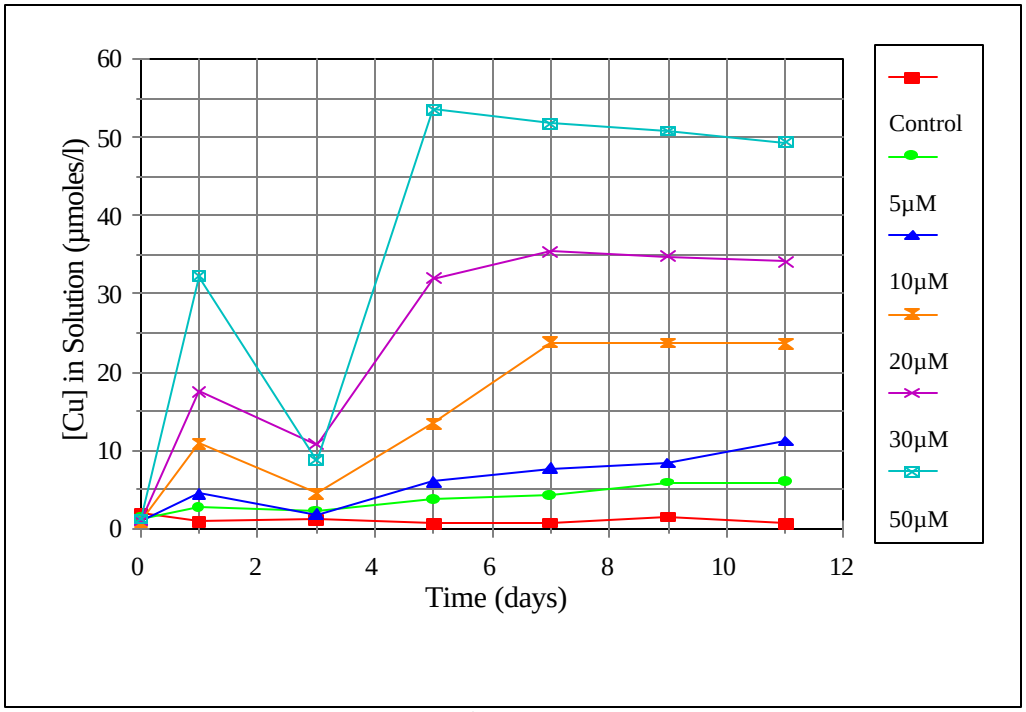


Figure 2.10: Copper left in solution over the 11 day period

2.3.2 ZINC

The results obtained for zinc showed essentially the same trends as those for copper. If the growth curve for zinc (Figure 2.11) is compared to that of copper (Figure 2.3) it can be seen that the copper appeared to take slightly longer before taking effect. Even in the 20, 30, and 50 µM groups, a slight increase in algal concentration was observed before decline began (Figure 2.3). In the case of zinc, the same groups started to decline immediately (Figure 2.11), with the exception of the 20 µM group. At first glance it would appear that, once the cells started deteriorating, death occurred more rapidly in the case of copper (Figure 2.3). However, as the starting concentration of *Spirulina* in the zinc experiment was far higher than in the copper experiment, the cultures took longer to deteriorate to the state of copper cultures. Figure 2.12 once again showed a positive correlation between culture strength and pH indicating that pH is directly related to cell function.

Figure 2.13 suggests that the tolerance level of *Spirulina* for zinc is approximately 5 µmoles/g, because as soon as the Zn accumulated exceeded 5 µmoles/g the cells started to die. In the case of copper (Figure 2.9) this occurred at approximately 7 µmoles/g.

In figure 2.14 it can be seen that the first readings taken after the addition of zinc showed a much lower metal concentration than that which was actually added. Figure 2.13 shows that a small proportion of zinc was taken up by the cells, but there was a large percentage unaccounted for. This suggests that a fairly large proportion of zinc precipitated as soon as it was added to the medium. These results suggest that a larger percentage of zinc was precipitated than copper (Figure 2.10). The cultures were only topped up with zinc on day nine, which would explain the sudden increase in Zn in solution (Figure 2.14). On day three there was 6.26 µmoles of zinc associated with the algae in the 50 µM flasks. By day five this value had decreased to 4.77 µmoles. This shows that the zinc associated with the algae decreased by 4.96 µmoles/l. This was probably due to cell death and release of metal that had already been accumulated, thus causing an increase in Zn in solution. This would explain the rise seen in Cu in

solution (Figure 2.10) between day three and five. Similar trends occurred in the other groups, which would explain the increases seen.

The deterioration of the cells can be seen in the photographs of *Spirulina* grown in 50 µM $ZnSO_4$ (Figures 2.15 to 2.18). Figures 2.15 and 2.16 show the state of the cells after two days in $ZnSO_4$. The *Spirulina* in these photographs looked much healthier than those at the same stage of the copper experiments (Figures 2.5 & 2.6). Very little damage had occurred to the filaments at this stage, they were just starting to unwind. This was probably due to the difference in the starting concentrations of the cultures. By day two in the zinc experiments, the cultures were already in a state of decline, but the algal concentration was still relatively high and therefore the culture would not yet have reached the state of the copper cultures of the same stage.

Figures 2.17 and 2.18 show the *Spirulina* after four days in $ZnSO_4$. Although the cells appeared to be very broken up at this stage, they were still a lot greener (Figure 2.17) and they looked a lot healthier (Figure 2.18) than those of the copper experiment (Figures 2.7 & 2.8). The cells in figure 2.18 have retained their shape completely, and don't appear to have lost as much of their cell contents as those in figure 2.8. This could be because the culture had not reached the same state of the copper cultures due to the increased starting concentration, but copper is also known to interfere with the photosynthetic electron transport, especially in photosystem II. In addition, copper toxicity interferes with pigment and lipid biosynthesis and consequently chloroplast ultrastructure, thus it negatively affects photosynthetic efficiency (Barón *et al*. 1995). Zinc has also been shown to inhibit photosynthesis, but it takes higher concentrations of zinc to have an equivalent effect on photosynthesis as a lower concentration of copper (Davies, 1983). Thus, the difference in colour between the cultures grown in copper (Figure 2.7) and zinc (Figure 2.17) could be due to the copper having a more marked effect on photosynthesis.

McBrien and Hassel (cited in Overnell, 1975) found that copper also increased the permeability of algal cells, whereas Overnell (1975) showed that zinc did not exhibit this effect. This could explain the loss of shape and slightly wrinkled effect of the cells that had been exposed to copper for four days (Figure 2.8). The *Spirulina* from the zinc experiments (Figure 2.18) did not exhibit this effect at all.

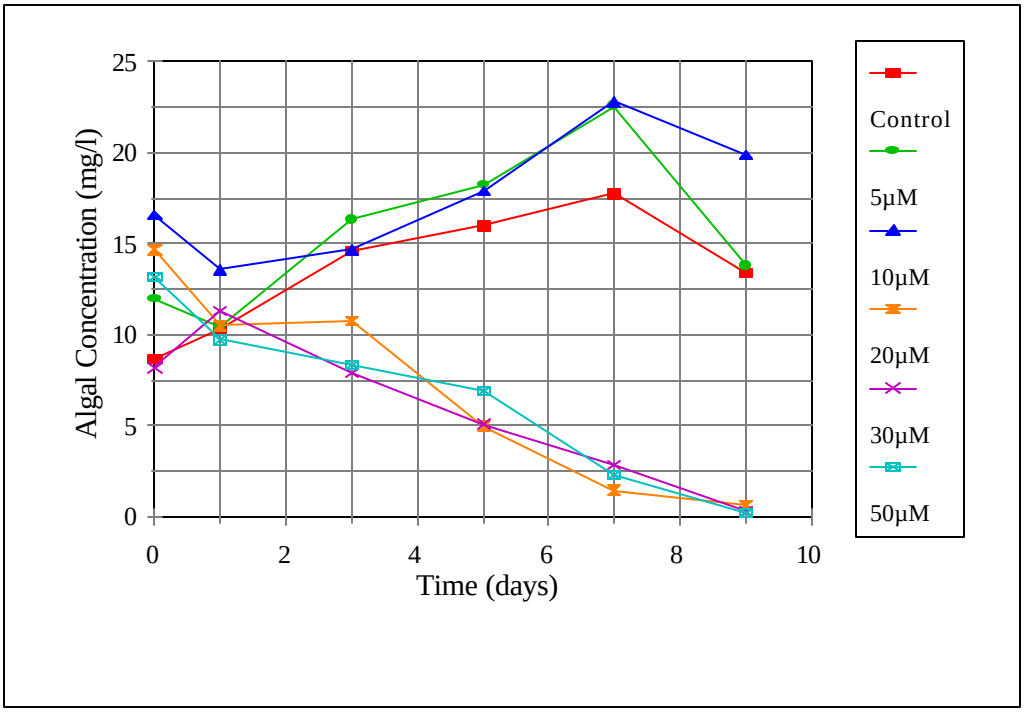


Figure 2.11: Growth curves of *Spirulina* cultures containing $ZnSO_4$

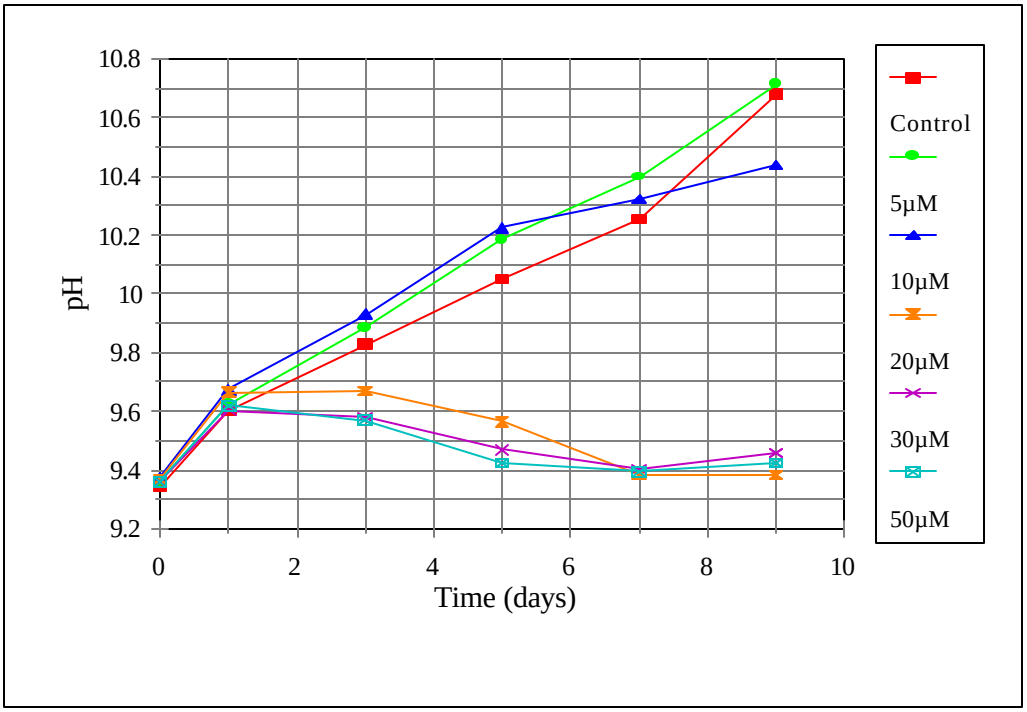


Figure 2.12: pH Profiles for zinc toxicity experiments

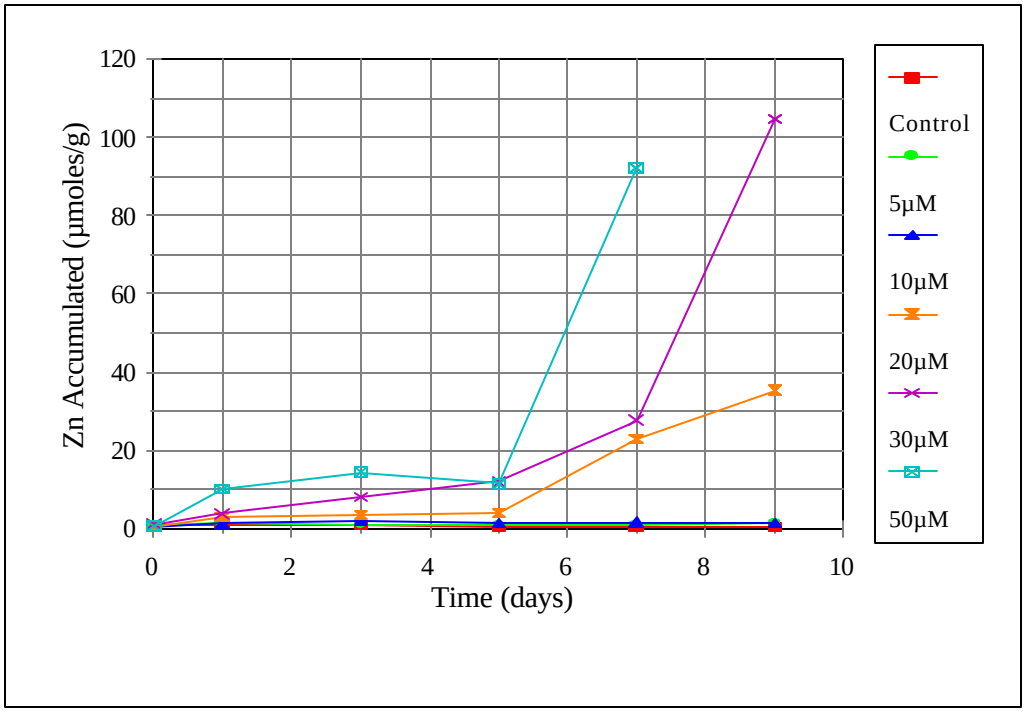


Figure 2.13: Zinc accumulated by *Spirulina* over 11 days

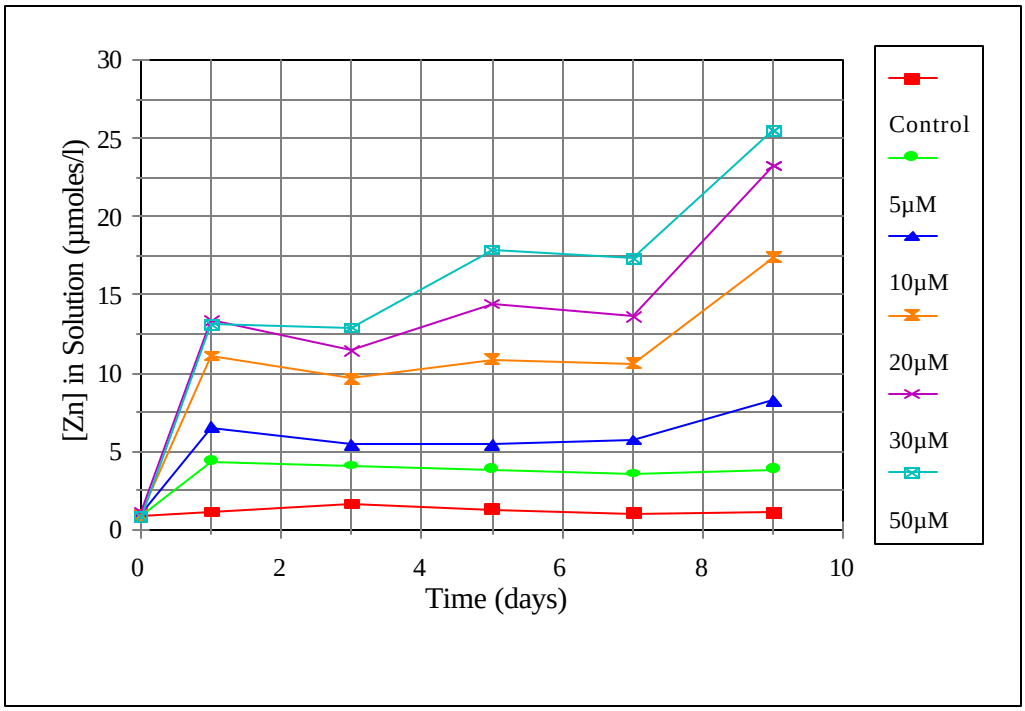


Figure 2.14: Zinc left in solution over 11 day period

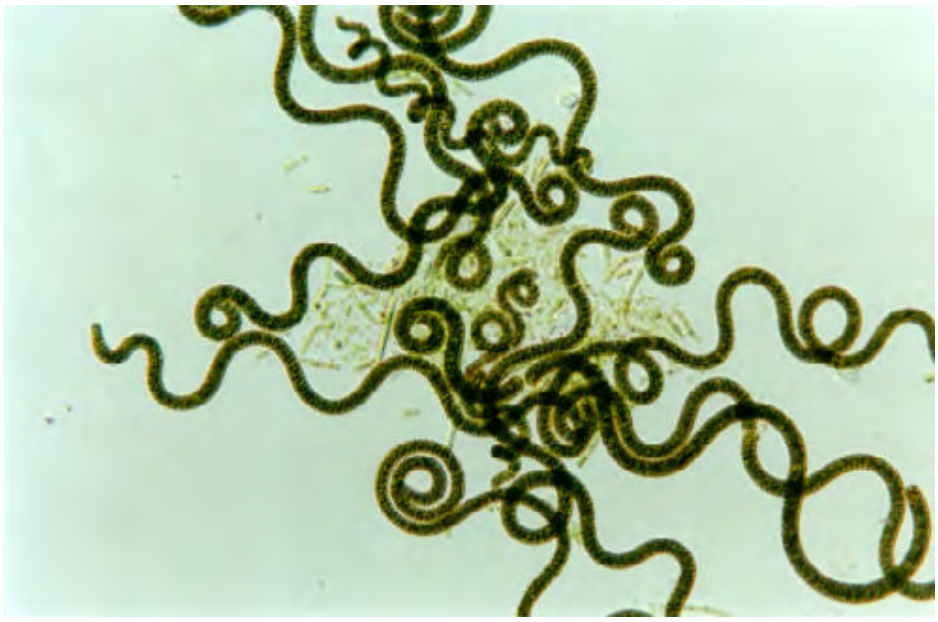


Figure 2.15: Light microscope preparation of *Spirulina* after 2 days in 50µM $ZnSO_4$

Figure 2.16: SEM preparation of *Spirulina* after 2 days in 50µM $ZnSO_4$

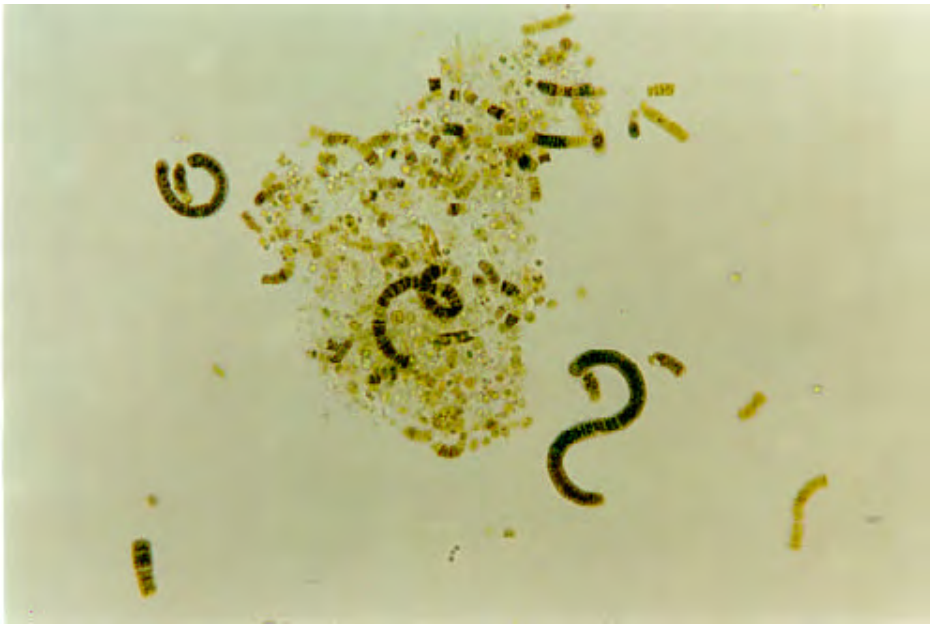


Figure 2.17: Light microscope preparation of *Spirulina* after 4 days in 50µM $ZnSO_4$

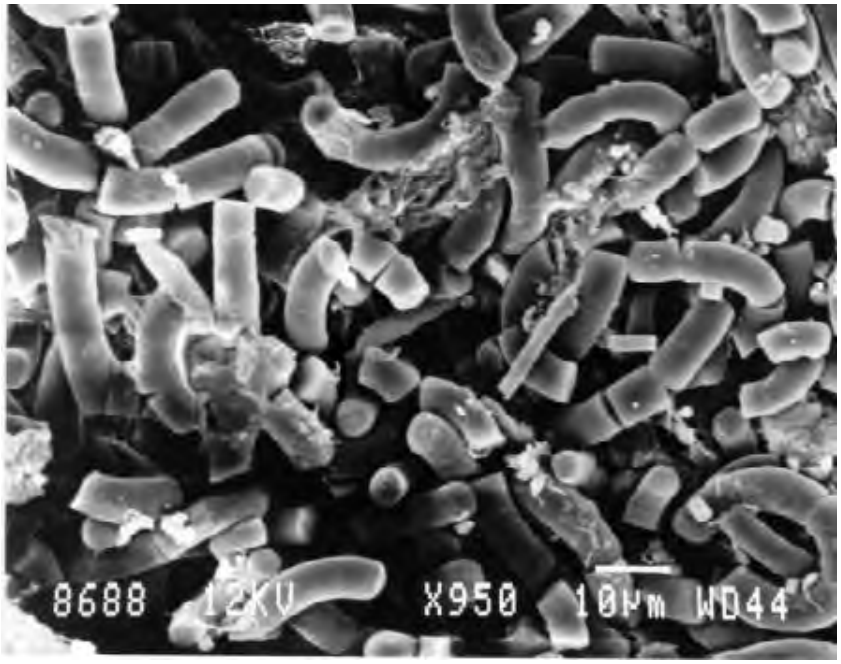


Figure 2.18: SEM preparation of *Spirulina* after 4 days in 50µM $ZnSO_4$

The results thus far suggest that *Spirulina* has approximately the same threshold levels for both zinc and copper, but the specific toxic effects vary between the two metals.

2.3.3 LEAD

The growth curves obtained from the lead experiments suggest that lead is not as toxic to *Spirulina* as copper or zinc. The first sign of any significant decline in algal concentration (Figure 2.19) was on day seven, at which stage growth in all groups, including the control started to decline. This suggests that the decline could be due to a depletion of nutrients in addition to lead toxicity. There was definitely some form of toxic effect seen in the 50 µM cultures (Figure 2.19), as there was no increase in algal concentration beyond day three, and the pH (Figure 2.20) of the 50 µM culture started to decrease from day three. These results indicate that there was no active growth in the 50 µM culture after day three. All the other cultures appear to have grown unaffected until day seven, when they all started to decline.

Once again there was a positive correlation between culture strength (Figure 2.19) and pH (Figure 2.20). A comparison of figure 2.20 with the pH graphs for copper and zinc (Figures 2.4 & 2.12) clearly shows the difference between lead and the other two metals with regards to pH, and hence cell function.

Although the lead did not appear to have a serious effect on the growth of *Spirulina*, it did cause drastic changes to the cell morphology (Figures 2.21 to 2.24). What, at first glance, appeared to be broken filaments in figure 2.21 were actually still connected. From figure 2.22 it is seen that the filaments seem to expel some of their cell contents, but still stay intact. By day four, a lot of debris is seen under the light microscope (Figure 2.23), but if compared to the day four photographs of copper and zinc (Figures 2.7 & 2.17) the appearance of the debris is different. In figure 2.7 and 2.17 pieces of broken filament could clearly be seen, but this was not the case with lead. The SEM photographs (Figures 2.22 & 2.24) suggest that the cell debris seen in figure 2.23 was cell contents alone, as the filaments were still more-or-less intact, although grossly distorted. Whole filaments could still be seen under the light microscope on day four (Figure 2.23).

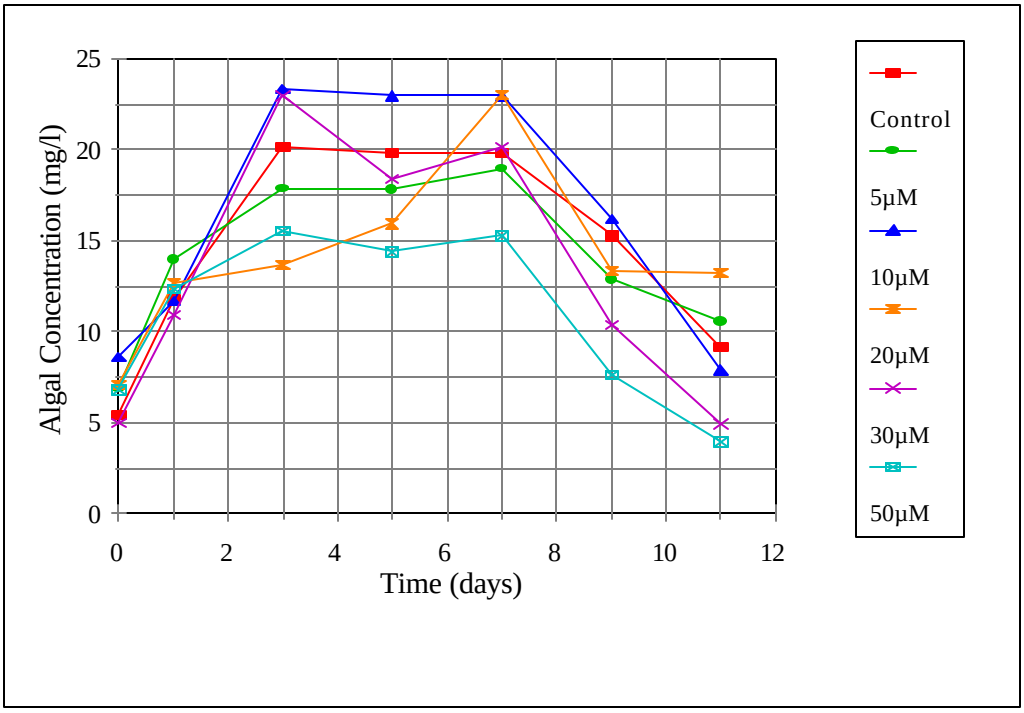


Figure 2.19: Growth curves of *Spirulina* cultures containing $Pb(NO_3)_2$

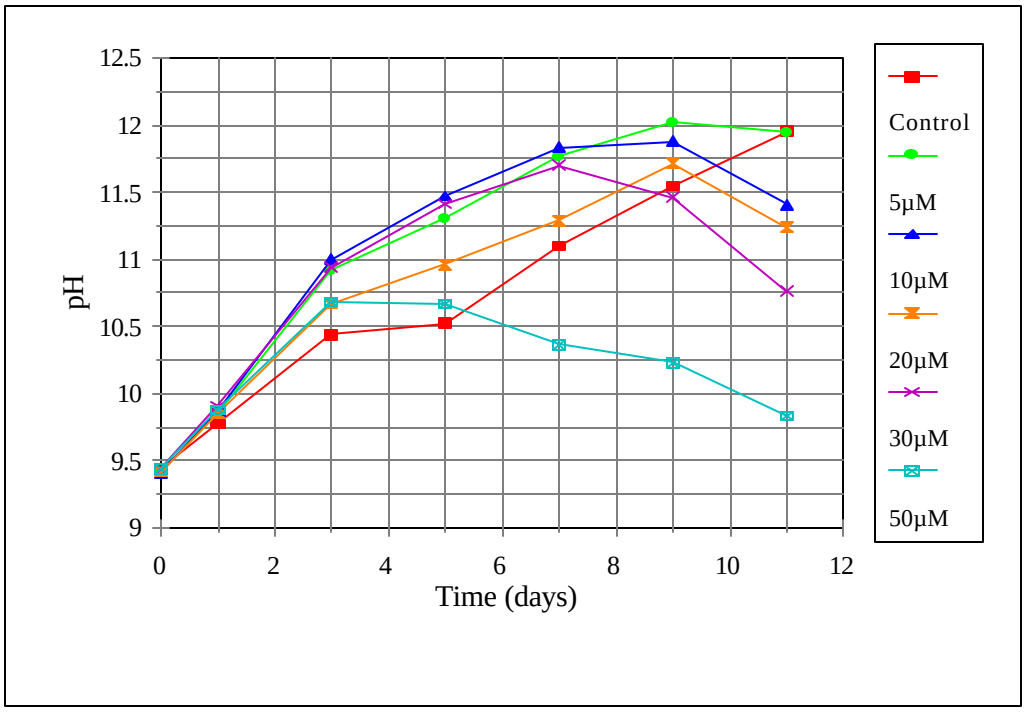


Figure 2.20: pH Profiles for lead toxicity experiments

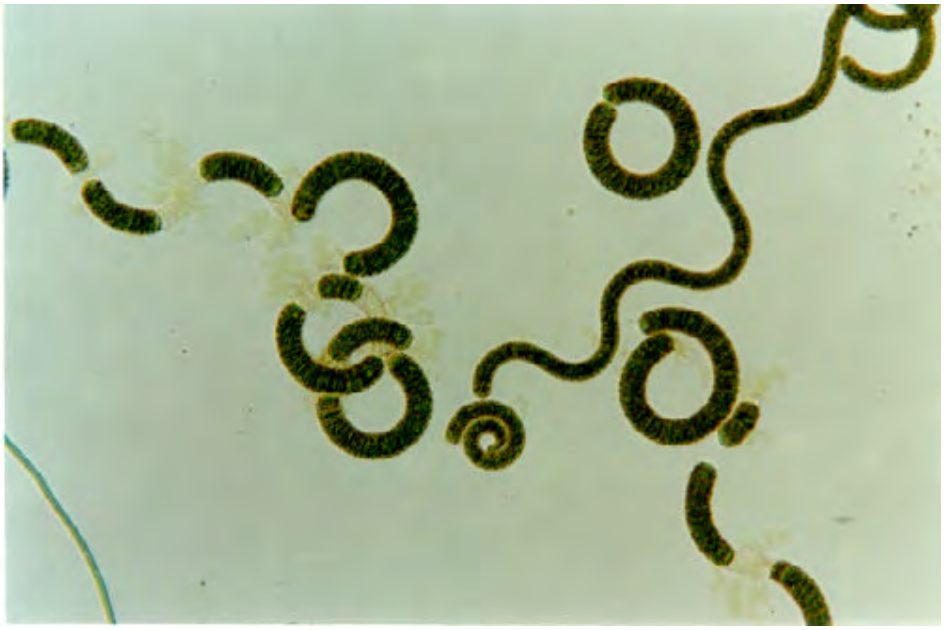


Figure 2.21: Light microscope preparation of *Spirulina* after 2 days in 50µM $Pb(NO_3)_2$

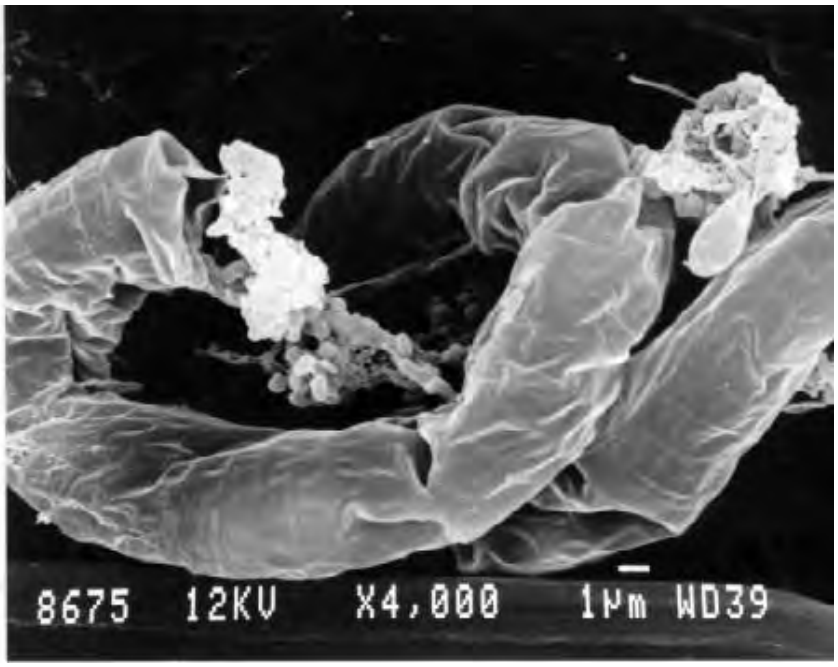


Figure 2.22: SEM preparation of *Spirulina* after 2 days in 50µM $Pb(NO_3)_2$

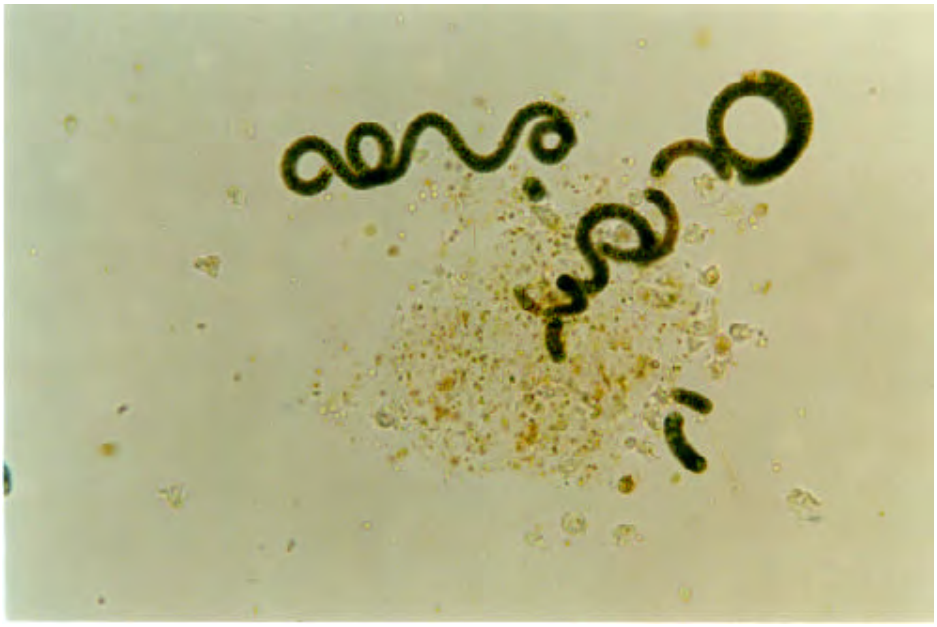


Figure 2.23: Light microscope preparation of *Spirulina* after 4 days in 50µM $Pb(NO_3)_2$

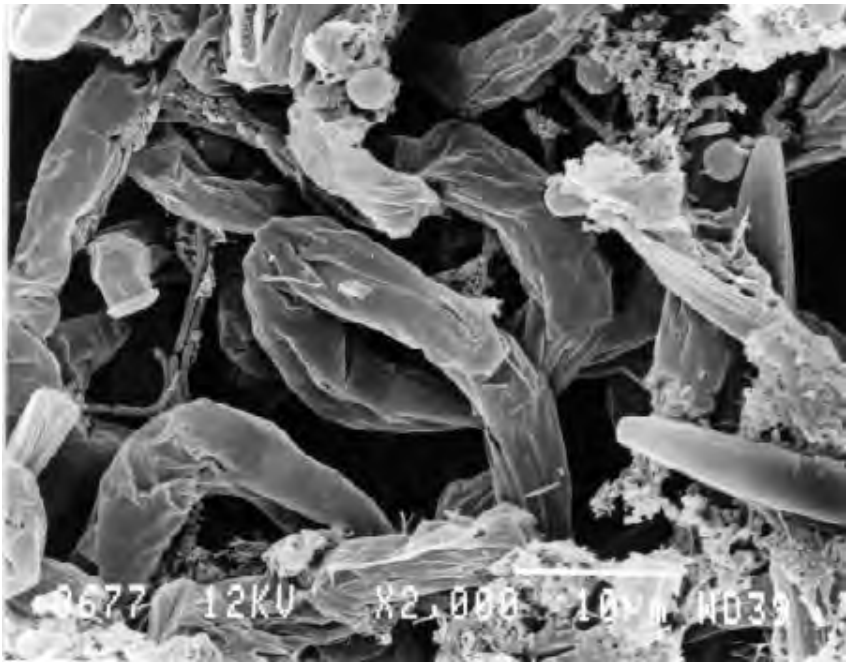


Figure 2.24: SEM preparation of *Spirulina* after 4 days in 50µM $Pb(NO_3)_2$

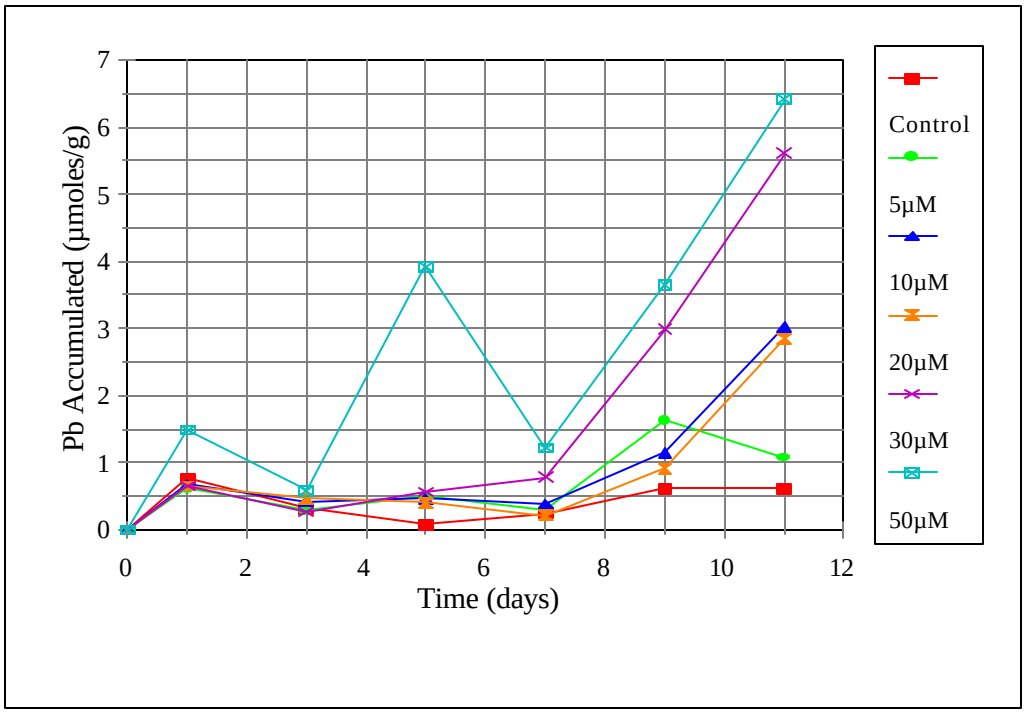


Figure 2.25: Lead accumulated by *Spirulina* over 11 days

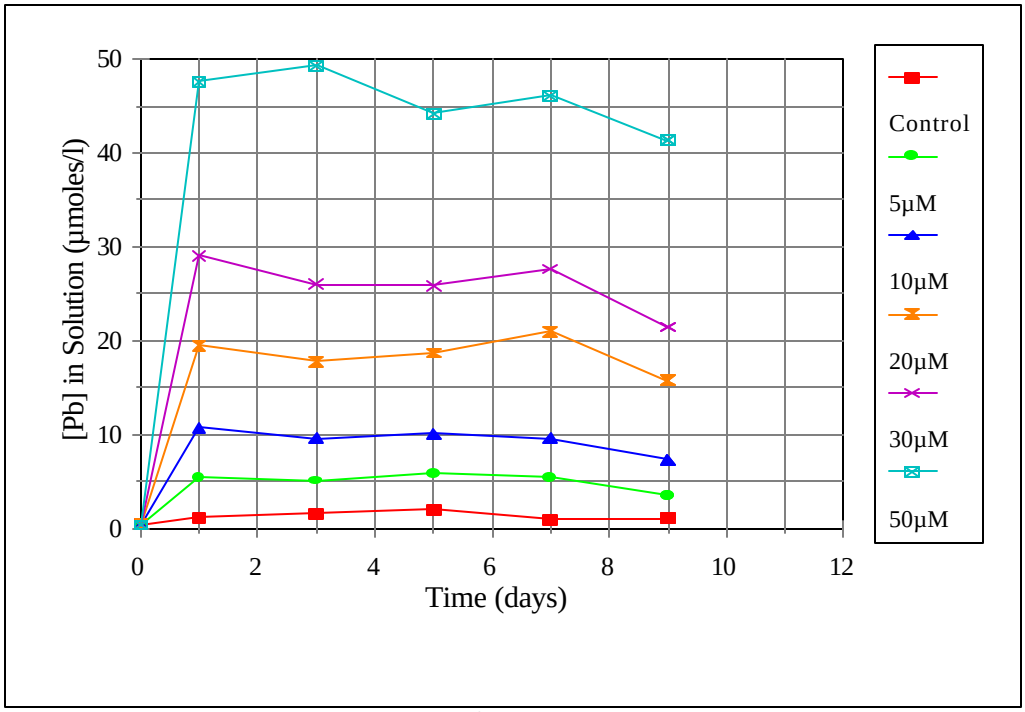


Figure 2.26: Lead left in solution over the11 day period

There did not appear to have been any precipitation of lead, as all the lead was accounted for (Figures 2.25 & 2.26). An uptake of about 5 µmoles/l occurred in the 50 µM culture between day three and day five (Figures 2.25 & 2.26). The sudden drop in the Pb accumulated by the 50 µM culture (Figure 2.25), between day five and day seven, could be due to the cell contents being expelled. Thus, any lead that had been taken up by the cells would have been released with the cell contents, probably complexed to soluble macromolecules. A very small decrease was seen in the Pb concentration in solution for the 30 µM culture, and even less for the 20 µM culture (Figure 3.26). This suggests that a very small proportion of the lead was taken up in the first two days after the metal addition. Virtually no uptake was observed for the other three groups. No additional metal was added during the course of these experiments, because the concentration of lead in solution (Figure 2.26) never decreased significantly enough to warrant further addition.

From these results it can be concluded that zinc does exert some form of toxic effect on *Spirulina*. The lead, however, affects the cells via a different mechanism to that of copper and zinc, which ultimately takes longer to affect the cells. It appears clear that the lead does not affect the photosynthetic pathway directly, as the copper and zinc do. This suggests that the decrease in chlorophyll *a*, recorded in the 50 µM culture could be a downstream result of the shutting down of other metabolic pathways.

In the cultures exposed to copper and zinc, where the photosynthetic pathway was directly affected, cell death may have resulted due to energy starvation as a result of inefficient photosynthesis. Samples were collected for ATP analysis to try and confirm this, but ATP could not be detected due to problems with the luminometer.

2.4 CONCLUSIONS

Spirulina was found to have a threshold level of about 30 µM for copper, zinc and lead. Copper and zinc appeared to have a direct effect on the photosynthetic pathway, thereby causing a rapid decline in cell growth. Lead on the other hand seemed to affect surface properties and hence took longer to cause deterioration in growth. It can hence be concluded that *Spirulina* has little or no potential as a biosorbent. It does,

however, have potential as a precipitation agent through its ability to maintain a high pH, possibly through the enzyme carbonic anhydrase. An interest in this process prompted an investigation into the enzyme CA, which will be discussed in the following chapters.

CHAPTER THREE

ASSAYS FOR CARBONIC ANHYDRASE

3.1 INTRODUCTION

Carbonic anhydrase (CA) has been studied for many decades, and is the subject of a large body of literature. This is due to its biological importance, and the fact that it has one of the highest turnover rates of any known enzyme. Ever since the enzyme was first discovered and purified in 1933, the quantitative determination of CA activity has been a stimulating analytical target. Many different analytical methods have been devised to assay for activity, but they all have serious disadvantages which strongly limit their use (Botré & Botré, 1990; Stemler, 1993).

Carbonic anhydrase plays a central role in the ability of *Spirulina* to increase the pH of the surrounding medium. This increase in pH leads to the precipitation of metal ions, and therefore, *Spirulina* can be utilised in the removal of these ions from contaminated effluents. A reliable assay is, however, required in order to screen *Spirulina*, as well as other potential bioremediation agents, for CA activity.

The standard and most widely used method is the Wilbur-Anderson method (1948), which measures the time taken for a decrease in pH when CO_2-saturated water is injected into a reaction mixture containing CA. The hydration of CO_2 liberates a proton, thus lowering the pH. This assay, however, requires non-physiological conditions. The temperature must be near 0°C, and the pH changes continuously during the assay. This method does not give a linear response over a broad enzyme range, and cannot accurately measure small differences in activity (Stemler, 1993). Introduction of whole plants, or parts thereof, interferes with measurements, and has been seen to cause significant negative values for CA activity (Giordano and Maberly, 1989). When used for determinations of external CA activity on macroalgae, the

method is insensitive, and there is a possibility that low external CA activities, which may still be significant, might be disregarded (Haglund *et al*, 1992).

A colorimetric version of the Wilbur-Anderson method, where an indicator is used to determine the endpoint, has also been used. A disadvantage of this method has been the inhibitory effects of the indicator (Rickli *et al*, 1964; Wilbur & Anderson, 1948).

A second method involves the use of a mass spectrophotometer. The reversible hydration of CO_2 results in the exchange of oxygen atoms between CO_2 and H_2O. This property can be used to measure CA activity by following the unlabelling of exogenous CO_2 enriched with ^{18}O. This is a sensitive and accurate method, which has been successfully used in liquid systems such as animal and algal cell suspensions, but it is slow and requires the dedicated use of a mass spectrophotometer (Silverman, 1973; Stemler, 1993; Peltier *et al*, 1995).

A third method, the stopped-flow, which measures the colour change of a pH-sensitive dye, also involves expensive equipment. In this case a spectrophotometer with a number of special accessories is required. This method has many advantages, but the equipment is inaccessible to most laboratories (Stemler, 1993).

The rate of CO_2 production from a buffered solution of HCO_3^- can also be measured by manometry (Botré & Botré, 1990; Sashidhar *et al*, 1990; Wilbur & Anderson, 1948). Small pressure changes are, however, often difficult to measure using this method. The Gilson differential respirometer, which has often been used for CA activity estimation, also suffers from this disadvantage (Sashidhar *et al*, 1990). Instead of determining pressure changes in the dehydration reaction, Sashidhar and associates (1990) used an infra-red gas analyser to determine the increase in CO_2 content. Although this method is accurate and reliable, the equipment is also not readily available to most laboratories.

Colorimetric assays have also been developed to measure the esterase and aldehyde hydration activities of CA. Although esterase activity can be determined with greater ease and accuracy than CO_2 hydration, these assays are typically 1000-fold less sensitive than other assays. This low sensitivity, and the fact that that a secondary and

perhaps non-physiological reaction is monitored, makes these methods undesirable for most studies (Armstrong *et al*, 1966; Mercado *et al*, 1997; Stemler, 1993).

Stemler (1993), and Katzman and colleagues (1994) have reported two different radiotracer assays, which show some promise. If radiolabelled bicarbonate is added to a system containing CA it should be converted to radiolabelled carbon dioxide, which, if successfully trapped, can be measured.

The aim of these experiments was to find a reliable method for assaying carbonic anhydrase, which could be used in a subsequent attempt to purify the enzyme from *Spirulina*. The radiotracer assays mentioned above were investigated, as well as the well-recognised Wilbur Anderson assay. Detection of protein by SDS polyacrylamide gel electrophoresis (SDS-PAGE) was also investigated.

3.2 METHODS AND RESULTS

3.2.1 RADIOTRACER ASSAY 1

3.2.1.1 MATERIALS

Commercially pure bovine carbonic anhydrase (CA), with an activity of 5200 Wilbur-Anderson Units (WAU) per milligram, was obtained from Sigma. Sodium [^{14}C] bicarbonate (specific activity: 55 mCi/mmol & radioactive concentration: 2.0 mCi/ml) was obtained from Amersham Life Sciences. Scintillator Plus^{TM} was purchased from Packard, and GF/B glass microfibre filters were obtained from Whatman. Ultra-pure deionised water, purified by a Milli-Q water system, was used for the preparation of all standards and buffers.

3.2.1.2 PREPARATION OF STANDARDS

A bovine CA stock solution of 150 WAU/ml was prepared. Aliquots of this stock solution were used to prepare 1 ml standards with concentrations ranging from 5 WAU/ml to 50 WAU/ml. 100 µl aliquots of the standards were used in each individual assay.

3.2.1.3 SAMPLE PREPARATION

Spirulina was grown in Zarrouk's media (Appendix A) under constant environment conditions. A 5 ml sample was taken and the chlorophyll extracted. A 10 ml sample was put on ice and sonicated twice for 15 seconds at 60% amplitude using a VibraCell sonicator.

3.2.1.4 ASSAY

Numerous assays were run in order to optimise the procedure with regards to the size and volumes used as well as fixation time of liberated $[^{14}C]O_2$.

Discs of 1.6 cm diameter were cut from Whatman GF/B glass microfibre filters using a No. 11 cork borer and placed at the bottom of a 1.6 cm diameter glass collecting vial. A 200µl sample of healthy *Spirulina* culture was placed on the centre of each disc to act as a CO_2 trap. A glass scintillation vial was then inoculated with 100 µl 20 mM Tris-HCl buffer (pH 8.2) and 100 µl of the sonicated sample (or commercial bovine CA in the case of the standards). The trap vial was then inverted over the sample vial, and the vials were sealed together using masking tape (Figure 3.1).

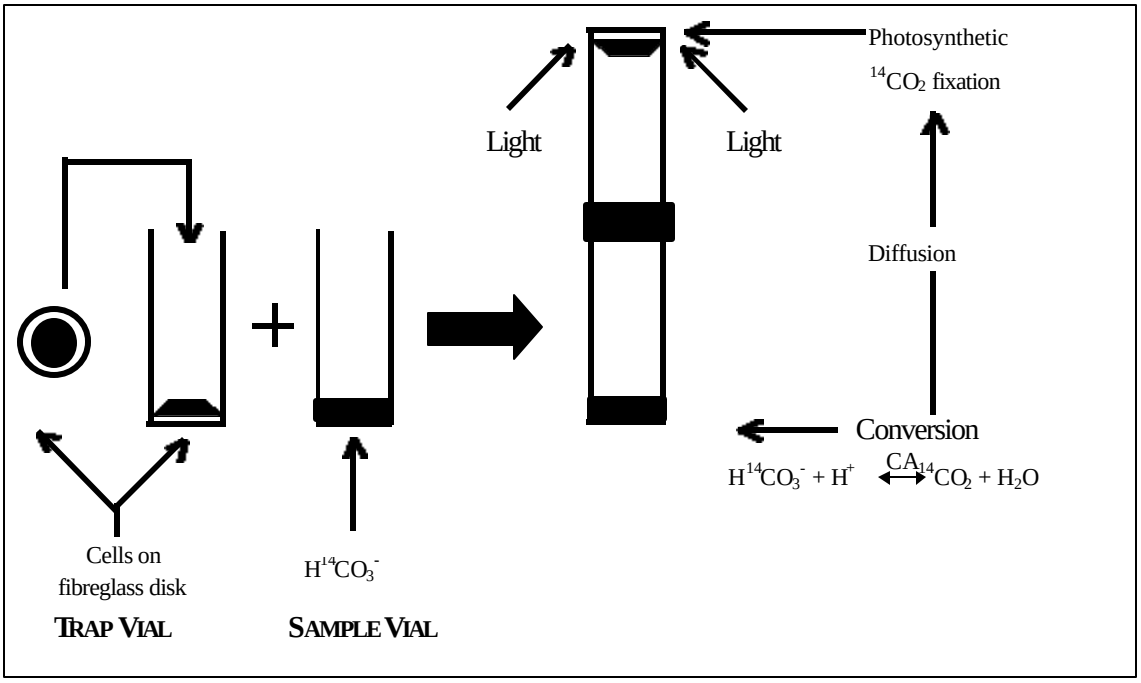


Figure 3.1: Diagram demonstrating the principle behind radiotracer assay 1

The entire assembly was placed in a beaker of ice with the sample side down. The beaker was placed on an orbital shaker at approximately 150 rpm. Four 20W cool white fluorescent lamps, two on either side of the apparatus, were turned on for a three minute pre-illumination period. The sample assembly was then removed from the apparatus, the trap vial removed and 2.75 µl NaH[^{14}C]O_3 (5.5 µCi) was added to the sample vial. The vials were then reassembled and placed back on ice on the orbital shaker. The lights were immediately turned on for a nine minute CO_2 fixation period, after which they were turned off and the sample assembly quickly removed, buried in ice and placed in the dark.

The sample assembly was then disassembled in a fume hood, and 10 µl was immediately removed from the sample vial and placed in a scintillation vial containing 5 ml scintillation fluid. To the trap vial, 100 µl glacial acetic acid was added and allowed to dry on a hot plate. When dry, the discs were removed and added to a scintillation vial containing 5 ml scintillation fluid. Radioactivity was quantified by liquid scintillation spectroscopy using a Beckman LS 9000 scintillation counter calibrated to read a full window and with external quench correction. Appropriate blanks were run simultaneously in order to determine the degree of quenching.

As a control, the degree of spontaneously (non-enzymatically) liberated [^{14}C]O_2 was assessed in parallel runs, by omitting any source of CA from the sample vial. In addition the fixed, total amount of radioactivity that was added in all assay runs was quantified, and the amount of [^{14}C]O_2 liberated (spontaneously or enzymatically) was calculated by determining the reduction in total radioactivity.

The preliminary results showed that sample sizes of 100 µl in a total volume of 200 µl, run for a nine minute fixation period, were the optimum conditions for this assay method. All subsequent assays were run, in duplicate, according to these parameters.

All results obtained, which were expressed in DPMs, were converted to moles. The results for the standards on the trap vials were used to plot a standard curve showing moles of [^{14}C]O_2 fixed versus Wilbur-Anderson Units. The results obtained were only linear for the first four points up to 1.5 WAU (r^2 = 0.986), which was in

agreement with the results obtained by Katzman and associates (1994), hence an extended graph, of the linear region only, was plotted (Figure 3.2).

The control that was assayed in the absence of any form of enzyme had a radioactivity of 1.39×10^{-11} moles in the trap vial, and 1.39×10^{-9} in the sample vial. The initial radioactivity added before the assay was 3.61×10^{-9} moles, thus a 61.5% removal of $NaH[^{14}C]O_3$ was observed. Of this, only 0.6% was fixed as $[^{14}C]O_2$ in the trap vial. This shows that there was a very high percentage of spontaneous liberation of the $^{14}[C]O_2$ in the absence of any enzyme and that a great deal of $[^{14}C]O_2$ was lost during the course of the assay. Remaining $[^{14}C]O_2$ was postulated to be present in the air.

As a comparison, a standard curve was plotted using the results from the sample vials (Figure 3.3). The results were converted to percentage liberation. The results obtained from the control were also converted to percentage liberation, and this value was subtracted from the standards in order to account for any spontaneous liberation that may have occurred.

Once the standard curves had been generated, a fixed amount of sonicated *Spirulina* was assayed for CA activity. The radioactivity obtained in the trap vial for the sample was 8.04×10^{-12} moles, which was too low a value to read off the standard curve.

The radioactivity obtained from the sample converted to a percentage removal of 70.09%. When the control value (61.5%) was subtracted, the sample showed a percentage removal of 8.59%, which converted to 0.9 WAU. The dry mass of the algae, calculated from the chlorophyll extractions, was found to be 5.13 mg. Therefore, the enzyme activity of the sample was 0.175 WAU/mg algae.

The value obtained from the second standard curve (Figure 3.3) is probably a more accurate estimation of activity, because spontaneous breakdown of $NaH[^{14}C]O_3$ and any loss of $[^{14}C]O_2$ was accounted for, assuming that any loss was the same as that in the control.

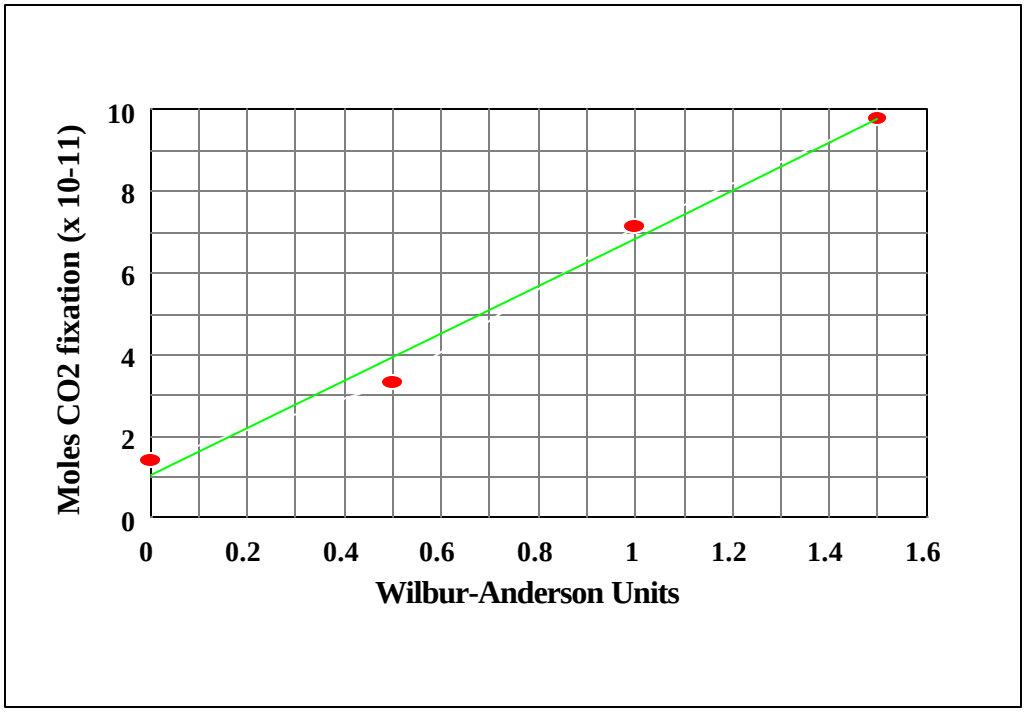


Figure 3.2: Correlation between CA and [^{14}C]O_2 fixation (r^2 = 0.94)

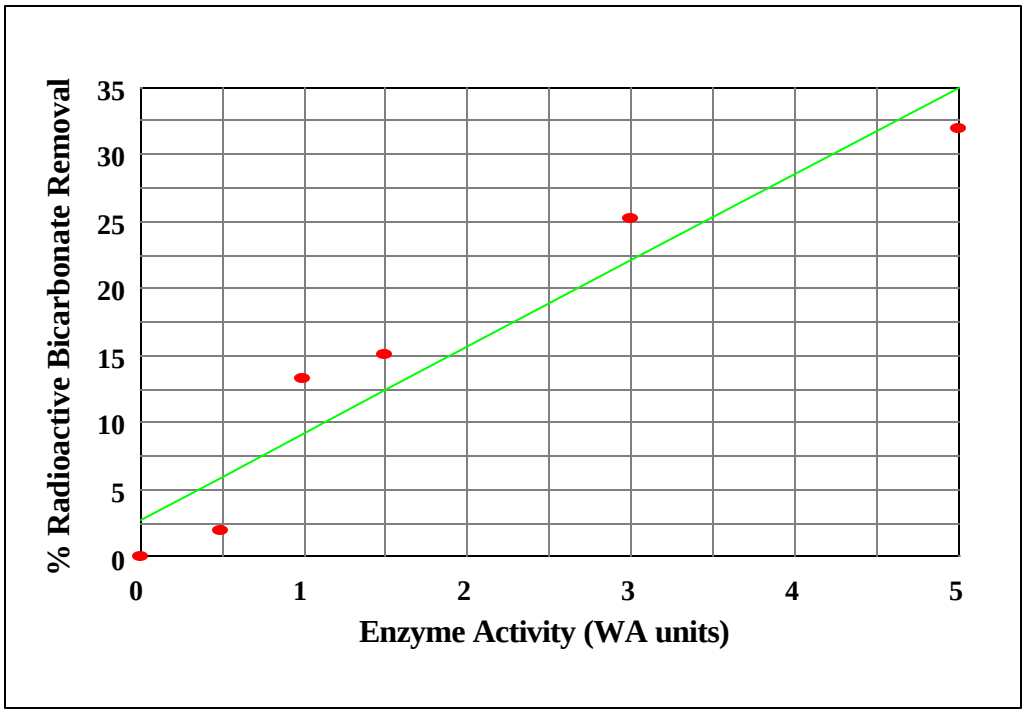


Figure 3.3: Correlation between CA activity and % liberation (r^2 = 0.96)

These results suggest that there was in fact some CA activity in the *Spirulina*, but there were several problems with the assay method, which may have led to the very inaccurate results. A great deal of variation was seen between duplicates of the same samples, and the reproducibility of the experiment was poor.

A very high percentage of the $NaH[^{14}C]O_3$ was lost due to spontaneous breakdown. This caused the control values to be very high in comparison to the sample values for the trap vial, which could have led to a great deal of the variability observed in the results. This problem would have to be resolved in order for this assay method to be effective. The buffer could also have caused the problem. It is possible that the Cl^- ions from the HCl in the buffer may have been enough to react with the $NaH[^{14}C]O_3$ and caused liberation of $[^{14}C]O_2$. The use of a different buffer, such as EPPS (Katzman *et al.*, 1994), could possibly help in reducing this problem.

The variability in the results may also have been due to the very small volumes used. When working with such small volumes, accuracy is a problem and small discrepancies can lead to relatively large variations in results, especially when working with radioactivity.

The masking tape may not have been effective at sealing the sample assembly apparatus, leading to some loss of $[^{14}C]O_2$. Some $[^{14}C]O_2$ may also have been lost when the sample assembly was opened up at the end of the assay.

This assay is limited by the CO_2 fixation rate in the trap vial. In these experiments, the CO_2 fixation was found to be very low. When the sample was assayed, only 0.3% of the $[^{14}C]O_2$ that was liberated was fixed. In the control, which contained no CA, 0.6% was fixed, which is very low, but higher than the sample. This explains why the sample from the trap was too low to read off the standard curve (Figure 3.2).

CA catalyses a reversible reaction (Shiraiwa *et al.*, 1993: Stemler, 1993). It is therefore possible that some of the $[^{14}C]O_2$ formed, may have converted back to $NaH[^{14}C]O_3$ due to some unforeseen physiological parameters. This could be a possible reason why the $[^{14}C]O_2$ fixation was so low.

A larger volume could not have been added to the trap vial because it had to be inverted over the sample vial. However if a more concentrated algal culture was used, the rate of the CO_2 fixation may have been higher. Optimisation of the apparatus could also lead to an increased rate of CO_2 fixation. If a system of blocks, similar to that used by Katzman *et al*. (1994) could be constructed, with mirrors placed such that the light is reflected up to the cells in the trap vial, photosynthesis could be increased, thereby increasing CO_2 fixation.

Due to the fact that the CO_2 fixation was not very effective, the necessity of the trap vial was considered. The most accurate results were obtained from sample vials, hence the possibility of a system without the CO_2 trap was considered. The disadvantage of this is that a certain amount of $[^{14}C]O_2$ may remain in solution as dissolved CO_2. Thus the radioactivity observed in the sample vial may include some dissolved $[^{14}C]O_2$ as well as the remaining $NaH[^{14}C]O_3$.

Numerous attempts were made to improve this assay system, but were unsuccessful. Although the results obtained were not very accurate, some CA activity was found. This prompted further investigation into another radiotracer based assay used by Stemler (1993).

3.2.2 RADIOTRACER ASSAY 2

3.2.2.1 MATERIALS

Commercially pure bovine CA, Sodium $[^{14}C]$ bicarbonate (specific activity: 55 mCi/mmol & radioactive concentration: 2.0 mCi/ml), Scintillator Plus^{TM} and the GF/B microfibre filters were the same as those for the previous experiment. Polyvinylidene difluoride (PVDF) transfer membranes were obtained from Millipore.

3.2.2.2 PREPARATION OF STANDARDS

Standards were prepared from a 150 WAU/ml stock solution of bovine CA, as for the previous experiment.

3.2.2.3 ASSAY

The second assay investigated is based on the diagram shown in figure 3.4. The main component of the assay system is a 65 mm high cylindrical glass reaction vessel with a diameter of 15 mm. A PVDF transfer membrane of the same diameter, which has a high permeability to gases, is attached to the bottom of the reaction vessel using Pratley Quickset. A GF/B glass filter (20 mm diameter) placed in the bottom of a scintillation vial is wet with 40 µl NaOH, which acts as a $[^{14}C]O_2$ trap. The glass reaction vessel containing a small stirrer bar is placed inside the scintillation vial. Reaction mixture containing buffer and CA (commercially pure bovine CA was used in all these experiments) is then added to the reaction vessel.

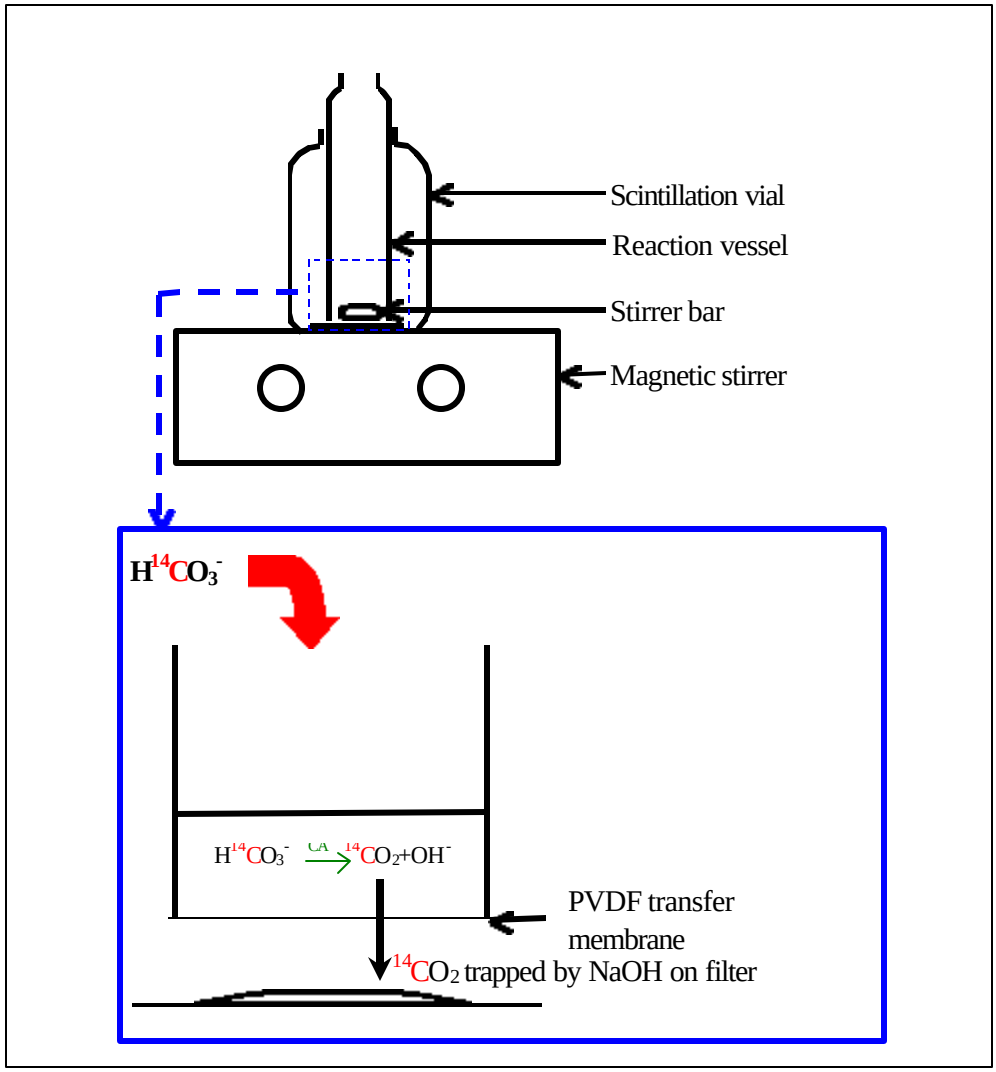


Figure 3.4: Diagram illustrating principle behind radiotracer assay 2

The scintillation vial and its contents are placed on a magnetic stirrer and 50 µl $NaH[^{14}C]O_3$ is injected into the reaction mixture at time zero. The radioactive bicarbonate is converted to $[^{14}C]O_2$ which diffuses across the membrane and is trapped by the NaOH-impregnated glass filter. The amount of $[^{14}C]O_2$ that is trapped in a short time is a function of the carbonic anhydrase activity in the reaction mixture. The reaction is stopped, usually after 5 or 10s, by simply removing the reaction vessel from the scintillation vial. Scintillor Plus™ (5 ml) is then added to the vial and radioactivity quantified as for the previous assay. Results are expressed in DPMs.

Various experiments were run in order to determine optimal parameters for the assay. After some experimentation it was decided to use a 200 µl reaction mixture containing 100 µl 20 mM Hepes buffer, pH 7.4, and to run the assay for 10s, using 20 mM $NaHCO_3$ such that 50 µl contained 0.2 µCi $NaH[^{14}C]O_3$. The results obtained from assays run in this manner, using commercially pure CA appeared to be relatively linear (r^2 = 0.95), and repeatable (Figure 3.5). However, due to the fact that this is an open system, the possibility that $[^{14}C]O_2$ was escaping into the air became a concern.

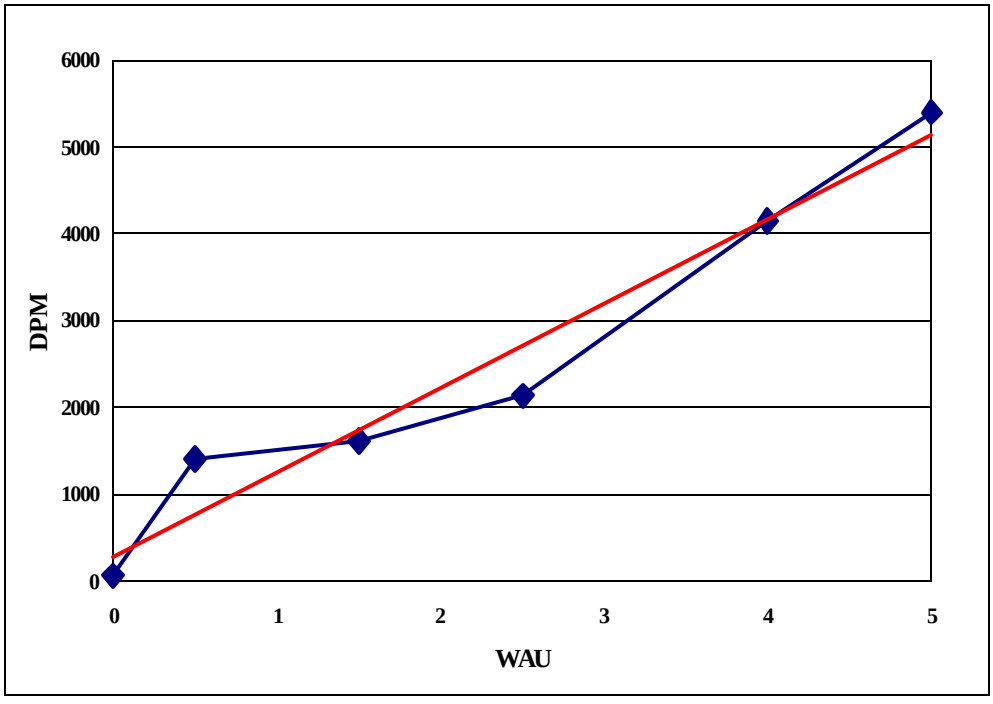


Figure 3.5: Results obtained from initial radiotracer 2 assays using commercially pure bovine CA (r^2 = 0.95)

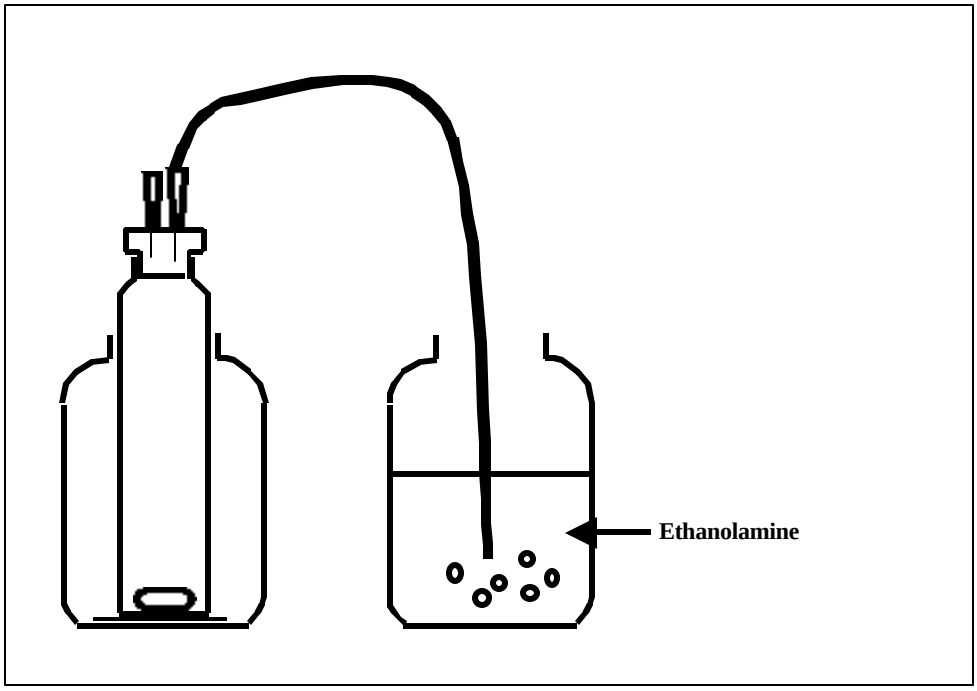


Figure 3.6: Diagram illustrating experiment to determine loss of $^{14}[C]O_2$

In order to determine how much $[^{14}C]O_2$ was being released into the air the reaction vessel was sealed with a rubber stopper with two needles placed in it (Figure 3.6). A piece of rubber tubing lead from the one needle into a second scintillation vial containing ethanolamine. The $NaH[^{14}C]O_3$ was injected through the second needle. Any $^{14}[C]O_2$ that would previously have escaped should now have bubbled through the tubing into the ethanolamine. Scintillation fluid was then added to the vial and radioactivity measured.

The results from this experiment indicated that very little $^{14}[C]O_2$ was escaping (Figure 3.7), the radioactivity found in the ethanolamine was just above the baseline count. It was, however, decided that, from a safety perspective, it was a good idea to use a sealed system anyway.

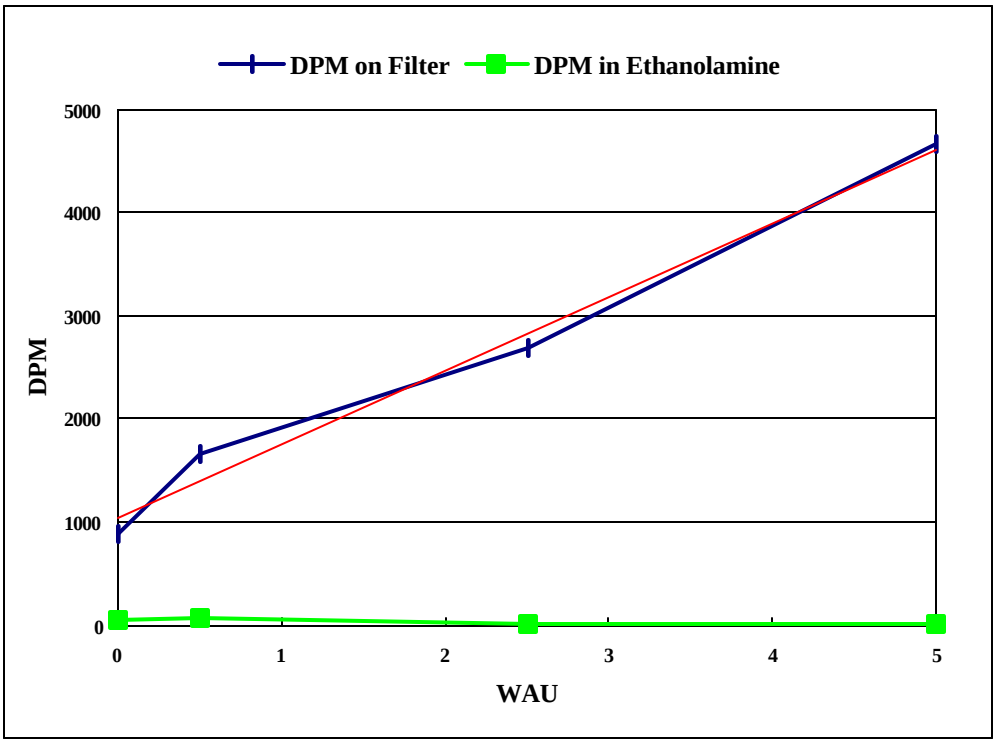


Figure 3.7: Results from experiment to determine loss of $^{14}[C]O_2$

Initially the $NaH^{14}[C]O_3$ was injected through a needle in the stopper using an appropriate syringe. When the initial experiments were repeated using this closed system, the results obtained showed no correlation to the CA concentrations used (Figure 3.8), with an r^2 value of only 0.001. It was therefore decided to use a Hamilton syringe to deliver the $NaH^{14}[C]O_3$, but again the results obtained were meaningless. It was therefore obvious that there was a problem with the delivery system in the sealed apparatus. When using small volumes, such as those in this assay, small discrepancies in delivery can lead to large differences in results.

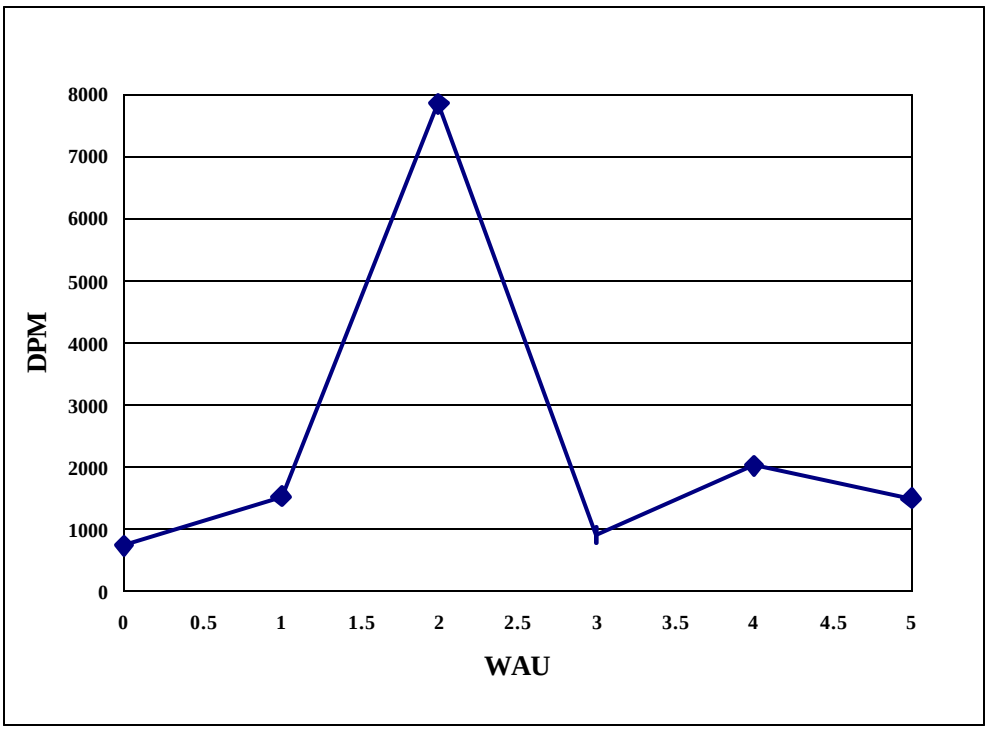


Figure 3.8: Results from experiments using a sealed system ($r^3 = 0.001$)

After spending considerable time experimenting unsuccessfully with the closed system it was decided to return to the original open system. One would have expected the results to be comparable to the original results (Figure 3.5), however, this was not the case (Figure 3.9). Regression analysis of the results gave a r^2 value of only 0.1 although there did appear to be a general increase in DPM's with increasing CA. After some analyses it was concluded that this method is not accurate for small differences in concentration, there are too many factors leading to variability in results. It was also concluded that the non-radioactive $NaHCO_3$ was outcompeting the radioactive, thus leading to an inaccurate assessment of CA activity.

Figure 3.9: New Results from Open System (r^2 = 0.10)

Figure 3.10: New results using pure NaH[^{14}C]O_3 (r^2 = 0.74)

It was hence decided to make up a new set of standards over a broader range of concentrations (2 – 200 WAU/ml), and at bigger increments. These were used in combination with pure radioactive NaH[^{14}C]O_3 (0.2 µCi/50 µl). The results are shown in figure 3.10.

Although the results were still not linear (r^2 = 0.74), they were better than for the previous experiment. The DPM's were, however, 10-fold lower than those in figure 3.9. This was probably due to the substrate concentration being too low (70 µM). Due to the decrease in non-radioactive NaH[^{14}C]O_3 the overall substrate concentration was decreased and was no longer saturating.

The assay was again repeated, this time using 2mM NaH[^{14}C]O_3 (5.5 µCi/50 µl). The results obtained showed DPMs comparable to those in figure 3.9, although the r^2 value of 0.75 was still low (Figure 3.11). This confirmed that substrate concentration was a problem, but if one were to use this assay with such high concentrations of NaH[^{14}C]O_3 it would not be economically viable. Radiolabelled compounds are very expensive and very large quantities would be used if assaying large numbers of samples.

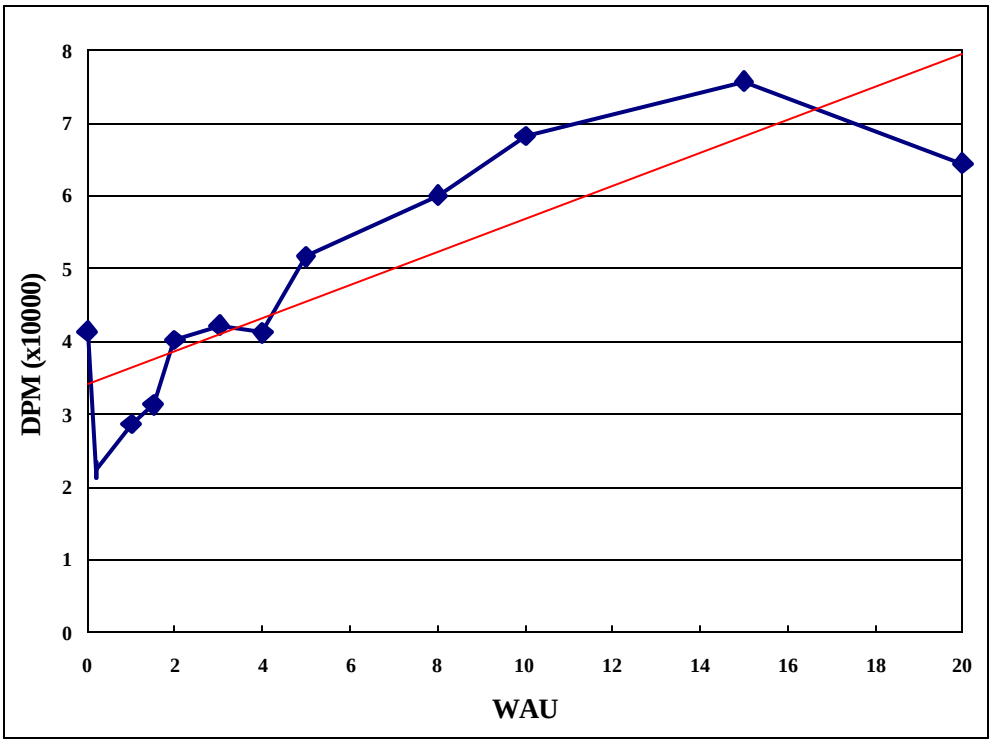


Figure 3.11: Results from assay using 2 mM NaH[^{14}C]O_3 (r^2 = 0.75)

A great deal of time had been spent on this assay system, and in addition to the expense, an accurate and reproducible protocol was still not possible. Consequently it was decided to investigate the commonly used Wilbur Anderson assay.

3.2.3 WILBUR-ANDERSON ASSAY

3.2.3.1 MATERIALS

New commercially pure bovine CA, with an activity of 8100 Wilbur-Anderson Units (WAU) per milligram, was obtained from Sigma Aldrich. Barbitone and sodium barbitone were purchased from Merck.

3.2.3.2 PREPARATION OF STANDARDS

Standards ranging in concentration from 10 to 150 WAU/ml were prepared from a bovine CA stock solution of 150 WAU/ml as for the previous assays. 100 µl aliquots of the standards were used in each individual assay.

3.2.3.3 SAMPLE PREPARATION

Spirulina was grown in Zarrouk's media under constant environment conditions. Cells were harvested by centrifugation at 2000g for 10 min in an Eppendorf centrifuge 5403. Pellets were washed in an equivalent volume of deionised water (Milli-Q System, Millipore) and centrifuged at 7 000g for 20 min. The pellets were then resuspended in an appropriate volume of 20 mM Veronal buffer (pH 8.3). Aliquots of 200 µl were used in each individual assay.

3.2.3.4 ASSAY

This assay was based on that of Wilbur and Anderson (1948) and modified according to Hiltonen and associates (1995) and Yang and associates (1985). CA activity was determined electrochemically by measuring the time taken for the pH to decrease from 8.2 to 7.2 when 2ml of ice-cold CO_2 saturated deionised water was added to 4

ml Veronal buffer (20mM, pH 8.3, 4ºC) containing the test material. One unit of activity (WAU) was defined as:

$$WAU = t_0 / t - 1$$

Where t_o and t were the times taken in enzyme-free buffer (control) and buffer containing the sample, respectively. CO_2 saturated water was prepared by bubbling CO_2 through 400 ml deionised water at 4ºC for 1 hour before use. All assays were performed in triplicate.

Initial studies using commercially pure bovine CA made up to known WAUs showed that the assay is not very accurate (Table 3.1). The margin of error appeared to be greater with higher WAUs. The standard error figures demonstrate that there was also a significant degree of variability within analogous samples (Table 3.1).

Table 3.1: Comparison between expected and observed CA activity units from commercially pure bovine carbonic anhydrase

Expected WAUs	Observed WAUs	Difference	Standard Error
1	1.296	0.296	0.351
2	4.107	2.107	0.496
3	7.791	4.791	0.256
4	10.114	6.114	0.560
5	12.579	7.579	0.570
15	21.179	6.579	0.748

Initial experiments on *Spirulina* also showed considerable variability between analogous samples. When a harvested *Spirulina* sample was assayed for CA activity a result of −0.253 WAU ± 0.225 was obtained. This experiment was repeated, and in addition a sample that had been homogenised by two passes through a French pressure cell at 1500psi, was also assayed. Once again negative results were obtained with a very high standard error (Table 3.2).

Table 3.2: Initial experiments to determine CA activity in *Spirulina*

Sample	WAU	Standard Error
Harvested Algae	-0.539	0.589
Homogenate	-0.483	0.796

Spirulina platensis has been found in waters containing salt concentrations ranging from 85 to 270 g/l, but the optimal growth appears to be at salt concentrations of 20 to 70 g/l (Ciferri, 1983; Richmond, 1986). Zarrouk's media in which our laboratory cultures were grown contained a NaCl concentration of 1g/l (Appendix A). It was hence reasoned that perhaps the cells were becoming stressed when washed with water in the harvesting step. This step was therefore replaced with a wash in 1g/l NaCl, and CA was again assayed in the harvested cells. Once again a negative result of –0.275 WAU ± 0.215 was obtained.

Until this point veronal buffer had been used as the control, and t_0 had always been lower than t, hence the negative results. Upon consultation with Professor David Husic (Lafayette College, Pennsylvania), it was decided to change the control to a sample, which had been boiled for 5 min in order to inactivate any CA. This would ensure that there were no extraneous factors present in the sample that were not in the control. This approach proved to be a success, and positive results were obtained (Table 3.3). The CA activity was, however, very low and the standard error very high.

Table 3.3: CA activity in *Spirulina* using new control

Sample	WAU	Standard Error
Harvested Algae	0.313	0.410
Homogenate	0.096	0.321

The ultimate aim of this project was to purify CA from *Spirulina*, and therefore preliminary purification steps were undertaken. CA activity was assayed at each step.

Harvested *Spirulina* cells were homogenised by French pressing as before, and then centrifuged at 11 500g for 20 min in an Eppendorf centrifuge 5403. The resulting supernatant was ultracentrifuged at 100 000g for 1 hour in a Beckman L-70

ultracentrifuge. All fractions were assayed for CA activity. This experiment was run twice, and the results are shown in figures 3.12 and 3.13.

The large standard error is clearly indicated in both cases. In addition, there was a great deal of variability in results between the two experiments. The first experiment showed an activity of 0.337 WAU ± 0.373 for the harvested algae, whereas experiment two gave a negative result. On the other hand experiment two gave a much higher result for the homogenate at 0.753 WAU ± 0.617, as opposed to 0.084 WAU ± 0.355 in experiment one. If CA is membrane bound, one would have expected to find the most CA activity in the first supernatant and the second pellet. The second pellet did appear to have the highest CA activity in both cases, although the CA activity for pellet 2 was four times higher in the second experiment than the first. One would also have expected the activity to be higher in supernatant one than in pellet two, as pellet two resulted from centrifuging supernatant one, and it was activity, not specific activity that was measured. These results are far from conclusive if one considers the vast margin of error. In all cases the standard error was close to, if not more than, the actual activity units. The variability between the two analogous experiments was also considerable.

It was hence concluded that this was a very unreliable method of determining the presence or activity of CA. It has been noted in the literature (Badger & Price, 1989; Hagalund *et al*, 1992; Raven, 1995), that this method can be fairly rough and insensitive, and very low, or no activity has been detected in cases where substantial activity has been detected using other methods such as mass spectrophotometry. If purification was to be successfully completed, a far more reliable method of detection was required.

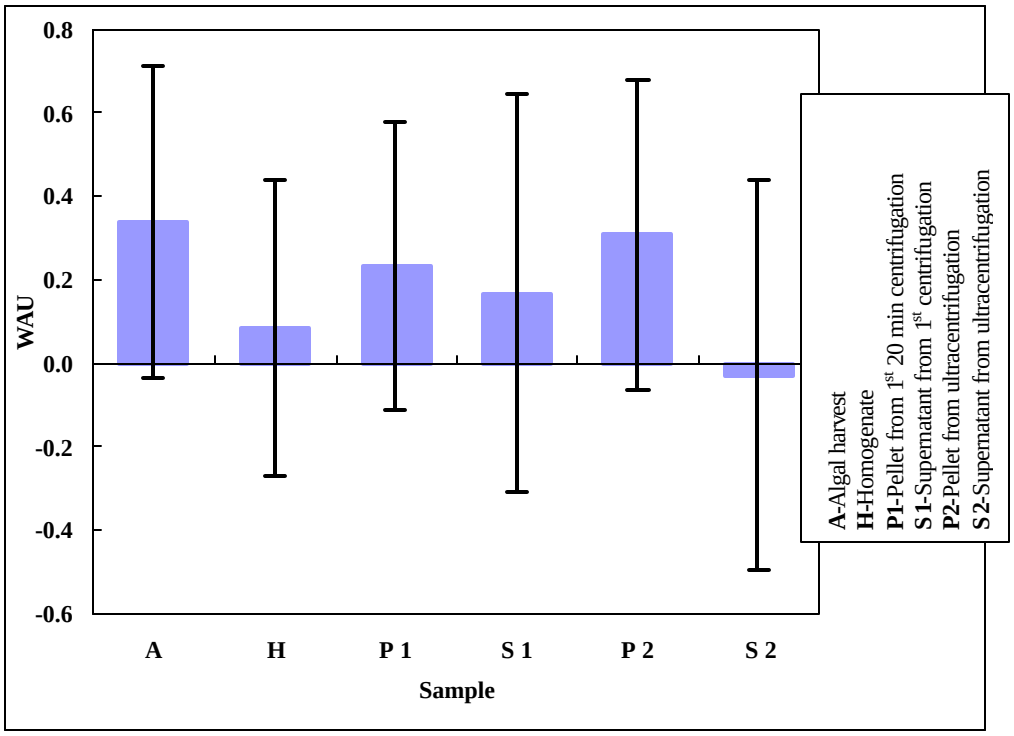


Figure 3.12: CA activity found in the first purification experiment

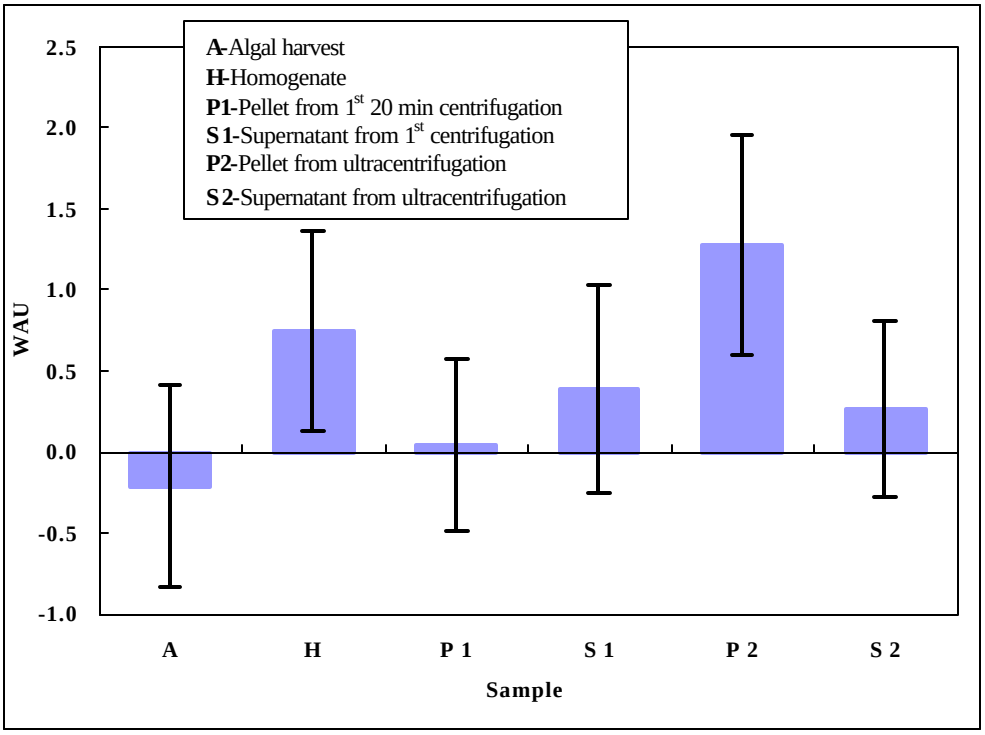


Figure 3.13: CA activity found in the second purification experiment

3.2.4 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

3.2.4.1 MATERIALS

Acrylamide, N, N'-methylene-bis-acrylamide, sodium dodecyl sulphate (SDS), SDS-70L Molecular weight markers N,N,N',N'-Tetramethylethylenediamine (TEMED), ammonium persulphate (APS), and β-mercaptoethanol were obtained from Sigma. Coomassie blue R250 and bromophenol blue were obtained from Saarchem. Bovine serum albumin (BSA) was purchased from Boehringer Mannheim. All other chemicals used were supplied by Merck or Saarchem. All water used was ultra-pure deionised water (Milli-Q system, Millipore)

3.2.4.2 SAMPLE PREPARATION

Spirulina cells were grown and harvested as described in 3.2.3.4, except that after the first centrifugation cells were washed in 1g/l NaCl, not deionised water.

3.2.4.3 PREPARATION OF MOLECULAR WEIGHT MARKERS

Sigma SDS 70L molecular weight markers ranging from 14 000 to 70 000 were used. The contents of the vial (3.5 mg) were mixed with 1.5 ml dissociation buffer (Appendix E) and aliquots were stored at -20°C. Carbonic anhydrase was made up to 0.5 mg/ml in 1x sample buffer, and bovine albumin to 0.7 mg/ml. Aliquots were stored at -20°C.

3.2.4.4 PROTEIN DETERMINATION

The protein concentration of the samples was determined using a modification of the method introduced by Bradford (1976). BSA was used as the external standard (Appendix F).

An aliquot of 200 µl protein sample was added to a test tube. This was made up to 1ml with 0.15M NaCl. Bradford's reagent (Appendix G) was added to make up a total volume of 6ml. This was vortexed immediately, followed by a waiting period of 5

min. The absorbance was then read at 595 nm in a Shimadzu UV 160A UV visible spectrophotometer, and protein concentration determined from the standard curve (Appendix F).

3.2.4.5 SDS-PAGE

A modification of the method of Laemmli (1970) was used for slab gel electrophoresis. A Hoefer Tall Mighty Small unit SE 280 was used to run 12% gels (4% stacking gel). Samples were mixed with an equal quantity of dissociation buffer, boiled for 5 min and then allowed to cool. Aliquots of 20 µl were loaded and gels were run at 120V (constant voltage) for 3.5 hours. Gels were stained for 1 hour with gentle agitation, followed by destaining for 1 hour. Gels were left in half strength destain overnight, and then photographed using Kodak Digital Science 1D™. All recipes can be found in Appendix E.

3.2.4.6 ENZYME PURIFICATION

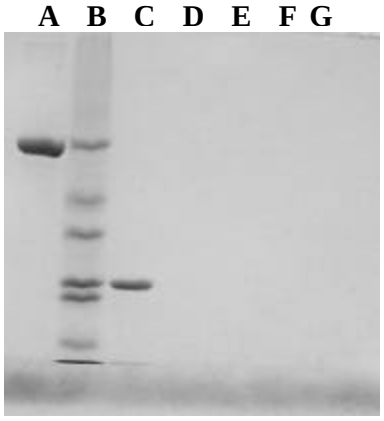
In the first experiment using this system, harvested cells were homogenised by two passes through a French pressure cell at 1500 psi. This homogenate was centrifuged at 11 500g for 20 min in an Eppendorf centrifuge 5403. The supernatant was subjected to 65% ammonium sulphate fractionation and then centrifuged at 9 500g for 15 min. The pellet was then resuspended in 15ml 0.1 M Tris-HCl (pH 8.5). Both the supernatant and the pellet were analysed using SDS-PAGE. In addition, samples were assayed for CA activity, using the Wilbur-Anderson method, and protein concentration was determined.

The CA activity in the final pellet, as determined by the Wilbur-Anderson method, was 0.252 WAU ± 0.424. The specific activity was calculated to be 12.51 WAU/ug protein (Table 3.4). When analysed by SDS-PAGE (Figure 3.14), however, no protein bands were seen. In addition to the high degree of variability, the Bradford assay used to determine protein concentration gave very unreliable results. The pellet fraction, which one would expect to have a high protein content, only gave a result of 0.02 µg/ml (Table 3.4). The Bradford (1976) protein assay is a colorimetric assay based on

the fact that Coomassie Brilliant Blue R250 exists in two colour forms, red and blue. The red form is converted to the blue form when the dye binds to the protein. This leads to a shift of the absorption spectrum from 465 nm to 590 nm. The increase of absorption at 595 nm is monitored (Bradford, 1976). As the algal samples have a strong green colour the absorption spectrum would be affected, hence giving inaccurate results. Due to the fact that a lower protein concentration than expected was obtained, the calculated specific activity would be too high.

Table 3.4: CA activity in *Spirulina* for comparison with SDS-PAGE

Sample	Activity (WAU)	Protein (µg)	Specific Activity (WAU/µg)
Algal Harvest	0.106 ± 0.076	0.0109	5.425
Homogenate	0.033 ± 0.108	0.0202	1.650
$(NH_4)_2SO_4$ Supernatant	0.024 ± 0.118	0.0201	1.203
$(NH_4)_2SO_4$ Pellet	0.254 ± 0.424	0.0201	12.513



A – Bovine Albumin Standard
B – Molecular Weight Markers
C – Carbonic Anhydrase Standard
D – Algal Harvest
E – Homogenate
F – $(NH_4)_2SO_4$ Supernatant
G – $(NH_4)_2SO_4$ Pellet

Figure 3.14: SDS-PAGE analysis of *Spirulina* samples

3.3 CONCLUSIONS

A great deal of time was spent on the two radiotracer assays, to little avail. Results were inaccurate and a reproducible protocol seemed unapproachable. Consequently, the Wilbur-Anderson method was investigated with little more success. Results were found to be inaccurate with a high degree of variability. Very little activity was found in *Spirulina*, probably due to the failings of the assay system. Many authors (Badger & Price, 1989; Hagalund *et al*, 1992; Raven, 1995) have noted the insensitivity of this assay, and Giordano and Maberly (1989) found that whole plants, or parts thereof interfered with measurements, sometimes causing significant negative values for CA activities.

Due to the problems associated with measuring CA activity it was decided to use only the SDS-PAGE method for detecting protein since this method, although not quantitative, appeared to be a reliable method of detecting the presence and purity of the protein.

CHAPTER FOUR

PURIFICATION OF CARBONIC ANHYDRASE

4.1 INTRODUCTION

Carbonic anhydrase has been purified from the cyanobacterium *Anabaena variabilis* (Yagawa *et al*, 1984), and the microalgae *Coccomyxa* (Hiltonen *et al*, 1995), *Chlamydomonas reinhardtii* (Husic, 1991; Husic *et al*, 1989; Kamo *et al*, 1990; Karlsson *et al*, 1995; Yang *et al*, 1985), and *Chlorella sorokiniana* (Satoh *et al*, 1998). Most of the publications documenting the purification of CA deal with the unicellular green alga *Chlamydomonas reinhardtii* (Figure 4.1). *Chlamydomonas* is a large genus of green flagellates belonging to the Chlorophyceae lineage. More than 600 species have been described world-wide from marine and freshwater, soil and even snow. *Chlamydomonas* is a single-celled, elliptical, biflagellate, green alga, approximately 10µm long. The thin cell wall is composed of complex polysaccharides, and is assembled by fusion of discrete particles. One or more chloroplasts, as well as a single nucleus occupy most of the cell volume. The genus *Chlamydomonas* contains several species that have become popular as research tools, but by far the most frequently used is *Chlamydomonas reinhardtii* (Bold & Wynne, 1985; Buchheim *et al*, 1991).

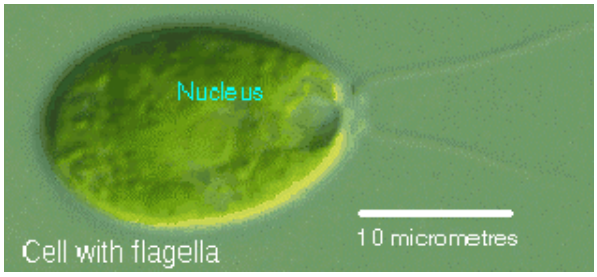


Figure 4.1: Diagram of *Chlamydomonas reinhardtii*

Aromatic sulfonamides are specific and potent inhibitors of carbonic anhydrase, and therefore present a logical choice for preparation of an insoluble support for affinity chromatography. Philip Whitney (1974) first demonstrated the use of a *p*-aminomethylbenzenesulfonamide affinity chromatography column for the purification of human erythrocyte CA. This method has since been adapted and is the method commonly used for the purification of CA from *Chlamydomonas reinhardtii* (Husic, 1991; Husic *et al*, 1989; Kamo *et al*, 1990; Karlsson *et al*, 1995; Yang *et al*, 1985), although preparation of the crude sample differs.

Due to the fact that the initial steps taken to purify CA from *Spirulina* were unsuccessful (section 3.2.4.6), it was decided to purify the enzyme from the well-studied *Chlamydomonas reinhardtii* using affinity chromatography. These methods are well documented, and if successful, could be modified for use on *Spirulina*.

4.2 METHODS AND RESULTS

4.2.1 PURIFICATION OF CA FROM *CHLAMYDOMONAS RHEINHARDTII*

4.2.1.1 MATERIALS

Chlamydomonas reinhardtii wild-type (cc-125) and the cell wall-deficient mutant cw-15mt^{+} (cc400) were obtained from the *Chlamydomonas* Genetics Centre at Duke University. Sypro® Orange Protein Stain was purchased from BIO-RAD, as was the Immun-Blot® Assay Kit Goat Anti-Rabbit IgG (H+L). Freunds adjuvant was obtained from Difco and *p*-aminomethylbenzenesulfonamide-agarose was purchased from Sigma. Snail acetone powder, lysing enzymes and polyoxyethylene ether W-1 were also purchased from Sigma and Macerase™ and Cellulysin™ were Calbiochem products. Polysulfone ultrafuge tubes with a molecular weight cut off (mwco) of 10 000 were purchased from MSI separations, inc. All other materials were the same as those listed in section 3.2.4.1.

4.2.1.2 SAMPLE PREPARATION

Both strains of *Chlamydomonas* were cultivated in Sueoka's (1959) minimal medium (Appendix H), under continuous light supplied by 2 fluorescent tubes (Osram cool white L20W/20S) at 24ºC. Cells were cultured on a Labcon orbital shaker at 132rpm. Twenty-four hours before harvesting, cultures were subjected to vigorous bubbling with air. Cultures were harvested by centrifuging at 3000g for 10 min. The cell pellets were then washed and centrifuged (3000g for 10 min) twice with ice-cold water.

4.2.1.3 CHLOROPHYLL DETERMINATION

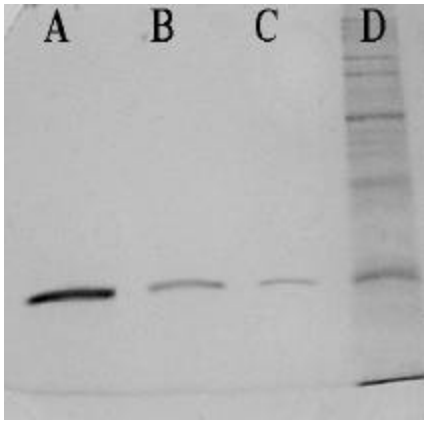
A 1 ml sample of algae was centrifuged at 2376g for 10 min in a Haraeus Biofuge. The pellet was resuspended in 1 ml 96% ethanol, and the absorbance measured at 750, 665 and 649 nm in a Shimadzu UV 160A UV visible spectrophotometer. Chlorophyll concentration was calculated according to the equations in appendix I (Wintermans & De Mots, 1965).

4.2.1.4 PHENOL/ETHER CONCENTRATION OF PROTEINS

Samples were concentrated before SDS-PAGE by adding 0.5 ml phenol to 1 ml of sample and vortexing for 20 seconds. These were then centrifuged at 16 000g for 5 min and the upper aqueous phase discarded. One millilitre of ether was then added and the samples were vortexed and centrifuged as above. The upper organic phase was removed and the last step was repeated (Sauve *et al*, 1995). Samples were then dried under nitrogen and resuspended in 50 µl dissociation buffer (Appendix E).

4.2.1.5 PRODUCTION OF ANTIBODY

In order to confirm the purity of the protein, bovine CA (Sigma) was analysed by SDS-PAGE. CA was dissolved in sample buffer to a concentration of 1 mg/ml. Aliquots of 1, 5 and 20 µl were made up to 20 µl with dissociation buffer, and boiled for 5 min. These samples, along with molecular weight markers, were run on a 12% gel for 3 hours at 100V. The gel was stained and photographed as described in section 3.2.4.5.



A – Bovine CA (1 mg/ml)
B – Bovine CA (250 µg/ml)
C – Bovine CA (50 µg/ml)
D – Molecular weight markers

Figure 4.2: SDS-PAGE analysis of commercial bovine CA

Figure 4.2 clearly shows the difference in concentration of the three samples. In addition there were no extra bands, confirming the purity of the commercial CA. A 1mg/ml solution of CA in phosphate buffered saline (PBS) was thus prepared, and mixed with an equal quantity of Freunds adjuvant until an emulsion formed. This emulsion was injected subcutaneously into a lab rabbit. After four weeks the rabbit was bled, and the blood was allowed to clot overnight. The serum was removed from the clot, and any remaining insoluble material was removed by centrifugation at 10 000g for 10 min at 4ºC. Aliquots of the serum were stored at –20ºC until required for Western blots.

4.2.1.6 WESTERN BLOTTING

Samples were run on a SDS-PAGE gel. The antigens were then electrophoretically transferred onto a PVDF membrane using a BIO-RAD mini-PROTEAN II cell at 100V for 2 hours. The membrane was removed from the apparatus and rinsed twice with TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5) for 5 min. Thereafter the membrane was incubated, with gentle agitation, in a 3% gelatine solution overnight in order for non-specific blocking to take place. Just as proteins transferred from the SDS-polyacrylamide gel can bind to the membrane, so can proteins in the immunological reagents used for probing. The sensitivity of Western

blotting depends on reducing this background of non-specific binding, by blocking potential binding sites with irrelevant proteins (Sambrook *et al*, 1989).

Once again the membrane was washed twice in TTBS, after which it was exposed to the first antibody for 2.5 hours. After another series of washes, the second, conjugated antibody (goat anti-rabbit IgG horseradish peroxidase conjugate) was added, and allowed to incubate for 2 hours. After 3 washes in TTBS the membrane was placed in colour development solution (4-chloro-1-naphthol) for 30 min. The membrane was then photographed using Kodak Digital Science 1D^{TM}.

4.2.1.7 DIFFERENTIAL CENTRIFUGATION

Initially the protocol used by Yang and associates (1985) for the purification of CA from *Chlamydomonas* was followed. One hundred millilitres of wild-type cells were harvested and resuspended in 15 ml 20 mM Veronal buffer, pH 8.3 (15.2 mg chlorophyll/ml), The cells were homogenised by two passages through a French pressure cell at 1500 psi and then ultracentrifuged at 100 000g for 1 hour. The pellet was resuspended in 4 ml Veronal buffer and all fractions were concentrated as described in section 4.2.1.4 and analysed by SDS-PAGE (Figure 4.3). For comparison, samples were also assayed for CA activity and protein content, using the Wilbur-Anderson and Bradford methods, respectively.

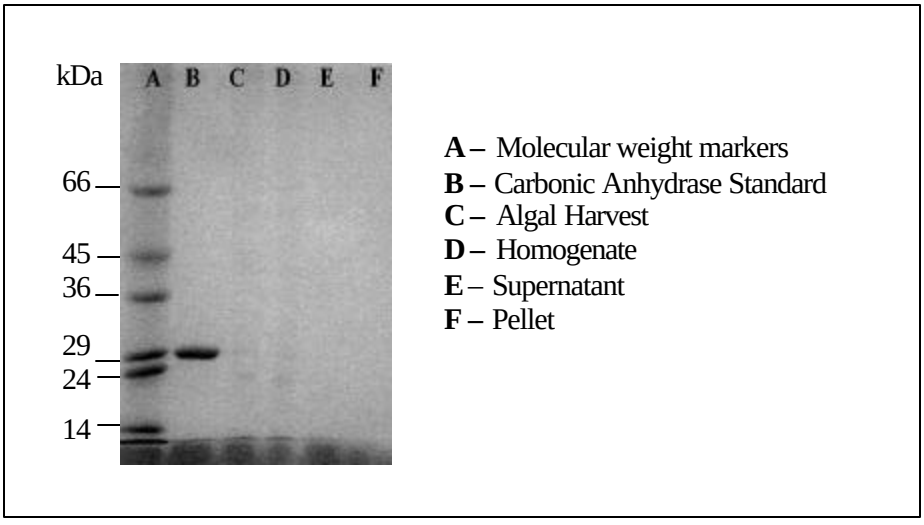


Figure 4.3: SDS-PAGE analysis of fractions obtained by differential centrifugation of *Chlamydomonas*

Table 4.1: CA activity in *Chlamydomonas* for comparison with SDS-PAGE

Sample	Activity (WAU)	Protein (µg)	Specific Activity (WAU/µg)
Algal Harvest	0.779 ± 0.348	1.584	0.491
Homogenate	1.083 ± 0.493	42.864	0.025
Supernatant	1.752 ± 0.630	25.098	0.070
Pellet	0.648 ± 0.153	15.170	0.009

Once again there was a very high standard error with the Wilbur–Anderson assay (Table 4.1), and the results from the Bradford assay were very improbable when compared with the SDS-PAGE gel (Figure 4.3). SDS-PAGE analysis only revealed two very faint bands in the algal harvest and homogenate fractions, and nothing in either fraction obtained after differential centrifugation. The Bradford results, however, gave the lowest protein content of 1.584 µg in the algal harvest, and the highest in the homogenate (42.864 µg), but the bands on the gel show similar quantities of protein in these two samples, which would be expected. The other two samples, which showed no bands on the gel, gave higher protein content than the algal harvest when analysed by the Bradford method. As explained in the previous chapter, the Bradford assay would be affected by the green colour of these samples. Due to the inaccuracy of both the activity and the protein assays, the specific activity results obtained were essentially meaningless.

SDS-PAGE analysis once again proved to be the most reliable method of detecting presence of protein, although not quantitative. However, only a small sample of algae had been harvested, hence the small quantity of protein detected. This experiment was therefore repeated, this time using 300 ml of wild-type cells. Harvested cells were resuspended in 15 ml 20 mM Veronal buffer, pH 8.3 (62.6 mg chlorophyll/ml). It was also decided to compare the sensitivity of the Coomassie staining with the BIO-RAD fluorescent reagent, Sypro® Orange. Two identical gels were therefore run, and one was stained as before with Coomassie Brilliant Blue. The second gel was stained for 30 min in Sypro® Orange and then rinsed in 7.5% acetic acid. The gel was visualised by ultraviolet illumination and photographed using Kodak Digital Science™.

Figure 4.4 shows the comparison between the gels stained with a) Coomassie Brilliant Blue and b) Sypro® Orange. There did not appear to be much difference in sensitivity between the two stains. The same bands could be seen in both, although they were much clearer in the Coomassie stained gel (a). It was hence decided to continue using Coomassie Brilliant Blue, even though the gels stained with Sypro® Orange could be visualised in a much shorter time. Sypro® Orange is also far more expensive than Coomassie Brilliant Blue.

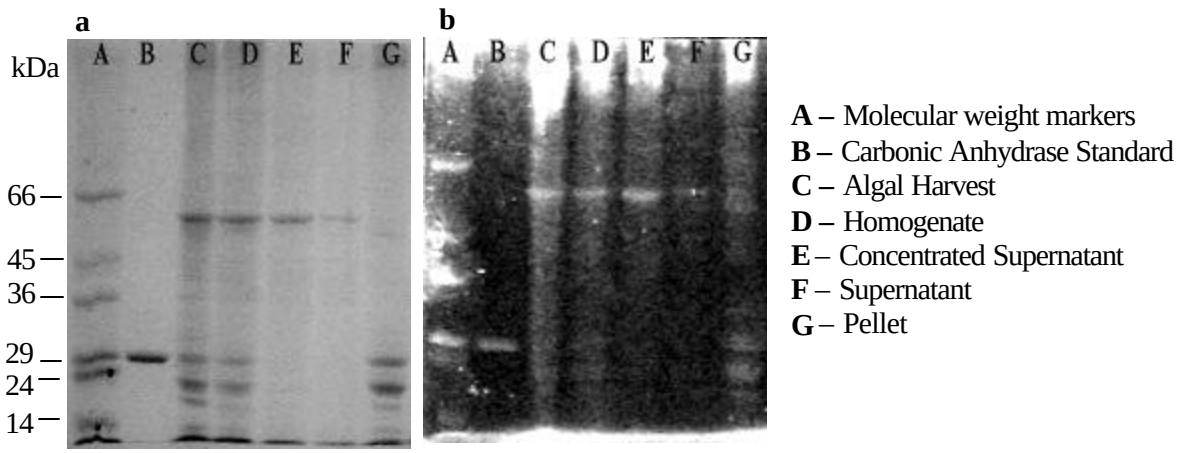


Figure 4.4: SDS-PAGE analysis of *Chlamydomonas* fractions stained with a) Coomassie Brilliant Blue and b) Sypro ® Orange

SDS-PAGE analysis revealed clear bands in all samples (Figure 4.4). A band in the 29 kDa region seen in the algal harvest, homogenate and pellet was suspected to be CA. These samples were therefore rerun on a SDS-PAGE gel, along with commercially pure CA. The proteins were then electrophoretically transferred onto a PVDF membrane, which was then analysed by Western blotting.

The antibody to bovine CA reacted with the CA standards as well as the commercial bovine CA, but not with the *Chlamydomonas* proteins (Figure 4.5). As bovine CA shows very little homology with that of *Chlamydomonas*, and Yang and associates (1985) reported that no cross-reactivity occurred between the two antisera, the bands of interest could still represent CA. Ideally one would perform Western blot analysis with antibody to *Chlamydomonas* CA, to confirm the identity of these proteins. Unfortunately such antibodies could not be obtained. However, due to the position of

the bands on the gel, it was assumed that they did in fact represent CA, and further steps were taken to purify the protein.

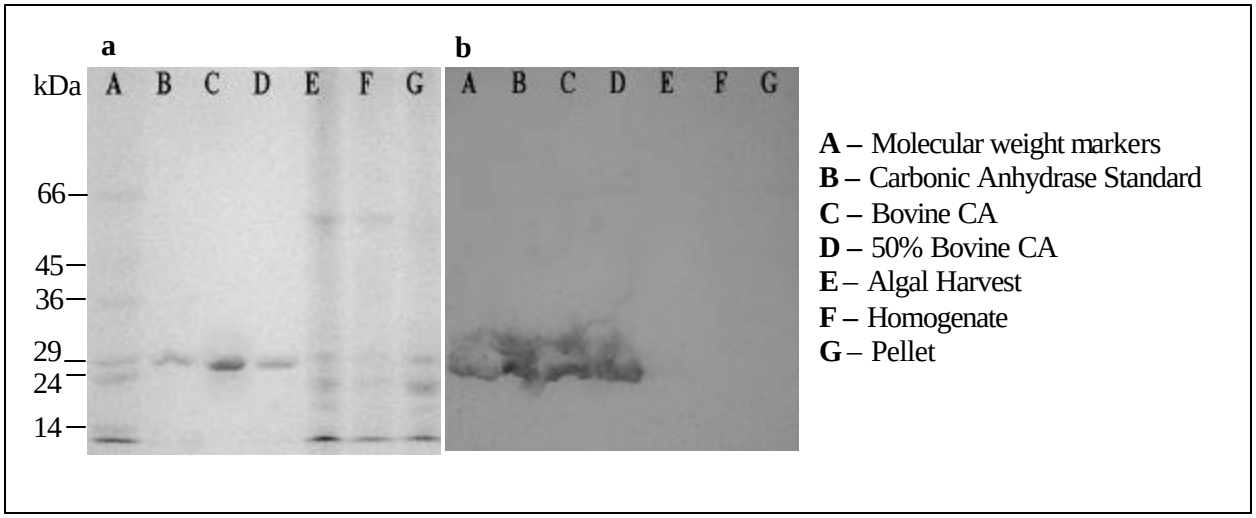


Figure 4.5: Western blot analysis of *Chlamydomonas* proteins a) SDS-PAGE gel, stained with Coomassie Brilliant Blue b) PVDF membrane after Western blotting

A 400 ml sample of *Chlamydomonas* was harvested, and resuspended in 25 ml 20 mM Veronal buffer, pH 8.3 (13.24 mg chlorophyll/ml). This was French pressed and centrifuged as described previously. The pellet was resuspended in 15 ml 0.1M Tris-HCl, pH 8.5, and subjected to ammonium sulphate fractionation. The 65 % protein precipitate was pelleted by centrifugation at 5 000g for 15 min, and resuspended in 10 ml of the same buffer. This was dialysed against 2 litres 50 mM Tris-HCl, pH 8.5. The buffer was changed 4 times over a period of 21 hours.

The dialysate was applied at a flow rate of about 0.5 ml/min to a 1 x 2 cm column of *p*-aminomethylbenzenesulfonamide-agarose, which had been washed and equilibrated with 50 mM Tris-HCl, pH 8.5. The column was washed with 15 ml 25 mM Tris-HCl; 22 mM Na_2SO_4 at pH 8.7, followed by 25 ml 25 mM Tris-HCl; 0.3 M $NaClO_4$ (sodium perchlorate) at pH 8.7. The CA protein was then eluted using 15 ml 0.1 M $NaC_2H_3O_2$ (sodium acetate); 0.5 M $NaClO_4$ at pH 5.6. The absorbance of all fractions was measured at 280 nm, and the elution profile plotted (Figure 4.6). Fractions 12 – 17 were combined and dialysed against 2 litres 20 mM Tris-HCl at pH 8.5 to remove the perchlorate, an inhibitor of the catalytic activity of the enzyme. The buffer was changed 3 times over a period of 16 hours. All fractions were concentrated and

analysed by SDS-PAGE (Figure 4.7). Two analogous sets of proteins were analysed. One set was concentrated by the phenol/ether method used previously, the others were concentrated to 100 µl by centrifugation at 1000g in polysulfone ultrafuge tubes.

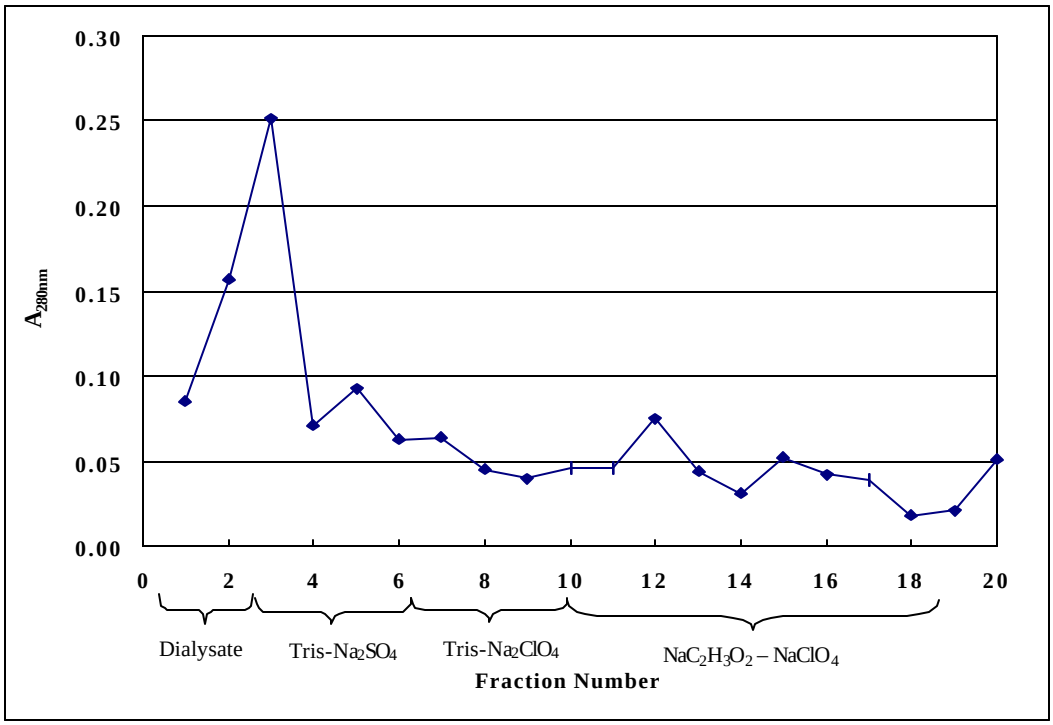


Figure 4.6: Elution profile obtained when dialysed *Chlamydomonas* pellet was applied to affinity chromatography column

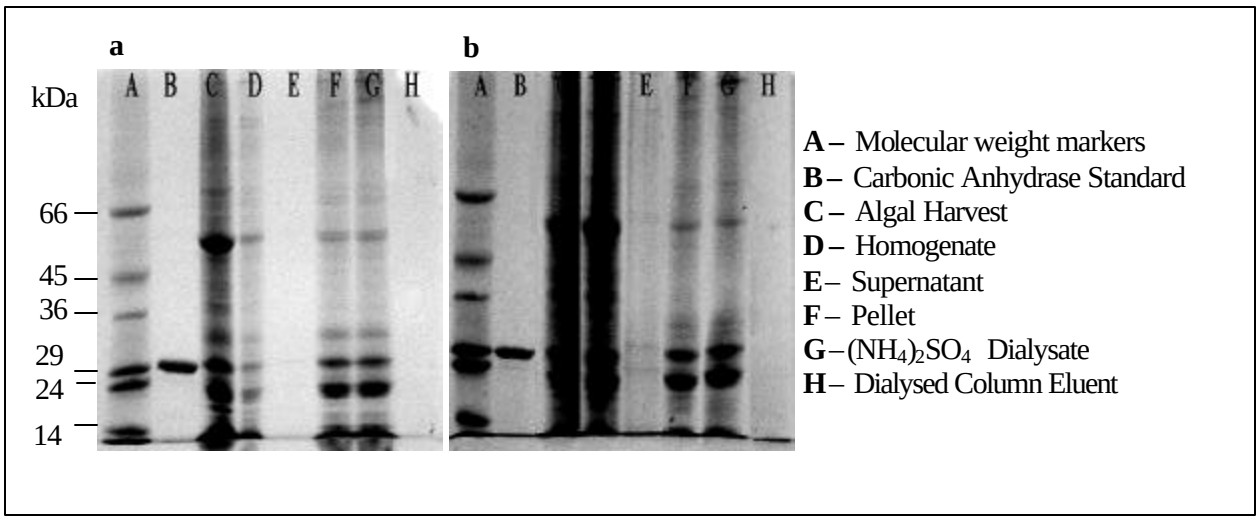


Figure 4.7: SDS-PAGE analysis of *Chlamydomonas* fractions at various stages of purification a) samples concentrated by centrifugation in polysulfone ultrafuge tubes b) samples concentrated by phenol/ether method.

It can be seen from figure 4.7 that the concentration by centrifugation in polysulfone ultrafuge tubes (a) yielded much clearer bands than the phenol/ether method (b). The polysulfone tubes were therefore used for concentration in all subsequent experiments.

Both gels clearly show the bands of interest (Figure 4.7). The proteins all remained associated with the pellet (Lane F) and were still present after ammonium sulphate fractionation and dialysis (Lane G). There were, however, no bands at all seen in the last lane, the dialysed column elluent. Figure 4.6 also showed very low absorbance readings at 280nm for all eluted fractions, which indicated that very little protein was being eluted from the column. The column did remain very green, even after elution with 15 ml 0.1 M $NaC_2H_3O_2$; 0.5 M $NaClO_4$ AT pH 5.6. The faction applied to the column still contained a significant amount of other protein besides the CA of interest (Lane G), which could have been causing interference and blocking the column. Ideally a relatively pure protein should be applied to an affinity chromatography column, and this was not the case. There was still too much interfering protein. It was thus decided to investigate a different option for purifying the CA before application to the column.

4.2.1.8 SALT-INDUCED DISSOCIATION OF CA

Husic and Quigley (1990) showed that extracellular CA could be dissociated from *Chlamydomonas* cells by treatment with 20 mM potassium phosphate buffer containing 0.4M KCl, and Husic (1991) has subsequently used this method in the purification of extracellular CA from *Chlamydomonas reinhardtii*. It was hence decided to investigate this protocol.

Five samples of 30 ml *Chlamydomonas* were harvested, and washed twice in 5 ml cold 20 mM potassium phosphate buffer at pH 7.4 (KPi buffer), followed by centrifugation for 5 min at 2000g. Each pellet was resuspended in 5 ml of cold KPi buffer (± 13 mg chlorophyll/ml) containing KCl (0; 0.2; 0.4; 0.8; 1M respectively), and allowed to stand on ice for 10 min. Each sample was centrifuged at 2000g for 5 min, and the supernatants removed. The pellets were resuspended in 1 ml KPi buffer containing the same concentrations of KCl, and allowed to stand on ice for a further

10 min. The samples were centrifuged as above, and the last step repeated. The supernatants were combined and concentrated to 100 µl in polysulfone ultrafuge tubes. Pellets were resuspended in 1 ml KPi buffer, and all samples were analysed by SDS-PAGE (Figure 4.8).

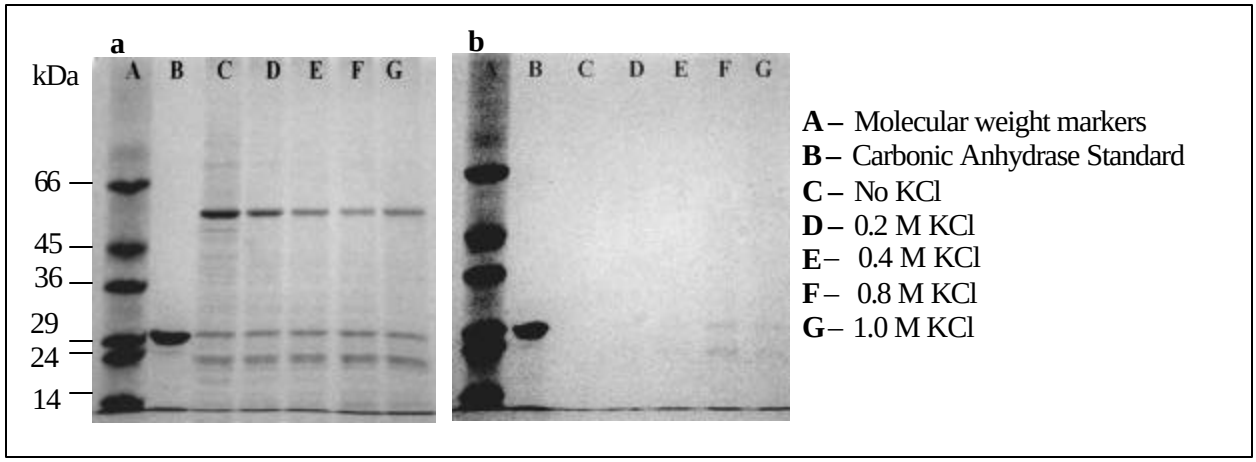


Figure 4.8: SDS-Page analysis of *Chlamydomonas* cells treated with KCl a) Pellets b) Supernatants

Figure 4.8 clearly shows that the protein remained associated with the pellet in all cases. Only a very small quantity of protein was dissociated in the samples containing 0.8 M and 1M KCl (Figure 4.8b, lanes F & G). This is double the concentration reported by Husic and Quigley (1990) to dissociate CA from *Chlamydomonas*.

The experiment was thus repeated, with slight modifications, using a larger sample volume and 0.8M KCl, as this concentration gave the best results in the previous experiment. Two samples of 150 ml *Chlamydomonas* were harvested and washed twice in 10 ml KPi buffer. The pellets obtained after washing were resuspended in 0.5 M KPi buffer containing 0.8 M KCl. Phosphate buffer is well known for its solubilising properties, and concentrations of 0.1 to 0.5 M have been noted to increase the total solubility of membrane proteins (Neugebauer, 1990), hence the higher concentration of KPi buffer used. The one sample (sample A), resuspended in 6 ml KPi buffer, was incubated on ice for 60 min with gentle agitation, and then centrifuged at 10 000g for 1 min (Thomas & McNamee, 1990). The pellet was resuspended in 10 ml 20 mM KPi, and the supernatant was concentrated to 100 µl.

The second sample (sample B), resuspended in 2 ml KPi buffer, was incubated on ice for 10 min followed by a 1 min centrifugation at 10 000g. The supernatant was removed, and the pellet resuspended in a further 2 ml KPi buffer containing 0.8 M KCl. Five cycles of incubation on ice and centrifugation followed. After the total incubation period, all the supernatants were combined and concentrated to 100 µl. The final pellet was resuspended in 10 ml 20 mM KPi buffer. All samples were analysed by SDS-PAGE (Figure 4.9).

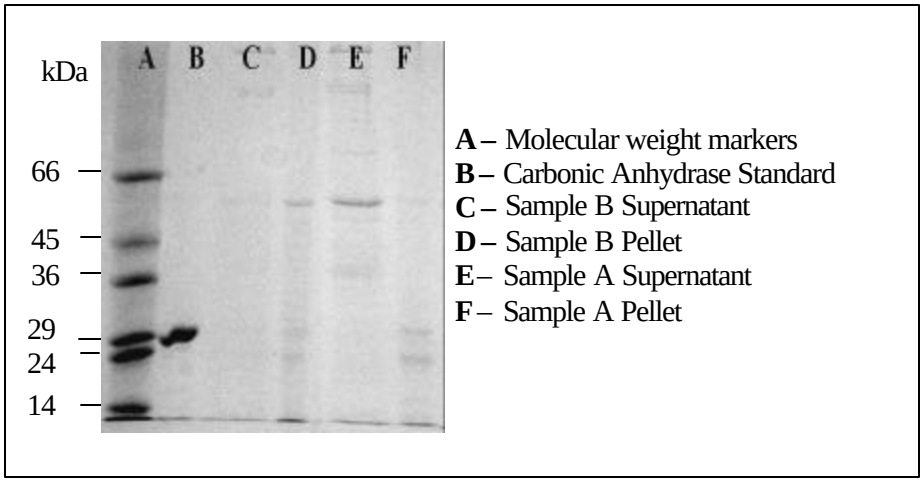


Figure 4.9: SDS-PAGE analysis of *Chlamydomonas* cells treated with KCl in 0.5 M KPi buffer

It can be seen from figure 4.9 that the protein of interest, in the 29 kDa region, remained associated with the pellet in both cases (Lanes D & F). Only a protein of approximately 56 kDa became dissociated from the cells in the case of sample A (Lane E). The increase in concentration of the phosphate buffer did not appear to have any effect on the solubilisation of the proteins in this case. The experiment was therefore repeated, using a combination of the methods above, and reverting to the original buffer concentration.

Two samples of 200 ml *Chlamydomonas* were harvested and washed in KPi buffer as above. The pellets were then resuspended in 5 ml 20 mM KPi buffer, one of which contained 0.8 M KCl. The other was run as a control. These were allowed to stand on ice for 10 min, after which they were centrifuged at 10 000g for 1 min. The pellets were resuspended in 2 ml of the same buffer, and the last step repeated. The pellets were once again resuspended in 2 ml buffer, and then incubated on ice for 30 min, followed by centrifugation. Supernatants were combined and concentrated to 100 µl. Pellets were resuspended in 5 ml KPi buffer, and all samples were analysed by SDS-PAGE (Figure 4.10).

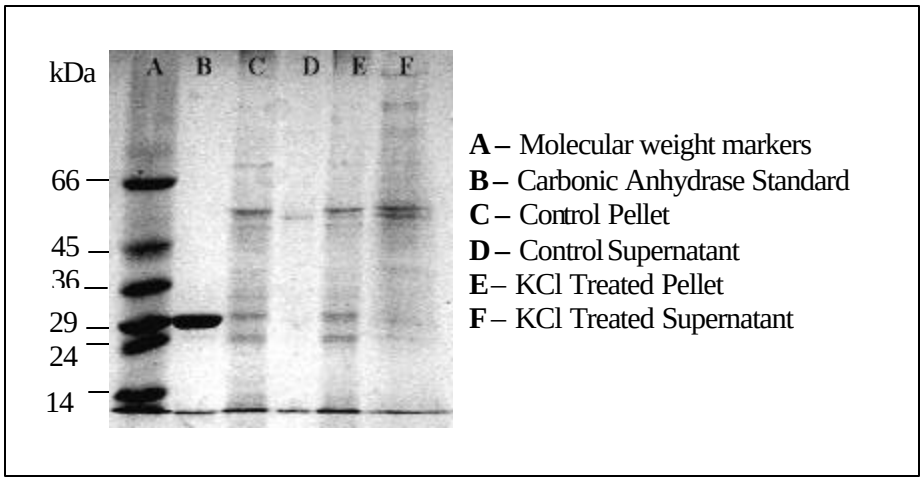


Figure 4.10: SDS-PAGE analysis of *Chlamydomonas* cells treated with KCl in the original KPi buffer

Figure 4.10 showed various proteins in the supernatant of the experimental sample (Lane F). There were two proteins of approximately 56 kDa, but the one was also seen in the supernatant of the control (Lane D), which could indicate that the protein is released into the surrounding medium. The protein could also be associated with the cells by very weak interactions, which may have been disrupted by the repeated centrifugation and resuspension. The other band is still present in the pellet in equivalent proportions, indicating that it was not all dissociated. A few larger proteins could also be seen in the pellet, although the bands were very faint. Only very faint bands could be seen in the 29 kDa region of interest, indicating that very little of the CA had been dissociated. These results were contrary to those reported by Husic and Quigley (1990) who observed that CA comprised over 70 % of the total protein released by treatment with 0.4 M KCl.

Due to the fact that little success had been achieved in dissociating the CA from the cells using the KCl treatment, but distinct bands were seen in the 29 kDa position in the pellet, it was decided to subject this fraction to affinity chromatography. The pellet fraction was made up to 8 ml with KPi buffer, and 7 ml of this was applied to 1 x 2 cm column of *p*-aminomethylbenzenesulfonamide-agarose, which had been washed and equilibrated with KPi buffer. The column, however, quickly became blocked and nothing would flow through. This sample was far too crude for treatment by affinity chromatography. It had not been homogenised at any stage, and hence still contained whole cells, which were probably too large to pass through the agarose gel matrix. On the whole this method had proved unsuccessful, and it was consequently decided to investigate an alternative purification procedure.

4.2.1.9 PREPARATION OF A MEMBRANE FRACTION

If the CA is bound to the plasma membrane, preparation of a suitable, enriched membrane fraction should be the first stage of isolating the enzyme (Thomas & McNamee, 1990). The method used by Ramwani and Mishra (1985) was optimised for this purpose.

A 400 ml sample of *Chlamydomonas* was harvested and resuspended in 20 ml sucrose solution (0.25 M sucrose; 1 mM EDTA; pH 7.4) (20 mg chlorophyll/ml). This was then homogenised by two passages through a French pressure cell at 1500 psi, and centrifuged at 1000g for 10 min. The pellet was resuspended in 10 ml sucrose solution and centrifuged as before. The supernatants were combined and centrifuged at 105 000g for 1 hour. The pellet was resuspended in 25 ml 50 mM Tris-HCl (1 mM EDTA; pH 7.4) and centrifuged at 3000g for 20 min. The final pellet was resuspended in 10 ml Tris-HCl to a concentration of approximately 1 mg/ml. This method yielded a membrane stock, which was free of whole cells, nuclei and soluble proteins (Thomas & McNamee, 1990).

Initially attempts were made to dissociate the CA from the membranes by KCl dissociation as described previously. Three aliquots of 1 ml of the stock membrane suspension were prepared. Aliquots of 1 ml 50 mM Tris-HCl, pH 7.4, containing 0.6, 0.8 and 1.0 M KCl were pipetted into appropriate tubes. These were incubated on ice

for 60 min, after which they were centrifuged at 105 000g for a further 60 min (Thomas & McNamee, 1990; Ramwani & Mishra, 1985). Pellets were resuspended in 1 ml 50 mM Tris-HCl, pH 7.4, supernatants were concentrated to 100 µl, and all samples were analysed by SDS-PAGE (Figure 4. 11).

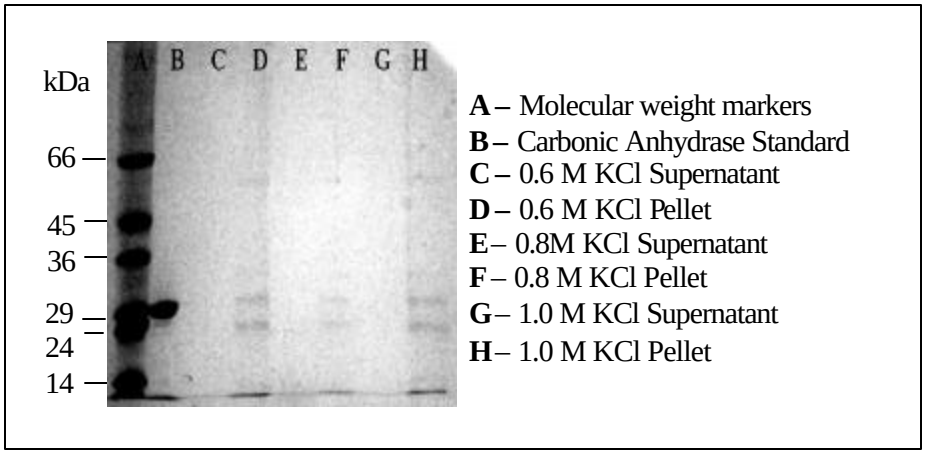


Figure 4.11: SDS-PAGE analysis of *Chlamydomonas* membrane fraction treated with KCl

Figure 4.11 clearly shows that the proteins of interest in the 29 kDa region were present in the membrane fraction. The KCl was still, however, unsuccessful at dissociating the CA from the membranes, as the proteins remained associated with the pellet. No protein was seen in any of the supernatants. Various other products were therefore investigated for their ability to solubilise the CA.

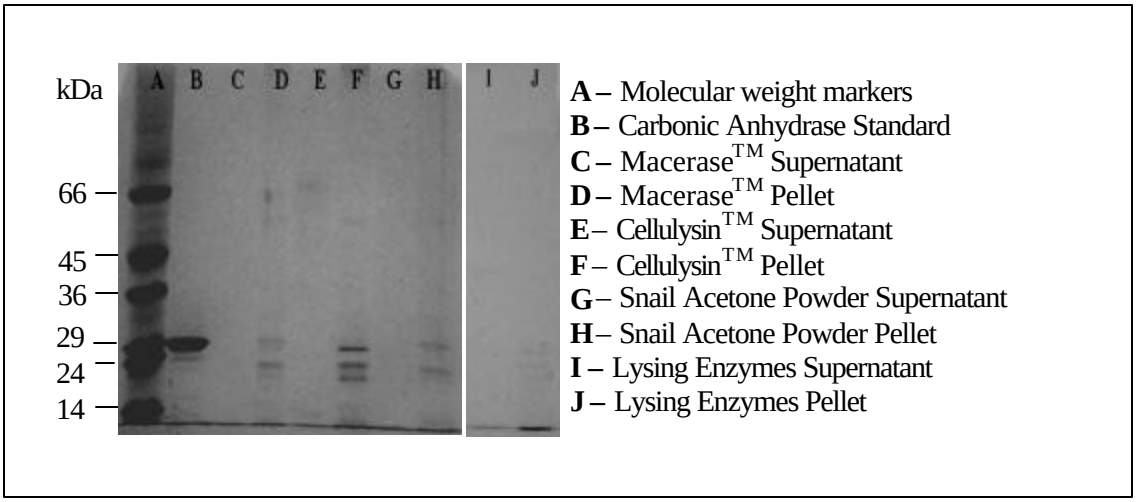


Figure 4.12: SDS-PAGE analysis of *Chlamydomonas* membrane fraction treated with alternative solubilising agents

Aliquots of 1 ml of membrane stock were incubated with 1 ml 1 M NaCl containing 0.3 % of the following solubilising agents: Snail acetone powder, Lysing enzymes, Macerase™ and Cellulysin™. After a 60 min incubation period, samples were centrifuged at 105 000g for 60 min.. Pellets were resuspended in 1 ml 50 mM Tris-HCl, and supernatants were concentrated to 100 µl before analysis by SDS-PAGE (Figure 4.12).

Once again no bands were seen in any of the supernatants, indicating that none of the products investigated were effective in solubilising the CA (Figure 4.12). Only very faint bands were seen in the pellet, which had been exposed to Lysing enzymes. It is possible that the enzymes may have denatured some of the protein.

The final method investigated for the solubilisation of CA, was the use of a detergent. The disadvantage of this being that extensive dialysis was required to remove the detergent before further purification steps, such as affinity chromatography and SDS-PAGE. As all solubilisation methods thus far had proved unsuccessful, this method was attempted.

Two aliquots of 1 ml of membrane stock were incubated with 1 ml 1 and 5 % polyoxyethylene ether W - 1 in 50 mM Tris-HCl, pH 7.4. A third, control was incubated with detergent-free buffer. The samples were centrifuged as before, once the 60 min incubation was complete. Both the pellets and supernatants were dialysed against 500 ml 50 mM Tris-HCL, pH 7.4. The buffer was changed every 4 hours for 60 hours. Upon completion of dialysis, the supernatants were concentrated to 100 µl and SDS-PAGE was carried out (Figure 4.13).

The detergent also proved unsuccessful as a solubilisation agent, as the CA bands were again only visible in the pellets. (Figure 4.13) The quantity of protein seen in the pellet from the 5 % sample did appear to be slightly less than in the control and 1 % pellets, indicating that a small quantity of protein may have been solubilised, but there were still no bands visible in the supernatant.

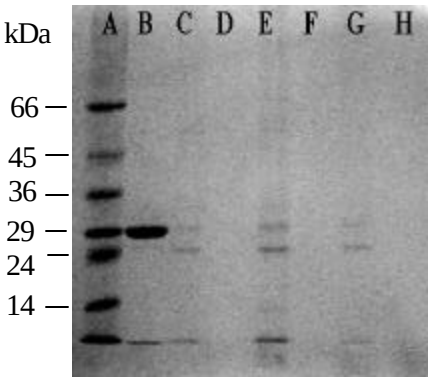


Figure 4.13: SDS-PAGE analysis of *Chlamydomonas* membrane fraction treated with polyoxyethelene ether W -1

Although no success had been achieved in solubilising the CA, the protein was clearly visible in the membrane fraction, when analysed by SDS-PAGE. This membrane fraction contained the CA in a purer form than the crude extracts originally applied to the affinity chromatography resin. It was therefore decided to attempt the purification directly from the membrane stock solution by affinity chromatography.

A membrane stock solution was prepared as previously described. A 5 ml sample was applied to a to 1 x 2 cm column of *p*-aminomethylbenzenesulfonamide-agarose, which had been washed and equilibrated with 20 mM Tris-HCL, pH 7.4. The column was washed with 25 ml 25 mM Tris-HCl; 22 mM Na_2SO_4 at pH 8.7, followed by 25 ml 25 mM Tris-HCl; 0.3 M $NaClO_4$ at pH 8.7. The CA protein was then eluted using 25 ml 0.1 M $NaC_2H_3O_2$; 0.5 M $NaClO_4$ at pH 5.6. The absorbance of all fractions was measured at 280 nm, and the elution profile plotted (Figure 4.14). Fractions 16, 17, 20 and 21 were combined and dialysed against 2 litres 20 mM Tris-HCl at pH 8.5. Fraction 7, which was eluted with 25 mM Tris-HCl; 0.3 M $NaClO_4$ at pH 8.7, had an absorbance value of 0.162. This was higher than the absorbance values for any of the fractions eluted with 0.1 M $NaC_2H_3O_2$; 0.5 M $NaClO_4$ at pH 5.6. It was therefore decided to subject this fraction to SDS-PAGE analysis as well. Consequently this fraction was also dialysed against 500 ml 20 mM Tris-HCl at pH 8.5. The buffer was changed 4 times over a period of 24 hours. After dialysis both samples were concentrated and analysed by SDS-PAGE (Figure 4.15).

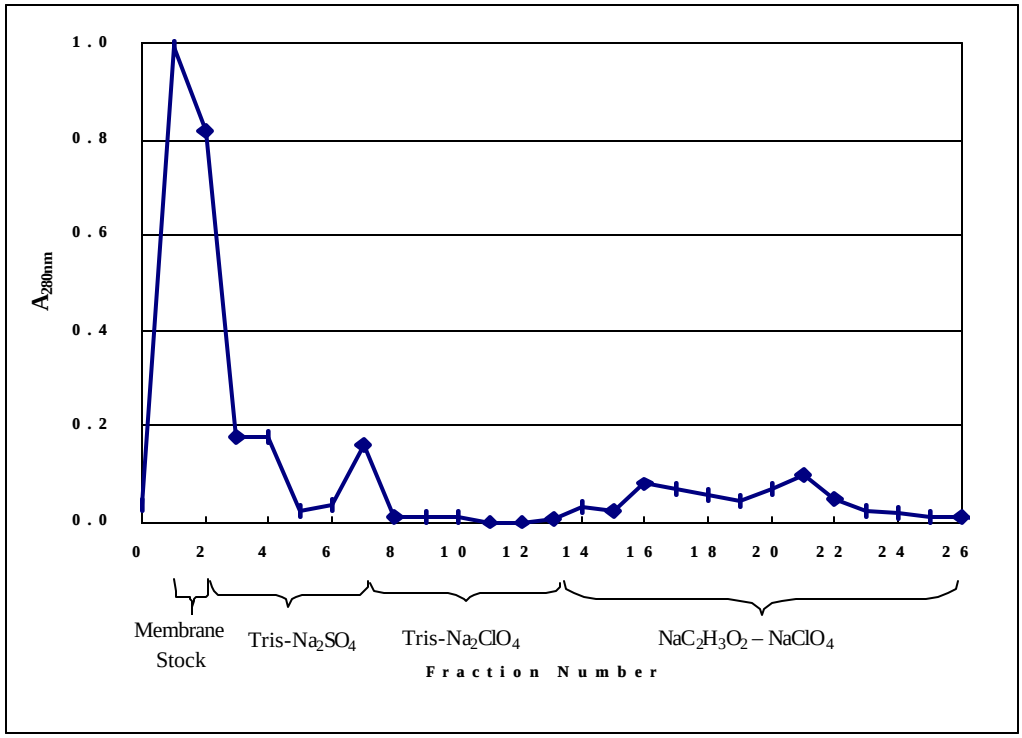


Figure 4.14: Elution profile of membrane stock after affinity chromatography

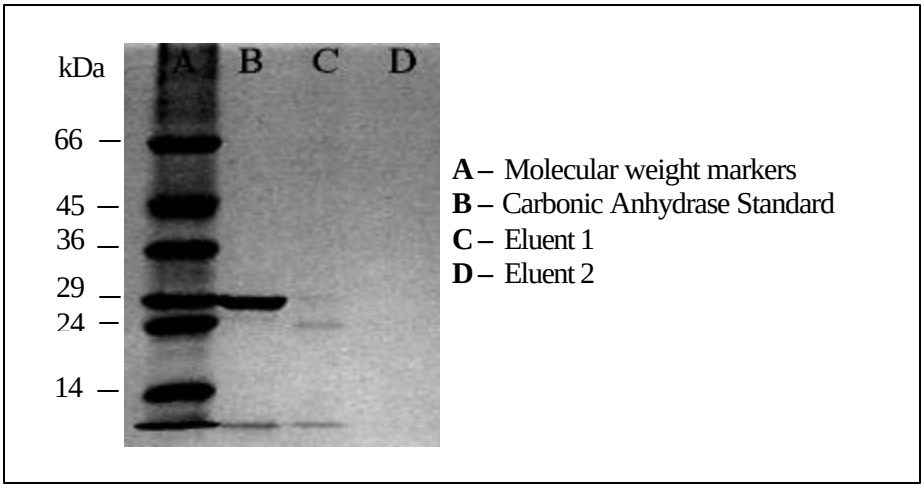


Figure 4.15: SDS-PAGE analysis of affinity chromatography eluents

Figure 4.15 shows that the CA was eluted with the 25 mM Tris-HCl; 0.3 M $NaClO_4$ at pH 8.7, as there are two bands clearly visible in fraction 7 (eluent 1). Nothing at all was seen in eluent 2, which were the combined fractions eluted with 0.1 M $NaC_2H_3O_2$; 0.5 M $NaClO_4$ at pH 5.6.

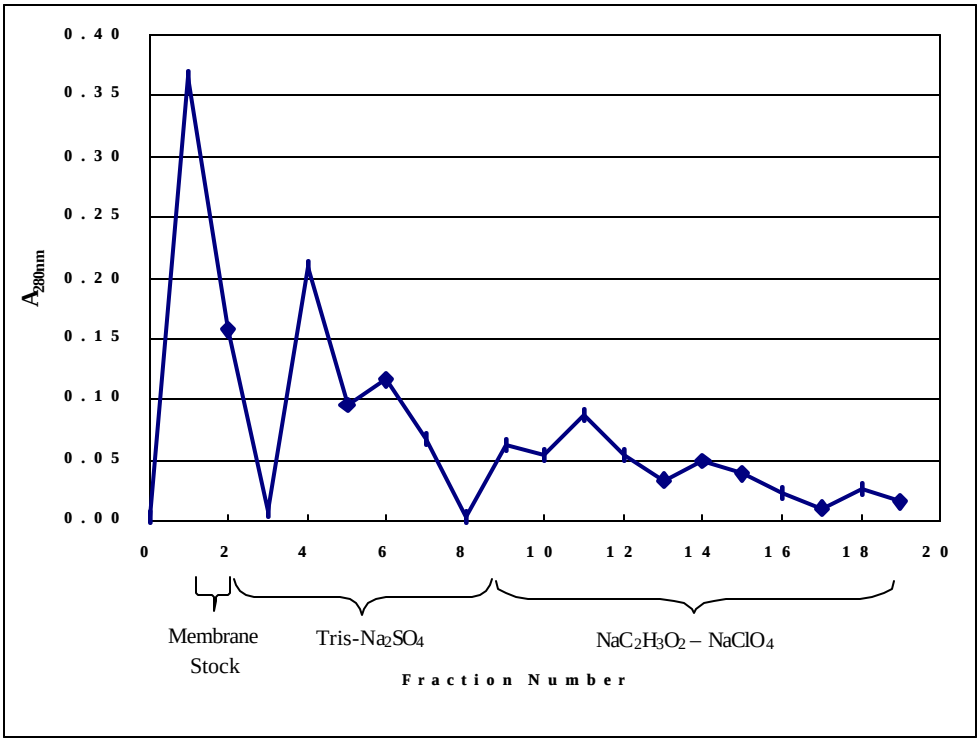


Figure 4.16: Elution profile of membrane stock, having omitted 25 mM Tris-HCl; 0.3 M $NaClO_4$ at pH 8.7

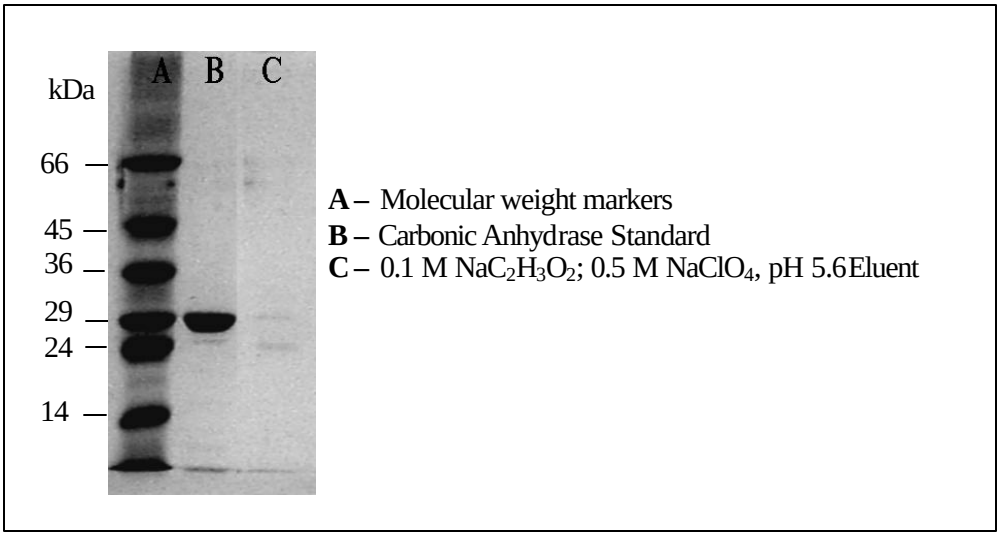


Figure 4.17: SDS-PAGE analysis of affinity chromatography eluent, having omitted 25 mM Tris-HCl; 0.3 M $NaClO_4$ at pH 8.7

The experiment was therefore repeated, but the wash with 25 mM Tris-HCl; 0.3 M $NaClO_4$ at pH 8.7 was omitted. After washing with 20ml 25 mM Tris-HCl; 22 mM Na_2SO_4 at pH 8.7, the CA was eluted with 20 ml 0.1 M $NaC_2H_3O_2$; 0.5 M $NaClO_4$ at

pH 5.6. The elution profile was plotted (Figure 4.16) and fractions 9 – 15 were combined and dialysed against 500 ml 20 mM Tris-HCl at pH 8.5.for 17 hours. The buffer was changed 4 times over this period. The dialysate was concentrated followed by analysis by SDS-PAGE (Figure 4.17).

Figure 4.17 shows that CA was successfully eluted by the 0.1 M $NaC_2H_3O_2$; 0.5 M $NaClO_4$ at pH 5.6. Two bands can be seen in the final sample, although fairly faint. The fact that the CA was eluted by both the 25 mM Tris-HCl; 0.3 M $NaClO_4$ at pH 8.7, and the 0.1 M $NaC_2H_3O_2$; 0.5 M $NaClO_4$ at pH 5.6, shows that the pH does not have a great effect on the elution of the protein. The presence of the $NaClO_4$ alone was enough to dissociate the CA from the *p*-aminomethylbenzenesulfonamide. The concentration of 0.3 M was also enough to elute the protein, it did not need to be increased to 0.5 M. The 25 mM Tris-HCl; 0.3 M $NaClO_4$ at pH 8.7 did in fact appear to be more effective at eluting the CA than the 0.1 M $NaC_2H_3O_2$; 0.5 M $NaClO_4$ at pH 5.6, as the bands obtained in figure 4.15 were far more distinct than those seen in figure 4.17. This indicates that more protein was eluted with the 25 mM Tris-HCl; 0.3 M $NaClO_4$ at pH 8.7.

CA was successfully isolated using this method. However, Western blot analysis using antibody to *Chlamydomonas* CA would be required to confirm the identity of CA. One can assume that the protein isolated in this manner was in fact CA due to the position on the SDS-PAGE gel, as well as the fact that it bound to the aminomethylbenzenesulfonamide, which is a specific and potent inhibitor of CA. It was also eluted by $NaClO_4$, another inhibitor of CA (Whitney, 1974). The fact that two bands were present in the final sample could indicate the presence of two subunits. Johansson & Forsman (1993) showed that pea CA appears on a SDS-PAGE gel as a doublet corresponding to masses of 25 kDa and 27 kDa.

4.2.1.10 CELL WALL-DEFICIENT MUTANT CW-15MT$^+$ OF *CHLAMYDOMONAS REINHARDTII*

The *Chlamydomonas reinhardtii* cell wall-deficient mutant cw-15mt$^+$ secretes CA into the surrounding medium (Coleman *et al*, 1984; Husic, 1991 XX). In order to isolate this CA 40 ml of cw-15mt$^+$ culture was centrifuged at 4100g for 10 min. Of the supernatant 20 ml was applied to a to 1 x 2 cm column of *p*-aminomethylbenzenesulfonamide-agarose, which had been washed and equilibrated with 20 mM Tris-HCL, pH 7.4. The column was washed with 20 ml 25 mM Tris-HCl; 22 mM Na_2SO_4 at pH 8.7, followed by 25 ml 25 mM Tris-HCl; 0.3 M $NaClO_4$ at pH 8.7. The CA protein was then eluted using 20 ml 0.1 M $NaC_2H_3O_2$; 0.5 M $NaClO_4$ at pH 5.6. The absorbance of all fractions was measured at 280 nm, and the elution profile plotted (Figure 4.18). Fractions 5, 7, 9 and 10 were combined (eluent 1), as well as 15 and 17 – 20 (eluent 2). The two samples were dialysed separately against 500 ml 20 mM Tris-HCl, pH 7.4. The buffer was changed 4 times over a 16 hour period. Both samples were concentrated, and were analysed by SDS-PAGE, along with a concentrated sample of the original supernatant (Figure 4.19).

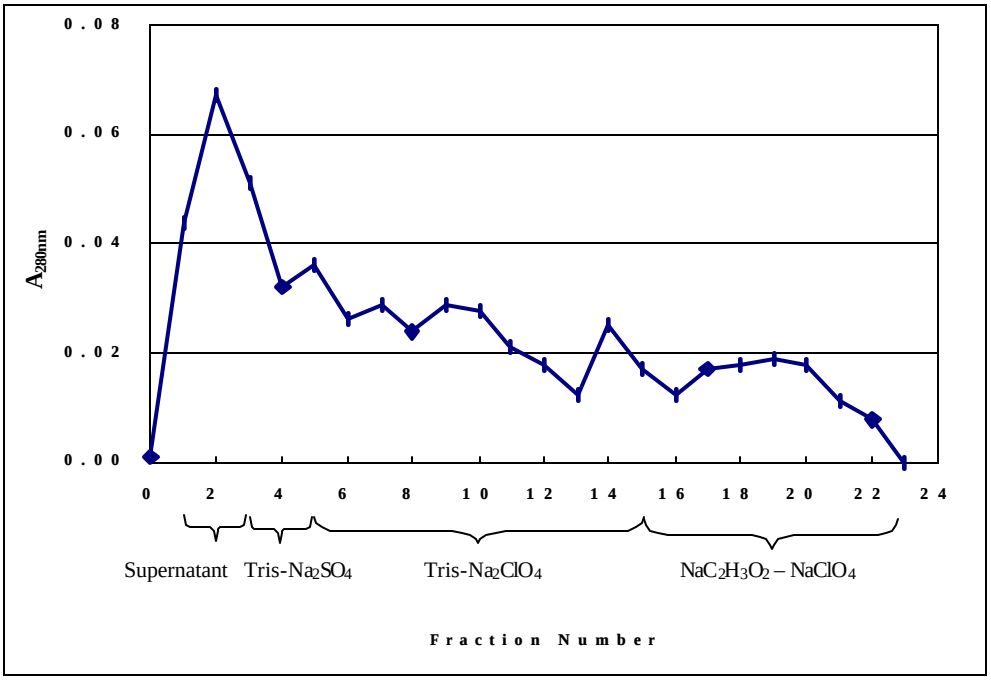


Figure 4.18: Elution profile of *Chlamydomonas* cw-15mt$^+$ after affinity chromatography

Figure 4.19 shows 3 proteins in the original supernatant, in, or close to, the 29 kDa region. This indicates that the CA is released into the medium, along with other proteins. There were, however, no proteins seen in either eluent fraction. This could possibly be due to the fact that the proteins were not concentrated enough. Only faint bands were seen in the original supernatant. This small quantity of protein may have become too diluted in the large eluent volume used during affinity chromatography, and hence was no longer detectable using SDS-PAGE.

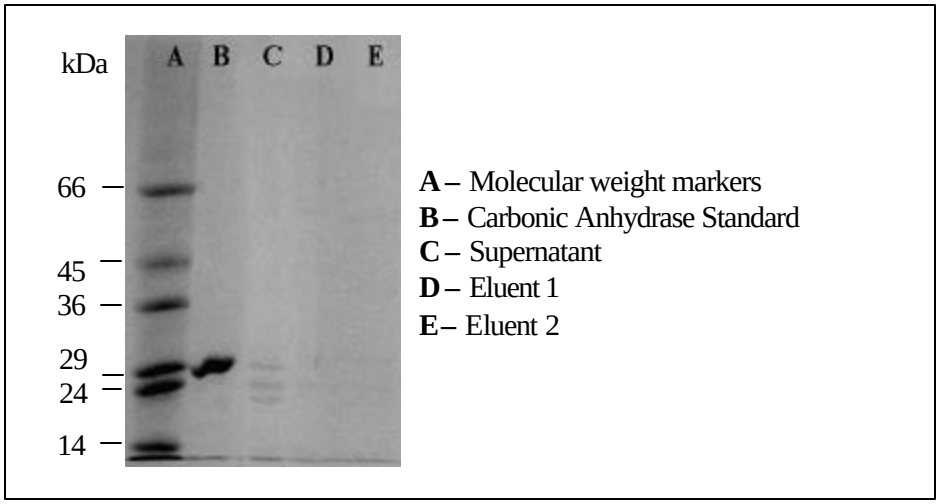


Figure 4.19: SDS-PAGE analysis of *Chlamydomonas* cw-15mt^{+} fractions

4.2.2 PURIFICATION OF CA FROM *SPIRULINA*

4.2.2.1 SAMPLE PREPARATION

Spirulina was cultured in Zarrouk's media under continuous light supplied by 2 fluorescent tubes (Osram cool white L20W/20S) at 24ºC. Cells were cultured on a Labcon orbital shaker at 132 rpm. Cells were harvested by centrifuging at 5500g for 10 min. Pellets were then washed twice in 20 ml cold NaCl (1g/l) and centrifuged at 7000g for 10 min.

4.2.2.2 SALT-INDUCED DISSOCIATION OF CA

A 200 ml sample of *Spirulina* was harvested and resuspended in 15 ml KPi buffer (20 mM potassium phosphate buffer at pH 7.4). This was then homogenised by two passages through a French pressure cell at 1500 psi, followed by centrifugation at

6000g for 15 min. The pellet was resuspended in 5 ml. Aliquots of 1 ml were incubated on ice with 2 ml KPi buffer containing 0, 0.8 and 1.0 M KCl. After a 15 min incubation samples were centrifuged at 10 000g for 5 min. The pellets were resuspended in 2 ml of the same buffer, and the incubation and centrifugation was repeated twice more. The final pellet was resuspended in 5 ml KPi buffer, and the combined supernatants were concentrated to 100 µl each. All samples were analysed by SDS-PAGE (Figure 4.20).

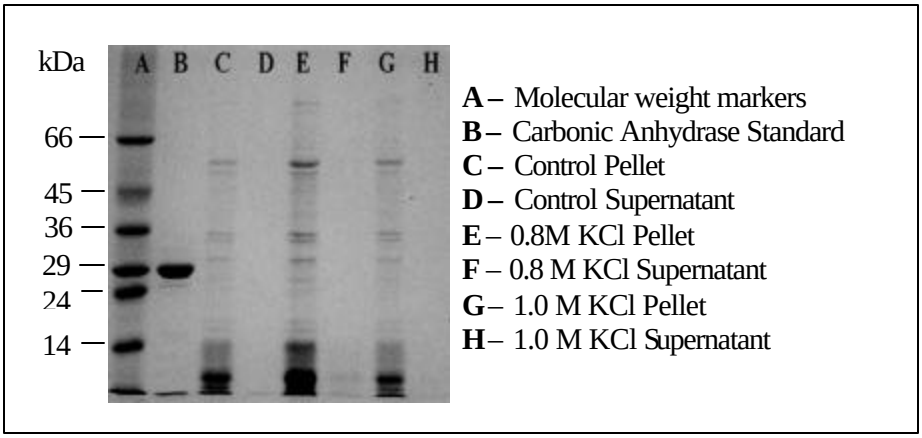


Figure 4.20: SDS-PAGE analysis of *Spirulina* cells treated with KCl

Figure 4.20 shows that the KCl was once again unsuccessful at dissociating the CA, as all the protein remained associated with the pellets, as they did in the case of *Chlamydomonas*. The proteins seen in the *Spirulina* pellet also differ from those seen in the case of *Chlamydomonas*. The protein assumed to be CA is slightly larger than in *Chlamydomonas*, and there are two proteins above it in the 36 kDa region. Either of these three proteins could represent CA, as molecular mass values of 27 – 42 kDa have been reported for CA (Bundy and Coté, 1980; Husic, 1991). *Spirulina* also has a protein in the 50 – 60 kDa range, as well as a high concentration of >5 kDa protein. A study conducted by Kamo and associates (1990) established the existence of a small subunit of CA of about 4 kDa in *Chlamydomonas reinhardtii*. It is possible that this band represents a subunit of CA, but we were unable to confirm this. It is also likely that this protein is completely unrelated to CA. As with *Chlamydomonas* it would be ideal to have an antibody to cyanobacterial CA in order to confirm the identity of CA, by Western blot analysis.

4.2.2.3 PREPARATION OF A MEMBRANE FRACTION

A membrane fraction was prepared from *Spirulina* in a similar manner to *Chlamydomonas*. A 250 ml sample of Spirulina cells were harvested and resuspended in 30 ml sucrose solution. The cells were homogenised by two passages through a French pressure cell at 1500 psi. They were then centrifuged at 1000g for 10 min. The pellet was resuspended in 15 ml sucrose solution, and centrifuged as before. The two supernatants were combined, and centrifuged at 105 000g for 1 hour. The pellet was resuspended in 25 ml 50 mM Tris-HCl (1 mM EDTA; pH 7.4) and centrifuged at 3000g for 20 min. The final pellet was resuspended in 10 ml 20 mM Tris-HCl, pH 7.4, to yield the membrane stock solution.

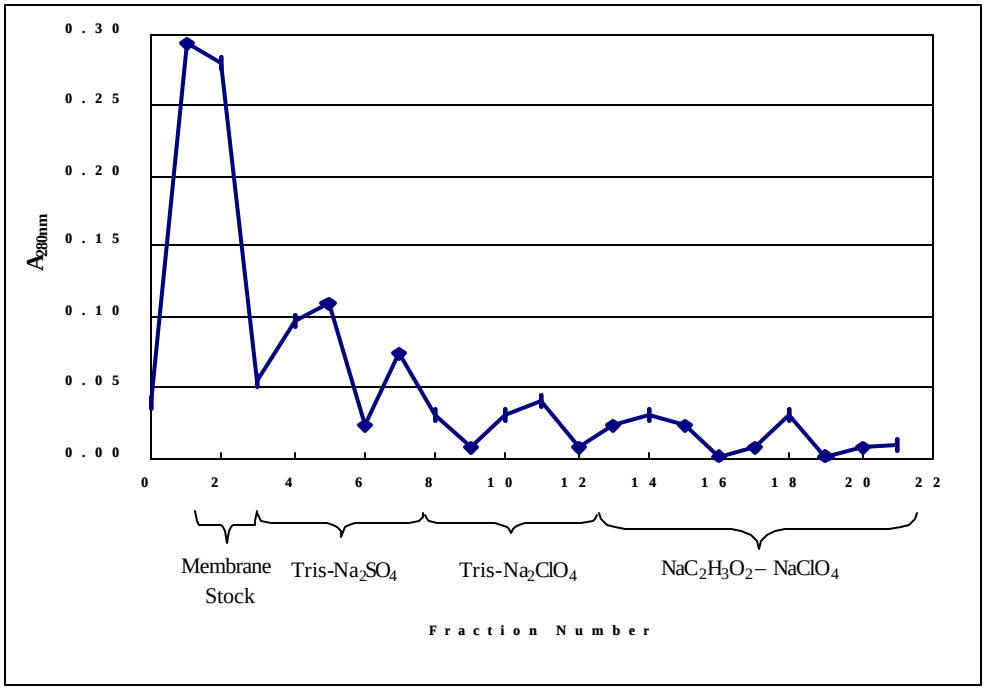


Figure 4.21: Elution profile of *Spirulina* membrane stock after affinity chromatography

Of the membrane stock, 3 ml was applied to a 1 x 2 cm column of *p*-aminomethylbenzenesulfonamide-agarose, which had been washed and equilibrated with 20 mM Tris-HCl, pH 7.4. The column was washed with 15 ml 25 mM Tris-HCl; 22 mM Na_2SO_4 at pH 8.7, followed by 25 ml 25 mM Tris-HCl; 0.3 M $NaClO_4$ at pH 8.7. The CA protein was then eluted using 15 ml 0.1 M $NaC_2H_3O_2$ 0.5 M $NaClO_4$ at pH 5.6. The absorbance of all fractions was measured at 280 nm, and the elution

profile plotted (Figure 4. 21). Fractions 13, 14 and 15 were combined (eluent 2), and dialysed against 500 ml 20 mM Tris-HCl, pH 7.4 for 24 hours. Fraction 7 (eluent 1) was also dialysed against 500 ml of the same buffer. The buffer was changed 4 times over the 24 hour period. Both fractions were concentrated and analysed by SDS-PAGE (Figure 4.22).

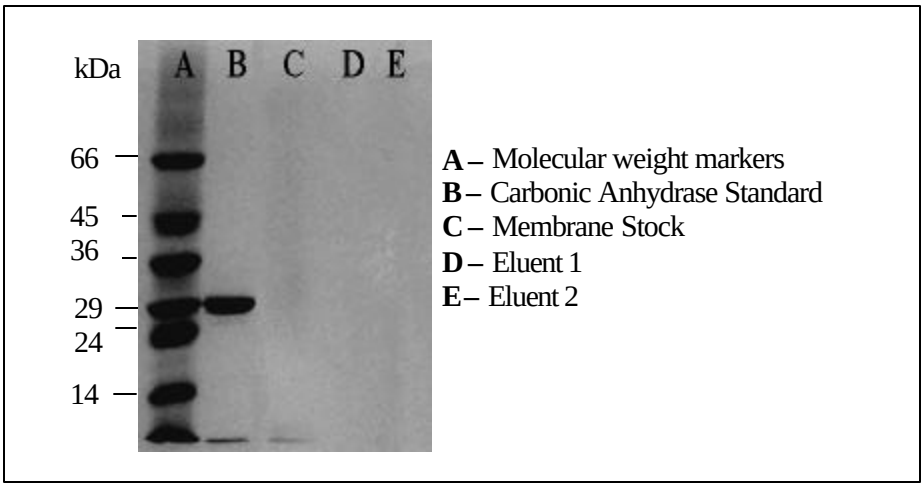


Figure 4.22: SDS-PAGE analysis of *Spirulina* membrane stock

No proteins were seen in either fraction eluted from the affinity column, and only a very faint smudge was seen in the membrane fraction (Figure 4.22). This could indicate that CA is not membrane bound in *Spirulina*. It could in fact be internal, although this would not account for the effect of increase in pH of the surrounding medium. It is, however, unlikely that no proteins were present in the membrane fraction. It is possible that the membrane stock was too dilute for the proteins to be detected. It is also possible that the protocol for membrane stock preparation may need to be adjusted slightly for *Spirulina,* due to the different ultrastructure.

No extracellular CA activity has been detected in most cyanobacteria analysed, although an activity closely associated with the membrane, or intrinsic to it, has been postulated as part of transport mechanisms leading to the entry of both CO_2 and HCO_3^-. A 'CA-like moiety' responsible for dehydration of HCO_3^- is part of a model system allowing equilibrated inorganic carbon (C_i) exchange across the membrane. An indirect proof for the likelihood of CA activity involvement in such systems is the

inhibition of C_i transport by the CA inhibitor ethoxyzolamide (EZA)(Bedu & Joset, 1991).

Espie and associates (1989) have demonstrated entry of CO_2 and HCO_3^- via independent systems and propose a model functioning without the mediation of CA. These authors do not, however, completely exclude a 'CA-like moiety' assisted model, which would best explain the inhibitory effect of EZA on the uptake of CO_2. A similar pattern of inhibition by EZA of CO_2 entry has been observed in *Synechocystis* PCC6803 strongly suggesting the presence of a zinc protein associated with this activity (Bedu & Joset, 1991). It has also been postulated (Fernley, 1988) that a 'CA-like moiety' is involved in C_I transport in A*nacystis nidulans.*

4.3 CONCLUSIONS

Affinity chromatography of a membrane stock prepared from *Chlamydomonas reinhardtii* yielded two bands. It was assumed that these bands represented two subunits of CA due to the position on the SDS-PAGE gel, as well as the fact that they were bound by the aminomethylbenzenesulfonamide and eluted by $NaClO_4$. If antibody to CA from *Chlamydomonas* had been obtainable, Western blot analysis would have been performed in order to confirm the identity of these proteins.

Unfortunately, purification of CA from *Spirulina* proved less successful. No proteins were visible in the membrane fraction, indicating that CA may not be membrane bound. It is more likely, however, that the membrane fraction was too dilute, or the procedure needs optimisation for *Spirulina*. It is also possible that it is not CA, but a 'CA-like moiety' that is involved in the alkalisation process, as few cyanobacteria appear to have external CA. This, however, would have been demonstrated in the membrane fraction.

Further analysis of *Spirulina* is required, as these results are very inconclusive. It is possible that further optimisation of available procedures will yield positive results with *Spirulina*. Another alternative is a molecular approach, which will be discussed in chapter five.

CHAPTER FIVE

CLONING CARBONIC ANHYDRASE

5.1 INTRODUCTION

As a result of the difficulties experienced with previous methods, an alternative method of identifying CA in *Spirulina*, involving standard molecular biology techniques was also considered. In terms of this procedure, DNA or protein sequences for CA from as many phylogenetic organisms as possible are aligned, and analysed for regions of homology. Based on regions of high homology in the DNA sequences, oligonucleotide primers can be designed for polymerase chain reaction (PCR). DNA isolated from *Spirulina* is then amplified and the PCR product obtained can be cloned into a plasmid vector (for example, pGEM®-T Easy, Promega). This is followed by ligation and transformation into a host cell. Once expressed, the genes can be sequenced and protein structure investigated. The most important consideration in designing these oligonucleotide primers is specificity, and therefore a very high degree of homology is required. Alternatively, degenerate primers can be designed from known amino acid sequences and cloned as above (Ausubel *et al*, 1983; Mathews & van Holde, 1990; Roe *et al*, 1996).

Another option involves the creation of a genomic library. This method is preferable if there is only one region of homology in the middle of the sequence of interest. Completely or partially digested genomic DNA is size fractionated and ligated into a plasmid vector. The plasmid library is then plated and the colonies transferred to a nitrocellulose membrane. The filters are then hybridised with a ^{32}P-labelled oligonucleotide probe, and the colonies containing the DNA of interest can be identified by autoradiography. The plasmid DNA can then be isolated and sequenced (Ausubel *et al*, 1983; Fukuzawa *et al*, 1990).

Various authors have cloned CA, although in most cases the polypeptide had been previously isolated and sequenced, at least partially. These amino acid sequences were then used to design degenerate primers. Eriksson and colleagues (1996), Fukuzawa and colleagues (1990 & 1991), and Karlsson and colleagues (1998) all constructed cDNA libraries from *Chlamydomonas reinhardtii* and screened them using radiolabelled degenerate primers designed as above. A cDNA encoding an intracellular β-CA has also been cloned and sequenced from the unicellular green alga *Coccomyxa* using similar techniques (Hiltonen *et al*, 1998). Fett and Coleman (1994) have isolated two cDNA clones from a *Arabidopsis thaliana* λYES cDNA library, using an internal restriction fragment of pea CA cDNA as a probe.

Soluble CA was purified from *Chlorella sorokiniana*, and the amino acid sequence determined. Based on this sequence, primers were designed for PCR. After two rounds of PCR and 5'-rapid amplification of cDNA ends (RACE), 342 bp of partial nucleotide sequence was obtained. Using this as a probe, a cDNA clone was screened from a cDNA library (Satoh *et al*, 1998).

CA has also been cloned from a *Synechocystis* PCC6803 subgenomic plasmid library (So and Espie, 1998). In this case oligonucleotide primers that shared high nucleotide sequence homology with the *ccaA* gene of *Synechococcus* PCC7942, and the *cynT* gene of *E. coli* were used to amplify a 349 bp DNA fragment of *Synechococcus* PCC7942. This was purified and used for Southern hybridisation analysis of a *Synechocystis* PCC6803 subgenomic library. Sequence analysis of the restriction fragment isolated from the library revealed three open reading frames (ORFs), one of which encoded a carboxysomal β-type CA.

5.2 SEQUENCE ALIGNMENT

The following CA sequences were obtained from Genbank using Entrez Nucleotide Query (National Institute of Health, USA): *Pisum sativum* (Accession number M63627), chloroplast CA from *Spinacia oleracea* (J05403), *Anabaena* PCC7120 CA (U72708), periplasmic CA from *Chlamydomonas reinhardtii* (AB026126), *Chlamydomonas reinhardtii* CA (E03319), soluble CA from *Chlorella sorokiniana*

(AB013804) and β-CA from *Coccomyxa* sp. PA. DNA and protein sequences were aligned using CLUSTAL W Multiple Sequence Alignment Program version 1.7, and observed for regions of homology.

Due to the fact that very little homology was observed between any of the sequences, suitable primers could not be designed. It was therefore decided not to pursue this approach. These procedures are also labour intensive and time consuming. A future option could be to use the *Synechocystis* PCC6803 β-CA sequence, identified by So and Espie (1998), as a primer for Southern hybridisation analysis of a *Spirulina* genomic library.

CHAPTER SIX

CONCLUSIONS

In determining the potential of *Spirulina* as a biosorbent the toxic effects of metals were investigated, and *Spirulina* was found to have a threshold level of about 30 µM for copper, zinc and lead. Results obtained in this study indicate that both copper and zinc have a direct effect on the photosynthetic pathway of *Spirulina*, causing cell deterioration and death. Lead, on the other hand, appears to affect structural properties, and thus takes longer to affect cell growth. Ultimately lead does lead to cell death.

Consequently, *Spirulina* does not appear to be a useful biomass for metal sorption but, although relatively low concentrations of metal may have a toxic effect on the algae, *Spirulina* may have potential as a precipitation agent. The role of *Spirulina* in the precipitation of heavy metals appears to be through its ability to maintain a high pH, possibly through the enzyme carbonic anhydrase. Hence an investigation of this enzyme was initiated.

A serious disadvantage associated with the study of CA is the lack of a reliable assay system. Many different assays have been devised, but they all have serious drawbacks limiting their use (Stemler, 1993). Hence, four different methods of detecting CA were investigated, with the aim of finding a reliable assay which could be used in a subsequent attempt to purify the enzyme from *Spirulina*.

Valuable time was spent testing two radiotracer assays that proved to be insensitive and results could not be reproduced. In addition, the radioactive material required was very expensive. Consequently, the standard Wilbur-Anderson method was investigated with little more success. Results were found to be inaccurate with a high degree of variability, and very little activity was found in *Spirulina*. The insensitivity of this assay has also been noted by other researchers (Badger & Price, 1989;

Giordano & Maberly, 1989; Hagalund *et al*, 1992; Raven, 1995). Finally the well-established analytical technique, SDS-PAGE was investigated. This proved to be the most reliable method of protein detection, although it was not quantitative. Consequently, this was the technique used in subsequent purification procedures.

Initial procedures employed to purify CA from *Spirulina* were unsuccessful. However, due to the fact that the techniques are well documented, it was decided to attempt purification from *Chlamydomonas*. If this proved successful the methods could be modified for use on *Spirulina*.

Although documented methods proved unsuccessful, affinity chromatography of *Chlamydomonas reinhardtii* membrane stock solution yielded two relatively pure bands. These bands were presumed to represent two CA subunits. However, confirmation of this by Western blot analysis, using antibody to *Chlamydomonas reinhardtii* CA, would be required. This could not be achieved because the appropriate antibody could not be obtained.

Unfortunately, purification of CA from *Spirulina* proved less successful. Further analysis of *Spirulina* is required, as results obtained were very inconclusive. It is possible that further optimisation of available procedures will yield positive results with *Spirulina*. It is also possible that it is not CA, but a 'CA-like moiety', that is involved in the alkalisation process, as few cyanobacteria appear to have external CA.

The possibility of cloning CA from a genomic library was also considered, but due to the fact that very little homology was observed between any of the sequences aligned, suitable primers could not be designed. These procedures are also labour intensive and time consuming.

REFERENCES

- Aderhold D., Williams C.J. and Edyvean R.G.J. (1996) The removal of heavy metal ions by seaweeds and their derivatives. *Bioresour. Technol.* **58:** 1-6.
- Ahuja P., Gupta R. and Saxena R.K. (1999) Zn^{2+} biosorption by *Oscillatoria auguistissima*. *Process Biochem.* **34:** 77-85.
- Aiking H., Stijnman A., Van Garderen C., van Heerikhuizen H. and Van't Riet J. (1984) Inorganic phosphate accumulation and cadmium detoxification in *Klebsiella aerogenes* NCTC 418 growing in continuous culture. *Appl. Environ. Microbiol.* **47:** 374-377.
- Aksu Z., Sag Y. and Kutsal T. (1992) The biosorption of copper (II) by *C. vulgaris* and *Z. ramigera*. *Environ. Technol.* **13:** 579-586.
- Aksu Z. and Açikel Ü. (1999) A single-staged bioseparation process for simultaneous removal of copper (II) and chromium (VI) by using *C. vulgaris*. *Process Biochem.* **34:** 589-599.
- Al-Moghrabi S., Goiran C., Allemand D., Speziale N. and Jaubert J. (1996) Inorganic carbon uptake for photosynthesis by the symbiotic coral-dinoflagellate association II. Mechanisms for bicarbonate uptake. *J. Exp. Mar. Biol. Ecol.* **199:** 227-248.
- Amaroso G., Weber C., Sültemeyer D. and Fock H. (1996) Intracellular carbonic anhydrase activities in *Dunaliella tetiolecta* (Butcher) and *Chlamydomonas reinhardtii* (Dangeard) in relation to inorganic carbon concentration during growth: further evidence for the existence of two distinct carbonic anhydrases associated with the chloroplasts. *Planta* **199:** 177-184.
- Armstrong J., Myers D.V., Verpoorte J.A. and Edsall J.T. (1966) Purification and properties of human erythrocyte carbonic anhydrases. *J. Biol. Chem.* **241:** 5137-5149.
- Atkins C.A., Patterson B.D. and Graham D. (1972a) Plant carbonic anhydrases: Distribution of types among species. *Plant Physiol.* **50:** 214-217.

- Atkins C.A., Patterson B.D. and Graham D. (1972b) Plant carbonic anhydrases: Preparation and some properties of monocotyledon and dicotyledon enzyme types. *Plant Physiol.* **50:** 218-223.
- Ausubel F.M., Brent R., Kingston R.E., Moore D.D., Seidman J.G., Smith J.A. and Struhl K. (1983) in *Current protocols in molecular biology*, 3rd Edition, Ch. 2, Wiley-Interscience, New York.
- Avery S.V. and Tobin J.M. (1993) Mechanism of adsorption of hard and soft metal ions to *Saccharomyces cerevisiae* and influence of hard and soft anions. *Appl. Environ. Microbiol.* **59:** 2851-2856.
- Badger M.B. and Price G.D. (1989) Carbonic anhydrase activity associated with the cyanobacterium *Synechococcus* PCC7942. *Plant Physiol.* **89:** 51-60.
- Badger M. B. and Price G.D. (1992) The CO_2 concentratng mechanism in cyanobacteria and microalgae. *Physiol. Plant.* **84:** 606-615.
- Badger M.B., Yu J.W. and Price G.D. (1991) Selection and analysis of mutants of the CO_2-concentrating mechanism in cyanobacteria. *Can. J. Bot.* **69:** 974-983.
- Badger M.R. and Andrews T.J. (1982) Photosynthesis and inorganic carbon usage by the marine cyanobacterium, *Synechococuus* sp. *Plant Physiol.* **70:** 517-523.
- Badger M.R., Basset M. and Comins H.N. (1985) A model for HCO_3^- accumulation and photosynthesis in the cyanobacterium *Synechococcus* sp. *Plant Physiol.* **77:** 465-471.
- Badger M.R., Palmqvist K. and Yu J.W. (1994) Measurement of CO_2 and HCO_3^- fluxes in cyanobacteria and microalgae during steady-state photosynthesis. *Physiol. Plant.* **90:** 529-536.
- Badger R.B., Andrews T.J., Whitney S.M., Ludwig M., Yellowlees D.C., Leggat W. and Price G.D. (1998) The diversity and coevelution of Rubisco, plastids, pyrenoids, and chloroplast-based CO_2-concentrating mechanisms in algae. *Can. J. Bot.* **76:** 1052-1071.
- Bakkaloglu I., Butter T.J., Evison L.M., Holland F.S. and Hancock I.C. (1998) Screening of various types of biomass for removal and recovery of heavy metals (Zn, Cu, Ni) by biosorption, sedimentation and desorption. *Wat. Sci. Technol.* **38:** 269-277.

- Barón M., Arellano J.B. and Gorgé J.L. (1995) Copper and photosystem II: A controversial relationship. *Physiol. Plant.* **94:** 174-180.
- Beardall J., Johnston A. and Raven J. (1998) Environmental regulation of CO_2-concentrating mechanisms in microalgae. *Can. J. Bot.* **76:** 1010-1017.
- Bedu S. and Joset F. (1991) Studies on the carbonic anhydrase activity in *Synechococcus* PCC6803 wild type and an acetazolamide-resistant mutant. *Can. J. Bot.* **69:** 1103-1108.
- Behravan G., Jonsson B.H. and Lindskog S. (1990) Fine tuning of the catalytic properties of carbonic anhydrase. *Eur. J. Biochem.* **190:** 351-357.
- Beveridge T. J. (1986) The immobilisation of soluble metals by bacterial walls. *Biotechnol. Bioeng. Symp.* **16:** 127-131.
- Bianchi M.F., Carbone M.L. and Lucchini G. (1981) Mn^{2+} and Mg^{2+} uptake in Mn-sensitve and Mn-resistant yeast strains. *Plant. Sci. Lett.* **22:** 345-352.
- Bold H.C. and Wynne M.J. (1985) in *Introduction to the algae,* 2nd edition, pp 84-93, Prentice-Hall, Englewood Cliffs, NJ.
- Borowitzka M.D. and Borowitzka L.J. (1990) in *Biology of Marine Plants,* Clayton M.N. and King R.J. (Eds), pp.385-399, Longman Cheshire, Melbourne.
- Botrè C. and Botrè F. (1990) Determination of carbonic anhydrase activity by a pCO_2 sensor. *Anal. Biochem.* **185:** 254-264.
- Bowes G.W. (1969) Carbonic anhydrase in marine algae. *Plant Physiol.* **44:** 726-732.
- Bradford M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
- Brady D. and Duncan J.R. (1994a) Bioaccumulation of metal cations by *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **41:** 149-154.
- Brady D. and Duncan. J.R. (1994b) Cation loss during accumulation of heavy metal cations by *Saccharomyces cerevisiae*. *Biotechnol. Letters* **16:** 543-548.
- Brady D. and Duncan. J.R. (1994c) Binding of heavy metals by the cell walls of *Saccharomyces cerevisiae*. *Enzyme Microbiol. Technol.* **16:** 633-637.

- Brady D., Stoll A.D., Starke L. and Duncan J.R. (1994a) Chemical and enzymatic extraction of heavy metal binding polymers from isolated cell walls of *Saccharomyces cerevisiae*. *Biotechnol. Bioeng*. **44:** 297-302.
- Brady D., Glaum D. and Duncan. J.R. (1994b) Copper tolerance in *Saccharomyces cerevisiae*. *Lett. Appl. Microbiol*. **18:** 245-250.
- Brady D., Letebele B., Duncan J.R. and Rose P.D. (1994c) Bioaccumulation of metals by *Scenedesmus, Selenastrum* and *Chlorella* algae. *Water SA*. **20:** 213-218.
- Brady M. J. and Tobin J.M. (1995) Binding of hard and soft metal ions to *Rhizopus arrhizus* biomass. *Enzyme Microbiol. Technol*. **17:** 791-796.
- Braus-Stromeyer S.A., Schnappauf G., Braus G.H., Göbner A.S. and Drake H.L. (1997) Carbonic anhydrase in *acetobacterium woodii* and other acetogenic bacteria. *J. Bacteriol.* **179:** 7197-7200.
- Buchheim M.A., Turmel M., Zimmer E.A. and Chapman R.L. (1991) Phylogenetic systematics of *Chlamydomonas* based on cladistic analysis of nuclear 18s rRNA sequence data. *J. Phycol.* **26:** 689-691.
- Bundy H.F. and Coté S. (1980) Purification and properties of carbonic anhydrase from *Chlamydomonas reinhardtii*. *Phytochemistry* **19:** 2531-2534.
- Cabral J.P.S. (1992) Selective binding of metal ions to *Pseudomonas syringae* cells. *Microbios*. **71:** 47-53.
- Çetinkaya Dönmez G., Aksu Z., Öztürk A. and Kutsal T. (1999) A comparative study of the effects of competing ions on the biosorption of metals. *Process Biochem.* **34:** 885-892.
- Chatterjee S., Asthana R.K., Tripathi A.K. and Singh S.P. (1996) Metal recovery by selected sorbents. *Process Biochem.* **31:** 457-462.
- Ciferri O. (1983) *Spirulina,* the edible microorganism. *Microbiol. Rev.* **47:** 551-578.
- Coleman C.R., Berry J.A., Togasaki R.K. and Grossman A.R. (1984) Identification of extracellular carbonic anhydrase of *Chlamydomonas reinhardtii*. *Plant Physiol*. **76:** 472-477.
- Corder S.L. and Reeves M. (1994) Biosorption of nickel in complex aqueous waste streams by cyanobacteria. *Appl. Biochem. Biotechnol.* **45/46:** 847-859.

- Cordery J., Wills A.J., Atkinson K. and Wills B.A. (1994) Extraction and recovery of silver from low-grade liquors using microalgae. *Minerals Engineering* **7:** 1003-1015.
- Cross R.H.M. (1987) in *A handbook on the preparation of biological material for electron microscopy,* pp 5-12 & 34-37, Rhodes University, Grahamstown.
- Cybulsky D. L., Nagy A., Kandel S.I., Kandel M. and Gornal G. (1979) Carbonic anhydrase from spinach leaves. *J. Biol. Chem.* **254:** 2032-2039.
- Darnall D.W., Green B., Henzl M.T., Hosea J.M., McPherson R.A., Sneddon J. and Alexander M.D. (1986) Selective recovery of gold and other metals from an algal biomass. *Environ. Sci. Technol.* **20:** 206-208.
- Davies A.D. (1983) in *Progress in phycological research,* Rourd F.E. and Chapman D.J. (Eds), pp 113-145, Elsevier Science Publishers.
- De Wet L.P.D., Schoonbee H.J., Pretorius J. and Bezuidenhout L.M. (1990) Bioaccumulation of selected heavy metals by the water fern *Azolla filiculoides* Lam. in a wetland ecosystem affected by sewage, mine and industrial pollution. *Water S.A.* **16:** 281-286.
- Department of Water Affairs (1986) in *Management of the water resources of the Republic of South Africa,* pp 1.3-1.15, Pretoria.
- Dionisio-Sese M.L. and Miyachi S. (1992) The effect of sodium chloride on carbonic anhydrase activity in marine microalgae. *J. Phycol.* **28:** 619-624.
- Dixon G.K., Patel B.N. and Merret M.J. (1987) Role of intracellular carbonic anhydrase in inorganic-carbon assimilation by *Porphyridium purpureum. Planta* **172:** 508-513.
- Duncan J.R., Brady D. and Wilhelmi B.S. (1997) Immobilisation of yeast and algal cells for bioremediation of heavy metals. In *Methods in Biotechnology, vol. 2: Bioremediation protocols,* Sheehan D. (Ed), pp 91-97, Humana Press Inc., Totowa, NJ.
- Durand-Chastel H. (1980) in *Algae Biomass,* Shelef G. and Soeder C.J. (Eds), pp 51-64, Elsevier/North-Holland Biomedical Press.
- Eccles H. (1999) Treatment of metal-contaminated wastes: why select a biological process? *Tibtech.* **17:** 462-465.

- Eriksson M., Karlsson J., Ramazanov Z., Gardeström P. and Samuelsson G. (1996) Discovery of an algal mitochondrial carbonic anhydrase and characterisation of a low-CO_2-induced polypeptide in *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA* **93:** 12031-12034.
- Espie G.S., Miller A.G. and Canvin D.T. (1989) Selective and reversible inhibition of active CO_2 transport by hydrogen sulfide in a cyanobacterium. *Plant Physiol.* **91:** 387-394.
- Espie G.S., Miller A.G. and Canvin D.T. (1991) High affinity transport of CO_2 in the cyanobacterium *Synechococcus* UTEX 625. *Plant Physiol.* **97:** 943-953.
- Espie G.S., Miller A.G., Kandasamy R.A. and Canvin D.T. (1991) Active HCO_3^- transport in cyanobacteria. *Can. J. Bot.* **69:** 936-944.
- Fehrmann C. and Pohl P. (1993) Cadmium adsorption by the non-living biomass of microalgae grown in axenic mass culture. J. *Appl. Phycol.* **5:** 555-562.
- Fernley R.T. (1988) Noncytoplasmic carbonic anhydrase. *Trends Biochem. Sci.* **13:** 356-358.
- Fett J.P. and Coleman J.R. (1994) Characterisation and expression of two cDNAs encoding carbonic anhydrase in *Arabidopsis thaliana*. *Plant Physiol.* **105:** 707-713.
- Flores-Moya A. and Fernández J.A. (1998) The role of external carbonic anhydrase in the photosynthetic use of inorganic carbon in the deep-water alga *Phyllariopsis purpurascens* (Laminariales, Phaeophyta). *Planta* **207:** 115-119.
- Ford T. and Mitchell R. (1992) in *Environmental Microbiology*, pp 83-101, Wiley Liss Inc.
- Fourest E. and Roux J.C. (1992) Heavy metal biosorption by fungal mycelial by-products: mechanism and influence of pH. *Appl. Microbiol. Biotechnol.* **37:** 399-403.
- Freskgård P.O., Carlsson U., Mårtensson L.G. and Jonsson B.H. (1991) Folding around the C-terminus of carbonic anhydrase II. *FEBS Lett.* **289:** 117-122.
- Fukuzawa H., Fujiwara S., Yamamoto Y., Dionisio-Sese M.L. and Miyachi S. (1990) cDNA cloning, sequence, and expression of carbonic anhydrase in *Chlamydomonas reinhardtii*: Regulation by environmental CO_2 concentration. *Proc. Natl. Acad. Sci. USA* **87:** 4383-4387.

- Fukuzawa H., Ishida S. and Miyachi S. (1991) cDNA cloning and gene expression of carbonic anhydrase in *Chlamydomonas reinhardtii*. *Can. J. Bot.* **69:** 1088-1096.
- Funke R.P., Kovar J.L. and Weeks D.P. (1997) Intracellular carbonic anhydrase is essential to photosynthesis in *Chlamydomonas reinhardtii* at atmospheric levels of CO_2. *Plant Physiol.* **114:** 237-244.
- Furla P., Bénazet-Tambutté S., Jaubert J. and Allemand D. (1998) Diffusional permeability of dissolved inorganic carbon through isolated oral epithelial layers of the sea anemone, *Anemonia viridis*. *J. Exp. Mar. Biol. Ecol.* **221:** 71-88.
- Gadd G.M. (1988) in *Biotechnology – A Comprehensive Treatise*, vol. 6b, Rehm H.J. and Reeds G. (Eds), pp 401-430, VCH, Weinheim.
- Gadd G.M. (1990) Heavy metal accumulation by bacteria and other microorganisms. *Experientia* **46:** 834-840.
- Gadd G.M. and White C. (1993) Microbial treatment of metal pollution - a workable biotechnology? *Tibtech.* **11:** 353-359.
- Gale N.L. and Wixon B.G. (1978) Removal of heavy metals from industrial effluents by algae. *Symposium*: *Biological recovery of metals from wastewater* **Ch. 24:** 259-273.
- Gardea-Torresdey T.L., Becker-Hapack M.K., Hosea J.M. and Darnall D.W. (1990) Effect of chemical modification of algal carboxyl groups on metal ion binding. *Environ. Sci. Technol.* **24:** 1372-1378.
- Garnham G.W., Codd G.A. and Gadd G.M. (1992) Kinetics of uptake and intracellular location of cobalt, manganese and zinc in the estuarine green alga *Chlorella salina*. *Appl. Microbiol. Biotechnol.* **37:** 270-276.
- Gekeler W., Grill E., Winnacker E. and Zenk M.H. (1988) Algae sequester heavy metals via synthesis of phytochelatin complexes. *Arch. Microbiol.* **150:** 197-202.
- Giordano M. and Maberly S.C. (1989) Distribution of carbonic anhydrase in British marine macroalgae. *Oecologia* **81:** 534-539.
- Goyal A., Shiraiwa Y., Husic H.D. and Tolbert N.E. (1992) External and internal carbonic anhydrases in *Dunaliella* species. *Mar. Biol.* **113:** 349-355.
- Hagalund K., Ramazanov Z., Mtolera M. and Pedersen M. (1992) Role of external carbonic anhydrase in light-dependent alkalisation by *Fucus serratus* L. and *Laminaria saccharina* (L.) Lamour. (Phaeophyta). *Planta* **188:** 1-6.

- Hammarström P., Kalman B., Jonsson B.H. and Carlsson U. (1997) Pyrene excimer fluorescence as a proximity probe for investigation of residual structure in the unfolded state of human carbonic anhydrase II. *FEBS Lett.* **420:** 63-68.
- Harris P.O. and Ramelow G.J. (1990) Binding of metal ions by particulate biomass derived from *Chlorella vulgaris* and *Scenedesmus quadricauda*. *Environ. Sci. Technol.* **24:** 220-228.
- Hart B.A., Bertram P.E. and Scaife B.D. (1979) Cadmium transport by *Chlorella Pyrenoidosa*. *Environ. Res.* **18:** 327.
- Hiltonen T., Björkbacka H, Forsman C., Clarke A.K. and Samuelsson G. (1998) Intracellular β-carbonic anhydrase of the unicellular green alga *Coccomyxa*. *Plant Physiol.* **117:** 1341-1349.
- Hiltonen T., Karlsson J., Palmqvist K, Clarke A.K and Samuelsson G. (1995) Purification and characterisation of an intracellular carbonic anhydrase from the unicellular green alga *Coccomyxa*. *Planta* **195:** 345-351.
- Holan Z.R. and Volesky B. (1994) Biosorption of lead and nickel by biomass of marine algae. *Biotechnol. Bioeng.* **43:** 1001-1009.
- Holan Z.R., Volesky B. and Prasetyo I. (1993) Biosorption of cadmium by biomass of marine algae. *Biotechnol. Bioeng.* **41:** 819-825.
- Horikoshi T., Nakajima A. and Sakaguchi T. (1979) Uptake of uranium by *Synechococcus elongatus*. *J. Ferment. Technol.* **57:** 191-197.
- Hulse J.H. (1982) Food science and nutrition: The gulf between the rich and the poor. *Science* **216:** 1291-1294.
- Humble A.V., Gadd G.M. and Codd G.A. (1997) Binding of copper and zinc to three cyanobacterial microcystins quantified by differential pulse polarography. *Wat. Res.* **31:** 1679-1686.
- Husic H.D. (1991) Extracellular carbonic anhydrase of *Chlamydomonas reinhardtii*: localisation, structural properties, and catalytic properties. *Can. J. Bot.* **69:** 1079-1087.
- Husic H.D. and Marcus C.A. (1994) Identification of intracellular carbonic anhydrase in *Chlamydomonas reinhardtii* with a carbonic anhydrase-directed photoaffinity label. *Plant Physiol.* **105:** 133-139.

- Husic H.D. and Quigley E.A. (1990) Salt-induced dissociation of carbonic anhydrase from intact cells of *Chlamydomonas reinhardtii*. *Plant Physiol.* **94:** 380-383.
- Husic H.D., Kitayama M., Togasaki R.K., Moroney J.V., Morris K.L. and Tolbert N.E. (1989) Identification of intracellular carbonic anhydrase in *Chlamydomonas reinhardtii* which is distinct from the periplasmic form of the enzyme. *Plant Physiol.* **89:** 904-909.
- Iglesias-Rodríguez M.D., Nimer N.A. and Merret M.J. (1998) Carbon dioxide-concentrating mechanism and the development of extracellular carbonic anhydrase in the marine picoeukaryote *Micromonas pusilla*. *New Phytol.* **140:** 685-690.
- Ingle R.K. and Colman B. (1975) Carbonic anhydrase levels in blue-green algae. *Can. J. Bot.* **53:** 2385-2387.
- Ishida S., Muto S. and Miyachi S. (1993) Structural analysis of periplasmic carbonic anhydrase 1 of *Chlamydomonas renhardtii*. *Eur. J. Biochem.* **214:** 9-16.
- Jebanathirajah J.A. and Coleman J.R. (1998) association of carbonic anhydrase with a Calvin cycle enzyme complex in *Nicotiana tabacum*. *Planta* **204:** 177-182.
- Johansson I.J. and Forsman C. (1993) Kinetic studies of pea carbonic anhydrase. *Eur. J. Biochem.* **218:** 439-446.
- Johansson I.J. and Forsman C. (1994) Solvent hydrogen isotope effects and anion inhibition of CO_2 hydration catalysed by carbonic anhydrase from *Pisum sativum*. *Eur. J. Biochem.* **224:** 901-907.
- Kamo T., Shimogawara K., Fukuzawa H., Muto S. and Miyachi S. (1990) Subunit constitution of carbonic anhydrase from *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* **192:** 557-562.
- Kaplan A., Schwarz R., Lieman-Hurwitz J. and Reinhold L. (1991) Physiological and molecular aspects of the inorganic carbon-concentrating mechanism in cyanobacteria. *Plant Physiol.* **97:** 851-855.
- Kapoor A. and Viraraghavan T. (1995) Fungal biosorption – an alternative treatment option for heavy metal bearing wastewaters: A review. *Bioresour. Technol.* **53:** 195-206.

- Kapoor A. and Viraraghavan T. (1998) Removal of heavy metals from aqueous solution using immobilised fungal biomass in continuous mode. *Wat. Res.* **32:** 1968-1977.
- Karlsson J., Clarke A.K., Chen Z.Y., Hugghins S.Y., Moroney J.V. and Samuelsson G. (1998) A novel α-type carbonic anhydrase associated with the thylakoid membrane in *Chlamydomonas reinhardtii* is required for growth at ambient CO_2. *EMBO J.* **17:** 1208-1216.
- Karlsson J., Hiltonen T., Husic H.D., Ramazanov Z. and Samuelsson G. (1995) Intracellular carbonic anhydrase of *Chlamydomonas reinhardtii*. *Plant Physiol.* **109:** 533-539.
- Katzman G.L., Carlson S.J., Marcus Y., Moroney J.V. and Togasaki R.K. (1994) Carbonic anhydrase activity in isolated chloroplasts of wild-type and high-CO_2-dependent mutants of *Chlamydomonas reinhardtii* as studied by a new assay. *Plant Physiol.* **105:** 1197-1202.
- Kerby N.W. and Stewart W.D.P. (1988) in *Biochemistry of the algae and cyanobacteria*, Rogers L.J. and Gallon J.R. (Eds), pp 319-334, Clarendon Press, Oxford.
- Kidd M. (1997) in *Environmental Law,* pp 83-84, Juta & Co. Ltd., Cape Town.
- Krishnan S.S., Cancilla A. and Jervis R.E. (1987) Industrial wastewater treatment for toxic heavy metals using natural materials as adsorbents. *J. Radioanal. Nucl. Chem.* **110:** 373-378.
- Kuyuack N. and Volesky B. (1988) Biosorbents for recovery of metals from industrial solutions. *Biotechnol. Bioeng.* **10:** 137-142.
- Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of a bacteriophage T4. *Nature* **227:** 680-685.
- Laube V.M., Mckenzie C.N. and Kushner D.J. (1980) Strategies of response to copper, cadmium and lead by a blue-green and a green alga. *Can. J. Microbiol.* **26:** 1300-1311.
- Lawrence J.R., Swerhone G.D.W. and Kwong Y.T.J. (1998) Natural attenuation of aqueous metalcontamination by an algal matt. *Can. J. Microbiol.* **44:** 825-832.
- Lee M.Y., Lee S.H., Shin H.J., Kajiuchi T. and Yang J.W. (1998) Characteristics of lead removal by crab shell particles. *Process Biochem.* **33:** 749-753.

- Lee S.H., Jung C.H., Chung H., Lee M.Y. and Yang J.W. (1998) Removal of heavy metals from aqueous solution by apple residues. *Process Biochem.* **33:** 205-211.
- Lindskog S. (1997) Structure and mechanism of carbonic anhydrase. *Pharmacol. Ther*. **74:** 1-20.
- Maeda H., Kobayashi M., Sakata T. and Hayashi R. (1997) Carbonic anhydrase activities of cyanobacterial phytoplankton in Lake Biwa. *Fisheries Sci.* **63:** 1054-1055.
- Mann H. and Fyfe W.S. (1984) An experimental study of algal uptake of U, Ba, V, Co, and Ni from dilute solutions. *Chem. Geol*. **44:** 385-398.
- Mann H. and Fyfe W.S. (1985) Uranium uptake by algae: experimental and natural environments. *Can. J. Earth Sci.* **22:** 1899-1903.
- Mann H. and Fyfe W.S. (1988) Biogeochemical cycling of the elements in some fresh water algae from gold and uranium mining districts. *Biorecovery* **1:** 3-26.
- Marcus Y., Volokita M. and Kaplan A. (1984) The location of the transporting system for inorganic carbon and the nature of the form translocated in *Chlamydomonas reinhardtii*. *J. Exp. Bot*. **35:** 1136-1144.
- Martel A., Yu S., Garcia-Reina G., Lindblad P. and Pederson M. (1992) Osmotic adjustment in the cyanobacterium *Spirulina platensis*: Presence of an α-glucosidase. *Plant Physiol. Biochem*. **30:** 573-578.
- Mathews C.K. and van Holde K.E. (1990) in *Biochemistry*, pp 898-904, The Benjamin/Cummings Publishing Company, Inc.
- McKnight D.M. and Morel F.M.M. (1979) Release of weak and strong copper-complexing agents by algae. *Linmol. Oceanogr*. **24:** 823-837.
- McKnight D.M. and Morel F.M.M. (1980) Copper complexation by siderophores from filamentous blue-green algae. *Limnol. Oceanogr*. **25:** 62-71.
- Mercado J.M., Figueroa F.L., Niell F.X. and Axelsson L. (1997) A new method for estimating external carbonic anhydrase activity in macroalgae. *J. Phycol*. **33:** 999-1006.
- Miller A.G., Espie G.S. and Canvin D.T. (1990) Physiological aspects of CO_2 and HCO_3^- transport by cyanobacteria: a review. *Can. J. Bot.* **68:** 1291-1302.

- Morel F.M.M., Hudson R.J.M. and Price N.M. (1991) Limitation of productivity by trace elements in the sea. *Limnol. Oceanogr*. **36:** 1742-1756.
- Moroney J.V. and Chen Z.Y. (1998) The role of the chloroplast in inorganic carbon uptake by eukaryotic algae. *Can. J. Bot.* **76:** 1025-1035.
- Moroney J.V. and Mason C.B. (1991) The role of the chloroplast in inorganic carbon acquisition by *Chlamydomonas reinhardtii*. *Can. J. Bot.* **69:** 1017-1024.
- Moroney J.V. and Tolbert N.E. (1985) Inorganic carbon uptake by *Chlamydomonas reinhardtii*. *Plant Physiol.* **77:** 253-258.
- Moroney J.V., Husic H.D. and Tolbert N.E. (1985) Effect of carbonic anhydrase inhibitors on inorganic carbon accumulation by *Chlamydomonas reinhardtii*. *Plant Physiol*. **79:** 177-183.
- Moroney J.V., Togasaki R.K., Husic H.D., and Tolbert N.E. (1987) Evidence that an internal carbonic anhydrase is present in 5 % CO_2-grown and air-grown *Chlamydomonas*. *Plant Physiol*. **84:** 757-761.
- Muraleedharan T.R., Iyengar L. and Venkobachar C. (1991) Biosorption: an attractive alternative for metal removal and recovery. *Current Science*. **61:** 379-385.
- Nakajima A., Horikoshi T. and Sakaguchi T. (1981) Studies on the accumulation of heavy metal elements in biological systems. *Eur. J. Appl. Microbiol. Biotechnol*. **12:** 76-83.
- Neugebauer J.M. (1990) Detergents: An overview. *Methods. Enzymol*. **182:** 239-253.
- Nourbakhsh M., Sag Y., Özer D., Aksu Z., Kutsal T. and Çaglar A. (1994) A comparative study of various biosorbents for removal of chromium (VI) ions from industrial waste waters. *Process Biochem.* **29:** 1-5.
- Oswald W.J. (1988) in *Microalgal Biotechnology*, Borowitzka M.D. and Borowitzka L.J. (Eds), Cambridge University Press, New York.
- Overnell J. (1975) The effect of heavy metals on photosynthesis and loss of potassium in two species of marine algae, *Dunaliella tertiolecta* and *Phaedoctylum tricornutum*. *Marine biology* **29:** 99-103.

- Özer A., Ibrahim Ekiz H., Özer D., Kutsal T. and Çaglar A. (1997) A staged purification process to remove heavy metal ions from wastewater using *Rhizopus arrhizus*. *Process Biochem.* **32:** 319-326.
- Palmqvist K., Sültemeyer D., Baldet P., Andrews T.J. and Badger M.R. (1995) Characterisation of inorganic carbon fluxes, carbonic anhydrase(s) and ribulose-1,5-biphosphate carboxylase-oxygenase in the green unicellular alga *Coccomyxa*. *Planta* **197:** 352-361.
- Park Y.I., Karlsson J., Rodjestvenski I., Pronina N., Klimov V., Öquist G. and Sammuelsson G. (1999) Role of a novel photosystem II-associated carbonic anhydrase in photosynthetic carbon assimilation in *Chlamydomonas reinhardtii*. *FEBS Lett.* **444:** 102-105.
- Peltier G., Cournac L., Despax V., Dimon B., Fina L., Genty B. and Rumeau D. (1995) Carbonic anhydrase activity in leaves as measured in vivo by ^{18}O exchange between carbon dioxide and water. *Planta* **196:** 732-739.
- Pempkowiak J. and Kosakowska A. (1998) Accumulation of cadmium by green algae *Chlorella vulgaris* in the presence of marine humic substances. *Environ. Int.* **24:** 583-588.
- Price G.D., Coleman J.R. and Badger M.B. (1992) Association of carbonic anhydrase activity with carboxysomes isolated from the cyanobacterium *Synechococcus* PCC7942. *Plant Physiol.* **100:** 784-793.
- Price N.M. and Morel M.M. (1990) Cadmium and cobalt substitution for zinc in a marine diatom. *Nature* **344:** 658-660.
- Puranik P.R., Modak J.M. and Paknikar K.M. (1999) A comparative study of the mass transfer kinetics of metal biosorption by microbial biomass. *Hydrometallurgy* **52:** 189-197.
- Ramwani R. and Mishra R.K. (1985) Purification of bovine striatal dopamine D-2 receptor by affinity chromatography. *J. Biol. Chem.* **261:** 8894-8898.
- Ratatore C. and Colman B. (1991) The localisation of active inorganic carbon transport at the plasma membrane in *Chlorella ellipsoidea*. *Can. J. Bot.* **69:** 1025-1031.
- Raven J.A. (1995) Photosynthetic and non-photosynthetic roles of carbonic anhydrase in algae and cyanobacteria. *Phycologia.* **34:** 93-101.

- Richmond A. (1986) in *Handbook of microalgal mass culture,* pp 117-145, 212-230, CRC Press Inc., USA.
- Rickli E.E., Ghazanfar S.A.S., Gibbons B.H. and Edsall J.T. (1964) Carbonic anhydrases from human erythrocytes. *J. Biol. Chem.* **239:** 1065-1077.
- Rivoallan A., Calmettes M. and Cohendet M. (1994) Recovery and recycling of heavy metals in industrial sludges. *Resources, Conservation and Recycling* **10:** 177-184.
- Roe B.A., Crabtree J.S. and Khan A.S. (1996) in *DNA isolation & sequencing,* pp 23-31, John Wiley & Sons, USA.
- Roy D., Greenlaw R.N. and Shane B.S. (1993) Adsorption of heavy metals by green algae and ground rice hulls. *J. Environ. Sci. Health* **A28:** 37-50.
- Sag Y., Açikel Ü., Aksu Z. and Kutsal T. (1998) A comparative study for the simultaneous biosorption of Cr (VI) and Fe (III) on *C. vulgaris* and *R. arrhizus*: application of the competitive adsorption models. *Process Biochem.* **33:** 273-281.
- Sambrook J., Fritsch E.F. and Maniatis T. (1989) in *Molecular cloning. A laboratory manual, 2nd edition,* pp 18.64-18.75, Cold Spring Harbour Laboratory Press.
- Sashidhar V.R., Kumur M.U., Prasad T.G., Seetharam A. and Devendra R. (1990) Determination of carbonic anhydrase activity using infra-red gas analysis. *Phytochem.* **29:** 55-58.
- Satoh A., Iwasaki T., Odani S. and Shiraiwa Y. (1998) Purification, characterisation and cDNA cloning of soluble carbonic anhydrase from *Chlorella sorokiniana* grown under ordinary air. *Planta* **206:** 657-665.
- Sauvé D.M., Ho D.T. and Roberge M. (1995) Concentration of dilute protein for gel electrophoresis. *Anal. Biochem.* **226:** 382-383.
- Schiewer S. and Volesky B. (1996) Modelling multi-metal ion exchange in biosorption. *Environ. Sci. Technol.* **30:** 2921-2927.
- Scott J.A. and Palmer S.J. (1990) Sites of cadmium uptake in bacteria used for biosorption. *Appl. Microbiol. Biotechnol.* **33:** 221-225.

- Scozzafava A. and Supuran T. (1998) Carbonic anhydrase inhibitors: novel compounds containing S-NH moieties: sulfenamido-sulfonamides, sulfenimido-sulfonamides and their interaction with isozymes I, II and IV. *J. Enzyme Inhib.* **13:** 419-442.
- Sharma D.C. and Forster C.F. (1993) Removal of hexavalent chromium using sphagnum moss peat. *Wat. Res.* **7:** 1201-1208.
- Shelef G., Azov Y., Moraine R. and Oron G. (1980) in *Algae Biomass,* Shelef G. and Soeder C.J. (Eds), pp 163-187, Elsevier/North-Holland Biomedical Press.
- Shiraiwa Y., Goyal A. and Tolbert N.E. (1993) Alkalisation of the medium by unicellular green algae during uptake of dissolved inorganic carbon. *Plant Cell Physiol.* **34:** 649-657.
- Silverman D.N. (1973) Carbonic anhydrase catalysed oxygen-18 exchange between bicarbonate and water. *Arch. Biochem. Biophys.* **155:** 452-457.
- Silverman D.N. (1991) The catalytic mechanism of carbonic anhydrase. *Can. J. Bot.* **69:** 1070-1078.
- Singh S.P. and Yadava V. (1985) Cadmium uptake in *Anacystis nidulans*: Effect of modifying factors. *J. Gen. Appl. Microbiol.* **31:** 39-48.
- Singh S.P., Singh R.K., Pandey P.K. and Pant A. (1992) Factors regulating copper uptake in free and immobilized cyanobacterium. *Folia Microbiol.* **37:** 315-320.
- Sly W.S. and Hu P.Y. (1995) Human carbonic anhydrases and carbonic anhydrase deficiencies. *Ann. Rev. Biochem.* **64:** 375-401.
- So A.K.C. and Espie G.S. (1998) Cloning, characterisation and expression of carbonic anhydrase from the cyanobacterium *Synechocystis* PCC6803. *Plant Mol. Biol.* **37**: 205-215.
- Srivastava A. and Srivastava P.C. (1990) Adsorption-desorption behaviour of zinc (II) at iron (III) hydroxide-aqueous solution interface as influenced by pH and temperature. *Environ. Pollut.* **68:** 171-180.
- Stemler A. (1993) An assay for carbonic anhydrase activity and reactions that produce radiolabelled gases or small uncharged molecules. *Anal. Biochem.* **210:** 328-331.
- Stokes P.M. (1983) in *Progress in Phycological Research,* Rowid F.E. and Chapman D.J. (Eds), pp 87-112, Elsevier Science Publishers.

- Strandberg G.W., Shumate S.E. and Parrott J.R. (1981) Microbial cells as biosorbents for heavy metals: accumulation of uranium by *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* **41:** 237-245.
- Sueoka N. (1959) Mitotic replication of deoxyribonucleic acid in *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA* **46:** 83-91.
- Sültemeyer D. (1998) Carbonic anhydrase in eukaryotic algae: characterization, regulation, and possible function during photosynthesis. *Can. J. Bot.* **76:** 962-972.
- Sültemeyer D., Amaroso G. and Fock H. (1995) Induction of intracellular carbonic anhydrase during the adaption to low inorganic carbon concentrations in wild-type and *ca-1* mutant cells of *Chlamydomonas reinhardtii*. *Planta* **196:** 217-224.
- Sültemeyer D., Miller A.G., Espie G.S., Fock H.P. and Canvin D.T. (1989) Active CO_2 transport by the green alga *Chlamydomonas reinhardtii*. *Plant Physiol.* **89:** 1213-1219.
- Sültemeyer D., Price G.D., Yu J.W. and Badger M.B. (1995) Characterisation of carbon dioxide and bicarbonate transport during steady-state photosynthesis in the marine cyanobacterium *Synechococcus* strain PCC7002. *Planta* **197:** 597-607.
- Summers A.O. (1992) The hard stuff: metals in bioremediation. *Curr. Opin. Biotechnol.* **3:** 271-276.
- Tashian R.E. (1989) The carbonic anhydrases: Widening perspectives on their evolution, expression and function. *Bioessays* **10:** 186-192.
- Thomas T.C. and McNamee M.G. (1990) Purification of membrane proteins. *Methods. Enzymol.* **182:** 499-519.
- Ting Y.P., Lawson F. and Prince I.G. (1991) Uptake of cadmium and zinc by the alga *Chlorella vulgaris:* A multi-ion situation. *Biotechnol. Bioeng.* **37:** 445-455.
- Tsezos M. and Volesky B. (1981). Biosorption of uranium and thorium. *Biotechnol. Bioeng.* **23:** 583-604.
- Tsezos M. and Volesky B. (1982) the mechanism of thorium biosorption by *Rhizopus arrhizus*. *Biotechnol. Bioeng.* **24:** 955-969.
- Tsezos M., Remoudaki E. and Angelatou V. (1996) A study of the effects of competing ions on the biosorption of metals. *Int. Biodeterior.Biodegrad.* **38:** 19-29.

- Tsuzuki M. and Miyachi S. (1991) CO_2 syndrome in *Chlorella*. *Can. J. Bot.* **69:** 1003-1007.
- Veglio F. and Beolchini F. (1997) Removal of metals by biosorption: A review. *Hydrometallurgy* **44:** 301-316.
- Venkobachar C. (1990) Metal removal by waste biomass to upgrade wastewater treatment plants. *Water Sci. Technol.* **22:** 319-320.
- Vilchez C., Garbayo I., Lobato M.V. and Vega J.M. (1997) Microalgae-mediated chemicals production and wastes removal. *Enzyme Microb. Technol.* **20:** 562-572.
- Villarejo A., Orús M.I., Ramazanov Z. and Martínez F. (1998) A 38-kilodalton low-CO_2-inducible polypeptide is associated with the pyrenoid in *Chlorella vulgaris*. *Planta* **206:** 416-425.
- Volesky B. (1987) Biosorbents for metal recovery. *Tibtech.* **5:** 96-101.
- Volesky B. (1986) Biosorbent materials. *Biotechnol. Bioeng. Symp.* **16:** 121-126.
- Volesky B. (1990) in *Biosorption of heavy metals*, pp 3-5, 8-9, 20-40, CRC Press, Inc., USA.
- Volesky B. (1992) Removal of heavy metals by biosorption. *American Chemical Society* 462-466.
- Volesky B. and Holan Z.R. (1995) Biosorption of heavy metals. *Biotechnol. Prog.* **11:** 235-250.
- Vonshak A., Kancharaska N., Bunnag B. and Tanticharoen M. (1996) Role of light and photosynthesis on the acclimation process of the cyanobacterium *Spirulina platensis* to salinity stress. *J. Appl. Phycol.* **8:** 119-124.
- Wase J. and Forster C. (1997) in *Biosorbents for metal ions*, pp 1-9, Taylor & Francis Ltd, London.
- Wehrheim B. and Wettern M. (1994) Biosorption of cadmium, copper and lead by isolated mother cell walls and whole cells of *Chlorella fusca*. *Appl. Microbiol. Biotechnol.* **41:** 725-728.
- Wells J.W., Kandel S.I., Kandel M. and Gornall A.G. (1975) The esterase activity of bovine carbonic anhydrase B above pH 9. *J. Biol. Chem.* **250:** 3522-3530.
- Whitney P.L. (1974) Affinity chromatography of carbonic anhydrase. *Anal. Biochem.* **57:** 467-476.

- Whitton B.A. and Say P.J. (1975) in *River Ecology*, pp 286-311, Blackwell Scientific Publications, Alden Press, Oxford.
- Wilbur K.M. and Anderson N.G. (1948) Electrometric and colorimetric determination of carbonic anhydrase. *J. Biol. Chem.* **176:** 147-154.
- Wilde E.N. and Benneman J.R. (1993) Bioremoval of heavy metals by the use of microalgae. *Biotech. Adv.* **11:** 791-812.
- Wilhelmi B.S. and Duncan J.R. (1995) Metal recovery from *Saccharomyces cerevisiae* biosorption columns. *Biotechnol. Letters* **17:** 1007-1012.
- Williams T.G. and Colman B. (1995) Quantification of the contribution of CO_2, HCO_3^-, and external carbonic anhydrase to photosynthesis at low dissolved inorganic carbon in *Chlorell saccharophila*. *Plant Physiol.* **107:** 245-251.
- Williams T.G. and Colman B. (1996) The effects of pH and dissolved inorganic carbon on external carbonic anhydrase activity in *Chlorella saccharophila*. *Plant Cell Environ.* **19:** 485-489.
- Wintermans J.F.G.M. and De Mots A. (1965) Spectrophotometric characteristics of chlorophylls *a* and *b* and their pheophytins in ethanol. *Biochim. Biophys. Acta* **109:** 448-453.
- Yagawa Y., Shiraiwa Y. and Miyachi S. (1984) Carbonic anhydrase from the blue-green alga (cyanobacterium) *Anabaena variabilis*. *Plant & Cell Physiol.* **25:** 775-783.
- Yang S., Tsuzuki M. and Miyachi S. (1985) Carbonic anhydrase of *Chlamydomonas*: Purification and studies on its induction using antiserum against *Chlamydomonas* carbonic anhydrase. *Plant & Cell Physiol.* **26:** 25-34.
- Zhao M. and Duncan J.R. (1997a) Batch removal of sexavalent chromium by *Azolla filiculoides*. *Biotechnol. Appl. Biochem.* **26:** 179-182.
- Zhao M. and Duncan J.R. (1997b) Column sorption and desorption of hexavalent chromium from aqueous solution and electroplating effluent using *Azolla filiculoides*. *Res. Environ. Biotechnol.* **2:** 51-64.
- Zhao M. and Duncan J.R. (1998a) Removal and recovery of nickel from aqueous solution and electroplating rinse effluent using *Azolla filiculoides*.**33:** 249-255.
- Zhao M. and Duncan J.R. (1998b) Bed-depth-service-time analysis on column removal of Zn^{2+} using *Azolla filiculoides*. *Biotechnol. Letters* **20:** 37-39.

- Zhou J.L. and Kiff R.J. (1991) The uptake of copper from aqueous solution by immobilized fungal biomass. *J. Chem. Technol. Biotechnol.* **52:** 317-330.
- Zhu X.L. and Sly W.S. (1990) Carbonic anhydrase IV from human lung. *J. Biol. Chem.* **265:** 8795-8801.
- Zielinski S., Buca M. and Famulski M. (1998) Precipitation-stripping processes for heavy metals. *Hydrometallurgy* **48:** 253-263.

APPENDICES

APPENDIX A

ZARROUK'S MEDIUM (2L[a])

NaCl	2.0 g
K_2SO_4	2.0 g
KNO_3	6.0 g
H_3PO_4[b]	0.5 ml
$NaHCO_3$	33.6 g
EDTA	0.16 g
$FeSO_4 \cdot 7H_2O$	0.02 g

[a] The volume is made up to 2 litres with Milli-Q deionised water and the pH adjusted to 9.3.

[b] The acid (H_3PO_4) is added after the salts have been dissolved to prevent it reacting with them.

APPENDIX B

ATOMIC ABSORPTION SPECTROPHOTOMETER SET-UP PARAMETERS FOR THE ANALYSIS OF COPPER, ZINC AND LEAD

	COPPER	ZINC	LEAD
BURNER TYPE	Air-acetylene	Air-acetylene	Air-acetylene
LAMP	Phototron Hollow Cathode (Cu)	Phototron Hollow Cathode (Zn)	Phototron Hollow Cathode (Pb)
LAMP CURRENT	4 mA	5 mA	4 mA
WAVELENGTH	324.7 nm	213.9 nm	217.0 nm
SLIT WIDTH	0.5 nm	0.5 nm	1.0 nm

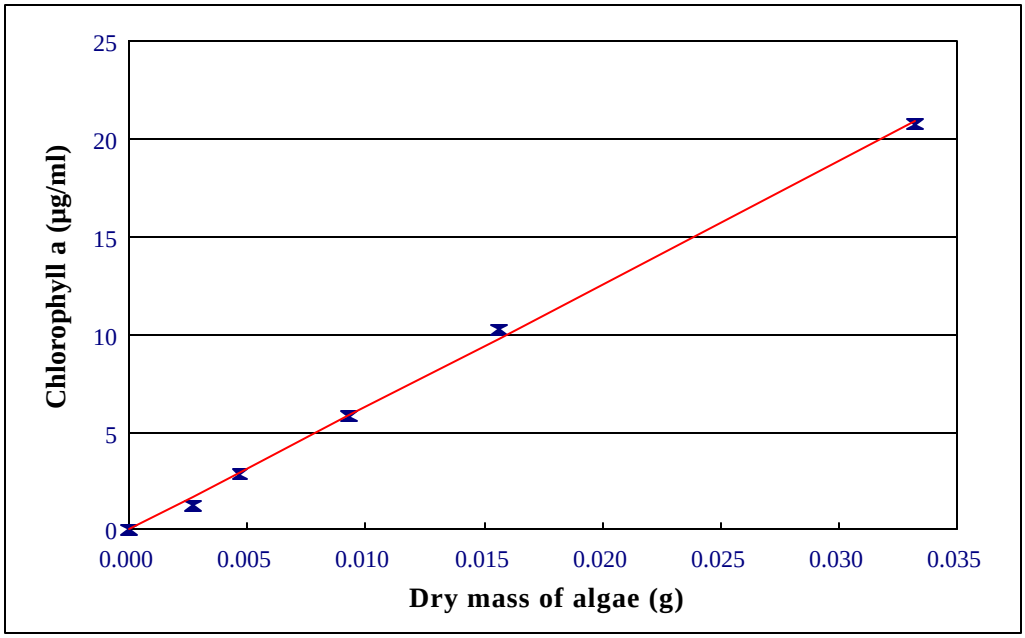
APPENDIX C

EQUATIONS FOR THE CALCULATION OF CHLOROPHYLL CONCENTRATIONS IN *SPIRULINA*

Chlorophyll *a*	$C_a = 11.24\ A_{661.6} - 2.04\ A_{644.8}$
Chlorophyll *b*	$C_b = 20.13\ A_{644.8} - 4.19\ A_{661.6}$
Chlorophyll *a* + *b*	$C_{a+b} = 7.05\ A_{661.6} + 18.09\ A_{644.8}$
Xanthophylls + Carotenoids	$C_{x+c} = 1000\ A_{470} - 1.90\ C_a - 63.14\ C_b / 214$

APPENDIX D

STANDARD CURVE FOR ESTIMATION OF ALGAL DRY MASS FROM CHLOROPHYLL *a* CONCENTRATIONS



APPENDIX E

SDS-PAGE RECIPES (Laemmli, 1970)

RESOLVING GEL BUFFER STOCK SOLUTION (1 M TRIS -HCl, PH 8.8)

Tris	60.6 g
Concentrated HCl	7.3 ml
Milli-Q deionised H_2O	500 ml

STACKING GEL BUFFER STOCK SOLUTION (1 M TRIS -HCl, PH 6.8)

Tris	60.6 g
Concentrated HCl	41.0 ml
Milli-Q deionised H_2O	500 ml

BATH BUFFER STOCK SOLUTION

Tris	30.3 g
Glycine	144.1 ml
SDS	10.0 g
Milli-Q deionised H_2O	500 ml

Dilute 1:10 with deionised H_2O before use.

ACRYLAMIDE STOCK SOLUTION

Acrylamide	150 g
N, N'-methylene-bis-acrylamide	4.0 g
Milli-Q deionised H_2O	500 ml

This recipe gives an acrylamide: bis ratio of 30: 0.8.

DISSOCIATION BUFFER

1 M Tris-HCl, pH 6.8	5.0 ml
10 % SDS	1.0 ml
β-mercaptoethanol	1.0 ml
Glycerol	2.0 ml
0.2 % Bromophenol Blue	0.5 ml
Milli-Q deionised H_2O	0.5 ml

Dilute 1:1 with deionised H_2O before use.

SLAB GEL FORMULATIONS

PLUG

Acrylamide Stock Solution	3.0 ml
10 % APS*	80 µl
TEMED	40 µl

* APS must be freshly prepared.

12 % RESOLVING GEL

Acrylamide Stock Solution	16 ml
1 M Tris-HCl, pH 8.8	15 ml
Milli-Q deionised H_2O	8.0 ml
10 % SDS	0.8 ml
10 % APS	0.3 ml
TEMED	20 µl

4 % STACKING GEL

Acrylamide Stock Solution	2.0 ml
1 M Tris-HCl, pH 6.8	1.9 ml
Milli-Q deionised H_2O	9.4 ml
80 % Glycerol	1.0 ml
10 % SDS	0.15 ml
10 % APS	0.1 ml
TEMED	20 µl

STAINING SOLUTION

Methanol	45 ml
Glacial Acetic Acid	10 ml
Coomassie Brilliant Blue	0.2 g
Milli-Q deionised H_2O	45 ml

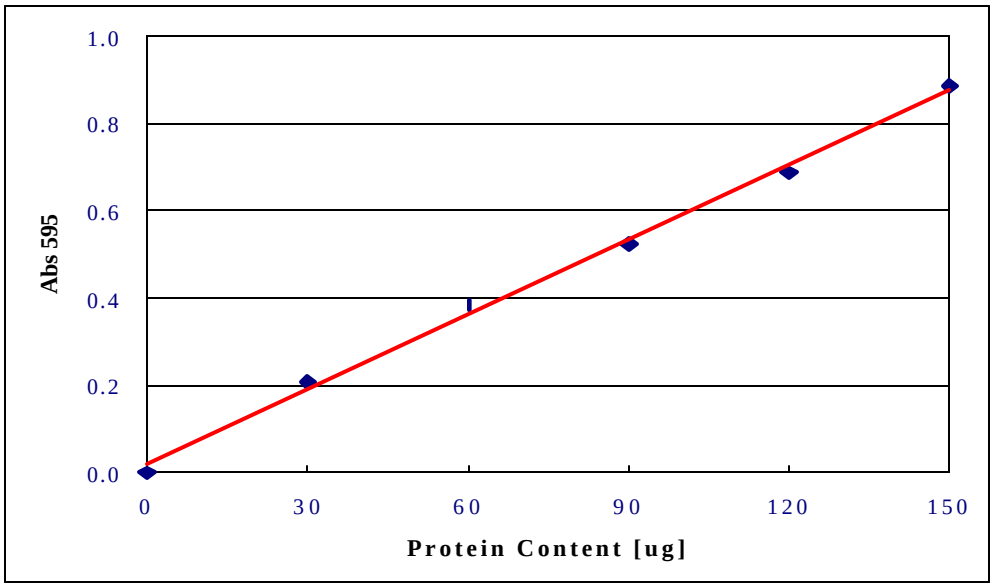
Dissolve the dye in a small amount of methanol, and then filter. Add the remaining methanol, $H_2O$and acetic acid.

DESTAINING SOLUTION

Methanol	450 ml
Glacial Acetic Acid	70 ml
Glycerol	100 ml
Milli-Q deionised H_2O	380 ml

APPENDIX F

A TYPICAL PROTEIN STANDARD CURVE USING BSA



APPENDIX G

BRADFORD'S REAGENT

95 % Ethanol	50 ml
85 % Phosphoric Acid	100 ml
Coomassie Brilliant Blue	100 mg
Milli-Q deionised H_2O	850 ml

Dissolve the Coomassie Brilliant Blue in ethanol, then add the phosphoric acid and make up to 1 litre with deionised water. Filter through Whatman # 1 filter paper and store in a brown bottle. Shake before use.

APPENDIX H

SUEOKA'S MINIMAL MEDIUM

NH_4Cl	0.50 g
$MgSO_4 \cdot 7H_2O$	0.02 g
$CaCl_2 \cdot 2H_2O$	0.01 g
K_2HPO_4	1.44 g
KH_2PO_4	0.72 g
Hunters Trace Element Solution*	1.0 ml
Milli-Q deionised H_2O	1.0 litre

* HUNTERS TRACE ELEMENT SOLUTION

Dissolve 50 g EDTA in 250 ml deionised H_2O. Heat to dissolve.

Dissolve the following, one by one, in order, heating to approximately 100ºC in 550 ml deionised H_2O.

BO_3H_3	11.4 g
$ZnSO_4 \cdot 7H_2O$	22.0 g
$MnCl_2 \cdot 4H_2O$	5.06 g
$FeSO_4 \cdot 7H_2O$	4.99 g
$CoCl_2 \cdot 6H_2O$	1.61 g
$CuSO_4 \cdot 5H_2O$	1.57 g
$Mo_7O_{24}(NH_4)_6 \cdot 4H_2O$	1.1 g

Mix the two solutions together. The resulting solution should be blue-green.

Heat to 100ºC. Cool slightly, but don't let the temperature drop below 80-90ºC.

Adjust the pH to 6.5 – 6.8 with 20 % KOH. Don't let the temperature drop below 70ºC until after the pH is adjusted.

Make up to 1 litre with deionised H_2O, and allow to stand at room temperature until the colour changes from green to purple (± 2 weeks).

Filter, with suction, through 3 layers of Whatman # 1 filter paper, and store in a brown bottle at 4ºC.

APPENDIX I

EQUATIONS FOR THE CALCULATION OF CHLOROPHYLL CONCENTRATIONS IN *CHLAMYDOMONAS*

Chlorophyll *a*	$C_a = 13.70\ (A_{665} - A_{750}) - 5.76\ (A_{649} - A_{750})$
Chlorophyll *b*	$C_b = 25.80\ (A_{649} - A_{750}) - 7.60\ (A_{665} - A_{750})$
Chlorophyll *a* + *b*	$C_{a+b} = 6.10\ (A_{665} - A_{750}) + 20.04\ (A_{649} - A_{750})$