THE TOXIC EFFECT OF HEAVY METALS ON ALGAL BIOMASS (*SPIRULINA SP.*) AND CARBONIC ANHYDRASE ACTIVITY, AN ENZYME WHICH IS CENTRAL TO ALGAL APPLICATION IN METAL PRECIPITATION

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ABSTRACT

Acid mine drainage (AMD) is a major pollution problem throughout the world, adversely affecting both surface and groundwaters. AMD is principally associated with the mining of sulphide ores. The most commonly associated minerals being sulphur, copper, zinc, silver, gold, lead and uranium. As conventional methods for removing heavy metals from wastewater are often prohibitively expensive, the implementation of biological processes for the removal of heavy metals has become a realistic practice.

The objectives of this project was firstly to establish the effect of copper, lead and nickel, heavy metals commonly found in AMD waters, on the enzyme carbonic anhydrase, which is an integral part of the carbon concentrating mechanism (CCM) and secondly, to determine the feasibility of using the alkalinity generated by *Spirulina* for the precipitation of heavy metals from solution.

Initially, batch flask experiments were performed and it was found that the algae were able to utilise the bicarbonate supplied in the medium, under CO_2 limiting conditions, through the induction of their CCM, resulting in the generation of carbonate.

The effect of the inhibitors, acetazolamide (AZ) and ethoxyzolamide (EZ), were also investigated in order to determine the importance of carbonic anhydrase (CA) in inorganic carbon accumulation and photosynthesis. Results obtained were consistent with those observed in literature and it was found that at 100μM AZ and EZ, complete inhibition of photosynthesis and carbonic anhydrase occurred, with no oxygen being evolved. The results obtained from the inhibitor experiments substantiate the findings that carbonic anhydrase is an important part of the CCM, and that the dehydration of bicarbonate to carbon dioxide and hydroxide ions, is in fact an enzymatic process regulated by the enzyme carbonic anhydrase and is essential for efficient photosynthesis.

The effect of heavy metals on *Spirulina* was also investigated. Lead, copper and nickel were all found to cause a reduction in the synthesis of chlorophyll *a*, which resulted in a decrease in photosynthetic efficiency and eventually death of the culture. The morphology of the algae was also severely affected by heavy metals, with degradation and almost complete disintegration of the algal filaments occurring. Using the Wilbur-Anderson assay method, carbonic anhydrase activity was found to be lower in the experimental flasks containing heavy metals, than the control flasks, reducing the algae's ability to utilise the bicarbonate in solution for effective photosynthesis.

The Wilbur-Anderson assay method did not prove to be a reliable method for measuring changes in enzyme activity as results were found to be erratic. Therefore attempts were made to use an oxygen electrode as an alternative method for determining the effects of various parameters on enzyme activity and photosynthesis, this proved to be more successful.

Because of the toxic effects of heavy metals on *Spirulina* it was decided that the use of the biogenic alkalinity generated by the algae for the precipitation of heavy metals may be successfully employed as an alternative method for bioremediation and metal recovery. Carbonate reacts readily with metals, therefore the carbonate produced by this algal system was used for the precipitation of metals. It was possible to categorise the precipitation reactions observed into three groups, namely those metals which, a) precipitate as hydroxides, b) precipitate as carbonates generated from the dissociation of bicarbonate and c) metals which can only precipitate if there is free carbonate present in solution.

ABBREVIATIONS

AA spec	Atomic absorption spectrophotometer
AMD	Acid mine drainage
ATP	Adenosine triphosphate
AZ	Acetazolamide
CA	Carbonic anhydrase
CCM	Carbon concentrating mechanism
Chl	Chlorophyll
C_i	Inorganic carbon
COS	Carbon oxysulphide
DIC	Dissolved inorganic carbon
EDS	Energy dispersive X-ray spectroscopy
EDTA	Ethylenediaminetetraacetic Acid
EDXA	Energy dispersive X-ray analysis
EPS	Extracellular polysaccharides
EZ	Ethoxyzolamide
GF/C	Glass fibre filters
HRAP	High rate algal ponding system
NADPH	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
PGA	Phosphoglycerate

P_i	Inorganic phosphate
PS-I	Photosystem I
PS-II	Photosystem II
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP	Ribulose-1,5-bisphosphate
SEM	Scanning electron microscopy
-SH	Sulfhydryl group
SRB	Sulphate reducing bacteria
TDS	Total dissolved solids
TEM	Transmission electron microscopy
WAU	Wilbur-Anderson units
WOC	Water-oxidising complex

CHAPTER ONE

REVIEW OF LITERATURE

1. Introduction

South Africa is a country rich in natural resources, with the critical exception of fresh water. While the climate varies from rain forest to desert, the typical climate of South Africa is semi-arid, experiencing an average rainfall of about 475mm per annum, which is well below the world average (DWAF, 1999). Rainfall patterns are variable and the distribution of rainfall is uneven, rainy conditions being experienced along a comparatively narrow region along the eastern and southern coastlines. In addition, high evaporation rates result in the run-off-to-rainfall ratio being amongst the lowest for any populated region on earth (DWAF, 1999; Kidd, 1997).

A population growth rate of 2-3% has resulted in an increase in human demand for water resources and there are a number of regions throughout South Africa, which are already relying on expensive transfer schemes, as water demand has far exceeded water availability. It has been estimated, from current water utilisation patterns, that by the year 2030 the demand for potable water in South Africa will exceed supply (Kidd, 1997; Odendaal, 1997; DWAF, 1999).

Coupled to the natural scarcity of water, is the decline in the quality of South Africa's water resources, primarily as a result of salinisation, as well as heavy metal pollution and eutrophication (Neytzell-De Wilde, 1992). Calcium, magnesium, sodium, chloride, sulphate and bicarbonate ions are largely responsible for salination of water systems and if present in high concentrations, may affect plants' and animals' water and nutrient uptake abilities.

It is known that trace amounts of certain metals are required for maintenance of some components or metabolic functions in the cell, but at elevated concentrations they become acutely toxic to living organisms. Pollution of the environment by toxic metals arises as a result of many activities, largely industrial and include processes such as mining, electroplating and nuclear power operations (Bryant *et al.*, 1992), although sources such as agriculture and sewage disposal (Table 1.1) also contribute to this problem (Gadd & White, 1993).

Table 1.1: Anthropogenic sources of toxic metals and related elements (Gadd & White, 1993)

Toxic metal	Fuel and power				Metals		Agriculture	Manufacturing	Waste disposal		Total^a
	Coal^b	Oil^b	Wood^b	Power	Non-ferrous	Iron and steel			Solid wastes	Sewage and sludge	
Arsenic	23830	57	210	820	19090	1945	7730	7550	793	12842	124820
Cadmium	8529	143	120	125	7371	156	3666	2450	1147	24319	38970
Chromium	308775	1408	-	5700	13350	15620	92680	50610	20336	61290	172480
Copper	5185	1959	900	720	16588	1601	411	33740	27970	40905	147370
Mercury	4670	-	180	1800	229	-	5245	1145	2420	498	11860
Manganese	1087212	1395	-	11400	17905	25000	230600	20515	25256	134055	1708270
Nickel	181262	27070	1200	10500	14631	5238	60246	7440	259	60710	380650
Lead	8158	250449	2100	720	54725	9732	192	9300	42100	16470	346150
Selenium	33878	4845	-	18000	13630	2	6566	4250	348	5904	88740
Vanadium	46925	76120	-	300	789	1151	25470	550	1295	676	230000
Zinc	11100	2159	-	18000	97287	34325	824935	85015	65100	103715	1729880

^{a}All quantities are tonnes per year

^{b}Direct yields from combustion, including lead derived from petrol and coal-ash disposal

Where no figure is given, the amount of metal emitted is negligible

Many of these pollutants are discharged into the environment mainly as solutes or particulates and may reach extremely high concentrations, eventually accumulating throughout the food chain, thus posing a serious threat to the environment, animals and humans (Giardi *et al.*, 2001). Once in the environment, metals may undergo transformation into various mobile forms and /or immobilization in an environmental sink.

The effects of metals on ecosystem function vary considerably and are of economic and public-health significance. As a result, environmental awareness and legal constraints on emissions are becoming increasingly strict, leading to a need for cost-effective emission control (Gadd & White, 1993).

2. Removal of heavy metals

Some of the main processes that remove, immobilize or detoxify heavy metals in the natural environment result from microbial activities. Biotechnological approaches to the abatement of toxic metal pollution consist of selectively using and enhancing these natural processes to treat wastes (Gadd & White, 1993). Microorganisms interact with toxic metals in a variety of ways (Figure 1.1), however, the three main processes used for treating liquid wastes containing toxic metals are biosorption, extracellular precipitation and uptake by metal-binding proteins and other specialist molecules derived from microbial cells (Gadd & White, 1993). These processes are not exclusive and several physico-chemical and biological processes may be involved. Biosorption and bioprecipitation are two of the most successfully utilized biotechnological processes to date.

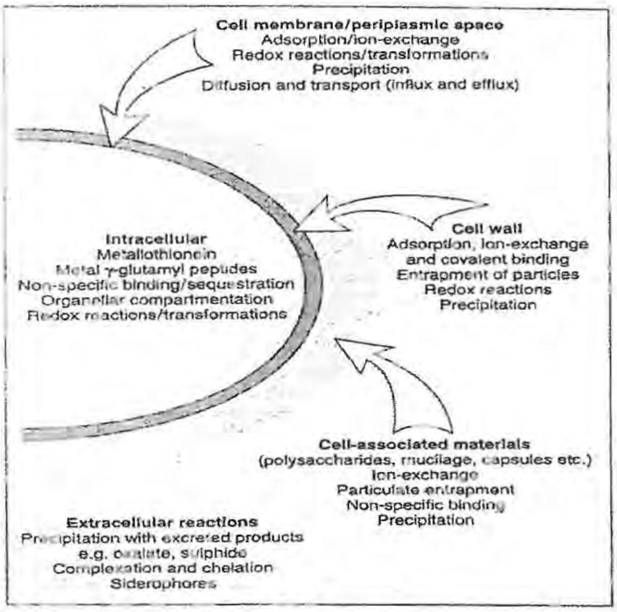


Figure 1.1: Processes contributing to microbial uptake and detoxification of heavy metals (Gadd & White, 1993)

2.1 Biosorption

Biosorption is the term used to define the ability of biomass (living or dead) to bind and concentrate heavy metals (Gadd & White, 1993; Volesky, 1992). The chemical composition of the cell is not the only factor on which biosorption is dependent, but also on external physico-chemical mechanisms and the solution chemistry of the metal. Most living-cell systems exploited to date have been used to decontaminate effluents containing metals at sub-toxic levels, as a result of the inactivation of living cells at high metal concentrations.

The use of freely suspended microbial biomass has disadvantages that include small particle size, low mechanical strength and difficulty in separating biomass and effluent, whereas immobilization of the biomass onto a support minimizes these disadvantages.

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 (Payne, 1997).

2.2 Microbial Metal Accumulation

Uptake capacities and mechanisms may vary widely among microorganisms due to their complex structure. 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 (Garnham *et al.*, 1992; Khummongkol *et al.*, 1982). The second, slower phase is metabolism-dependent transport across the cell membrane (Wilde & Benemann, 1993; Terry & Stone, 2002). This only occurs in living cells, and may be accompanied by toxic symptoms.

2.3 Metal-binding proteins and peptides

Virtually all biological material has a high affinity for toxic metals, however, some biomolecules function specifically to bind metals and are induced by their presence (Ahner & Morel, 1995; Ahner *et al.*, 1995; Gadd & White, 1993). First identified in fission yeast, phytochelatins (Pawlik-Skowronska, 2001) have been shown to be the major intracellular metal binding peptides of plants, algae and some fungi (Ahner *et al.*, 1995). The primary function of phytochelatin is thought to be metal detoxification as demonstrated by:

- the binding properties of the peptides
- their induction upon metal exposure, and
- the control of phytochelatin synthetase by metal concentrations

2.4 Cell wall components and exopolymers

The microbial exopolymers that have received the most attention in relation to metal binding are those that form capsules or slime layers. The majority of these exopolymers are composed of polysaccharide, glycoproteins and lipopolysaccharide, which may be associated with protein (Gadd & White, 1993).

2.5. Metal precipitation

South Africa has been and still is one of the world's leading producers of precious metals and minerals and the pollution of surface waters by acid mine drainage (AMD) and acidic effluents from refineries is a serious problem in the country (van Hille *et al*, 1999).

Current technology for the removal of heavy metals from wastewater includes precipitation, ion exchange with synthetic resins and adsorption with activated carbon. Precipitation of heavy metals as hydroxides from basic solutions has been widely used in industry, however, precipitation alone usually cannot reduce heavy metal concentrations far enough to meet current water quality standards (Bryant *et al.*, 1992). Ion exchange with synthetic resins or adsorption with activated carbon can be highly efficient in heavy metal removal, but is relatively expensive when used on a large scale. The adsorbent is usually regenerated by desorbing the metal ions to another liquid stream, producing a high –volume secondary waste that must also be treated or stored (Bryant *et al.*, 1992). Other less frequently used processes include electrodialysis and reverse osmosis (Wilde & Benemann,1993).

The conventional processes for the treatment of acidic, metal laden effluents from industry involves neutralisation by the addition of alkaline chemicals such as limestone, lime, sodium hydroxide, sodium carbonate or magnesia; which increases the pH and causes precipitation of metals (van Hille *et al.*, 1999). However, the continuous cost of reagents required for these processes makes them generally not financially feasible and recently there has been a trend toward the implementation of passive treatment schemes with minimal operational and maintenance requirements (Terry & Stone, 2002).

The majority of active and passive treatment systems depends on precipitation as the primary method for metal removal from acidic effluents, by the addition of inorganic hydroxide or carbonate salts, by the action of microbially generated sulphide or a combination of the two (van Hille *et al*, 1999).

Research conducted thus far, on the use of algae for the removal of metal ions in solution, has predominantly concentrated on their ability to adsorb or accumulate the metal. Little research has focused on the possibility of using the alkalinity generated by the algae for the precipitation of heavy metals.

3. Significance and origin of algae

Algae are common and normal inhabitants of surface waters and are encountered in every water supply that is exposed to the sunlight. After the first isolation by Turpin in 1827 from a freshwater stream, species of *Spirulina* have been found in a variety of environments (Ciferri, 1983; Lu & Vonshak, 2002). While a few of the algae are found in soil and on surfaces exposed to air, the great majority of them are truly aquatic and grow in the waters of ponds, lakes, reservoirs, streams, oceans, salt pans and thermal springs (Ciferri, 1983; Palmer, 1980; Price *et al.*, 1998). Thus, the organism appears to be capable of adaptation to variable habitats and colonises certain environments in which life for other microorganisms is, if not impossible, very difficult.

3.1 Morphology and taxonomy

Algae are polyphyletic and range in size from picoplankton, which are only 0.2-2.0μm in diameter to giant kelps with fronds of up to 60m in length (Lembi & Waaland, 1988). Of interest to this study is the algae *Spirulina*, which was easily obtainable from a source in Wellington in the Western Cape. *Spirulina* is a multicellular, filamentous cyanobacterium. Under the microscope, *Spirulina* appears as blue-green filaments composed of cylindrical cells arranged in unbranched, helicoidal trichomes (Figure 1.2) (Ciferri, 1983). The filaments are motile gliding along their axis. The helical shape is maintained only in liquid media and in solid media the filaments become true spirals (Ciferri, 1983). The diameter of the cells ranges from 1 to 3μm in the smaller species and from 3 to 12μm in the larger ones.

Cyanobacteria (traditionally known as blue-green algae) contain a different chlorophyll (chlorophyll a), use water as an electron donor, release O_2 and use both photosystems I and II. It has been suggested that cyanobacteria evolved from anaerobic photosynthetic bacteria as some facultatively use sulphide (H_2S or Na_2S) as an electron donor under anaerobic conditions (Lembi & Waaland, 1988).

The cyanobacteria are prokaryotic in cell structure and therefore are not included among the algae by some scientists, but most continue to consider them algae, albeit prokaryotic algae due to the presence of chlorophyll-a-dependant, oxygen-evolving photosynthesis, their algal-like forms and ecological relationships (Lembi & Waaland, 1988). In structural diversity, blue-green algae range from unicells to branched and unbranched filaments to unspecialised colonial aggregations and are possibly the most widely distributed of any group of algae. The ability of some blue-greens to fix nitrogen is unique among the algae and makes them a very important part of the earth's nitrogen cycle.

Blue-green algae, develop readily or may even grow best in water with a high pH. A high water temperature tends to favour the proliferation of these algae, and certain species of blue-green algae are the most heat tolerant (Palmer, 1980). Algae sometimes contribute to corrosion either directly in localised places where they are growing or through their modification of the water by physical or chemical changes.

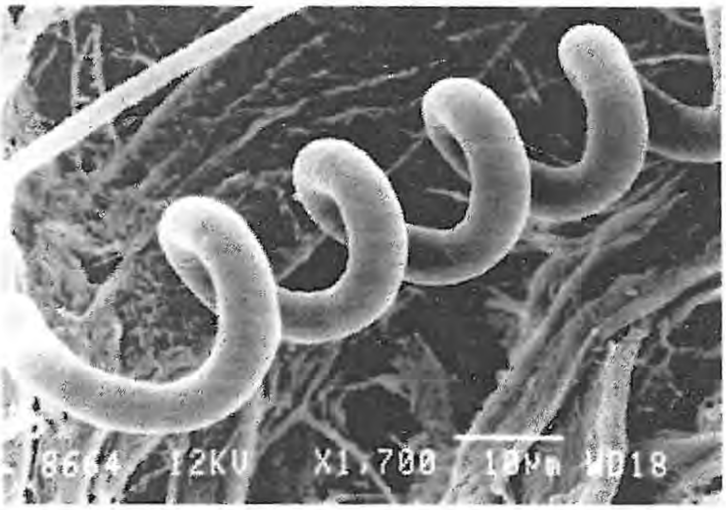


Figure 1.2: SEM preparation of healthy *Spirulina* (Payne, 1997)

3.2 The carbon dioxide equilibria in water

Algae differ from the other groups of small or microscopic organisms in that they possess an internal green pigment called chlorophyll, sometimes hidden or partially masked by other pigments, which enables them in the presence of sunlight to combine water and carbon dioxide to form starch or related substances, and to release oxygen into the water. In algae and other green plants, the rate of photosynthesis is normally faster than that of respiration, therefore more oxygen is released than used and they absorb more carbon dioxide than they release.

An important chemical effect of algae is the continuous removal of CO_2 from the water during the daylight hours as a result of photosynthesis. This process brings about an alteration in the relative amounts of soluble carbonic acid, partially soluble bicarbonates and the nearly insoluble monocarbonates, often causing some of the latter to precipitate. These changes in CO_2 also tend to change the pH of the water. The pH rises as the algae increase their photosynthetic activity during the daylight hours and the pH then decreases at night when the algae are not carrying out photosynthesis but are releasing CO_2 during respiration.

Carbon dioxide reacts with water to form a weak acid, carbonic acid (2). This in turn dissociates into bicarbonate (3) and carbonate ions (4). The carbonate ions react with alkaline earth ions, particularly calcium, to form solid carbonates.

$CO_2(gas) \Leftrightarrow CO_2(aq)$ (1)

$CO_2\ (aq) + H_2O \Leftrightarrow H_2CO_3$ (2)

$H_2CO_3 \Leftrightarrow H^+ + HCO_3^-$ (3)

$HCO_3^- \Leftrightarrow H^+ + CO_3^{2-}$ (4)

Below pH8 equations (2) and (3) can essentially be written as:

$CO_2 + H_2O \Leftrightarrow H^+ + HCO_3^-$

Above pH 8 the following reaction predominates:

$CO_2 + OH^- \Leftrightarrow HCO_3^-$

From the rate constants and the inorganic carbon equilibria it appears that CO_2 can serve as a carbon source below pH values of about 8. Above pH8 however, the dehydration of HCO_3^- apparently becomes limiting and the algae must take up HCO_3^- (Round & Chapman, 1982).

3.3 The interaction of biogenic alkalinity and heavy metals

It is thought that under CO_2-limiting conditions the algae are able to utilise the bicarbonate present in the medium, through the induction of the enzyme carbonic anhydrase, resulting in a release of CO_2 (which is taken up by the algal cell for photosynthesis) and OH^- ions into solution. More than half of the OH^- ions react with HCO_3^- ions in solution to form carbonate, which then complex with metal ions, causing them to precipitate as carbonates. Some of the remaining OH^- ions react with free H^+ ions in solution, resulting in an increase in the pH of the surrounding medium (Figure 1.3).

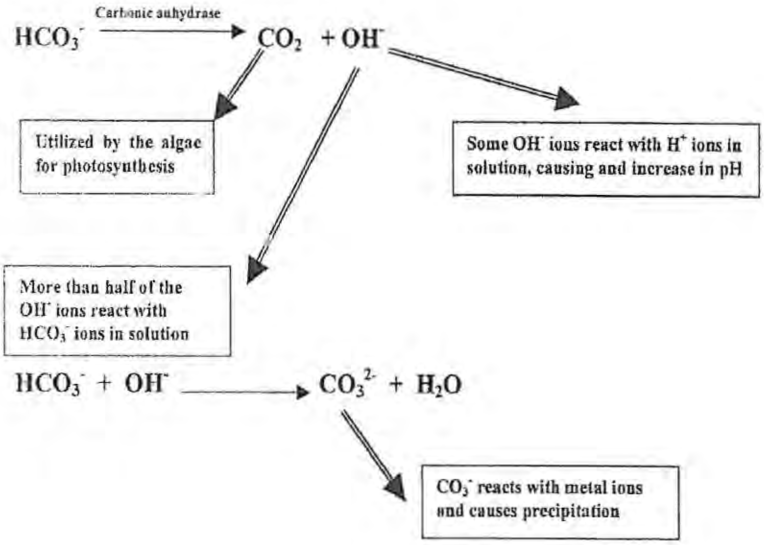


Figure 1.3: Proposed interaction of biogenic alkalinity and heavy metals

4. The effects of heavy metals on algal metabolism

From the standpoint of environmental pollution, metals may be classified into the following three groups: (1) noncritical; (2) toxic, but very insoluble or very rare; (3) very toxic and relatively accessible (Table 1.2). However, some heavy metals, such as manganese, iron, copper and zinc are essential micronutrients required for growth in trace concentrations, but if found in higher concentrations, can be incredibly toxic (Moenne, 2001).

Table 1.2: Classification of elements according to toxicity and availability (Wood, 1974)

Noncritical	Toxic but very insoluble or very rare	Very toxic and relatively accessible
Na C F	Ti Ga Hf La	Be As Au Co
K P Li	Zr Os W Rh	Se Hg Ni Te
Mg Fe Rb	Nb Ir Ta Ru	Tl Cu Pd Pb
Ca S Sr	Re Ba	Zn Ag Sb Sn
H Cl Al		Cd Bi Pt
O Br Si N		

The order of metal toxicity to algae varies with the algal species and the experimental conditions, but generally the order is Hg>Cu> Cd>Ag>Pb>Zn (Gimmler *et al.*,1991).

4.1. Mercury

Mercury, the most toxic metal, interacts with enzyme systems and inhibits their activities, especially enzymes with reactive sulfhydryl (-SH) groups (Gimmler *et al.*, 1991). The toxic effects of mercury on algae generally include (1) cessation of growth in extreme cases, (2) inhibition of photosynthesis (Overnell, 1975), (3) reduction in chlorophyll content, and (4) increased cell permeability and loss of potassium ions from the cell (Rai *et al.*, 1981). Mercury compounds are known to inhibit photosynthesis. They interrupt the flow of electrons at multiple sites, such as plastocyanin (Katoh & Takamiya, 1964; Kimimura & Katoh, 1972), at the reaction center of photosystem I (PSI) according to Kojima *et al.* (1987) and at iron sulphur (Golbeck *et al.*, 1977) as well as ferredoxin nicotinamide-adenine-dinucleotide-phosphate (NADP) and oxidoreductase (Honeycutt & Krogmann, 1972) sites. It has been suggested that the multiple effects of mercury arise from the interaction of mercury ions with the sulfhydryl groups of proteins (Passow *et al.*, 1961; Bernier *et al.* 1993). Mercury inhibits photosystem II (PSII) in algal cells and thylakoid membranes by inhibiting the water splitting system, inactivating some of the PSII reaction centers (El-Sheekh, 1999).

4.2. Copper

Even though copper is an essential micronutrient because it participates in photosynthetic electron transport and also plays a role as a cofactor of several oxidising enzymes (Fathi & El-Shahed, 2000), it is the second most toxic metal if present in high concentrations. Copper toxicity is dependent on the ionic activity (ie. concentration of free Cu^{2+}), and not the total copper concentration (Sunda & Guillard, 1976). However, some organic copper complexes, especially the lipid soluble ones, are much more toxic than ionic copper (Stauber & Florence, 1987), because they can diffuse directly through the membrane into the cell (Terry & Stone, 2002).

The mechanism, of Cu toxicity has been described by Stauber & Florence (1987) as a chronological sequence in microalgae. They propose that initially the Cu binds to the cell via carboxylic and amino residues in the membrane protein, interfering with cell permeability or the binding of essential metals. Copper affects the permeability of the plasmalemma (McBrien & Hassall, 1965; Terry & Stone, 2002), causing loss of K^+ (Overnell, 1975) from the cell and changes in cell volume. Next copper may be transported to the cytoplasm and then to the chloroplasts, where it inhibits photosynthesis by uncoupling electron transport to $NADP^+$. As the ionic concentration increases, copper is bound to chloroplast membranes and other cell proteins, causing degradation of chlorophyll and other pigments. Copper may also exert its toxicity in subcellular organelles, interfering with mitochondrial electron transport, respiration, ATP production and photosynthesis in the chloroplasts. At still higher concentrations, copper produces irreversible damage to chloroplast lamellae, preventing photosynthesis and eventually causing cell death.

4.3. Cadmium

Some physiological investigations have been conducted on cadmium toxicity on phytoplankton but there has been little research on macrophytes. Lobban and Harrison (1997) reported on Cd uptake and its effects on growth, pigment content and carbon assimilation in *Ulva lactuca* and *Laminaria saccharina.* The Cd concentration that caused a growth rate reduction of 50% was $900\mu g.L^{-1}$. Lobban and Harrison (1997) concluded that the long-term effects are more serious than is immediately evident and that the exposure time is important in determining the extent of the effects. It was found, that at sub-lethal concentrations, sharp reductions in photosynthesis and growth rates were observed (Gimmler *et al.*, 1991; Leborans & Novillo, 1996; Terry & Stone, 2002), because photosystems of algae can be damaged by excessive amounts of cadmium resulting in a reduction of photosynthetic pigments, such as chlorophyll *a* (Terry & Stone, 2002). Lobban and Harrison (1997) concluded from these observations that cadmium inhibits one or more steps in protein synthesis and thus leads to enzyme deficiencies and a series of secondary effects. In *in vitro* experiments with Cd-treated isolated protoplasts, Weigel (1985) showed that the main target of Cd action is the Calvin cycle where a decrease in PGA formation was observed.

Previous observations by Siedlecka *et al.* (1997), from fluorescence experiments, confirmed that the dark reactions of photosynthesis are most susceptible to cadmium toxicity and that "feedback-control" also inhibits photosynthetic electron transport.

4.4. Lead and Zinc

Lead (Pb) is a non-essential element, which becomes toxic to algae at very low concentrations (10-50μg/L). The toxic concentrations of lead for some algal metabolisms are: In *Phaeodactylum tricornutum* photosynthetic inhibition occurs at 10mg/L (Woolery & Lewin, 1976), *Skeletonema costatum* cells experience cellular chemical composition changes at 10μg/L due to the loss of permeability of the plasmalemma and *Nitzschia closterium* has an EC_{50} of 29.4μmol/L (Maeda & Sakaguchi, 1990). Methylated lead is invariably more toxic than its inorganic form. This toxicity is probably due to the non-polar nature of these organometalic compounds which allows them to diffuse rapidly into and through cell membranes.

In studies carried out by Danilov & Ekelund (2001), they determined that the maximal values of oxygen evolution in case of treatment with copper, lead and zinc were lower than in the control, which can be seen as evidence of the lowered capacity for photosynthesis by *Euglena gracilis* cells.

Zinc is an essential micronutrient for algae, but causes inhibition of cell division and destruction of chlorophyll at high concentrations (Skowronski & Rzeczycka, 1980; Stauber & Florence, 1990).

4.5 Nickel

Nickel is a ubiquitous trace metal and occurs in soil, water, air and in the biosphere where its content in the Earth's crust is about 0.008% (http://www.ukmarinesac.org.uk). The primary sources of nickel emissions into the ambient air are the combustion of coal and oil for heat or power production, incineration of waste and sewage sludge, nickel mining and electroplating.

Rai and Raizoda (1987) found that exposure of *Nostoc muscorum* to different concentrations of nickel brought about reduction in growth, carbon fixation and an increase in the loss of Na^+ and K^+ ions.

Similar work performed on *Chlorella vulgaris* by Mallick and colleagues (1990) found nickel to cause a decrease in growth rate, uptake of NO_3^- and NH_4^+ and photosynthesis. Statistical analysis of their data confirmed that $^{14}CO_2$ uptake was the most sensitive parameter compared to others in metal toxicity assessment.

Mallick and Rai (1992) demonstrated a concentration dependent inhibition of carbon fixation, O_2 evolution, photosynthetic electron transport and ATP content of *Anabaena doliolum* and *Chlorella vulgaris*. Although the mode of inhibition of the photosynthetic electron transport chain of both the algae was similar, photosytem II (PSII) depicted greater sensitivity to the test metals used (Cu, Ni and Fe), than photosystem I (PSI). Later studies performed on *Chlorella vulgaris* by Rai *et al.* (1994), indicated that nickel also increased the K^+ efflux from the cell, resulting in changes in membrane permeability.

4.6. Polyvalent anions

Polyvalent anions, such as the polynuclear tungstate or WO_4^{2-}, bind to the plasma membrane of cells permitting an increase of H^+ influx from the medium into the cells, causing an internal acidification and consequently an inhibition of photosynthesis (Gimmler *et al.*, 1991).

5. An overview of photosynthesis

Algae differ from the other groups of small or microscopic organisms, in that they are able in the presence of sunlight to combine water and carbon dioxide to form starch or related substances, and to release oxygen into the water (Palmer, 1980). In general, this process is not a characteristic of animals but is common to all types of green plants.

The algae make possible important chemical changes and metabolic activities in the water through their release of oxygen during daylight hours. The oxygen in the water is then utilised by fish, crustaceans and insects for respiration. Oxygen release by algae and O_2 uptake by reaeration are the two primary sources for renewal of O_2 in flowing streams and turbulent water (Palmer, 1980).

Photosynthesis encompasses two major groups of reactions. The "light reactions", which involve the capture of light energy and its conversion to chemical potential as ATP and NADPH. The light reactions consist of three processes: energy absorption, energy trapping and generation of chemical potential. As well as the "dark reactions", which include the sequence of reactions by which this chemical potential is used to fix and reduce inorganic carbon.

Light reaction of photosynthesis

Pigment molecules absorb the quantum of energy when hit by a photon and are chemically excited for a fraction of a second before releasing that energy. The close packing of pigment molecules into light-harvesting pigment complexes allows most of the energy to be passed from molecule to molecule until it reaches the reaction center (Raven *et al.*, 1999). An electron is boosted out of the chlorophyll in the reaction center, giving the electron a large redox potential. The electron is then captured by an electron acceptor and passed along an electron transport chain consisting of several compounds capable of undergoing redox reactions. The reaction centers and electron-transport chain are arranged in the thylakoid membrane in such a way that a proton gradient develops across the membrane, the membrane being impermeable to H^+. This gradient is relieved through complex ATPase particles, in which the energy of the gradient is used to phosphorylate adenosine diphosphate (ADP).

There are two photosystems in most cells and they are connected by electron-transport compounds. Photosystem II (PS-II) is able to split water, producing electrons, protons and waste oxygen (Giardi *et al.*, 2001; Matoo *et al.*, 1999). The electrons are finally used to reduce $NADP^+$ in non-cyclic phosphorylation (Raven *et al.*, 1999). Non-cyclic phosphorylation is when electrons boosted out of chlorophyll *a* in PS-II (P680) are given another boost in PS-I (P700). In cyclic phosphorylation, which can take place in for example, blue-green algal heterocysts all the time and other algal cells sometimes, only PS-I is active and the electrons cycle back to PS-I, generating ATP but not NADPH (Figure 1.4).

ATP and NADPH are the end products of the light reactions and they contain the energy saved from light and are used to fix inorganic carbon in the Calvin cycle in the stroma of the chloroplast.

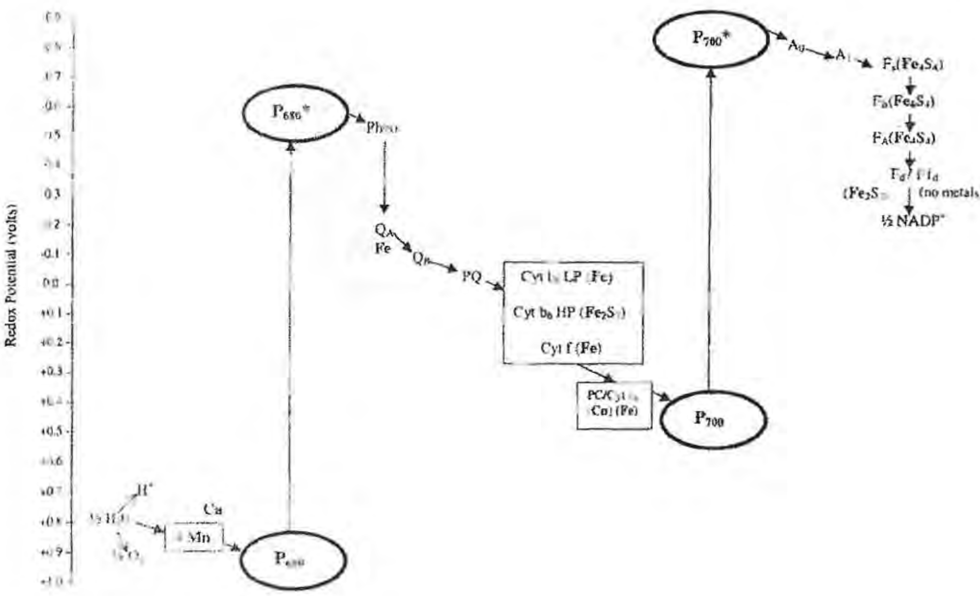


Figure 1.4: The "Z scheme" of non-cyclic electron transport, showing the involvement of trace metals in various redox components in relation to their standard redox potential (Raven *et al.*, 1999)

Dark reaction of photosynthesis

The Calvin cycle does not directly require light, although some of the enzymes involved are stimulated by light. The steps in the Calvin cycle are called the "dark reactions" of photosynthesis, but in fact the pathway functions only during the light.

In the Calvin cycle, carbon dioxide and ribulose-1,5-bisphosphate (RuBP) react in the presence of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) to give two molecules of phosphoglycerate (PGA). Subsequent steps, using ATP and NADPH in a pathway almost the reverse of glycolysis, convert PGA to glyceraldehyde-P and dihydroxyacetone-P and then put these together to give fructose-1,6-bis-P (Raven *et al.*, 1999). The fructose-1,6-bis-P formed is utilised in two ways, some is used to produce low-molecular-weight free sugars or sugar alcohols and the rest, together with glyceraldehyde-P and dihydroxyacetone-P, enter a complex series of carbon transfers that regenerate RuBP. For net production of one hexose molecule, six turns of a cycle and six CO_2 molecules are needed.

6. Bicarbonate uptake, proton and hydroxyl fluxes in cyanobacteria

Cyanobacteria have a very high affinity for CO_2, but the enzyme RuBisCO which is responsible for the fixation of this CO_2, has a very low affinity, with a $K_{0.5}(CO_2)$ of about 200uM (Badger, 1980). It thus became obvious that cyanobacteria must possess the means to raise the CO_2 concentration around the active site of RuBisCO very considerably above that in the extracellular medium. This was because the observed rates of photosynthesis at alkaline pH were significantly higher than could be supported by the spontaneous conversion of HCO_3^- into CO_2 and OH^- in the external solution (Al-Moghrabi *et al.*, 1996).

CO_2 is the substrate for RuBisCO, the enzyme responsible for the fixation of CO_2 into triose-phosphates in photosynthesis (Round & Chapman, 1982). The general criterion used to determine whether bicarbonate is the species taken up by the plant, is that if there is a substantial photosynthetic rate at high pH where CO_2 is at very low concentration, then HCO_3^- must be taken up (Round & Chapman, 1982). If HCO_3^-, is taken up by a plant, then it must be converted to CO_2, which in turn is taken up by the cell for photosynthesis, and OH^- ions, which remain in the medium. The resulting OH^- ions in the solution are responsible for the alkalization of the culture medium (Raven, 1995; Shiraiwa *et al.*, 1993; van Hille *et al.*, 1999).

The enzyme carbonic anhydrase (CA) catalyses this reaction and has been found to be universally distributed in all plant cells. It appears that separate cytoplasmic and chloroplastic carbonic anhydrases exist (Round & Chapman, 1982; Moroney *et al*, 1985).

There is also good evidence that carbonic anhydrase facilitates the diffusion of CO_2 across membranes.

Changes in external pH can be used to measure dissolved inorganic carbon (DIC) uptake in both microalgae and macroalgae. It was found that O_2 production was concomitant with alkalisation of the medium, with a stoichiometry close to 1:1 between the appearance of OH^- and O_2 in seawater (Al-Moghrabi *et al.*, 1996). Similar results were obtained in freshwater cyanobacteria, freshwater macroalgae and marine macroalgae. Alkalisation of the water surrounding marine algae during photosynthesis is a consequence of the conversion of HCO_3^- to $CO_2 + OH^-$ either in the extracellular medium by a membrane- bound CA or in the intracellular medium following the activity of a HCO_3^- pump and the release of OH^- formed inside the cells due to CO_2 fixation (Al-Moghrabi *et al.*, 1996).

Active transport of HCO_3^- and CO_2

There have been many arguments as to whether the accumulation of DIC by cyanobacteria is due to active CO_2 transport or to active HCO_3^- transport. It is now known that both DIC species are actively transported, quite possibly by separate transport systems. Investigations by Miller and Colman (1980) on the cyanobacterium, *Coccochloris peniocystis*, showed a lack in extracellular carbonic anhydrase activity. The lack of this enzyme meant that if cells of *C. peniocystis* were unable to transport HCO_3^-, then their rate of CO_2 fixation, in a closed system, would be limited by the rate of CO_2 uptake (Miller *et al.*, 1990). However, the observed rates of CO_2 fixation were as much as 50-fold higher than the maximum possible rate of HCO_3^- to CO_2 conversion when the pH of the medium was above 8.3. As a result, it was obvious that most of the observed CO_2 fixation must have been supported by HCO_3^- influx across the plasmalemma. Miller *et al.* (1984) determined that the electrical potential difference across the membrane in illuminated cyanobacteria was in the range of −120mV, hence the substantial influx of HCO_3^- must have been a result of active HCO_3^- transport.

A direct line of evidence for active HCO_3^- transport was later shown by Badger and Andrews (1982), where they used mass spectrometry to monitor DIC uptake. Working with a marine species of *Synechococcus*, they found that although the cells possessed an active CO_2 transport system, the rate of HCO_3^- transport was so rapid that nett leakage of CO_2 occurred. This leakage was driven by the rapid rate of intracellular dehydration of the accumulated HCO_3^- that soon exceeded the scavenging capacity of the CO_2 transport system (Miller *et al.*, 1990).

Additional experiments carried out by Badger and Andrews (1982) using mass spectrometry, demonstrated the existence of active CO_2 transport. It was evident that upon illumination in a closed system the marine *Synechococcus* utilised CO_2 so rapidly that the CO_2 concentration in the medium fell almost to zero, as the spontaneous dehydration rate of the HCO_3^- remaining in the medium was too slow to maintain the equilibrium CO_2 concentration. This rapid fall in the extracellular CO_2 concentration could not be accounted for simply by CO_2 fixation and had to be due to the operation of an active CO_2 transport system (Miller *et al.*, 1990).

Scavenging role of active CO_2 transport

The CO_2 utilised was soon followed by a CO_2 influx in the marine *Synechococcus*, causing the extracellular CO_2 concentration to rise above the initial equilibrium value. In other species this phenomenon is not observed, indicating that there is an active CO_2 transport system which is able to scavenge the CO_2 that must leak from the cells as a consequence of HCO_3^- accumulation and dehydration (Miller *et al.*, 1990). In these species, the extracellular CO_2 concentration remains very low even when rapid transport of HCO_3^- occurs (Espie *et al.*, 1988; Miller *et al.*, 1987).

The role of the active CO_2 transport system in returning leaked CO_2 to the cells can be seen when the system is selectively inhibited by carbon oxysulphide (COS), which is a structural analog of CO_2. COS has been found to selectively inhibit CO_2 transport without having much effect on HCO_3^- transport. When HCO_3^- is given to illuminated cells in the presence of COS, the HCO_3^- accumulates in the cells as a consequence of HCO_3^- transport and is dehydrated to form CO_2 that leaks from the cells. With CO_2 transport inhibited, the concentration of CO_2 in the medium rises above its equilibrium value (Miller *et al.*, 1990).

In the absence of COS, with the CO_2 transport system operating normally, the CO_2 that leaks from the cells as a consequence of intracellular HCO_3^- dehydration is efficiently scavenged by the CO_2 transport system.

Simultaneous CO_2 and HCO_3^- transport

As both CO_2 and HCO_3^- are substrates for active transport in cyanobacteria, the question arises as to whether they are transported by the same or separate systems. CO_2 and HCO_3^- are structurally and chemically very different. The CO_2 molecule is uncharged, lipid soluble and susceptible to nucleophilic attack, whereas the anionic HCO_3^- is none of the above (Miller *et al.*, 1990). As a result, one would envisage HCO_3^- binding more readily to a transport protein than would CO_2. Carboxylases have evolved to use either CO_2 or HCO_3^-, but never both. The active site of CA is known to interact with both CO_2 and HCO_3^-, but its affinity for CO_2 is so low that it seems a poor model for the transport of CO_2 in cyanobacteria (Espie *et al.*, 1988).

Miller *et al.* (1990) found that there was very little kinetic interaction between CO_2 and HCO_3^- transport in *Synechococcus.* The rate of CO_2 transport is little affected by increasing HCO_3^- concentrations and the onset of rapid HCO_3^- transport does not decrease the rate of ongoing CO_2 transport (Espie *et al.*, 1988).

7. Role of carbonic anhydrase (CA)

Graham *et al.* (1971) were the first to suggest that CA might play an important role in the control of the photosynthetic process. Today, its involvement in the photosynthetic process is well documented in marine algae and cyanobacteria. However, the exact role of CA in photosynthesis is not yet elucidated.

Carbonic anhydrase (CA) is a zinc-containing metalloenzyme that catalyses the interconversion of dissolved CO_2 and H_2CO_3 or HCO_3^- via enhanced rates of hydration (hydroxylation) / dehydration (dehydroxylation) (Dionisio-Sese, 1992; Mercado *et al*, 1997; Sultemeyer, 1998). The enzyme has been shown to occur in many animals, plants, eubacteria, archebacteria and viruses (Karlsson *et al.*, 1998, http://www.plantphys.umu.se). CA is involved in various physiological processes such as carboxylation-decarboxylation reactions, transport of CO_2 and HCO_3^- across membranes either by passive or active mechanisms, pH regulation, ion exchange and efficient virus replication (Karlsson *et al.*, 1998; Sultemeyer, 1998).

According to Hewett-Emmett and Tashian (1996), CA can be classified into three CA gene families, designated α–, β–, γ–CAs indicating that the enzyme has evolved independently at least three times (Sultemeyer, 1998, http://www.plantphys.umu.se). The α-type are primarily found in animals, but homologues have also been identified in the bacterium *Neisseria gonnorhoeae* and the green alga *Chlamydomonas reinhardtii*. This is the most extensively studied CA family (Table 1.3) and includes the biochemically well characterised mammalian CA isozymes, crystal structures of which have been solved to high resolution (http://www.plantphys.umu.se).

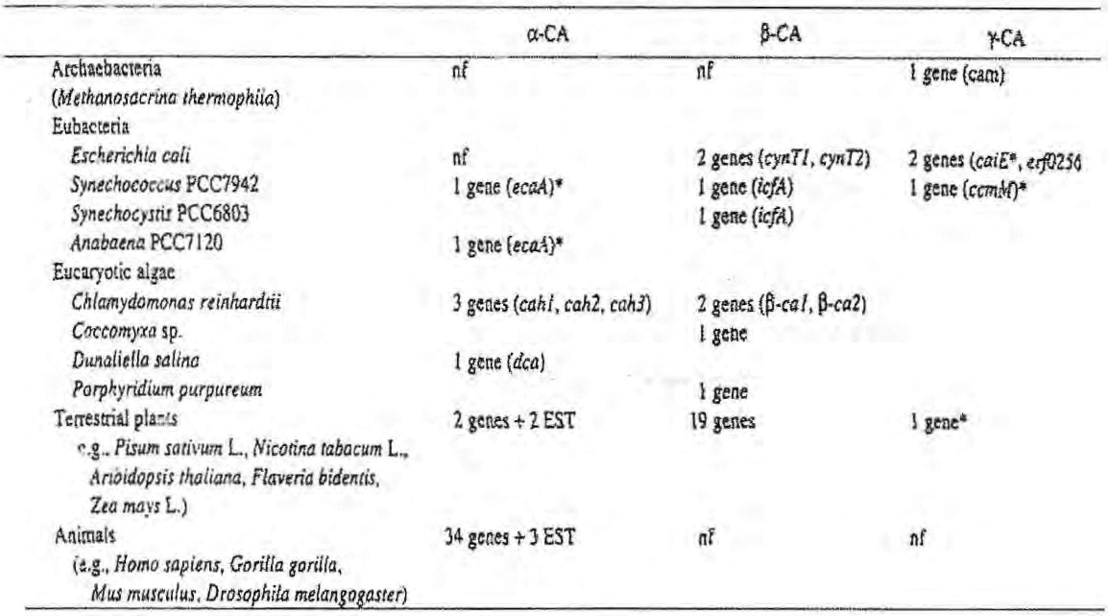
Conversely, gamma-CA is a newly discovered gene family, with the enzyme from *Methanosarcina thermophila* being the only gamma-CA isolated and characterised to date (http://www.plantphys.umu.se). Related sequences have been found in several eubacteria and in *Arabidopsis thaliana*, but it is not known whether they encode functional CAs (Sultemeyer, 1998).

CAs belonging to the beta-CA family have been found in both C_3 and C_4 monocot and dicot plants, in the mitochondria of *C.reinhardtii* and in various eubacteria (Sultemeyer, 1998). Among the dicot species, the sequence similarity between the different beta-CAs is around 80% (60% identity) (http://www.plantphys.umu.se). The homology is slightly lower when comparing the monocot and dicot homologues, but the similarity remains considerable (>70%). In contrast, the beta-CAs found in prokaryotes are more variable and exhibit low sequence similarity to the plant homologues (30%).

Despite this phyletic diversity all the enzymes function via Zn-H_2O / Zn-OH complexes, amplifying the rate and increasing the specificity of the reaction catalysed by Zn alone (Raven, 1995).

Carbonic anhydrase is required in living organisms, because the spontaneous interconversion between CO_2 and HCO_3^- is rather small compared with the flux rates in various physiological processes. For instance, CO_2 fixation by some photosynthetically active organisms may be some 10^4 times faster than the uncatalysed rate of CO_2 formation from HCO_3^- (Badger & Price, 1994). Therefore, the conversion of HCO_3^- to CO_2 has been thought to be the physiological role of CA in plants. Modern methods of molecular biology have indicated the occurrence of multiple forms of CAs in plants, including green algae (Table 1.3), where the enzyme is located in different cellular compartments and is therefore probably involved in different metabolic pathways.

Table 1.3: Number of DNA sequences with high similarity to CA found in Archebacteria, animals, eubacteria and plants. *nf, non-functional* (Sultemeyer, 1998).



	α-CA	β-CA	γ-CA
Archaebacteria (*Methanosacrina thermophila*)	nf	nf	1 gene (cam)
Eubacteria			
Escherichia coli	nf	2 genes (*cynT1*, *cynT2*)	2 genes (*caiE**, *erf0256*
Synechococcus PCC7942	1 gene (*ecaA*)*	1 gene (*icfA*)	1 gene (*ccmM*)*
Synechocystis PCC6803		1 gene (*icfA*)	
Anabaena PCC7120	1 gene (*ecaA*)*		
Eucaryotic algae			
Chlamydomonas reinhardtii	3 genes (*cah1*, *cah2*, *cah3*)	2 genes (β-*ca1*, β-*ca2*)	
Coccomyxa sp.		1 gene	
Dunaliella salina	1 gene (*dca*)		
Porphyridium purpureum		1 gene	
Terrestrial plants (e.g., *Pisum sativum* L., *Nicotina tabacum* L., *Arabidopsis thaliana*, *Flaveria bidentis*, *Zea mays* L.)	2 genes + 2 EST	19 genes	1 gene*
Animals (e.g., *Homo sapiens*, *Gorilla gorilla*, *Mus musculus*, *Drosophila melangogaster*)	34 genes + 3 EST	nf	nf

In most of these organisms the physiological function of CA is embedded in a process known as the CO_2 concentrating mechanism (CCM), which is highly active under conditions where the inorganic carbon (C_i) concentration in the surrounding medium is limited. The primary purpose of the CCM is to increase the CO_2 concentration around the primary CO_2 –fixing enzyme, RuBisCO, to favour the carboxylation reaction at the expense of the oxygenase reaction, thereby achieving efficient photosynthesis even at low CO_2 concentrations (Badger *et al.*, 1994; Badger & Price, 1992; Price *et al.*, 1998; Sultemeyer, 1998).

8. Carbon concentrating mechanism (CCM)

It is clear that both cyanobacteria and microalgae possess an environmental adaptation for survival at low CO_2 concentrations, this is known as a CO_2 concentrating mechanism which elevates CO_2 around the active site of the primary photosynthetic carboxylating enzyme RuBisCO (Al-Moghrabi *et al.*, 1996; Badger *et al.*, 1994; Price *et al.*, 1998). This process actively transports and accumulates inorganic carbon within the cell and then uses this pool to provide elevated CO_2 concentrations which in turn allow them to perform efficient photosynthesis in their aquatic environments (Price *et al.*, 1998).

8.1 Carbon concentrating mechanism in eukaryotic algae

Most of our understanding of the CCM in eukaryotic algae, including the roles of CAs, has been derived from studies with *Chlamydomonas reinhardtii*, and therefore, the model of the CCM illustrated in Figure 1.5 (Badger & Price, 1992) shows the situation for this organism. In eukaryotic algae, the chloroplast is the central location of CO_2 fixation, and contains the entire photosynthetic C_3 cycle, elements of the C_2 cycle, as well as components of the CCM (Moroney & Chen, 1998). The extracellular inorganic carbon has to pass at least two membrane systems (plasma membrane and chloroplast envelope) before it reaches the site of carboxylation. With the expression of a highly active CCM a severalfold stimulation of CA activity is generally observed indicating that this enzyme is an important component of the CCM (Beardall *et al.*, 1998; Nimer *et al.*, 1994; Patel & Merret, 1986; Williams & Colman, 1996).

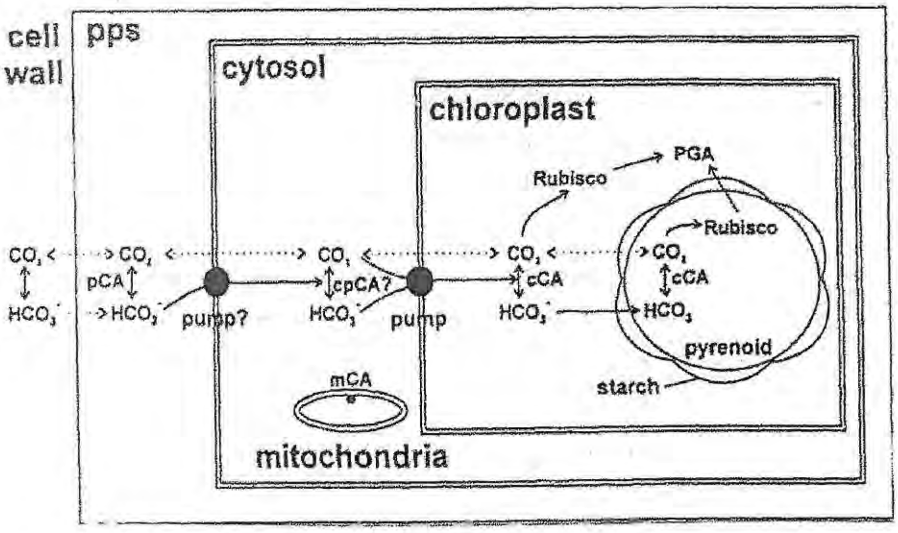


Figure 1.5: Proposed model for the CO_2 concentrating mechanism of *Chlamydomonas reinhardtii* as a model organism for microalgae (Badger & Price, 1992).

8.2 Carbon concentrating mechanism in cyanobacteria

It appears that the site of CO_2 elevation in cyanobacteria occurs within a unique microcompartment known as the carboxysome, which is a proteinaceous polyhedral body that contains most, if not all, of the RuBisCO within the cell (Price *et al.*, 1998). Studies have shown that mutations that cause a misdirecting of RuBisCO result in cells with poorly functioning CCMs, indicating the importance of the localisation of RuBisCO within the carboxysome (Orus *et al.*, 1995; Schwarz *et al.*, 1995).

Current models of CCM can be put into two groups according to the inorganic carbon species crossing the cell membrane. The first group is based on CO_2 diffusion (passive diffusive uptake) in which HCO_3^- acts as an external reservoir for CO_2 production by active dehydration (Figure 1.6). It has been suggested that the active component may be a proton-translocating ATPase. This outwardly directed pump would acidify the extracellular medium thereby converting HCO_3^- into CO_2 (Al-Moghrabi *et al.*, 1996).

Dehydration may also be achieved by an extracellular CA. This enzyme catalyses the reversible hydration-dehydration of CO_2 and then can potentially increase the CO_2 flux across the membrane 2 to 5 times (Al-Moghrabi *et al.*, 1996).

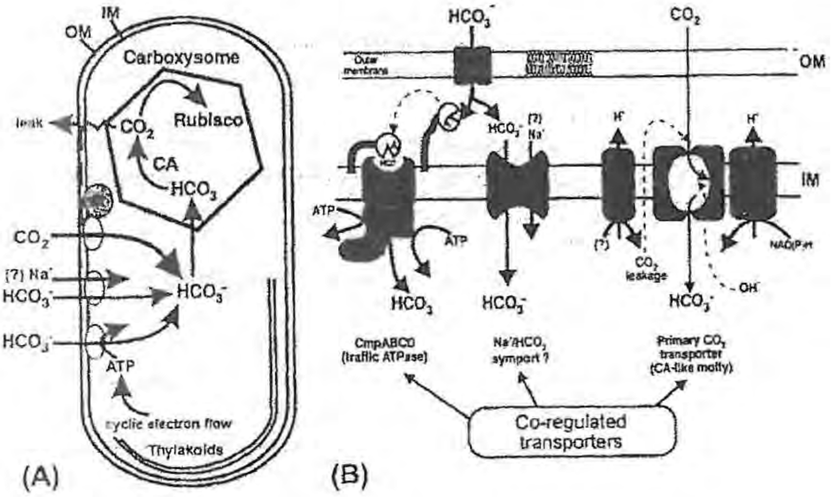


Figure 1.6: Basic components of the CCM in *Synechococcus* species (A and B). Three types of transporters are shown: i) A primary CO_2 transporter that delivers HCO_3^- inside the cell owing to a CA-like event inside the transporter. ii) A Na^+-independent, high affinity HCO_3^- transporter that is powered by ATP. iii) A constitutive Na^+-dependent HCO_3^- transporter; Na^+ involvement may be direct (HCO_3^- - Na^+ symport) or indirect (Na^+- H^+ antiport and pH regulation). OM, outer membrane; IM inner membrane (Price *et al.*, 1998)

The alternative theory, which has been proposed, is that CO_2 can be transported via a carrier protein in the marine cyanobacterium, *Synechococcus* sp. and Rotatore *et al.* (1992) also hypothesized the presence of a CO_2-ATPase in the green alga *Eremosphaera viridis*. This model assumes that HCO_3^- can cross the plasma membrane via a carrier protein. A primary electrogenic pump (HCO_3^--ATPase) is thought to play a major role in DIC uptake by many cyanobacteria and algae (Al-Moghrabi *et al.*, 1996). HCO_3^- can also enter the cells by a H^+ / HCO_3^- symport or by an OH^- / HCO_3^- antiport mechanism secondary to a H^+ extrusion pump or by a Na^+ /HCO_3^- symport (Al-Moghrabi *et al.*, 1996). However, in spite of a large number of studies, no specific protein for such transport of HCO_3^- has yet been identified.

The cyanobacterial CCM provides several key benefits:

- it provides a competitive survival advantage under C_i limitation, allowing both CO_2 and HCO_3^- species to be exploited;
- nitrogen allocation costs for RuBisCO are lessened, providing another competitive advantage with respect to N availability; and
- photorespiratory costs are dramatically lessened, providing a competitive advantage with respect to efficiency of photosynthesis (Price *et al.*, 1998).

There are metabolic costs associated with active accumulation of C_i, but these are generally outweighed by the other advantages conferred by a CCM.

The essential features of the cyanobacterial CCM include:

- an active transport mechanism for CO_2 and HCO_3^- species with resultant accumulation of HCO_3^- within the cell and
- generation of elevated CO_2 levels within a RuBisCO-containing microcompartment known as the carboxysome (Figure 1.6A and Figure 1.6B)
- another essential feature of a CCM is that an effective mechanism for minimizing leakage from the site of elevation has to be employed, and this feature is associated in some way with the carboxysome (Price *et al.*, 1998).

9. Environmental regulation of CO_2-concentrating mechanisms in microalgae

The CO_2 - concentrating mechanism (CCM) found in marine and freshwater microalgae has profound effects on the ability of these organisms to assimilate dissolved inorganic carbon (DIC) from the environment. There have been few observations of the effect of environmental factors on CCM activity in microalgae that can cast light on the extent to which carbon acquisition by these organisms might be regulated, *in situ*, in aquatic ecosystems.

9.1. CO_2 concentration

Numerous experiments have demonstrated that carbonic anhydrase and the CCM are inducible by growth of cells in low levels of CO_2, but is generally repressed when the concentrations of CO_2 in the gas phase is >1% (Beardall *et al.*, 1980; Beardall *et al.*, 1991; Sultemeyer, 1998). Although there are not many natural habitats for photosynthesising microalgae in which CO_2 concentrations reach values as high as 1%, many environmental factors will influence both the absolute CO_2 or DIC concentration and the ratio of CO_2 to other inorganic C species in the aqueous phase (Beardall *et al.*, 1998). Aside from the CO_2 concentration in the gas phase in equilibrium with the medium, there are many environmental factors such as pH, temperature, salinity, and cell density and aeration rate of cultures (Beardall *et al.*, 1998) that, perhaps less directly, control the availability of CO_2. Mayo *et al.* (1986) suggest that the controlling factor governing CCM expression for cyanobacteria is the HCO_3^- concentration in the external medium.

9.2 Cell density and light-dependent DIC consumption

Intensive photosynthetic activity will decrease the DIC concentration in water and, in poorly buffered situations, will increase the pH, leading to a decrease in the CO_2 to bicarbonate ratio as well as a reduced absolute CO_2 concentration (Beardall *et al.*, 1998). The process will be more marked in high-density cultures where the rate of DIC consumption may exceed the rate of replenishment from the atmosphere.

This appears to be a problem when doing culture work and makes it somewhat difficult to extrapolate from such studies to what might be happening in a natural ecosystem where cell densities are rarely as high. However, it appears that there are situations, especially in poorly buffered fresh waters, where considerable CO_2 depletion can occur.

While cyanobacteria respond to DIC depletion by an increased capacity of cells for direct active transport of CO_2 and HCO_3^- and hence DIC accumulation, many studies on eukaryotic microalgae suggest that such conditions also lead to enhanced expression of external carbonic anhydrase activity (Beardall *et al.*, 1998; Nimer *et al.*, 1994; Patel & Merrett, 1986; Williams & Colman, 1996). This allows rapid equilibration between CO_2 and HCO_3^- at the cell surface and provides a mechanism for indirect bicarbonate use and improved CO_2 supply at the plasmalemma (Beardall *et al.*, 1998). Alternatively, some microalgae appear to have a capacity for direct active transport of bicarbonate.

Aeration will tend to restore the equilibration of the aqueous phase with CO_2 in the atmosphere and will thus counteract the effects of CO_2 depletion from photosynthetic activity.

9.3 pH

The equilibration between the various inorganic carbon species in solution (CO_2, HCO_3^- and carbonate) will be affected by pH. Increasing pH will lead to a greater bicarbonate to CO_2 ratio and, if air equilibrium is maintained, to increased total inorganic carbon concentrations (Beardall *et al.*, 1998). The latter phenomenon is not common in natural waters that are well buffered, but is a possible complication in laboratory experiments where cultures are grown at elevated pH with good aeration and stirring. In the absence of air equilibration, high pH will reduce the CO_2 available to algae to very low concentrations. This occurs frequently in poorly buffered freshwater systems.

Low pH values are associated with rapid rates of uncatalysed CO_2 supply from bicarbonate, and activity of external carbonic anhydrase is usually less in low pH media than in high pH media (Beardall *et al.*, 1998).

According to Williams & Colman (1996), external carbonic anhydrase activity in *Chlorella saccharophila* was suppressed by growth at high dissolved inorganic carbon and at acid pH (growth was suppressed at pHs below 7.0, with total repression occurring at pH 5.0) (Beuf *et al.*, 2000). In well aerated systems, the total DIC levels at elevated pH may become so high that rates of CO_2 supply from air-equilibrated cultures are higher than those at low pH (with a lower total DIC in solution) (Beardall *et al.*, 1998).

9.4 Salinity

Salinity is one of the major constraints on species diversity and productivity of natural populations of microalgae (Booth & Beardall, 1991; Fisher *et al.*, 1996). Some species of microalgae have a narrow salinity tolerance, whilst others can survive and proliferate in environments of widely differing salinity (Booth & Beardall, 1991).

Although it has been shown that in some species HCO_3^- transport is Na^+ dependent, the Na^+ concentration required is very low (< 25mM) (Booth & Beardall, 1991). Ritchie *et al.* (1996), have conclusively shown that the Na^+ effect is not via the use of Na^+ as a driving ion for Na^+: HCO_3^- co-transport. Therefore salinity is more likely to influence carbon acquisition via indirect effects on the DIC system in water through changes in dissociation constants and CO_2 solubility (Beardall *et al.*, 1998; Raven, 1991). Thus, freshwaters contain DIC at about 50% of the concentration found in marine systems at a comparable pH and temperature, and the proportion of DIC as CO_2 decreases (for a given pH) with increasing salinity (Beardall *et al.*, 1998).

It was also found that the affinity in photosynthesis for CO_2 was more pronounced in cells grown at higher NaCl concentrations. The activity of carbonic anhydrase accessible at the cell surface was observed to increase substantially with increasing NaCl concentration (Booth & Beardall, 1991). From the results, it was decided that increases in external carbonic anhydrase activity correlated closely with increased affinity for CO_2 and HCO_3^- in photosynthesis, reflecting the importance of external carbonic anhydrase for the effective functioning of the CCM in *D. salina*.

9.5 Temperature

Elevated temperatures have been found to increase the metabolic activity of algae and result in a decrease in CO_2 solubility (Raven, 1991). These elevations result in lower values for pKa_1 and pKa_2 and at a given carbonate alkalinity, there will be a shift in equilibrium of C_i species (CO_2: HCO_3^- :CO_3^{2-}) from CO_2 plus bicarbonate towards carbonate (Beardall *et al.*, 1998). Despite the decreased bicarbonate concentration at elevated temperature, the rate of CO_2 supply from the uncatalysed dehydration of bicarbonate increases as the temperature rises (Beardall *et al.*, 1998). This is likely to more than compensate for the decreased solubility of CO_2 at elevated temperatures.

9.6 Light

Inorganic carbon transport and accumulation is an active process, which is activated and energised by photosynthesis. The ATP necessary for carbon transport is believed to be derived from electron flow associated with photosystem I (PSI) (Beardall *et al.*, 1998). Raven and Lucas (1985) attempted to quantify the cost of operation of a CCM and proposed an energetic cost of 1 mol of ATP per mole inorganic carbon transported. This corresponds to an overall requirement of 3.9 ATPs for operation of the CCM in addition to the 3 ATPs and 2 reduced nicotinamide adenine dinucleotide phosphates (NADPH) necessary for CO_2 fixation via the Calvin cycle (Beardall *et al.*, 1998).

It must be recognised that one of the principle effects of changes to photon flux for growth is a change in the rate of CO_2 assimilation (Raven, 1991) and hence the potential rate of removal of inorganic carbon from the medium (Beardall *et al.*, 1998). If air equilibrium is not maintained, increased photosynthetic rates at higher photon fluxes can lead to decreased DIC concentrations in the medium and to subsequent enhancement of CCM activity (Beardall *et al*, 1998; Bedell, 1990). Light limitation of growth will thus regulate the capacity of cells to transport DIC, although the CCM is never completely repressed and quite low growth rates are required before any effect of photon flux is noticeable. However, the more immediate photon flux under which transport activity is measured does appear to have a major influence on CCM activity.

The greatest concern, is the effect of high light intensities, which cause photoinhibition or photoinactivation due to the impairment of photosynthetic processes, resulting in decreased algal growth yield or sometimes death of the entire biomass (Richmond, 1986). Photoinhibition occurs because supraoptimal intensities of light cause a destruction of the photosynthetic pigments as a result of oxygen and light-dependent bleaching (photooxidation) of cells (Bedell, 1990).

Clearly then, photon flux can have significant effects not only on the CCM activity of microalgal and cyanobacterial cells, but also on the capacity of these cells for DIC transport (Beardall *et al.*, 1998). The efficiency of carbon acquisition in the natural environment is thus likely to be strongly regulated by light.

9.7 Nutrients

9.7.1 Nitrogen

Beardall *et al.* (1982) have shown that *Chlorella emersonii* grown under nitrogen-limited conditions, possessed a CCM and the physiological characterisitics that this confers, irrespective of the CO_2 concentration during growth. It has been suggested that the induction of the CCM by nitrogen limitation was a response that improved the nitrogen efficiency of the cells (Beardall *et al.*, 1991). Beardall *et al.* (1982) pointed out that photorespiration is not only a source of inefficiency in carbon dioxide assimilation, but also in nitrogen metabolism due to the NH_4^+ released during the glycine to serine conversion in mitochondria and postulated that the induction of the CCM under nitrogen limitation could be related to suppression of this NH_4^+ release.

A simpler explanation is also feasible, namely that induction of the CCM under nitrogen limitation would result in higher rates of CO_2 assimilation for a given (limited) amount of RuBisCO than would occur in the absence of the CCM.

Under nitrogen sufficiency, high CO_2 grown cultures showed repression of CCM capacity. The induction of a CCM by low rates of nitrogen supply improved the efficiency of nitrogen use by microalgal cells (Raven, 1991) and allowed the cells to maintain reasonable rates of CO_2 fixation with less investment of N in RuBisCO (Beardall *et al.*, 1998). It is therefore likely that nitrogen supply can have an impact on the extent of CCM activity and because of the differing energetic requirements for assimilation, the source of inorganic nitrogen will also have an influence. Nitrogen assimilation, especially nitrate, imposes high energetic demands, so there is likely to be an interaction between N and photon flux in regulation of inorganic carbon acquisition.

9.7.2 Phosphorous

Inorganic phosphate (P_i) plays a central role in cellular energy transduction (Beardall *et al.*, 1998). As a result, phosphate limitation can have an important impact on various aspects of metabolism. Since carbon acquisition in most microalgae is an active process, requiring ATP, it might be expected that phosphate limitation could have an indirect regulatory influence on CCM activity.

Beardall *et al.* (1998) looked at the effects of P limitation on the ability of *Chlorella emersonii* to take up and assimilate CO_2. Research showed that the photosynthetic capacity decreased under P limitation and, in contrast to N limitation, the activity of the CCM appeared to be partially repressed in P-limited cultures. Internal CO_2 pools in phosphate limited cultures were approximately half of those found in phosphate –replete cultures growing at maximum rates.

9.7.3 Other nutrients

- Iron

Iron is one of the most important trace metals as it forms an essential part of cytochromes and ferredoxins, which are involved in membrane-associated reactions in photosynthesis, chlorophyll biosynthesis (Benesova *et al.*, 2000), respiration and nitrate reduction (Beardall *et al.*, 1998). A reduction in Fe availability might therefore impinge upon a cell's ability to provide the ATP necessary for transport of DIC.

- Zinc

Zinc is an important component of CA, which is involved both in diffusive CO_2 entry and in CCMs (Beardall *et al.*, 1998). Zinc limitation would therefore reduce the capacity of microalgae for indirect use of HCO_3^- via CA and thereby influence their ability to accumulate DIC.

The capacity of microalgal cells for carbon transport and accumulation is controlled by many environmental factors. Primarily, regulation is brought about by changes in the CO_2 supply to the cells and these can be affected by factors such as salinity, temperature and biological activity, as well as by changes in the DIC concentration in the medium. Restriction in the capacity to generate ATP, either by decreased light availability or through nutrient limitation, can also restrict the capacity of cells for DIC accumulation.

10. Objectives of this study

From research carried out in this field, it has been established that the algae and the cyanobacteria have an effective environmental adaptation, known as a CO_2 concentrating mechanism (CCM), for survival at low CO_2 concentrations. This mechanism functions to actively transport and accumulate inorganic carbon within the cell, and use this inorganic pool to elevate CO_2 concentrations around the primary CO_2-fixing enzyme RuBisCO.

A considerable amount of research has been conducted on algae as a bioremediation agent, but the research has been focussed mainly on the ability of some species to adsorb or accumulate metal ions from solution. The production of extracellular components, such as chelatins and polysaccharides, have also been extensively studied and are capable of complexing metal ions. It has been documented that certain algal species are able to increase the alkalinity of the surrounding medium as a by-product of their inorganic carbon accumulating mechanism, but very little work has been done to investigate the potential use of the akalinity generated for the precipitation of metals (van Hille *et al*, 1999).

The objective of this project was two-fold, firstly to establish the effect of copper, lead and nickel, heavy metals commonly found in AMD waters, on the enzyme carbonic anhydrase, which is an integral part of the carbon concentrating mechanism. Secondly, to determine the feasibilty of using the alkalinity generated by the algae for the precipitation of heavy metals from solution.

CHAPTER TWO

MATERIALS AND METHODS

2.1 General procedures

The following general procedures were observed throughout all experimental work:

i) Milli-Q water was used for the preparation of all media and Atomic Absorbance Spectrophotometer metal standards

ii) Borosilicate glassware was used for all experiments because of its negligible metal binding capacity

iii) All glassware was deionized by washing with 15% nitric acid and then rinsed in water

iv) Chemicals from various manufactures were used, mainly Saarchem and Merck

v) All experiments were carried out in triplicate, unless otherwise stated

2.2 Biomass

The *Spirulina sp.* used in this study, was originally obtained from Mr Len Dekker in Wellington in the Western Cape, and was initially cultured in five litre round bottom flasks containing Zarrouk's medium, in the Metals Laboratory, Rhodes University. Zarrouk's medium (Appendix A), is a chemically defined medium used specifically for the cultivation of *Spirulina.*

Once there was enough biomass, a 50L algal raceway (Figure 2.1) was started and maintained in a constant environment room set at 28°C and illuminated overhead by 3 fluorescent lights.

Figure 2.1: A 50 litre raceway used for the culturing of *Spirulina sp.*

Biomass was harvested by filtering a volume of the stock culture from the algal raceway through a nylon cloth (mesh size 0.2mm) and then washing with Zarrouks medium containing no $NaHCO_3$ to rid the culture of any excess bicarbonate. The algae were then resuspended in Zarrouks medium containing no bicarbonate to form a concentrated slurry from which appropriate dilutions could be made if required. Fresh biomass was harvested for each experiment.

2.3 Determination of algal concentration

Spirulina is a filamentous cyanobacterium, so cell counts could not be used to determine culture concentration. A chlorophyll extraction method and a photometric method were used to accurately determine the chlorophyll concentration within a sample. Chlorophyll plays an essential role in the photosynthetic pathway and was as a result a good indication of the growth of the culture.

2.3.1 Photometric method

Aliquots (2ml) of the culture were removed and thoroughly mixed on a vortex mixer for 5 seconds to ensure even distribution and the optical density (OD) was determined at 660nm. Samples were read in triplicate.

2.3.2 Chlorophyll extractions

Algal slurry (10ml) was filtered using 25mm Whatmann GF/C filters and the algal laden disks were then transferred to foil-covered McCartney bottles containing 10ml of acetone. The bottles were covered to prevent light degradation of chlorophyll *a*. The disks were then crushed using a glass rod and the bottles were stored overnight at 4°C. The following day, samples were again filtered to remove crushed paper and cell debris and then read on a Shimadzu UV-visible spectrophotometer at the following absorbancies: 661.6nm, 644.8nm, 470nm. From the absorbance readings obtained, the chlorophyll concentrations could be calculated using the equations specified in Appendix B (Lichtenhaler, 1987).

2.4 Determination of carbonate species in solution

Titrations were carried out on 10ml of filtered media (to remove the algae) using 0.02N H_2SO_4 until the bicarbonate and carbonate endpoints were reached, these being pH 8.3 and pH 4.5 respectively. The volume of acid required to reach these endpoints was recorded and then the alkalinity as calcium carbonate ($CaCO_3$) was determined using the following equation:

$CaCO_3$ alkalinity = volume of acid titrated (ml) x 10(100/10)

The table in Appendix C was then used to determine the molarities of the carbonate species in solution.

2.5 Determination of the effect of *Spirulina* on the chemical composition of the surrounding medium

2.5.1 Batch flask cultures

Conical flasks (500ml) containing 270ml of Zarrouk's media (Appendix A), of varying concentrations of $NaHCO_3$ (namely 2.1; 4.2; 8.4 and 16.8g/L), were used. The algae were harvested as described in Section 2.2 and 30ml of the concentrated algal slurry was used to inoculate the flasks. The pH was adjusted to 8.3 and titrations were carried out, as described in Section 2.4, to determine the carbonate species in solution at the beginning of the experiment. The flasks were then stoppered and sparged with nitrogen for 15 seconds to flush the media and headspace of any O_2 and CO_2. These cultures were grown under constant environment conditions (28°C – constant illumination) on a shaker at 138rpm in the Departmental constant environment room at Rhodes University. The experiment was done in triplicate.

Two sets of control flasks were prepared, containing Zarrouk's media with no $NaHCO_3$ present. The flasks were also seeded with 30ml of concentrated algae. The one set of flasks were sparged with N_2 and stoppered and the other set had a cotton wool bung inserted but was otherwise exposed to the air.

Another set of control flasks were prepared containing 16.8g/L $NaHCO_3$, but no algae. These flasks were to demonstrate spontaneous dehydration of HCO_3 in the absence of algae.

2.5.2 The effect of carbonic anhydrase inhibitors on inorganic carbon accumulation

To determine the effect of carbonic anhydrase inhibitors on the algae's ability to change the chemical composition of the surrounding medium, triplicate flasks containing 16.8g/L $NaHCO_3$ and either 200µM acetazolamide (AZ) or ethoxyzolamide (EZ) were prepared. The flasks were also seeded with 30ml concentrated algae, sparged and stoppered as above.

Every second day, chlorophyll extractions (Section 2.3.) and titrations (Section 2.4.) were performed on all flasks and pH was measured. The flasks were then resparged.

2.6 The effect of heavy metals on bicarbonate utilisation, photosynthesis and carbonic anhydrase activity

2.6.1 Harvesting of biomass

Biomass was harvested as described in Section 2.2 and batch flasks were prepared as discussed in Section 2.5.1, with the exception that the highest $NaHCO_3$ concentration was used (i.e. 16.8g/L) and the EDTA and $FeSO_4$ were 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.

To determine the effect of heavy metals on the cultures, a range of concentrations of metals were investigated. An appropriate volume of a 5000µM stock solution of copper sulphate ($CuSO_4$), was added to each flask to make the final concentrations up to 50, 100, 300, 500μM. Chlorophyll extractions (section 2.3), titrations (section 2.4) and pH readings were performed on a daily basis.

Light microscope (section 2.6.2), scanning electron microscopy (SEM) (section 2.6.3) and transmission electron microscopy (TEM) preparations (section 2.8.2) of the algae were made at various stages.

The experiments were repeated using 5000μM stock solutions of nickel sulphate ($NiSO_4$) and lead nitrate ($Pb(NO_3)_2$).

2.6.2 Light microscopy

Samples were prepared for light microscopy by mounting 25μl of biomass on a glass slide and covering with a glass coverslip. The slides were photographed at the Electron Microscopy Unit, Rhodes University, using an Olympus BX50 camera-microscope unit attached to an Olympus PM-30 exposure control unit.

2.6.3 Scanning electron microscopy (SEM)

Algal specimens were prepared by filtering 5ml of sample through a Whatmann GF/C filter and then washing the filter with 5ml phosphate buffered saline to rid the culture of excess salt. The specimens were fixed in cold 2.5% gluteraldehyde in 0.1M phosphate buffer for 12 hours. After fixation, the specimens were washed in phosphate buffer again, followed by a 30% to 100% ethanol dehydration sequence, for 10min at each concentration. Samples were then incubated, in a fume hood, in increasing concentrations of amyl acetate for 15min each, prior to critical point drying. The samples were then mounted on copper studs, coated in gold and observed using a JEOL JSM-80 SEM (Cross *et al.*, 1987).

2.6.4 Carbonic anhydrase activity assays (Wilbur-Anderson method)

2.6.4.1 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.

2.6.4.2 Preparation of Standards

Standards ranging in concentration from 1 to 15 WAU/ml were prepared from a bovine CA stock solution of 150 WAU/ml. 100 µl aliquots of the standards were used in each individual assay.

2.6.4.3 Sample Preparation

5ml samples were pipetted into test tubes from each flask containing metal solutions. These were left to stand until the *Spirulina* had floated to the top of the tube (this was done as the salinity of the media and the filamentous nature of the algae made it difficult to centrifuge down without damaging the cells). Once the algae had surfaced, 600µl aliquots of the concentrated sample were used in each individual assay.

2.6.4.4 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 (10mM, pH 8.3, 4°C) 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 800ml deionised water at 4°C for 2 hours before use (to ensure saturation). All assays were performed in triplicate.

2.7 Measurement of the efficiency of photosynthetic oxygen evolution under various conditions using a Rank Oxygen Electrode

2.7.1 Setting up the electrode

The base of the incubation vessel was detached by unscrewing the perspex locking nut, and sufficient saturated KCl solution was added to wet the Ag and platinum electrodes. A $1cm^2$ piece of lens tissue was cut and a 1mm diameter hole was made with a pair of sharp scissors in the middle of the lens tissue. This was then placed over the platinum electrode so that the hole was over the electrode. Then a piece of teflon membrane (manufactured by Yellow Springs Instrument Company) of a similar size was placed over the lens tissue and locked into place by putting the incubation vessel in place and screwing down the locking nut. Care had to be taken to ensure that no air bubbles were trapped and that the membrane was not twisted. The reaction chamber was enclosed within a water jacket and the temperature was maintained as desired (in this case, 25°C).

2.7.2 Operation and calibration

2.7.2.1 Calibrating the oxygen electrode

The Ag-AgCl electrode was connected to the positive side of the potential divider and the platinum to the negative. The oxygen read-out meter was set to zero using the appropriate control. Air-saturated water of known oxygen concentration was added to the incubation vessel and the perspex disc, which prevents any gaseous exchange with the outside environment, was placed into position. This air-saturated water served as a reference solution. The magnetic stirrer was only switched on after ensuring that no air bubbles had been trapped. The sensitivity control on the chart recorder was adjusted to give a suitable deflection for measurement. Once a suitable sensitivity had been determined, the reference solution was removed using a pipette, rinsed three times and experimental samples were then analysed.

2.7.2.2 Testing for electrode leakage

In theory, a perfect electrode will give zero output when the oxygen tension in the solution is zero. In practice, small faults in the sealing of the platinum electrode into the insulator can occur, and this will cause some leakage (residual) current to flow in the absence of oxygen. To test if the teflon membrane was "leaky" (which leads to noise), a few crystals of sodium dithionite were added to the chamber containing air-saturated water. Sodium dithionite chemically removes all the oxygen in the solution, so any remaining signal will then be caused by electrode leakage current. The electrode current should fall within 5 seconds to zero or nearly so; if not, the membrane may need changing and electrical connections should be checked.

2.7.3 Experimental preparation

2.7.3.1 Harvesting of biomass

Biomass was harvested by centrifuging 10ml samples at 5000rpm for 20 minutes in a Heraeus benchtop centrifuge and resuspending the pellet in 5ml Zarrouks medium (Appendix A) containing no $NaHCO_3$. The resuspended sample was then added to the reaction chamber where various experiments were performed. Chlorophyll extractions (Section 2.3.2) were carried out before all experiments, to determine the chlorophyll concentration of the samples and all oxygen evolution rates were calculated per mg Chl.

2.7.3.2 Preparation of reagents

- 0.1M $NaHCO_3$

This stock solution was prepared by dissolving the appropriate amount of $NaHCO_3$ in a 1L volumetric with Milli-Q water and adjusting the pH to 8.3.

- Inhibitors

20mM Acetazolamide

Acetazolamide (20mM) was prepared by weighing out 0.0444g of AZ, manufactured by Sigma, and dissolving it in a volumetric flask with methanol. This was then transferred to a brown bottle to prevent light degradation of the inhibitor.

20mM Ethoxyzolamide

Ethoxyzolamide (20mM) was prepared by weighing out 0.0517g of EZ, manufactured by Sigma, and dissolving it in a volumetric flask with methanol. This was then transferred to a brown bottle to prevent light degradation of the inhibitor.

- Bovine carbonic anhydrase

A 10µg/ml stock solution of bovine carbonic anhydrase was prepared freshly and stored at 4°C until required.

2.7.3.3 Determining the response time of an oxygen electrode

A very rough check on the response time of an oxygen electrode can be made, by switching off the stirrer. Once the electrode output had fallen three recorder units, the stirrer was switched back on at the same speed as before, and the time taken to recover 90% of the former signal was measured.

2.7.3.4 Determination of the effect of varying light intensities on oxygen evolution

Photosynthesis versus irradiance determinations was made with a Rank oxygen electrode (Rank Brothers, Cambridge, England) at 25°C for each culture. Cell suspensions (prepared as discussed in Section 2.7.3.1) were placed in the electrode chamber. The cells were exposed to 7 different photon fluxes (ranging from 67,10 to 192,7 $\mu mol.m^{-2}s^{-1}$) which correlated to a specific distance the light source was placed from the chamber (ranging from 19,5 to 2,5 cm away) as measured by a light meter. The oxygen-electrode tracing obtained was then used to calculate the rates of oxygen evolution at varying light intensities.

2.7.3.5 Investigating rates of photosynthetic oxygen evolution as a function of external bicarbonate concentration in the absence and presence of bovine carbonic anhydrase

Once again, samples were prepared as discussed in Section 2.7.3.1 and inorganic carbon dependent photosynthetic oxygen evolution was monitored using the oxygen electrode. The light intensity at the surface of the oxygen electrode chamber was the saturating light determined in Section 2.7.3.4 at a temperature of 25°C. Cells were allowed to deplete endogenous carbon sources, as measured by the cessation of O_2 evolution. Photosynthetic rates were obtained subsequently by varying the concentration of $NaHCO_3$ added (ranging from 0 to 4000μM).

The experiments were repeated as above at varying $NaHCO_3$ concentrations, but this time in the presence of 10μg/ml of bovine carbonic anhydrase, and this was used as a measure of active CO_2 uptake.

2.7.3.6 Assessing the spontaneous dehydration of HCO_3^- in the absence of *Spirulina*

In these experiments, the objective was to investigate any difference in the rate of O_2 evolution, in the absence of *Spirulina* compared with that obtained in Section 2.7.3.5 at varying $NaHCO_3$ concentrations. Aliquots of Zarrouk's media (5ml) containing no *Spirulina* were placed in the electrode chamber, the light was switched on, at the intensity determined in Section 2.7.3.4. Varying concentrations of $NaHCO_3$ were added to the media and the O_2 traces were then used to determine if there was any noted difference between catalysed and uncatalysed dehydration of HCO_3^-.

2.7.3.7 The effect of carbonic anhydrase inhibitors on photosynthetic oxygen evolution

The inhibitors 6-ethoxyzolamide (EZ) and acetazolamide (AZ) were used. It is generally assumed that AZ cannot penetrate the cell and therefore only acts on surface-accessible CA (Moroney *et al.*, 1985), although this assumption has been questioned (Williams & Turpin, 1987). The EZ penetrates into the cell, inhibiting external and internal CA. Both AZ and EZ were dissolved in methanol at a concentration of 20mM.

Algal samples were once again prepared as discussed in Section 2.7.3.1 and inorganic carbon dependent photosynthetic oxygen evolution was monitored using the oxygen electrode. The light intensity at the surface of the oxygen electrode chamber was the saturating light determined in Section 2.7.3.4 at a temperature of 25°C. Cells were allowed to deplete endogenous carbon sources. The light was then switched off for a period of three minutes. When the light was again switched on after a period of respiration, varying concentrations of the inhibitors (20-100μM) were added to the culture and they were allowed to photosynthesise again. After a few minutes 2mM $NaHCO_3$ was added to the electrode chamber to see if an increase in oxygen evolution was observed.

2.8 Heavy metal uptake by *Spirulina*

2.8.1 Metal analysis by Atomic Absorption Spectrophotometry (AA spec)

A 5ml sample was removed from each of the flasks (described in Section 2.6.1) containing varying concentrations of lead, copper and nickel and filtered through 0.45μm OSMONICS nylon membrane filters, before analyzing the samples for residual metal on the Atomic Absorption Spectrophotometer. Standard curves for each metal were generated on the AA spec by making appropriate dilutions of a concentrated (1000ppm) metal solution. Metal concentrations of the samples were then analysed at the end of each experiment on a GBC 909AA atomic absorption spectrophotometer linked to a GBC integrator. From the results obtained, the percentage uptake was determined.

2.8.2 Transmission electron microscopy (TEM) preparation for Energy Dispersive X-ray Analysis (EDXA)

Algal specimens were prepared by centrifuging 2ml of sample at 5000rpm in an Eppendorf centrifuge 5403 and then washing the pellet with 2ml phosphate buffered saline to rid the culture of excess salt. The specimens were fixed in cold 2.5% gluteraldehyde in 0.1M phosphate buffer for 12 hours. After fixation, the specimens were washed in phosphate buffer again, followed by a 30% to 100% ethanol dehydration sequence, for 10min at each concentration. Samples were then incubated, in a fume hood, in increasing concentrations of resin for 30min each. The sample was then left in 100% resin overnight in the fridge. The following day fresh resin (100%) was added to the eppendorfs containing the samples and incubated at 60°C for 36hrs. The samples were then sectioned using a RMC MT7 ultramicrotome. The sections were then mounted onto the relevant grids and examined under a JEOL JEM 100 CX transmission electron microscope at the University of Port Elizabeth (Cross, 1987).

2.9 Metal precipitation experiments

2.9.1 Experimental preparation

Biomass was harvested as described in Section 2.2 and 50mls of the concentrated biomass was used to seed each of the two 1L flasks containing 450ml of full strength Zarrouk's media (pH adjusted to 8.3). The algae were cultured under day/night cycles at 25°C on a shaker set at 138rpm. The one flask was stoppered and flushed with nitrogen, while the other one had a cotton bung inserted. The experiment was performed in triplicate.

Every second day titrations (Section 2.4) and metal precipitation experiments (Section 2.9.1.3) were performed.

2.9.1.1 Metal solutions

Metal solutions for precipitation experiments were made by dissolving an appropriate amount of a metal salt in Milli-Q water to give a concentration of 500μM. The following metal salts were used:

- Nickel sulphate ($NiSO_4$)
- Ferrous sulphate ($FeSO_4.7H_2O$)
- Magnesium chloride ($MgCl_2$)
- Ferric nitrate ($Fe(NO_3)_3.9H_2O$)
- Lead nitrate ($Pb(NO_3)_2$)
- Copper sulphate ($CuSO_4$)
- Calcium chloride ($CaCl_2$)

2.9.1.2 Determination of the volume of filtered media to be added to metal solutions

Titrations were carried out on the Zarrouk's media before inoculating with algae, to determine the bicarbonate alkalinity in the media. It was decided that a concentration of 30μmoles was required for the precipitation experiment, which meant that 147μl of the filtered media would be added to the metal solutions in the precipitation experiment.

2.9.1.3 Experimental procedure

147μl of filtered media from the 1L flasks was added to 100ml conical flasks containing 60mls of each of the metal solutions. The flasks were left to react for 30 minutes, after which the pH was recorded. A 5ml sample was then removed with a syringe and filtered through a 0.45μm OSMONICS nylon membrane filter, before analysing the samples on the Atomic Absorption Spectrophotometer (AA spec). Previous precipitation studies have shown that there was no binding of the metal to the filters, as results for centrifuged and filtered samples were almost identical.

2.9.1.4 Metal analysis by Atomic Absorption Spectrophotometry

Standard curves for each metal were generated on the Atomic Absorption Spectrophotometer spec by making appropriate dilutions of a concentrated (1000ppm) metal solution as previously described in Section 2.8.1.

2.9.1.5 Chemical modelling

Once the data had been accumulated, the alkalinity and pH results were used for chemical and precipitation modelling, using the chemical speciation software, MINTEQA2.

CHAPTER THREE

DETERMINATION OF THE EFFECT OF *SPIRULINA* ON THE CHEMICAL COMPOSITION OF THE SURROUNDING MEDIUM

3.1. Introduction

It is well known that algae are capable of causing eutrophication of many water systems. However, they are also recognised as an important part of these systems, as they have the capacity for modifying pH, alkalinity, colour and turbidity (Palmer, 1980). Some types are considered a nuisance in a water supply, but other types have been effectively used for the improvement of a water supply.

An important chemical effect of algae is the continuous removal of CO_2 from the water during the daylight hours as a result of photosynthesis. This process brings about an alteration in the relative amounts of soluble (unbound) carbonic acid, partially soluble (half bound) bicarbonates and the nearly insoluble (bound) monocarbonates, often causing the latter to precipitate (Booth & Beardall, 1998; Palmer, 1980). These changes in CO_2 also tend to change the pH of the water. The pH rises as the algae increase their photosynthetic activity during daylight hours, and the pH then decreases at night when the algae release CO_2 in respiration (Booth & Beardall, 1998; Williams & Colman, 1996).

Carbonic anhydrase activities in relation to inorganic carbon concentration were investigated by Amoroso *et al.* (1996) in *Dunaliella tertiolecta* and *Chlamydomonas reinhardtii*. According to Price *et al.* (1998), efficient photosynthesis is maintained by the operation of an inducible CO_2 concentrating mechanism (CCM). It has also been suggested that the inorganic carbon (C_i) transport system and CA form two essential components of the CCM (Badger, 1987). In low-C_i cells of *D. tertiolecta*, the C_i transport system was found to be responsible for an increase in the internal C_i concentration of up to 20-fold over that in the external medium (Amoroso *et al.*, 1996). Both C_i species, HCO_3^- and CO_2, have been suggested to serve as substrates for the uptake process, with CO_2 being preferentially taken up by low-C_i cells. Similar to *C. reinhardtii*, the utilization of CO_2 by low-C_i cells of *D. tertiolecta* is facilitated by an external CA that dehydrates HCO_3^- to CO_2, which in turn is then transported into the cell (Amoroso *et al.*, 1996; Moroney *et al.*, 1985). The significance of the CA-mediated HCO_3^- utilisation is probably more important at alkaline than at neutral conditions (Amoroso *et al.*, 1996; Moroney *et al.*, 1985; Price *et al.*, 1998).

3.2. Motivation and Aims

An extensive amount of research has been conducted, investigating the effect of CO_2 on the carbon concentrating mechanism (CCM) present in algae. When algae experience conditions of limited carbon dioxide, they are able to utilise inorganic carbon, in the form of bicarbonate, through the use of this mechanism, for photosynthesis. CA is the enzyme responsible for the dehydration of HCO_3^- to CO_2 and OH^-. This results in a decrease in acidity, as the resulting OH^- ions in solution are responsible for the alkalization of the culture medium and a shift in the bicarbonate/carbonate equilibrium results in more carbonate in the medium.

The major focus of this chapter is to determine the effect the algae used in this study have on the chemical composition of their surrounding medium by quantifying bicarbonate utilisation and carbonate production.

3.3. Results and Discussion

3.3.1 Batch flask cultures

Batch flask cultures were prepared using varying concentrations of $NaHCO_3$ to quantify bicarbonate utilization and to investigate the effect of the algae on carbonate speciation of the medium over a period of about two weeks. The culture flasks were stoppered and sparged with nitrogen to ensure removal of any CO_2 and O_2 in solution, resulting in low-C_i cells. The algal CCM was induced by the limitation of CO_2 and as a result utilisation of bicarbonate was made possible by the enzyme, CA, which is known to be responsible for the dehydration of HCO_3^- to CO_2 and OH^-.

3.3.2. Chlorophyll extraction

Every second day, chlorophyll extractions were carried out on all the batch flasks until a decrease in chlorophyll *a* concentration was observed and the flasks were then discarded (Figure 3.1). The chlorophyll *a* concentrations obtained were used as an indication of the health of the cultures. As *Spirulina* is filamentous, cell counts cannot be carried out, so chlorophyll extractions are a possible method of estimating the concentration of algae in the medium.

Chlorophyll is one of the most important chelates in nature and is capable of channeling the energy of sunlight into chemical energy through the process of photosynthesis. In photosynthesis, the energy absorbed by chlorophyll transforms carbon dioxide and water into carbohydrates and oxygen (Raven *et al.*, 1999). The chemical energy stored by carbohydrates drives biochemical reactions in nearly all living organisms.

In the photosynthetic reaction, carbon dioxide is reduced by water and chlorophyll assists in the transfer of electrons. When chlorophyll absorbs light energy, an electron in chlorophyll is excited from a lower energy state to a higher energy state (Raven *et al.*, 1999). In this higher energy state, this electron is more readily transferred to another molecule, which starts a chain of electron-transfer steps, which ends with an electron transferred to carbon dioxide (Giardi *et al.*, 2001; Mattoo *et al.*, 1999). Thus, chlorophyll is at the center of the photosynthetic oxidation-reduction reaction between carbon dioxide and water.

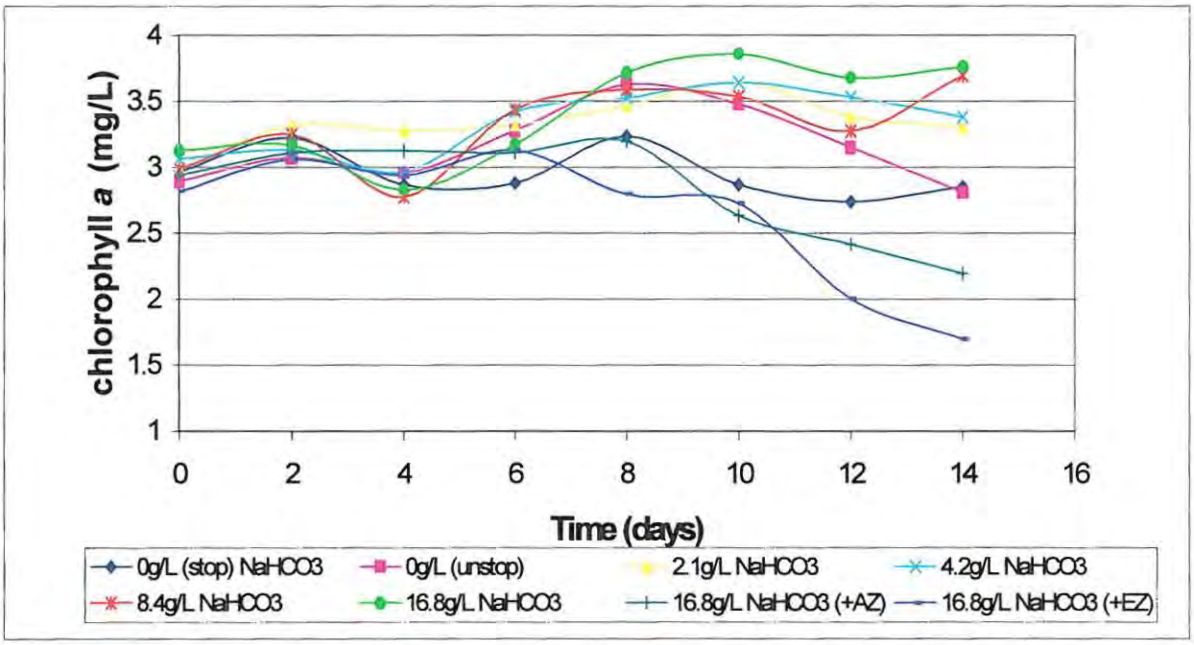


Figure 3.1: Chlorophyll *a* concentrations at varying concentrations of $NaHCO_3$

From Figure 3.1 it can be seen that the cultures supplied with the highest concentration of $NaHCO_3$, had the highest chlorophyll concentrations. Chlorophyll *a* is considered to be at the center of the photosynthetic reaction and is therefore responsible for supplying the algae with the necessary energy required for growth.

The set of control flasks, which contained no bicarbonate and were sparged and stoppered, showed a decrease in chlorophyll *a* concentration over the two-week period of study, as they were not supplied with an inorganic carbon source for growth. The chlorophyll *a* concentrations in these flasks were seen to decrease as the culture died off, indicating that the method of chlorophyll extraction is a reasonably suitable method for determining the health of the cultures.

The other set of control flasks, which were also not supplied with an inorganic carbon source, but were stoppered with a cotton wool bung (essentially the flasks were "unstoppered" as gaseous exchange with the outside environment could take place through the cotton wool bung), showed a higher increase in chlorophyll concentration over a period of a few days. This was due to the algae's ability to utilise the small amount of CO_2 which entered the "unstoppered" flasks. Because the amount of CO_2 going into solution was not sufficient to maintain the photosynthetic demand of the algae, they too began to die off and a decline in the chlorophyll *a* concentrations of those flasks was observed.

The flasks containing the carbonic anhydrase inhibitors acetazolamide (AZ) and ethoxyzolamide (EZ) showed a decline in chlorophyll *a* concentration. It appears that EZ had a more significant effect on chlorophyll *a* concentration that AZ. This may be due to the fact that AZ is thought to not penetrate the cell and therefore only acts on surface-accessible CA, whereas EZ penetrates into the cell, inhibiting both internal and external CAs. This means that the inhibitors reduce or prevent the amount of CO_2 converted from HCO_3^-, thus reducing the CO_2 concentration around the primary carboxylating enzyme, RuBisCO, resulting in inefficient photosythesis. As a result, there is not sufficient CO_2 present to maintain the photosynthetic requirements of the algae and they begin to die.

When the chlorophyll *a* concentrations began to decrease, the colour of the culture was observed to change from a dark blue-green colour to yellowish-green at this stage. This is probably due to the carotenoids, which are yellow or orange in colour. Their colour is usually masked by the predominant chlorophyll *a*, but as the nutrients are used up chlorophyll synthesis and accumulation are inhibited (Richmond, 1986), hence the decrease in the chlorophyll concentration and the observed colour change of the culture.

3.3.3. Determination of the carbonate species in solution and measurement of pH

Titrations were performed on 10ml of filtered algal solution from each flask using 0.02N H_2SO_4, and the molarities of the carbonate species in solution were determined as described in Section 2.4.

The pH of the batch flasks investigated was adjusted to 8.3 at the start of the experiment, to ensure that there was only bicarbonate in solution. From the carbon dioxide equilibria equations it has been determined that below values of pH 8.3, CO_2 appears to serve as the main carbon source, whereas above pH 8.3, HCO_3^- appears to be the predominant species in solution utilised by the algae for photosynthesis. The alkalinities (Figure 3.3 and Figure 3.4) were determined and the pH (Figure 3.2) of the cultures were recorded over a two week period.

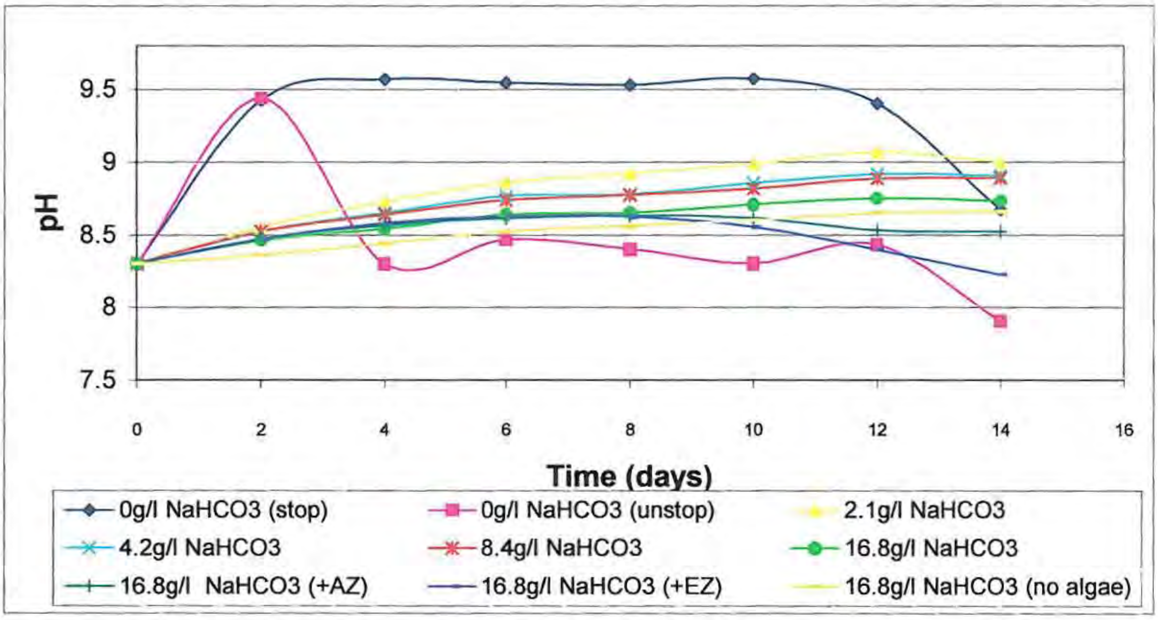


Figure 3.2: The pH of the batch flasks containing varying concentrations of $NaHCO_3$

Figure 3.2-3.4 will be discussed in conjunction with one another. Referring to Figure 3.2, it can be seen that there was an initial rapid increase in the pH of the control flasks containing medium with no bicarbonate, but after 2 days the pH began to decrease. The increase in pH was most likely as a result of the utilisation of traces of DIC in the water and carry over from the cell suspension. Because the control flasks contained no bicarbonate to buffer the pH, the increase in pH was more pronounced.

In the control flasks, which were stoppered with a cotton wool bung, gaseous exchange with the external environment could occur, enabling the culture to utilise the small amounts of CO_2 entering the flasks. The decrease in pH occurs in both sets of control flasks when the respiration rate exceeds the photosynthetic rate. Photosynthesis is inhibited as a result of this and more CO_2 is released into the medium, forming carbonic acid.

The flasks which were supplied with varying concentrations of bicarbonate demonstrated that alkalisation of the medium was dependent on the concentration of the external bicarbonate. An initial, reasonably rapid increase in the pH was observed and then the pH of the flasks containing the higher concentrations of bicarbonate leveled off due to the bicarbonate/carbonate buffering capacity of the medium. This observation is demonstrated in Figure 3.2, where it can be seen that the flasks containing 16.8g/L $NaHCO_3$ had a less rapid rate of pH increase as compared to flasks containing 2.1g/L $NaHCO_3$, because of the effective buffering capacity of the medium. Once all the DIC has been utilised, the pH of the medium begins to level off and may even decrease when the respiration rate exceeds the photosynthetic rate.

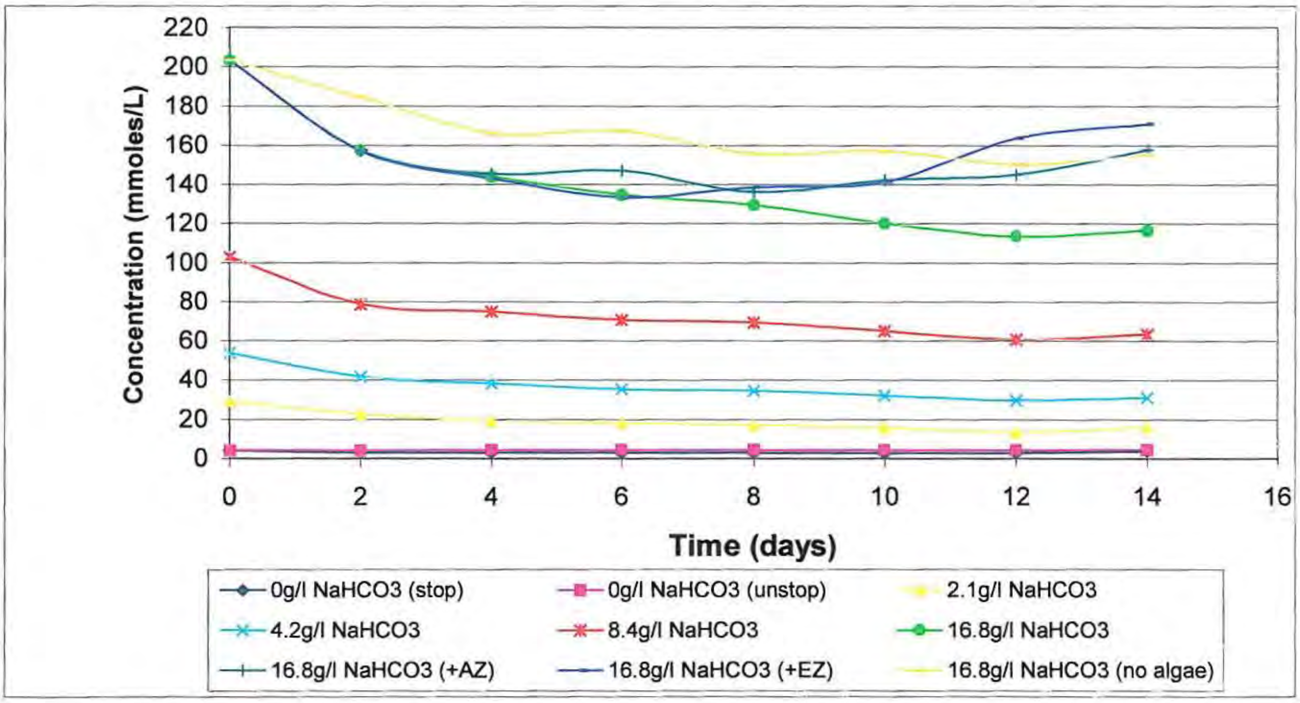


Figure 3.3: Bicarbonate speciation in solution (pH of media initially adjusted to 8.3)

The trend of carbonate speciation in solution is represented in Figure 3.3 (bicarbonate alkalinity) and Figure 3.4 (carbonate alkalinity). Initially, as the pH of the medium was

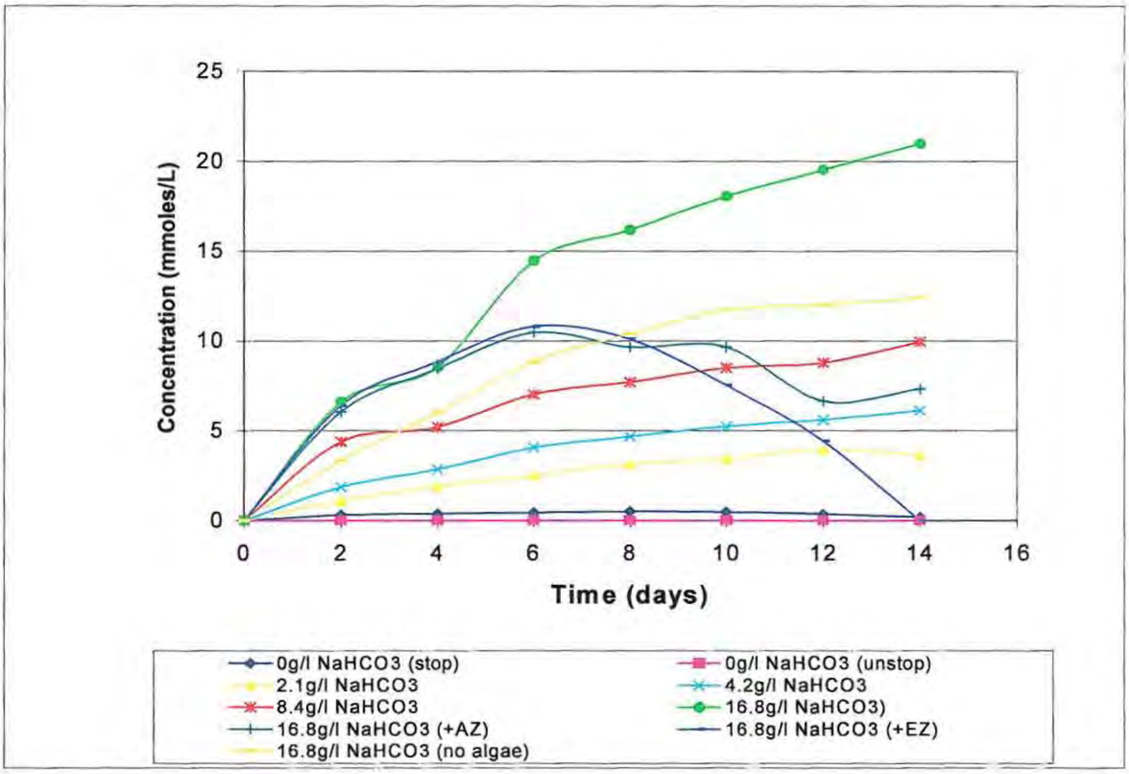


Figure 3.4: Carbonate speciation at varying concentrations of $NaHCO_3$

adjusted to 8.3 using H_2SO_4, it meant that there was only bicarbonate in solution and no carbonate was present. However, the carbon concentrating mechanism (CCM) in the algae was induced when the flasks were sparged with nitrogen to remove any CO_2 and O_2. As a result the algae were then able to utilise the bicarbonate for photosynthesis, thus reducing the bicarbonate alkalinity in solution. The use of bicarbonate is made possible by the enzyme, CA, which is responsible for the dehydration of HCO_3^- to CO_2 and OH^-. The CO_2 released is in turn utilised by the cell for photosynthesis, and some of the OH^- ions remain in solution and are responsible for the increase in pH as they react with free

H^+ ions in solution (Figure 3.2). The remaining OH^- ions react with some of the HCO_3^- in solution, according to the following equation, $HCO_3^- + OH^- \Leftrightarrow CO_3^{2-} + H_2O$, to form carbonate, resulting in an increase in the carbonate alkalinity. As bicarbonate is being utilised in two ways, the bicarbonate alkalinity is decreasing more than twice the rate the carbonate alkalinity is increasing (this is demonstrated in Figure 3.3 and 3.4).

Referring to Figure 3.3, it is obvious that the spontaneous dehydration of HCO_3^- is significantly slower than the active dehydration of HCO_3^- by the algae. It is also interesting to note that the algae were not as capable of utilising the HCO_3^- in solution upon the addition of the carbonic anhydrase inhibitors AZ and EZ. Again it appears from these results that the effect of EZ is greater than AZ on the algae's ability to utilise HCO_3, probably due to the fact that it inhibits both external and internal carbonic anhydrases. Whereas AZ inhibits mostly surface-accessible CA, which means that any HCO_3^- entering the algal cell passively or actively through mechanisms not involving CA, may be converted enzymatically to CO_2 by internal CA.

Corresponding to a decrease in bicarbonate alkalinity is an increase in carbonate alkalinity (Figure 3.4), the reasons for which have been explained above. Again it is evident that the active dehydration of HCO_3^- is considerably more effective than spontaneous dehydration and as a result, the carbonate alkalinity is greater in the flasks containing algae than those without. The flasks to which inhibitors were added, initially showed an increase in carbonate alkalinity, but as the cultures began to die off, very little bicarbonate was utilised and as a result no carbonate alkalinity was present.

To confirm the results already observed, the pH and alkalinity data from an experiment containing 16.8g/L $NaHCO_3$ were used for carbonate speciation modeling, using the chemical speciation software, MINTEQA2. MINTEQA2 is unable to predict photosynthesis, nevertheless, the titration data for HCO_3^- and CO_3^{2-} were used as input data to obtain a basic idea of the predicted chemical speciation characteristics observed using this system. The modelled data obtained confirmed that there was a rapid decrease in bicarbonate concentration as the algae photosynthesised, while at the same time there was a corresponding increase in the carbonate concentration in the medium, though not as rapid (Figure 3.5).

The pH (Figure 3.6) was also predicted to increase, due to alkalisation of the medium as the result of OH^- release caused by dehydration of the bicarbonate supplied in the external medium. As bicarbonate was supplied in the medium, the first three carbon dioxide equilibria reactions discussed on page 10 are suppressed, as the bicarbonate does not need to be generated by dissociation of carbonic acid (H_2CO_3). As a result no carbonic acid should be present in solution, and this is confirmed by the modelled results.

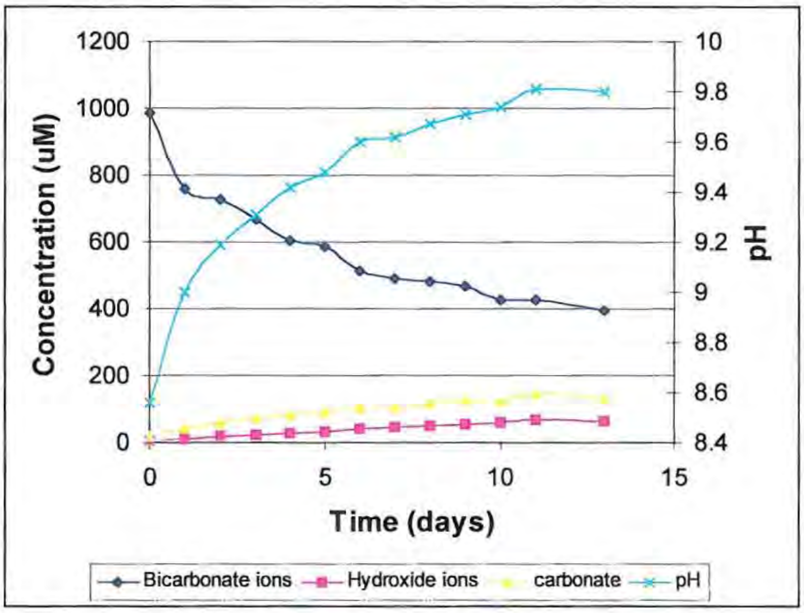


Figure 3.5: Predicted carbonate species in solution and pH readings (MINTEQA2)

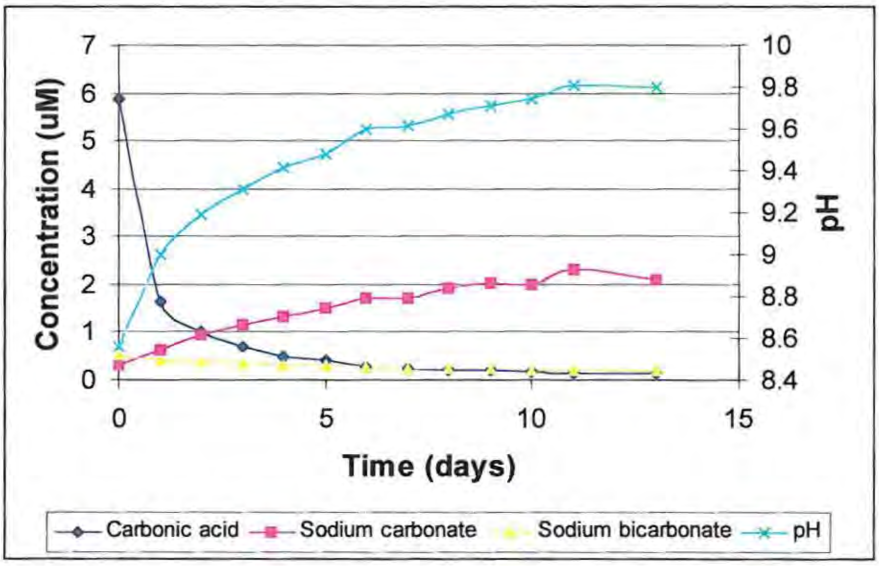


Figure 3.6: Predicted carbonate species in solution using the software MINTEQA2

These results confirm that under carbon dioxide limiting conditions algae are able to utilise bicarbonate supplied in the medium, through the use of their CCM, for photosynthesis. The hydroxide ions formed, contribute to the alkalisation of the medium, thus increasing the pH, and the remaining OH^- ions bind with HCO_3^- to form carbonate, which reacts readily with metals. When considering all the results discussed in this chapter, it is possible to assume that the use of algae for the precipitation of metal may be a feasible option and warranted further research. Hence, it was important to determine the effect that heavy metals would have on bicarbonate utilisation by the algae and on the enzyme, carbonic anhydrase, which we propose to be a key enzyme in the interaction of biogenic alkalinity with heavy metal ions.

CHAPTER FOUR

THE EFFECT OF HEAVY METALS ON BICARBONATE UTILISATION, PHOTOSYNTHESIS AND CARBONIC ANHYDRASE ACTIVITY

4.1 Introduction

The order of metal toxicity to algae varies with the algal species and the experimental conditions, but generally the order is Hg>Cu> Cd>Ag>Pb>Zn (Gimmler *et al.*,1991). The toxic effects of some of these metals will be mentioned briefly below, but greater detail can be found in Chapter 1 (Section 4).

Mercury is the most toxic of all the heavy metals and is known to interact with enzyme systems, especially enzymes with reactive sulfhydryl (-SH) groups thereby inhibiting their functions (Gimmler *et al.*, 1991). Mercury is also known to inhibit photosystem II (PSII) in algal cells and thylakoid membranes by inhibiting the water splitting system, inactivating some of the PSII reaction centers (El-Sheekh, 1999).

Copper is an essential micronutrient because it participates in photosynthetic electron transport and also plays a role as a cofactor of several oxidising enzymes (Fathi & El-Shahed, 2000) but it is the second most toxic metal if present in higher concentrations. Copper has been found to exert its toxicity in subcellular organelles, interfering with mitochondrial electron transport, respiration, ATP production and photosynthesis in the chloroplasts. At still higher concentrations, copper produces irreversible damage to chloroplast lamellae, preventing photosynthesis and eventually causing death.

Cadmium is a serious pollutant for plants and animals and it was found, that at sub-lethal concentrations, sharp reductions in photosynthesis and growth rates were observed (Gimmler *et al.*, 1991; Leborans & Novillo, 1996; Terry & Stone, 2002), because photosystems of algae can be damaged by excessive amounts of cadmium resulting in a reduction of photosynthetic pigments, such as chlorophyll *a* (Terry & Stone, 2002). Cadmium also inhibits one or more steps in protein synthesis and thus leads to enzyme deficiencies and a series of secondary effects.

Lead (Pb) is a non-essential element, which becomes toxic to algae at very low concentrations (10-50μg/L). Methylated lead is invariably more toxic than its inorganic form as its non-polar nature allows them to diffuse rapidly into and through cell membranes.

Zinc is an essential micronutrient for algae, but causes inhibition of cell division and destruction of chlorophyll at high concentrations (Skowronski & Rzeczycka, 1980; Stauber & Florence, 1990).

Nickel is a ubiquitous trace metal and occurs in soil, water, air and in the biosphere where its content in the Earth's crust is about 0.008% (http://www.ukmarinesac.org.uk), however, if present in high concentrations can be extremely toxic to algae. Nickel has been found to cause reduction in algal growth (Rai & Raizoda, 1987; Mallick *et al.*, 1990), carbon fixation, photosynthesis and an increase in the loss of Na^+ and K^+ ions resulting in changes in membrane permeability (Mallick & Rai, 1992).

4.2 Motivation and Aims

An extensive amount of research has been conducted, investigating the effect of heavy metals on eukaryotic algae, but little is known about the effect on cyanobacterial systems.

The carbon concentrating mechanism (CCM) present in algae is an essential mechanism induced when the inorganic carbon concentration in the surrounding medium is limited. The primary purpose of the CCM is to increase the CO_2 concentration around the primary CO_2-fixing enzyme, RuBisCO, to favour the carboxylation reaction at the expense of the oxygenase reaction, thereby achieving efficient photosynthesis even at low CO_2 concentrations.

The major focus of this chapter is to determine the effect that heavy metals has on the morphology of the algae, bicarbonate utilisation and the enzyme carbonic anhydrase, which is a major component of the CCM. Three metals were investigated, copper, lead and nickel as these are commonly found in acid mine drainage (AMD) waters.

4.3 Results and discussion

4.3.1 Batch flask cultures

Batch flask cultures were prepared using a range of concentrations of heavy metals. The effect these metals had on carbonate speciation of the medium, cell morphology and enzyme activity was investigated over a period of a week.

4.3.2 The effect of copper, lead and nickel on chlorophyll *a* concentrations

The trends in chlorophyll *a* concentration observed were very similar in the case of all three metals. As an example to illustrate the trend, only the result of the cultures grown in varying concentrations of nickel is shown (Figure 4.1).

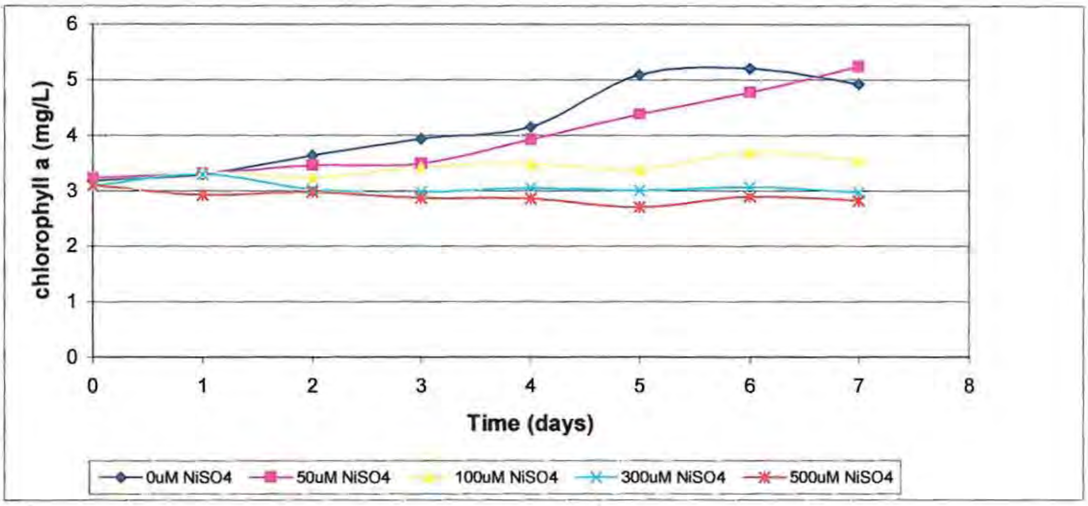


Figure 4.1: Chlorophyll *a* concentrations were used as an indication of the health of the algal cultures when grown in the presence of varying $NiSO_4$ concentrations

It is evident from Figure 4.1 and the data from the other metals (not shown as explained) that heavy metals have a significant effect on the synthesis of photosynthetic pigments. It has been noted before that excessive concentrations of metals become toxic to algal cells and often damage photosystems of algae resulting in a reduction of photosynthetic pigments, such as chlorophyll *a*, consequently preventing photosynthesis and eventually causing death.

The chlorophyll *a* concentration in the control flasks were seen to be higher than those in the flasks containing the highest metal concentration. It appears that the concentration of chlorophyll *a* is dose dependent, so the higher the concentration of metal in solution, the lower the chlorophyll *a* concentration. The control cultures demonstrated a steady increase in chlorophyll *a* concentration, whereas the flasks containing 300 and 500μM of metal, plateaued and would have eventually decreased as the cultures began to die due to the inhibition of chlorophyll synthesis and accumulation. A change in the colour of the culture from a blue-green to a yellowish colour was also observed at higher metal concentrations, again this was probably due to the carotenoids, which are usually masked by the predominant chlorophyll *a*.

4.3.3 Indirect method of determining the effect of heavy metals on the enzyme carbonic anhydrase

Due to some of the difficulties experienced using direct measurement of CA activity, such as the need for expensive equipment which is not readily accessible to the majority of laboratories and long retention times, an indirect method which measures bicarbonate utilisation and carbonate production was used to determine the effect of heavy metals on the enzyme carbonic anhydrase.

4.3.3.1 Lead

It is interesting to note that the flasks containing lead showed a very unusual trend. It was found that the flasks containing the highest metal concentrations utilised more bicarbonate from solution (Figure 4.2), whereas inorganic carbon utilisation was generally less in the control flasks containing no added metal. This was unusual, as one would expect bicarbonate utilisation to be greater in the control flasks containing no metal, with a decrease in C_i utilisation occurring with an increase in metal concentration.

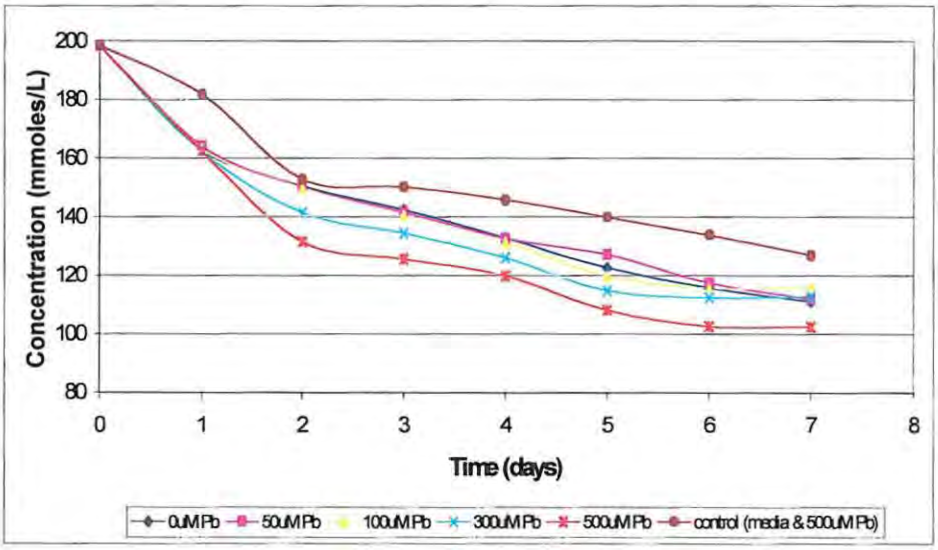


Figure 4.2: The bicarbonate alkalinity present in solution at varying concentrations of lead

From results obtained in Chapter 7 (metal precipitation studies), it became clear that the possible reason for the unusual trend in bicarbonate utilisation in the presence of lead was due to the fact that Pb is a strong acid metal which is able to drive the bicarbonate equilibrium resulting in the dissociation of HCO_3^- to form CO_3^{2-} and H^+. This is probably why bicarbonate utilisation is greater at higher Pb concentrations. As a result of this, a greater carbonate alkalinity would be expected at higher metal concentrations, which is not what was observed (Figure 4.3). When metal precipitation reactions were carried out using algal overflow (Chapter 7), a 40-100% precipitation of the lead from solution was observed. So, it is likely that the Pb drives the dissociation of HCO_3^- to form CO_3^{2-}, which then binds and precipitates out as lead carbonate. That is possibly the reason for a lower carbonate alkalinity at higher metal concentrations compared to the control flasks containing no metal (ie. no Pb is present to bind to the carbonate formed, therefore a higher carbonate alkalinity is observed).

Another set of control flasks was prepared, these contained 300ml of full strength Zarrouks medium and the highest concentration of metal. These flasks demonstrated the spontaneous dehydration of HCO_3^- in the absence of algae. It is clear from both Figures 4.2 and 4.3 that spontaneous dehydration of bicarbonate occurs at a much slower rate than does active dehydration by the enzyme carbonic anhydrase present within the algal cell, resulting in a slower rate of carbonate formation in comparison with the control containing *Spirulina.*

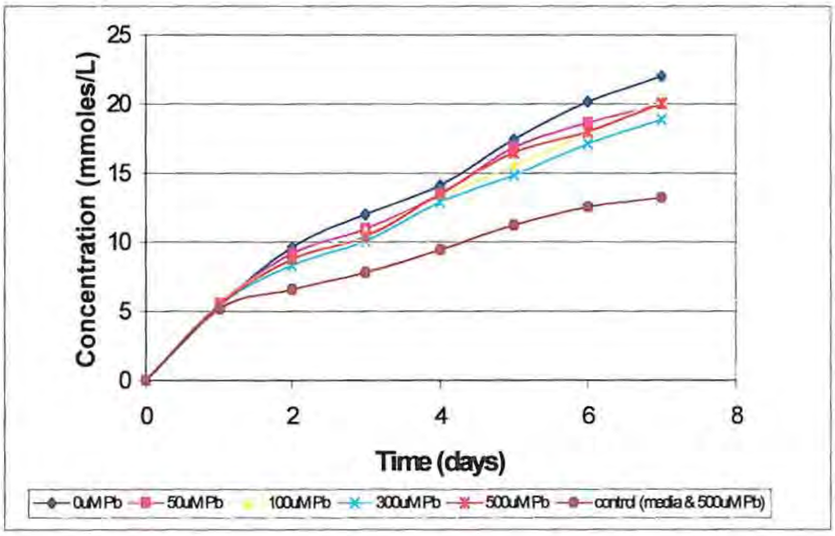


Figure 4.3: Carbonate alkalinity present in solution at varying concentrations of Pb

Another set of control flasks was prepared containing 300ml of full strength Zarrouks medium and the highest concentration of metal. These flasks demonstrated a spontaneous dehydration of HCO_3^- in the absence of algae. It is clear from both Figure 4.2 and 4.3 that spontaneous dehydration of bicarbonate occurs at a much slower rate than does active dehydration by the enzyme carbonic anhydrase present within the algal cell, resulting in a slower rate of carbonate formation in comparison with the control containing *Spirulina*.

4.3.3.2 Copper

The bicarbonate alkalinity usage is different for copper when compared to that with lead. Copper is known to precipitate out as a hydroxide and is not able to drive the dissociation of HCO_3^- (Chapter 7), as a result, the observed trend of bicarbonate utilisation is an expected one (Figure 4.4). From the graph it is evident, that the control flasks containing no added metal, utilised more bicarbonate from solution and that inorganic carbon utilisation was generally less with an increase in metal concentration. This may indicate that the copper added may have a significant effect on the enzyme carbonic anhydrase, causing a reduction in the enzymes ability to efficiently utilise the bicarbonate in solution.

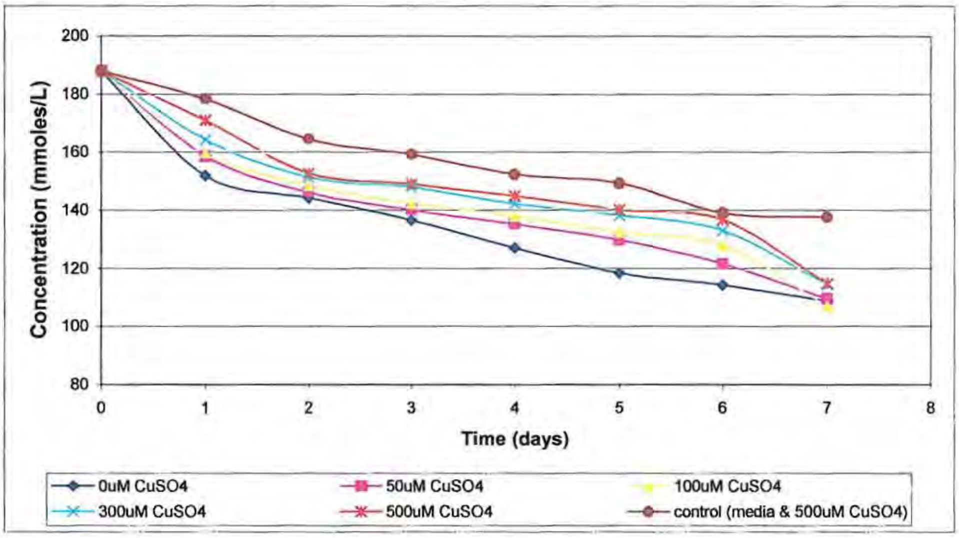


Figure 4.4: Bicarbonate alkalinity present in solution at varying concentrations of $CuSO_4$

From Figure 4.5, it is evident that there was a corresponding increase in carbonate production, with the control cultures generating more carbonate than cultures containing higher concentrations of metals.

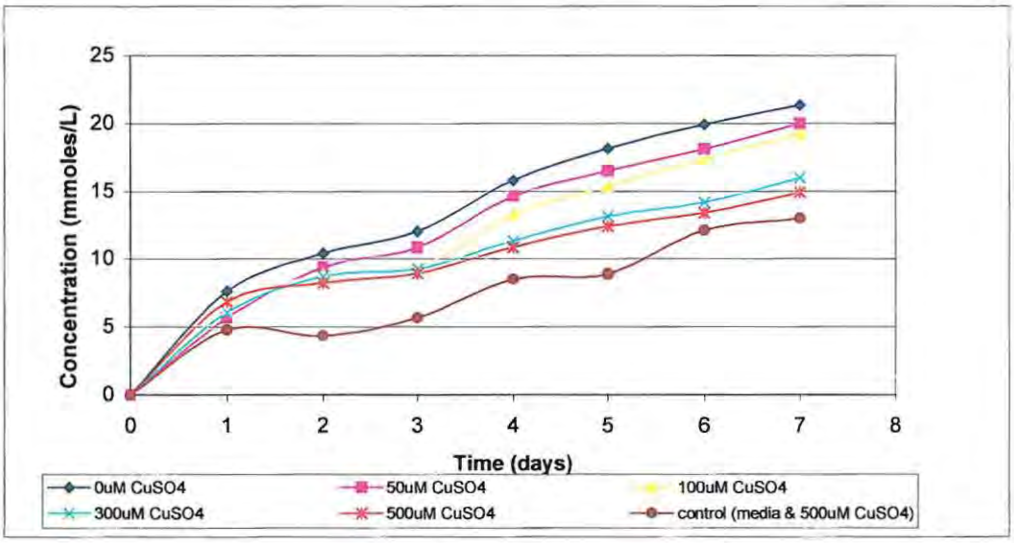


Figure 4.5: Carbonate alkalinity present in solution at varying $CuSO_4$ concentrations

4.3.3.3 Nickel

The bicarbonate usage in the various flasks containing a range of nickel concentrations (Figure 4.6) was very similar, which leads one to suspect that perhaps nickel did not have as significant an effect on the enzyme carbonic anhydrase. As a result the algae were still able to efficiently utilise the bicarbonate ions in solution. When considering the pH profile (Figure 4.7) of these cultures, it was evident that there wasn't a significant difference in the pH at varying metal concentrations, which is another indication that nickel does not appear to be affecting carbonic anhydrase. It is important to recap that carbonic anhydrase is the enzyme responsible for the dehydration of HCO_3^- to CO_2 and OH^- ions. This results in a decrease in acidity, as the resulting OH^- ions in solution are responsible for the alkalisation of the culture medium (ie. pH increase). So, if the nickel were to have an effect on the CCM, the bicarbonate utilisation would be greatest in the control flasks containing no metal and a corresponding increase in pH would be observed and the effect of the metal would be concentration dependent, with bicarbonate utilisation and pH being lower with increasing metal concentration.

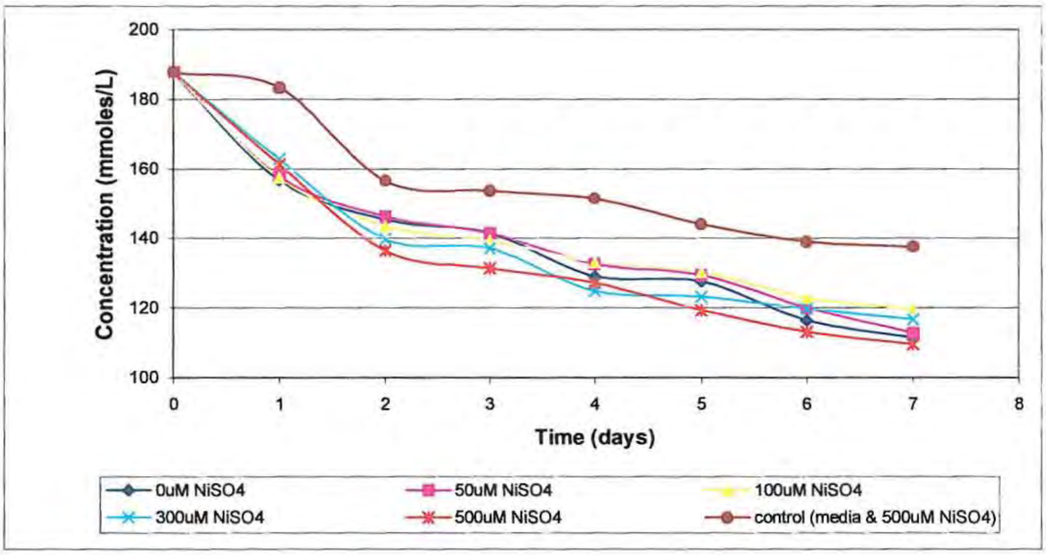


Figure 4.6: Bicarbonate alkalinity present in solution at varying $NiSO_4$ concentrations

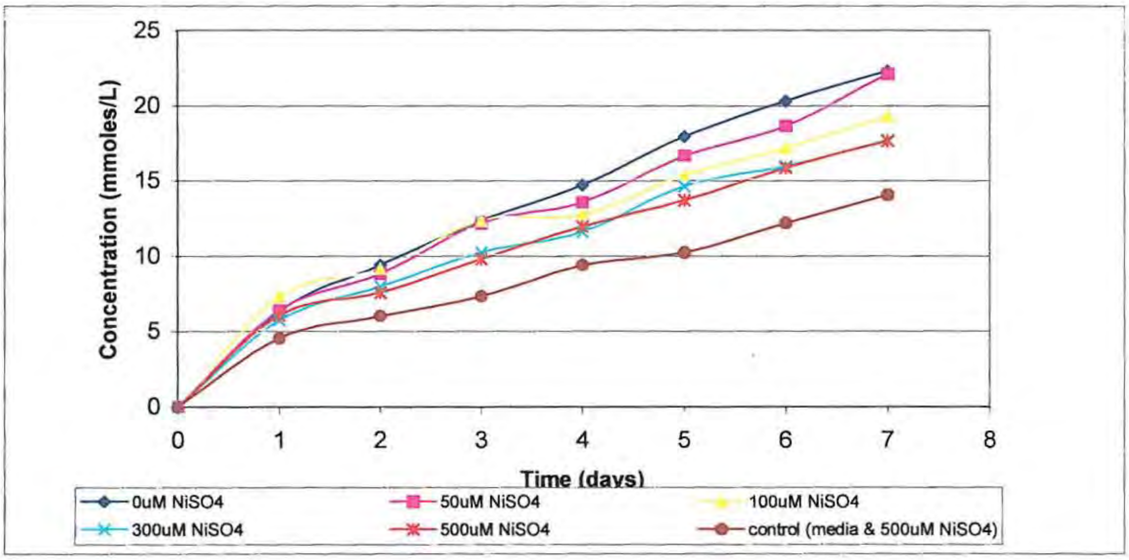


Figure 4.7: Carbonate alkalinity present in solution at varying $NiSO_4$ concentrations

Referring to Figure 4.7, it appears that the carbonate alkalinity is greater in the control flasks and decrease slightly with increasing metal concentration. Perhaps the nickel is binding to carbonate ions formed and precipitating out, resulting in a lower carbonate alkalinity at higher metal concentrations.

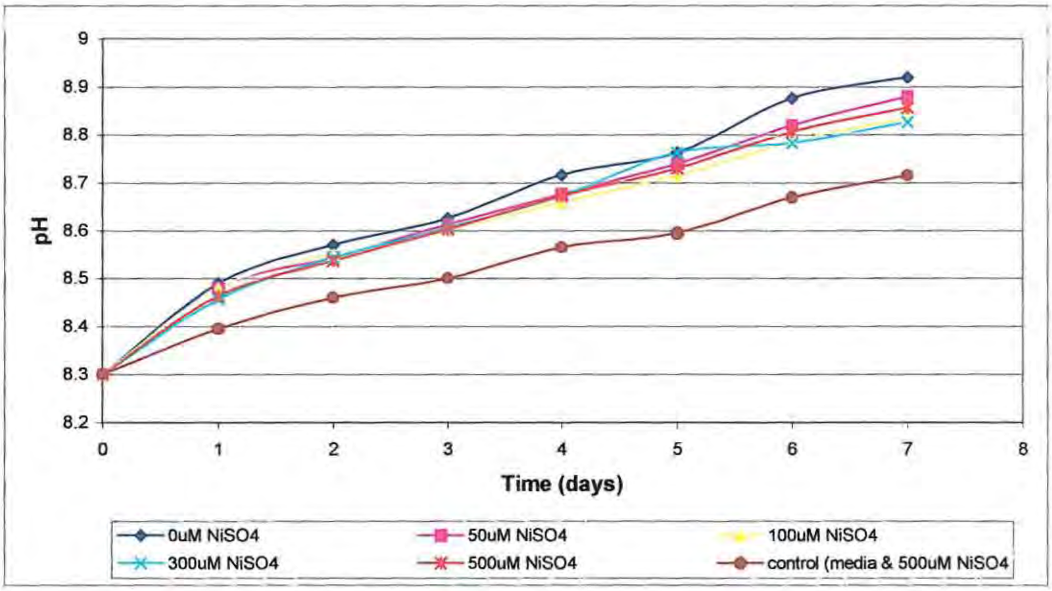


Figure 4.8: pH profile of the cultures at varying $NiSO_4$ concentrations.

It can clearly be seen in Figure 4.8 that there doesn't appear to be a significant difference in the pH of the cultures at varying concentrations of nickel.

4.3.4 Direct method of determining the effect of heavy metals on the enzyme carbonic anhydrase (Wilbur-Anderson assay method)

The quantitative determination of carbonic anhydrase activity has been a stimulating analytical target ever since the enzyme was discovered and first purified in 1933 (Botre & Botre, 1990). Since then, a great number of different analytical techniques have been devised in an attempt to determine the speed of the reactions catalysed by CA. Despite the interest in this enzyme, all of the means of assaying for activity have serious disadvantages.

Perhaps the most widely used and economically viable assay for CA is the Wilbur-Anderson method (Wilbur & Anderson, 1948). It measures the time needed for a pH change from 8.2 to 7.2 when CO_2-saturated water is injected into a reaction mixture that contains CA (Botre & Botre, 1990). The hydration of CO_2 liberates a proton, thus lowering the pH. A second method involves the use of a mass spectrometer and the catalysed isotopic exchange between CO_2 and H_2O is normally measured when one of these reactants is initially labelled with ^{18}O. This is a sensitive and accurate method but is slow and requires the dedicated use of a mass spectrometer. A third method, the stopped-flow, also involves the use of expensive spectrometers and equipment and measures the colour change of a pH sensitive dye. This method has many advantages but the equipment is not accessible to most labs. A few radiotracer assays have been developed, however these usually have long reaction times (in excess of 90 min). In this laboratory, Rosemary Payne (2000) attempted to develop these assays for use with *Spirulina*, but these attempts proved to be unsuccessful.

4.3.4.1 Commercially pure bovine CA

Initial studies using commercially pure bovine CA made up to known Wilbur-Anderson Units (WAU) showed that the assay was reasonably accurate (Table 4.1). Although it has been suggested that the method does not give a linear response over a broad enzyme range (Stemler, 1993), it was decided that the Wilbur-Anderson enzyme assay, would be used to determine the carbonic anhydrase activity of cultures exposed to varying concentrations of metals. An added advantage is that the method is inexpensive when assaying large numbers of samples.

Table 4.1: A comparison between the expected and observed CA activity units from commercially pure bovine carbonic anhydrase

EXPECTED WAU	OBSERVED WAU	DIFFERENCE
1	0.878	-0.122
2	2.603	0.603
3	4.517	1.517
4	5.963	1.963
5	7.042	2.042
6	6.049	0.049
7	7.718	0.718
10	10.420	0.420
15	19.035	4.035

4.3.4.2 Lead

Wilbur-Anderson enzyme assays were performed on each flask on a daily basis. It was evident that the enzyme activity of the cultures in the control flasks were much higher than those which had been exposed to lead as no or little activity was detected in these cultures after day 3 (Figure 4.2). A decrease in the activity of the control flasks was observed over time, which was not really expected, as under low CO_2 conditions the algae are known to induce the expression of CA as part of their carbon concentrating mechanism (CCM), to maintain efficient photosynthesis (Table 4.2).

Table 4.2: The Wilbur-Anderson assay was used to determine the carbonic anhydrase activity of *Spirulina* cultures at varying concentrations of lead

Metal Concentration (μM)	Enzyme activity (WAU/mg chla)		Rate of change in enzyme activity
	DAY 1	**DAY 7**	
0	16.617	5.867	-1.5965
50	5.167	0	-0.985
100	4.645	0	-0.7982
300	4.225	3.285	-0.822
500	5.796	3.943	-0.9967

4.3.4.3 Copper

The carbonic anhydrase activity assays proved to be very erratic when trying to determine the effect of varying concentrations of copper on the enzyme. The only significant trends which were observed was that again the control cultures appeared to have a greater initial activity than the flasks containing copper, but this activity was found to decrease over time (Table 4.3).

Table 4.3: The Wilbur-Anderson assay was used to determine the carbonic anhydrase activity of *Spirulina* cultures at varying concentrations of copper

Metal Concentration (μM)	Enzyme activity (WAU/mg chla)		Rate of change in enzyme activity
	DAY 1	**DAY 7**	
0	7.701	6.494	-0.9883
50	5.482	5.036	-0.4059
100	4.629	5.074	-0.0234
300	5.513	1.576	-0.4678
500	5.786	0	-0.1775

The activity results for the flasks exposed to 300μM and 500μM copper, did indicate a decrease in CA activity from day 2, with very little activity occurring on day 7. Other than those mentioned, no significant trends were observed.

4.3.4.4 Nickel

Table 4.4: The Wilbur-Anderson assay was used to determine the carbonic anhydrase activity of *Spirulina* cultures at varying concentrations of nickel

Metal Concentration (μM)	Enzyme activity (WAU/mg chla)		Rate of change in enzyme activity
	DAY 1	**DAY 7**	
0	8.150	5.650	-0.4988
50	6.802	0	-0.5164
100	4.802	0	-0.4531
300	4.135	0	-0.9975
500	5.204	0	-0.4932

On the first day, the activity observed in all the flasks ranged from 4 to 8 WAU/mg chla, with the highest activity occurring in the control flasks and the lower activities observed in the flasks containing the highest metal concentrations (Table 4.4). By day 7, no activity was detected in the flasks containing nickel. Compared to the control flasks for lead and copper, the nickel control cultures appeared to maintain a reasonably stable activity over the period of study, this can be seen by the very low rate of change in enzyme activity (-0.4988). At all nickel concentrations, quite a drastic decrease in carbonic anhydrase activity was observed from day 4 onwards.

From the activity results above, a few similar trends were evident for all three metals investigated. Firstly, the control cultures demonstrated a higher activity than the cultures exposed to varying concentrations of metal. Secondly, even though the control cultures showed higher activities than those of the experimental flasks, a decrease was observed over the period of study (this is indicated by the rate of change in enzyme activity), this was not expected as the expression of carbonic anhydrase is induced when low CO_2 conditions are experienced. Thirdly, no or very little enzyme activity was detected from about day 3 in the flasks containing 300μM and 500μM metal. However, the Wilbur-Anderson assay, did not prove to be a reliable method of measuring changes in CA activity.

It has been mentioned that all the methods used to determine carbonic anhydrase activity have some disadvantages, which have strongly limited there use for adequate reproduction of a physicochemical model system of the physiological conditions under which the enzyme has been observed to operate. Stemler (1993) highlighted some of the disadvantages observed when using the Wilbur-Anderson assay method. Firstly, the assay requires nonphysiological conditions and is performed at temperatures below 4°C. Secondly he found that the method did not give a linear response over a broad enzyme range. And most importantly, the method was found incapable of accurately measuring small differences in activity (Stemler, 1993). As a result it was decided that perhaps an oxygen electrode could be used as an alternative and more reliable method for assaying CA enzyme activity. This is described in the next chapter.

4.3.5 The effect of heavy metals on cell morphology

To investigate the effect of heavy metals on cell morphology, light microscopy and scanning electron microscopy (SEM) was performed on control cultures and cultures containing the highest metal concentration on Day 0 and 7.

Unfortunately, light microscopy of the algal cells did not really demonstrate clearly an effect of any of the metals on the external morphology of the cells, probably as a result of low magnification. However, it was observed that there were more, shorter fragments in the flasks containing metals than the control flasks. In some cases a slight loss in chlorophyll could be seen in some of the cells, but otherwise, the photographs didn't particularly indicate any significant effect.

Due to the higher magnification one can achieve using SEM, this method was found to be more reliable in identifying the effect of the metals on the external morphology of the cells.

4.3.5.1 Lead

Referring to Figure 4.9, which is a SEM photograph of *Spirulina* grown in the absence of lead and comparing this with Figure 4.10, which is a SEM preparation of *Spirulina* exposed to 500μM $Pb(NO_3)_2$, it can be clearly seen that the lead has caused degradation of the cells and almost complete disintegration of the filaments. In some areas complete distortion of segments has occurred, this could possibly be due to the lead affecting the permeability of the plasmalemma, causing loss of K^+ ions from the cell and changing the cell volume and turgor pressure within the cell. The control cultures did not demonstrate any of the above symptoms, the filaments were found to be intact and no distortion or degradation of the cells occurred.

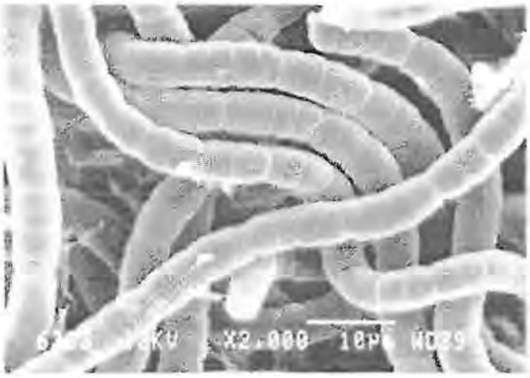


Figure 4.9: SEM photograph of a control culture of *Spirulina*, which was not exposed to $Pb(NO_3)_2$ after 7 days

Figure 4.10: A SEM preparation of *Spirulina* exposed to 500μM $Pb(NO_3)_2$ for a week

4.3.5.2 Copper

Figure 4.11 is a SEM photograph of *Spirulina*, which has not been exposed to any copper and Figure 4.12 is a preparation of the algae exposed for 7 days in 500μM $CuSO_4$. The control cultures in Figure 4.11 show no signs of disintegration of the filaments, as is observed in Figure 4.12. It is interesting to note how severely the copper has affected the cells in Figure 4.12, where there were very few, if any, intact filaments. At a higher magnification in Figure 4.13, it was evident how the copper appeared to affect the permeability of the cell, resulting in a change in cell volume and turgor pressure, causing the wrinkled effect observed.

McBrien& Hassall (1965) and Terry & Stone (2002) have documented in their research that copper affects the permeability of the plasmalemma, causing a loss of K^+ from the cell and this is probably responsible for the change in morphology of the cells. Once copper is transported into the cells, it inhibits photosynthesis by uncoupling electron transport to $NADP^+$ (Stauber & Florence, 1987). Fathi & El-Shahed (2000) found that copper severely reduced the growth, chlorophyll *a*, protein, sugar and amino acid contents in *Scenedesmus bijuga* in a concentration dependent manner. This reduced sugars content was thought to have contributed to suppression of protein accumulation by a shortage of carbon skeletons. Due to the inhibition of photosynthesis, production of essential components is reduced and this is likely to be responsible for the disintegration of the algal filaments in Figure 4.12 and 4.13 as less carbohydrates (Pace *et al.*, 1977) and proteins are being invested into the cell walls.

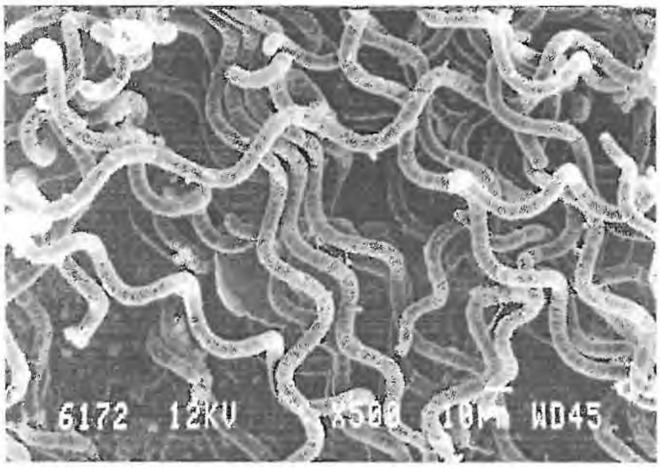


Figure 4.11: SEM photograph of a control culture of *Spirulina*, which was not exposed to $CuSO_4$ after 7 days

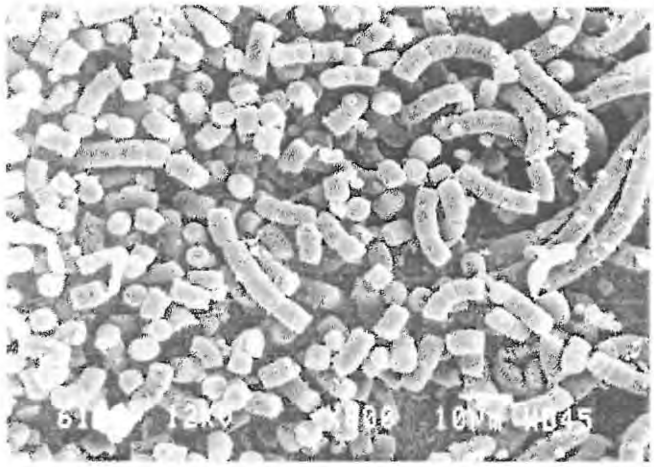


Figure 4.12: A SEM preparation of *Spirulina* exposed to 500μM $CuSO_4$ for a week

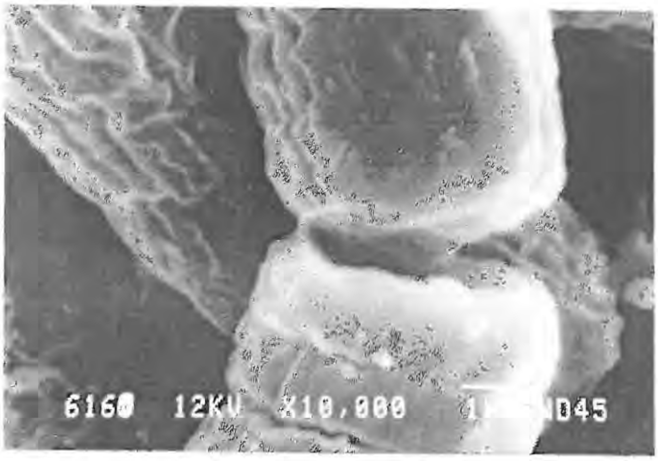


Figure 4.13: A higher magnification of the wrinkled exterior of *Spirulina* filaments after been exposed to $CuSO_4$ for a week.

4.3.5.3 Nickel

A SEM photograph of the control cultures (Figure 4.14) again showed that the cultures were still healthy and filaments were intact after 7 days. When comparing this photograph with that of Figure 4.15, it was evident that the density of the culture was less than that of the control after a week's exposure. And the filaments of the algae exposed to 500μM $NiSO_4$ demonstrated similar physiological responses to those observed when cultures were exposed to lead and copper.

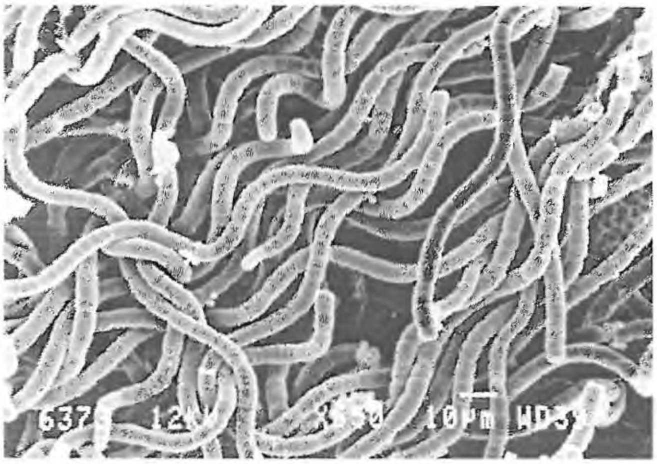


Figure 4.14: A SEM preparation of *Spirulina,* which was not exposed to any nickel during the period of study

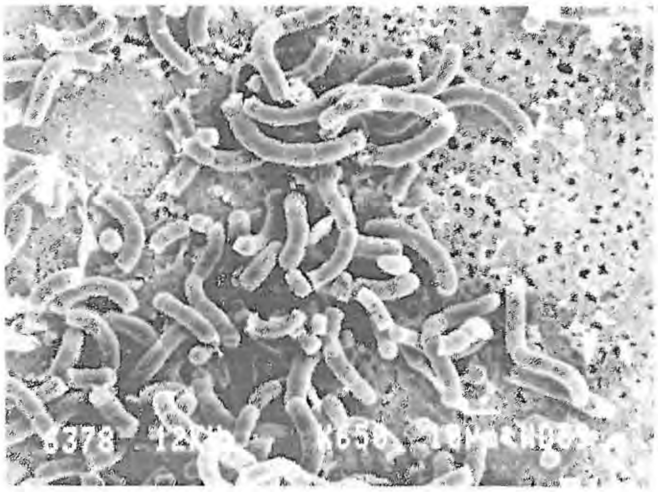


Figure 4.15: A SEM preparation of *Spirulina* exposed to 500μM nickel for a week

It is evident from the results presented in this chapter that the exposure of *Spirulina* to heavy metals such as lead, copper and nickel (which are commonly found in AMD waters), appeared to have significant effects on cell morphology, photosynthesis (due to the reduction in photosynthetic pigments), carbonate speciation of the medium and more importantly the activity of the enzyme carbonic anhydrase, which is an integral component of the carbon concentrating mechanism.

CHAPTER FIVE

MEASUREMENT OF THE EFFICIENCY OF PHOTOSYNTHETIC OXYGEN EVOLUTION UNDER VARIOUS CONDITIONS USING A RANK OXYGEN ELECTRODE

5.1 Introduction

Oxygen-evolving photosynthesis is the global process where light is absorbed by photosynthetic pigments and converted into chemical energy in plants, algae and cyanobacteria. Photosynthetic water oxidation takes place in photosystem II (PSII), a large multisubunit protein complex, located in the thylakoid membranes of chloroplasts and cyanobacteria, which functions as a light-driven water-plastoquinone oxidoreductase (Villarejo *et al.*, 2002). Despite significant progress in the biochemical, biophysical and structural characterisation of PSII (Nield *et al.*, 2000; Zouni *et al.*, 2001) details of water oxidation remain largely unknown (Renger, 2001). Oxygen evolution is catalysed by the water-oxidising complex (WOC), a manganese cluster and PSII extrinsic proteins on the lumenal side of thylakoid membranes (Villarejo *et al.*, 2002). The electrons and protons released from the water oxidation are transported in a vectorial manner and used to reduce NADP and to generate ATP that reduces CO_2 to simple sugars.

However, CO_2 (or its ionic species bicarbonate) is not only involved in photosynthetic reactions as the ultimate electron acceptor but also participates in the regulation of the photochemical reactions (Villarejo *et al.*, 2002). More than 40 years ago, Warburg and Krippahl (1958) discovered that bicarbonate could stimulate electron flow in the Hill reaction, but ever since then the effect of bicarbonate on PSII activity has been a matter of debate among the scientific community (Van Rensen *et al.*, 1999). The requirement for bicarbonate at the acceptor side of PSII is now well established (Van Rensen *et al.*, 1999). However, the bicarbonate stimulation of PSII activity on the donor side still remains controversial and has yet to be fully elucidated (Klimov and Baranov, 2001).

Carbonic anhydrase (CA) is a zinc metalloenzyme, accelerating the interconversion of CO_2 and HCO_3^- by a factor of $>10^6$. It was proposed earlier that a thylakoid CA might in some way be involved in the bicarbonate effect in PSII (Moubarak-Milad & Stemler, 1994).

Results presented by Villarejo *et al.* (2002) provided for the first time, strong evidence that the novel carbonic anhydrase (Cah3), identified in the thylakoid lumen of *Chlamydomonas reinhardtii* (Karlsson *et al.*, 1998), was required for the optimal function of the WOC. It was found that, in the absence of this CA activity, the manganese cluster became unstable and the electron donation side of PSII became impaired (Villarejo *et al.*, 2002). It was therefore concluded, that the catalysed interconversion between the inorganic carbon species at the donor side of PSII is essential for the optimal function of the WOC.

Photosynthesis cannot easily be followed by infra-red gas analysis. $^{14}CO_2$ fixation can provide invaluable information about products, but it does not lend itself to continuous measurement. For these reasons, routine measurements of photosynthesis are usually carried out with an oxygen electrode, which can easily detect changes of the order of 0.01μmol in the oxygen content of a suspension.

An oxygen electrode (Figure 5.1) is a special form of electrochemical cell, in which a current is generated that is proportional to the activity of oxygen present in a solution. In principle, the electrode consists of two wires, one made of platinum and one of silver coated with silver chloride. These wires dip into a solution of electrolyte, e.g. KCl. An electrical potential is applied across the wires, with the platinum electrode being made negative with respect to the silver, causing the following reactions:

At the silver electrode:

$4\ Ag \longrightarrow 4\ Ag^+ + 4e^-$

At the platinum electrode:

$O_2 + 2e^- + 2H^+ \longrightarrow H_2O_2$

$H_2O_2 + 2e^- + 2H \longrightarrow 2H_2O$

If the wires are connected to a battery, a current will flow from the silver to the platinum. Under appropriate conditions, the magnitude of the current will be linearly proportional to the oxygen activity in solution. If no oxygen is present, the reaction at the platinum electrode will not occur and no current should flow (Walker, 1991).

The platinum and reference electrodes are shielded from the solution by a thin teflon membrane which is permeable to oxygen. The membrane is kept in place by a rubber "O" ring. The platinum cathode consumes oxygen and to maintain a stable oxygen gradient across the membrane, the solution needs to be stirred with a small magnetic stirrer bar.

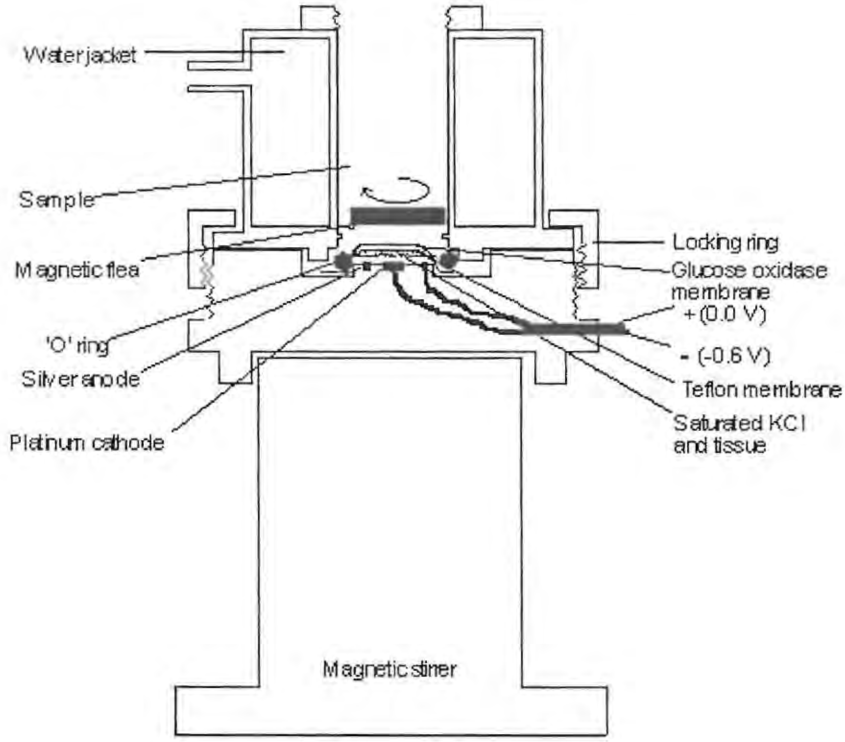


Figure 5.1: The oxygen electrode unit is a high precision laboratory instrument ideally suited for measuring the kinetics of oxygen uptake or evolution (http://www.sbu.ac.uk)

5.2 Motivation and Aims

Algae differ from the other groups of small or microscopic organisms, in that they possess an internal green pigment called chlorophyll, sometimes hidden or partially masked by other pigments. Chlorophyll enables them in the presence of sunlight to combine water and carbon dioxide to form starch or related substances, and to release oxygen into the water (Palmer, 1980). In algae and other green plants, the rate of photosynthesis is normally faster than that of respiration, therefore more oxygen is released than used and they absorb more carbon dioxide than they release.

The algae make possible important chemical changes and metabolic activities in the water through their release of oxygen during daylight hours. The oxygen released by the algae into the water is then utilised by fish, crustaceans and insects for respiration. Oxygen release by algae and O_2 uptake by reaeration are the two primary sources for renewal of O_2 in flowing streams and turbulent water (Palmer, 1980).

The major focus of this chapter is to determine if an oxygen electrode can be successfully used to determine the efficiency of photosynthetic oxygen evolution in *Spirulina* under various conditions and to establish if this method is a possible alternative and more reliable method, albeit it an indirect one, for determining the effects of certain parameters on carbonic anhydrase activity and the CCM.

5.3 Results and discussion

5.3.1 Determination of the response time of an oxygen electrode

The 90% response time of an oxygen electrode is defined as the length of time required for the electrode to make a 90% response to an instantaneous change in oxygen tension in the solution. There are a number of factors, which may affect the response time, namely the thinness and composition of the membrane, the size of the cathode and the rate of stirring.

The response time curve was determined by switching off the stirrer and then switching it on again once the recorder output had fallen, and determining the time taken to recover 90% of the former signal. The response time of this oxygen electrode was found to be approximately 40 seconds (Figure 5.2).

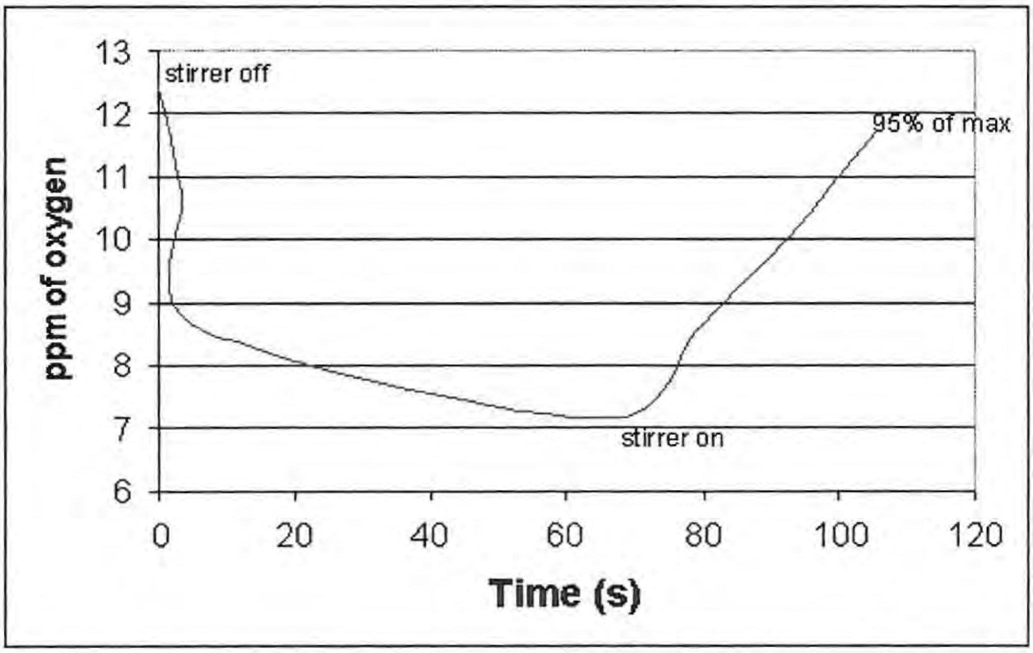


Figure 5.2: The response time curve of the oxygen electrode

The electrode output falls when the stirrer is switched off because oxygen diffusion across the membrane is not fast enough to balance the oxygen consumption at the cathode. When the stirrer was switched on again, the time taken to recover 90% of the former signal was found to be about 40 seconds. Walker (1991) reported that using modern electrode designs and a 0.5-1.0 mm cathode, a response time of 1-10 seconds is normal at 25°C. The membrane used for these experiments was a fresh membrane, so it is not evident why the response time is so much longer than that suggested by Walker. Thinner membranes are known to give increased response speeds, so perhaps the teflon membrane used was thicker than average.

5.3.2 Determination of the effect of varying light intensities on oxygen evolution

Light is one of the major limiting environmental factors affecting algal growth. For the practical purposes of growing algal biomass, the outdoor light problems are twofold: (1) either there is not enough light or (2) there is so much light under certain conditions that the algal cells are damaged by the light.

Insufficient light usually causes a decrease in algal biomass yields due to lower photosynthetic rates, but this seldom causes cell damage unless the culture is so thick that some of the cells are never exposed to the light. On the other hand, photooxidation and photoinhibition resulting from the high light intensities create far worse problems due to damaged cells. Photoinhibition or photoinactivation causes an impairment of photosynthetic processes which causes decreased algal growth yield or sometimes death of the entire biomass. Photoinhibition occurs because supraoptimal intensities of light cause destruction of the photosynthetic pigments as the result of an oxygen- and light-dependent bleaching (photooxidation) of cells.

Spirulina was exposed to seven different photon fluxes or light intensities (ranging from 67.10 to 192.7 μmol.m^{-2}s^{-1}) which correlated to a specific distance the light source was placed from the chamber (ranging from 19.5 to 2.5cm away). It appears from Figure 5.3, that the saturating light for photosynthesis was found to be 168.4 μmol.$m^{-2}s^{-1}$, which correlates to 5.5cm away from the chamber. The oxygen evolution rate was found to be less at higher light intensities, probably due to photoinhibition of the cells. At lower light intensities, the rate of oxygen evolution dropped significantly as insufficient light caused a reduction in photosynthetic rates.

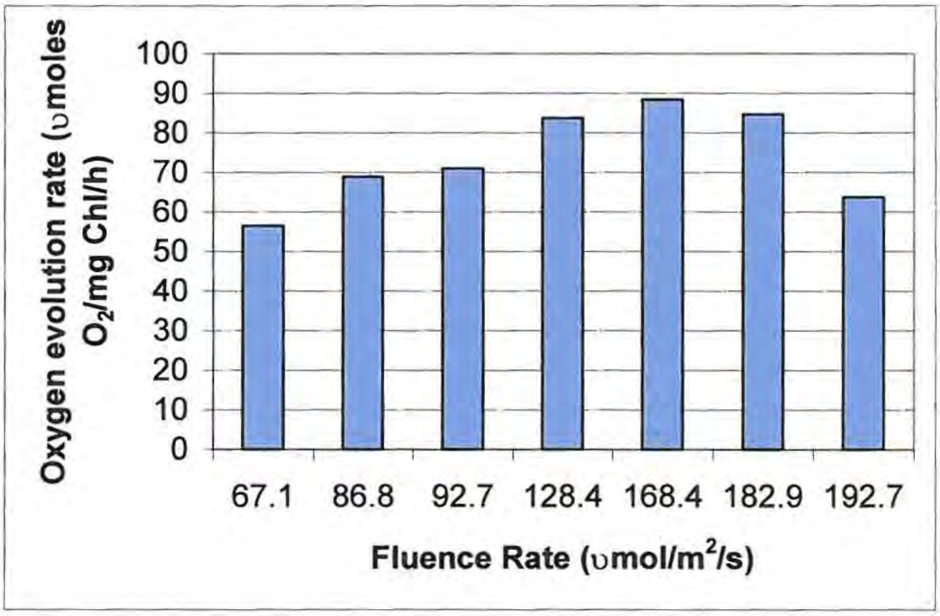


Figure 5.3: Nett photosynthesis measured as O_2 evolution rate in *Spirulina* illuminated at different light intensities

5.3.3 Assessment of the spontaneous dehydration of bicarbonate ions in the absence of *Spirulina*

From *in vitro* kinetic studies performed by Badger (1980) on cyanobacterial RuBisCO, it was determined that virtually no CO_2 fixation would occur if the intracellular CO_2

concentrations were as low as that used in most experimental solutions. It thus became obvious that cyanobacteria must possess the means of raising the CO_2 concentration around the active site of RuBisCO considerably above that in the extracellular medium. This was because the observed rates of photosynthesis at alkaline pH were significantly higher than could be supported by the spontaneous conversion of HCO_3^- into CO_2 and OH^- in the external solution.

The intention of the present experiments was to observe if there was a reduction or cessation in oxygen evolution in the absence of *Spirulina*. Oxygen evolution studies were therefore performed using varying concentrations of bicarbonate (0-4000μM) in the presence and absence of the algae.

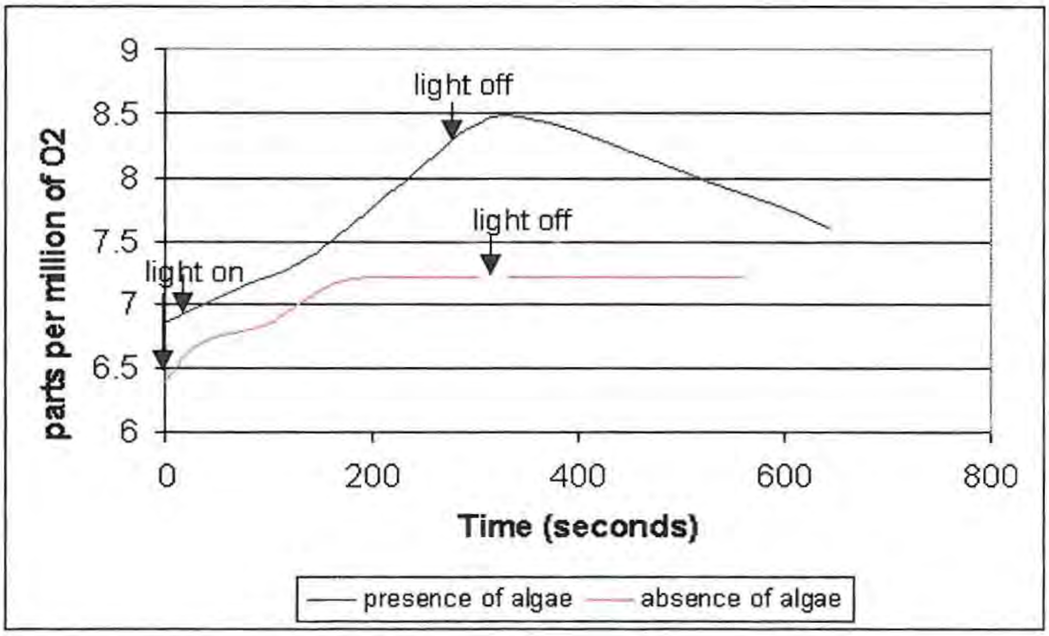


Figure 5.4: Oxygen electrode tracing demonstrating the difference between oxygen evolution in the presence and absence of *Spirulina*. The light was initially switched on for approximately 5 minutes and then switched off to determine the effect on oxygen evolution.

Figure 5.4 clearly indicates that the absence of *Spirulina* in the media, results in the cessation of oxygen evolution during the period of study. Identical graphs were obtained for all concentrations of bicarbonate used during the oxygen evolution experiments. These observations were as a result of there being no algae to utilise the bicarbonate provided in solution for photosynthesis, therefore very little or no oxygen was being evolved.

A reduction in the rate of oxygen evolution occurs if the light source is switched off in the presence of algae, as they begin to utilise the oxygen in solution for respiration. Switching off of the light source was found to have no effect on the rate of oxygen evolution in the absence of *Spirulina*.

5.3.4 Rates of photosynthetic oxygen evolution as a function of external bicarbonate concentration in the absence and presence of bovine carbonic anhydrase

It has already been discussed that carbonic anhydrase is required in living organisms, because the spontaneous interconversion between CO_2 and HCO_3^- is small compared with the flux rates in various physiological processes. Badger and Price (1994) found that CO_2 fixation by some photosynthetically active organisms were some 10^4 times faster than the uncatalysed rate of CO_2 formation from HCO_3^-. Therefore, the conversion of HCO_3^- to CO_2 has been thought to be the physiological role of CA in plants.

Bozzo *et al.* (2000) found that when CA (10μg/ml) was added to a sample of *Chlorella kessleri*, the oxygen evolution rate was 1.5-fold greater than the control samples. This indicated active CO_2 transport, since the addition of excess CA maintains the CO_2 supply available to the cells. Therefore, oxygen evolution studies were carried out at varying bicarbonate concentrations in the presence and absence of 10μg/ml bovine carbonic anhydrase to determine if the addition of the enzyme had any significant effect on the evolution of oxygen in *Spirulina* (Figure 5.5).

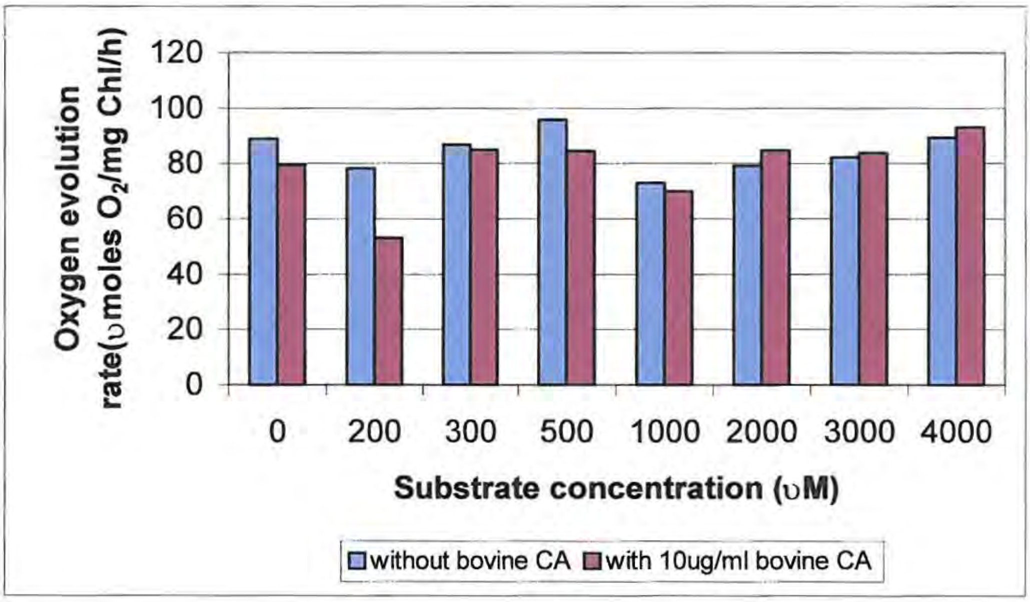


Figure 5.5: Photosynthetic rates of oxygen evolution as a function of external bicarbonate concentration in the absence and presence of 10μg/ml bovine carbonic anhydrase

Unlike the results obtained by Bozzo *et al.* (2000), it appears from Figure 5.5 that the addition of bovine carbonic anhydrase had no significant effect on the rate of oxygen evolution when compared with the controls. However, the rate of oxygen evolution was found to be lower in the presence of carbonic anhydrase at the lower bicarbonate concentrations and higher at higher bicarbonate concentrations. Perhaps, this is due to the increased dehydration of bicarbonate ions at higher substrate concentrations by bovine CA.

5.3.5 The effect of carbonic anhydrase inhibitors on photosynthetic oxygen evolution

5.3.5.1 Acetazolamide

Results from the present study indicate in Figure 5.6a, that the rate of oxygen evolution in the control experiment is very similar (note the scale on the graph) under all conditions. A similar pattern of oxygen evolution was observed at all AZ concentrations (Figure 5.6b-f), the control samples were found to be higher than the experimental samples incubated with the enzyme acetazolamide. On the addition of 2mM $NaHCO_3$, a drastic increase in the rate of oxygen evolution was observed.

At 100μM AZ, complete inhibition of photosynthesis appeared to occur, with no oxygen being evolved. These results suggest that AZ inhibits the enzyme carbonic anhydrase, responsible for the dehydration of HCO_3^- to CO_2 and OH^-, resulting in the algae's inability to photosynthesise, due to a lack in inorganic carbon accumulation during periods of CO_2 limitation.

If HCO_3^- is taken up by a plant or algae, then it must be converted to CO_2, which is in turn taken up by the cell for photosynthesis, and OH^-, which remain in the medium. The resulting OH^- ions in solution are responsible for the alkalisation (i.e. pH increase) of the culture medium (Raven, 1995; Shiraiwa *et al.*, 1993; van Hille *et al.*, 1999). The enzyme, CA, is responsible for this reaction.

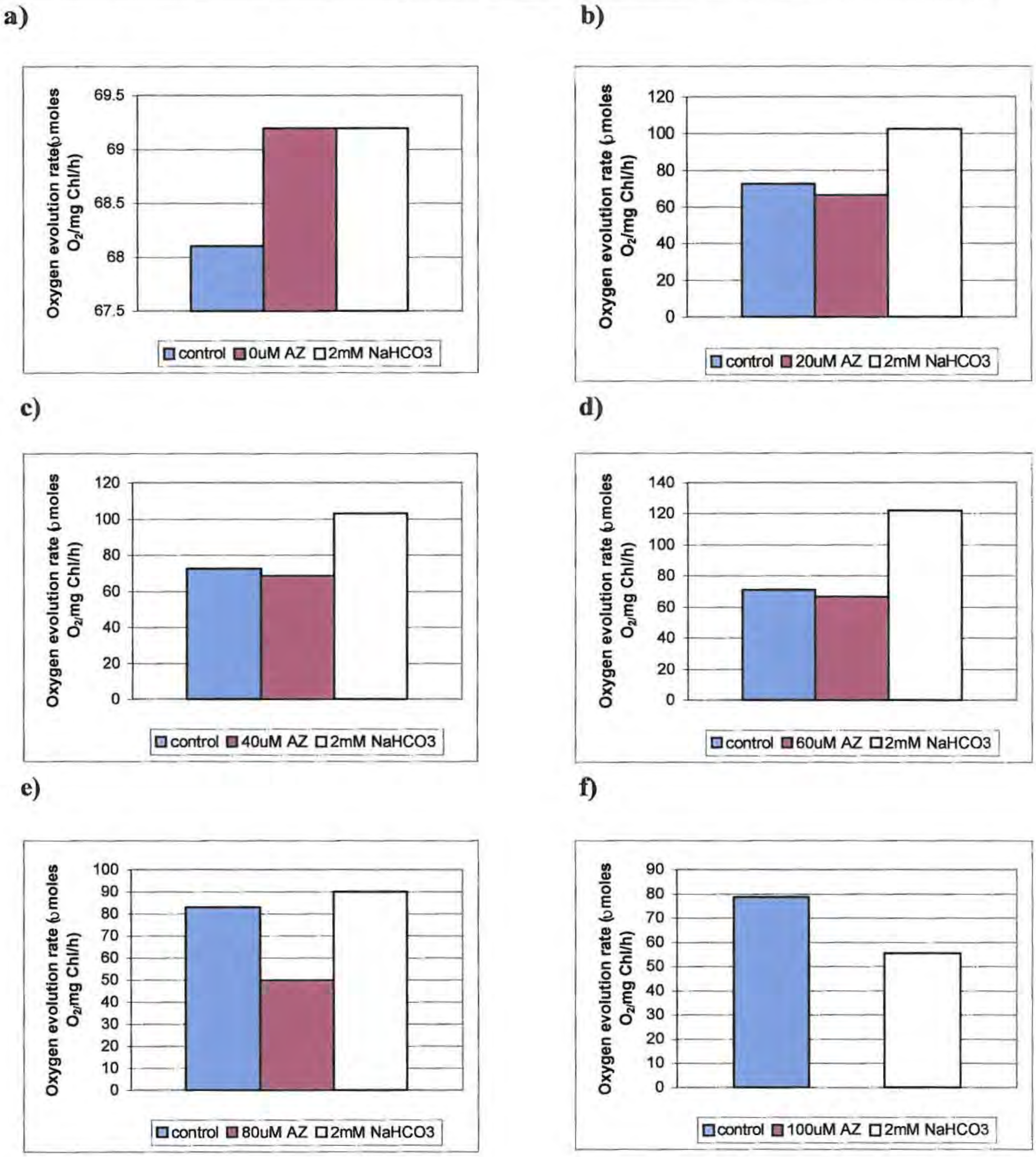


Figure 5.6: The rate of oxygen evolution observed when varying concentrations of the inhibitor, acetazolamide, was incubated with *Spirulina*: a) 0µM; b) 20µM; c) 40µM; d) 60µM; e) 80µM and f) 100µM. Control experiments were carried out at each concentration and the rate of oxygen evolution was determined in the absence of AZ. 2mM $NaHCO_3$ was then added to the chamber to see if the addition of a high concentration of the substrate resulted in an increase in oxygen evolution.

Previous experiments performed (Figure 5.7), substantiate that AZ affects the algae's ability to photosynthesise and is able to inhibit the alkalisation of the medium (due to the inability of the algae to utilise inorganic carbon) at a concentration of 50μM as compared to the control experiments. These results are consistent with those observed in literature, which have found that alkalisation of the medium was almost completely inhibited by 50μM AZ, with complete inhibition occurring at 100μM (Moroney *et al.*, 1987; Sultemeyer *et al.*, 1989; Sultemeyer *et al.*, 1990; Williams & Turpin, 1987).

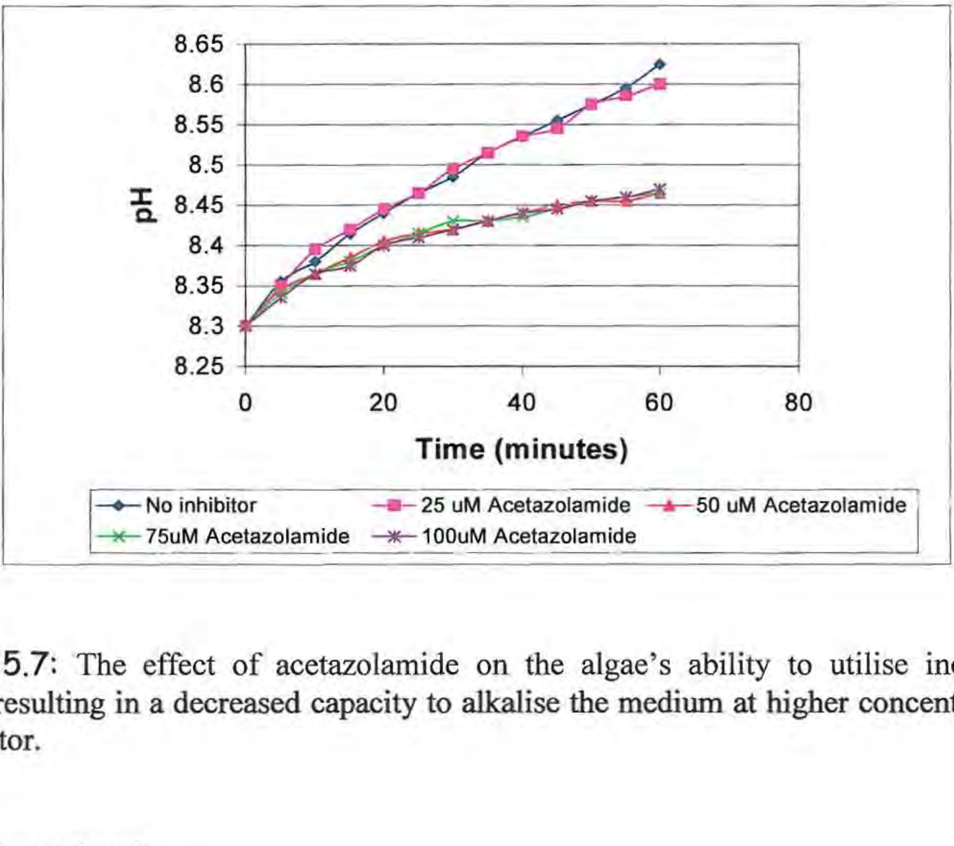


Figure 5.7: The effect of acetazolamide on the algae's ability to utilise inorganic carbon, resulting in a decreased capacity to alkalise the medium at higher concentrations of inhibitor.

5.3.5.2 Ethoxyzolamide

In *C. reinhardtii*, the physiological importance of internal CA has been concluded from the response of photosynthesis to ethoxyzolamide (EZ), a CA inhibitor, which penetrates the plasma membrane. Ethoxyzolamide causes a decrease in the affinity for external C_i

and an over-accumulation of internal C_i, indicating that cells were unable to use the intracellular C_i pool (Amoroso *et al.*, 1996).

A similar pattern was observed in this study when the rate of O_2 evolution was determined in the presence of the membrane-permeable inhibitor, ethoxyzolamide (EZ). Again it was found that the trend in oxygen evolution was similar at all EZ concentrations (Figure 5.8b-f), with the concentration of oxygen being slightly greater in the control samples than those incubated with EZ. When 2mM $NaHCO_3$ was added to the chamber, the rate of oxygen evolution did not appear to increase significantly above that observed at lower EZ concentrations (i.e. 20 and 40μM), but quite a drastic increase in evolved oxygen was observed at the higher EZ concentrations.

From Figure 5.8f, it appears that complete inhibition of photosynthesis occurred at 100μM EZ as no oxygen was evolved at the higher inhibitor concentration. The results obtained for EZ were almost identical to those observed for AZ. As discussed before, the reason for this is that EZ inhibits the enzyme CA, which is responsible for converting HCO_3^- to CO_2, thereby affecting the algae's ability to photosynthesise at low inorganic carbon concentrations.

Interestingly, it was found that the amount of oxygen evolved after the addition of 2mM $NaHCO_3$ was generally greater in the experiments where AZ was used and the rate of oxygen evolution was less when EZ was used. It appears that EZ had a more significant effect on oxygen evolution than AZ, probably due to the fact that AZ is an impermeable inhibitor and is therefore thought to only affect external or membrane-bound CAs, whereas EZ penetrates into the cell, inhibiting both internal and external CAs (Pollock & Colman, 2001). This means that the amount of CO_2 converted from HCO_3^- is greatly reduced in the presence of EZ, resulting in inefficient photosynthesis and reduced oxygen evolution.

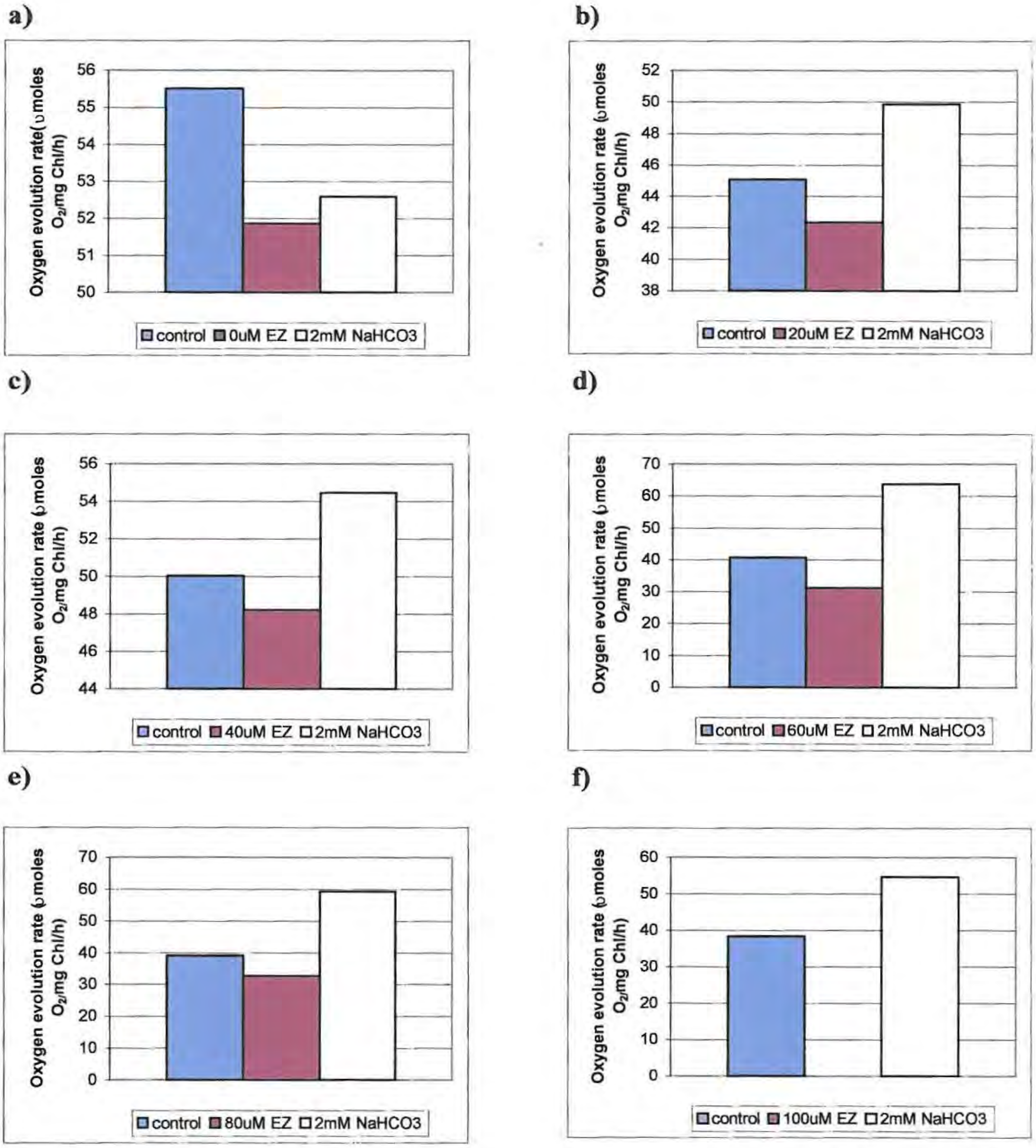


Figure 5.8: The rate of oxygen evolution observed when varying concentrations of the inhibitor, ethoxyzolamide, was incubated with *Spirulina*: a) 0µM; b) 20µM; c) 40µM; d) 60µM; e) 80µM and f) 100µM. Control experiments were carried out at each concentration and the rate of oxygen evolution was determined in the absence of EZ. 2mM $NaHCO_3$ was then added to the chamber to see if the addition of a high concentration of the substrate resulted in an increase in oxygen evolution.

Several attempts have been made to explain the function of CA in algae but the experimental evidence is rather scarce. As *Spirulina* are autotrophs they are dependent on the import of inorganic carbon into the cells to be used in the photosynthetic pathway. In plants, which are constantly exposed to air, carbon is freely available as atmospheric carbon dioxide (CO_2). In the presence of low levels of dissolved CO_2, the algae are able to employ an inorganic carbon accumulating mechanism. The induction of this mechanism is primarily dependent on light and a reduction in the levels of inorganic carbon.

Moroney *et al.* (1985) studied the effect of carbonic anhydrase inhibitors on inorganic carbon accumulation by *C. reinhardtii*. They used membrane-permeable and impermeable inhibitors of carbonic anhydrase to assess the roles of extracellular and intracellular carbonic anhydrase on the inorganic carbon concentrating system in *C. reinhardtii*.

Acetazolamide (AZ), ethoxzolamide (EZ) and a membrane-impermeable, dextran bound sulfonamide were found to be potent inhibitors of extracellular carbonic anhydrase measured with intact cells. At pH 5.1 however, where CO_2 is the predominant species of inorganic carbon, both AZ and the dextran-bound sulphonamide had no effect on the concentration of CO_2 required for the half-maximal rate of photosynthetic O_2 evolution or inorganic carbon accumulation (Moroney *et al.*, 1985).

These results are consistent with the hypothesis that CO_2 is the species of inorganic carbon which crosses the plasmalemma and that extracellular carbonic anhydrase is required to replenish CO_2 from HCO_3^- at high pH. The data obtained also implicates a role for intracellular carbonic anhydrase in the inorganic carbon accumulating system, and indicates that both AZ and the dextran-bound sulphonamide inhibit only the extracellular enzyme (Moroney *et al.*, 1985).

The results obtained in these experiments correlate with observations made in Chapter 3 when *Spirulina* was cultured in the presence of the carbonic anhydrase inhibitors, AZ and EZ. It was evident that these sulphonamides inhibit the enzyme CA, which is essential for the formation of CO_2 from HCO_3^-, which as a result affects the algae's ability to photosynthesise at low inorganic carbon concentrations, ultimately resulting in the death of the algal cells. Therefore it could be said that the oxygen electrode can be successfully used to determine the effect of various parameters on oxygen evolution in *Spirulina*, as well as the effect on the enzyme carbonic anhydrase, which is an integral part of the CCM.

CHAPTER SIX

HEAVY METAL UPTAKE BY *SPIRULINA*

6.1 Introduction

Capacities and mechanisms of uptake of metals may vary widely among microorganisms due to their complex structure. Although both living and dead cells are capable of metal accumulation, there are considerable differences in the mechanisms involved in each 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 (Garnham *et al.*, 1992; Khummongkol *et al.*, 1982). The second, slower phase is metabolism-dependent transport across the cell membrane (Wilde & Benemann, 1993; Terry & Stone, 2002). 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 (Ting, 1991). With some metals such as lead, uranium and thorium, the accumulation by microbial biomass is surface-based with little, or no intracellular uptake, unless by diffusion (Garnham *et al.*, 1992). The metal ions may be preferentially located within specific organelles and/or bound to proteins such as metallothionein once inside the cells. The composition of the medium and the presence of chelators produced by the microorganisms in the presence of metals are all capable of affecting metal uptake in growing cultures. As a result, several mechanisms of uptake may operate simultaneously and/or sequentially in a microbial system (Gadd, 1988; Gadd, 1990; Garnham *et al*, 1992; Veglio & Beolchini, 1997; Vilchez *et al*, 1997).

6.1.1 Metabolism-Independent Metal Accumulation

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). In some cases dead biomass has a greater ability to bind heavy metals, probably due to the exposure of previously masked binding sites (Wilde & Benemann, 1993). These mechanisms vary, and any one or a combination may be operational, including, ion exchange, physical adsorption, complexation and microprecipitation. Metabolism-independent metal accumulation is a relatively rapid process, approaches equilibrium within a few seconds (Khummongkol *et al.*, 1982), can be reversible and is not a limiting factor in bioremoval kinetics (Wilde & Benemann, 1993). This mechanism is most common in bioremoval kinetics and as a result the biomass has all the characteristics of an ion exchange resin or an activated carbon, implying many advantages in industrial application (Veglio & Beolchini, 1997; Volesky, 1987).

Cell walls of microbial biomass, mainly composed of polysaccharides, proteins and lipids, offer particularly abundant metal-binding functional groups (Kuyucak & Volesky, 1988; Veglio & Beolchini, 1997; Volesky, 1987). The polymers which constitute the cell wall are rich in phosphoryl, carboxyl, aromatic and hydroxyl groups (Ehrlich, 1986) which bind cationic metals (Christ *et al.*, 1981).

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 uptake by bacterium *Zoogloea ramigera* and the alga *Chlorella vulgaris* (Aksu *et al.*, 1992), for chromium uptake by fungi *Ganoderma lucidum* and *Aspergillus niger* (Venkobachar, 1990), as well as for cadmium accumulation by marine algae (Holan *et al.*, 1993). Physical adsorption is furthermore responsible for copper, nickel, zinc, cadmium and lead uptake by *Rhizopus arrhizus* (Fourest & Roux, 1992; Zhou & Kiff, 1991).

Metal removal from solution may also take place through complex formation on the cell surface after interaction between the metal and active sites. 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).

6.1.2 Metabolism-Dependent Metal Accumulation

Metabolism-dependent uptake of metal ions is a slower metal binding process in which additional metal ion is bound, often irreversibly (Khummongkol *et al.*, 1982; Ting *et al.*, 1991; Xue *et al.*, 1988). This "slow" phase of metal uptake can be due to a number of mechanisms, including covalent bonding, surface precipitation (Wood & Wang, 1983), redox reactions (Hosea *et al.*, 1986), crystallisation on the cell surface or, most often, diffusion into the cell interior and binding to proteins and other intracellular sites (Wilde & Benemann, 1993). Low temperatures, the absence of an energy source, metabolic inhibitors, and uncouplers inhibit rates of uptake. The physiological state of the cells and the nature and composition of the growth medium may influence rates of intracellular uptake (Gadd, 1988; Gadd 1990).

There are several examples where general adsorption plays a significantly greater role than energy-dependent uptake. This is particularly true for filamentous fungi and those organisms possessing extracellular polysaccharide (Singh *et al.*, 1999), slime or mucilage, where high biosorptive capacities mask low rates of intracellular uptake (Gadd, 1988; Gadd, 1990).

6.1.3 Energy Dispersive X-ray Analysis

6.1.3.1 Description of the technique

Energy dispersive x-ray spectroscopy (EDS) is a chemical microanalysis technique performed in conjunction with a scanning electron microscope (SEM). The technique utilises x-rays that are emitted from the sample during bombardment by the electron beam to characterise the elemental composition of the analysed volume (http://www.mee-inc.com). Features or phases as small as about 1μm can be analysed.

When the sample is bombarded by the electron beam of the SEM, electrons are ejected from the atoms comprising the sample's surface. A resulting electron vacancy is filled by an electron from a higher shell, and a x-ray is emitted to balance the energy difference between the two electrons (http://www.ou.edu).

The EDS x-ray detector measures the number of emitted x-rays versus their energy. The energy of the x-ray is characteristic of the element from which the x-ray was emitted. A spectrum of the energy versus relative counts of the detected x-rays is obtained and evaluated for qualitative and quantitative determinations of the elements present in the sampled volume (http://www.mee-inc.com).

6.1.3.2 The Lithium drifted Silicon (SiLi) detector

The detector used in EDX is the Lithium drifted Silicone detector. This detector must be operated at liquid nitrogen temperatures. When a x-ray strikes the detector, it will generate a photoelectron within the body of the Si. As this photoelectron travels through the Si, it generates electron-hole pairs. The electrons and holes are attracted to opposite ends of the detector with the aid of a strong electric field. The size of the current pulse generated depends on the number of electron-hole pairs created, which in turn depends on the energy of the incoming x-ray. Thus, a x-ray spectrum can be acquired giving information on the elemental composition of the material under examination (http://www.uksaf.org).

6.1.3.3 Analytical information

1) Qualitative analysis

Elements with atomic numbers from that of beryllium to uranium can be detected. The minimum detection limits vary from about 0.1 weight percent to a few percent depending on the element and matrix.

2) Quantitative analysis

Quantitative results are readily obtained without standards. The accuracy of standardless quantitative analysis is highly sample dependent. Greater accuracy is obtained using known standards with similar structure and composition as the unknown sample.

3) Line profile analysis

The SEM electron beam is scanned along a preselected line across the sample while x-rays are detected for discrete positions along the line. Analysis of the x-ray energy spectrum at each position provides plots of the relative elemental concentration for each element versus position along the line.

4) Elemental mapping

Characteristic x-ray intensity is measured relative to lateral position on the sample surface. Variations in x-ray intensity then indicate the relative elemental concentrations across the surface. Maps are recorded using image brightness intensity as a direct function of the local concentration of the element(s) present. Lateral resolution of about 1μm is possible.

6.1.3.4 Advantages of using EDXA

Electron microscopic analysis of elements provides some advantages compared to wet chemical analysis (http://www.ou.edu):

- x-ray sampling is non-destructive
- natural heterogeneity is retained
- analysis can be conducted on microgram quantities
- adjacent structures can be directly compared
- excellent probe size (20 to 200 nm)

6.1.3.5 Typical applications of EDXA

- surface contamination analysis
- corrosion evaluations
- coating composition analysis
- rapid material alloy identification
- small component material analysis
- phase identification and distribution (http://www.mee-inc.com)

6.2 Motivation and Aims

The results presented in Chapter 4 indicate that the cell morphology, photosynthetic ability, bicarbonate utilisation and carbonic anhydrase activity in *Spirulina* were severely affected on exposure to heavy metals such as lead, copper and nickel.

The aim of this chapter was to determine the percentage uptake of various metals from solution by *Spirulina* and using Energy Dispersive X-ray Analysis (EDXA) to determine if there was integration of heavy metals into the algal cell.

6.3 Results and Discussion

6.3.1 Heavy metal uptake by *Spirulina*

It is important to note that when the various metals were added to the algal containing media, there was some initial precipitation, especially at the higher metal concentrations. Therefore, the results presented have been calculated taking precipitation into account. This means, that the trends in % uptake of metal, is the uptake of what remains in solution.

Lead was found to precipitate out the most, with about 40-100% precipitating within a few minutes of being added to the Zarrouks medium. Copper and nickel were also found to precipitate out, though the initial concentrations were less than that of lead, and were found to be 30-40% and 20% respectively.

The trends in % uptake were found to be similar among all the metals investigated. The highest % uptake of metal was found to occur at the lowest initial metal concentrations and the lowest % uptake occurred in the flasks containing the higher concentrations of metal (Figure 6.1-6.3). This is similar to the findings of Fathi and El-Shahed (2000) who performed studies on *Scenedesmus biguja* and found that the lower the copper concentration in the culture medium, the higher the activity of the uptake and the capacity of the cells to accumulate copper.

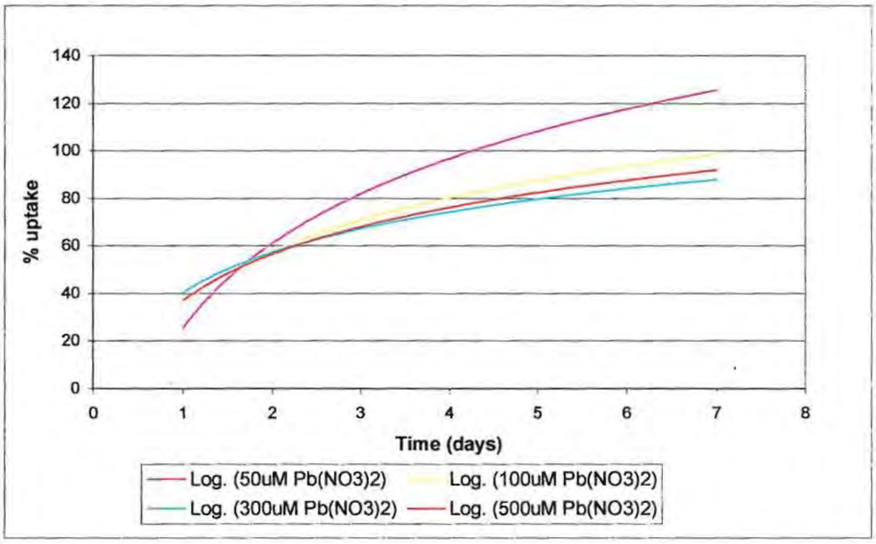


Figure 6.1: The trend in % uptake of lead at varying initial concentrations over time

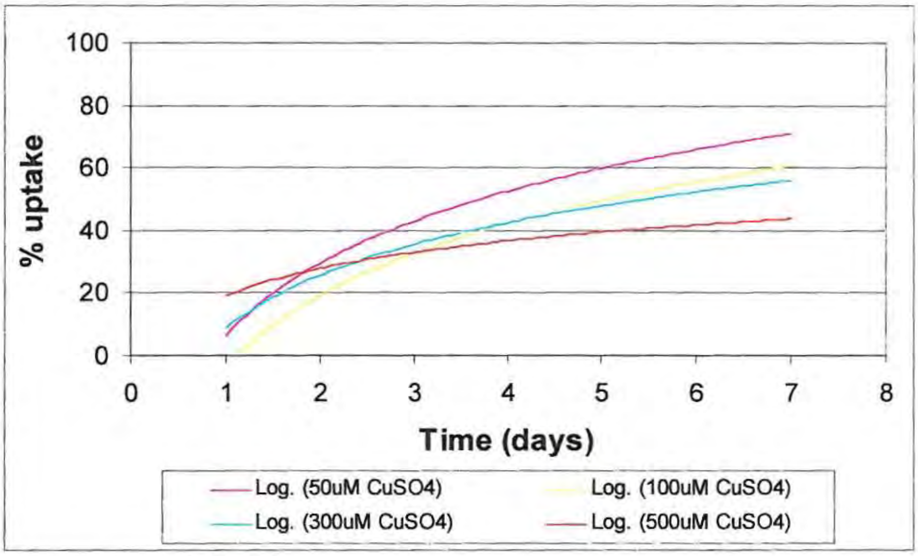


Figure 6.2: The trend in % uptake of copper at varying initial concentrations over time

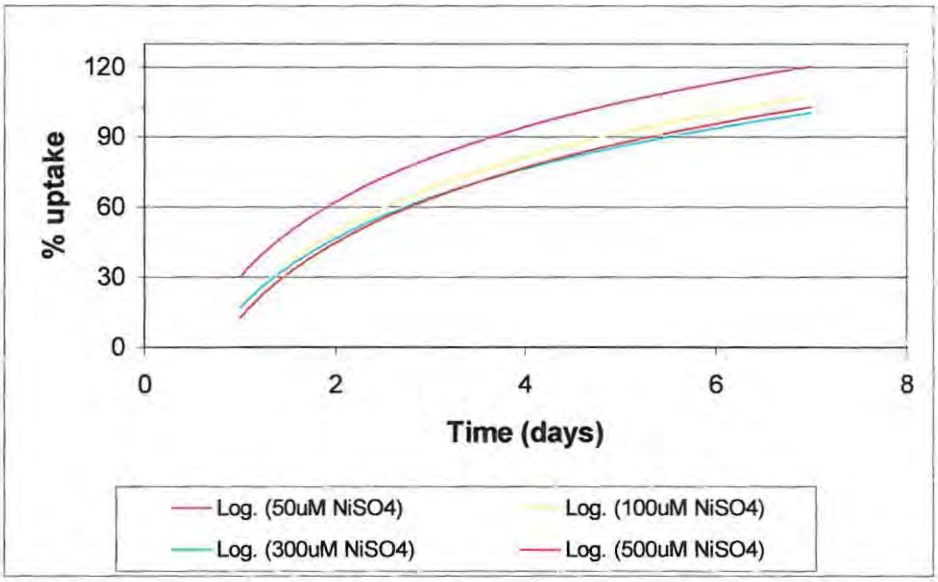


Figure 6.3: The trend in % uptake of nickel at varying initial concentrations over time

The possible reasons for this observation, is that at lower metal concentrations, there are more binding sites available for metal uptake, and as a result, there is a greater, relative, % uptake of metal by the cells compared to higher metal concentrations. At the higher metal concentrations, the remaining metal ions in solution (i.e. after initial precipitation has occurred), probably do adhere to binding sites on the algal surface, but as there are only a limited number, not all of the metal will be removed from solution.

The other possible explanation is that the algal cells are not as affected by the lower metal concentrations and as a result are able to actively accumulate the heavy metal ions from solution (metabolism-dependent uptake), whereas the cellular processes of the algae exposed to higher concentrations of metal are severely affected, thus reducing their ability to accumulate lead, copper and nickel from solution.

It is evident that the trend in % uptake at all metal concentrations is an increase over the period of study. This could be as a result of a number of factors. Firstly, due to metabolism-dependent uptake of heavy metals by the algal cells or an increase in surface adsorption due to the increase in surface area of the cells as they grow (Khummongkol *et al.*, 1982; Garnham *et al.*, 1991). Thirdly, the metals cause degradation of the algae over time and as a result more intracellular binding sites are exposed for biosorption.

6.3.2 Determination of integration of heavy metals into the algal cell using Energy Dispersive X-ray Analysis (EDXA)

Energy Dispersive X-ray Analysis (EDXA) samples were prepared from the control cultures and from those flasks containing the highest concentration of metal (ie. Pb; Cu; Ni), to determine if there was any uptake and integration of the metals into the algal cells.

6.3.2.1 Lead

The EDS spectrum for the lead control samples indicated that there was no lead present in the samples (Figure 6.4). This was expected, as these control cultures had not been exposed to lead. Two other elements were found to be present and the highest elemental concentration detected from the relative counts was nickel. When mounting the sections onto grids, a metal grid that is not the metal of interest has to be chosen. In this case, lead samples were mounted onto nickel grids, hence nickel was the dominant element detected. Iron was also detected in the samples. The iron found could not have been from the media, as this was excluded at the beginning of the experiment, as it tends to precipitate out in the absence of the metal chelator EDTA. However, it is possible that the iron had integrated into the cell during its growth phase in the algal raceway, and as a result could be detected.

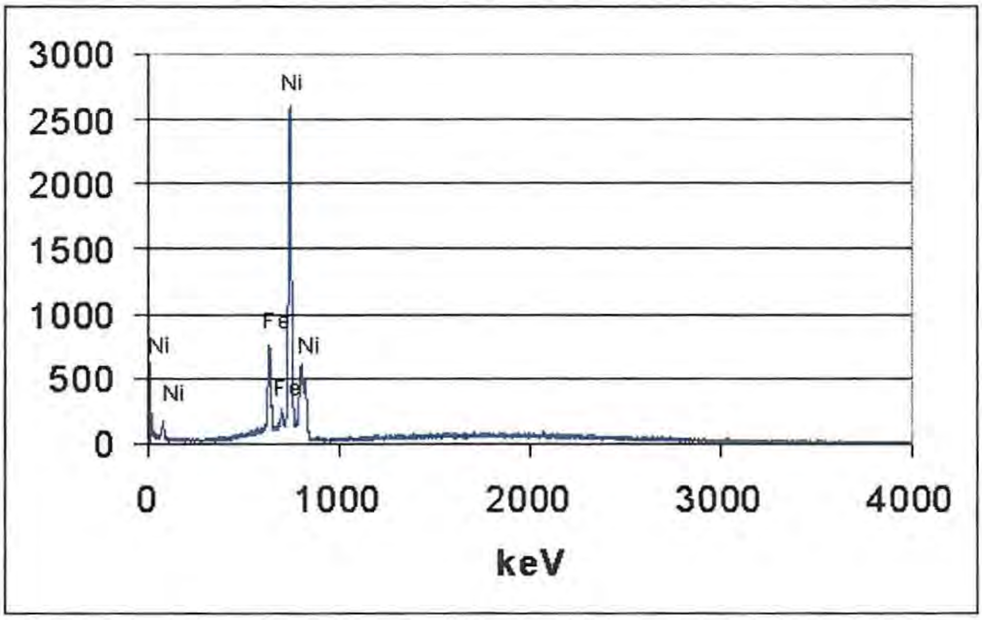


Figure 6.4: The energy dispersive spectrum (EDS) of the control specimens of *Spirulina* not exposed to lead

Comparing the control EDS spectrum to that of the specimens exposed to the highest concentration of lead (ie. 500μM), it was evident that the elements identified were identical to those found in the control specimens. The only exception was that very low amounts of lead were identified in the experimental samples (Figure 6.5). This indicates that there was some internalisation of the metal by the algae, but the method does not give any indication where this accumulation was occurring within the algal cell. The lead detected was not surface-bound metal, as EDXA is not a surface science technique. When EDXA is used, the x-rays are generated in a region about 2 microns in depth, so only metal that has been internalised will be detected.

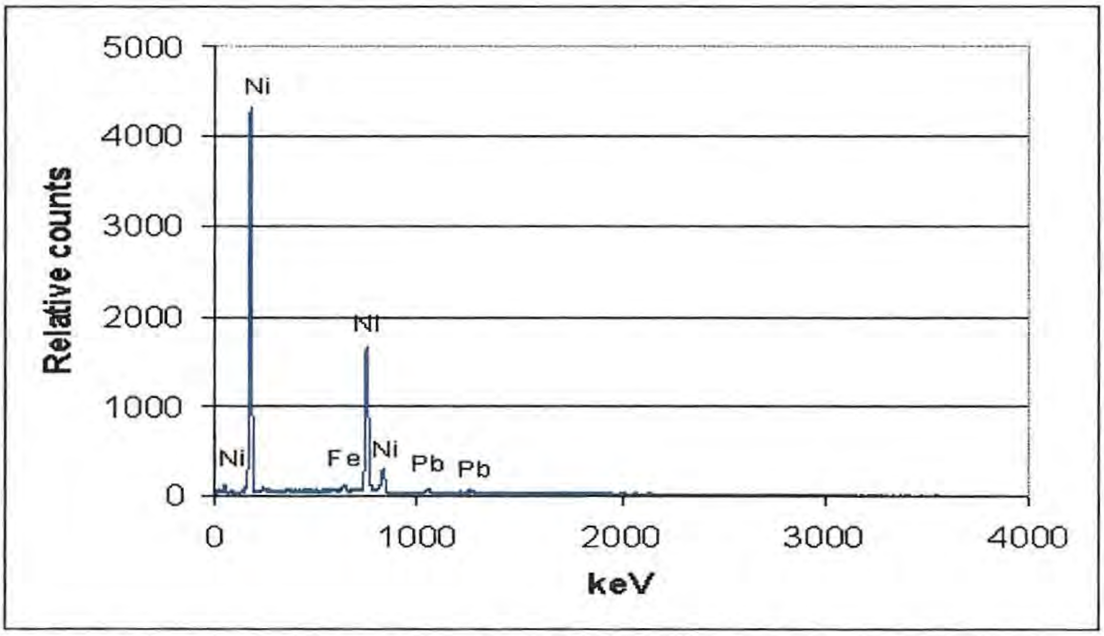


Figure 6.5: The energy dispersive spectrum (EDS) of the experimental specimens of *Spirulina* exposed to 500μM lead.

6.3.2.2 Copper

A number of elements were detected to be present within the control specimens for the copper experiment, but no copper was detected. The control samples were found to contain oxygen, aluminium, silica, calcium, nickel and iron (Figure 6.6). It was interesting to note that the control samples for the copper experiment contained a greater selection of elements than the control specimens from the lead experiment. The samples for the copper experiment were prepared and sectioned better than all the other samples, which means that there was probably a better sample area for the electron beam to assess.

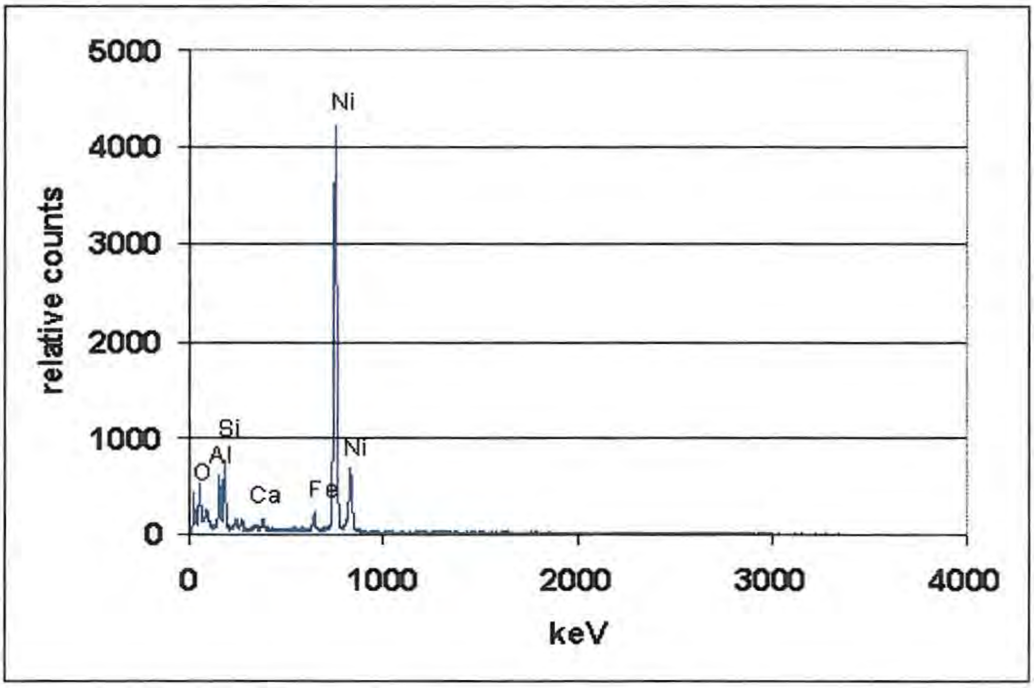


Figure 6.6: The energy dispersive spectrum (EDS) of the control specimens of *Spirulina* not exposed to copper

The experimental samples for copper indicate the presence of similar elements as those found in the control specimens (with the exception of calcium). It appears from Figure 6.7, that there was significant uptake and internalisation of copper into the algal cells. Initially, it was thought that perhaps the electron beam was detecting copper, which had not been washed off during the rinse stage of TEM preparation and was bound to the surface of the cells. However, EDXA is not a surface science technique and the x-rays are generated in a region about 2 microns in depth (http://www.uksaf.org). Copper was also detected in samples that had been prepared for EDXA from the flasks exposed to 300μM copper (Figure 6.8), though lower relative counts were observed, it was an indication that copper was indeed being internalised by the cells.

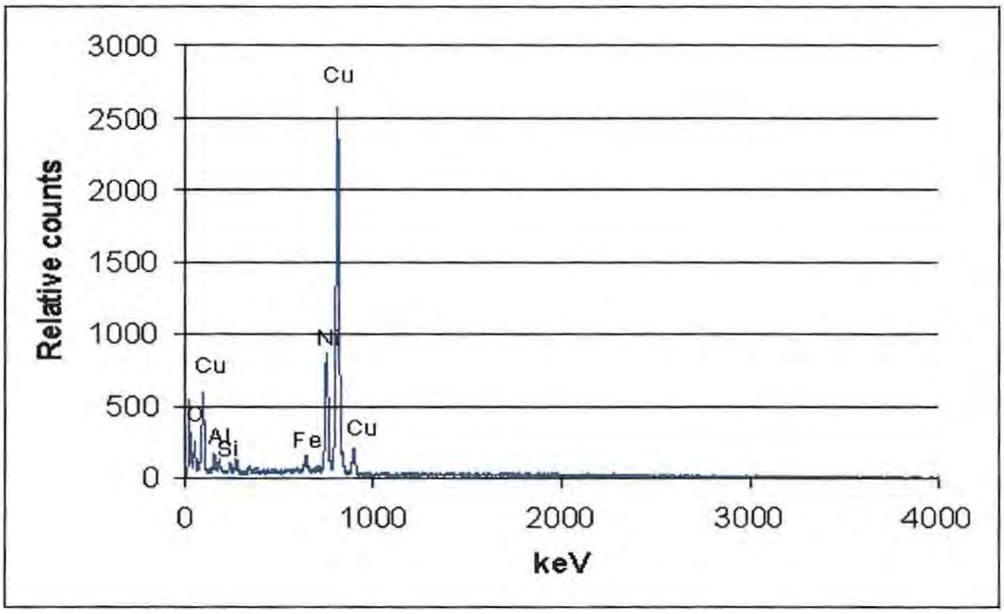


Figure 6.7: The energy dispersive spectrum (EDS) of the experimental specimens of *Spirulina* exposed to 500μM copper

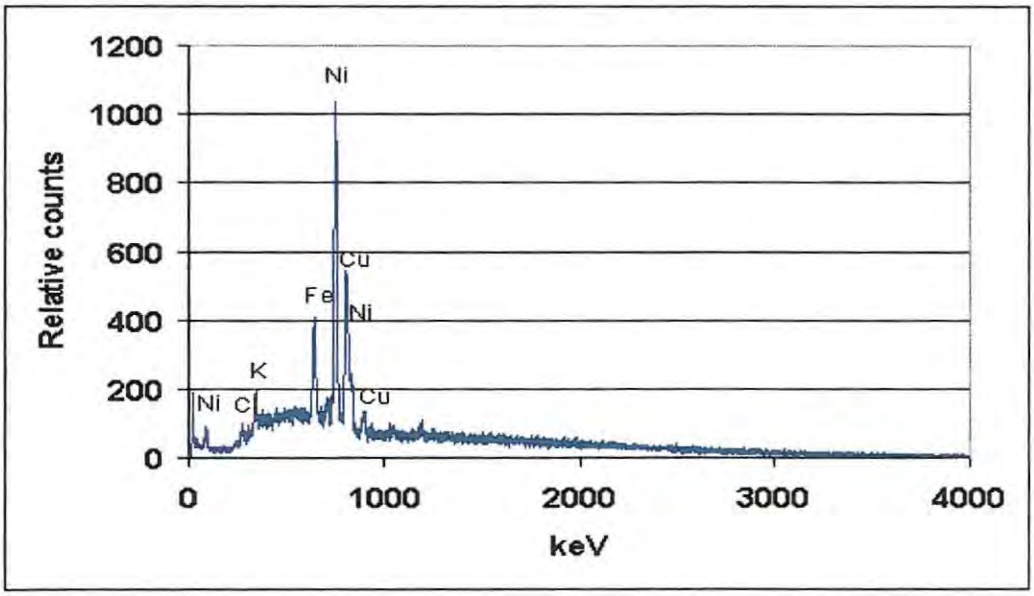


Figure 6.8: The energy dispersive spectrum (EDS) of the experimental specimens of *Spirulina* exposed to 300μM copper

6.3.2.3 Nickel

Unfortunately, there are no results for the nickel control samples, as the sections were cut too thinly and the beam burnt right through the resin. Results were obtained for the experimental samples (Figure 6.9), but they indicate that there was no nickel internalisation into the cells. Four predominant elements were identified, copper, calcium, titanium and iron. As the metal under study was nickel, sections were mounted onto copper grids, this is the reason for the high counts obtained for copper. Titanium is a reasonably obscure element to be detected within the cell, and none of the other samples were found to contain it, so it was assumed that it was an artifact from TEM preparation.

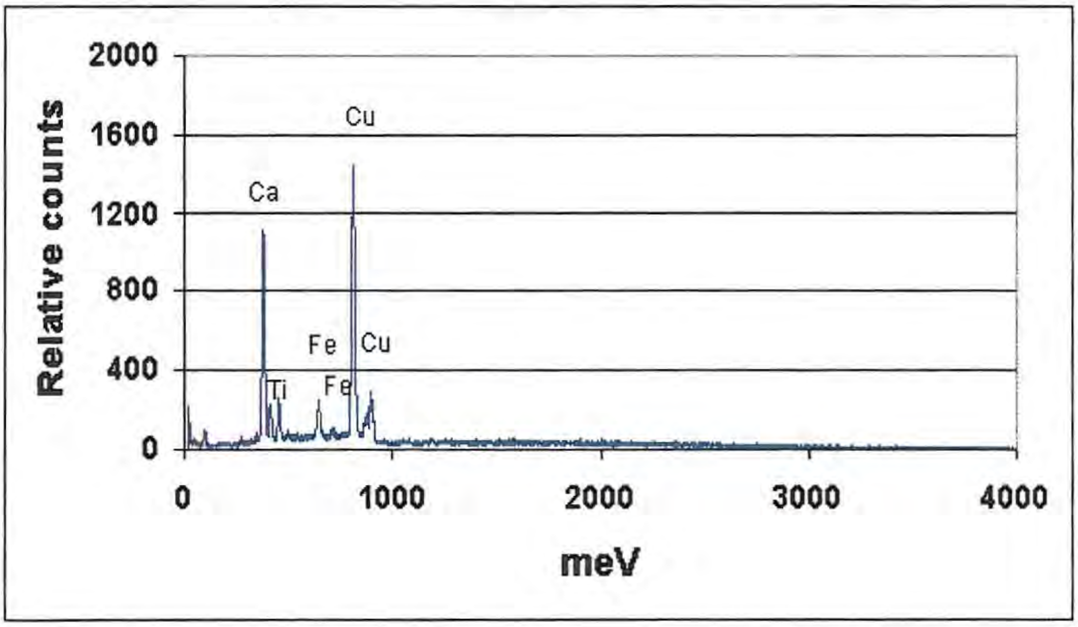


Figure 6.9: The energy dispersive spectrum (EDS) of the experimental specimens of *Spirulina* exposed to 500μM nickel.

It appears from the results that there was uptake of all three metals investigated, by the alga, *Spirulina*, with the greatest % uptake occurring with lead and nickel. However, the EDS spectrums indicated that copper was the metal found to have been internalised the most by the algal cells. A small amount of lead was also found to have been accumulated internally by the cells, but no nickel was detected.

Considering the toxic effects that high concentrations of heavy metals have had on the photosystems and the general health of the algal culture (Chapter 4), it was evident that *Spirulina* would not be a suitable bioremediation agent if used as a biosorbent. As a result, an alternative bioremediation application of *Spirulina* was examined, namely, using the biogenic alkalinity generated by the algae for the precipitation of metal.

CHAPTER SEVEN

THE USE OF THE ALKALINITY GENERATED BY *SPIRULINA* FOR THE PRECIPITATION OF METALS

7.1 Introduction

Due to their mobility in natural water ecosystems and their toxicity to higher life forms, heavy metal ions in surface and groundwater supplies have been prioritised as major inorganic contaminants in the environment. Even if they are present in dilute, undetectable quantities, their recalcitrance and consequent persistence in water bodies imply that through natural processes such as biomagnification, concentrations may become elevated to such an extent that they begin exhibiting toxic charactersitics (Atkinson *et al.*, 1998). These metals can either be detected in their elemental state which implies that they are not subject to further biodegradative processes or bound in various salt complexes. In either instance, metal ions cannot be mineralised. Apart from environmental issues, technological aspects of metal recovery from industrial wastewaters must also be considered (Wyatt, 1988). Metal resources are non-renewable and natural reserves are becoming depleted. It is therefore imperative that those metals considered environmentally hazardous, or which are of technological importance, strategic significance or economic value, be removed/recovered at their source using appropriate treatment systems (Atkinson *et al.*, 1998).

7.1.1 Chemical and physical treatment processes

Conventional chemical (precipitation/neutralisation) or physical (ion exchange, activated carbon sorption and membrane technology) treatment techniques are inherently problematic in their application to metal-bearing waste streams. Chemical treatment methods can prove costly, as the active agent cannot be recovered for reuse in successive treatment cycles. Also, the end-product is usually a low-volume, highly concentrated metal-bearing sludge that is difficult to dewater and dispose of (Atkinson *et al.*, 1998). After treatment, total dissolved solids (TDS) values of the waste-water may still be unacceptably high due to the poor compaction properties of the sludge cake (Atkinson *et al.*, 1998). Addition of natural or synthetic flocculants may be required to assist with precipitation. Introduction of chemicals increases the conductivity/salinity of water through the production of soluble sulphates and chlorides. This is of particular concern in countries such as South Africa where these waters are reused a number of times (Atkinson *et al.*, 1998). Chemical dosing is a direct function of soluble metal concentrations and must be closely monitored to correlate with diurnal loading patterns.

Application of membrane technology to metal-bearing waste streams has several major drawbacks. Apart from the expense, membranes are also unable to resist certain types of chemicals and pH values, and are prone to deterioation in the presence of micro-organisms. Compaction, scaling, short operation life and applicability only to feed streams with low concentrations of metal ions are some other problems which can be encountered with membrane installation (Kuyucak, 1997). Skilled labour is required for their successful operation and certain quantities of ions can always be detected in the permeate (Kuyucak, 1997).

7.1.2 The Biological Solution

New technologies are required that can reduce heavy metal concentrations to environmentally acceptable levels at affordable costs. Bioremoval has the potential to contribute greatly to the achievement of this goal. Bioremoval is therefore defined as the accumulation and concentration of pollutants from aqueous solutions by the use of biological materials, thus allowing the recovery and/or environmentally acceptable disposal of the pollutants (Wilde & Benemann, 1993). Microorganisms have the ability to influence metals' mobility in the environment by modifying their chemical and/or physical charactersitics.

The removal of metals from various aqueous streams using biosorptive and bioaccumulative processes has received significant attention. Biosorptive processes are essentially pseudo-ion exchange processes in which the metal ion is exchanged for a counterion attached to the biomass or resin (Eccles, 1999). Biosorption usually involves more than one functional group on the biomass and is dependent on the pH of the liquid and the chemical characteristics of the metal.

The use of metabolically active systems may offer the greatest potential for remediation. For example, the application of sulphate reducing bacteria (SRB) to sulphate-containing wastes has been studied for many years in substantial detail (Barnes *et al.*, 1991, Dvorak *et al.*, 1991, Gyure *et al.*, 1990, Hammack *et al.*, 1993, Hammack *et al.*, 1994), and the potential application of the biogenic alkalinity generated by the algae, *Spirulina*, for the precipitation of heavy metals when bicarbonate is utilised as a carbon source for photosynthesis. This will be discussed in greater detail in the present chapter.

7.2 Motivation and Aims

A considerable amount of work has been conducted on algae as a bioremediation agent, but this research has focussed on the ability of the algae to adsorb or accumulate metal ions from solution (van Hille *et al*, 1999). These methods are usually limited by the toxicity caused to algal systems by the presence of even relatively low concentrations of metals in solution. It has been documented that algae are able to alter the alkaline species of the surrounding medium through the use of their inorganic carbon accumulating mechanism (this has also been demonstrated in Chapters 3). Very little research has been conducted on the use of the alkalinity generated for the precipitation of metals. This chapter will focus on the feasibility of using this alkalinity generating system for metal precipitation. This will be done by using both a modeling system (MINTEQA2) for predictive precipitation and actual experimental precipitation.

7.3 Results and Discussion

7.3.1 Determination of carbonate species in solution

The results for the change in carbonate species over the two week period of study have already been discussed in Chapter 3, Figure 3.3 and Figure 3.4.

7.3.2 Comparison of predicted MINTEQA2 modeling results with experimental results

The alkalinity and pH values recorded from the 16.8g/L $NaHCO_3$–containing experimental flask was used for carbonate speciation modeling using the chemical speciation software, MINTEQA2 and the predicted results can be seen in Chapter 3 (Figure 3.5). These predicted results coincide with those obtained from the experimental flasks.

These values were again used to model what we would expect to find after a metal precipitation reaction occurred, and to determine the effect of the metal on the chemical species in solution. From the results, it was possible to categorise the precipitation reactions into three different groups, namely, those which:

- Precipitate as hydroxides
- Precipitate as carbonates, and are able to generate CO_3^{2-} from the dissociation of HCO_3^- in solution
- Precipitate as carbonates only if there is free CO_3^{2-} present in solution

According to the conditions specified, the chemical speciation software program, MINTEQA2, is able to predict the formation of a number of super-saturated compounds which could possibly precipitate from the reaction. Many of these predicted compounds will take days or even weeks to form, therefore only the compounds which were most likely to form in a reasonably short period of time were considered.

It is important to note that after the metal had been added to solution, samples were removed and filtered through 0.45µm filters (refer to Chapter 2), to remove the precipitate from solution before analysing the samples on the Atomic Absorption Spectrophotometer (AA spec). Previous experiments (data not shown) using centrifugation and filtration to remove the precipitate from the samples yielded almost identical results for all the metals tested, indicating that there was no binding of the metal to the filters.

A variety of metals were selected for the precipitation experiments, these included metals commonly found in mine waters, such as lead, copper, nickel and iron as well as ions responsible for contributing to the salination of water-sytems such as calcium and magnesium, to determine if this system could efficiently be used to remove these metals from waste-waters.

The precipitation results obtained from MINTEQA2 were categorised as follows:

Copper and nickel were grouped into the same category, as they both precipitate from solution as hydroxides.

7.3.2.1 Copper

From the predictive data obtained, a very low concentration of OH^- ions (4.683×10^{-8} M) were expected to be present in solution at the start of the experiment. Cu then reacted with these free OH^- ions in solution and precipitated out as copper hydroxide ($Cu(OH)_2$). An imbalance in the chemical equilibrium occurred as a result of the removal of OH^- from solution, resulting in the dissociation of water to restore the OH^- imbalance, and the subsequent release of H^+ into solution. These H^+ ions then react with HCO_3^- ions in solution to from carbonic acid, which is why the concentration of H_2CO_3 is so high on Day 0. It is important to note that before adding the filtered algal media to the metal solution, there was only HCO_3^- present in solution and not carbonic acid, as the pH was initially adjusted to 8.3, which means that the predominant species in solution was bicarbonate. Only once the metal had been precipitated, was there formation of H_2CO_3.

As the bicarbonate is utilised by the algae for photosynthesis, there is a decrease in the available carbon pool over time, resulting in an overall decrease in the concentration of H_2CO_3,(i.e. there are less HCO_3^- ions present in solution to combine with H^+ ions released from the dissociation of water) as shown in Appendix D.

The amount of copper precipitated on Day 0 is about 50%. The copper precipitation then decreases to 43% on day 1 and then remains reasonably constant over the period of study as an equilibrium is reached (Appendix D). The observed removal of copper in the filtered samples was initially found to be lower than those predicted by MINTEQA2, but increased the last four days (Figure 7.1).

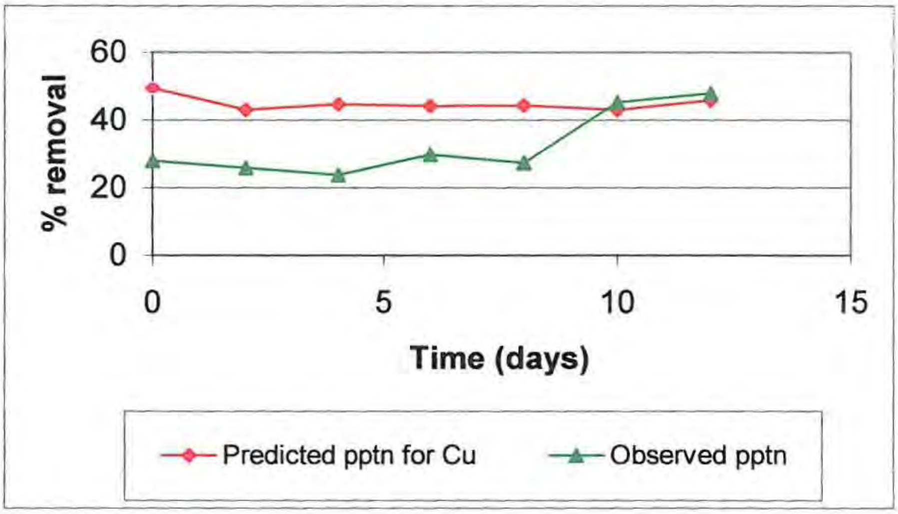


Figure 7.1: Predicted and observed precipitation results for copper

The predicted pH, after the precipitation reaction has occurred, remains at about 6.2 and the observed pH appeared to remain relatively constant at a pH of 6.35 (Figure 7.2). As the ratio of HCO_3^- to H_2CO_3 remains constant, i.e. 1.3 mol H_2CO_3:1 mol HCO_3^-, there is very little change in the amount of H^+ ions present in solution and as a result there is no excessive variation in pH.

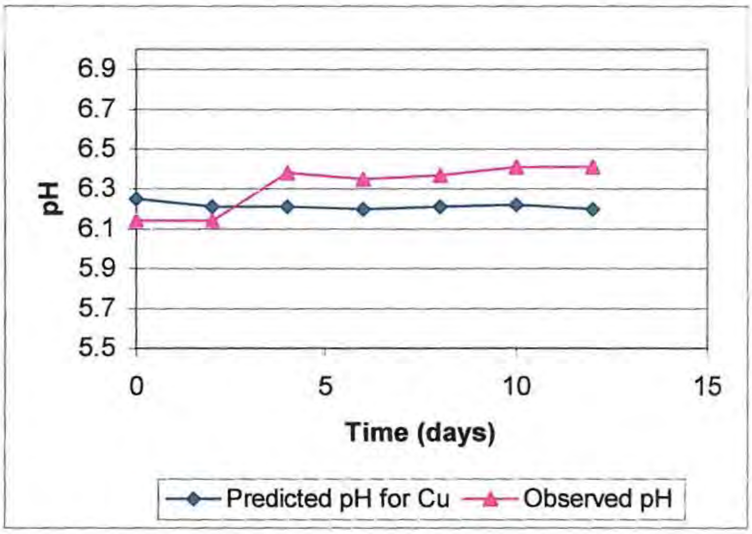


Figure 7.2: The predicted and observed pH values for copper recorded after precipitation

7.3.2.2 Nickel

The MINTEQA2 programme indicates that no precipitation of nickel should occur, however a small percentage, typically no higher than 20% was observed and most probably occurred as a result of complexation with soluble organic carbon such as extracellular polysaccharides (EPS). A small amount of EPS is usually continuously produced by the algae, but during nutrient and environmental stress, the amount of EPS produced has been found to increase (Gadd & White, 1993).

7.3.2.3 Iron and Lead

Iron and lead precipitate as carbonates, but the presence of CO_3^{2-} in solution is not initially required for precipitation to occur, as they are strong acid metals which are able to drive the dissociation of HCO_3^- to form CO_3^{2-}.

Any CO_3^{2-} in solution at the start of the experiment will react with the metals iron and lead as shown in Appendix E and F and precipitate as $PbCO_3$ and $FeCO_3$. Lead and iron are both reasonably strong acid metals and are able to drive the bicarbonate equilibrium, resulting in the dissociation of HCO_3^- to form CO_3^{2-} and H^+. The metals then react with the CO_3^{2-} formed and precipitate as metal carbonates. As the pH of the medium at the onset of the experiment is 8.3, one would not expect any H_2CO_3 to be present in solution, only bicarbonate ions. However, once metal precipitation has occurred, the H^+ generated reacts with excess HCO_3^- in solution to form carbonic acid (the ratio of carbonic acid to bicarbonate ions is initially very different until an equilibrium is reached) and this process is responsible for the high H_2CO_3 concentration at the start of the experiment (Appendix E and F). The concentration of the carbonic acid is seen to decrease over time, because as the algae utilise the bicarbonate in solution for photosynthesis, the available carbon pool is decreased.

The percentage of Pb^{2+} and Fe^{2+} precipitated on Day 0 is predicted to be 92.1% and 46.7% respectively (Appendix F and E). In the experimental flasks a visible, white precipitate formed almost instantly on the addition of the algal overflow to the metal solution. The lead samples were initially filtered through 0.45μm nylon membrane filters, but it was found that only 40-55% of the lead was being removed. This was a lot lower than the predictive results obtained, which indicated 70-92% removal. As a result of these observations, 0.22μm nylon membrane filters were used to filter out any lead in colloidal form (a precipitate small enough to pass through the filters with a larger pore size) and an increase in lead removal was noticed (Figure 7.3).

There is a higher precipitation of Pb^{2+}, as it is a stronger acid metal than Fe^{2+} and is more effective at dissociating HCO_3^- to form CO_3^{2-}. As a result, more CO_3^{2-} is available to react with the Pb^{2+} ions. The amount of Pb^{2+} precipitated over time should show a reduction due to the decrease in the available carbon pool. The percentage of Pb precipitated in the experimental flasks when filtered with 0.22μm filters was observed to be slightly higher than the predicted results (Figure 7.3).

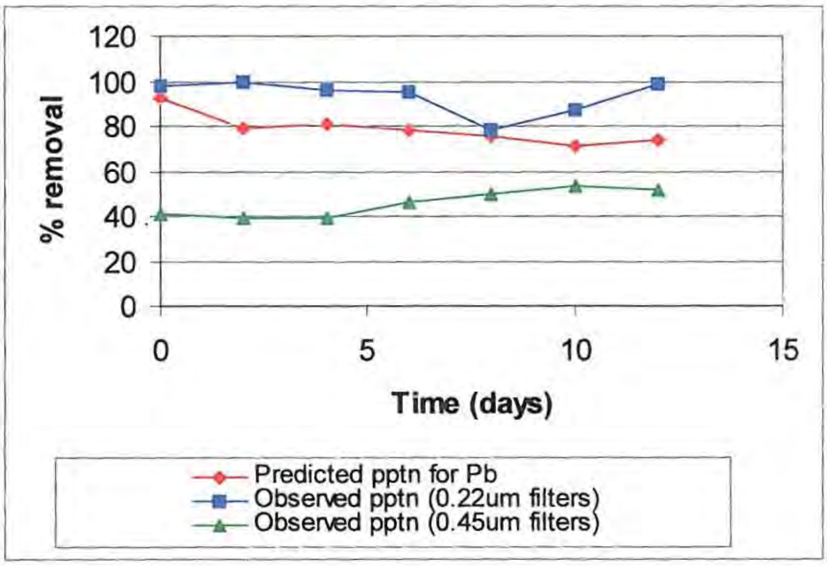


Figure 7.3: Comparison of the predicted and observed precipitation results for Pb^{2+}

The percentage removal of Fe^{2+} was predicted to be 46.7%, and the observed precipitation results were found to be almost identical to the precipitation results predicted by the modeling software MINTEQA2 (Figure 7.4).

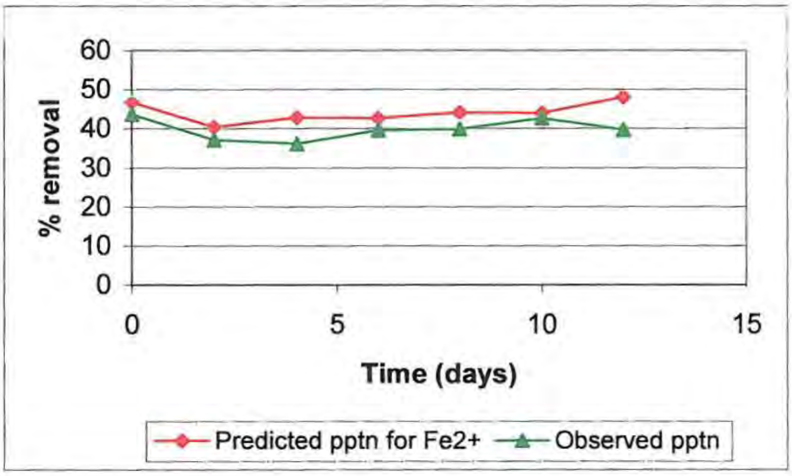


Figure 7.4: Comparison of the predicted and observed precipitation results for Fe^{2+}

The precipitation pH of lead remains constant, as the ratio of HCO_3^- to H_2CO_3 remains relatively constant and there is very little change in the amount of H^+ ions present in solution and as a result there is no excessive variation in pH (Figure 7.5).

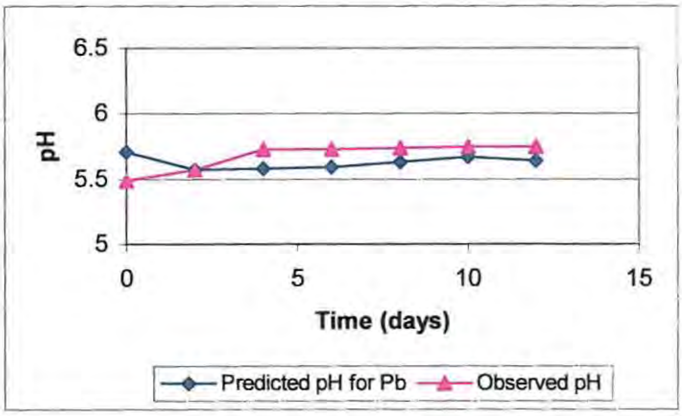


Figure 7.5: The predicted and experimental precipitation pH of Pb^{2+}

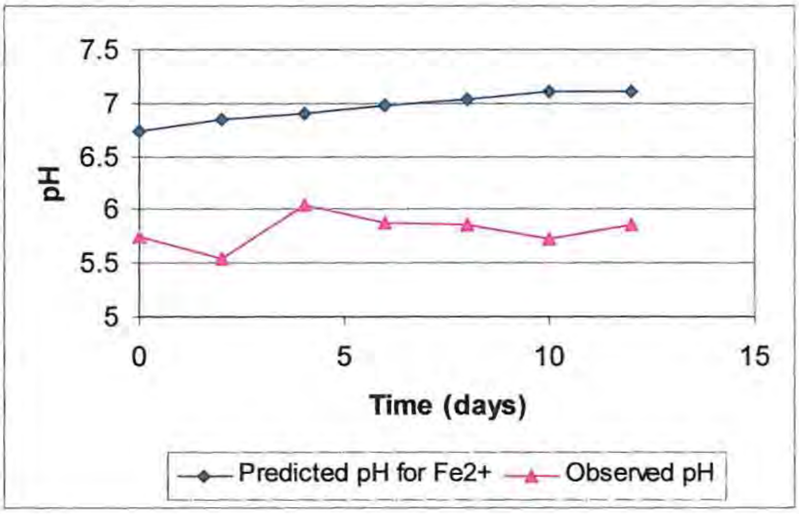


Figure 7.6: The predicted and experimental precipitation pH of Fe^{2+}

A slight increase in the pH recorded from the Fe^{2+} precipitation flasks was observed as a result of the higher bicarbonate to carbonic acid ratio. This means that the concentration of basic ions in solution was higher than the acidic ions, resulting in an increase in the pH (Figure 7.6).

7.3.2.4 Magnesium and Calcium

These metals are weak acid metals and precipitate as carbonates. However, the presence of free carbonate in the medium is required for precipitation, as they are not strong enough to drive the dissociation HCO_3^- to CO_3^{2-} and H^+.

According to the model, no precipitation of Mg or Ca (Appendix G and H respectively) should occur on day 0, as the pH of the medium was initially adjusted to 8.3, which means the only DIC species in solution is bicarbonate. As the algae begin to utilise the bicarbonate in the medium, CO_2 (which is taken up by the cell) and OH^- are released into solution. More than half of the OH^- ions react with HCO_3^- in solution to form carbonate, which then complex with the metal ions, causing them to precipitate as carbonates. Some of the remaining OH^- react with free H^+ in solution and this removal of H^+ from solution results in an increase in pH (Appendix G and H).

An increase in the pH of the experimental flask was observed for both Mg and Ca, as a result of the formation of OH^-, from the dehydration of HCO_3^-, reacting with free H^+ in solution causing removal of H^+ from solution resulting in an increase in pH (Figure 7.7 and 7.8).

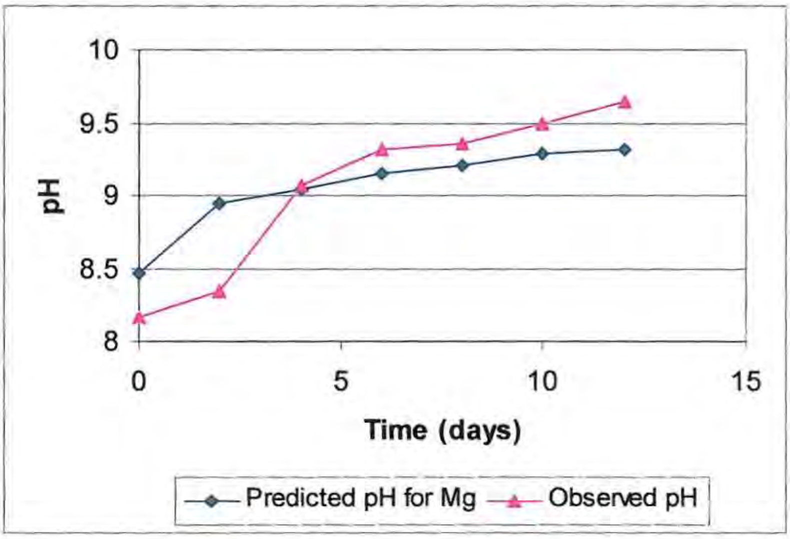


Figure 7.7: The predicted and experimental precipitation pH of Mg

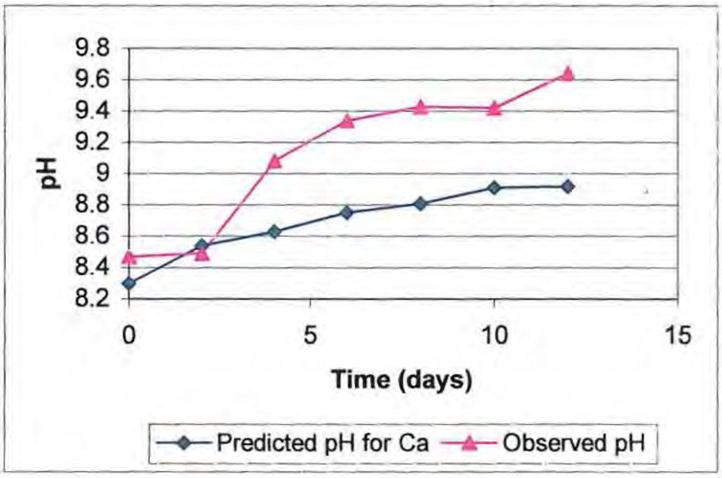


Figure 7.8: The predicted and experimental precipitation pH of Ca

MINTEQA2 predicts that there should be no precipitation at the start of the experiment, as there should be very little free CO_3^{2-} present in solution to complex the metal ions. As the concentration of carbonate in the medium increases, the predictive model indicates that an increase in metal precipitation from 0-26% in Mg (Appendix G) and 0-35% in Ca (Appendix H) should occur. Experimental results for Mg indicate a very erratic trend in precipitation with a maximum precipitation of 13% occurring over the period of study (Figure 7.9). Soluble organic carbons such as EPS, which are known to chelate metals, may be responsible for the precipitation observed at the start of the experiment.

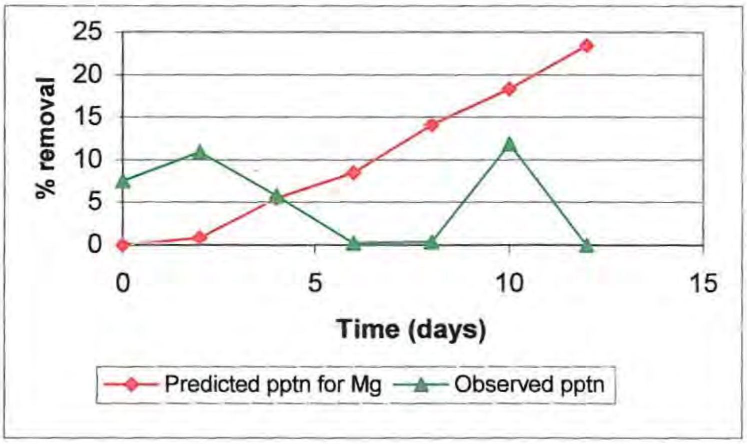


Figure 7.9: Comparison of the predicted and observed % precipitation results for Mg

The experimental results for Ca indicate that there is 40% precipitation on Day 0 and a maximum of 47% occurring on Day 8. The experimental results were found to be higher than the predicted % precipitation results (Figure 7.10), this could possibly be due to the release of algal chelating agents into the medium.

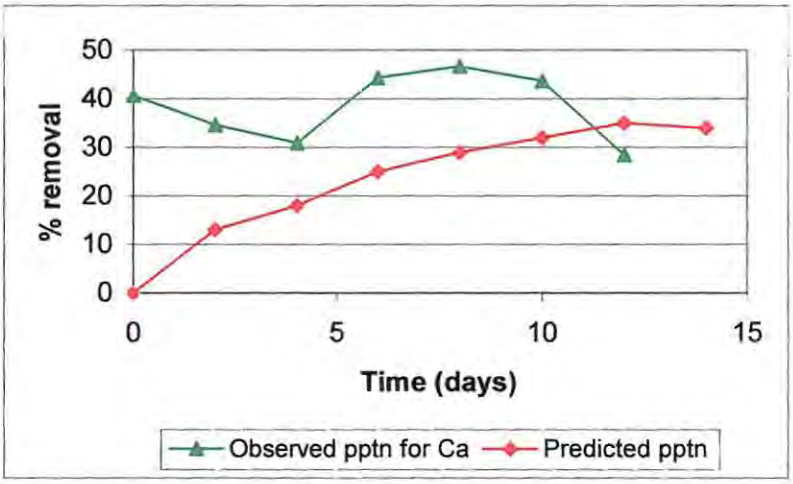


Figure 7.10: Comparison of the predicted and observed % precipitation results for Ca

When comparing the experimental results with those obtained from the MINTEQA2 modelling programme it can be seen that they correlate quite strongly with one another. A difference, which was observed, is that the amount of lead removed increased when a filter with a smaller pore size was used. This was presumably due to the removal of a very fine lead precipitate.

A limitation of using an algal system, in which the cells are in direct contact with the metal-containing solution, is that the algal culture is only effective over a short period of time because of the effects of heavy metal toxicity (this is clearly seen in Chapter 4). To overcome this problem, it would be more beneficial to keep the culture separate from the metal-containing solution or effluent. Therefore, in an application of this system for bioremediation or metal recovery, it appeared to be more feasible to use a system whereby metal precipitation occurred when the alkaline water from an algal growth vessel was transported to a reaction vessel containing the effluent. Such a system has been designed and utilised in other studies in our laboratory (van Hille *et al*, 1999).

CHAPTER EIGHT

CONCLUSIONS

Inorganic contaminants present in waste streams may be removed by one of three methods, namely, physical, chemical or biological. Chemical and physical techniques have traditionally been employed to remediate such streams. However, due to the crisis of rapidly deteriorating potable water quality, legislation governing the level of contaminants is becoming progressively stricter and penalties are incredibly high if these laws are contravened. Therefore, alternative methods for the treatment of these contaminated waters have been investigated.

Effluent treatment processes are designed to ensure that when wastewaters are discharged into natural water courses, any adverse effects are reduced or prevented. The extent of any such effect will be a function of the volume and composition of the influent wastewater and the dilution capacity of the receiving stream. It is for this reason that similarly operated processing facilities may be required to meet different effluent discharge standards according to their location (Atkinson *et al.*,1998).

The impact of industry on water sources is immense and it is only through promotion of good pollution prevention practices that contamination and deterioation of these waters will decrease.

Current technology for the removal of heavy metals from wastewater includes precipitation, ion exchange and adsorption, usually using synthetic resins or activated carbon. However, the continuous cost of reagents required for these processes makes them less financially feasible and alternative, cost-effective treatments are being implemented, which have low operational and maintenance requirements.

The main objectives of this project was to investigate the inorganic carbon accumulating mechanism in *Spirulina*, to determine the effect of heavy metals on the cell morphology and carbonic anhydrase activity and to determine if the carbonate generated by the algae, under conditions of limited carbon dioxide, from bicarbonate would be a feasible method for metal precipitation.

Initial batch flask experiments were carried out to determine the change in carbonate species over the period of study. The results obtained from the experimental flasks exhibited similar trends to those predicted by the chemical speciation modelling program, MINTEQA2. A decrease in the bicarbonate concentration was observed when the algae were grown under carbon dioxide limiting conditions, as a result of the induction of their carbon concentrating mechanism (CCM). Some of the hydroxide ions formed, contribute to the alkalisation of the medium, and bind to free H^+ ions in solution resulting in an increase in the pH of the surrounding medium. The remaining OH^- ions bind with some of the HCO_3^- to form carbonate, which reacts readily with metals.

The effect of heavy metals on the algal cell was also investigated to try and determine the importance of the enzyme, carbonic anhydrase (CA), on the inorganic carbon accumulating mechanism.

It was found that heavy metals had a significant effect on the synthesis of photosynthetic pigments. The chlorophyll *a* concentration in the control flasks was found to be significantly higher than those in the flasks containing the highest metal concentration. The reduction of photosynthetic pigments, such as chlorophyll *a*, resulted in a decrease in photosynthetic efficiency, eventually causing the death of the culture.

Heavy metals were also found to affect bicarbonate utilisation and carbonate formation. In the case of lead, an unusual trend in bicarbonate utilisation was observed with the control flasks, containing no metal, utilising less bicarbonate than the flasks containing higher metal concentrations. Data obtained in Chapter 7 indicated that the possible explanation for this was that lead was able to drive the dissociation of HCO_3^- to H^+ and CO_3^{2-}, increasing bicarbonate utilisation. The CO_3^{2-}ions would then bind with lead and precipitate out as lead carbonate, resulting in a lower carbonate alkalinity in the experimental flasks containing lead.

The expected trend of bicarbonate utilisation and carbonate formation was observed when copper was investigated. It was found that inorganic carbon utilisation was less with an increase in metal concentration and carbonate formation was greater in the control flasks, indicating that the copper may have a significant effect on the enzyme carbonic anhydrase, causing a reduction in the enzymes ability to utilise bicarbonate efficiently.

Nickel did not appear to have a significant effect on bicarbonate usage. However, the carbonate alkalinity was found to be greater in the control flasks and decreased slightly with an increase in metal concentration. It was thought that the nickel was binding to the carbonate ions formed and precipitating out, lowering the carbonate alkalinity at higher nickel concentrations.

Lead, copper and nickel were all found to have an adverse effect on the morphology of the algal cells when comparing SEM photographs of the experimental cultures with those from the control flasks. In each case, the metal was found to cause degradation of the cells and almost complete disintegration of the algal filaments, however this was not observed in the control cultures.

The Wilbur-Anderson enzyme assay method was used as a direct method for determining the effect of heavy metals on the enzyme CA. The trends in enzyme activity for the three metals showed some similarities, with the control cultures demonstrating a higher enzyme activity than the cultures exposed to varying concentrations of metal. At all metal concentrations a drastic decrease in CA was observed over the period of study. However, results were erratic and the Wilbur-Anderson assay did not prove to be a reliable method for measuring changes in CA activity.

As a result, attempts were made to determine if an oxygen electrode could be successfully used to determine the efficiency of photosynthetic oxygen evolution in *Spirulina* under various conditions and to establish if this method was a possible alternate and more reliable method, albeit an indirect one, for determining the effects of certain parameters, such as light intensity, the addition of bovine CA and inhibitors, on CA activity.

Spirulina was exposed to seven different photon fluxes, ranging from 67.10 to 192.7μmol.m^{-2}.s^{-1}, and the saturating light for photosynthesis was found to be 168.4μmol.m^{-2}.s^{-1}. The oxygen evolution rate was found to be less at higher light intensities, probably due to photoinhibition. And photosynthesis rates decreased at very low light intensities due to insufficient light available to sustain photosynthesis.

Experiments were performed to determine if a reduction or cessation in oxygen evolution in the absence of *Spirulina* occurred. It was found that when the light was switched off in the presence of the algae, oxygen evolution was reduced as the algae utilised the oxygen in solution for respiration, no effect however was found to occur in the absence of *Spirulina*.

Attempts were made to establish if the addition of bovine carbonic anhydrase at varying bicarbonate concentrations increased the rate of oxygen evolution. Bozzo *et al.* (2000) found that the oxygen evolution rate was 1.5-fold greater than the control when bovine

CA was added to a sample of *Chlorella kessleri*, this however was not found to be the case with *Spirulina*.

The effect of the inhibitors, acetazolamide and ethoxyzolamide, were also investigated to try and determine the importance of CA in inorganic carbon accumulation and photosynthesis. Results obtained were consistent with those observed in literature and it was found that at 100μM AZ and EZ, complete inhibition of photosynthesis and CA occurred, with no oxygen being evolved. It was also evident from observations that EZ appeared to have a more significant effect on oxygen evolution than AZ, probably due to the fact that AZ is an impermeable inhibitor, only affecting external or membrane-bound carbonic anhydrases, whereas EZ penetrates into the cell inhibiting both internal and external carbonic anhydrases. This means that the amount of CO_2 converted from HCO_3^- is greatly reduced in the presence of EZ and inefficient photosynthesis causes reduced oxygen evolution.

From the above results, it was concluded that the oxygen electrode could be successfully used as an alternate and more reliable method for determining the effects of various parameters on CA and photosynthesis.

It was also important to establish if there was any uptake and integration of nickel, lead and copper into the cells. The results indicated that there was uptake of all metals investigated, with the greatest % uptake occurring with lead and nickel. However, the EDS spectrums indicated that copper was the metal found to be internalised the most by *Spirulina*. A small amount of lead was found to have integrated into the cells but no nickel was detected.

From the results obtained in Chapter 4 it was evident that heavy metals adversely affected the photosystems and morphology of *Spirulina*. Due to this an alternative method using biogenic alkalinity for the precipitation of heavy metals was considered, as *Spirulina* could not be successfully implemented as a bioremediation agent if used as a biosorbent.

As it had been established in the batch flask cultures that algae are capable of affecting the carbonate species present in solution, metal precipitation experiments were performed to try and determine if the carbonate produced in the medium could be used for the precipitation of metals. The results observed in the experimental flasks were very similar to those predicted in the modelling programme, MINTEQA2.

It was possible to categorise the precipitation reactions observed into three different groups, namely those which:

1. Precipitate as hydroxides (Cu and Ni)
2. Precipitate as carbonates and are able to generate carbonate from the dissociation of bicarbonate (Pb and Fe)
3. Precipitate as carbonates only if there is free carbonate present in solution (Mg)

It appears that this algal-based method of metal precipitation would also be most suitable for the removal of Mg and Ca, as no precipitation of these metals occurs if carbonate is not present in solution. Therefore, as the algae utilise the bicarbonate supplied in the medium, when conditions of low carbon dioxide are experienced, an increase in the carbonate concentration is observed, the carbonate then complexes with the metal ions in solution resulting in the precipitation of the metals. Pb and Fe are strong acid metals capable of dissociating bicarbonate, to form carbonate, therefore all that would be required to precipitate these metals is the addition of bicarbonate.

The addition of bicarbonate in the form of the sodium salt on a large scale basis as the carbon source in an algal alkalinity reaction system is costly and leads to an increase in downstream processing. As a result our laboratory has been investigating the use of an integrated system, where the generation of alkalinity in the form of bicarbonate, by the anaerobic digestion of organic carbon by a range of bacteria has been utilised by the algae in a high rate algal ponding system (HRAP) and the alkaline overflow was then used for the precipitation of metals in a reaction vessel (Figure 8.1).

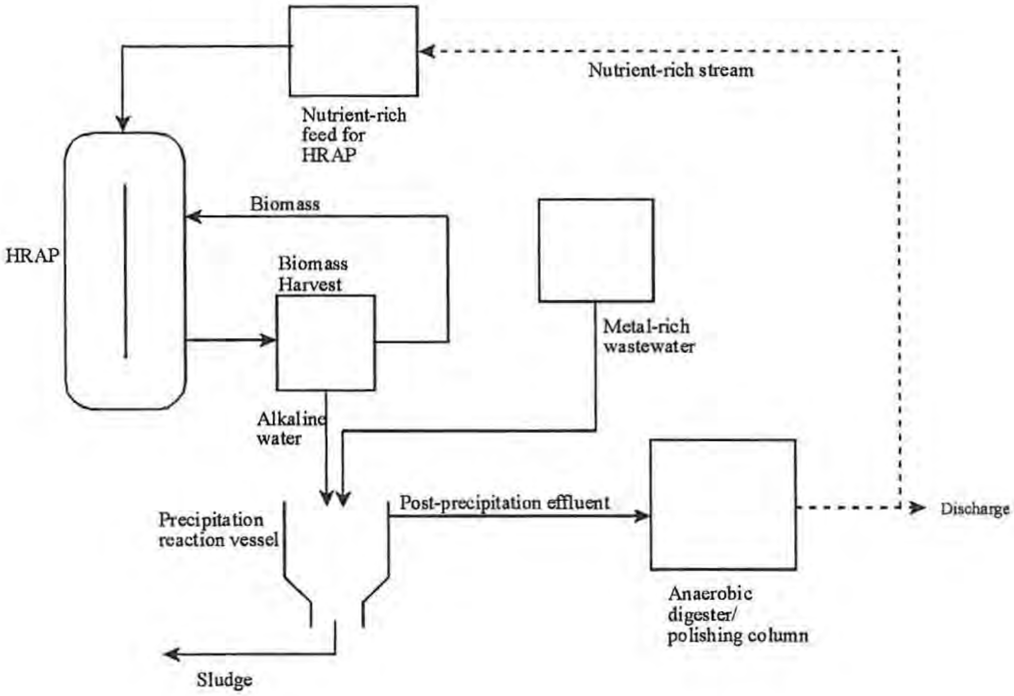


Figure 8.1: An integrated system for the precipitation of heavy metals, where the nutrient-rich stream from the anaerobic digester is utilised by the algae in a HRAP, resulting in an alkaline water which can then be utilised for the precipitation of heavy metals (van Hille, 2002)

It is clear that there are several biotechnological approaches for treating metal-containing wastes, which offer potentially efficient and cost-effective alternatives to existing treatment technologies. Whether the full potential of these biotreatments is realised depends on further investment and exploitation by receptive industries.

REFERENCES

Ahner B.A. & Morel F.M.M. (1995) Phytochelatin production in marine algae. 2. Induction by various metals, *Limnol. Oceanogr.* **40:** 658-665

Ahner B.A., Kong S. & Morel F.M.M. (1995) Phytochelatin production in marine algae. 1. An interspecies comparison, *Limnol. Oceanogr.* **40:** 649-657

Aksu Z., Sag Y. and Kutsal T. (1992) The biosorption of copper (II) by *C. vulgaris* and *Z. ramigera, Environ. Technol.* **13:** 579-586.

Al-Moghrabi S., Goiran C., Allemand D., Speziale N. & Jaubert J. (1996) Inorganic carbon uptake for photosynthesis by the symbiotic coral-dinoflagellate association II. Mechanisms for bicarbonate uptake, *J.E.M. Bio. Eco.* **199:** 227-248

Amoroso G., Weber C., Sultemeyer D. & Fock H. (1996) Intracellular carbonic anhydrase activities in *Dunaliella tertiolecta* and *Chlamydomonas reinhardtii* 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

Atkinson B.W., Bux F. & Kasan H.C. (1998) Considerations for application of biosorption technology to remediate metal-contaminated industrial effluents, *Water SA* **24:** 129-134

Badger M.R. (1980) Kinetic properties of Ribulose 1,5-bisphosphate carboxylase/oxygenase from *Anabaena variabilis, Arch. Biochem. Biophys.* **231:**233-242

Badger M.R. & Andrews T.J. (1982) Photosynthesis and inorganic carbon usage by the marine cyanobacterium, *Synechococcus* sp., *Plant Physiol.* **70:** 517-523

Badger M.R. (1987) The CO_2 concentrating mechanism in aquatic phototrophs. In: Hatch M.D., Boardman N.K. (eds) The biochemistry of plants: A comprehensive treatise. Academic Press, New York, vol 10, pp 132-218

Badger M.R. & Price G.D. (1992) The CO_2 concentrating mechanism in cyanobacteria and microalgae, *Plant Physiol.* **84:** 606-615

Badger M.R. & Price G.D. (1994) The role of carbonic anhydrase in photosynthesis, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **45:** 369-392

Badger M.R., Palmqvist K. & Yu J-W. (1994) Measurement of HCO_3^- and CO_2 fluxes in cyanobacteria and microalgae during steady-state photosynthesis, *Physiologia Plantarum* **90:** 529-536

Barnes L.J., Janssen F.J., Sherren J.H., Versteegh J.H., Koch R.O. & Scheeren P.J.H. (1991) A new process for the recovery of microbial recovery of sulphate and heavy metals from contaminated waters extracted by a geohydrological control system, *Chem. Eng. Res. and Des.* **69:** 184-186

Beardall J., Raven J.A. & Griffiths H (1980) Transport of inorganic carbon and the CO_2 concentrating mechanism in *Chlorella emersonii*, *J. Phycol.* **17:** 134-141

Beardall J., Griffiths H. & Raven J.A. (1982) Carbon isotope discrimination and the carbon dioxide accumulating mechanism in *Chlorella emersonii*, *J. Exp. Bot.* **33:** 729-737

Beardall J., Roberts S. & Millhouse J. (1991) Effects of nitrogen limitation on uptake of inorganic carbon and specific activity of Rubisco in green microalgae, *Can. J. Bot.* **69:** 1146-1150

Beardall J., Johnston A. & Raven J (1998) Environmental regulation of CO_2-concentrating mechanisms in microalgae, *Can. J. Bot.* **76:** 1010-1017

Bedell G.W. (1990) Propagation of freshwater algal biosorbents, In: Volesky B. (Ed) Biosorption of heavy metals, CRC Press, Boca Raton, Florida, pp 360-368

Benesova J., Nickova K., Ferimazova N. & Stys D. (2000) Morphological and physiological differences in *Synechococcus elongatus* during continuous cultivation at high iron, low iron and iron deficient medium, *Photosynthetica* **38:** 233-241

Bernier M., Popovic R. & Carpentier R. (1993) Mercury inhibition at the donor side of photosystem II is reversed by chloride, *FEBS Letters* **321:** 19-23

Beuf L., Kurano N. & Miyachi S. (2000) Effect of external pH on inorganic carbon assimilation in unicellular marine green algae, *Phycol. Res.* **48:** 47-54

Booth W.A. & Beardall J. (1991) Effects of salinity on inorganic carbon utilisation and carbonic anhydrase activity in the halotolerant alga *Dunaliella salina* (Chlorophyta), *Phycologia* **30:** 220-225

Booth W.A. & Beardall (1998) Biodiversity of marine plants in an era of climatic change, *Bot. Mar.* **141:**113-123

Botre C. & Botre F. (1990) Determination of carbonic anhydrase activity by a pCO_2 sensor, *Anal. Biochem.* **185:** 254-264

Bozzo G.G., Colman B. & Matsuda Y. (2000) Active transport of CO_2 and bicarbonate is induced in response to external CO_2 concentration in the green alga *Chlorella kessleri*, *J. of Experi. Bot.* **51:**1341-1348

Bryant P.S., Petersen J.N., Lee J.M. & Brouns T.M. (1992) Sorption of heavy metals by untreated Red Fir sawdust, *Appl. Biochem. Biotechnol.* **34/35:** 777-781

Cabral J.P.S. (1992) Selective binding of metal ions to *Pseudomonas syringae* cells, *Microbios.* **71:** 47-53.

Christ R.H., Oberholser K., Shank N. & Nguyen M. (1981) Nature of binding between metallic ions and algal cell walls, *Environ. Sci. Technol.*, **15:** 1212-1220

Ciferri O. (1983) *Spirulina*, the edible microorganism, *Microbiol. Reviews* **47:** 551-578

Cross R.H.M., Botha T. & Pinchuck S. (1987) A handbook on preparation of biological material for electron microscopy, Rhodes University, Grahamstown, pp 5-12, 34-37

Danilov R.A. & Ekelund N.G.A. (2001) Responses of photosynthetic efficiency, cell shape and motility in *Euglena gracilis* (Euglenophyceae) to short-term exposure to heavy metals and pentachlorophenol, *Water, Air and Soil Pollution* **132:** 61-73

Dionisio-Sese M.L. (1992) The effect of sodium chloride on carbonic anhydrase activity in marine microalgae, *J. Phycol.* **28:** 619-624

Department of Water Affairs and Forestry (DWAF) (1999) Draft water conservation and demand management national strategy framework. Department of Water Affairs and Forestry, pp 7-28

Eccles H. (1999) Treatment of metal-contaminated wastes: why select a biological process?, *Tibtech* **17:** 462-465

Ehrlich H.L. (1986) What types f microorganisms are effective in biobleaching, bioaccumulation of metals, ore beneficiation and desulphurisation of fossil fuels?, *Biotechnol. Bioeng. Symp.* **16:** 227-238

El-Sheekh M.M. (1999) Inhibition of the photosynthetic electron transport in the unicellular green alga *Chlorella kessleri* by mercury at multiple sites, *Cytobios.* **98:** 25-37

Espie G.S., Miller A.G., Birch D.G. & Canvin D.T. (1988) Simultaneous transport of CO_2 and HCO_3^- by the cyanobacterium *Synechococcus* UTEX 625, *Plant Physiol.* **87:** 551-554

Fathi A.A. & El-Shahed A.M. (2000) Response of tolerant and wild strains of *Scenedesmus biguja* to copper, *Biologia Plantarum* **43:** 99-103

Fisher M., Gokhman I., Pick U. & Zamir A. (1996) A salt-resistant plasma membrane carbonic anhydrase is induced by salt in *Dunaliella salina*, *J. Biol. Chem.* **271:** 17718-17723

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.

Gadd G.M. (1988) Accumulation of metals by microorganisms and algae In: Rehm H.J. and Reeds G. (eds), Biotechnology – A Comprehensive Treatise VCH, Weinheim, vol 6b, pp 401-430

Gadd G.M. (1990) Heavy metal accumulation by bacteria and other microorganisms, *Experientia* **46:** 834-840.

Gadd G.M. & White C. (1993) Microbial treatment of metal pollution- a working biotechnology?, *Tibtech* **11:** 353-359

Garnham G.W., Codd G.A. & Gadd G.M. (1991) Effect of salinity and pH on cobalt biosorption by the estuarine microalga *Chlorella salina*, *Biology of metals*, **4:** 151-157

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.

Giardi M.T., Koblizek M. & Masojidek (2001) Photosystem II-based biosensors for the detection of pollutants, *Biosensors & Bioelectronics* **16:** 1027-1033

Gimmler H., Treffny B., Kowalski M & Zimmermann (1991) The resistance of *Dunaliella acidophila* against heavy metals: The importance of the zeta potential, *J. Plant Physiol.* **138:** 708-716

Golbeck J.H., Leins S. & San Pietro A. (1977) Isolation and characterisation of a sub-chloroplast particle enriched in iron sulphur protein P_{700}, *Arch. Biochem. Biophys.* **178:** 140-150

Graham D., Atkins C.A., Reed M.L., Patterson B.D. & Smillie R.M. (1971) Carbonic anhydrase, photosynthesis and light-induced pH changes, In: Hatch M.D., Osmond C.B., Slayter R.O. (eds). Photosynthesis and photorespiration, Wiley-Interscience, New York, pp 267-274

Gyure R.A., Konopka A., Brooks A. & Doemel W. (1990) Microbial sulphate reduction in acidic (pH 3) stripmine lakes, *FEMS Microbiol. Ecol.* **73:** 192-202

Hammack R.W., Dvorak D.H. & Edenborn H.M. (1993) The use of biogenic hydrogen sulphide to selectively recover copper and zinc from severely contaminated mine drainage, In: Torma A.E. & Way J.E. (eds). Biohydrometallurgical Technologies: Proceedings of the International Biohydrometallurgy Symposium, The Minerals, Metals and Materials Society, pp 631-639

Hammack R.W., Dvorak D.H. & Edenborn H.M. (1994) Selective metal recovery using biogenic hydrogen sulphide: Rio Tinto mine, Nevada, In Proceedings of the International land Reclamation and Mine Drainage Conference on the Abatement of Acidic Mine Drainage, pp 24-29

Hewett-Emmett D & Tashian R.E. (1996) Functional diversity, conservation and convergence in the evolution of the α-, β-, γ-carbonic anhydrase gene families, *Mol. Phylogenet. Evol.* **5:** 50-77

Hiltonen T., Karlsson J., Palmqvist K., Clarke A.K. and Samuelsson G. (1995) Purification and characterization of an intracellular carbonic anhydrase from the unicellular green algae *Coccomyxa*, *Planta* **195:** 345-351

Holan Z.R., Volesky B. and Prasetyo I. (1993) Biosorption of cadmium by biomass of marine algae, *Biotechnol. Bioeng.* **41:** 819-825.

Honeycutt R.C. & Krogmann D.W. (1972) Inhibition of chloroplast reactions with phenylmercuric acetate, *Plant Physiol.* **49:** 376-380

Hosea M., Greene B., McPherson R., Henzl M., Alexander M.D. & Darnall D.W. (1986) Accumulation of elemental gold on the alga *Chlorella vulgaris*, *Inorganic Chimica Acta* **123:** 161-165

Karlsson J., Clarke A.K., Chen Z-Y., Hugghins S.Y., Park Y-II., Husic H.D., Moroney J.V. & 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, *The EMBO Journal*, **17:** 1208-1216

Katoh S. & Takamiya A. (1964) Nature of copper-protein binding in spinach plastocyanin, *J. Biochem.* **55:** 378-387

Khummongkol D., Canterford G.S. & Fryer C. (1982) Accumulation of heavy metals in unicellular algae, B*iotechnology and Bioeng.* **4:** 22643-22660

Kidd J. (1997). In: Environmental law, Juta & Co. Ltd., Cape Town, pp 83-84

Kimimura M. & Katoh S. (1972) Studies of electron transport associated with photosystem I. Functional site of plastocyanine, inhibitory effects of $HgCl_2$ on electron transport and plastocyanine in chloroplasts, *Biochim. Biophys. Acta* **283:** 279-292

Klimov V.V. and Baranov S.V. (2001) Bicarbonate requirement for the water-oxidising complex of photosystem II, *Biochim. Biophys. Acta* **1503:** 187-196

Kojima Y., Hiyama T. & Sakurai H. (1987) Effect of mercurials on iron sulphur centers of PSI of *Anacystis nidulans*, In: Biginns J. (ed). Progress in photosynthesis research, Nijhoff/Junk, The Hague, pp 57-60

Kuyucak N. & Volesky B. (1988) Biosorbents for recovery of metals from industrial solutions, *Biotechnol. Lett.* **10:** 137-143

Kuyucak N. (1997) Feasibility of biosorbents application, In: Volesky B. (ed) Biosorption of heavy metals, CRC Press, Boca Raton, pp 371-378

Leborans G.F. and Novillo A. (1996) Toxicity and bioaccumulation of cadmium in *Olisthodiscus luteus*, *Water Resources* **30:** 57-62

Lembi C.A. & Waaland J.R. (1988) Algae and human affairs, Cambridge University Press, Cambridge

Lichtenthaler H.K. (1987) Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes, *Methods in Enzymology*, Academic Press Inc, pp350-382

Lobban C.S. & Harrison P.J. (1997) Seaweed ecology and physiology, Cambridge University Press, United Kingdom, pp 259-265

Lu C. and Vonshak A. (2002) Effects of salinity stress on photosystem II function in cyanobacterial *Spirulina platensis* cells, *Physiologia Plantarum* **114:** 405-413

Maeda S. & Sakaguchi T. (1990) Accumulation and detoxification of toxic metal elements by algae. In: Akatsuka I. (eds). Introduction to applied phycology, SPB Academic Publishing, The Hague, Netherlands, pp 109-117

Mallick N., Singh A.K. & Rai L.C. (1990) Impact of bimetallic combinations of Cu, Ni and Fe on growth rate, uptake of nitrate and ammonium, $^{14}CO_2$ fixation, nitrate reductase and urease activity of *Chlorella vulgaris*, *Biol. Met.* **2:** 223-228

Mallick N. & Rai L.C. (1992) Metal induced inhibition of photosynthesis, photosynthetic electron transport chain and ATP content of *Anabaena doliolum* and *Chlorella vulgaris*: interaction with exogenous ATP, *Biomed. Environ. Sci.* **5:** 241-50

Mattoo A., Giardi M.T., Raskind A. and Edelman M (1999) Transport of inorganic and the CO_2 concentrating mechanism, *Plant Physiol.* **107:** 454-463

Mayo W.P., Williams T.G., Birch D.G. & Turpin D.H. (1986) Photosynthetic adaptation by *Synechococcus leopoliensis* in response to exogenous dissolved inorganic carbon, *Plant Physiol.* **80:** 1038-1040

McBrien D.C.H. & Hassall K.A. (1965) Loss of cell potassium by *Chlorella vulgaris* after contact with toxic amounts of copper sulphate, *Physiologia* **18:** 1059-1065

Mercado J.M., Figueroa F.L. & Niell F.X. (1997) A new method for estimating external carbonic anhydrase activity in macroalgae, *J. Phycol.* **33:** 999-1006

Miller A.G. and Colman B. (1980) Evidence for HCO_3^- transport by the blue-green alga (cyanobacterium) *Coccohloris peniocystis*, *Plant Physiol.* **65:** 397-402

Miller A.G., Turpin D.M. & Canvin D.T. (1984) Na^+-requirement for growth, photosynthesis and pH regulation in the alkalotolerant cyanobacterium *Synechococcus leopoliensis*, *J. Bacteriol.* **159:** 100-106

Miller A.G., Espie G.S. & Canvin D.T. (1987) Active transport of CO_2 by the cyanobacterium *Synechococcus* UTEX 625: Measurement by mass spectrometry, *Plant Physiol.* **86:** 677-683

Miller A.G., Espie G.S. & Canvin D.T. (1990) Physiological aspects of CO_2 and HCO_3^- transport by cyanobacteria: a review, *Can. J. Bot.* **68:** 1291-1302

Moenne A. (2001) Eukaryotic metallothioneins: proteins, gene regulation and copper homeostasis, *Biol. Mar.* **42:** 125-135

Moroney J.V., Husic H.D. & Tolbert N.E. (1985) Effect of carbonic anhydrase inhibitors on inorganic carbon accumulation by *Chlamydomonas reinhardtii*, *Plant Physiol.* **79:** 177-183

Moroney J.V., Kitayama M., Togasaki R.K. and Tolbert N.E. (1987) Evidence for inorganic carbon transport by intact chloroplasts of *Chlamydomonas reinhardtii*, *Plant Physiol.* **83:** 460-463

Moroney J.V. & Chen Z-Y. (1998) The role of the chloroplast in inorganic carbon uptake by eukaryotic algae, *Can. J. Bot.* **76:** 1025-1034

Moubarark M.A. & Stemler A. (1994) Oxidation-reduction potential dependence of photosystem II carbonic anhydrase in maize thylakoids, *Biochemistry* **33:** 4432-4438

Neytzell – De Wilde, F.G. (1992) Reassessment of the strategy with respect to industrial effluent discharge with special reference to advanced technology treatment methods: Phase 1: Industrial effluent discharge problem areas, WRC Report number 407/1/92, Water Research Commission, Pretoria, SA

Nield J., Kruse O., Ruprecht J., Fonseca P., Buchel C. & Barber J. (2000) 3D structure of *Chlamydomonas reinhardtii* and *Synechococcus elongatus* photosystem II complexes allows for comparison of their OEC organisation, *J. Biol. Chem.* **275:** 27940-27946

Nimer N.A., Guan Q. & Merrett M.J. (1994) Extra- and intra-cellular carbonic anhydrase in relation to culture age in a high calcifying strain of *Emiliania huxleyi* Lohmann, *New Phytol.* **126:** 601-607

Odendaal P. (1997) The future role and functions of the Water Research Commission, Proceedings of the Water Institute of Southern Africa biennial conference and exhibitions, Cape Town, 4-7 May

Orus M.I., Rodriquez M.L., Martinez F. & Marco E. (1995) Biogenesis and ultrastructure of carboxysomes from wild type and mutants of *Synechococcus* sp. strain PCC 7942, *Plant Physiol.* **107:** 1159-1166

Overnell J. (1975) The effect of heavy metals on photosynthesis and loss of cell potassium in two species of marine algae, *Dunaliella tertiolecta* and *Phaedactylum tricornutum*, *Marine Biology* **29:** 99-103

Pace F., Ferrara R. and Del Carratore G. (1977) Effects of sub-lethal doses of copper sulphate and lead nitrate on growth and pigment composition of *Dunaliella salina Teod. Bull. of Environ. Contam. & Toxic.* **17:** 679-685

Palmer C.M. (1980) Algae and water pollution, Castle House Publications Ltd. England, pp 1-5

Passow H., Rothstein A. & Clarkson T.W. (1961) The general pharmacology of the heavy metals, *Pharmacol. Rev.* **13:** 185-224

Patel B.N. and Merrett M.J. (1986) Regulation of carbonic anhydrase activity, inorganic carbon uptake and photosynthetic biomass yield in *Chlamydomonas reinhardtii*, *Planta* **169:** 81-86

Pawlik-Skowronska B. (2001) Phytochelatin production in freshwater algae *Stigeoclonium* in response to heavy metals contained in mining water; effects of some environmental factors, *Aquatic toxicology* **52:** 241-249

Payne R.A. (1997) Unpublished Thesis, Rhodes University, Grahamstown, South Africa

Payne R.A. (2000) *Spirulina* as a bioremediation agent: Interaction with metals and involvement of carbonic anhydrase, Unpublished Thesis, Rhodes University, Grahamstown, South Africa

Pollock S.V. & Colman B. (2001) The inhibition of the carbon concentrating mechanism of the green alga *Chlorella saccharophila* by acetazolamide, *Physiologia Plantarum*, **111:** 527-532

Price G.D., Sultemeyer D., Klughammer B., Ludwig M. & Badger M.R. (1998) The functioning of the CO_2 concentrating mechanism in several cyanobacterial strains: a review of general physiological characteristics, genes, proteins and recent advances, *Can. J. Bot.* **76:** 973-1002

Rai L.C., Gaur J.P. and Kumar H.D. (1981) Mercury toxicity to *Chlorella vulgaris*, *Biol. Rev.* **56:** 99-106

Rai L.C. & Raizoda M. (1987) Toxicity of nickel and silver to *Nostoc muscorum*: interaction with ascorbic acid, glutathione and sulphur-containing amino acids, *Ecotoxicol. Environ. Saf.* **14:** 12-21

Rai P.K., Mallick N. & Rai L.C. (1994) Effect of Cu and Ni on growth, mineral uptake, photosynthesis and enzyme activities of *Chlorella vulgaris* at different pH values, **7:** 56-67

Raven J.A. (1991) Implications of inorganic carbon utilisation: ecology, evolution and geochemistry, *Can. J. Bot.* **69:** 908-924

Raven J.A. (1995) Photosynthetic and non-photosynthetic roles of carbonic anhydrase in algae and cyanobacteria, *Phycologia* **34:** 93-101

Raven J.A. & Lucas W.J. (1985) Energy costs of carbon acquisition. In: Lukas W.J. & Berry J.A. (eds) Inorganic carbon uptake by aquatic photosynthetic organisms. American Society of Plant Physiologists, Rockville, Md, pp 305-324

Raven J.A., Evans M.C.W. & Korb R.E. (1999) The role of trace metals in photosynthetic electron transport in O_2-evolving organisms, *Photosynthesis Research* **60:** 111-149

Renger G. (2001) Photosynthetic water oxidation to molecular oxygen: apparatus and mechanism, *Biochim. Biophys. Acta* **1503:** 210-228

Richmond A. (1986) Handbook of microalgal mass culture, pp 117-145, 212-230, CRC Press Inc., USA

Ritchie R.J., Nadolny C. & Larkum A.W.D. (1996) Driving forces for bicarbonate transport in the cyanobacterium *Synechococcus* R-2(PCC7942), *Plant Physiol.* **112:** 1573-1584

Rotatore R., Colman B. and Kuzma M. (1992) The active uptake of carbon dioxide by the marine diatoms *Phaeodactylum ticornutum* and *Cyclotella sp.*, *Plant Cell Environment* **18:** 913-918

Round F.E. & Chapman D.J. (1982) Progress in phycological research, Volume 1, Elsevier Biomedical Press, Amsterdam, pp 137-144

Schwarz R., Reinhold L. & Kaplan A. (1995) Low activation state of Rubisco in carboxysome-defective *Synechococcus* mutants, *Plant Physiol.* **108:** 183-190

Shiraiwa Y., Goyal A. & Tolbert N.E. (1993) Alkalisation of the medium by unicellular green algae during uptake of dissolved inorganic carbon, *Plant Cell Physiol.* **34:** 649-657

Siedlecka A., Krupa Z., Samuelsson G., Oquist G. & Gardestrom P. (1997) Primary carbon metabolism in *Phaseolus vulgaris* plants under Cd/Fe interaction, *Plant Physiol. Biochem.* **35:** 951-957

Singh N., Asthana R.K., Kayastha A.M., Pandey S., Chaudhary A.K. & Singh S.P. (1999) Thiol and exopolysaccharide production in a cyanobacterium under heavy metal stress, *Process Biochem.* **35:** 63-68

Skowronski T. and Rzeczycka M. (1980) Effect of high zinc concentrations on the growth of *Stichococcus bacillaris* and *Chlorella vulgaris*, *Acta Microbiol.Poll.* **29:** 389-396

Stauber J.L. & Florence T.M. (1987) Interactions of copper and manganese: A mechanism by which manganese alleviates copper toxicity to the marine diatom, *Nitschia closterium* (Ehrenberg), *Aquatic Toxicol.* **9:**356-369

Stauber J.L. and Florence T.M. (1990) Mechanisms of toxicity of zinc to the marine diatom *Nitschia closterium*, *Mar. Biol.* **105:** 519-524

Stemler A. (1993) An assay for carbonic anhydrase activity and reactions that produce radiolabelled gases or small uncharged molecules, *Analytical Biochem.* **210:** 328-331

Sultemeyer D. (1998) Carbonic anhydrase in eukaryotic algae: characterisation, regulation and possible function during photosynthesis, *Can. J. Bot.* **76:** 962-972

Sultemeyer D.F., Miller A.G., Espie G.S., Fock M.P. and Canvin D.T. (1989) Active CO_2 transport by the green alga *Chlamydomonas reinhardtii*, *Plant Physiol.* **89:** 1213-1219

Sultemeyer D.F., Fock H.P. and Canvin D.T. (1990) Mass spectrometric measurements of intracellular carbonic anhydrase activity in high and low C_i cells of *Chlamydomonas*, *Plant Physiol.* **94:** 1250-1257

Sunda W.G. and Guillard R.R. (1976) Relationship between cupric ion activity and the toxicity of copper to phytoplankton, *J. Mar. Res.* **34:** 511-529

Terry P.A. & Stone W. (2002) Biosorption of cadmium and copper contaminated water by *Scenedesmus abundans*, *Chemosphere* **47:** 249-255

Ting Y.P. (1991) Uptake of Cadmium and Zinc by the alga *Chlorella vulgaris*: II. Multi-ion situation, *Biotech. & Bioeng.* **37:** 445-455 van Hille R.P., Boshoff G.A., Rose P.D. & Duncan J.R. (1999) A continuous process for the biological treatment of heavy metal contaminated acid mine water, *Resour. Conserv. & Recycl.* **27:** 157-167

van Hille, R.P. (2002) Biological generation of reactive alkaline species and their application in a sustainable bioprocess for the remediation of acid and metal contaminated wastewaters, Unpublished thesis, Rhodes University, Grahamstown, South Africa

Van Rensen J.J.S., Xu C. & Govindjee (1999) Role of bicarbonate in photosystem II, the water-plastoquinone oxido-reductase of plant photosynthesis, *Plant. Physiol.* **105:** 585-592

Veglio F. & 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., Shutova T., Moskvin O., Forssen M., Klimov V.V. & Samuelsson G. (2002) A photsystem II-associated carbonic anhydrase regulates the efficiency of photosynthetic oxygen evolution, *The EMBO Journal* **21:**1930-1938

Volesky B. (1987) Biosorbents for metal recovery, *Trends Biotechnol.* **5:** 96

Volesky B. (1992) Removal of heavy metals by biosorption, *American Chem. Soc.* 462-466.

Walker D.A. (1991) Measurement of oxygen and chlorophyll fluorescence. In: Scurlock S.P., Long S.P., Hall D.O. & Coombs J. (eds) Techniques in bioproductivity and photosynthesis, Pergamon Press, Oxford, England, pp 95-98

Warburg O. & Krippahl G. (1958) Hill-Reaktionen, *Z. Naturforsch* **13b:** 509-514

Weigel H.J. (1985) The effect of Cd^{2+} on photosynthetic reactions of mesophyll protoplasts, *Plant Physiol.* **63:** 192-200

Wilbur K.M. & Anderson N.G. (1948) Electrometric and colorimetric determination of carbonic anhydrase, *J. Biol. Chem.* **176:** 147-154

Wilde E.W. & Benemann J.R. (1993) Bioremoval of heavy metals by the use of microalgae, *Biotech. Adv.* **11:** 781-812

Williams T.G. & Turpin D.H. (1987) The role of external carbonic anhydrase in inorganic carbon acquisition by *Chlamydomonas reinhardtii* at alkaline pH, *Plant Physiol.* **83:** 92-96

Williams T.G. & Colman B. (1996) The effects of pH and dissolved inorganic carbon on external carbonic anhydrase activity in *Chlorella saccharophila, Plant, Cell and Environment* **19:** 485-489

Wood L. (1974) Toxicity and availability of elements, *Science* **183:** 1048-1052

Wood J.M. and Wang H.K. (1983) Microbial resistance to heavy metals, *Environ. Sci. Technol.* **17:** (Abstr.) 582

Woolery M. & Lewin R.A. (1976) The effects of lead on algae. IV. Effects of Pb on respiration and photosynthesis of *Phaedodactylum tricornutum* (Bacilariophyceae), *Wat. Air Soil Pollut.* **6:**25-31
Wyatt J.M. (1988) Biotechnological treatment of industrial wastewater, *Microbiol. Sci.* **5:** 186-191

Xue H.B., Stumm W. and Sigg L. (1988) The binding of heavy metals to algal surfaces, *Water Res.* **22:** 917-925

Yang S.H., Tsuzuki M. and Miyachi S. (1985) Carbonic anhydrase of *Chlamydomonas*: Purification and studies on its induction using antiserum raised against *Chlamydomonas* carbonic anhydrase, *Plant Cell Physiol.* **26:** 25-34

Zarrouk C. (1966) Contribution a l'etude d'une cyanophycee. Influence de divers facteurs physiques et chimiques sur la croissance et la photosynthese de *Spirulina maxima*. PhD Thesis, University of Paris, France, pp 74

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.

Zouni A., Witt H.T., Kern J., Fromme P., Krauβ N., Saenger W. and Orth P. (2001) Crystal structure of photosystem II from *Synechococcus elongatus* at 3.8 A resolution, *Nature* **409:** 739-743

Websites

http://www.mee-inc.com

http://www.ou.edu

http://www.plantphys.umu.se

http://www.sbu.ac.uk

http://www.ukmarinesac.org.uk

http://www.uksaf.org

Appendices

Appendix A

Preparation of Zarrouk's medium for the cultivation of *Spirulina*

Reagent	Amount Added (for 1L[a])
NaCl	40.0g
K_2SO_4	1.0g
KNO_3	3.0g
H_3PO_4[b]	0.25ml
$NaHCO_3$[c]	16.8g
EDTA	0.08g
$FESO_4.7H_2O$	0.01g
Hunters trace element buffer	1ml

[a] The volume is made up with Milli Q distilled water

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

[c] Varying concentrations of $NaHCO_3$ were used (16.8g/L; 8.4g/L; 4.2g/L; 2.1g/L)

Appendix B

Equations for the calculation of chlorophyll concentrations

Pigments	*Equation*
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 C

Proportion of alkaline material in waters in terms of three types of alkalinity [a]

Result of titration	Caustic or hydroxide alkalinity	Carbonate alkalinity [b]	Bicarbonate alkalinity [c]
Colourless to phenolphthalein Alkaline to methyl orange	0	0	Total alkalinity to methyl orange
Phenolphthalein alkalinity < one half methyl orange alkalinity	0	Twice phenolphthalein alkalinity	Total alkalinity to methyl orange – carbonate alkalinity
Phenolphthalein alkalinity = one half methyl orange alkalinity	0	Methyl orange alkalinity	0
Phenolphthalein alkalinity > one half methyl orange alkalinity	Twice phenolphthalein alkalinity– methyl orange alkalinity	Twice the difference between methyl orange and phenolphthalein alkalinity	0
Phenolphthalein alkalinity = methyl orange alkalinity	Total alkalinity	0	0

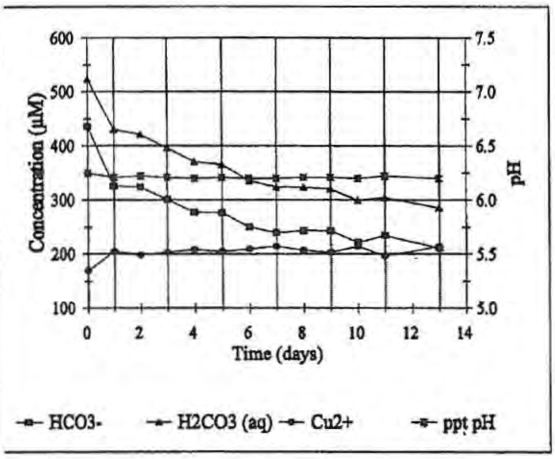
[a] Alkalinity expressed as calcium carbonate

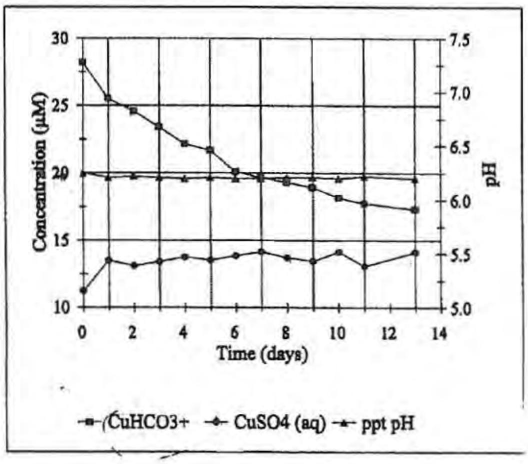
[b] Molarity of carbonate in solution (mM) = carbonate alkalinity/100

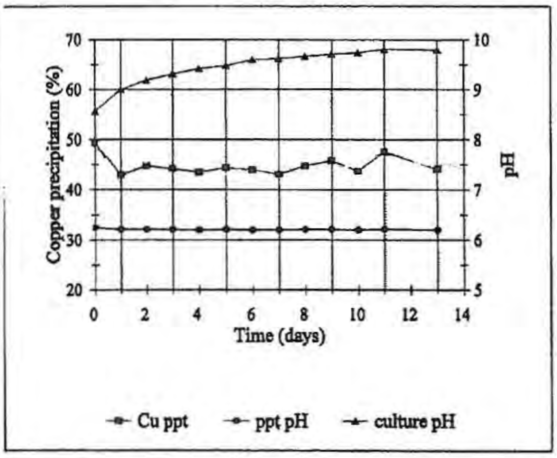
[c] Molarity of bicarbonate in solution (mM) = (bicarbonate alkalinity x 84/50)/84

Appendix D

Predicted effects Cu^{2+} would have on carbonate speciation, using MINTEQA2

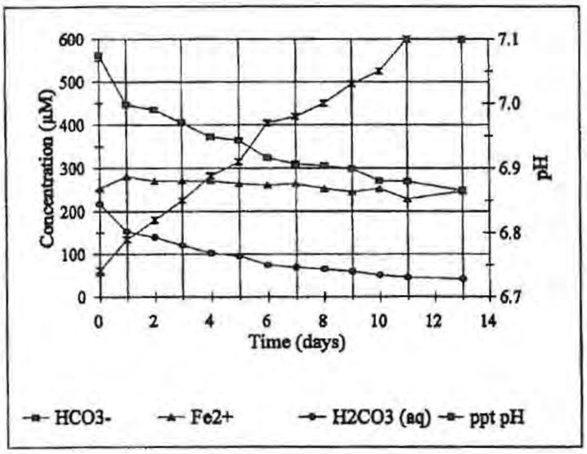


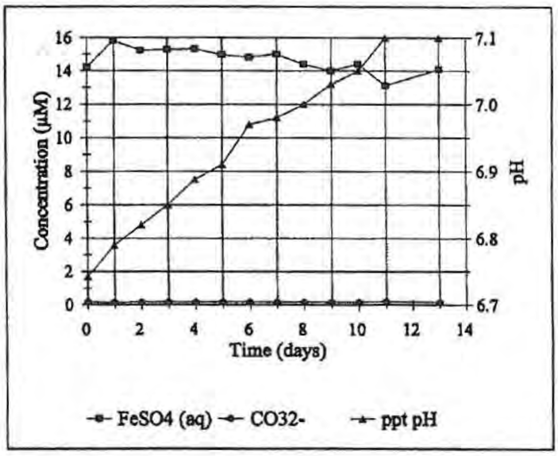


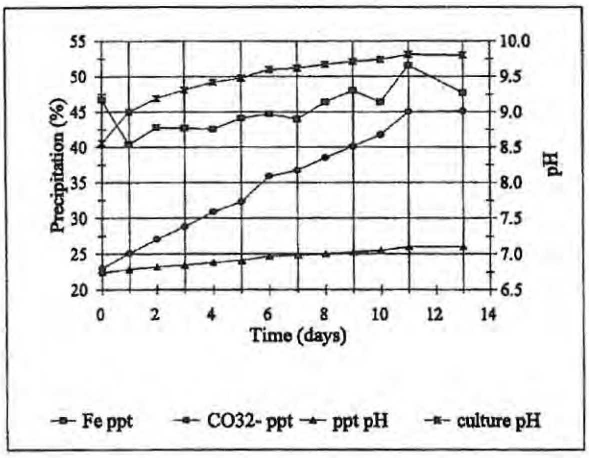


Appendix E

Predicted effects Fe^{2+} would have on carbonate speciation, using MINTEQA2

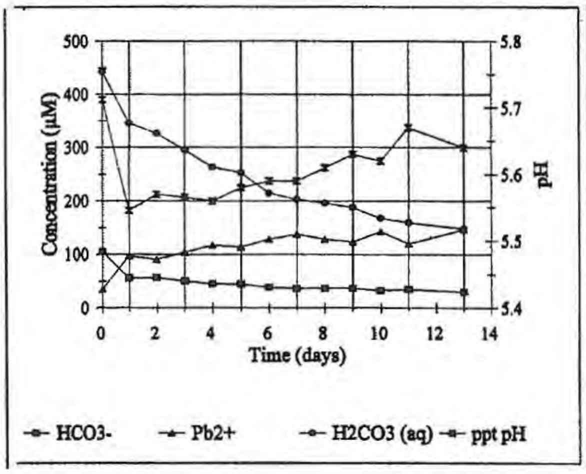


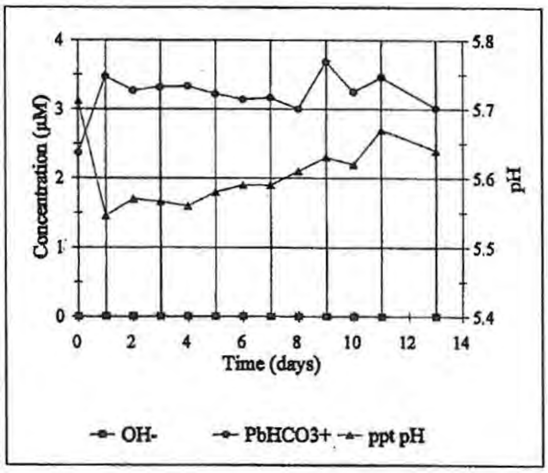




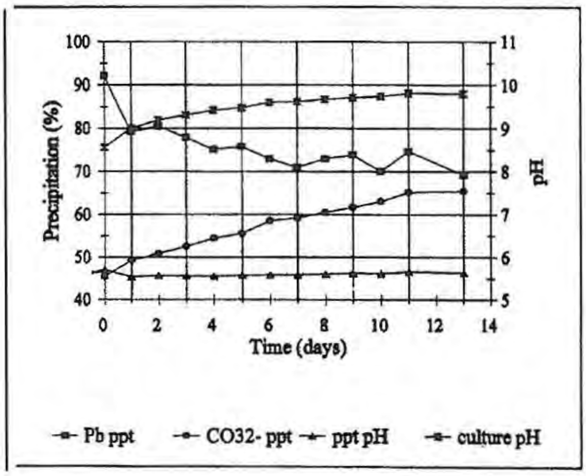
Appendix F

Predicted effects Pb^{2+} would have on carbonate speciation, using MINTEQA2



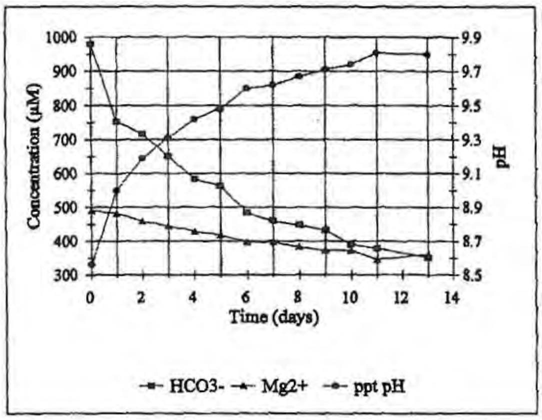


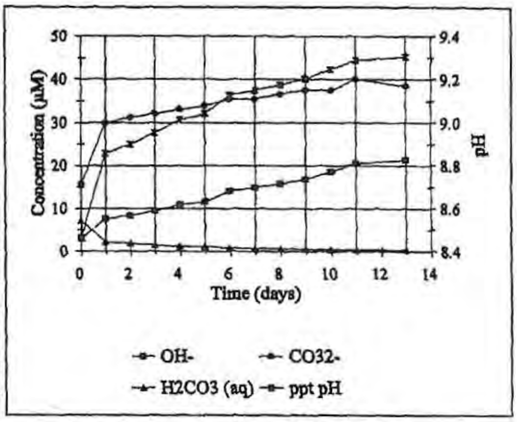
Free CO_3^{2-} concentration < $1x10^{-9}$ throughout experiment

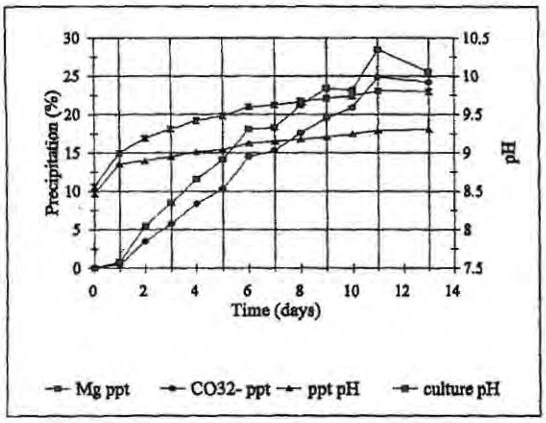


Appendix G

Predicted effects Mg^{2+} would have on carbonate speciation, using MINTEQA2







Appendix H

Predicted effects Ca^{2+} would have on carbonate speciation, using MINTEQA2