DEVELOPMENT OF INTEGRATED BIOSORPTION SYSTEMS FOR THE REMOVAL AND/OR RECOVERY OF HEAVY METALS FROM MINING AND OTHER INDUSTRIAL WASTEWATERS, AND DETERMINATION OF THE TOXICITY OF METALS TO BIOREMEDIATION PROCESSES

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Development of integrated biosorption systems for the removal and/or recovery of heavy metals from mining and other industrial wastewaters, and determination of the toxicity of metals to bioremediation processes

Report to the Water Research Commission

by

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EXECUTIVE SUMMARY

Heavy metal contamination of wastewaters is a major source of environmental pollution and represents a loss of a vital resource since water availability and quality will be a significant factor in the future socio-economic growth of South Africa. The unsuitability of metal-contaminated water for reuse in industrial processing is also an important economic consideration for many industries because of the escalating cost of fresh water supplies. Numerous studies, included those in our laboratory, have shown that biotechnology-based processes may provide effective and inexpensive means of removing heavy metals from water when compared to more traditional methods such as ion-exchange and chemical precipitation.

The objectives of this study were to:

1. Provide a means of cost-effective treatment of heavy metal containing effluents, both for the removal of metals to reduce environmental pollution and to facilitate effective disposal of such effluents, and for the possible recovery and the recycling of the metals in certain instances.
2. Provide a technology for the potential reuse in industrial operations of water that is currently disposed of.
3. Evaluate the potential toxicity of heavy metals to micro-organisms such as bacteria and algae used in bioremediation processes.
4. Train researchers through the participation in the project in the technology of bioremediation of water systems contaminated with heavy metals and thereby develop a local expertise in this field of biotechnology.
5. Develop expertise and capacity in this field of technology amongst black and female students and assist in developing a research culture in this field at institutions such as technikons which were not previously involved in such initiatives.
6. Transfer technology to collaborating industries in the first instance.

In initial studies on the binding and accumulation of heave metals by biomass of the alga, *Spirulina* sp., metal binding was rapid with saturation reached in 30 minutes, and followed an affinity series of Pb>Cu>Zn>>Fe. The binding capacity of the *Spirulina* for each of the metals was relativity low when compared to a range of other biosorbents. The treatment of an effluent from a base metal mine by direct contact with *Spirulina* cultures was effective for

a period of time, although most of the metal removal could be attributed to alkaline precipitation as a result of the high carbonate content of the growth medium.

The toxicity data show that copper and zinc are both acutely toxic to *Spirulina* with threshold toxicity levels of below 10 μmoles of metal per gram of biomass. The mechanism of toxicity appears different, with the results suggesting copper affects the photosynthetic pigments and the integrity of the cell membrane, resulting in chlorophyll breakdown and the leakage of cell contents. Lead was less toxic, but did induce considerable changes in cell morphology with the cells becoming highly distorted. Lead has been reported to affect membrane stability and the SEM images suggest that leakage of water and cellular components induced a loss of turgor pressure in the lead-treated cells. The algae were therefore not suitable for application in a treatment system in which they came into direct contact with the toxic metals.

A significant finding was the ability of the algae to increase the pH of the surrounding medium. This occurred as a result of the accumulation of inorganic carbon, for bicarbonate, as a response to low concentrations of carbon dioxide in the medium, The release of a hydroxide ion into solution led to the increase in pH. The increase in pH was shown to be due to a reduction in acidity, rather than an increase in alkalinity. The enzyme carbonic anhydrase was shown to be pivotal in this system. Attempts to determine the enzyme activity directly were unsuccessful, due to the inherent inaccuracy of the assay system. An indirect method of determining enzyme activity, by measuring changes in the carbonate species equilibrium, was developed. Under optimal conditions *Spirulina* was able to reduce the acidity by an amount equivalent to the addition of 3670 μmoles NaOH $g^{-1}h^{-1}$. Predictive modelling showed that this enhanced the potential of the medium to effect metal precipitation.

In order for such as system to be sustainable, a readily available source of bicarbonate is needed, Clearly the continuous addition of bicarbonate salts would not be a cost-effective solution. The biological generation of bicarbonate by sulphate reducing bacteria (SRB) was investigated in another study. While sulphate reducing bacteria have been the focus of a considerable amount of research, the majority of this has focused on desalination and the production of sulphide for metal precipitation. For most carbon sources, the amount of bicarbonate generated per unit of sulphate reduced is significantly greater than the sulphide

produced. The SRB reactor designed for bicarbonate production generated a ratio of bicarbonate to sulphide of 6:1, three times higher than expected.

Pilot plant studies were subsequently undertaken and demonstrated the potential of an integrated biological system for the treatment of acid mine drainage. The *Spirulina* based system was effective, but suffered from problems associated with maintaining the algae in suspension and preventing biomass loss. These problems were overcome by using *Oscillatoria* in the simulated stream reactors and the system was operated without a loss of efficiency for 90 days. The anaerobic digested component of the integrated system was able to consistently generate bicarbonate at a rate of 4200 µmoles/l/day. The combination of the nitrogen sparging step and the action of the algae was able to reduce the bicarbonate concentration by over 50% and increase the carbonate concentration from 0.07 mM to 12.65 mM. In terms of metal removal, the selective precipitation of over 99% of copper and lead, using hydrogen sulphide gas, was consistently achieved. The blending of the hydrogen sulphide treated acid mine drainage and the algal overflow at an acidity to alkalinity ratio of 1:2 resulted in the precipitation of all the heavy metal, except manganese, to concentrations below the acceptable environmental risk level. It was shown that a portion of the overflow stream from the precipitator could be enriched with a carbon source, ammonia and phosphate, and used as a feed for the anaerobic digester. The remainder of the stream could be discharged to a final polishing step, possibly an anaerobic wetland.

The integrated biological treatment system performed well, effectively treating an effluent modelled closely on the quality of the water being discharged from the East Rand Basin. The cost of such a system would be considerably less than a "high tech" physico-chemical system. This, coupled with the potential long term sustainability of a biological system, would make it a potentially attractive option for the treatment of future acid mine drainage discharges.

In terms of the objectives of this study, the potential toxicity of heavy metals in effluents to organisms used in bioremediation systems was demonstrated and a system of non-contact between the effluent and the organisms was designed. A cost-effective, "low-tech" treatment system for heavy metal containing effluents was also demonstrated and also provided for a

potential technology for reuse in industrial operations of water which currently disposed. In respect of training of researchers and capacity development, this study facilitated the development of local expertise in this field of biotechnology. A number of black and female students participated and were trained on this project as were staff and students at the Vaal Triangle Technikon.

In summary, there are no major obstacles to the scaling up of an integrated biological system such as the one proposed here. The individual reactor components are relatively "low-tech", as are the required control mechanisms. The system has the additional advantage that once the biological components are established, the system is essentially self-sustaining, provided a suitable carbon source is provided. It has been previously noted that considerable research is being conducted into the utilisation of low cost and waste carbon sources as feeds for sulphate reducing systems. The results presented in this study clearly indicate that this type of system could be a viable treatment option for the uncontrolled, long term discharge of acid mine drainage from abandoned mines.

This technology is in the process of being transferred to two major mining concerns. Further studies on the implementation of the process will need to examine the exact composition of the effluents and to model the associated water chemistry in order to optimise the reactor design and operation.

This report will deal with experimental studies designed to achieve these. An overview of the previous studies and literature related to this field is presented followed by a series of chapters daeling with the toxicity of heavy metals to algae, the possible direct treatment of acid mine drainage by algal biomass, and algal modification of the aqueous environment to facilitate heavy metal precipitation. The final chapter deals with the use of pilot scale integrated biological systems for the treatment of acid mine drainage. Each chapter has a relevant introduction section followed by a detailed description of materials and methods, results and conclusions.

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The steering committee responsible for this project consisted of the following persons:

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CHAPTER 1: LITERATURE REVIEW

1.1 Water as a resource in South Africa

South Africa (SA) is a developing country that is water scarce and water stressed. While water conservation (WC) and demand management (DM) are topics that are commonly referred to in water resources literature in South Africa, the need and value of WC/DM principles have only recently been fully recognised and appreciated. The realities of the new democratic South Africa demand improved management of the country's limited water resources (DWAF, 1999).

The demand on South Africa's limited water resources is growing as a result of a high population growth rate, a developing economy and the urgent need to supply water services to millions of people who have previously been without them. The sustainability of SA's water resources is threatened both in terms of quantity and quality. If the current patterns of water usage are maintained, all renewable water resources in SA will be fully committed (Odendaal, 1997; DWAF, 1999). Already there are a number of regions in the country that rely on expensive transfer schemes as water demand has far exceeded water availability.

Some of the reasons contributing to the challenges of ensuring sustainable water resources for the future are:

- While the climate varies from rain forest to desert, the typical climate of SA is semi-arid, with the average rainfall, 475mm per annum, being just over half the global average.
- Rainfall patterns are variable, with droughts followed by floods commonly occurring.
- The distribution of rainfall is uneven, with 60% of the river flow arising from only 20% of the land area.
- Limited groundwater.
- Some metropolitan and industrial growth centres have developed around mineral deposits and are far from major water resources.
- South Africa's average evaporation rate exceeds its precipitation rate.
- A population growth rate of 2-3% per annum.
- A large backlog of housing and service delivery.

While alternative water sources, such as desalination and icebergs, are possible they are prohibitively expensive in view of South Africa's socio-economic needs. Therefore, the need to develop effective WC/DM strategies is acute (DWAF, 1999).

Reducing water demand reduces water withdrawals impacting on the environment and results in increased stream flows and reduced demand on groundwater sources. Current land and water utilisation in South Africa is having a detrimental effect on the ecology of rivers, lakes, wetlands and estuaries. These threatened ecosystems need to be protected from the over utilisation of water resources and the continued development of new dams (DWAF, 1999).

The protection of existing water resources through conservation measures is of great importance. Examples include:

- The removal of alien vegetation, which has reduced surface runoff and the yield of existing resources. Catchments cleared of invading alien plants show improved yields of up to 10%.
- Rehabilitation of wetlands.
- Protection of groundwater resources.
- Minimising pollution of water resources.

1.2 Water resource policy and legislation

Management of natural resources and the environment has progressed rapidly in South Africa in recent years. This is a result of the global trend towards increased knowledge of the environment and the need to protect it, as well as a massive change in the political perspectives in the country (Harris *et al.*, 1999).

The Department of Water Affairs and Forestry has reviewed water laws, both locally and abroad in preparation for new legislation designed to reflect democratic principles and equitable resource access by all. The development of new legislation presented an ideal opportunity to achieve the objectives of providing more equitable access to water resources and consolidate much of the recent development in water resource thinking (Harris *et al.*, 1999; Singh and Constantinides, 2000).

The Department's slogan of "some for all, forever" succinctly captures the goal for water

resource management and service provision in South Africa. It recognises that water is an essential, finite resource and indicates a fundamental commitment to equitable allocation. Finally it acknowledges the goal of sustainable use.

1.2.1 Ecological integrity

Ecosystems have an inherent recovery capacity in response to variable conditions, such as floods and droughts, but this ability is finite. As the maximum recovery capacity is assumed to exist in a natural, unmodified system, the degree to which a specific component of the water resource differs from its natural state is important in estimating its ability to recover. The degree of modification of a particular part of a water resource can be assessed by measuring components of its ecological integrity (Harris *et al.*, 1999).

The ecological integrity of a water source is its ability to maintain a balanced, integrated composition of physico-chemical habitat characteristics and biotic components on a temporal and spacial scale. These should be comparable to the natural characteristics of ecosystems in the region. Ecological integrity implies the ecosystem's structure and functions are not impaired by anthropogenic stresses. A resource will only be able to provide for long term water uses if its ecological integrity is assured (Harris *et al.*, 1999, Singh and Constantinides, 2000).
Many of South Africa's water resources have already been modified by use and development and are no longer in their natural state, but this does not mean they are no longer sustainable. In such cases the effects of these uses need to be determined and those uses that can be sustained by the water resource, so that its integrity remains at an acceptable level, need to be identified (Harris *et al.*, 1999).

The concept of a water "Reserve" has been defined and is intended to protect water resources, so that basic human needs can be met and ecological functions and processes can be sustained. The Reserve consists of two parts, the basic human needs reserve and the ecological reserve. The basic human needs reserve provides for the essential needs of individuals served by the water resource in question and includes water for drinking, for food preparation and for personal hygiene. The ecological reserve relates to the water required to protect the aquatic ecosystem of the water resource. The Reserve for all or part of any significant water resource is determined by the Minister of Water Affairs and Forestry (National Water Act, 1998).

Components of ecological integrity, such as the chemical and physical characteristics of the water, the quantity of water, the habitat and the structure and function of the associated biotic communities are measured to ensure the Reserve does indeed protect the ability to meet human needs and sustain ecological functions. The ongoing monitoring and assessment of the condition of the water resources, their response to impacts and the state of the Reserve are critical to the management and protection of resources. The aim is for management decisions to be made on the basis of sound scientific and technical information and understanding (Harris *et al.*, 1999; Singh and Constantinides, 2000).

1.2.2 Policy for protecting water resources

The enactment of the National Water Act (1998) and the Water Services Act (1997) made various requirements and provisions for the implementation of water conservation and demand management principles. The Water Services Act sets out a framework to ensure the provision of basic water supply and sanitation and a regulatory framework for water services institutions. The National Water Act ensures that the nation's water resources are protected, used, developed, conserved, managed and controlled in ways which take into account, amongst other factors, promoting the efficient, sustainable, and beneficial use of water in the public interests. Furthermore, the Act gives the Minister broad powers to make regulations limiting or restricting the purpose, manner or extent of water use as well as the attachment of conditions relating to water resource protection to every authorisation or licence issued. Finally the Act requires the establishment of a national water resource strategy and a catchment management strategy, to provide for the development, management and protection of water resources and catchments (DWAF, 1999).

The policy integrates resource-directed measures for protection (such as resource quality objectives) with source-directed measures (such as effluent standards). It includes:

- Setting water resource-based objectives which clearly define acceptable values for water resources for each of the components (chemical, physical, biological) of ecological integrity.
- Use of source-directed standards which clearly define acceptable values for waste discharge or impact generation and encourage movement towards minimisation of waste disposal and impacts.

- Where source-directed standards cannot be met in the short term, a temporary exemption from the standards could be considered if an impact assessment indicates that the water resource-based objectives could still be met.

Before any potentially hazardous waste can be disposed of, a waste disposal site permit needs to be issued by the Department of Water Affairs and Forestry. The applicant is required to adhere to a series of "minimum requirements" before a permit is issued. The objectives of the minimum requirements are to ensure the protection of SA's water resources, to ensure a nationally uniform approach to waste disposal and to make South African waste management practices internationally acceptable (DWAF, 1998).

The minimum requirements are enforceable under the National Water Act (1998) and the Environment Conservation Act (1989). They allow for the suspension and revocation of disposal permits and the recovery of remediation costs from any person responsible for the pollution of water resources.

A cornerstone of the minimum requirements is the determination of the estimated environmental concentration (EEC) for particular hazardous substances. The EEC is calculated based on acceptable risk criteria. The aquatic environment has been chosen to quantify the risk to man and the environment. Since the aquatic environment is extremely sensitive to contamination and pollution it is possible to prove that if the aquatic environment is not at risk (being within acceptable risk), that man and more robust ecosystems should also not be exposed to unacceptable risk. The acceptable risk levels have been set at one tenth of the LC_{50} (the concentration determined to kill 50% of specific warm and cold water fish or aquatic invertebrate species) and have been statistically shown to result in a mortality incidence of 1 in 300 000 in the aquatic environment (DWAF, 1998). The acceptable risk values for the elements of interest to this study are listed in appendix A.

1.3 Water pollution

Human activities have resulted in the widespread pollution of the natural environment by both organic and inorganic contaminants. This study concentrates on inorganic contaminants, particularly heavy metals and salinity.

A study by the Council for Scientific and Industrial Research (CSIR) in 1991 indicated that the quality of South Africa's water resources is declining, primarily as a result of salinisation, but also as a result of metal pollution and eutrophication (Neytzell-De Wilde, 1992). The major ions responsible for salinity related problems are calcium, magnesium, sodium, chloride, sulphate and bicarbonate. High concentrations of these ions may detrimentally affect plant and animal health by affecting water and nutrient intake. While a portion of these ions may enter waters naturally, by leaching from sedimentary deposits, the major source is anthropogenic, primarily as a result of mining and related industries and to a lesser extent agriculture (Backeburg *et al.*, 1996; Casey *et al.*, 1996).

The heavy metals consist of a group of elements with densities greater than five. Many of these elements are essential micronutrients, but at elevated concentrations they become acutely toxic to living organisms. The incidence of heavy metal pollution in surface and groundwaters is a direct result of industrial activities, primarily mining, refining, electroplating and manufacturing.

Heavy metals have a profound effect on aquatic ecosystems. At low (sub-lethal) concentrations they are accumulated by many microbial species and are increasingly concentrated at higher levels in the food chain. Higher concentrations of acutely toxic metals such as copper, lead, zinc, cadmium, nickel and mercury have a devastating effect on aquatic organisms. Their effect on the organisms is diverse and includes the denaturation of proteins (Gadd and Griffiths, 1978), the inactivation of enzymes (Teisseire and Vernet, 2000), the retardation of electron transport chains and photosynthesis (Asthana *et al.*, 1992; Abalde *et al.*, 1995; Barón *et al.*, 1995; Jin *et al.*, 1996) and the destabilisation of membrane structures (Brady and Duncan, 1994; Franklin *et al.*, 2000). While metals such as iron, aluminium and manganese are less toxic to aquatic organisms, they form bulky hydroxide and oxide precipitates in oxygen-rich waters. These precipitates can still have a serious effect on aquatic invertebrates by coating their gills and breathing apparatus as well as reducing adhesion points to the substrate (Younger, 1995; Jooste and Thirion, 1999).

The major source of both salinity and heavy metal contamination of water resources in SA is acid mine drainage, originating from operating and abandoned mines as well as rock piles and tailings.

1.4 Treatment strategies

1.4.1 Chemical precipitation

Chemical treatment strategies for AMD remediation have two primary goals, the neutralisation of pH and the precipitation of heavy metals. The most commonly employed chemical treatment method is the addition of lime. The lime may be added in the unslaked, hydrated ($Ca(OH)_2$) or dolomitic ($CaMg(CO_3)_2$) form. Each form has associated advantages and disadvantages, for example hydrated lime reacts faster than the dolomitic form, but is almost three times as expensive and produces greater sludge volumes (Maree *et al.*, 1992).

All heavy metals encountered in AMD waters form complexes with hydroxide species. These complexes affect the solubility of the particular mineral. Considering the generic divalent metal ion (M^{2+}) it is clear that up to three hydroxide complexes can be formed (MOH^+, $M(OH)_2^0$ and $M(OH)_3^-$) depending on the pH. While the positive charged complex has meaning considering the aqueous phase alone, the remaining two only have significance in determining metal ion solubility (Loewenthal *et al.*, 2001). The two phase equilibrium diagram (Figure 1.1) for ferrous iron illustrates this phenomenon. This has implications for lime dosing, with a relatively specific amount needing to be added to achieve optimal metal removal. From figure 1.1 it is evident that optimal $Fe(OH)_2$ precipitation is only achieved in the region of pH 11 and that below pH 10 the Fe^{2+} and $Fe(OH)^+$ species dominate.

As ferrous iron is the dominant heavy metal in most AMD discharges and the efficient precipitation of $Fe(OH)_2$ requires a high pH, a modified process is most commonly employed. The high density sludge (HDS) process involves the initial oxidation of the ferrous iron to the ferric form at a high oxygen concentration and a relatively low pH. The ferric iron subsequently precipitates and settles (at pH 6) as amorphous ferric hydroxide which upon dehydration changes to FeOOH and Fe_2O_3 as the final products. The sludge is recycled and contacted with lime in order to induce crystallisation. This process has proven effective for iron removal, but has little capacity to remove heavy metals and salinity (Loewenthal *et al.*, 2001).

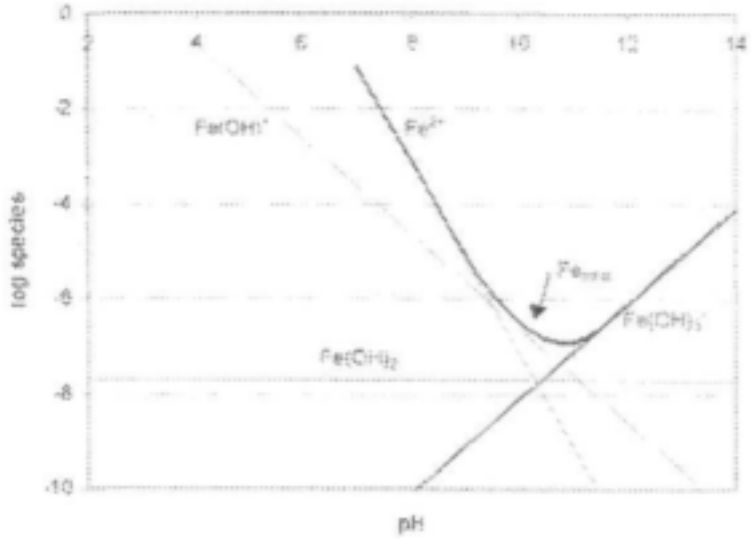


Figure 1.1. Two phase equilibrium diagram for ferrous iron.

An alternative to the HDS process is a sequential process where neutralisation and the precipitation of some heavy metals as hydroxides is achieved by lime addition and ferrous iron is removed by sulphide addition. A range of sulphide salts has been utilised, but barium sulphide (BaS) is preferential as the barium will not persist in solution in the presence of sulphate ions. Therefore, the use of BaS can reduce the salinity of the AMD (Larson *et al.*, 1973). The precipitation of ferrous iron by sulphide addition is not without disadvantages, the main one being the formation of a colloidal precipitate that has poor settling characteristics. The potential of biologically generated hydrogen sulphide has also been investigated and will be discussed in more detail later.

Based on the shortcomings of the above processes a number of authors have proposed an alternative system, the Ferrite Process, which is capable of removing iron and heavy metals in a single process, without the necessity of further sludge treatment (Wang *et al.*, 1996; Barrado *et al.*, 1998; Loewenthal *et al.*, 2001). The term "ferrite" refers to magnetic oxides containing other metals in addition to iron. The general chemical formula of ferrites is MFe_2O_4, where M is any divalent ion with an unhydrated ion radius of between 0.6 an 1.0Å.

Ferrite is formed via partial oxidation of ferrous species at pH values of greater than 7. The reaction has been shown to proceed through two pathways, depending on the pH and rates of oxidation. At pH values of 7-10 and relatively slow oxidation rates, ferrites form through the Green Rust (GR) path. At pH values of 10.5 and greater they form from the FeOOH phase.

Green rusts are unstable green-blue compounds consisting of a mixture of ferrous and ferric ions accompanied by Cl^-, SO_4^{2-} or CO_3^{2-} and OH^- ions. Ferrite formation through the GR pathway at pH<10 has been shown to result in relatively high residual iron and heavy metal concentrations in solution, as well as inferior sludge settling properties (Perez *et al.*, 1998; Loewenthal *et al.*, 2001).

Ferrite formation at pH>10 showed excellent removal efficiencies (>99%) and good sludge characteristics. Barrado *et al.* (1998) proposed the following reactions for ferrite formation from ferrous at pH>10.5:

1) In the absence of heavy metals and controlled oxidation rate:

$$3FeSO_4 + 6NaOH + 0.5O_2 \rightarrow Fe_3O_4 + 3Na_2SO_4 + 3H_2O \quad (1)$$

2) In the presence of heavy metals and controlled oxidation rate:

$$xM^{+n} + 3FeSO_4 + 6NaOH + 0.5O_2 \rightarrow M_xFe_{3-x}O_4 + 3Na_2SO_4 + 3H_2O + xFe^{+n} \quad (2)$$

Fe^{+n} represents the total concentration of iron whose precipitation as ferrite is impeded by the other metal cations present in the water. If the pH is maintained at greater than 10 these ferrous and ferric ions will react again to form magnetite (Fe_3O_4).

The ferrite process is most efficient where the iron to heavy metals ratio is 10-20:1. To achieve this, $FeSO_4$ is added to the solution. The pH is raised to over 10 at 65°C and oxygen is introduced to effect the partial oxidation of ferrous iron. The resulting precipitates are stable and can be easily recovered by magnetic filtration. In addition, as the heavy metals are incorporated into the lattice structure they are not susceptible to leaching from the sludge upon ageing. The major disadvantage of the standard ferrite process is the high temperature, which introduces prohibitive energy costs. The major focus of research in this field is on developing an efficient ambient temperature process (Loewenthal *et al.*, 2001).

1.4.2 Adsorption and ion exchange

One of the major drawbacks of treatment systems based on chemical precipitation is their poor efficiency when treating effluents with low metal concentrations. The removal of inorganic pollutants from dilute aqueous media can be achieved by adsorption and ion exchange mechanisms. Adsorption refers to the binding of charged species in solution to reactive groups, of opposite charge, on a solid support. Much of the research in recent years has focussed on the use of cells or sorbents derived from biological material (Kratochvil and Volesky, 1998). A wide range of biosorbents have been tested, including yeast (Brady and Duncan, 1994; Ashkenazy *et al.*, 1997), fungi (Tobin *et al.*, 1990; 1993), algae (Ting *et al.*, 1989; Holan *et al.*, 1993; Leusch and Volesky, 1995; Kratochvil and Volesky, 1998) and the aquatic fern *Azolla* (Zhao and Duncan, 1998; Sanyahumbi *et al.*, 1998). The most important metal binding sites on the surface of biological material are the carboxyl, phosphoryl, amino and thiol groups.

Ion exchange refers to the replacement of toxic heavy metal ions in solution by more benign counter-ions that balance the surface charge of the solid exchanger. The ion exchange resins are derived from both natural (zeolite) and synthetic (synthetic polymers) sources and can be manufactured to contain single or multiple functional groups, depending on the application (Ahmed *et al.*, 1998; Chiarle *et al.*, 2000).

Both biosorbents and ion exchange resins have been successfully utilised in low volume effluent treatment systems. The most common reactor configurations are the packed and fluidised bed reactors, as well as a number of column designs. Their application to high volume effluents such as AMD are limited by reactor design and flow dynamics, with a typical unit containing between 20 and 100kg of biomass (Gadd and White, 1993).

1.4.3 Membrane technologies

A number of membrane based technologies have been developed for the treatment of wastewaters polluted with heavy metals. Both electrodialysis and tubular reverse osmosis reactors have demonstrated the ability to desalinate non-scaling mine waters, with the former producing water fit for human consumption. The majority of AMD waters contain high concentrations of sodium and sulphate, leading to scale formation on the membrane surface. There have been some advances in the field with the development of the SPARRO (Slurry

Precipitation and Recycle Reverse Osmosis) process, which partially overcomes the scaling problem and reduces operation costs (Juby *et al.*, 1996). The construction and operation costs of membrane reactors, coupled with the volume of polluted water discharges from South African mines limit the large scale application of this technology.

1.4.4 Active biological treatment systems

Active biological systems for the treatment of AMD are primarily dependent on the action of sulphate reducing bacteria (SRB). The bacteria are able to utilise sulphate as the terminal electron acceptor to oxidise organic carbon, producing bicarbonate and hydrogen sulphide as by-products. The biochemical pathways involved are complex and will be discussed in greater detail in the relevant chapter of this thesis. A simplified overview of the reaction is shown below:

$$2CH_2O + SO_4^{2-} \rightarrow 2HCO_3^- + H_2S \qquad (3)$$

The form of the alkalinity and the sulphide will depend on the pH and aqueous chemistry of the system. The sulphide reacts with metals in solution to form insoluble metal sulphides and the bicarbonate alkalinity helps to raise the pH of the AMD.

The SRB are grown in purpose built bioreactors under controlled conditions. A number of reactor designs have been tested, including anaerobic filters (Chian and De Walle, 1983), packed bed anaerobic reactors (Maree *et al.*, 1987), mixed systems (Maree and Hill, 1989), fluidised bed systems (van Houten *et al.*, 1994), sequencing batch reactors (Herrera *et al.*, 1991) and upflow anaerobic sludge blanket systems (Barnes *et al.*, 1991). A wide range of simple and complex organic carbon sources have been investigated.

There have been few successful industrial scale applications of this technology, although Shell Research Ltd successfully ran a pilot plant at the Budelco BV zinc refinery in the Netherlands, which was later scaled up to a system capable of treating 7Ml day^{-1}. The system uses a selected, but undefined consortium of sulphate reducing bacteria, with ethanol as the carbon source. The final concentration of all heavy metals was reduced to the ppb range and the sulphate concentration was reduced by over 80% (Barnes *et al.*, 1991).

1.4.5 Passive treatment systems

Natural processes commonly ameliorate mine drainage pollution. As contaminated mine drainage flows through receiving systems (streams, rivers and lakes) its toxicity decreases naturally as a result of biological and chemical reactions as well as by dilution with uncontaminated waters. The low pH typical of many mine drainages increases as the water mixes with less acidic water or through dissolution of carbonate minerals. Metals contained in the mine water then precipitate as oxides or hydroxides under the aerobic conditions found in most surface waters. Ferric iron, aluminium and to a lesser extent manganese are the first metals to precipitate out (Hedin *et al.*, 1994).

The passive treatment of mine water developed from pilot scale plants to full scale field implementation during the 1980's. Passive treatment technologies take advantage of natural chemical and biological processes. Ideally these systems require no addition of chemicals and little or no operation and maintenance inputs. Passive systems depend on processes that are slower than those of active treatments and thus require longer retention times and larger areas to achieve similar results (Hedin *et al.*, 1994).

The goal of passive systems is to enhance natural processes so that they occur within the treatment area and not in the receiving water body. There are two factors which determine if this can be achieved, the kinetics of the contaminant removal processes and the retention time within the system. The retention time for a particular site is often determined by the available land area. However, the kinetics of contaminant removal processes can often be affected by manipulating the environmental conditions that exist within the passive treatment system. Efficient manipulation of contaminant removal processes requires that the nature of the rate-limiting aspects of each removal process be understood (Hedin *et al.*, 1994). The development of passive systems from an experimental concept to full scale field application occurred during the 1990's. While over 200 wetlands have been operating in the Appalachia region of the US, their application has been limited to acid waters with low metal content (Gazea *et al.*, 1996).

1.4.5.1 History of passive treatment

The interest in passive systems was sparked by research in the late 1980's which indicated that natural *Sphagnum* wetlands improved the quality of mine drainage without incurring any obvious

ecological damage (Wieder and Lang, 1992). A number of experimental wetlands were constructed to mimic the *Sphagnum* moss wetlands. However, *Sphagnum* moss was not readily available, proved difficult to transplant, and had the tendency to accumulate metals to toxic levels within several months (Spratt and Wieder, 1988). Despite these initial setbacks research continued and eventually a design evolved that proved tolerant to years of exposure to contaminated mine drainage and was effective at lowering the concentrations of dissolved metals. They typically consisted of a series of small wetlands (<1 ha) vegetated with cattails (*Typha latifolia*) (Wieder, 1989).

During the development of the most recently applied passive treatment schemes the importance of anaerobic processes in metal removal was identified. In such systems a complex ecosystem is not needed and the treatment cells operate effectively without plants. Pretreatment systems have also been developed which involve contacting the acidic waters with limestone in an anoxic environment before it enters a settling pond or wetland system (Gazea *et al.*, 1996).

1.4.5.2 Mechanisms for contaminant removal

A number of physical, chemical and biological processes are known to occur within passive treatment systems to reduce metal concentrations and neutralise the acidity of the influent water. The relative importance of the various processes is difficult to assess due to a lack of accurate quantitative data (Gazea *et al.*, 1996).

The simplest mechanism encountered in a passive wetland system is the dilution of contaminants by inflows of uncontaminated water. Large volume inflows may cause significant changes in the water chemistry that might be mistakenly attributed to biological or chemical processes (Hedin *et al.*, 1994).

After accounting for dilution there are four major processes which can occur in passive systems to reduce the acidity and lower the concentration of metal ions in solution. These are:

- oxidation and hydrolysis
- metal removal by plants, algae and organic substances
- reduction
- limestone addition

1.4.5.3 Oxidation and hydrolysis

The mechanism of oxidation of ferrous to ferric iron has been previously covered. This process occurs abiotically, but can also be catalysed by bacteria. The kinetics of both mechanisms are pH dependent. At pH values of 5 and above the kinetics of oxidation can be described by the following equation:

$$-(d[Fe^{2+}]/dt) = k_1[Fe^{2+}][OH^-]^2 {}_PO_2 \quad (4)$$

where $k_1 = 8.0 \times 10^{13}$ min^{-1} atm^{-1} mol^{-2} l^2 and ${}_PO_2$ = partial pressure of oxygen

From the $[OH^-]^2$ it is clear that for each unit the pH is raised, above 5, the reaction rate increases 100-fold. Under these conditions the most important role of the constructed wetland is to provide a retention time that is sufficient for the dissolved iron to oxidise and precipitate (Gazea *et al.*, 1996).

At lower pHs (pH < 3.5) the kinetics of abiotic oxidation is described by the following equation:

$$-(d[Fe^{2+}]/dt) = k_2[Fe^{2+}]_PO_2 \quad (5)$$

where $k_2 = 1.0 \times 10^{-7}$ min^{-1} atm^{-1}

Under these conditions the oxidation is clearly slow and the contribution of iron oxidising bacteria becomes important. Species such as *Acidothiobaccillus* and *Leptobacillus ferrooxidans* have a pH optimum of between pH 1.5-3.5. They are able to increase the ferrous iron oxidation rate by several orders of magnitude. In experimental aerobic wetland systems the numbers of iron oxidising bacteria has been positively correlated with iron oxidation and removal (Gazea *et al.*, 1996).

As ferrous iron is oxidised to ferric, it is subjected to hydrolysis that results in the rapid precipitation of ferric hydroxide and the release of proton acidity, the mechanisms of which have been discussed previously. Based on the solubility of ferric hydroxide ($K_{sp} = 6.0 \times 10^{-38}$) it is clear that under equilibrium conditions negligible concentrations of dissolved ferric iron exist at pH values above 3. Natural or constructed aerobic wetlands that receive neutral iron containing

waters typically discharge acid water (pH 2-3) as a result of iron oxidation and hydrolysis (Gazea *et al.*, 1996).

Manganese is the second important component of most acid mine drainages that can be removed by oxidation and hydrolysis. Typically manganese is discharged to the surface as the Mn^{2+} ion and for efficient hydroxide precipitation to occur requires the oxidation of the Mn^{2+} to a higher oxidation state. The exact mechanisms occurring in aerobic wetlands have not been fully elucidated, but it has been suggested that Mn^{2+} is oxidised to either the trivalent or tetravalent form, which then precipitates as MnOOH (Hedin *et al.*, 1994). The proposed reaction is shown below:

$$4Mn^{2+} + O_2 + 6H_2O \rightarrow 4MnOOH + 8H^+ \quad (6)$$

Over time the MnOOH is likely to oxidise to the thermodynamically more stable MnO_2. As with iron, the oxidation of manganese is strongly dependent on pH and occurs very slowly at pH values below 8. The process may be accelerated by the action of manganese oxidising bacteria, which are most readily found at pHs of 7 and above (Gazea *et al.*, 1996). The bacteria oxidise Mn^{2+} to MnOOH or the manganate ion (Mn^{4+}).

In alkaline environments manganese can also precipitate in the carbonate form. In the presence of atmospheric oxygen the carbonate may oxidise further to the more stable oxide as shown below:

$$Mn^{2+} + HCO_3^- \rightarrow MnCO_3 + H^+ \quad (7)$$

$$2MnCO_3 + O_2 \rightarrow 2MnO_2 + 2CO_2 \quad (8)$$

Data collected from wetland applications has shown that when a minewater contaminated with both iron and manganese passed through the system the manganese removal was far less effective than the iron and occurred sequentially, not simultaneously (Hedin *et al.*, 1994). There are two main factors which contribute to this phenomenon. Both mechanisms are pH dependent, but the oxidation of iron occurs more readily and is optimal at lower pH values. The oxidation and hydrolysis of iron releases further proton acidity which makes the conditions for manganese oxidation even less favourable. The second factor is the reduction of oxidised manganese by ferrous iron:

$$MnOOH + Fe^{2+} \rightarrow FeOOH + Mn^{2+} \quad (9)$$

The net result is that manganese removal in passive aerobic systems is a slow and inefficient process, even in situations where ferrous iron concentrations are low (Gazea *et al.*, 1996).

1.4.5.4 Metal removal by plants and algae.

Emergent vegetation is one of the components of aerobic wetland systems. While accumulation of metals in the plant tissues plays only a minor role in metal removal the presence of plants has a number of associated benefits. Plants are able to diffuse oxygen from their roots, generating localised oxidising zones within the substrate which promote metal removal by oxidation.

Decaying plant material is an important component of a number of other processes which occur in the wetland. It is used as an organic carbon source for sulphate reducing bacteria and contains carboxylic and phenolic acids which are able to complex metal ions from solution. In addition, the presence of emergent vegetation enhances the aesthetics of the treatment system and reduces erosion and dispersal of metal precipitates by wind during dry periods (Gazea *et al.*, 1996).

A considerable amount of research has been conducted on the ability of algae to reduce the concentration of metals in solution. This is achieved by adsorption onto charged functional groups on the cell surface and by accumulation of the metal inside the cell. The mechanisms involved will be discussed in greater detail in chapter two.

1.4.5.5 Reduction processes

Bacterial sulphate reduction under anaerobic conditions can be an important factor in determining the final water quality discharged from the passive treatment system. Sulphate reduction occurs in the organic substrate layer, where bacteria such as members of the *Desulfovibrio* species use sulphate to oxidise organic matter and release bicarbonate and hydrogen sulphide (Kalin *et al.*, 1991).

Bacterial sulphate reduction is limited to certain environmental conditions. The process is curtailed at pH values below 4 and in the presence of oxidising agents such as O_2, Fe^{3+} and Mn^{4+}.

Typically the conditions which favour bacterial sulphate reduction can be maintained in an anoxic wetland environment (Gazea *et al.*, 1996).

The hydrogen sulphide reacts with some dissolved metal ions to form insoluble metal sulphides, which may then precipitate. The removal of dissolved metals in the sulphide form depends on the pH, the solubility of the specific metal sulphide and the concentration of reactants.

While low pH values inhibit the growth and activity of SRB, their activity results in an increase in alkalinity and pH in the surrounding micro-environment. As a result they have been found to be active in sediments below extremely acidic waters (Herlihy *et al.*, 1987). They are also tolerant of relatively high concentrations of toxic heavy metals, with exposure to zinc, lead and nickel at concentrations of 60mg/l not resulting in significant inhibition.

While sulphate reduction is the most important source of alkalinity in recently commissioned wetlands, there is evidence to suggest that in more established wetlands the reduction of ferric iron to ferrous iron is the major source of alkalinity (Vile and Wieder, 1993).

1.4.5.6 Limestone addition

The addition of limestone may be employed where the mine drainage has a particularly high acidity. The limestone dissolves to produce calcium and bicarbonate alkalinity, which neutralises acidity and buffers the pH. The solubility of limestone depends on pH, temperature and carbon dioxide concentration. When acid water, pH < 6.4, contacts limestone, the limestone reacts according to the equation:

$$CaCO_3 + 2H^+ \rightarrow Ca^{2+} + H_2CO_3^* \qquad (10)$$

Dissolved carbon dioxide, conventionally noted as $H_2CO_3^*$, is a weak acid and continues to react with limestone, producing calcium and bicarbonate alkalinity which is available for acid neutralisation reactions (Gazea *et al.*, 1996).

In practice, the effectiveness of this process is reduced where the influent water is high in ferrous iron and contacts limestone in an oxidising environment. The resulting formation of ferric hydroxide precipitates leads to coating of the limestone surface and reduces dissolution to almost

nil. In anoxic environments, where the iron is maintained in the ferrous form and no precipitate forms at pH < 5.5, limestone addition can be an effective method of reducing acidity (Gazea *et al.*, 1996).

1.4.6 Types of passive treatment systems

There are three principal types of passive technologies developed for the treatment of mine discharges. They are:

1. aerobic wetland systems
2. anaerobic organic substrate systems
3. anoxic limestone drains

1.4.6.1 Aerobic wetland systems

Aerobic wetlands have been effectively used to treat net alkaline waters, which typically contain enough alkalinity to buffer the acidity produced by metal hydrolysis reactions. The system relies on oxidation reactions and metals precipitate primarily as hydroxides, oxyhydroxides and oxides. The aerobic wetland cells are designed to retard the flow of water long enough for metal oxidation and hydrolysis to occur and for the resulting precipitates to settle. The hydrolysis process releases proton acidity, which retards the oxidation rate. In some cases an amount of crushed limestone is initially added to mediate this effect. Ideally the pH of the wetland is maintained at between 5.5 and 6.5, which enhances the precipitation of iron, manganese and aluminium, the primary constituents of net alkaline minewaters (Younger, 1995; Gazea *et al.*, 1996).

The design is similar to that of a "natural" wetland and consists of basins or channels with a relatively impermeable bottom layer to prevent seepage. This is covered with soil or a similar medium suitable for supporting vegetation. The efficiency of aerobic wetlands for removing iron is limited by dissolved oxygen concentrations. To enhance efficiency, wetlands are often designed with features to enhance aeration, such as steps or waterfalls, followed by quiescent areas. Each aeration step provides sufficient oxygen to remove 50mg/l Fe^{2+}, so where iron concentrations are higher, a series of aeration steps needs to be incorporated (Hedin *et al.*, 1994). The water depth is typically shallow (10-50cm) to further enhance aeration (Younger, 1995), but

not consistent throughout the wetland, with deeper (1-2m) areas providing regions for sludge accumulation (Hedin *et al.*, 1994). The length to width ratio is typically over 10 to ensure sufficient residence time. The presence of vegetation helps to regulate the water flow, introduces additional oxygen into the water and sediment, assists in the removal of iron flocs and reduces the erosion potential of the precipitates (Gazea *et al.*, 1996).

1.4.6.2 Anaerobic (compost) wetlands

The compost wetland is one of the systems available for the treatment of net acidic mine waters. In these cases the system is required to generate sufficient alkalinity to neutralise excess acidity. Compost wetlands generate alkalinity through a combination of bacterial activity and limestone dissolution. The SRB require a rich organic substrate in which anaerobic conditions will develop (Hedin *et al.*, 1994; Gazea *et al.*, 1996).

A wide range of organic substrates have been tested and are typically low-cost natural products and wastes, such as horse and cow manure, spent mushroom compost, hay bales, peat, wood chips or sawdust. Some substrates, such as mushroom compost contain limestone, while those that don't are usually supplemented with limestone. The substrate is placed in a non-compacted layer, typically 30-45cm thick A compost loading of 250-300kg.m^{-2} is usually employed (Hedin *et al.*, 1994). The bacteria reduce sulphate to assist in the oxidation of labile organic carbon. The sulphide which is released reacts with metal ions in solution and the bicarbonate assists in neutralising excess acidity. As the bacterial metabolism is influenced by temperature, the efficiency of the system is affected by seasonal fluctuations in temperature. The loss of efficiency in winter may be reduced by limestone addition and by directing the water flow through the compost layer, rather than over the surface (Younger, 1995; Gazea *et al.*, 1996).

1.4.6.3 Anoxic limestone drains

Anoxic limestone drains (ALDs) have become an increasingly popular passive "pre-treatment" technology (Younger, 1995). Essentially these consist of trenches filled with limestone gravel or cobbles (10cm diameter) that are overlayed with a plastic sheet and a layer of clay to exclude oxygen. They are used to raise the pH of net acidic waters by carbonate dissolution prior to metal and/or acidity removal in wetlands (Younger, 1995; Gazea *et al.*, 1996).

Anoxic limestone drains are operated under saturated conditions to further minimise the infiltration of atmospheric oxygen. Their dimensions vary considerably, with most of the early systems consisting of long, narrow (0.6-1m wide) trenches (Hedin *et al.*, 1994), although more recently systems have been installed that are 10-20m wide, without a significant reduction in efficiency.

The mass of limestone required to neutralise a certain discharge for a specific period of time can be calculated based on the minewater flow rate and the alkalinity-generating performance of the ALD. Research has shown that a 14 hour contact time between the minewater and the limestone is required to achieve a maximum concentration of alkalinity (275-300mg/l). To achieve a contact time of 14 hour approximately 3000kg of limestone is required for each litre per minute of minewater flow. In the above situation the limestone would dissolve at a rate of 160kg per year for each litre per minute of minewater flow (Hedin *et al.*, 1994).

The major problem associated with the use of ALDs is the armouring of the limestone surfaces by iron and aluminium precipitates. If the influent minewater contains over 2mg/l of Fe^{3+} or Al^{3+}, or has a high dissolved oxygen content the efficiency of the ALD system decreases rapidly as a result of armouring, although this is not common in discharges originating from deep level mining, where the vast majority of Fe and Al is in the divalent state (Hedin *et al.*, 1994; Gazea *et al.*, 1996).

The precipitation of metals should ideally not occur within the ALD, so its primary function is to add alkalinity to the water. Therefore they need to be utilised in conjunction with other passive treatment components.

1.4.7 Wetland size

The sizing design for wetlands has been developed by Hedin *et al.* (1994) and is based on two key principals:

(1) The pollutant loading which the wetland will receive is calculated as:

Loading (g/day) = Q (l/min) x concentration (mg/l) x 1.44 (11)

where Q is the peak flow rate

Where the water is net alkaline, iron is typically the main polluting component. Iron removal occurs most effectively by oxidation processes in aerobic wetlands. However, where waters are net acidic, iron removal must be accompanied by the neutralisation of total acidity (proton and mineral) in order to generate a satisfactory final effluent. This is best achieved using an anaerobic wetland. Based on this, loadings are determined with reference to iron alone in the case of net alkaline waters and total acidity loadings where net acid waters are to be treated (Hedin *et al.*, 1994; Younger, 1995).

(2) The minimum size of a constructed wetland is determined by dividing the appropriate loading by an empirical removal rate (RR), which has been determined by the US Bureau of Mines based on existing systems. That is:

$$\text{Wetland size } (m^2) = \text{Loading } (g/d) / \text{RR } (g/d/m^2) \quad (12)$$

Hedin *et al.* (1994) recommended two distinct suites of RR values based on the legal conditions relating to the remediation of the site. Where strict compliance with discharge standards is required a "conservative criterion" (CC) must be used. In this situation a removal rate of 10g of iron per m^2 d^{-1} should be assumed when designing aerobic wetlands receiving net alkaline waters and an acidity removal rate of 3.5g total acidity per m^2 d^{-1} when designing an anaerobic wetland to treat net acidic discharges. The use of these strict compliance criteria will yield large wetland areas and as such will only be popular where legal obligations exist. In many cases the pollution is associated with long abandoned mines, where liability cannot be traced to an existing company. In these cases application of the CC may prove too costly so a second set of RR values, based on "reasonable improvement criteria" (RIC) has been proposed. The RR values are 20 for aerobic wetlands and 7 for anaerobic wetlands, resulting in smaller, less expensive wetlands (Hedin *et al.*, 1994; Younger, 1995).

1.4.8 Integrated biological treatment systems

Both active and passive biological systems have been used for the treatment of acid mine drainage, but there are serious constraints to their widespread implementation, particularly in SA. The land area necessary to treat high volume AMD discharges has restricted the use of passive

systems, particularly as the affected areas are typically surrounded by industrial development. The three major factors constraining the active biological treatment approach are the reactor configuration used, the cost of construction and the cost and availability of the carbon source and the electron donor for the microbial reduction processes (Rose *et al.*, 1998).

Research at Rhodes University has focused on the utilisation of cheap or waste carbon sources for sulphate reduction and the integration of SRB and algal based technologies for AMD treatment (Rose *et al.*, 1998; Boshoff, 1999; van Hille *et al.*, 1999). Algal waste stabilisation pond (WSP) technology has been developed over the past 40 years (Mara *et al.*, 1996). While WSPs have been used to treat a diverse range of effluents little attention has focused on their potential to treat AMD.

Rose *et al.* (1998) reported on the treatment of tannery effluent in an Algal Integrated Ponding System (AIPS), similar to the type developed by Oswald (1991). The system consisted of a facultative pond, containing both an anaerobic and an aerobic component. Efficient sulphate reduction and metal precipitation was observed in the anaerobic compartment, while the aerobic cap was responsible for the re-oxidation of excess sulphide, which eliminated potential odour and toxicity concerns. This work led to the consideration of algal ponding as a possible system for the treatment of AMD.

Boshoff (1999) developed an integrated biological system for the treatment of acid mine drainage, utilising tannery effluent or primary sewage sludge as the carbon source for sulphate reduction.

1.5. General research hypothesis

The mining industry has been a cornerstone of the South African economy for over a century. The abandonment of mines due to ore depletion and economic conditions has created a serious environmental problem that is likely to intensify in the near future and persist for several centuries.

While chemical treatment strategies are being successfully employed for the remediation of acid mine drainage, they are expensive and therefore not a feasible long-term strategy. Based on the predicted volume of the discharges and the level of contamination the land area required for

complete treatment by passive biological systems would be prohibitive. In addition, passive systems do not allow flexibility in terms of operational control.

The reactive alkaline species, sulphide, hydroxide, carbonate and bicarbonate, that are used to drive precipitation in chemical treatment systems can be generated biologically by the action of sulphate reducing bacteria and algae. The bacteria are able to utilise sulphate and a carbon source to generate sulphide and bicarbonate, which in turn can be used as a source of carbon for algal photosynthesis. The algae split the bicarbonate into carbon dioxide, which is accumulated, and a hydroxide ion which is released into the surrounding medium. The kinetics of the biological reactions are such that the reactive alkaline species can be generated at a suitable rate to drive an effective treatment process. Such an integrated biological treatment system, based on the biological generation of reactive alkaline species, offers a number of potential advantages over conventional methodologies. The systems depend on the same naturally occurring biological and geochemical reactions as passive systems, but the compartmentalisation of different components allows for a greater degree of optimisation and finer control. This will result in reduced land usage and an increased potential to deal with fluctuations in discharge volume and quality. The reactor systems described in this study are cheaper to construct and require less inputs than those employed in other active biological systems.

If correctly implemented, an integrated biological treatment system based on biogenic alkalinity offers the potential for a low-cost, sustainable solution to the problem of long-term discharge of acid and metal contaminated water from abandoned mines, rock piles and tailings.

CHAPTER 2: DIRECT TREATMENT OF AMD BY ALGAL BIOMASS - EFFICIENCY AND TOXIC EFFECTS

2.1 Introduction

During the last decade a considerable amount of attention has been focused on the ability of both viable and non-viable algal biomass as a potential tool for the extraction and recovery of toxic and valuable heavy metals from aqueous solutions. The studies have focused on both unicellular algae (Gardea-Torresdey *et al.*, 1990; Roy *et al.*, 1993; Pascucci, 1993; Garnham *et al.*, 1993; Wilde and Benemann, 1993) and cyanobacteria (Karamushka *et al.*, 1995) as well as macroalgae (Kratochvil *et al.*, 1995; Fourest and Volesky, 1996) and complex algal mats (Lawrence *et al.*, 1998).

The biosorption of the metal by the algal biomass arises from the coordination of the ions to different functional groups in or on the algal cell. These groups are found on proteins, lipids and carbohydrates and include amino, thioether, sulfhydral, carboxyl, carbonyl, imidazole, phosphate, phenolic, hydroxyl and amide moieties (Gardea-Torresdey *et al.*, 1990).

The specific binding mechanism depends on the species of metal ion, the specific organism and the chemical composition of the metal ion solution. Several researchers have demonstrated the release of protons during metal accumulation, suggesting an ion-exchange mechanism (Greene *et al.*, 1987; Crist *et al.*, 1994a; 1994b), while electrostatic interactions and covalent bonding have also been demonstrated (Volesky, 1987).

Many heavy metals are required by algae in trace concentrations as essential micronutrients and as such the organisms have developed specific transport pathways for accumulating these metals. At elevated concentrations these metals become toxic to the cells. Copper and zinc have both been used to control nuisance algal blooms, due to their high toxicity to cyanobacteria in particular (Meador *et al*, 1998). As a result, algae are often used as indicator organisms in toxicity testing protocols (Lam *et al.*, 1999). The effect of metal toxicity is therefore an important consideration in the development of algal based bioremediation systems.

Spirulina sp. was chosen for the initial algal studies as it was readily available and its cultivation on a large scale has been documented (Ciferri, 1983). In addition, it has been used on an industrial scale to treat municipal waste and is able to tolerate a wide range of pHs and salinities (Vonshak *et al.*, 1996). Due to its filamentous nature it is easy to harvest by filtration, using a nylon mesh, which reduces the problems associated with solid-liquid separation.

2.1.1 Characteristics of Spirulina

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

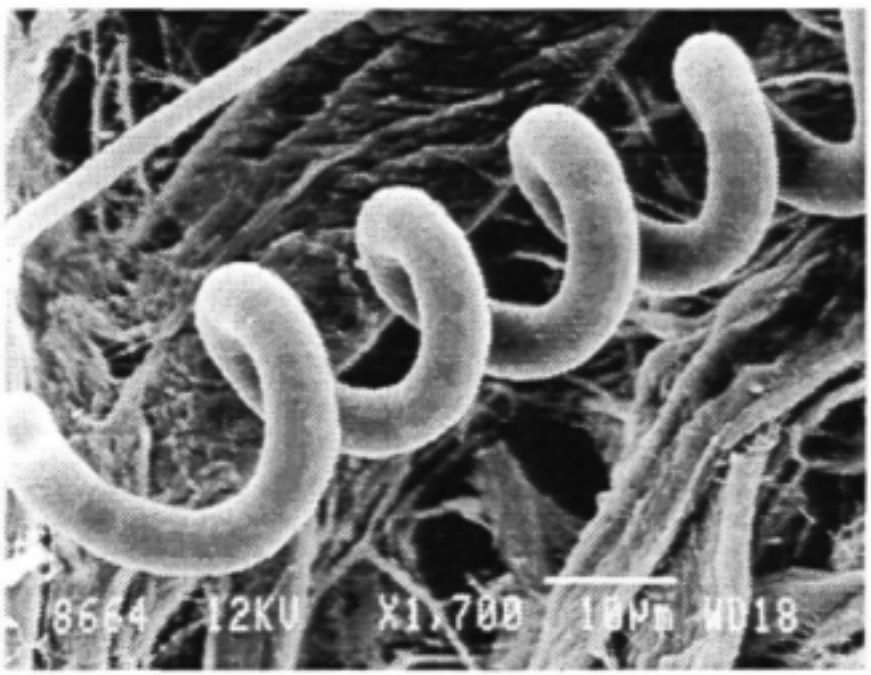


Figure 2.1: Scanning electron micrograph of healthy *Spirulina*.

The cell wall of *Spirulina* is composed of four distinct layers. The outer layer consists of structural material analogous to the Gram negative bacterial cell wall. Below this is a layer of protein fibrils helically bound to the trichomes. The second layer is composed of peptidoglycan, which folds towards the inside of the filament. This second layer, together

with the inner fibril layer give rise to the septum, which separates the individual cells in the filament (Ciferri, 1983; Richmond, 1986).

2.2 Research aims

The primary aims of this section of the project were:

1. To evaluate *Spirulina* as a potential biosorbent for the heavy metals encountered in the mine effluent. To this end the adsorption capacity and kinetics of binding need to be determined.
2. To evaluate the efficiency of *Spirulina* as a treatment agent for the removal of heavy metals from an actual mine effluent.
3. To determine toxicity threshold levels for the various toxic heavy metals, above which the integrity of the treatment system would be compromised.

2.3 Materials and methods

2.3.1 Algal biomass

The *Spirulina* and *Oscillatoria* (expanded on in chapters 2 and 3) used in this study were cultivated under constant environment conditions (constant illumination at 27°C). The algal cultures were maintained in Zarrouk's chemically defined medium (Appendix B). Algae used in the experiments were harvested by filtering a portion of the stock culture through a nylon cloth (mesh size 0.2mm), washing twice with distilled water and resuspended in a 1g/l NaCl solution to form a concentrated slurry from which appropriate dilutions were made.

2.3.2 Determination of algal concentration

Both *Spirulina* and *Oscillatoria* are filamentous cyanobacteria, so cell counts could not be used to determine culture concentration. A chlorophyll extraction method and a photometric method were developed which could be accurately correlated to algal dry mass.

2.3.2.1 Dry mass determination

Porcelain crucibles were labelled, dried at 60°C for 3 hours and individually weighed. Volumes of 1, 2, 5, 7, 10 and 20ml were removed from the algal culture, spun down at 5000rpm for 5 minutes, and washed twice with distilled water. The washed cells were transferred to the crucibles and dried at 60°C for 12 hours, after which the dry mass was determined. Determinations were performed in triplicate and mean values used. A curve of dry mass against initial volume was plotted, with an r^2 value of 0.950 (Appendix C).

2.3.2.2 Photometric determination

Sample volumes of 1, 2, 5, 7, 10 and 20ml were removed from the stock culture and diluted to a final volume of 20ml with distilled water. The samples were thoroughly mixed to ensure even distribution and the optical density (OD) determined at 660nm. Determinations were made in triplicate and mean values were used to plot a curve of OD against initial volume, with an r^2 value of 0.998 (Appendix D).

2.3.2.3 Chlorophyll extractions

Similar volumes as previously used were filtered through a Whatman GF/C filter. The algal laden filters were transferred into foil covered bottles containing 10ml of acetone and crushed. The samples were stored at 4°C for 24 hours, after which they were filtered again and the absorbence determined at 661.6, 644.8 and 470nm. Chlorophyll concentrations were calculated from the equations given in Appendix E (Lichtenhaler, 1987). Extractions were performed in triplicate and mean values were used to plot a curve of chlorophyll *a* against initial volume, with an r^2 value of 0.995 (Appendix F).

The data from the OD_{660} measurements and chlorophyll extractions were plotted against the dry mass data and were found to be directly proportional (r^2 values of 0.972 and 0.965 respectively), indicating that algal mass could be accurately determined by either the photometric or chlorophyll extraction method.

2.3.3 Analytical techniques

All pH measurements were made using a Cyberscan 2500 pH meter. Metal analysis was performed using a GBC 909AA atomic absorption (AA) spectrophotometer, linked to a GBC integrator (Individual program parameters shown in Appendix G). Optical density measurements were made using a Shimadzu Mini 1240 UV-VIS spectrophotometer. All glassware was washed in 15% nitric acid followed by repeated rinses in distilled water.

2.3.3.1 Acid digests

Acid digests were performed to determine the amount of metal taken up by the algal cells. A 3ml sample was removed and filtered through a 0.45µm cellulose acetate filter. The filtrate was retained and used to determine the amount of metal remaining in solution. The filters containing the algae were placed in Pyrex boiling tubes to which 0.2ml 55% nitric acid was added. These were boiled for approximately 30 minutes (until all the nitrous oxide had dissipated). Once cooled 4ml of distilled water was added to each tube and the metal concentration was determined by AAS.

2.3.3.2 Carbohydrate determination

Carbohydrate determinations were performed to quantify extracellular polysaccharide. A sample was removed from the culture and filtered through a 0.45µm nylon membrane filter. 1ml of phenol reagent and 5ml 32% sulphuric acid were added to 1ml of the filtrate and mixed. The absorbance was read at 490nm and the results converted to glucose equivalents using a standard curve of glucose against absorbance (Appendix H).

2.3.3.3 Light microscopy

All light microscope preparations were made by mounting 25µl of sample on a glass slide. These were photographed at the Electron Microscopy Unit, Rhodes University, using an Olympus BX50 camera-microscope attached to an Olympus PM-30 exposure control unit.

2.3.3.4 Scanning electron microscopy (SEM)

Specimens were prepared either by filtering 5ml of sample through a Whatman GF/C filter or by evaporating a small volume onto a glass cover slip. The specimens were fixed in cold 2.5% gluteraldehyde in 0.1M phosphate buffer for 12 hours. After fixation the specimens were washed twice in phosphate buffer, followed by a 30% to 100% ethanol dehydration sequence, for 10min at each concentration. Specimens were then incubated in increasing concentrations of amyl acetate, prior to critical point drying. The dried specimens were mounted on copper stubs, coated in gold and observed using a JEOL JSM-80 SEM (Cross, 1987).

2.3.4 Adsorption isotherms

Adsorption isotherms were conducted, in triplicate, at 20°C for five hours. Viable *Spirulina*, equivalent to 100mg dry mass, was added to 100ml of the metal solutions (Cu^{2+}, Zn^{2+}, Fe^{2+}, Pb^{2+}) and shaken at 140rpm on a rotary shaker. Isotherm experiments were conducted at pH 6 and initial metal concentrations (C_0) varied from 20 to 1000μM. A pH of 6 was selected so that metal precipitation as hydroxides did not occur. After 5 hours a 5ml sample was removed, filtered through a 0.45 μm nylon membrane filter and the metal remaining in solution was determined by AAS. The Langmuir sorption model was used to estimate the maximum metal uptake (X_m), where these could not be reached in the experiment (Holan *et al.*, 1993).

$$C_e/q_e = 1/(X_m b) + C_e/X_m \qquad (1)$$

Where X_m represents the maximum adsorption capacity and b is the Langmuir constant, which is the ratio of sorption/desorption rates (Holan *et al.*, 1993). q_e is the metal uptake (mg metal/g of biomass) at equilibrium and C_e represents the equilibrium concentration of metal (mg/l). The Langmuir model makes four basic assumptions (Faust and Aly, 1987):

1. The molecules are adsorbed on definite sites on the surface of the adsorbent.
2. Each site can accommodate only one molecule.

3. The area of each site is a fixed quantity, determined solely by the geometry of the surface.
4. The adsorption energy is the same at all sites.

2.3.5 Adsorption kinetics

Viable *Spirulina* (300mg dry mass) was mixed with 300ml of metal solution at concentrations between 100 and 500μM. The solutions were shaken at 140 rpm for 4 hours. Samples were taken at varying intervals and analysed as previously described.

2.3.6 Effluent treatment studies

Acid mine effluent was obtained from Black Mountain, a base metal mine in the Northern Cape Province, South Africa. The effluent was characterised by a low pH (1.99) and high concentrations of iron (1700 μM), copper (17 μM), lead (7 μM) and zinc (33 μM). A complete effluent profile is shown in Appendix I.

Algal cultures were established in 400 ml Zarrouk's medium, modified by omitting the iron and EDTA. The flasks were divided into five groups (three flasks per group), a control group and four experimental groups, to which increasing volumes of raw effluent were added. Chlorophyll extractions, heavy metal analysis (Cu, Fe, Zn and Pb) and pH measurements, as described previously, were performed on day 0. The flasks were incubated on a rotary shaker at 140rpm under constant environment conditions (constant illumination at 27 °C).

On day 1 chlorophyll extractions were performed, after which the raw effluent was added to the flasks in the experimental groups (group 1 – 10 ml, group 2 – 20 ml, group 3 – 30 ml and group 4 – 50 ml). This was repeated on successive odd-numbered days. Prior to effluent addition the volume of the flasks was adjusted to 400 ml, either by abstraction or addition of distilled water.

On day 2 and successive even-numbered days a 5ml sample was removed, filtered through a 0.45 μm nylon membrane filter and the filtrate used to determine the concentration of heavy metals in solution.

The experiment was conducted over a 44 day period. The Zarrouk's media has a high pH and contains 16.8 g/l of sodium bicarbonate which would lead to alkaline precipitation of the heavy metals in the effluent. To determine the effect of the medium the experiments were repeated without the addition of algae to any of the flasks.

Data were analysed by determining the number of µmoles of each metal in the experimental flasks at day 0. This was determined each time heavy metal analysis was performed and allowed the metal removal to be evaluated for each two day period, as well as cumulatively over the duration of the experiment.

2.3.7 Toxicity studies

The loss of viability of the algal cultures during the effluent studies prompted the determination of the toxic effects of copper, lead and zinc on *Spirulina*. The following experiments were performed in triplicate.

Eighteen 500 ml flasks were inoculated with 30ml of concentrated algal slurry and 270 ml of Zarrouk's medium. A chlorophyll extraction, an acid digest and a pH reading were performed on each experimental flask. The cultures were allowed to stabilise for 24 hours under constant environment conditions.

On day 1 the appropriate volume of a 1 M $CuSO_4$ was added to each flask to make the final concentrations up to 5, 10, 20, 30 and 50 µM. Chlorophyll extractions, acid digests and pH readings were performed. This was repeated every second day thereafter. On day 4 additional copper was added to return the concentration in solution to the original levels. Carbohydrate determinations were performed every second day. Light microscope and EM preparations of the algae were made at various stages.

The experiments were repeated using zinc (stock 1 M $ZnSO_4$) and lead (stock 1 M $PbCl_2$).

2.4 Results and discussion

2.4.1 Adsorption isotherms

The adsorption isotherm data for all three metals tested showed relatively good compliance to the Langmuir equation (Table 2.1). The correlation coefficients, r^2, for all the metals except iron were greater than 0.92, indicating a good relationship between the equilibrium metal concentration (C_e) and the metal uptake (q_e). The model assumes that no movement of adsorbed molecules occurs across the surface (Faust and Aly, 1978). As viable biomass was used in this study it is likely that there was some internalisation of adsorbed metal. This is evident from the kinetic data which indicate that there was a small increase in bound metal after equilibrium appeared to have been reached. The correlation coefficients would probably have been higher if the experiments had been performed over a shorter time period. The kinetic data (Figure 2.2) indicated that by five hours some internalisation of metal, particularly copper and zinc, would have occurred. There appeared to be a slight increase in metal removal after two hours, by which time adsorption equilibrium seemed to have been reached. This internalisation of metal would free additional binding sites and result in the overall binding being higher than would be expected assuming monolayer binding.

Table 2.1: Langmuir constants of metal-Spirulina binding (mean values, n = 3).

Metal	Temperature (°C)	X_m (mg/g)	Correlation (r^2)
Copper (Cu^{2+})	20.0	9.91	0.93
Zinc (Zn^{2+})	20.0	8.76	0.94
Lead (Pb^{2+})	20.0	65.36	0.96
Iron (Fe^{2+})	20.0	1.68	0.86

The isotherm data were substituted into the Langmuir equation to calculate the maximum adsorption capacity of *Spirulina* in terms of the various metals. Of the metals tested the capacity was highest for lead, followed by copper and zinc, with iron adsorbing poorly to the biomass. While the adsorption capacity of lead appeared to be almost 10 times higher than copper and zinc it must be remembered that the capacity is expressed in terms of mg/g and the atomic mass of lead is considerably higher than copper or zinc. In terms of molar concentrations, the capacity for lead is just over double that of copper and zinc. This sequence is similar to that obtained by Pascucci (1993) using *Chlorella vulgaris*, Roy *et al.*

(1993) using *Chlorella minutissima* and Hammaini *et al.* (1999) using activated sludge. Comparing the data from this study with published results for both algal and other biosorbents (Table 2.2) it is clear that *Spirulina* is not the most effective adsorbent. This is partly due to the filamentous nature of *Spirulina*, which leads to a smaller surface area to volume ratio than that of unicellular algae or yeast.

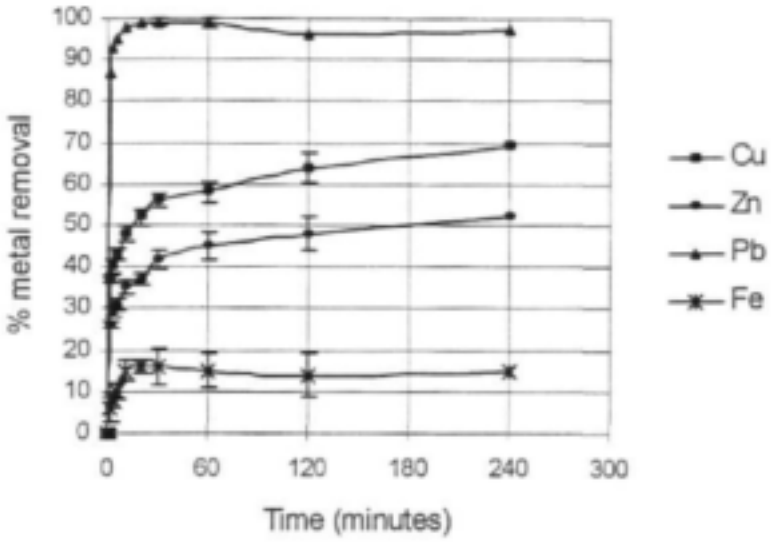


Figure 2.2: Percentage metal accumulation in batch flasks by *Spirulina* as a function of time (n=3 ± SD). Initial metal concentration 200µM. Data for individual metals obtained from separate experiments and are plotted on a single set of axes for comparative purposes.

Table 2.2: Metal uptake capacity of various adsorbents.

Metal	Adsorbent	X_m (mg/g)	Source
Copper	*Spirulina* sp.	9.91	This study
	Activated sludge	18.8	Hammaini *et al.* (1999)
	Sargassum fluitants	61.5	Kratochvil *et al.* (1995)
	AMT-BIOCLAIM™	152.0	Brierely *et al.* (1985)
Zinc	*Spirulina* sp.	8.76	This study
	Activated sludge	15.4	Hammaini *et al.* (1999)
	AMT-BIOCLAIM™	137.0	Brierely *et al.* (1985)
Lead	*Spirulina* sp.	68.36	This study
	Azolla filiculoides	100.0	Sanyahumbi (1998)
	Penicillium chrysogenum	116.0	Niu *et al.* (1993)
	Activated sludge	142.9	Hammaini *et al.* (1999)
	Sargassum fluitants	219.6	Fourest and Volesky (1996)
	AMT-BIOCLAIM™	601.0	Brierely *et al.* (1985)

2.4.2 Adsorption kinetics

The kinetic data indicate that in all cases initial binding of the metal to algae was rapid, with over 80% of the total accumulation occurring within the first 30 minutes. Figure 2.2 combines the data for all four metals at an initial concentration of 200μM. At lower concentrations the percentage removal was higher for all groups and the trends could not be seen as clearly. The kinetics of lead binding was the most rapid with over 95% of the total removal occurring within the first 60 seconds. This is consistent with the results of Sanyahumbi (1998). The kinetics of copper and zinc adsorption were slightly slower than that of lead. These are consistent with the results of Wilhelmi (1997), obtained using *Saccharomyces cerevisiae*. There appears to be a slight bi-phasic shape to the copper and zinc curves, suggesting some metal accumulation into the cells.

2.4.3 Effluent treatment studies

The treatment of raw AMD by direct contact with the algal culture was effective, although the algal cultures only remained viable for a short period of time. Figure 2.3 shows that the algal cultures in all groups remained healthy for the first seven days after which they began to decline rapidly. The decline in algal viability was most noticeable in the groups receiving the higher doses of AMD. Group one declined at a slower rate, while the control flasks essentially followed a standard growth curve pattern. The chlorophyll extractions were stopped after day 25 as most of the cultures in the experimental groups had died.

The pH data (Figure 2.4) mirrored the algal viability, with the pH in all flasks increasing by a similar degree over the first nine days. However, once the algal cultures began to deteriorate the pH no longer increased and subsequent addition of effluent resulted in a proportional decrease in pH. The pH of the flasks in the control group continued to increase until the cultures began to decline, due to nutrient depletion. The legend for Figures 2.3 to 2.7 is explained in Table 2.3 below.

Table 2.3: Key to legends for Figures 2.3 to 2.7.

Group	Description of dosing on day 1 and subsequent odd-numbered days
Control	10 ml Zarrouk's medium
Group 1	10 ml raw mine effluent
Group 2	20 ml raw mine effluent
Group 3	30 ml raw mine effluent
Group 4	50 ml raw mine effluent

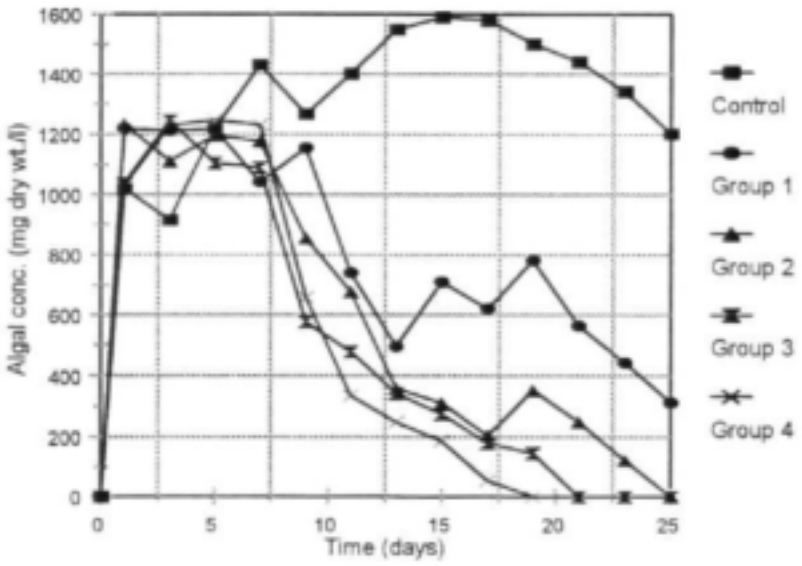


Figure 2.3: Graph showing mean (n = 3) algal concentration vs. time for effluent treatment studies. Viable algal mass was determined using the linear relationship between Chlorophyll *a* and dry mass. Chlorophyll determinations terminated after day 25 due to death of most cultures.

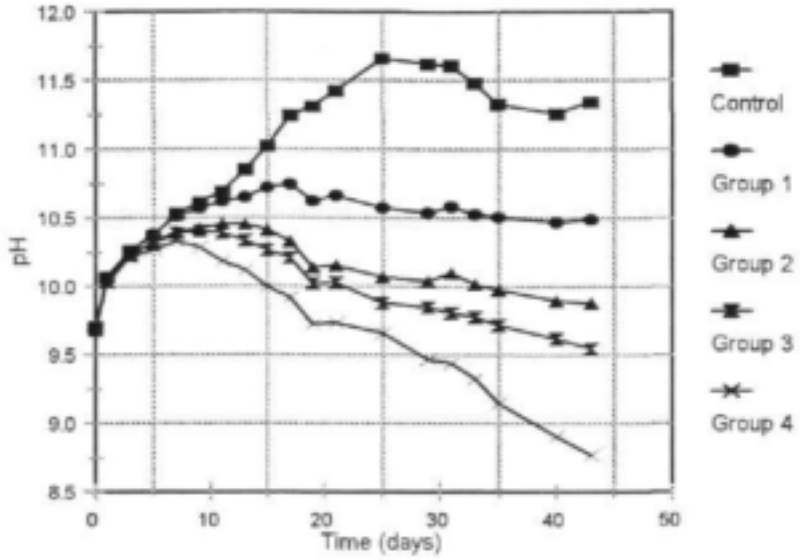


Figure 2.4: Graph of pH vs. time for effluent treatment studies (mean values, n = 3).

Metal removal essentially appeared to be determined by pH. Due to the high pH and bicarbonate content of the algal growth medium the pH in all the groups did not fall significantly below 9.5 at any stage and only group four dropped below pH 9. As a result the metal removal percentages were high for all metals, especially the iron where over 99% was removed in all the groups. Copper showed an unusual trend (Figure 2.5), with a high percentage removal for the first eight days, most likely as a result of both precipitation and adsorption. However, there was a significant drop in efficiency after day eight, which coincided with the deterioration of the algal cultures (Figure 2.3). After day 14 the percentage removal began to increase again, particularly in groups 2, 3 and 4, which coincided with the complete deterioration of the relevant algal cultures. The algal cultures in the group one flasks, although declining, had not died completely and the copper removal remained lowest in this group.

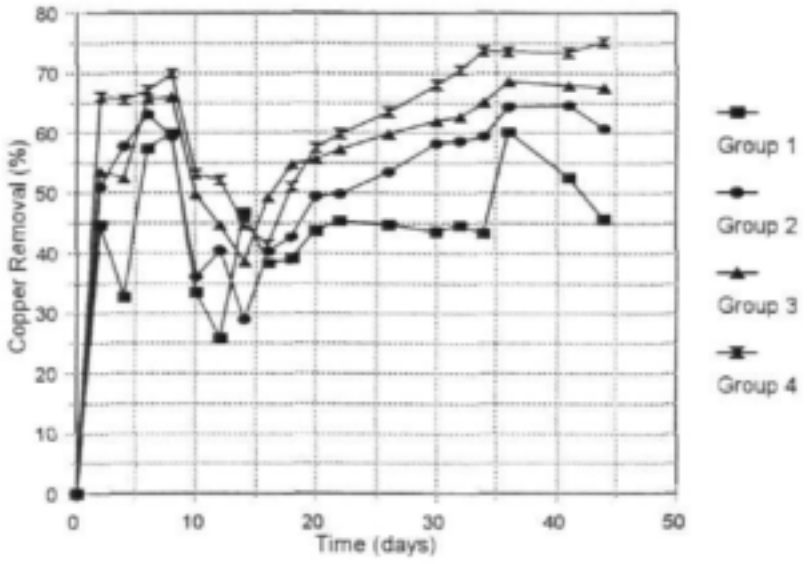


Figure 2.5: Graph of cumulative copper removal vs. time for the effluent treatment studies (mean values, n = 3).

The trend observed for the copper suggests the release of exopolysaccharides and thiols by the metal stressed algae. These substances have been shown to chelate metals in solution, rendering them unavailable for accumulation (Kaplan *et al.*, 1987). Singh *et al.* (1999) found that the exposure of the cyanobacterium *Nostoc spongiaeforme* to elevated concentrations of copper, nickel and mercury significantly increased the production of both thiols and exopolysaccharide. Exposure to 5μM copper resulted in a 50% increase in thiol production relative to the controls. A more significant increase in exopolysaccharide production was found. Kidambi *et al.*, (1995) showed that the gene responsible for alginate production in *Pseudomonas syringae* was transcriptionally activated by the presence of copper. A similar process may occur in cyanobacteria.

Once the affected cultures died the production of the metal complexing agents would cease and the copper added to the system during subsequent effluent additions would be free to precipitate. This would explain the increase in cumulative removal observed after the cultures had died. Group one showed the lowest copper removal, which can be attributed to the fact that the culture did not die completely.

The removal of zinc and lead showed very similar patterns. Zinc removal is shown in Figure 2.6.

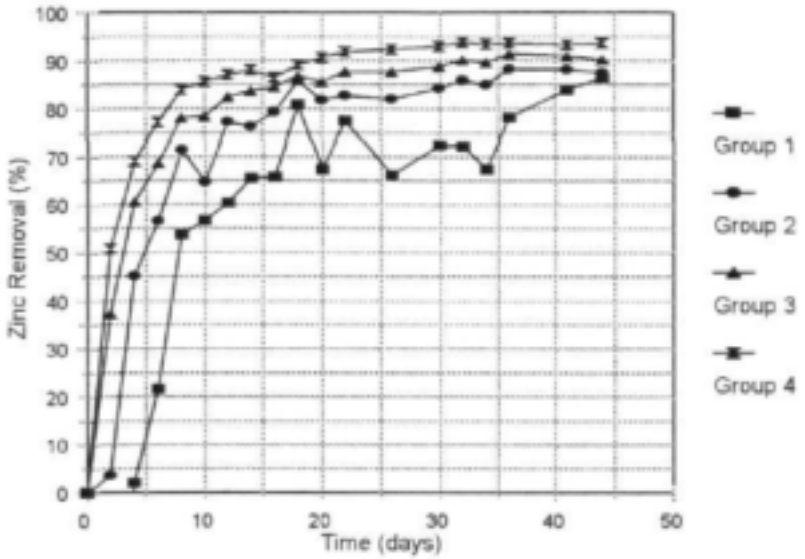
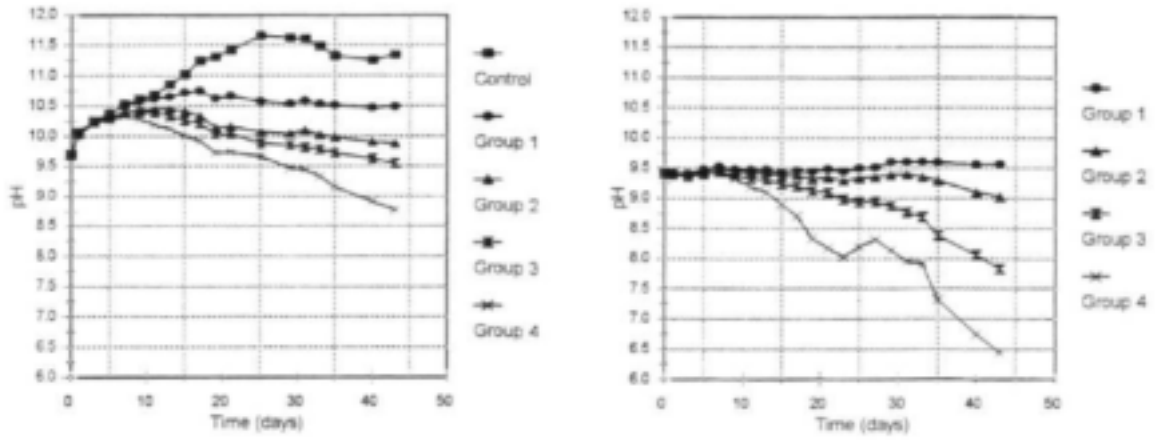


Figure 2.6: Graph of cumulative zinc removal vs. time for effluent treatment studies (mean values, n = 3).

Metal removal can be attributed primarily to pH dependent alkaline precipitation as a result of the high bicarbonate content of the growth medium. The extracellular elements have been shown to have the highest affinity for copper, which would explain why the removal of the other metals was not affected. In all groups the amount of zinc and lead remaining in solution was similar, but as each group received an increasing dosage of effluent the percentage removal appears lower for group 1. The results suggest that precipitation is effective to a lower threshold level.

The results for the algal free control show a similar degree of metal removal as obtained in the experimental flasks. The copper flasks did not show the reduced metal removal between days 8-16, which further supports the assumption that thiols and polysaccharides were responsible for copper chelation in the experimental flasks. The rate of pH change between the experimental and control flasks was however, significantly different (Figure 2.7).



(a) (b)

Figure 2.7: Comparison of pH change vs. time for experimental (a) and algal free control (b) flasks (mean values, n = 3).

From the results it is clear that the presence of the algae has a significant effect on both maximum pH achieved and the rate of pH decrease. In both cases the volume of media added initially was the same, so the total amount of bicarbonate alkalinity (16.8 g/l) present at the start was the same. The algae are able to modify the aqueous chemistry of their surrounding medium by utilising bicarbonate ions as a source of carbon dioxide for photosynthesis (Shiraiwa *et al.*, 1993). The remaining hydroxide ion is released back into the medium and results in the pH increase observed in the control and group one in Figure 2.7(a). The process does not increase the alkalinity, but rather reduces the acidity of the system. This phenomenon, and its potential application to AMD treatment will be discussed in chapter four.

As the pH in the group four flasks dropped below pH 7.5 the efficiency of the zinc removal decreased dramatically and a further drop in the pH to below 6.8 resulted in zinc that had been previously precipitated being remobilised.

2.4.4 Toxicity studies

2.4.4.1 Copper

The results for the algal concentration and pH are shown in Figures 2.8 and 2.9. From the results it is clear that for the first three days all six groups exhibited essentially similar behaviour. Both the algal concentration and the pH increased steadily. Between days three and five the effects of acute copper toxicity become clearly evident, with the 20, 30 and 50μM copper groups showing a significant decline in both algal concentration and pH. By day 11 all the cultures exposed to 20-50μM Cu had no filaments and few cells still intact. By contrast, the control and 5μM cultures appeared unaffected, while the 10μM cultures had begun to deteriorate.

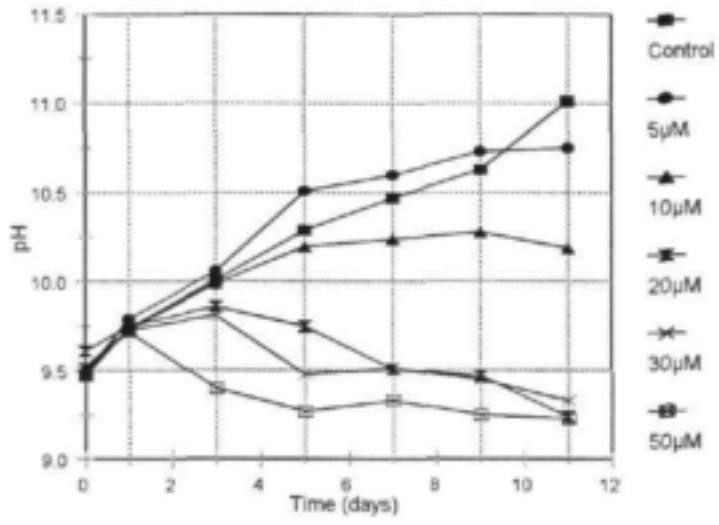


Figure 2.8: Mean pH vs. time for copper toxicity study (n = 3).

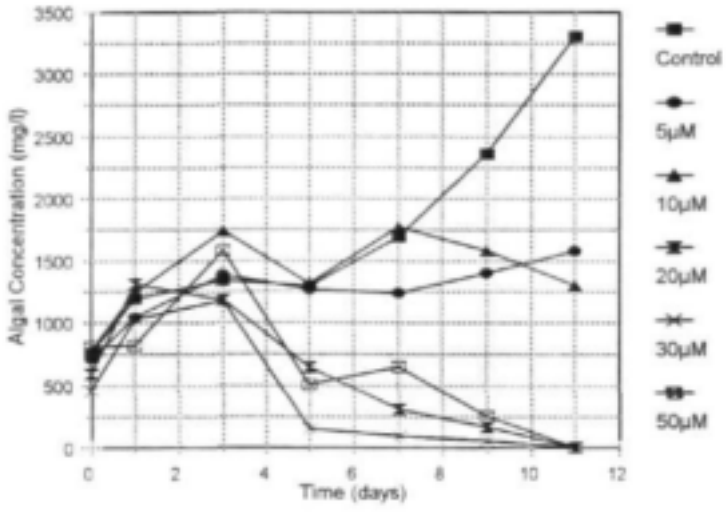


Figure 2.9: Mean algal concentration vs. time for copper toxicity study (n = 3). Algal concentration determined from chlorophyll extractions.

The effect of the copper on the cell morphology is shown in Figures 2.10 to 2.13. A sequential degradation process can be observed, beginning with the unravelling of the coiled filaments (Figure 2.10) and progressing to complete disintegration of the filaments. After two days of exposure to copper at a concentration of 50µM the filaments had begun to uncoil and several had started to break up. After four days the disintegration process had progressed appreciably and large amounts of cell debris were visible (Figure 2.12). This appeared to be accompanied by the loss of a proportion of the cell contents, which resulted in some distortion of the individual cells (Figure 2.13), possibly as a result of reduced turgor pressure. Brady *et al.* (1994) found similar changes in the morphology of the yeast *Saccharomyces cerevisiae* following exposure to elevated concentrations of copper, with the cell surface becoming convoluted and the overall cell size decreasing slightly.

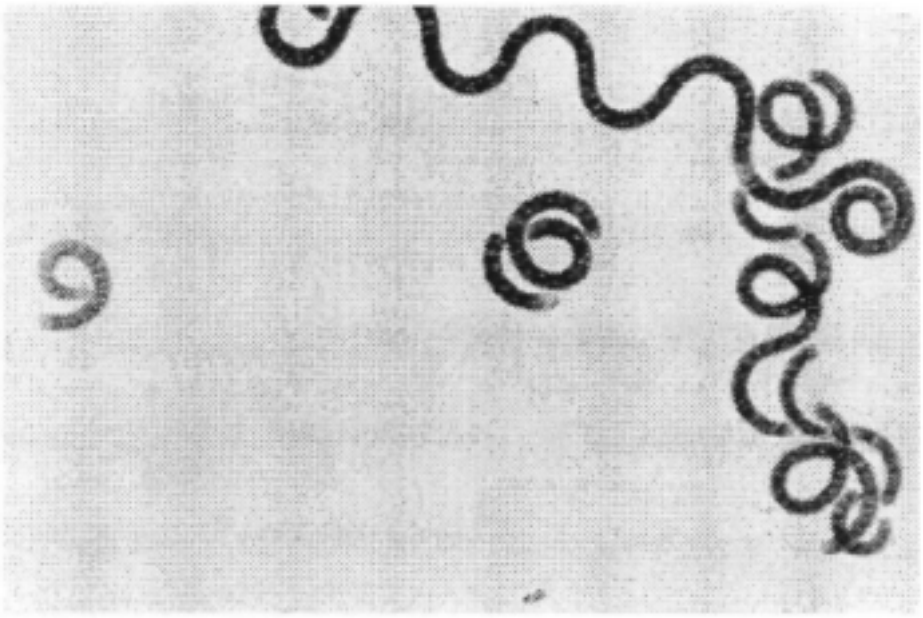


Figure 2.10: Light microscope preparation of *Spirulina* after two days exposure to 50μM Cu^{2+} showing uncoiling of the helical trichome.

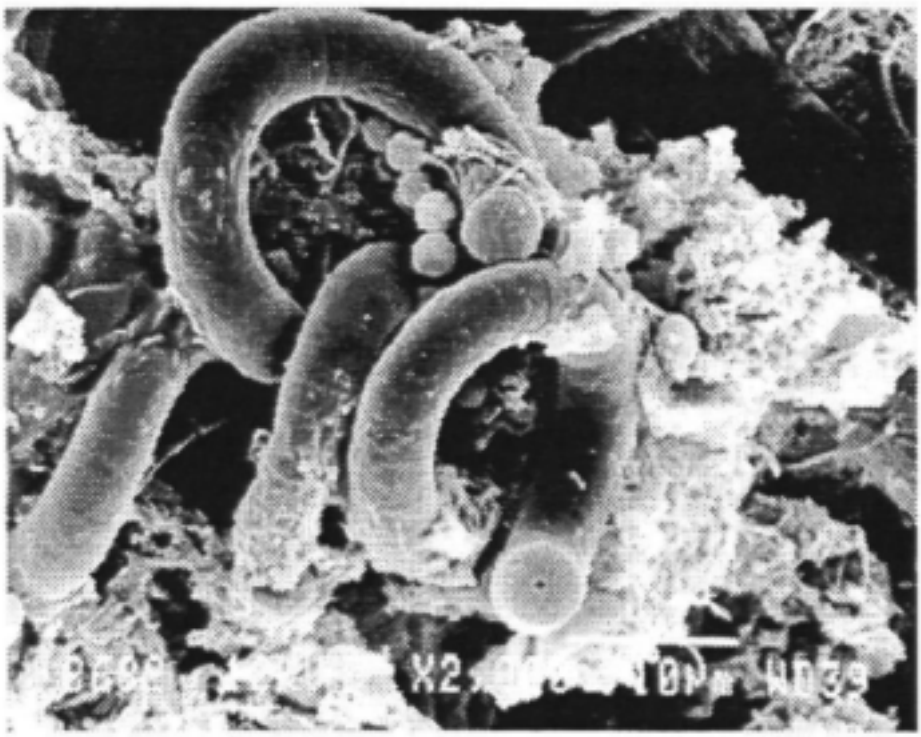


Figure 2.11: SEM preparation of *Spirulina* after two days exposure to 50μM Cu^{2+}.

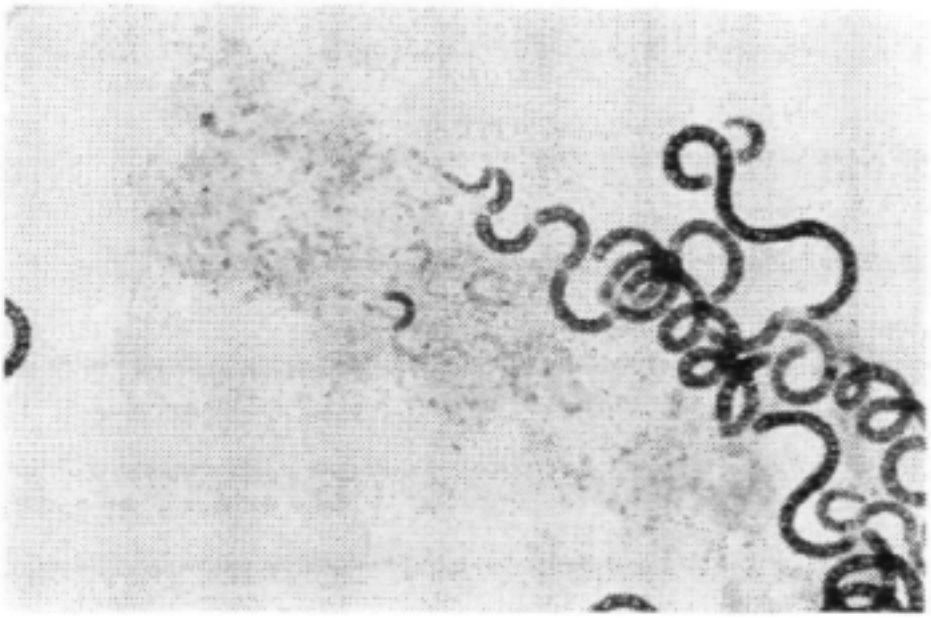


Figure 2.12: Light microscope preparation of *Spirulina* after four days exposure to 50μM Cu^{2+} showing yellowing and disintegration of filaments.

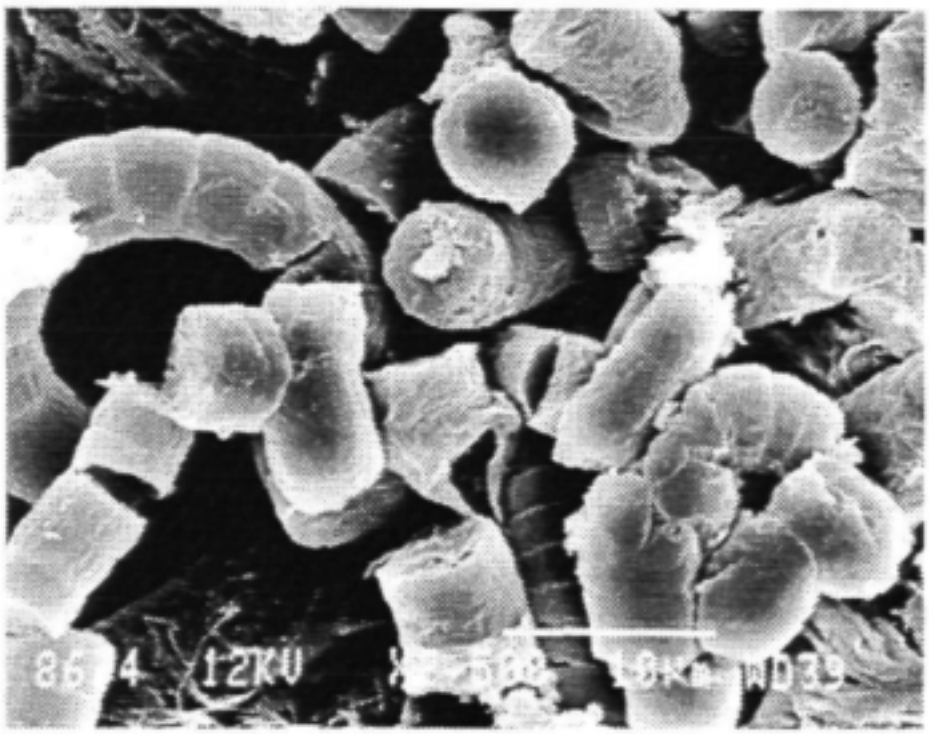


Figure 2.13: SEM preparation of *Spirulina* after four days exposure to 50μM Cu^{2+} showing extensive disruption of the filaments and distortion of the individual cells.

The results from the acid digests, showing the amount of copper associated with the cell and remaining in solution are shown in Figures 2.14 and 2.15. Figure 2.14 shows that there was a rapid increase in the copper accumulation after day three in the 20-50μM groups, but if one compares Figure 2.14 to the graph of algal concentration (Figure 2.9) it can be seen that day three is when the cultures began to deteriorate. The data in Figure 2.14 are expressed as μmoles of copper per gram of dry mass, which is determined from the chlorophyll *a* values. As the cells begin to die the chlorophyll concentration decreased, so while the actual mass was not affected the estimate of dry mass, based on the chlorophyll data, was reduced. Therefore, the graph only gives an accurate representation of the copper accumulation in the 20, 30 and 50μM groups up to day three. The data suggest that the threshold level for copper accumulation is approximately 7μmoles/g, because as soon as the amount of copper accumulated exceeded this value the cells began to die. The copper concentration in the cells in the control, 5 and 10μM groups never exceeded this value and the cultures remained viable after 11 days.

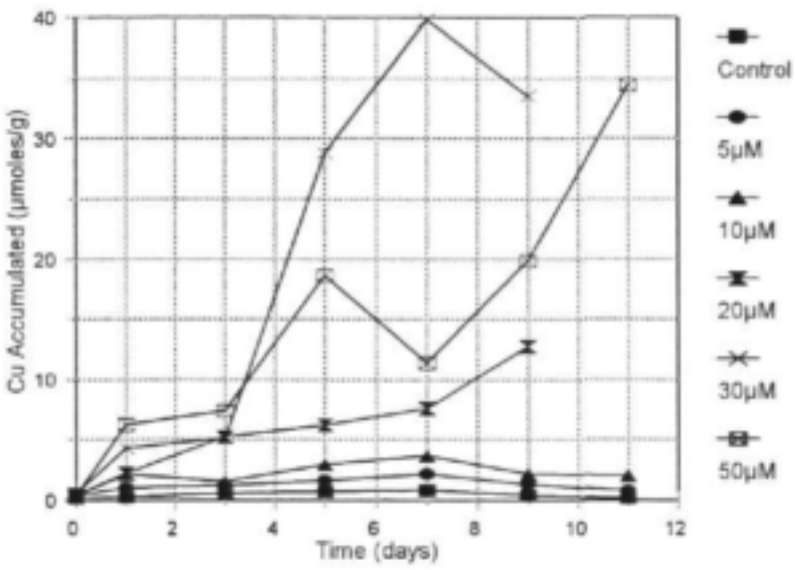


Figure 2.14: Copper accumulation by *Spirulina* over an 11 day period (mean values, n = 3).

Figure 2.15, showing copper concentration in solution, gives a clearer indication of what occurred. The peaks seen on day one were as a result of the initial spiking of the cultures. There was a steady decrease in the copper in solution in all groups as a result of accumulation

by the cells. On day four, additional copper was added to restore the concentration of metal in solution to the initial levels. Following second dosing there was no significant decrease in the copper concentration. This is most likely due to the binding of copper to extracellular polysaccharides released by the algae. The bound copper stayed in solution, but couldn't be bound by the algae.

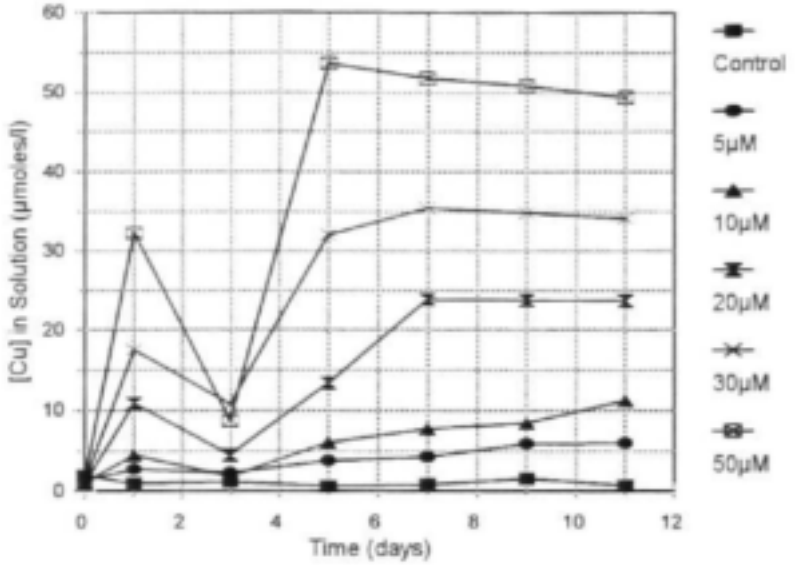


Figure 2.15: Concentration of copper remaining in solution over 11 day period. Initial dosing occurred on day 0 and subsequent dosing on day 4 (mean values, n = 3).

Figure 2.16 clearly shows a significant increase in extracellular carbohydrate following the addition of copper to the medium. The increase is proportional to the dosage with copper. The concentration of extracellular carbohydrate observed in the copper stressed cultures are in the same order of magnitude as observed by Singh *et al.* (1999) with *Nostoc spongiaeforme*. The increase in extracellular carbohydrate is less significant and slower in the control and 5µM cultures and is most likely due to nutrient stress. Brady *et al.* (1994) found that yeast cells which had been serially cultured in a medium containing copper showed reduced biosorptive capacity when exposed to high concentrations of copper.

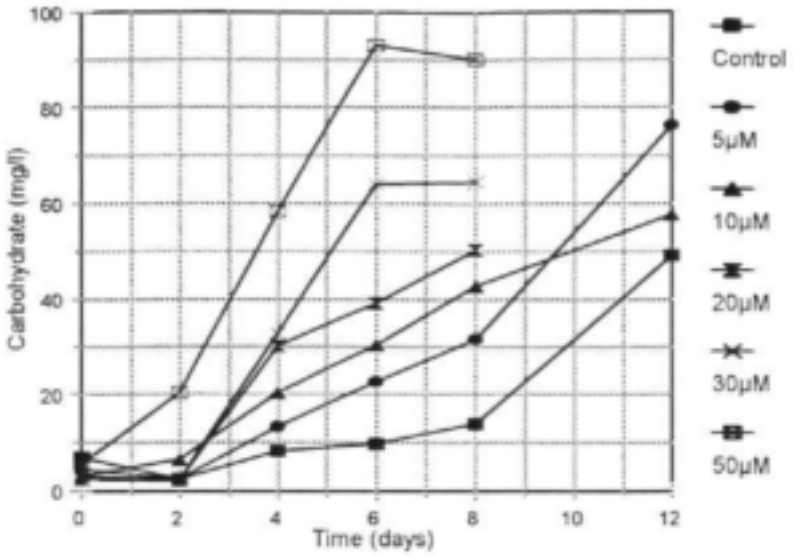


Figure 2.16: Extracellular carbohydrate concentration vs. time during copper toxicity studies. Carbohydrate concentration is expressed as mg/l glucose equivalents (mean values, n = 3).

2.4.4.2 Zinc

The results obtained for zinc showed the same basic trends as in the copper experiments. The onset of the toxic effects was rapid in the 20, 30 and 50 µM groups (Figure 2.17) and although the affected groups took longer to die completely, this can be accounted for by the higher initial algal concentration. The studies on the different metals were performed consecutively and there was an initial acclimation period during which the algal growth rate in individual flasks may have differed. It was therefore not possible to ensure that the algal concentration at the time of metal dosing was the same in each flask. Figure 2.18 once again showed a strong positive correlation between algal culture strength and pH, indicating that pH is directly related to metabolic function.

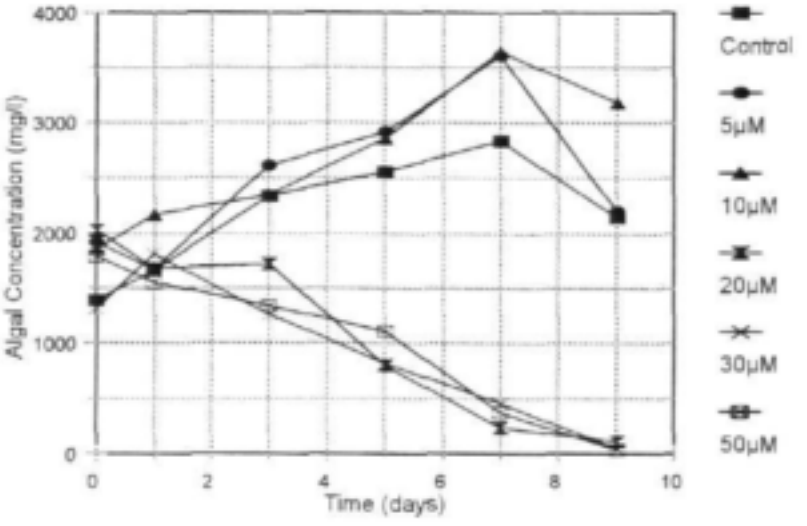


Figure 2.17: Mean algal concentration vs. time for zinc toxicity study (n = 3). Algal concentration determined from chlorophyll extractions.

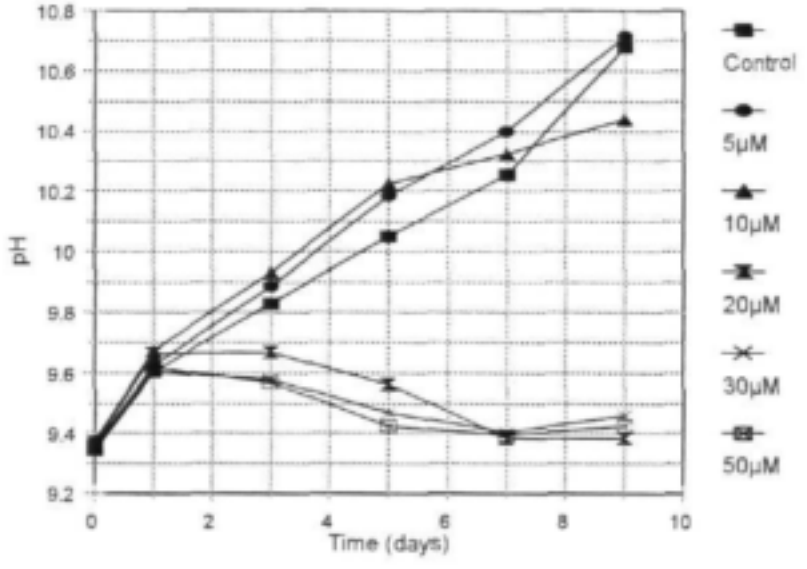


Figure 2.18: Graph of pH vs. times for zinc toxicity study (mean values, n = 3).

Figure 2.19, showing zinc accumulation by the culture, suggests that the *Spirulina* is capable of tolerating approximately 5 µmoles/g of zinc, as the cultures began to deteriorate once the concentration exceeded this. The reason for the dramatic increase in zinc accumulation seen in the 30 and 50µM groups after day five and seven respectively, is the same as for the similar trend observed during the copper study.

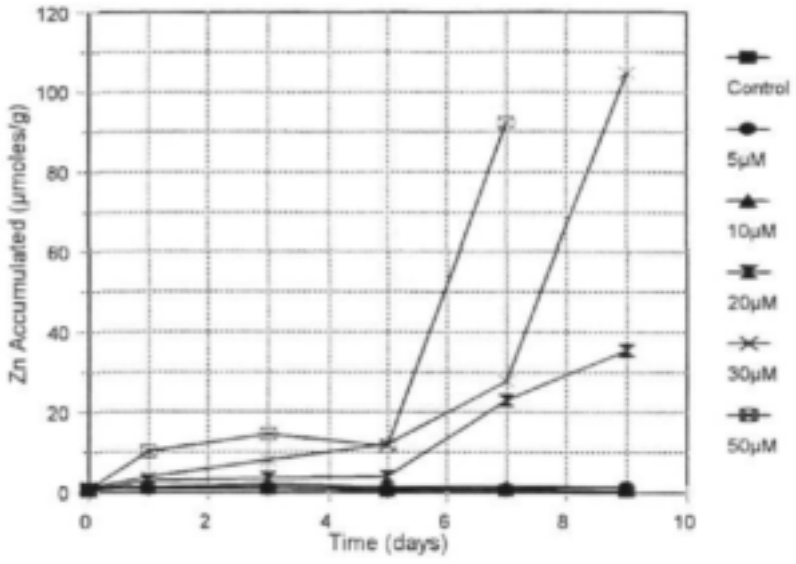


Figure 2.19: Zinc accumulated by *Spirulina* over 11 day period (mean values, n = 3).

The zinc in solution (results not shown) measured on day one was lower than the copper in the corresponding experiments and the reduction could not be attributed entirely to algal accumulation. This suggests that a portion of the zinc had either precipitated or been adsorbed to colloidal material, although the lack of significant amounts of extracellular carbohydrate (maximum concentration 30 mg/l for 10µM culture) provides evidence for precipitation. There was a 5-10 % increase in the zinc in solution between days three and five in the 20, 30 and 50µM groups, which was most likely as a result of metal being released as the cells began to disintegrate.

The deterioration of the cells can be seen in the photographs of the *Spirulina* grown in 50µM Zn^{2+} (Figures 2.20 and 2.21). The Figures show the state of the cells after two days. At this point the cells looked healthier than at the corresponding time when challenged with copper. This could be as a result of the higher initial algal concentration. After four days (Figures 2.22 and 2.23) the cells showed a greater degree of morphological damage, although they had not yellowed to the extent seen in the copper experiments. In addition the smooth appearance on the cell wall suggests that there has not been significant loss of cell contents.

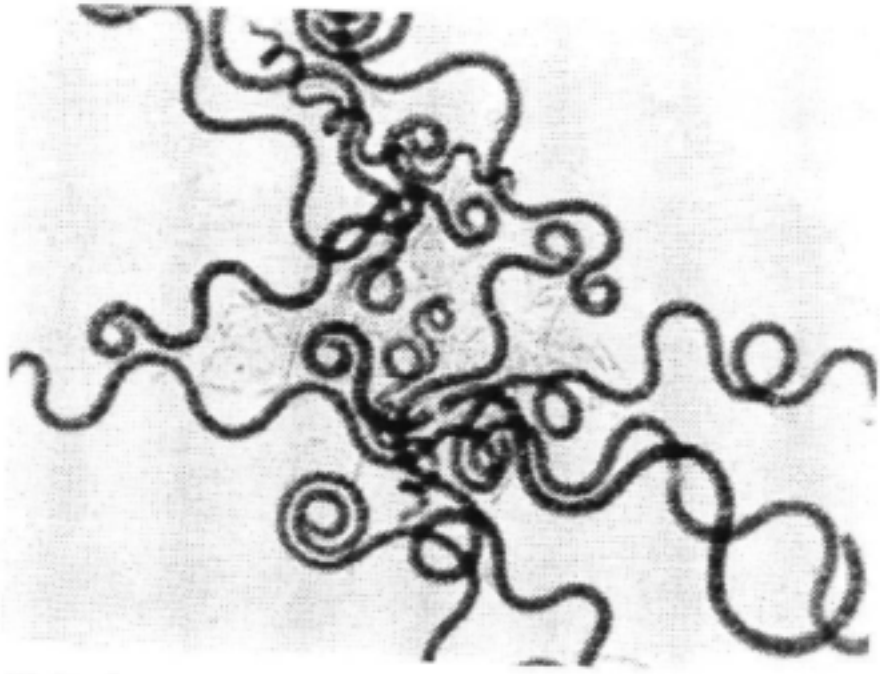


Figure 2.20: Light microscope preparation of *Spirulina* after exposure to 50μM Zn^{2+} for two days.

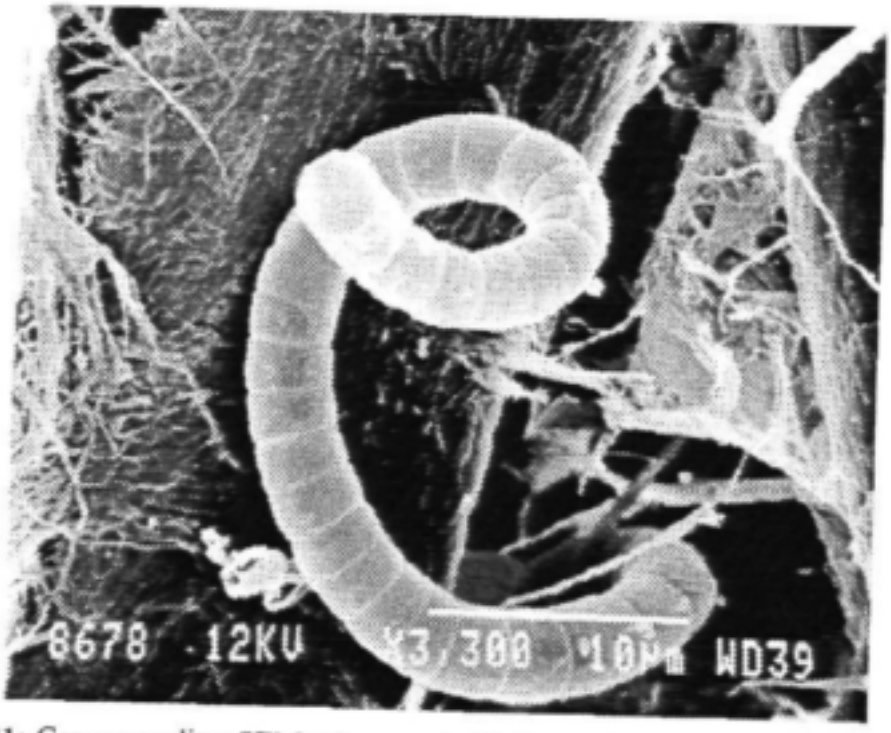


Figure 2.21: Corresponding SEM micrograph. Light specks on the cell surface represent electron dense regions possibly indicating metal accumulation.

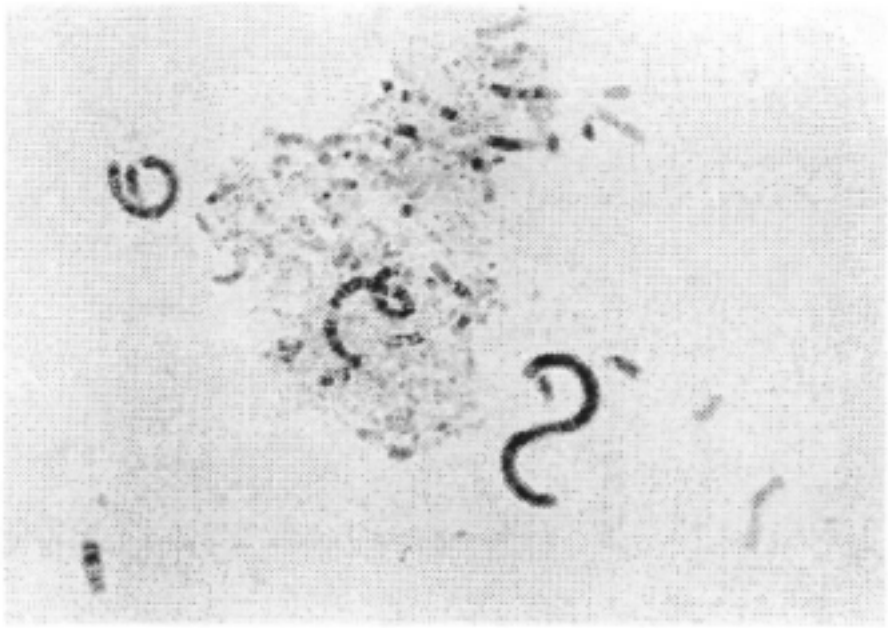


Figure 2.22: Light microscope preparation of *Spirulina* after exposure to 50μM Zn^{2+} for four days showing reduced degradation of photosynthetic pigments relative to copper treated cells.

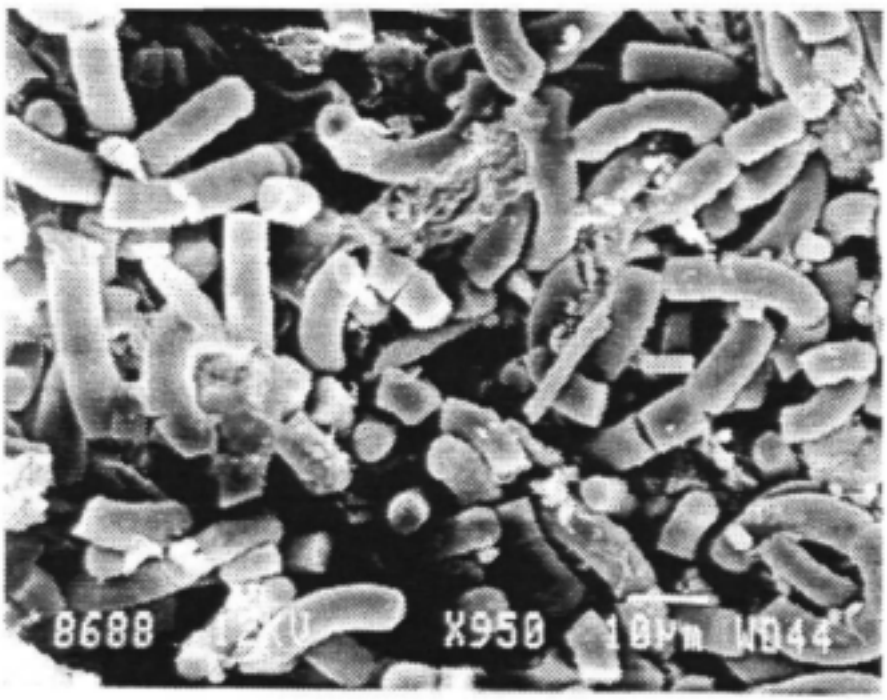


Figure 2.23: Corresponding SEM micrograph showing extensive filament degeneration without distortion of cell shape.

Copper is known to affect photosynthetic electron transport, especially in photosystem II. In addition, copper has been shown to interfere with pigment and lipid biosynthesis and consequently chloroplast ultrastructure (Stauber and Florence, 1987; Guanzon *et al.*, 1994; Barón *et al.*, 1995). While zinc has also been shown to inhibit photosynthesis, it requires higher concentrations to have an equivalent effect on photosynthesis, as compared to copper (Davies, 1983). Therefore, the difference in colour between the cultures grown in zinc and copper could be due to the more acute affect of copper on the photosynthetic pigments.

Copper has also been shown to increase the permeability of algal cells, whereas zinc did not (Overnell, 1975). This would explain why the surface on the copper treated cells became distorted after a number of days, presumably as a result of the loss of turgor pressure.

2.4.4.3 Lead

The growth curve data (Figure 2.24) indicated that lead is not as toxic to *Spirulina* as copper or zinc. The first sign of any significant decline in algal concentration occurred on day 7, at which point all groups, including the control, began to decline. This suggests that nutrient depletion was a contributing factor. Lead clearly had an inhibitory effect at high concentrations, as the cultures subjected to 50μM Pb^{2+} showed a reduced increase in algal concentration (Figure 2.24) and a decrease in pH after day three (results not shown). This indicates that there was no active growth in that group after day three. All the other cultures appear to have been affected to a reduced degree until day seven when all began to decline. As a result a threshold level could not be determined for lead.

Lead has been shown to inhibit the electron transport chain (ETC) in *Spirulina platensis*, but had relatively little effect on the photsynthetic pathway compared to copper (Murthy and Mohanty, 1995). Results of work conducted by Murthy and Mohanty (1995) are summarised in Table 2.4. The inhibition of the ETC would result in reduced energy being available for cellular metabolism, which would reduce cell growth and division. The results for the lead studies are consistent with this. The high level of inhibition of PSII by copper of similar concentrations to those used in this study provides further evidence to support the data presented in section 2.4.4.1.

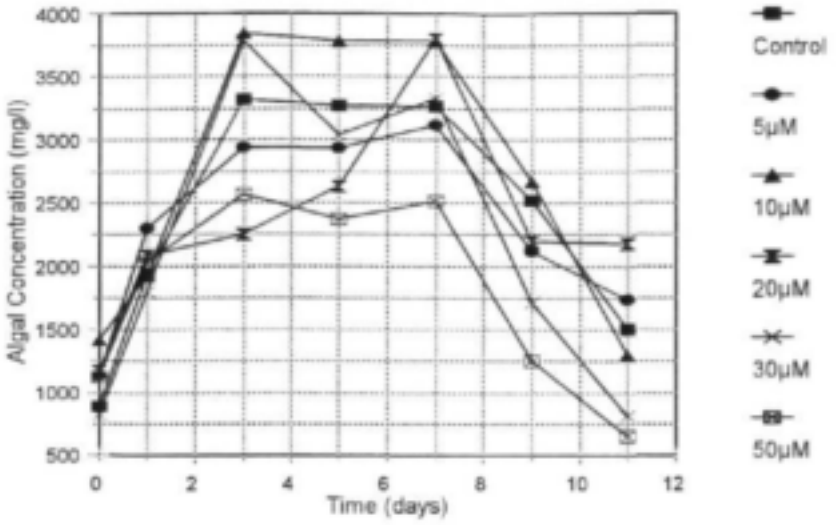


Figure 2.24: Mean algal concentration vs. time for lead toxicity study (n = 3). Algal concentration determined from chlorophyll extractions.

Table 2.4: Effect of various concentrations of copper and lead on the electron transport chain (ETC) and photosystem II (PSII) activity in *Spirulina platensis* (Murthy and Mohanty, 1995).

Metal salt	Conc. (µM)	Inhibition of ETC (%)	Conc. (µM)	Inhibition of PSII (%)
Control	0	0	0	0
$CuCl_2$	6	5	6	7
	18	26	18	50
$Pb(NO_3)_2$	6	21	18	6
	18	35	36	16

While lead did not have the same level of acutely toxic effect on the algae it did induce gross changes in cell morphology (Figures 2.25 and 2.26). The filaments do not appear to break apart, but become shrivelled in appearance. As the experiment proceeded an increasing amount of cellular debris became apparent. As there was little evidence of gross cellular disintegration it appears the debris consisted of cellular contents that had leaked out of the cells. Due to the saline nature of the growth medium, water would not diffuse into the cells resulting in a loss of turgor pressure and the cells developing a shrivelled appearance.

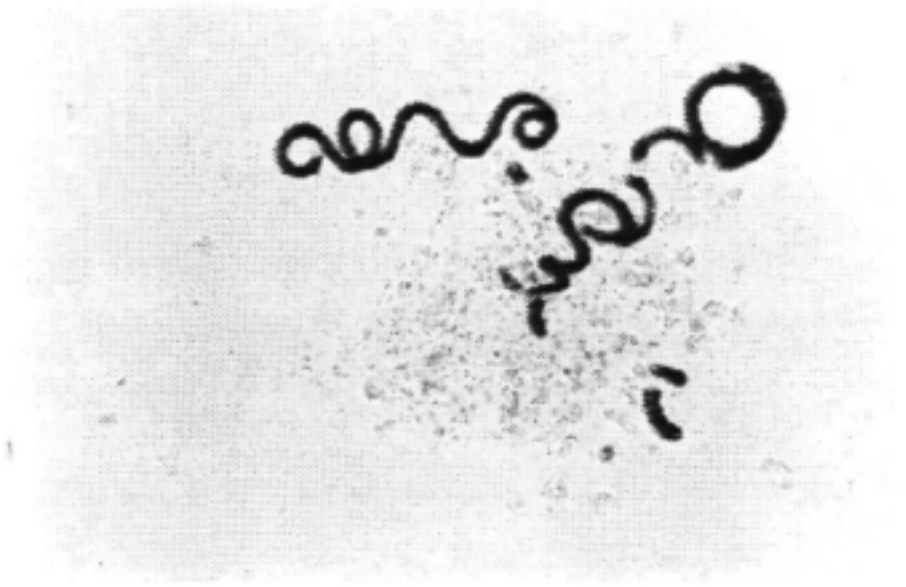


Figure 2.25: Light microscope preparation of *Spirulina* after exposure to 50 μM Pb^{2+} for four days.

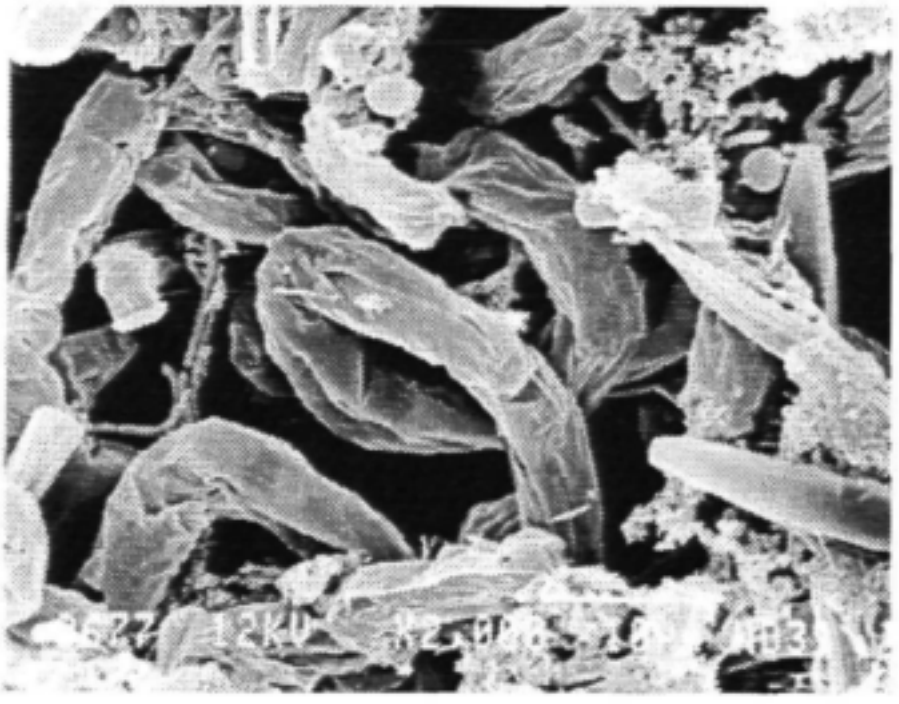


Figure 2.26: Corresponding SEM micrograph showing intact filaments, but grossly distorted cell surface.

2.5 Conclusions

This study shows that using *Spirulina*, in a direct algal pond treatment system, for an effluent containing even moderate concentrations of toxic heavy metals, such as copper and zinc, is not a viable option.

The biosorption data indicate that the adsorption of heavy metals to the *Spirulina* biomass complies well ($r^2 > 0.92$) with the Langmuir monolayer model, until active transport across the cell wall begins to occur. The kinetics of metal adsorption follows a similar pattern to that reported for other biosorbents and the affinity series of Pb>Cu>Zn conforms to published results obtained using a variety of biomass types. The adsorption capacity of *Spirulina* is significantly lower than what has been reported for most other algal and fungal species. This was true for all the metals tested.

The treatment of an effluent from a base metal mine by direct contact with *Spirulina* cultures was effective for a period of time, although the majority of metal removal could be attributed to alkaline precipitation as a result of the high bicarbonate content of the growth medium.

The exposure of the algae to copper induced the excretion of copper complexing polysaccharides. The copper-polysaccharide complex remained in solution, but the copper in the complex did not appear available for algal binding or accumulation. In terms of effluent treatment the presence of high concentrations of polysaccharide is not desirable as it prevents effective copper removal from solution. Zinc and lead did not stimulate significant polysaccharide secretion.

The toxicity data show that copper and zinc are both acutely toxic to *Spirulina*, with threshold toxicity levels of below 10 µmoles of metal per gram of biomass. The mechanism of toxicity appears different with the results suggesting copper affects the photosynthetic pigments and the integrity of the cell membrane, resulting in chlorophyll breakdown and the leakage of cell contents. Lead was less toxic, but did induce considerable changes in cell morphology with the cells becoming highly distorted. Lead has been reported to affect membrane stability and

the SEM images suggest that leakage of water and cellular components induced a loss of turgor pressure in the lead treated cells.

A significant finding was the ability of the algae to modify the aqueous chemistry of the surrounding medium through the utilisation of bicarbonate ions as a source of carbon dioxide for photosynthesis. The net result is an increase in pH and decrease in acidity of the system. This could be clearly seen by comparing the rate of pH change between the experimental and control groups in the effluent treatment study. The potential application of the phenomenon in wastewater treatment will be investigated in Chapter four.

CHAPTER 3: ALGAL MODIFICATION OF THE AQUEOUS ENVIRONMENT - THE ROLE OF CARBONIC ANHYDRASE

3.1 Introduction

The data presented in the previous Chapter showed that *Spirulina* was able to increase the pH of the surrounding medium and that, when dosed with an acidic effluent, the rate of pH decrease was significantly slower in flasks containing algae than the corresponding controls. This phenomenon, driven by the enzyme carbonic anhydrase, presents very real possibilities for the remediation of acidic effluents.

3.1.1 Carbonic anhydrase

Carbonic anhydrase (CA), also referred to as carbonate hydrolyase or carbonic dehydratase is a zinc containing metalloenzyme which catalyses the reversible interconversion of carbon dioxide (CO_2) and bicarbonate (HCO_3^-) with a maximum turnover number in excess of 10^6 s^{-1} (Braus-Stromeyer *et al.*, 1997; Eriksson *et al.*, 1996; Hiltonen *et al.*, 1998; So and Espie, 1998; Sültemeyer, 1998).

CA is a ubiquitous enzyme that has been isolated from a diverse range of organisms, including animals, plants, eubacteria, archaebacteria and viruses. To date no fungal CAs have been identified (Eriksson *et al.*, 1996; Hiltonen *et al.*, 1998; Sültemeyer, 1998). CA has been implicated in multiple and diverse physiological processes, including pH homeostasis, facilitated diffusion of CO_2, interconversion of CO_2 and HCO_3^-, ion transport, photosynthesis, efficient virus replication, calcification, bone resorption and the synthesis of urea, glucose and lipids (Braus-Stromeyer *et al.*, 1997; Eriksson *et al.*, 1996; Hiltonen *et al.*, 1998; So and Espie, 1998; Sültemeyer, 1998; Tashian, 1989).

The enzyme is classified into three independent CA gene families, namely α, ß and γ. There is little similarity in the DNA sequence between the three families, indicating that the genes have evolved independently at least three times. The α family is found primarily in animals, although homologues have been found in the bacterium *Neisseria gonorrhoeae* and the

periplasmic space of the green algae *Chlamydomonas reinhardtii*. This is the most extensively studied CA family and includes the mammalian CA isozymes. The ß family was originally isolated from the stroma of higher plants, but has subsequently been identified in various bacteria and algal species. The γ family has only recently been discovered with only one representative characterised to date (Eriksson *et al.*, 1996; Hiltonen *et al.*, 1998; Sültemeyer, 1998).

3.1.2 Structure of carbonic anhydrase

3.1.2.1 α-carbonic anhydrases

Mammalian CAs are divided into three groups, I, II and III, which share extensive homology in primary structure. All have a molecular mass of approximately 29kDa. In addition, crystal structures have shown that the secondary and tertiary structures are also similar (Tashian, 1989; Silverman, 1991).

Typically they are ellipsoidal molecules, approximately 5 x 4 x 4 nm, which could be considered single-domain proteins were it not for the loosely connected amino terminal region of about 24 amino acids. A 10-stranded, twisted ß-sheet is the dominant tertiary structure (Figure 3.1), which divides the molecule into two halves. The upper half includes the N-terminal helical region and the active site, while the lower half contains a large hydrophobic core. The ß-sheets, with the exception of two pairs of parallel strands, are antiparallel and are connected by hairpin loops and some helices. A number of relatively short helices are present on the surface of the molecule (Freskgård *et al.*, 1991; Lindskog, 1997).

The active site is located in a large, cone shaped cavity that penetrates close to the centre of the molecule. The zinc ion occurs at the base of the cleft (Figure 3.1) and is tetrahedrally co-ordinated to the imidazole rings of three histidine residues. The fourth co-ordination position is occupied by a water molecule. The ionisation of the water molecule is critical to the catalytic pathway (Silverman, 1991). The water ligand is hydrogen bonded to the side chain

of Thr_{199}, which in turn is hydrogen bonded to the side chain of Glu_{106}. These two amino acids are conserved in all α-CAs.

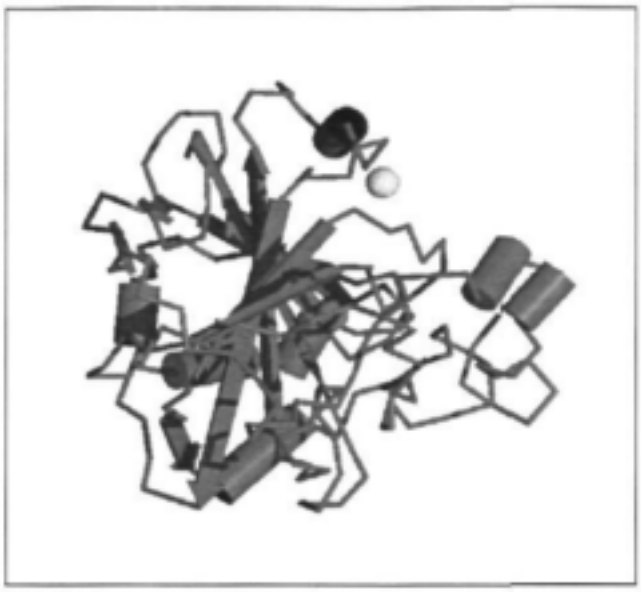


Figure 3.1: Tertiary structure of human carbonic anhydrase II. Cylinders represent α-helices and arrows the ß-sheets. Zinc ion shown in green.

3.1.2.2 ß-carbonic anhydrases

While no crystal structures of ß-CAs have yet been determined, they differ considerably from α-CAs in several respects. Their primary structure is entirely different which has made it impossible to identify any active site residues from sequence alignments (Johansson and Forsman, 1993). The ß-CAs contain a greater number of cysteine residues, compared to the α-CAs. Furthermore the oligomeric structure is different, although there has been some debate as to whether the molecule is a hexamer (Silverman, 1991) or an octomer (Johansson and Forsman, 1993). The ß-CAs also appear to have a greater number of α-helices compared to the α-CAs, which are composed primarily of ß-sheets.

3.1.3 Catalytic mechanism

The catalytic mechanism has been extensively studied using human carbonic anhydrase II (HCA II) and can be divided into two parts. The first part involves the interconversion of CO_2

and HCO_3^- (Equation 1). This is controlled by the catalytically active zinc-bound water molecule, which can be hydrolysed to a hydroxide ion. The second part is the regeneration of the active form of the enzyme (E), which involves the transfer of a proton from the catalytic site to the surrounding medium (Equation 2). The zinc bound hydroxide ion attacks a carbon dioxide molecule to form a metal bound bicarbonate ion, which is then displaced by a water molecule (Equation 1). The zinc bound hydroxide is regenerated by a rate-limiting transfer of a zinc-bound water proton to the His-64 residue, which delivers the proton to a buffer base (Equation 2). The reactions are shown in greater detail in Figure 3.2 (Lindskog, 1997).

$$EZnOH^- + CO_2 \rightleftharpoons EZnH_2O + HCO_3^- \quad (1)$$

$$EZnH_2O + B \rightleftharpoons EZnOH^- + BH^+ \quad (2)$$

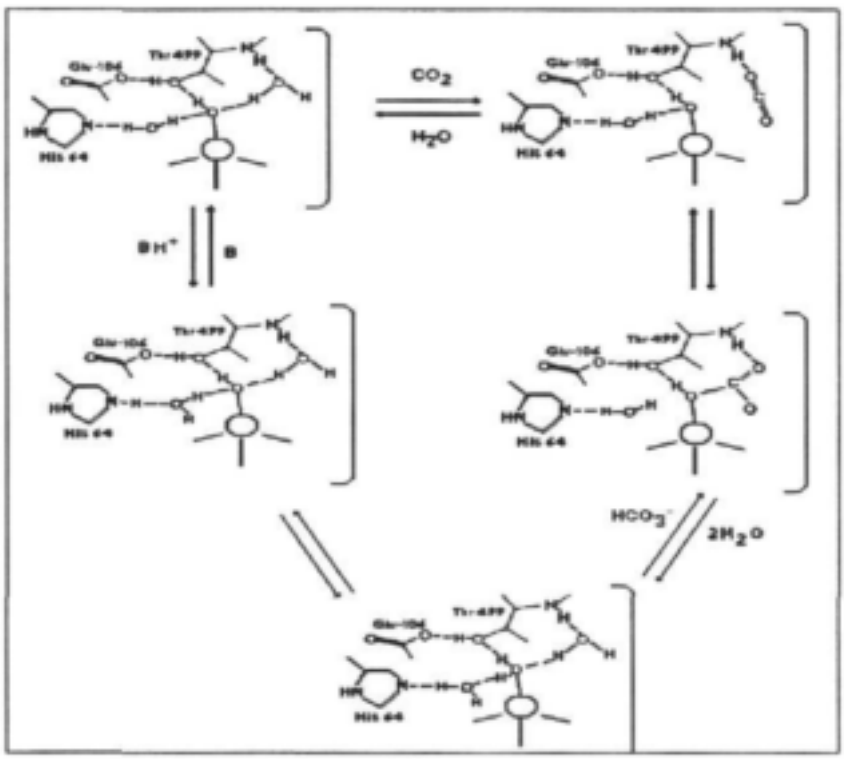


Figure 3.2: Schematic representation of the catalytic mechanism of HCA II (Lindskog, 1997).

3.1.3.1 ß-carbonic anhydrases

The catalytic capabilities of the ß-CAs are similar to those of the α-CAs, despite their different evolutionary origin. The kinetic parameters relating to the hydration of carbon dioxide by pea carbonic anhydrase indicated that the enzyme was as efficient as HCA II, although the observed kinetic patterns were more complex. The results were consistent with a zinc-hydroxide mechanism and the presence of a buffer-dependent proton-transfer step, although the proton transfer mechanism appeared different (Johansson and Forsman, 1993; Lindskog, 1997).

3.1.4 Inhibitors of carbonic anhydrase

The most extensively studied CA inhibitors are the sulfonamides. Aromatic and some heterocyclic sulfonamides, possessing the general formula $R\text{-}SO_2NH_2$ (or $R\text{-}SO_2NH(OH)$) are powerful and specific inhibitors of most CAs. They bind to the zinc ion as anions, via the nitrogen atom of the sulfonamide group (Lindskog, 1997; Moroney *et al.*, 1985). The NH group of the ionised sulfonamide replaces the zinc-bound water molecule and hydrogen bonds to the OH group of Thr_{199}.

Most monovalent anions are able to inhibit CA function, although the dissociation constants of the enzyme-anion complex vary considerably, from a few μM for CN^- to about 1M for F^-. The monovalent anions interfere with the hydrogen bonds formed between the zinc-bound water and the associated amino acid side chains (Lindskog, 1997).

3.1.5 Carbonic anhydrase in microalgae and cyanobacteria

The primary function of CA in algae, as in higher plants, is to increase the concentration of carbon dioxide around the photosynthetic enzyme ribulose biphosphate carboxylase (Rubisco). Both microalgae and cyanobacteria live in an aqueous medium where the concentration of carbon dioxide surrounding the cells is significantly less than that surrounding aerial plants. In order to maintain optimal photosynthetic rates these organisms have developed a carbon concentrating mechanism (CCM) that functions to raise the

intracellular carbon dioxide concentration to a level far in excess of that present in the surrounding environment. Carbon dioxide derived from this internal pool is used by Rubisco to initiate the first reaction in the Calvin cycle, the carboxylation of ribulose biphosphate. The inorganic carbon (C_i) transport system and CA are critical to the functioning of the CCM. The enzyme is induced by low levels of dissolved CO_2 and light (Badger and Price, 1992; Beardall *et al.*, 1998; Moroney and Chen, 1998; Sültemeyer, 1998).

3.1.5.1 Microalgae

The majority of the research into algal carbonic anhydrase has been conducted using *Chlamydomonas reinhardtii*, and the data accumulated have led to a broad understanding of the CCM in eukaryotic algae. A model of the CCM is shown in Figure 3.3 (Sültemeyer, 1998). This shows that the inorganic carbon needs to cross the cell wall, the plasma membrane and the membranes of the chloroplast envelope before it reaches the pyrenoid containing the Rubisco (Badger and Price, 1992).

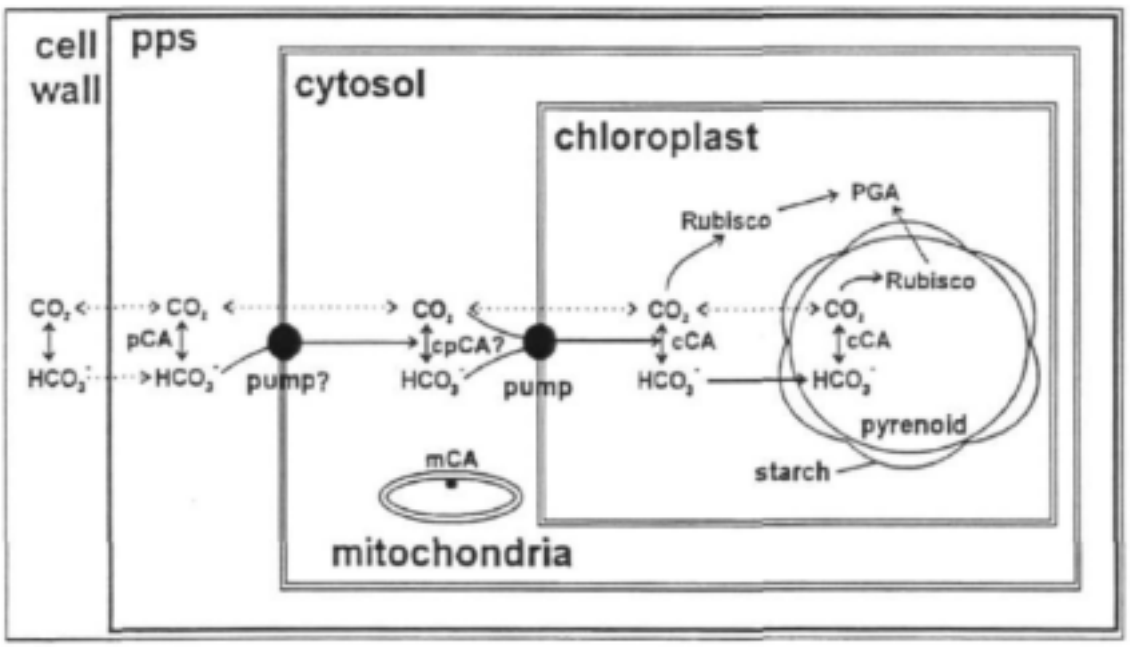


Figure 3.3: Proposed model for the carbon concentrating mechanism of *Chlamydomonas reinhardtii* as a model organism for microalgae (Sültemeyer, 1998).

There has been considerable debate as to which inorganic carbon species is actively transported across the membranes. Historically, it was assumed that bicarbonate was the species involved, but recent research has indicated that an active CO_2 uptake system exists in some organisms (Sültemeyer *et al.*, 1989) and that plastids are able to actively transport both CO_2 and HCO_3^- (Amaroso *et al.*, 1996).

Both extracellular and intracellular forms of the enzyme have been isolated from a variety of algal species (Williams and Coleman, 1996; Flores-Moya and Fernández, 1998). It is believed that the extracellular form accelerates the extracellular equilibration of CO_2 and HCO_3^- to provide CO_2 at a sufficient rate to enter the cell and serve as a substrate for photosynthetic reduction. The periplasmic enzymes are particularly important for the acquisition of inorganic carbon under alkaline pH conditions, where HCO_3^- is the predominant species (Husic and Marcus, 1994).

3.1.5.2 Cyanobacteria

Cyanobacteria are structurally different from eukaryotic algae, lacking discrete chloroplasts. The photosynthetic pigments are concentrated in carboxysomes. CA activities have been found to be lower and more variable in cyanobacteria. The enzyme appears to be either granular or soluble in nature, depending on the species (Badger and Price, 1989).

The mechanisms by which C_i transport occurs in cyanobacteria is still not well understood. A number of methods have been used to try and discriminate between transport of CO_2 and HCO_3^- and a general picture has emerged which indicates that the C_i transport system can utilise both species as substrates. The transport of CO_2 is, however, considered constitutive while the ability to accumulate HCO_3^- appears to be induced as a response to low external CO_2 concentrations. Regardless of the species accumulated from the external medium, it is HCO_3^- that is delivered into the cytosol. This HCO_3^- penetrates the carboxysomes, which contain the vast majority of the cell's Rubisco. In the carboxysomes specifically located CAs generate CO_2 in the immediate vicinity of the Rubisco (Al-Moghrabi *et al.*, 1996; Badger and Price, 1992; Sültemeyer *et al.*, 1995).

3.1.6 Implications for aqueous species equilibrium

Carbonic anhydrase, which is a critical component of the CCM of algae and cyanobacteria, is able to increase the CO_2 concentration around the photosynthetic enzyme Rubisco. The enzyme catalyses the interconversion of CO_2, H_2CO_3 and HCO_3^- via enhanced rates of hydration/dehydration. The simplified reactions involved in this process are as follows:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \quad (3)$$

$$H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \quad (4)$$

$$HCO_3^- \rightarrow CO_2 \text{ (inside cell)} + OH^- \text{ (outside cell)} \quad (5)$$

In the presence of elevated concentrations of bicarbonate in the surrounding medium equations (3) and (4) become negligible and equation (5) dominates. This leads to an accumulation of hydroxide ions in the medium, resulting in an increase in pH (Shiraiwa *et al.*, 1993). The increased pH shifts the bicarbonate/carbonate aqueous equilibrium towards carbonate formation. This phenomenon has been termed "alkalisation" (Haglund *et al.*, 1992; Shiraiwa *et al.*, 1993), but a closer look at the chemistry reveals that the total alkalinity of the system does not change, rather the increase in pH represents a decrease in the acidity of the system. The net result of the accumulation of inorganic carbon, from bicarbonate, by algae and cyanobacteria is an increase in pH and the shift in carbonate species equilibrium from bicarbonate to the more reactive carbonate and hydroxide species.

This Chapter will examine the efficiency of this mechanism and evaluate its potential application in the generation of reactive alkaline species for the remediation of metal contaminated acidic wastewaters.

3.2 Research aims

The primary aims of this section of the project were:

1) To extract carbonic anhydrase from *Spirulina* and determine enzyme activity.
2) To quantify the generation of reactive alkaline species from bicarbonate.

3) To evaluate the potential for remediation of heavy metal contaminated acidic wastewaters.

3.3 Materials and methods

3.3.1 Algal biomass

Algal biomass was obtained from stock cultures as described in Chapter 2.

3.3.2 Carbonic anhydrase assay

3.3.2.1 Assay system

The assay system was based on that of Wilbur and Anderson (1948) and modified according to Hiltonen *et al.* (1995) and Yang *et al.* (1985). The enzyme activity was determined electrochemically by measuring the time required for the pH to decrease from 8.2 to 7.2 when 2ml of ice-cold CO_2 saturated deionised water was added to 4ml of Veronal buffer (20mM, pH 8.3, 4°C) containing the test material. One unit of activity (Wilbur Anderson Unit (WAU)) was defined as:

$$WAU = t_0/t - 1 \qquad (6)$$

where t_0 was the time taken in an enzyme-free buffer (control) and t was the time taken in the experimental samples. Commercially pure bovine carbonic anhydrase, with an activity of 8100 WAU, was obtained from Sigma Aldrich. Carbonic anhydrase standards (10-150 WAU/ml) were prepared by diluting the stock solution. 100µl aliquots of standard were used in each individual assay. Carbon dioxide saturated water was prepared by bubbling CO_2 through 400ml of double distilled water (ddH_2O) at 4°C for one hour just prior to use.

3.3.2.2 Sample preparation and partial purification

Initial whole cell assays using *Spirulina* yielded negative values so after consultation with Professor David Husic (Lafayette College, Pennsylvania) it was decided to use a sample which had been boiled for five minutes as a control to remove any variable that may have resulted from the presence of algae in the assay system.

Algal samples were prepared by centrifuging 1ml of culture at 2000g for 10min in an Eppendorf 5403 centrifuge. Pellets were washed in an equivalent volume of 1g/l NaCl (to avoid osmotic shock) and centrifuged at 7000g for 20min. The pellets were then resuspended in an appropriate volume of 20mM Veronal buffer (pH 8.3). A 200µl aliquot was used for each individual determination.

Partial purification was achieved by subjecting the sample to two passes through a French pressure cell at 1500psi. The homogenate was centrifuged at 11 500g for 20min in an Eppendorf 5403 centrifuge. The resulting supernatant was ultracentrifuged at 100 000g for 60min in a Beckman L-70 ultracentrifuge. All fractions were assayed for CA activity.

3.3.3 Titration method for determining the effect of algae on the aqueous chemistry

3.3.3.1 Analytical technique

An indirect method for assaying carbonic anhydrase activity by analysing the effect of the algae on the aqueous chemistry of the medium was developed as a result of the inaccuracies of the Wilbur Anderson method. A titration-based system was developed to compare the effect of inorganic carbon accumulation by algae to the addition of sodium hydroxide (NaOH). The experiments were performed using 50mM sodium bicarbonate ($NaHCO_3$) in 1 g/l NaCl. The number of moles of hydroxide ions required to increase the pH of 50 ml of bicarbonate solution from 7.0 to 11.5 was determined. Titrations were repeated five times and the mean data used to generate formulae for the relevant conditions. The results showed that there were two phases over which the relationship between OH^- added and pH change were essentially linear. Regression analysis allowed the generation of the following formulae:

pH 7-9: μmoles OH^- = (ΔpH x 2.804) x 100 (r^2 = 0.97) (7)

pH 9-11.5: μmoles OH^- = (ΔpH x 10.819) x 100 (r^2 = 0.99) (8)

3.3.3.2 Batch experiments

For the batch experiments 50 ml of 50 mM $NaHCO_3$ in 1 g/l NaCl, adjusted to pH 7, was inoculated with viable algae (20-500 mg dry mass equivalent) that had been washed twice with 1 g/l NaCl to remove any traces of alkalinity. The culture was agitated at 120rpm, using a magnetic stirrer and flea. The experiments were initiated by switching on a bank of six cold, white fluorescent lights. pH measurements were taken each minute for the first 30 minutes and at five minute intervals thereafter. The temperature was maintained between 25 and 27 °C. Control experiments were conducted without addition of algae and in the absence of light. All experiments were repeated five times and mean values reported. The experiments were repeated using 50mg of algae, but after 60 minutes the pH was reduced to 8.3 by the addition of 0.02 N sulphuric acid (H_2SO_4). Following the pH adjustment the reaction was again initiated by illumination and the pH change recorded.

3.3.3.3 Effect of substrate concentration

Flasks were set up as for section 3.3.3.2 (five replicates), but at bicarbonate concentrations of 5, 10, 25 and 50 mM. The pH was adjusted to 8.3 to ensure maximum alkalinity as bicarbonate. The experiment was conducted for 30 hours, with pH measurements recorded hourly for the first 5 hours and at five hour intervals thereafter.

3.3.3.4 Inhibitor studies

Flasks were set up as in section 3.3.3.2 (five replicates) and the initial pH adjusted to 8.3. The effect of the CA inhibitor acetazolamide (AZ) was determined by dosing the cultures with the appropriate volume of 10mM AZ stock to achieve inhibitor concentrations of 25, 50, 75 and 100 μM. The experiment was initiated as before and the pH recorded at five minute intervals for 60 minutes.

3.3.4 Alkalinity assay

A second indirect method was used to determine the change in the aqueous alkaline species. A suiTable volume of sample was filtered through a 0.45 µm GF/C filter to remove algal biomass. The pH was recorded after which 0.02 N sulphuric acid (H_2SO_4) was added using a burette. The pH was monitored continuously and the volume of acid added was recorded at pH 8.3 and 4.5. The total alkalinity was calculated using the equation below:

Total alkalinity as mg/l $CaCO_3$ equivalents = ml acid added x (1000/sample vol) (9)

The alkaline species could theoretically be determined by using the volume of acid required to reduce the pH to 8.3 and comparing it to the amount needed to reach pH 4.5, but the results for the OH^- component proved inaccurate. A more accurate picture was obtained by using the aqueous species modelling software, MinteqA2, to predict the species present at a specific pH and total alkalinity.

3.3.5 Analysis and modelling of long term changes in aqueous chemistry

Spirulina cultures were set up in two litre culture flasks of Zarrouk's medium with an initial bicarbonate concentration of 16.8 g/l. The cultures were maintained at 25 °C with a light/dark cycle of 12 hours. Each day the pH of the cultures were recorded and a 10 ml sample was removed, filtered through a 0.45 µm GF/C filter and the alkalinity of the filtrate determined. The pH and alkalinity data were entered into Minteq to generate a comprehensive model of aqueous species present in the solution.

3.4 Results and discussion

3.4.1 Carbonic anhydrase assay

The Wilbur Anderson assay method did not prove accurate, even when used to test the commercially pure enzyme. In all cases the calculated enzyme activity was significantly higher than what was predicted from the dilutions (Table 3.1), with the degree of error

generally increasing with increased enzyme concentration, with the exception of the highest concentration.

Table 3.1: Comparison between expected and observed carbonic anhydrase activity units from commercially pure bovine carbonic anhydrase (n=5).

Expected activity WAUs	Observed activity WAUs	Standard deviation	Difference
1.000	1.296	0.351	0.296
2.000	4.107	0.496	2.107
3.000	7.791	0.256	4.791
4.000	10.114	0.560	6.114
5.000	12.579	0.570	7.579
15.000	21.179	0.748	6.579

The results of the carbonic anhydrase assays performed on the various fractions obtained from the partial purification are shown in Figure 3.4. The results clearly show that the Wilbur Anderson assay method is not reliable. The standard deviations are unacceptably high, in most cases exceeding the mean value. The highest enzyme activity was found in pellets resulting from the ultracentrifugation step (P2). This would suggest that the enzyme is membrane bound, but if this was the case the supernatant from the first centrifugation step (S1) should have had the highest activity as P2 is derived from S1. A number of authors have noted that the Wilbur Anderson method can be fairly rough and insensitive, with very little or no activity being detected in situations where substantial activity has been detected using alternative methods such as mass spectroscopy (Badger and Price, 1989; Haglund *et al.*, 1992; Raven, 1995). The methods however, require a pure enzyme preparation.

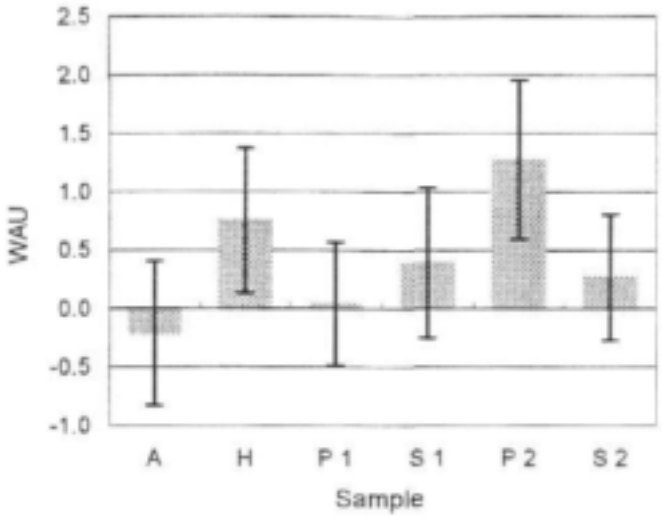


Figure 3.4: Carbonic anhydrase activity of partially purified fractions determined by Wilbur Anderson method. A = harvested algae, H = algal homogenate, P1 = pellet from 1st centrifugation, S1 = supernatant from 1st centrifugation, P2 = pellet from ultracentrifugation and S2 = supernatant from ultracentrifugation (n=5 ± SD).

3.4.2 Batch experiments

For the batch experiments 100mg of algae provided optimal results. The combined data from the batch experiment replicates are shown in Figure 3.5. The net pH change over the 60 minute period was 1.31 units. By substituting the pH change into the equation derived from the standard curve this represents the equivalent of adding 367 µmoles of OH^- ions. Therefore, it can be estimated that 1g of *Spirulina* (dry mass) is capable of generating the equivalent of 3670 µmoles of OH^- ions per hour under the experimental conditions.

No increase in pH occurred in the absence of algae or in the dark (results not shown). Figure 3.6 confirms the reduction of acidity during the accumulation of inorganic carbon. After the first 60 minutes the pH was adjusted back to 8.3 by the addition of 1.6ml of 0.02N H_2SO_4. The phenomenon can be explained in terms of acid and alkaline potential of the various species. The effect on alkalinity and acidity of the addition of various chemicals to water is summarised in Table 3.2.

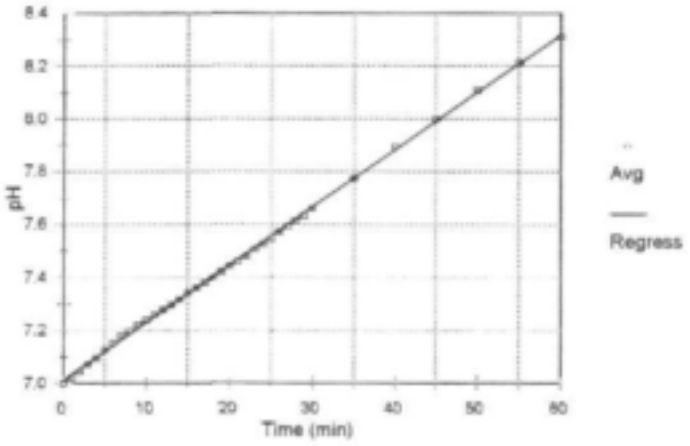


Figure 3.5: Mean data for pH increase vs. time for batch experiments (n=5, SD<0.1 for all points, $R^2 = 0.999$).

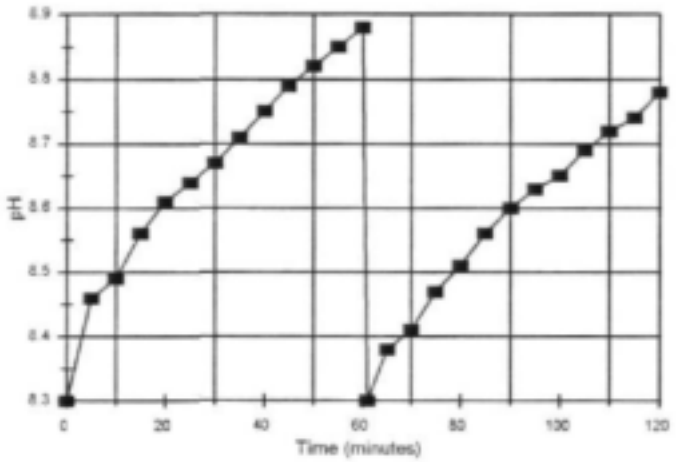


Figure 3.6: Change in pH vs. time as a result of inorganic carbon accumulation. pH adjusted to 8.3 after 60 minutes by adding H_2SO_4 (mean values, n = 5).

Table 3.2: Effect on alkalinity and acidity of the addition of various chemicals to water.

X ppm of chemical added (expressed as $CaCO_3$)	**Alkalinity change (as ppm $CaCO_3$)**	**Acidity change (as ppm $CaCO_3$)**
NaOH	+ X	- X
$NaHCO_3$	+ X	+ X
CO_2	0	+ X

In the case of the carbonic anhydrase catalysed utilisation of bicarbonate by algae, the substrate (HCO_3^-) has an acid and an alkaline potential. The bicarbonate is split into CO_2 and OH^-, with the CO_2 being utilised for photosynthesis. This removes the acid potential from the right hand side of the equation. The hydroxide ion is released into the medium so the net change in the acid/alkaline potential of the medium from +1 acid and +1 alkaline to +1 alkaline and -1 acid. Therefore, the term "alkalisation" is not strictly correct as the alkaline potential has not changed, rather the acidity of the system has been reduced and hence the pH has increased. The OH^- ions that are released into the medium affect the equilibrium between HCO_3^- and CO_3^{2-}, shown below.

$$HCO_3^- \rightleftharpoons CO_3^{2-} + H^+ \qquad (10)$$

The acid ionisation constant (K_a) for HCO_3^- (4.8×10^{-11}) suggests that at pH values below 8.3 all the carbonate alkalinity will be in the bicarbonate form. In the presence of a strong base, such as OH^- ($K_b = 1$), the reaction is driven to the right. The released protons equilibrate with the OH^- ions.

The validity of the reaction series proposed above can be tested by using Minteq to model the predicted pH at the end of the experiment. The proposed mechanism suggests that for each mole of carbon dioxide that is derived from bicarbonate, a further mole of bicarbonate is converted to carbonate as a result of the release of hydroxide ions into solution. Using the data shown in Figure 3.5 (first 60 minutes) it can be calculated that the pH change was equivalent to adding 160µmoles of OH^- ions. The proposed changes in alkaline species are shown in Table 3.3. The pH is determined by the ions in solution. Therefore, if the proposed system is correct, the equilibrium pH predicted by Minteq using the ion concentrations from Table 3.3 should be the same as the measured pH. Minteq predicted an equilibrium pH of 9.06 which is not significantly different ($P = 0.065$, $n = 5$) from the experimental mean. The slightly lower pH measured in the experimental flasks is most likely as a result of the system being open, which would allow some carbon dioxide to diffuse into the medium and reduce the pH.

Table 3.3: Proposed changes in alkaline species induced by algal uptake of inorganic carbon.

Component	Time = 0	Time = 60min	Change
pH	8.30	8.89	+0.69
Na^+ (μM)	3 355	3 355	0.000
Cl^- (μM)	855	855	0
HCO_3^- (μM)	2500	2180	-320
CO_3^{2-} (μM)	0	160	+160

3.4.2.1 Effect of substrate concentration

The effect of initial substrate concentration on the rate of pH change induced by algal accumulation of inorganic carbon is shown in Figure 3.7, which shows that the initial rate of pH increase is rapid. The pH change during the first hour is highest where the bicarbonate concentration is lowest. This is expected because the higher concentrations have a higher capacity to buffer change in pH. The effect of substrate limitation is clearly visible in the 0.5 and 1mM cultures. It has previously (section 3.4.2) been calculated that, under similar reaction conditions, *Spirulina* is capable of utilising up to 367 μmoles of HCO_3^- per hour. It has also been demonstrated that a maximum of 50 % of the bicarbonate is available for accumulation as the other 50% is converted to carbonate as a result of the release of hydroxide ions. The 5 and 10 mM $NaHCO_3$ cultures initially contained only 250 and 500μmoles of HCO_3^- ions respectively, so it is clear that this would rapidly become limiting and the pH would cease to increase. The decrease in pH observed in the 5 mM cultures most likely occurred due to the release of carbon dioxide as a result of cellular respiration.

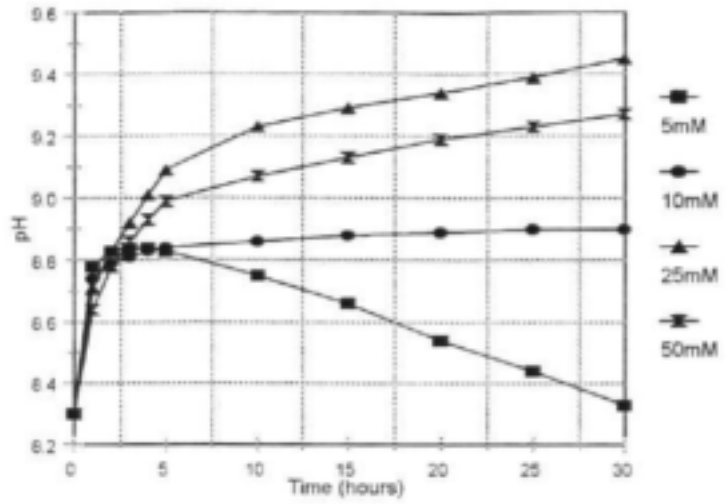


Figure 3.7: The effect of bicarbonate concentration on the rate of pH change induced by the utilisation of inorganic carbon by 100 mg *Spirulina* (mean values, n = 5).

3.4.2.2 Inhibitor studies

The effect of the addition of a carbonic anhydrase inhibitor to the cultures is shown in Figure 3.8. Acetazolamide (AZ) is a specific inhibitor of external CA. The molecule cannot enter the cell, so only affects enzyme that is associated with the cell wall and membrane. The results indicate that AZ has a significant effect on carbonic anhydrase at a concentration of 50μM and above, although the effect did not appear proportional to the concentration of inhibitor. The fact that there is still an increase in pH in the cultures grown at AZ concentrations of over 50μM suggests that both an internal and external CA enzyme are present in *Spirulina*. Sültemeyer *et al.*, (1998) has demonstrated that direct HCO_3^- transport can still occur in cells with an external CA. The HCO_3^- ions that are transported across the membrane can be utilised by internal CA in the region of the carboxysomes. The OH^- ions generated inside the cell will still be released to the external environment, resulting in an increase in pH.

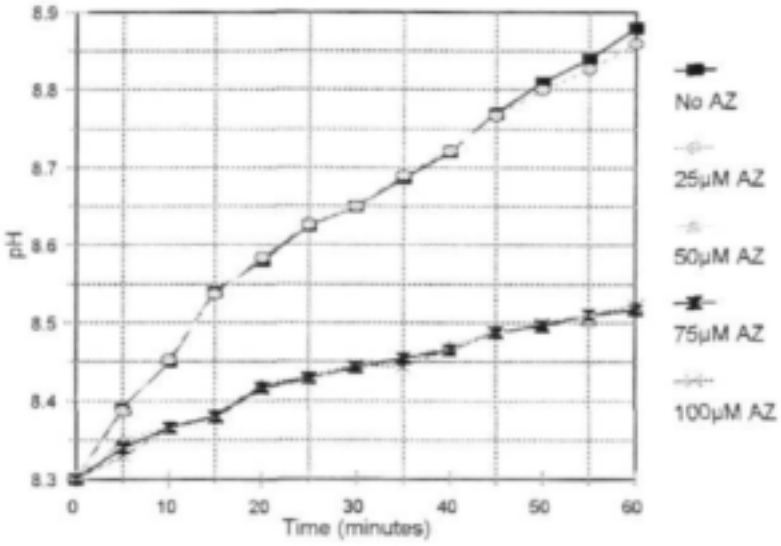


Figure 3.8: The effect of acetazolamide on carbonic anhydrase activity in *Spirulina* (mean values, n = 5).

3.4.3 Assessment of bioremediation potential

The effect of the change in carbonate species, induced by the algal accumulation of inorganic carbon, on the metal precipitation potential was assessed by direct measurement and predictive modelling. The two litre *Spirulina* cultures were maintained for a period of 13 days, at which point the cultures began to show signs of nutrient depletion. Over this period the pH increased from 8.6 to 9.8. Figure 3.9, which shows the change in alkaline species in solution over the experimental period, indicates a reduction of over 70% in the bicarbonate concentration by the end of the experimental period. There was a corresponding increase in the carbonate concentration. The molar relationship of carbonate generated relative to bicarbonate reduced is slightly below 50%. This is consistent with the proposed mechanism, as half the carbon is incorporated into the cells during photosynthesis. The increase in pH represents an increase in the concentration of OH^- ions relative to H^+ ions, but this could not be determined by the alkalinity assay. To accurately determine the hydroxide concentration the data from Figure 3.9 was entered into Minteq and the pH regulated speciation determined. The results are summarised in Figure 3.10.

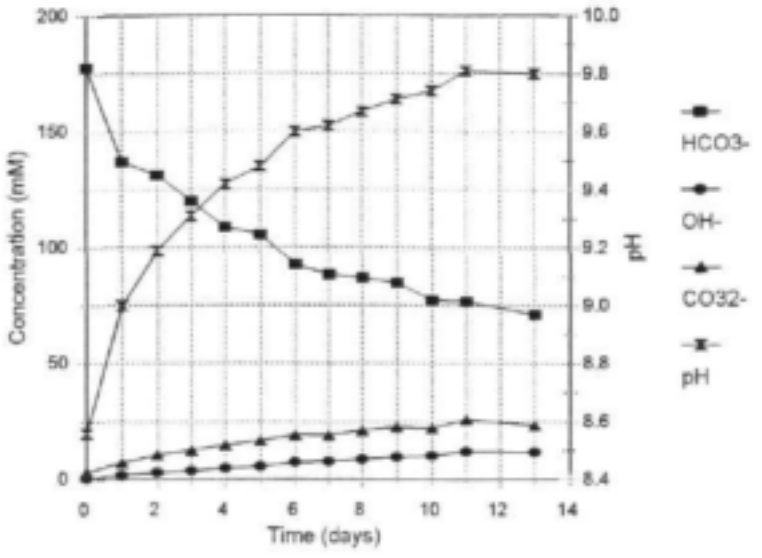


Figure 3.9: Changes in pH and alkaline species induced by cultivation of *Spirulina* in 2l Zarrouk's medium (16.8g/l $NaHCO_3$). Alkaline species determined by titration.

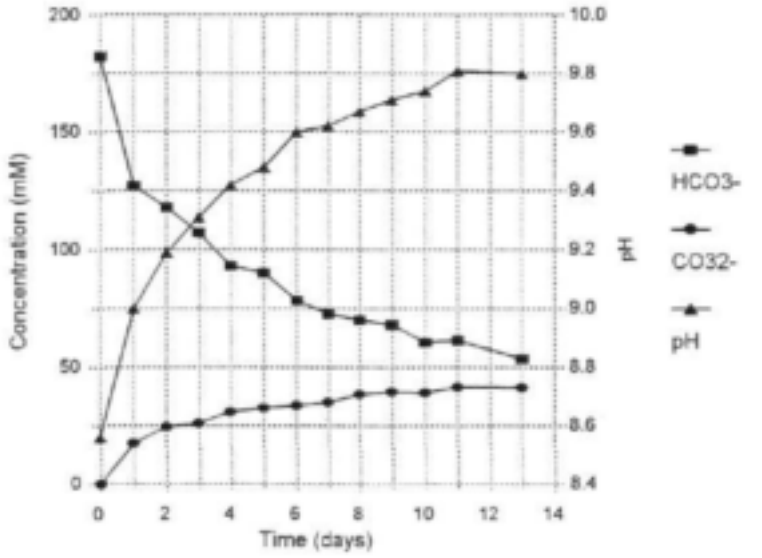


Figure 3.10: Changes in pH and alkaline species induced by cultivation of *Spirulina* in 2l Zarrouk's medium (16.8g/l $NaHCO_3$). Alkaline species determined by MinteqA2.

Comparisons of Figure 3.8 and Figure 3.9 show that predicted species at the specific pH values determined by the modelling software differ somewhat from those determined by the alkalinity titration method. The increase in hydroxide ions is expected due to the increase in pH and as the titration method cannot account for the hydroxide ions, the concentration of carbonate is overestimated.

Further modelling was conducted, using the Minteq programme, to determine the predicted effect of the change in alkaline species and pH on the precipitation of a range of metals commonly found in mining and industrial effluents. It was calculated that 333µl of filtered medium from day 0 would provide sufficient HCO_3^- to react with 60ml of 500µM metal solution, with a base to metal ratio of 2:1. From day 0-13 the precipitation was modelled by calculating the concentration of the various species that would result from adding 333µl of algal filtrate from the specific day to 60ml of metal solution. The change in predicted precipitation from day 0 to day 13 is shown in Table 3.4.

The predicted results show that for copper and nickel, which precipitate as hydroxides, the generation of reactive alkaline species will not increase the efficiency of precipitation. Iron, manganese, lead and zinc are predicted to precipitate as carbonates. These metals are acidic, which means that they are able to drive the dissociation of bicarbonate to carbonate and a proton. This explains the predicted precipitation on day 0, prior to the formation of significant amounts of carbonate. The biologically mediated generation of carbonate and hydroxide ions is likely to increase the efficiency of precipitation by 15-20%. Calcium and magnesium are also predicted to precipitate as carbonates, but they are not acidic and are only predicted to precipitate once the action of the algae has resulted in the formation of free carbonate ions (Greenwood and Earnshaw, 1984). The modelled data confirm the potential of the algal mediated modification of alkaline species to enhance metal precipitation and acid neutralisation.

Table 3.4: Percentage precipitation, predicted by Minteq, of various metals by the addition of 333 µl of algal filtrate to 60 ml of 500 µM metal solution. Initial bicarbonate to metal ratio of 2:1.

Metal salt	% precipitation (d0)	% precipitation (d13)	Precipitated species
$CaCl_2$	0.0	39.7	$CaCO_3$
$CuSO_4$[1]	82.8	68.4	$Cu_3(OH)_2CO_3$
	(47.5)	(47.6)	$Cu(OH)_2$
$FeSO_4$	46.7	47.7	$FeCO_3$
$MgCl_2$	0.0	25.6	$MgCO_3$
$MnCl_2$	41.4	51.8	$MnCO_3$
$NiCl_2$[2]	0.0	0.0	-
$PbCl_2$	49.8	61.4	$PbCO_3$
$ZnSO_4$	31.5	46.2	$ZnCO_3.H_2O$

[1] The predicted precipitating species, malachite, while thermodynamically the most sTable is kinetically unlikely to form in a short time. The results in parentheses are for $Cu(OH)_2$ which should be favoured kinetically. This will be further investigated in Chapter 6.

[2] At the 2:1 ratio of base to metal, nickel is not predicted to precipitate, although 53.8 % of the nickel in solution is in the form $NiCO_{3(aq)}$ which increased to 51.7 % by day 13. Doubling the bicarbonate concentration resulted in a predicted precipitation of 12.2 % as $Ni(OH)_2$

3.5 Conclusions

The results presented in this chapter clearly show that the Wilbur Anderson method of assaying carbonic anhydrase activity is not consistently accurate, particularly when applied to raw and partially purified material. The activity of the enzyme could be indirectly assessed by measuring the rate of pH increase of a solution containing a predetermined concentration of bicarbonate. This could be compared to results from standard titrations using sodium hydroxide which allowed the expression of the enzyme activity in terms of a hydroxide equivalent.

The experiments indicated that a *Spirulina* concentration of 2000mg dry mass/l (100mg in 50ml) was optimal and resulted in acidity reduction equivalent to the addition of 3670µmoles OH^- g^{-1} $hour^{-1}$. This efficiency will most likely be somewhat reduced under environmental conditions. Ideally the results should be expressed in terms of OH^- equivalents per mg enzyme, but attempts in this laboratory to isolate and purify carbonic anhydrase from *Spirulina* have proved unsuccessful (Payne, 2000).

There is strong evidence to suggest that the proposed mechanism for the accumulation of inorganic carbon and the subsequent modification of alkaline species in solution is correct. The aqueous species modelling software MinteqA2 was used to predict the pH after various time intervals based on the expected change in alkaline species. The results were not significantly different to those recorded experimentally.

Inhibitor studies performed using various concentrations of acetazolamide clearly indicated significant inhibition of carbonic anhydrase at inhibitor concentrations of 50μM and above. Acetazolamide cannot enter the cell, so the fact that there was a significant, although reduced, increase in pH in the cultures subjected to high doses of inhibitor suggests that *Spirulina* has both an internal and external carbonic anhydrase. Furthermore, the data suggest that *Spirulina* has some capacity to directly transport bicarbonate ions into the cell.

Experiments conducted with larger volumes and for up to 13 days indicated that *Spirulina* was able to increase the pH to almost 10. Alkalinity measurements confirmed that despite the increase in pH there was no increase in alkalinity. The algae were able to reduce the initial bicarbonate concentration by up to 70 % through carbon dioxide accumulation and by conversion to carbonate and hydroxide. Predictive modelling was conducted assuming the addition of 333 μl of filtered medium to 50 ml of 500 μM metal solution, an alkalinity to metal ratio of 2:1. The results indicated that over the 13 day period the potential of the medium to precipitate iron, manganese, lead and zinc increased by 15-20%, while the potential to precipitate the hardness causing metals, calcium and magnesium, increased from zero to up to 40%. There was no effect on the precipitation of copper and nickel which are thought to precipitate as hydroxides. Further modelling and experimental data to determine the optimal mixing regime for the effective remediation of a complex effluent will be discussed in Chapter 4.

From the data it can be concluded that the biological modification of alkaline species can significantly enhance the ability of alkaline streams to reduce acidity and precipitate metals. The requirements for such a system are a source of bicarbonate alkalinity and conditions conducive to the culture of algae. On an industrial scale the provision of bicarbonate as a chemical salt would not be a sustainable or cost effective option.

CHAPTER 4: AN INTEGRATED BIOLOGICAL SYSTEM AND PILOT PLANT STUDIES

4.1 Introduction

The previous chapters have indicated that an extensive body of literature exists relating to the biology, ecology and physiology of a wide range of organisms, which have been evaluated in terms of their ability to reduce aqueous metal and sulphate concentrations. Similarly, a number of reactor configurations and electron donors have been investigated for the cultivation of sulphate reducing bacteria (Maree *et al.*, 1987; Maree and Hill, 1989; Barnes *et al.*, 1991; Hammack and Edenborn, 1992; van Houten *et al.*, 1994) and the ability of large scale algal ponds to treat a number of domestic and industrial effluents has also been demonstrated (Oswald, 1990; Mara *et al.*, 1996; Rose *et al.*, 1996).

Notwithstanding this, there has been very little work published on the integration of algal and bacterial processes for the treatment of industrial effluents. The major contribution in this field has come from Professor Peter Rose and his research group at Rhodes University. The pioneering work was undertaken by Boshoff *et al.* (1996) and Dunn (1998) and concentrated on the co-disposal of sewage sludge and saline wastewaters, derived from the tanning process. The results showed that sewage sludge and tannery effluent could be used effectively as electron donors and carbon sources, particularly for sulphate-salinity reduction applications. The work progressed further and resulted in the conceptual development of the 'Algal Sulphate Reducing Process for Acid and Metal Wastewater Treatment' - the ASPAM Process (Rose *et al.*, 1998).

Initial plans to scale up the ASPAM process using tannery effluent as the electron donor were put on hold as a result of the Grootvlei mine incident, described in Chapter 2. The initial results obtained by Boshoff *et al.* (1996), Dunn (1998) and Whittington-Jones (2000), on the accelerated hydrolysis of primary sewage sludge indicated the potential to use an integrated system, utilising sewage sludge as the carbon source, to treat the water being pumped from Grootvlei. This led to the conceptualisation of the patented Rhodes BioSURE Process, which was later piloted on site (Rose and Hart, 1998). The pilot plant was able to treat

approximately 140 000l of mine water per day, with a sulphate removal efficiency of between 14.8% and 55.3% during the initialisation phase, stabilising between 65-70% once the system had been optimised (Corbett, 2001).

While the ASPAM and BioSURE Processes both contain SRB and algal components, in both cases the role of the algae is primarily as a polishing step, to remove trace amounts of metal, oxidise any remaining sulphide and reduce nutrients. The metal precipitation in these processes is achieved by blending the recycled, sulphide-rich, treated water with the AMD. Metal precipitation is presumed to occur as sulphides and possibly carbonates (Corbett, 2001).

The results described in other studies, comparing the metal precipitation achieved using sulphide-rich anaerobic digester overflow to carbonate-rich algal overflow, clearly indicate the benefit of utilising the sulphide-free system, particularly to achieve iron removal. The two integrated systems described in this chapter, while essentially containing the same components as the ASPAM and BioSURE Processes, differ in that the role of the algae is far more integral and the metals are not precipitated as sulphides. The exception to this is where gaseous hydrogen sulphide is used to achieve selective precipitation of some metals, prior to the blending with the algal overflow.

4.2 Research aims

The primary aims of this section of the project were:

1. To evaluate a number of reactor configurations for the continuous treatment of acidic, metal-laden effluents at a laboratory pilot scale.
2. To assess the suitability of enriched, partially treated effluent as a feed for the sulphate reducing bacteria.
3. To assess the accuracy of using MinteqA2 to model the metal precipitation occurring in reaction vessel during the continuous operation phase.

4.3 Treatment of a base metal effluent by a *Spirulina* based system

The results shown in Chapter 3 demonstrated the potential of a *Spirulina* based system for the treatment of AMD, although a direct contact system did not prove sustainable due to the acute toxic effects of copper and zinc. The ability of algae to generate reactive alkaline species through the accumulation of inorganic carbon was demonstrated in Chapter 4. This led to the development of a non-contact system for the continuous treatment of AMD, described below.

4.3.1 Process description

A schematic representation of the non-contact system is shown in Figure 4.1.

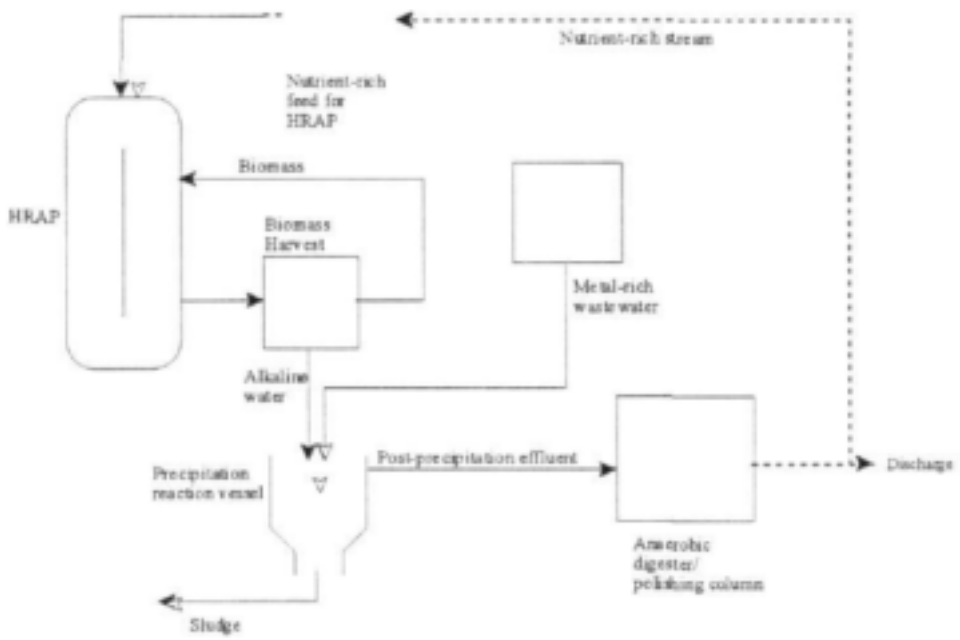


Figure 4.1: A generalised description of a process for the treatment of acidic, metal-laden effluents (van Hille *et al.*, 1999).

The nutrient and bicarbonate-rich algal feed is pumped into the high rate algal pond (HRAP), where the bicarbonate is utilised by the algae to obtain carbon for photosynthesis. The reactions involved result in an increase in pH and a shift in carbonate species equilibrium

towards carbonate and hydroxide. The overflow from the HRAP passes through a biomass harvest and recycle step and the aqueous alkaline component passes into the precipitation reaction vessel, where it is blended with raw effluent. The rate of effluent addition is determined by calculating the optimum mixing ratio, based on the alkalinity of the HRAP overflow and the acidity of the effluent. The resulting metal sludge settles at the bottom of the cone shaped reaction vessel, from where it can be drained off. The clarified liquid can either be discharged or if necessary passed through a polishing step to remove trace amounts of metals. If the initial effluent is high in sulphate it will be necessary to pass the post precipitation effluent through a sulphate reduction step as no sulphate is removed during the alkaline precipitation step.

4.3.2 Materials and methods

4.3.2.1 Analytical techniques

The determination of pH, acidity, alkalinity, chlorophyll content, sulphate, sulphide, phosphate, ammonia and metal concentrations were conducted as described in previous chapters. In all cases samples from the individual reactors were performed in triplicate and mean values are presented.

4.3.2.2 Effluent pH profile

A pH profile was performed on the base metal effluent (appendix I) to determine the pH dependency of the metal precipitation. Twelve 100ml samples of the effluent were removed and the pH of the samples were increased by the addition of 1M NaOH. A pH range of 2-13 was used. All samples were allowed to react for 60 minutes after which a sample was removed and filtered through a 0.45µm nylon membrane filter. The concentration of metal in the filtrate was determined by AA spectroscopy and the percentage metal removal was determined. The pH profile was performed in triplicate.

4.3.2.3 Control reactors

Prior to the continuous operation phase, a number of control reactors were set up, similar to those described in section 2.3.6. The initial algal culture was established in Zarrouk's medium which has a high alkalinity due to the sodium bicarbonate. To determine the metal precipitation potential of this in the continuous system, two five litre reactors were set up. The first contained a culture of *Spirulina* in Zarrouk's medium and the second, only media. In both cases tap water was pumped into the reactors at a rate of 1ml/min. The overflow from the reactors passed into the reaction vessel, into which the effluent was pumped at a rate of 1ml/min. The pH and algal concentration in the 5l growth vessel were measured daily. The pH and metal concentrations in the reaction vessel were also measured on a daily basis.

4.3.2.4 Continuous operation

For the continuous operation of the system, the *Spirulina* was maintained in a 30 l, oval, raceway pond. The algae were maintained in suspension through the action of a paddle wheel. The algal culture was fed at a rate of 1.4 ml/min on the liquid overflow of an anaerobic digester treating primary sewage sludge, obtained from the Grahamstown municipal sewage treatment works. Overflow from the algal growth vessel was passed through a filtration step (nylon mesh, 0.2 mm mesh size). The filtrate passed into the reaction vessel and the algal biomass was returned to the growth vessel. Effluent was pumped into the reaction vessel, at a rate of 1.4ml/min, where metal precipitation occurred. The overflow from the reaction vessel was collected and supplemented with yeast extract (1 g/l), 60 % sodium lactate (6 ml/l), ammonium chloride (1 g/l) and potassium dihydrogen orthophosphate (0.5 g/l). The mixture was pumped into a 20 l anaerobic digester, seeded with an SRB consortium, at a rate of 2. 8ml/min. The overflow from the reactor was sampled daily to determine pH, sulphate, sulphide and metal concentrations.

4.3.3 Results and discussion

4.3.3.1 Effluent pH profile

The results of the pH profile are shown in Figure 4.2 and indicate that for effective precipitation of all four metals to be achieved, the pH in the reaction vessel would need to be maintained above pH 8. The pH profile suggests that should the pH decrease below 8 the efficiency of zinc removal would be affected first, followed by copper, lead and eventually iron. This sequence is consistent with theoretical values (Greenwood and Earnshaw, 1984) and modelled predictions generated by MinteqA2.

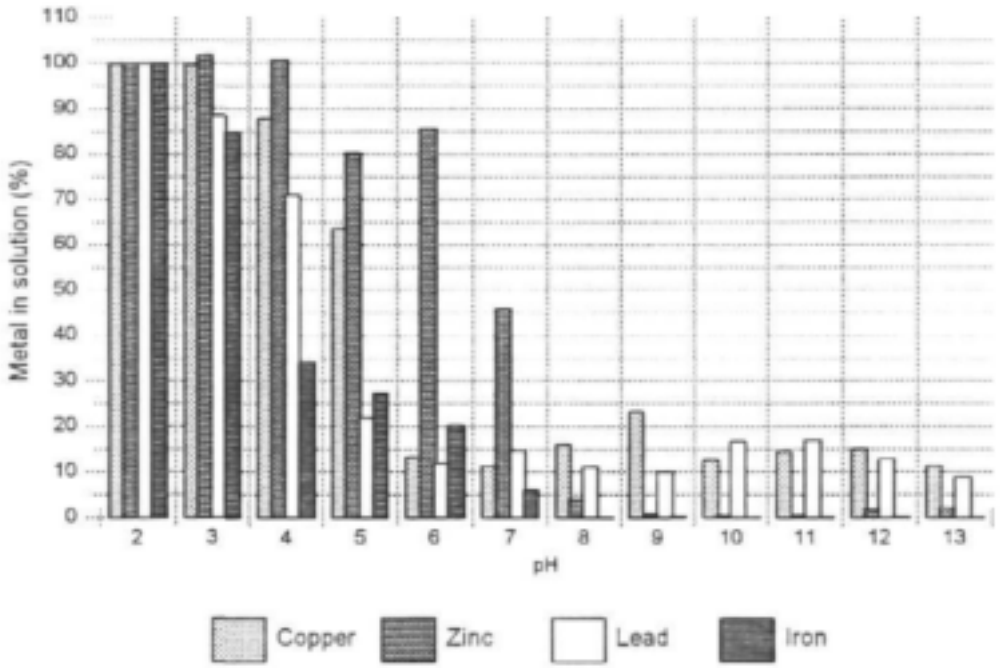


Figure 4.2: pH profile of effluent from the Black Mountain base metal mine illustrating the percentage of each metal remaining in solution following the increase of the pH from 1.99 by the addition of NaOH. (mean values, n = 3).

4.3.3.2 Control reactors

The algal concentration in the 5 l growth vessel remained relatively constant during the first five days, during which time the pH increased from 9.00 to 9.85, due to the accumulation of

inorganic carbon. After day five, the algal concentration began to decrease, primarily as a result of nutrient stress, but also as a result of biomass washout due to the nylon mesh not being a totally effective filter. The pH decreased steadily over the same period, to pH 9.03 on day 14, due to replacement of alkaline Zarrouk's medium with tap water. The pH change in the algal free control was more dramatic, with the pH decreasing from 9.00 to 7.67 over the corresponding period.

Figures 4.3 and 4.4, showing the changes in pH and metal removal observed in the reaction vessel, clearly demonstrate the contribution of the algae to the efficiency of the process. In both cases the pH in the reactor vessel decreases, although the decrease in the algal free control system is far more rapid and significant. In both cases, the initial concentration of alkalinity was the same. The results presented in Chapter 4 show that accumulation of inorganic carbon, from bicarbonate, by the algae does not lead to an increase in total alkalinity but rather reduces the acidity of the system. The rate of effluent addition and hence acidity addition was the same in both systems. Therefore, the overflow entering the reaction vessel had the same alkalinity in both cases, but the overflow from the system containing the algae had a greatly reduced acidity, allowing it to buffer the acidity of the effluent more effectively and for a longer period of time.

The metal removal is essentially pH dependent and follows the trend observed in the pH profile (Figure 4.2). In both systems there was a proportional decrease in zinc removal after the pH fell below 7.5. The zinc removal in the algal free system was negative on days 9 and 10 and the copper removal on day 10, after the pH had dropped to 4, as a result of some precipitated metal redissolving. The removal of lead and iron remained constant in the system containing algae for all 14 days, but began to decrease after day eight in the algal free system, as the pH fell below 6.

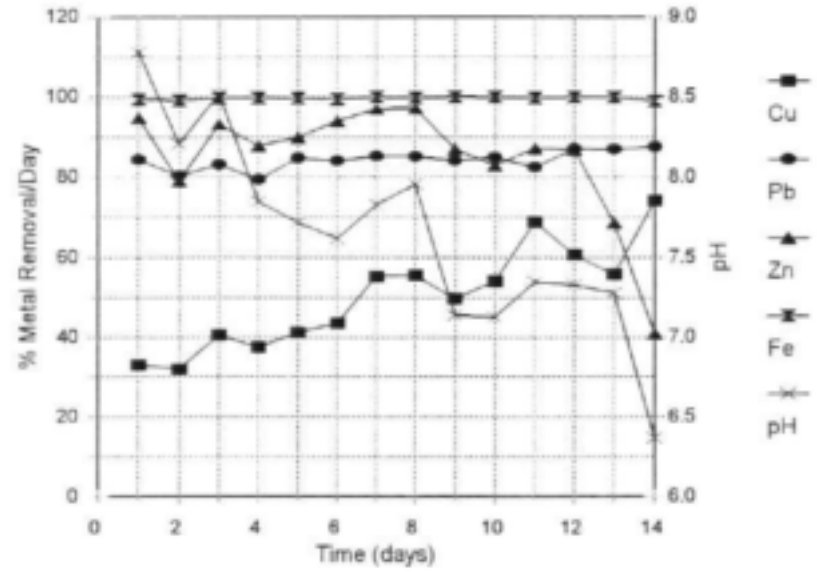


Figure 4.3: Relationship between metal removal for each 24 hour period and pH in the reaction vessel for the control system containing algae. Metal removal calculated by analysing the reaction vessel overflow.

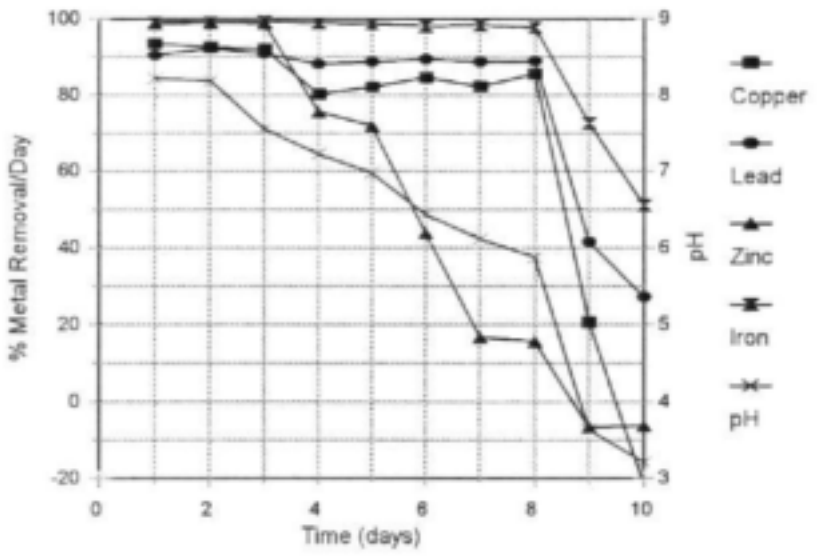


Figure 4.4: Relationship between metal removal for each 24 hour period and pH in the reaction vessel for the algal free control system. Metal removal calculated by analysing reaction vessel overflow.

The copper removal in the system containing algae showed an opposite trend, with a low initial percentage removal, which increased as the experiment progressed. This can be attributed to the presence of copper complexing extracellular polymeric substances (EPS), as described in Chapter 3. They have been shown to be released as a result of nutrient stress, which would be induced as the tap water diluted the growth medium (Singh *et al.*, 1999). At low concentrations of EPS the complex remains soluble, but as the concentration increases it forms insoluble aggregates (Boshoff, unpublished results). As the experiment progressed the nutrient stress became more acute and hence the EPS production increased, accounting for the increase in percentage copper removal.

4.3.3.3 Continuous operation

The results of the continuous operation of the system are summarised in Table 4.1 and indicate that the system was able to run effectively for a 30 day period, reducing the concentration of all four heavy metals to below the acceptable risk criteria determined by the Department of Water Affairs and Forestry (1998).

Table 4.1: Summary of the performance of the *Spirulina* based integrated process for the treatment of a base metal effluent. The results (mean ± SD) represent a combination of the daily measurements over a 30 day period.

Component	Units	Effluent	After RV[1]	After AD[2]	% reduction	AR[3]
pH	-	1.99	8.26 ± 0.37	8.12 ± 0.16	-	-
Sulphate	mg/l	4415	2236 ± 63	295 ± 27	89.1	-
Copper	µM	54.90	1.52 ± 0.11	1.42 ± 0.09	94.3	1.6
Iron	µM	1771.8	8.3 ± 4.1	4.7 ± 2.5	99.5	161.2
Lead	µM	11.30	0.27 ± 0.08	0.27 ± 0.06	95.1	0.5
Zinc	µM	109.50	2.98 ± 0.68	3.37 ± 0.46	93.8	10.7

[1]RV = reaction vessel, [2]AD = anaerobic digester, [3]AR = accepTable risk level.

It is clear from Table 4.1 that the majority of the metal removal occurs as precipitation in the reaction vessel as a result of mixing the effluent with the alkaline overflow from the algal growth vessel. During the continuous operation phase the algae were fed on overflow from an anaerobic digester treating sewage sludge. This feed had a bicarbonate alkalinity of between 1100 and 1300 mg/l $CaCO_3$ equivalents and also contained sufficient nitrogen and phosphorous to prevent the algae from suffering nutrient stress. As a result, the overflow

from the algal growth vessel contained EPS concentrations low enough (18 ± 13mg/l) not to affect copper precipitation.

It is also clear that the alkaline precipitation step did not reduce sulphate concentration, other than by dilution, resulting from the mixing of the two streams. This necessitated an additional anaerobic digestion step. The combination of the dilution and anaerobic digestion steps resulted in an 89 % reduction in the sulphate concentration.

The advantage of the system described above, stems from the fact that neither the algal or bacterial components come into direct contact with the effluent. This allows both components to operate under optimal conditions, without any stress related to toxic heavy metals or acidic pH values. The system did, however, have a number of shortcomings. The most significant were the problems related to using an algal species which was suspended in the liquid medium. This necessitated constant agitation, which would increase the operational cost, as well as a solid-liquid separation stage, to avoid biomass loss. The nylon-mesh filter was not entirely effective and by day 30 the biomass concentration in the algal growth vessel was 32 % lower than at the start, which led to a reduction in efficiency. In addition, if the system where to be scaled up the nylon-mesh filter would not be appropriate. A second shortcoming lay in the fact that the system had no potential to facilitate the selective recovery of potentially valuable metals.

A second system that depended on the same basic principles, but overcame the problems described above, was developed. The *Spirulina* was replaced by *Oscillatoria* sp., another filamentous cyanobacterium, which has a similar CA enzyme system and adheres firmly to solid surfaces. A selective precipitation, utilising gaseous hydrogen sulphide, was incorporated for the recovery of copper and lead. The modified system is described below.

4.4 Treatment of a synthetic acid mine drainage by an *Oscillatoria* sp. based system

4.4.1 Process description

The design of the continuous system, utilising *Oscillatoria* (Figures 4.5 and 4.6) rather than *Spirulina* to generate carbonate and hydroxide alkalinity, is illustrated in Figure 4.7. The system essentially contains the same components as those described in section 4.3, but utilises biogenic hydrogen sulphide to selectively precipitate copper and lead, as metal sulphides, prior to the precipitation of the bulk of the metals in the reaction vessel.

The high rate algal pond has been replaced by a simulated stream reactor, in which the algae are attached to stones lining the surface of the reactor. The bicarbonate rich overflow from the anaerobic digester flows over the algae by means of gravity.

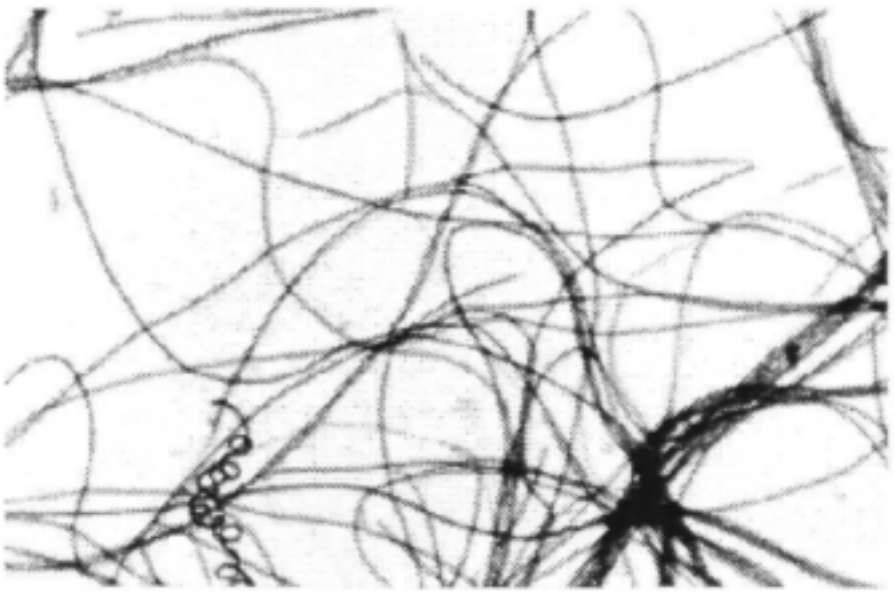


Figure 4.5: Light microscope preparation of *Oscillatoria* sp.

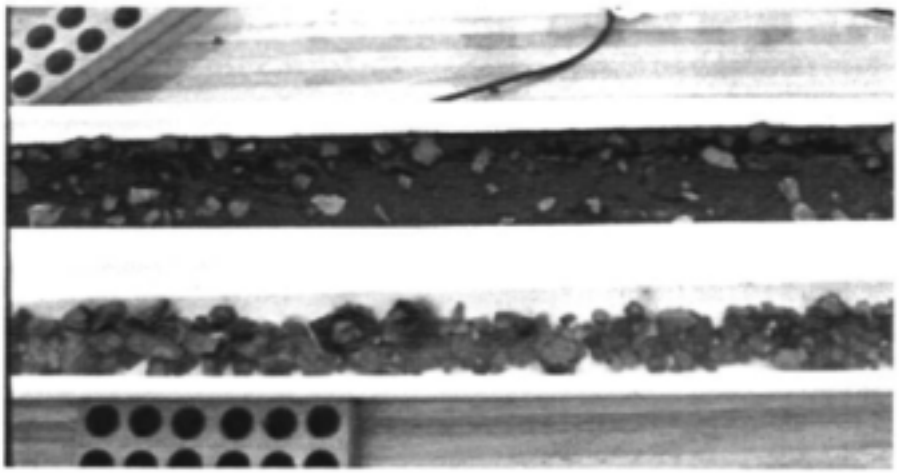


Figure 4.6: Seeding of the *Oscillatoria* culture in the simulated stream (gutter) reactors showing the adhesion of the algae to the solid supports and the subsequent extension of the filaments.

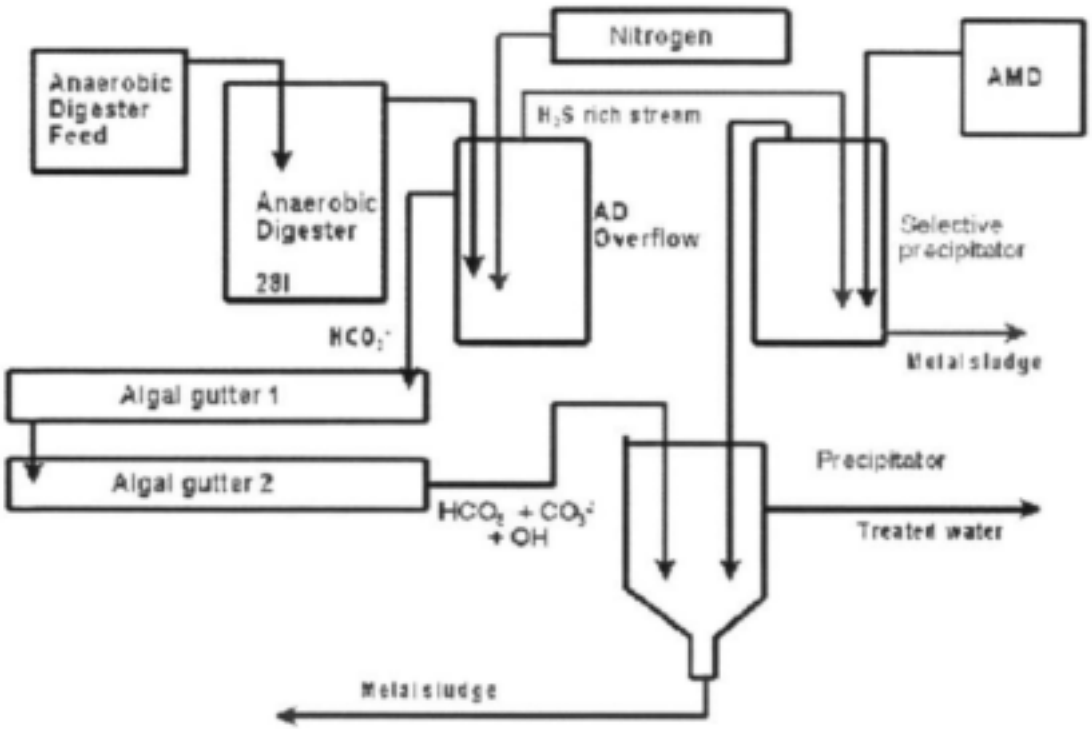


Figure 4.7: Schematic diagram illustrating the integrated biological system for the treatment of acidic, metal-laden wastewaters. Blue lines represent liquid streams and red lines gaseous streams.

4.4.2 Materials and methods

4.4.2 Materials and methods

4.4.2.1 Analytical techniques

The determination of pH, acidity, alkalinity, sulphate, sulphide and metal concentrations were conducted as described in previous chapters. In all cases samples from the individual reactors were performed in triplicate and mean values are presented.

4.4.2.2. Operational parameters

The pilot plant was operated for a period of 100 days, the first 10 of which were used to optimise the various parameters. The full bench-scale pilot system is shown in Figure 4.8. Modified Postgate B medium was fed into the 28 l anaerobic digester at a rate of 2 ml/min. The overflow from the anaerobic digester was collected in a stoppered, 2 l Schott bottle which was periodically sparged with nitrogen at a rate of 40 ml/s for 60 minutes. The hydrogen sulphide rich gas stream was passed into an airtight, 10 l selective precipitation vessel, where copper and lead were removed (Figure 4.9). The nitrogen sparged ADO was fed into the first algal gutter at a rate of 2 ml/min. The combined volume of the algal gutters was 7 l, giving a retention time of 2.4 days. The overflow from the algal gutters was blended with the acid mine drainage, exiting the selective precipitation vessel, into the precipitation vessel. The rate of blending was controlled to ensure an acidity to alkalinity ratio in the precipitation vessel of 1:2. The metal precipitate formed in the precipitation vessel settled in the cone at the base of the reactor, from where it could be removed, and the clarified liquid was drawn off through a port situated 10cm below the top of the reactor. The clarified liquid could be subjected to a final polishing step, prior to discharge.

Figure 4.8: Bench scale integrated biological system showing the various components, a = anaerobic digester feed, b = 28l anaerobic digester, c = algal gutters, d = selective precipitation vessel, e = acid mine drainage, f = nitrogen source and g = precipitation vessel.

Figure 4.9: The selective precipitation vessel showing the formation of the brown copper and lead sulphide precipitate.

The following measurements were taken on a regular basis: anaerobic digester - pH, sulphate, sulphide and alkalinity; algal gutters - pH and alkalinity; selective precipitation vessel - pH and dissolved metal concentration; overflow from precipitation vessel - pH, alkalinity, sulphate and dissolved metal concentration.

4.4.3 Results and discussion

The results from the analyses performed on the overflow from the 28l anaerobic digester over the 100 day operation period are shown in Figure 4.10. The initial optimisation period, day 0 to 10, was split into two phases. For the first seven days the reactor was operated as a batch system. This phase was characterised by a rapid drop in the sulphate concentration and a corresponding increase in the pH and aqueous sulphide concentration. Figure 4.11 shows that, over the same period, there was a rise in the bicarbonate alkalinity in the reactor. The ratio of alkalinity to sulphide was initially very high (53:1), but dropped rapidly as the sulphate reducing bacteria became established. From day seven onward the medium was pumped into the reactor at a constant rate of 2 ml/min. This resulted in an initial increase in the sulphate concentration, until a steady state was reached by day 10.

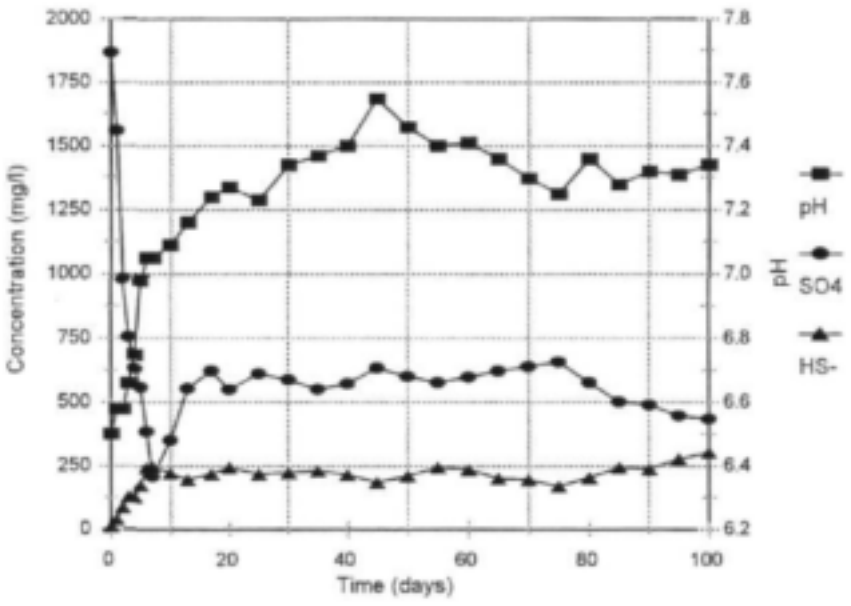


Figure 4.10: pH, sulphate and sulphide concentrations recorded in the overflow from the 28 l anaerobic digester over the 100 day pilot study period. The reactor was operated as a batch system for 7 days after which medium was pumped in at a constant rate of 2 ml/min.

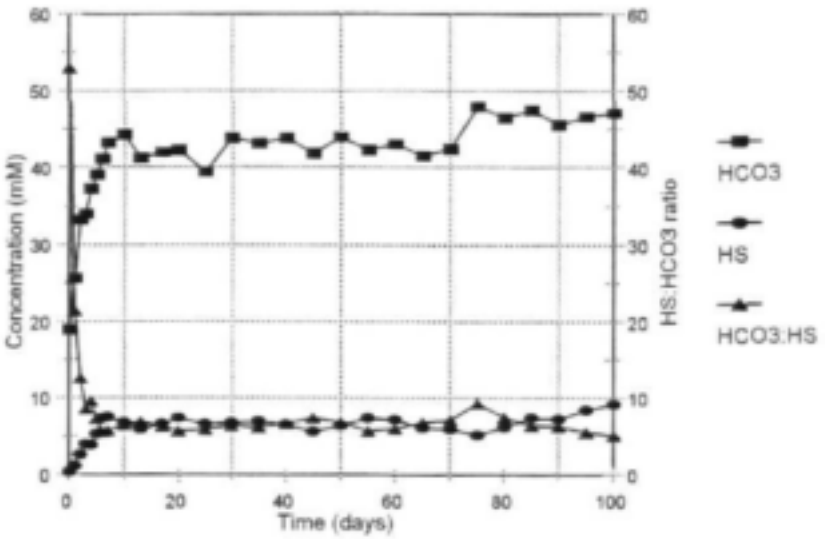


Figure 4.11: Bicarbonate, sulphide and bicarbonate to sulphide ratio recorded in the overflow from the 28l anaerobic digester over the 100 day pilot study period.

During the 90 day period when the reactor was essentially in a steady state, the concentration of sulphate and sulphide, as well as the pH and alkalinity, fluctuated within a narrow range. The mean values for the parameters measured, between day 10 and day 100, are shown in Table 4.2.

Table 4.2: Mean (± SD) values for pH, sulphate, sulphide, bicarbonate and the ratio of bicarbonate to sulphide recorded for the anaerobic digester overflow, for the period between day 10 and day 100.

Component	mg/l	mM
pH	7.32 ± 0.10	-
Sulphate	559.62 ± 74.66	5.80 ± 0.80
Sulphide	224.46 ± 29.04	6.80 ± 0.88
Bicarbonate	2192.54 ± 95.45	43.85 ± 2.24
HCO_3^- to HS^- ratio	6.55 ± 0.88	

This represented a mean sulphate conversion rate of 141.96 ± 15.61 mg l^{-1} day^{-1} (1478.75 ± 162.58 µmoles l^{-1} day^{-1}) for the steady state period. This value is lower than that reported by Boshoff (1999) for a stirred tank reactor, fed tannery effluent. Introducing some form of agitation to enhance the mass transfer within the reactor could significantly increase the

sulphate conversion rate, but this would have significant cost implications on a larger scale. The mean bicarbonate generation was calculated as 252.60 ± 10.61 mg l^{-1} day^{-1} (4200.50 ± 176.40 μmoles l^{-1} day^{-1}), which proved sufficient to maintain optimum performance of the algal system.

The sparging of the anaerobic digester overflow with nitrogen resulted in a reduction in aqueous sulphide of between 62 and 68 % and an associated increase in pH from approximately 7.3 to between 8.44 and 8.71. This increase in pH initiated the shift in carbonate species equilibrium from bicarbonate to carbonate and effectively provided a "head start" for the algal system. The combined performance of the two gutter reactors, as measured from the overflow of the second gutter, over the 100 day trial period is summarised in Figure 4.12.

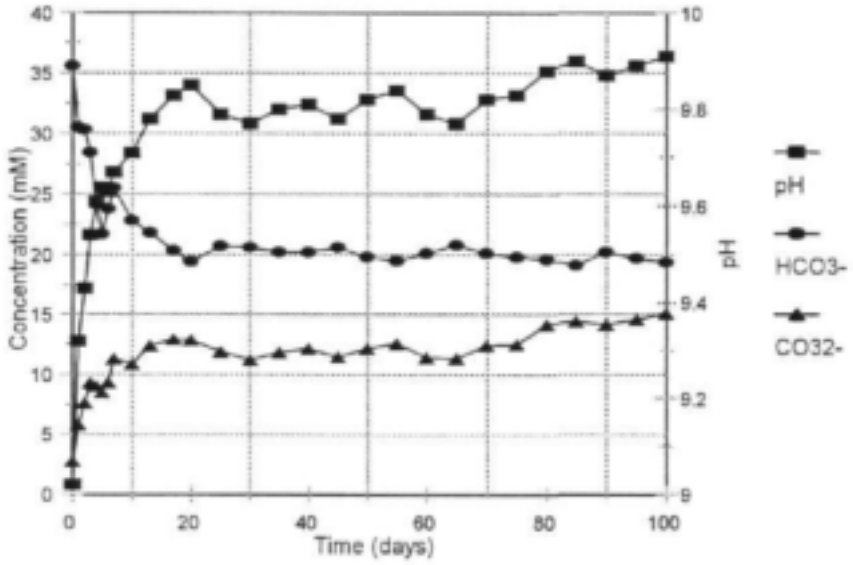


Figure 4.12: pH, bicarbonate and carbonate concentrations recorded in the overflow from the second algal gutter reactor over the 100 day pilot study period.

The algal culture was initially established in Zarrouk's defined medium, which accounts for the high initial bicarbonate concentration. During the first seven days, while the anaerobic digester was in the batch phase, the liquid in the gutters was re-circulated. Tap water was added to the system to overcome the effect of evaporation and as such there was little change in the total alkalinity of the system. Figure 4.12, however, shows that carbonate species equilibrium changed dramatically during this period, as a result of the algal accumulation of

inorganic carbon. The ratio of bicarbonate to carbonate decreased from 13.04 to 2.54. The change from batch to continuous operation of the anaerobic digester resulted in the continuous feed to the algal reactors and the re-circulation was terminated. A small increase in the bicarbonate concentration, resulting from the influx of anaerobic digester overflow, can be seen between days 7 and 9, after which a steady state is established. The total alkalinity in the gutter reactors was consistently higher than that of the anaerobic digester overflow, which can be accounted for by the evaporation of a certain fraction of the liquid. During the 90 day steady state phase, the gutter reactors showed a reduction in the bicarbonate concentration of over 50 %, relative to the anaerobic digester overflow. The combined effect of the sparging of the digester overflow and the action of the algae resulted in a mean increase in carbonate concentration from 0.07 ± 0.01 mM (ADO) to 12.65 ± 1.20 mM, or 5070 ± 410 μmoles l^{-1} day^{-1}.

The selective precipitation stage consistently removed over 99% of the copper and lead from solution, as metal sulphides. The effect on the aqueous concentration of the other metals was negligible. The precipitation of copper and lead was accompanied by a reduction in the mean pH, over the trial period, from 3.12 ± 11.64 to 2.96 ± 5.38.

The mean overflow rate from the second algal gutter was 1.76 ± 0.15 ml/min, with the fluctuation resulting from differing evaporation rates. This resulted in a mean overflow volume of 2.53 l/day, which at a mean alkalinity concentration of 4017 mg/l $CaCO_3$ equivalents, constituted the addition of 10179 mg of alkalinity to the precipitation vessel. As the mean acidity of the artificial acid mine drainage was 760 mg/l $CaCO_3$ equivalents, this facilitated the treatment of 6.7 l/day of acid mine drainage at an acidity to alkalinity ratio of 1:2.

The results of the metal removal in the selective precipitator and the precipitation vessel, from day 10 to day 100, are summarised in Table 4.3.

The data from Table 4.3 clearly show that for all the heavy metals, with the exception of manganese, the concentration in the precipitation vessel overflow stream is below the

acceptable environmental risk criteria laid down by the Department of Water Affairs and Forestry (1998).

Table 4.3: Mean (± SD) results for the heavy metal precipitation, from the artificial acid mine drainage, achieved from day 10 to day 100.

Metal	ArtAMD[1] conc. (μM)	Sel. Prep[2]. conc. (μM)	% removal	Precipitator concentration (μM)	Combined % removal[3]	AER[4] conc.
Cu	315 ± 6	1.3 ± 0.6	99.6	0.7 ± 0.8	99.8	1.6
Fe	3760 ± 32	3748 ±36	0.3	8.7 ± 4.6	99.8	161.2
Mn	728 ± 17	730 ± 11	< 0.1	234 ± 21	67.9	5.5
Ni	341 ± 9	336 ± 12	1.5	15.2 ± 5.7	95.5	19.4
Pb	97 ± 2	0.1 ± 0.2	99.9	0.1 ± 0.3	99.9	0.5
Zn	306 ± 22	298 ± 13	2.6	3.1 ± 2.2	99.0	10.7

[1]artAMD = artificial acid mine drainage, [2]Sel. prep. = selective precipitator, [3]the combined percentage removal includes precipitation in both the selective precipitator and the bulk precipitation reactor, as well as a dilution factor associated with blending the streams together, [4]AER = acceptable environmental risk.

The concentrations of calcium (2720 ± 62 μM), magnesium (4867 ± 131 μM) and sulphate (1860 ± 24 mg/l) in the precipitation vessel overflow stream were still appreciable, although the pH of 7.86 ± 0.09 was within prescribed limits. Much of the magnesium and sulphate was derived from the initial anaerobic digester feed. If the anaerobic digester could be run on enriched, partially treated acid mine drainage, these concentrations could be significantly reduced.

4.5 Cultivation of SRB on enriched, partially treated artAMD

4.5.1 Materials and methods

Three 2l anaerobic digesters were set up on partially treated artificial acid mine drainage that had been enriched with KH_2PO_4 (0.5 g/l), NH_4Cl (1.0 g/l), yeast extract (1.0 g/l) and sodium lactate (6 ml of a 60% solution). The reactors were inoculated with a 10 % (v/v) inoculum from the 28 l anaerobic digester and shaken at 120 rpm on an orbital shaker. The reactors were operated as batch systems for 13 days, and pH, sulphate, sulphide and alkalinity were measured daily. The concentration of metals remaining in solution was determined after seven days.

4.5.2 Results and discussion

The performance of the reactors is summarised in Figure 4.13, which shows that the SRB were able to successfully grow on the enriched AMD, despite the high manganese concentration. The reactors had essentially achieved maximum (95.8 %) sulphate reduction by day 10. During this period, the pH had increased from 7.20 to 7.40 and the sulphide and alkalinity levels had stabilised at approximately 250 mg/l and 3300 mg/l $CaCO_3$ equivalents respectively. These values are consistent with the levels observed during the steady state operation of the 28 l reactor and suggest that the addition of nutrients to the partially treated acid mine drainage could generate a feed suitable to run the entire system.

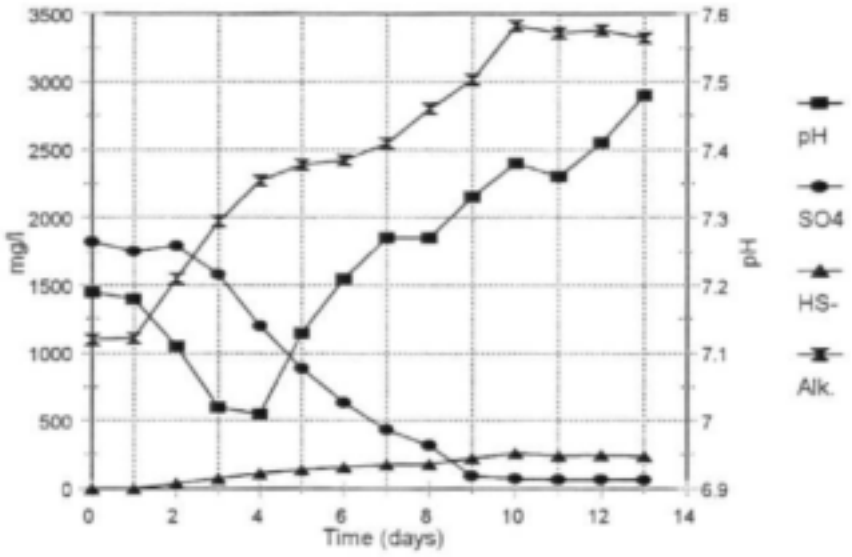


Figure 4.13: Performance of a sulphate reducing system with lactate enriched, partially treated acid mine drainage as the electron donor and carbon source (mean values, n = 3).

The analysis of the metal content on day seven showed a dramatic decrease in the manganese content, from a mean value of 226 µM to 4.5 µM, which is below the acceptable environmental risk level. Similarly, the mean concentration of calcium and magnesium in the three reactors had fallen to 1078 µM (43.2 mg/l) and 3402 µM (82.7 mg/l) respectively.

The blending of 6.7 l of acid mine drainage with 2.5 l of algal overflow would theoretically generate 9.2 l of partially treated acid mine drainage each day. Diverting 30 % of this stream

and enriching it with nutrients could create an effective feed for the large anaerobic digester. The remaining 70 % of the stream could be polished to final discharge quality, perhaps utilising a small anaerobic wetland.

The attempts to model the precipitation reactions occurring due to the blending of the acid mine drainage with the algal overflow did not prove successful. The results showed the same inaccuracies as explained in Chapter 5.

4.6 Scale-up considerations

Treating a volume of acid mine drainage similar to the predicted discharge volume from the East Rand Basin, would require the scaling-up of all parameters of the system proposed above, by a factor of approximately five million. Assuming no significant loss in the efficiency of the system, this would require an anaerobic digester with a volume of $140m^3$, being fed at a rate of 60 kl/h. Some form of active mixing would be required to achieve efficient mass transfer in a reactor of such size. It would be possible to use the gas stream exiting the selective precipitator to facilitate this in an airlift system, as the gas would be oxygen-free. The simulated stream (algal reactor) would require a volume of 35 Ml. The stream would need to be relatively shallow, with an approximate depth of 50 cm. This would require a land area of 70 m^2. If a portion of the partially treated minewater was supplemented with a carbon source and used as the feed for the main anaerobic digester, this would result in the discharge of 32.2 Ml/day of treated water, with an elevated manganese and sulphate content. Due to the absence of high concentrations of iron and acidity (pH ± 7.8), this stream could be effectively treated to strict discharge criteria in an anaerobic wetland of approximately 200 000 m^2.

The formulation of a cost estimate for such a system fell beyond the scope of this project. However, the individual components of such a system are significantly more "low-tech" than the systems covered in Chapter 2. In addition, the potential to use a waste carbon source to provide the nutrients for the anaerobic digestion would have a significant cost benefit. The system is also able to recover all the copper and lead from the minewater, which would result in the generation of revenue from their sale. The fact that the system is entirely dependent on

biologically mediated processes to generate the reactive anionic species means that, once operational, the system could become essentially self-sustaining.

4.7 Conclusions

The results from the two pilot plant studies illustrated the potential of an integrated biological system for the treatment of acid mine drainage. While the *Spirulina* based system was effective in the treatment of the base metal effluent, the problems associated with maintaining the culture in suspension and preventing the loss of excessive biomass, introduced practical problems. These problems would be exaggerated should the system have been scaled up.

The modification of the algal vessel design to accommodate *Oscillatoria*, which adhered firmly to the solid supports, removed the problem of solid-liquid separation. The addition of a selective precipitation step allowed the recovery of all the copper and lead from the artificial acid mine drainage, while reducing the sulphide concentration of the anaerobic digester overflow to a level which was not toxic to the algae.

After a 10 day optimisation period the integrated system was operated for 90 days, without any loss of efficiency. The anaerobic digester operated at a mean sulphate conversion rate of 479 μmoles l^{-1} day^{-1}, while generating bicarbonate at a rate of 4200 μmoles l^{-1} day^{-1}. The bicarbonate to sulphide ratio remained consistent at approximately 6:1.

The combination of the nitrogen sparging and the action of the algae raised the pH of the anaerobic digester overflow from 7.3 to 9.7. The concentration of bicarbonate in the algal overflow was over 50% less than the anaerobic digester overflow and the concentration of carbonate had increased from 0.07 mM to 12.65 mM. This represented a significant reduction in acidity.

The selective precipitation of copper and lead by gaseous hydrogen sulphide resulted in the removal of over 99 % of each metal from solution. The gaseous sulphide has little effect on the other metals. The blending of the post H_2S-treated acid mine drainage with the algal overflow, at an acidity to alkalinity ratio of 1:2, resulted in the precipitation of iron, nickel

and zinc to concentrations below the acceptable environmental risk levels. Only manganese was not efficiently removed. The mean pH of the overflow from the precipitator was 7.86, which was within acceptable limits.

The precipitator overflow did still contain appreciable amounts of calcium, magnesium and sulphate. The enrichment of this stream with ammonia, phosphate and lactate resulted in an effective feed for the sulphate reducing bacteria. The treatment of the enriched stream in a second anaerobic digester resulted in the almost complete removal of the manganese and sulphate, and a reduction in the calcium and magnesium concentrations. The enrichment of 30% of the precipitator overflow could provide the feed for the large anaerobic digester. Due to low acidity and iron content of the precipitator overflow, the remaining 70 % could easily be polished by an anaerobic wetland.

As shown in the previous chapter, the ability of MinteqA2 to generate an accurate model of such a complex precipitation system was limited.

There are no major obstacles to the scaling up of an integrated biological system such as the one proposed here. The individual reactor components are relatively "low-tech" as are the required control mechanisms. The system has the additional advantage that once the biological components are established, the system is essentially self-sustaining, provided a suitable carbon source is provided. It has been previously noted that considerable research is being conducted into the utilisation of low cost and waste carbon sources as feeds for sulphate reducing systems. The results presented in this chapter clearly indicate that this type of system could be a viable treatment option for the uncontrolled, long-term discharge of acid mine drainage from abandoned mines.

CHAPTER 5: GENERAL CONCLUSIONS

Increasing urbanisation, industrialisation and the provision of water to a large sector of the population who were previously deprived are placing an ever-increasing burden on South Africa's limited water reserves. The Government has recently introduced pro-active legislation, aimed at ensuring the management and conservation of these water resources for future generations.

The contamination of surface and groundwater reserves by heavy metals is a major concern. Historically these metals have originated from active mining operations, manufacturing and urban run-off. While leaching mine dumps and abandoned coal mines are a factor, the volume of these discharges are limited. However, economic pressure on the gold mining industry has resulted in the decommissioning of an increasing number of mines, particularly on the East Rand. In the near future all gold mining on the East Rand and eventually throughout the Witwatersrand will cease. As a consequence, the dewatering of the vast underground basins, which have resulted from mining operations, will stop and the groundwater will rebound. The rising groundwater will dissolve acid generating salts, which formed from the oxidation of sulphide minerals (mainly pyrite) under non-saturated conditions. This will most likely result in the dramatic deterioration of the groundwater quality, and where the groundwater discharges to the surface, a serious and long term environmental problem.

The conventional methods for treating acid mine drainage have focused on chemical precipitation, typically by lime addition, in a high density sludge process. While such systems are effective, they are expensive and are not a feasible long term option. There has been an increasing amount of interest shown in biological systems, ranging from active, sulphate reducing bacteria systems, to passive, artificial wetlands for acid mine drainage remediation. This project focused on the development of an integrated biological system, driven by the biological generation of reactive alkaline species, for the long term, sustainable treatment of acid mine drainage.

The initial work concentrated on the binding and accumulation of heavy metals by the blue-green alga, *Spirulina* sp. Metal binding was rapid and the binding affinity series of Pb > Cu > Zn >> Fe was consistent with published data for other algal species. The binding capacity was, however,

lower than that determined for most other microbial biomasses. Furthermore, the acute toxicity of copper and zinc resulted in the rapid deterioration of the algal culture. This precluded the algae from being used in a treatment system where they came into direct contact with toxic metals. The algae were able to increase the pH of the surrounding medium when external bicarbonate concentrations were high.

The ability of the algae to modify the aqueous environment was studied in greater detail. The phenomenon is controlled by the enzyme carbonic anhydrase, which plays an integral part in the carbon concentrating mechanism of the algae. In the presence of light and reduced concentrations of external carbon dioxide, the algae are able to utilise bicarbonate as a source of carbon dioxide for photosynthesis. This results in the release of a hydroxide ion to the external medium and an increase in pH. The process does not increase the alkalinity of the medium, but rather reduces the acidity by driving the equilibrium between bicarbonate and carbonate towards carbonate formation. Bicarbonate is able to act as both an acid and a base, and is considered to have one acid and one basic potential. Carbonate has only one basic potential. Experimental evidence showed that *Spirulina* was able to reduce the acidity of the medium by an amount equivalent to the addition of 3670 μmoles of hydroxide per gram of biomass per hour, at an optimal biomass concentration of 2000 mg dry mass/1.

Attempts to quantify the enzyme activity directly were unsuccessful, due to the inaccuracies of the Wilbur-Anderson method. An indirect method was developed which analysed the change in carbonate species equilibrium. The carbonic anhydrase inhibitor, acetazolamide, significantly reduced, but did not completely inhibit the enzyme activity at external concentrations of 50 Mm and above. The inhibitor cannot enter the cell so the fact that there was an incomplete inhibition of enzyme activity, even at inhibitor concentrations in excess of 100μM, suggests the presence of both an internal and external carbonic anhydrase enzyme. Experiments conducted with increased volumes (2l) of algal culture, showed a pH increase from 8.3 to almost 10 and a 70 %. reduction in the bicarbonate concentration over a 13 day period.

Predictive modelling, using MinteqA2, on the potential of the algal treated, liquid medium to precipitate a variety of metals showed that over the 13 day period of the experiment, the potential

of the medium to precipitate iron, manganese, lead and zinc increased by 15-20 %. Over the same period, the potential to precipitate calcium and magnesium increased from 0 up to 40 %. This highlighted the potential of using the algal mechanism in a remediation context.

In order for such a system to be sustainable, a readily available source of bicarbonate is needed. Clearly the continuous addition of bicarbonate salts would not be a cost effective solution. The biological generation of bicarbonate by sulphate reducing bacteria was investigated in another study. While sulphate reducing bacteria have been the focus of a considerable amount of research, the majority of this has focused on desalination and the production of sulphide for metal precipitation. For most carbon sources, the amount of bicarbonate generated per sulphate reduced is significantly greater than the sulphide produced.

In this parallel study a bacterial consortium was isolated from an anaerobic pond at the Grahamstown sewage treatment works and maintained in a lactate based medium. Morphological and physiological data suggested the presence of at least two species of *Desulfovibrio* and one of *Desulfotomaculum*. Results obtained from experiments utilising glucose as a carbon source suggested the presence of acetogenic bacteria in the consortium, as well as the absence of acetate utilising SRB, such as *Desulfobacter* species. The consortium was able to reduce sulphate at a rate of up to 500mg l^{-1} day^{-1} in an agitated, 2l reactor. The sulphate reduction rate dropped to 135mg l^{-1} day^{-1} in a 28l, non-agitated reactor. This was most likely due to reduced mass transfer. The ratio of bicarbonate to sulphide generated in the reactor was 6:1, which was three times higher than expected. This could be attributed to the loss of sulphide, as hydrogen sulphide gas, to the headspace as well as the oxidation of sulphide to elemental sulphur. Additional bicarbonate could also have been produced in sulphate poor microenvironments within the non- agitated reactor. In these environments, substrate level phosphorylation would dominate.

The mechanisms involved in the precipitation of individual metal species and a complex acid mine drainage, by reactive alkaline species were investigated in other studies. The precipitation of individual metal species by the addition of hydroxide, bicarbonate and carbonate was relatively straightforward and a combination of precipitate analysis and information from the modelling allowed the determination of the precipitated species.

The bench scale pilot operation of the integrated biological systems highlighted their potential for the remediation of acid mine drainage effluents. The *Spirulina* based system was effective, but suffered from problems associated with maintaining the algae in suspension and preventing biomass loss. These problems were overcome by using *Oscillatoria* in the simulated stream reactors and the system was operated without a loss of efficiency for 90 days. The anaerobic digester component of the integrated system was able to consistently generate bicarbonate at a rate of 4200μmoles l^{-1} day^{-1}. The combination of the nitrogen sparging step and the action of the algae was able to reduce the bicarbonate concentration by over 50% and increase the carbonate concentration from 0.07mM to 12.65mM. In terms of metal removal, the selective precipitation of over 99% of copper and lead, using hydrogen sulphide gas, was consistently achieved. The blending of the hydrogen sulphide treated acid mine drainage and the algal overflow at an acidity to alkalinity ratio of 1:2 resulted in the precipitation of all the heavy metal, except manganese, to concentrations below the acceptable environmental risk level. It was shown that a portion of the overflow stream from the precipitator could be enriched with a carbon source, ammonia and phosphate, and used as a feed for the large anaerobic digester. The remainder of the stream could be discharged to a final polishing step, possibly an anaerobic wetland.

There are no obvious obstacles preventing the scale-up of the integrated biological system presented here. As such, the use of biologically generated reactive alkaline species has been shown to provide a possible treatment option for the long term, sustainable remediation of future, high volume, acid mine drainage discharges.

CHAPTER 6: REFERENCES

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CHAPTER 7: APPENDICES

Appendix A: Risks and hazards

Acceptable risk levels and hazard ratings of the metals encountered in this study. Guidelines determined by the Department of Water Affairs and Forestry (1998)

Acceptable environmental risk			
Metal	ppm	μM	Hazard rating
Copper	0.1	1.6	2
Iron	9.0	161.2	3
Lead	0.1	0.5	2
Manganese	0.3	5.5	2
Nickel	1.14	19.4	2
Zinc	0.7	10.7	2

Explanation of hazard rating

Hazard rating	Oral toxicity - LD_{50} (mg/kg)	Dermal toxicity - LD_{50} (mg/kg)
1	Up to 5	Up to 40
2	5-50	40-200
3	50-200	200-1000
4	>200	>1000

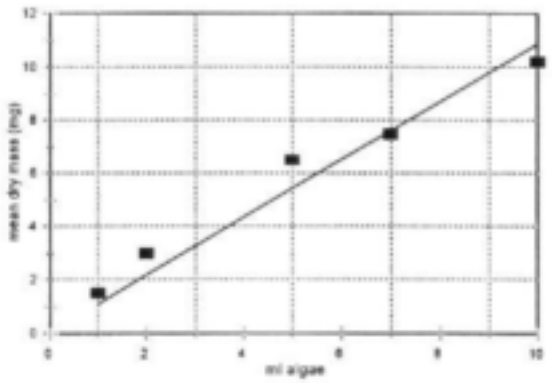
Appendix B: Zarrouk's medium for the cultivation of *Spirulina* sp.

Component	Mass/volume
NaCl	1 g/l
K_2SO_4	1 g/l
KNO_3	3 g/l
H_3PO_4	0.25 ml/l
$NaHCO_3$	16.8 g/l
EDTA	0.08 g/l
$FeSO_4.7H_2O$	0.01 g/l

The H_3PO_4 was added after the salts had been dissolved.

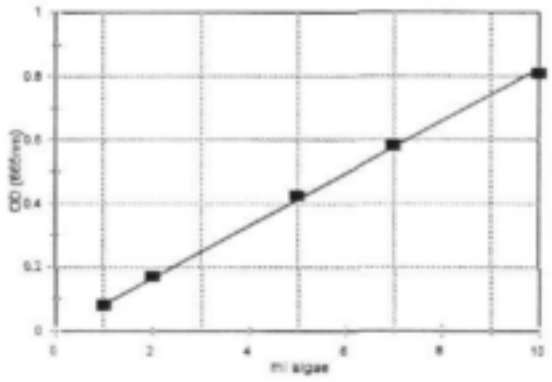
In experiments where metal precipitation was measured the EDTA was omitted from the media as it acts as a metal complexing agent.

Appendix C: Standard curve of dry mass of *Spirulina*.



Standard curve of dry mass of *Spirulina* against volume of algal culture dried (n = 5, r^2 = 0.950)

Appendix D: Standard curve of optical density.



Standard curve of optical density (660nm) against volume of algae (n = 5, r^2 = 0.998)

Appendix E: Chlorophyll concentration calculations.

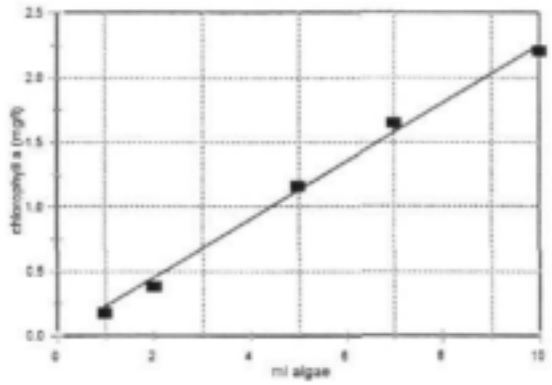
Equations for calculating chlorophyll concentrations (mg/l) from acetone extraction data

Chlorophyll *a* : $C_a = 11.24A_{661.6} - 2.04A_{644.8}$

Chlorophyll *b* : $C_b = 20.13A_{644.8} - 4.19A_{661.6}$

Xanthophylls and Carotenoids : $C_{x+c} = (1000A_{470} - 190C_a - 63.4C_b)/214$

Appendix F: Standard curve of chlorophyll *a*.

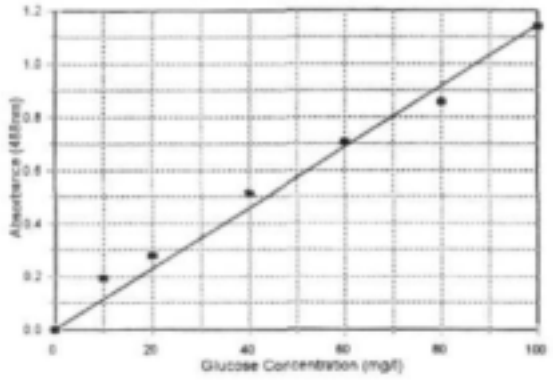


Standard curve of chlorophyll *a* against volume of algae used for extraction (n = 5, r^2 = 0.995)

Appendix G: Atomic absorption spectrophotometer program parameters for individual metal windows.

Range	Wavelength	Slit width
Calcium: Nitrous oxide-acetylene, lamp current 10.0mA		
10-100µM	422.7nm	0.5nm
Copper: Air-acetylene flame, lamp current 3.0mA		
2-50µM	324.7nm	0.5nm
100-500µM	217.9nm	0.2nm
500-2500µM	222.6nm	1.0nm
Iron: Air acetylene flame, lamp current 7.0mA		
10-150µM	248.3nm	0.2nm
50-1000µM	372.0nm	0.2nm
500-2500µM	386.9nm	0.2nm
Potassium: Air-acetylene flame, lamp current 6.0mA		
25-125µM	769.9nm	0.5nm
Magnesium: Air-acetylene flame, lamp current 3.0mA		
20-500µM	202.6nm	1.0nm
Manganese: Air-acetylene flame, lamp current 5.0mA		
25-100µM	280.1nm	0.2nm
100-500µM	403.1nm	0.2nm
Sodium: Air-acetylene flame, lamp current 5.0mA		
4000-15000µM	330.2nm	0.5nm
Nickel: Air-acetylene flame, lamp current 4.0mA		
25-125µM	232.0nm	0.2nm
Lead: Air-acetylene flame, lamp current 5.0mA		
10-100µM	217.0nm	1.0nm
50-250µM	283.3nm	0.5nm
250-2000µM	261.4nm	0.5nm
Zinc: Air-acetylene flame, lamp current 5.0mA		
2-50µM	213.9nm	0.5nm

Appendix H: Standard curve for carbohydrate determinations.



Standard curve for carbohydrate determinations (n = 5, r^2 = 0.984)

Appendix I: Analysis of effluent from the Black Mountain base metal mine.

pH = 1.99

Component	Concentration (mg/l)	Concentration (μM)
Chemical Oxygen Demand (COD)	148	-
Calcium as Ca	890	22 206
Magnesium as Mg	121	4 978
Copper as Cu	1.08	17.00
Lead as Pb	1.52	7.34
Zinc as Zn	2.20	33.64
Iron as Fe	95	1 701
Chloride as Cl	621	17 516
Sulphate as SO_4	3620	37 708
Nitrate as N	1.4	100
Total hardness as $CaCO_3$	2718	-
Calcium hardness as $CaCO_3$	2220	-
Magnesium hardness as $CaCO_3$	498	-
Bicarbonate as $CaCO_3$	0	-

Appendix J: Research Products

Papers

van Hille RP, Rose PD and Duncan JR. (in press) Water chemistry relative to SRB reactors and algal alkalinity generation. *Appl. Micro. Biotech.*

Antunes APM, Duncan JR, Watkins GM and Maclear A. (2002). The removal of gold from solution by the viable microbial biomass *Phomas sp. Res. Environ. Biotech.* **3**, 220-229.

Conference attendance

K. Rashamuse, C. Whiteley, D. Sanyahumbi and B. Pletschke. Enzymatic bioaccumulation of platinum from aqueous solution using sulphate reducing bacteria. *WISA 2002 Biennial Conference and Exhibition*, ICC, Durban, South Africa. 19 to 23 May. WRC CD-ROM ISBN: 1-86845-844-X. Paper no. 162.

Students / Degrees

Mamta Bhagat, BSc Honours. November 2002. The use of biogenic alkalinity and sulphidogenic streams in the precipitation of metal ions.

Cherie-Lyn Mack, BSc Honours. November 2002. Recovery of platinum (IV) from aqueous solutions utilizing the non-viable biomass *Azolla filiculoides*.

Roland Smith MSc. April 2002. An investigation into the biological treatment of platinum refinery effluent.

Leigh Nightingale MSc. Submitted January 2003. The toxic effect of heavy metals on algal biomass (*Spirulina* sp.) and CA activity, an enzyme which is central to algal application in metal precipitation.

Other related WRC reports available:

Assessment and application of imported biomass for the bioremediation of heavy metal effluents

FM Swalaha, S Datadin and BB Choonawala

The presence of heavy metal ions in surface water continues to be most pervasive environmental issue of present time. Heavy metal contamination exists in aqueous streams of many industries such as metal plating facilities, mining operations and tanneries. The release of heavy metal pollutants by the industrial sector into the sewage system has a negative impact on the environment in a number of ways. Water quality is affected, small quantities of metal become incorporated into the food chain and this is toxic to both humans and animals, and surface and groundwater can become contaminated due to leaching of metals.

This Report is divided into three parts. The first part reports on the batch testing of the acquired biomass technologies performance, where the maximum metal uptake and mechanisms were determined. The second part reports on the behaviour of the immobilised biomass in a continuous packed bed laboratory scale reactor and the third part consisted of an on-site pilot scale study to determine field operating conditions and efficiencies.

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