DEVELOPMENT OF INTEGRATED BIOLOGICAL PROCESSING FOR THE BIODESALINATION OF SULPHATE- AND METAL-RICH WASTEWATERS

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ABSTRACT

The substantial pollution threat to the South African environment from acid mine drainage (AMD) effluents has been well documented. Due to the juvenile nature of acidity in these flows, any remediation strategies implemented will need to function effectively and at low cost for long periods of time. The widespread use of sulphate reducing biological systems for the treatment of such effluents, and in particular large volume flows, has been limited. The supply of inexpensive electron donor and carbon sources, as well as appropriate reactor designs capable of handling large volume flows, have been identified as among the principal factors limiting development of this technology. The broad aim of the research programme reported here was to undertake an evaluation of the feasibility of an algal-bacterial integrated ponding system for the treatment of AMD, and the waste stabilisation pond (WSP) as an appropriate reactor design for this application.

The study attempted to demonstrate the feasibility of individual unit operations in a proposed process train using complex organic carbon serving as the electron donor source for the sulphate reducing bacteria (SRB). Studies were undertaken as laboratory and pilot-scale investigations.

Tannery effluent was shown to be a functional carbon source for biological sulphate reduction, with effective removal of sulphate and organics being recorded. In turn, the use of biological sulphate reduction for the treatment of tannery effluent was demonstrated. Algal biomass was shown in laboratory studies to function as an effective carbon source for biological sulphate reduction.

It is known that micro-algae produce large quantities of photosynthate which is released to the growth medium under conditions of physiological stress. The potential for the use of photosynthate production in high rate algal ponding systems and its manipulation and use as a sustainable carbon source for sulphate reduction was investigated. Growth of a mixed culture of

Dunaliella under conditions of light, temperature and salinity stress demonstrated production of large quantities of organic carbon. However, growth was inhibited at high temperatures. An elevation of salinity levels led to a decrease in growth of *Dunaliella*, but to increased organic carbon production. *Spirulina* spp., on the other hand, grew well at higher temperatures but showed the highest organic carbon production, and release to the medium, under low light conditions.

These results led to a proposed process for the integration of algal ponding into an integrated system for the treatment of AMD. The algal biomass may be fed into the anaerobic digester as a carbon source, or it may be passed into a High Rate Algal Pond (HRAP) where it is stressed to enhance the organic carbon content. This can then be fed into the anaerobic digester as a carbon source.

The impact of high levels of sulphide in the water feeding to the algal growth compartment was investigated. *Spirulina* spp. isolated from a tannery waste stabilisation pond was shown to be a sulphidophilic strain of cyanobacterium, capable of being adapted to high concentrations of sulphide. *Dunaliella salina* on the other hand was less tolerant. These results demonstrated the practical use of algal biomass providing an oxygen-rich cap for odour control on the surface of the facultative pond as well for the secondary treatment of sulphide-rich overflow to the High Rate Algal Pond.

The ability of micro-algae to elevate the pH of their surrounding environment was evaluated as a functional precipitant and neutralisation reagent for acidic metal containing wastewater. *Spirulina* spp. was shown to perform effectively. *D. salina* was less functional in this environment. *Anacystis* spp. was effective in elevating the pH of a defined medium as well as a zinc-rich effluent. These results indicated the practicality of a neutralising function for algal ponds in the treatment of AMD. Metal removal in the system was found to be a combined function of sulphide precipitation, removal by binding to micro-algal biomass and extracellular polymeric substances.

The feasibility of waste stabilisation ponding technology use for the treatment of large volume AMD effluents was provisionally demonstrated. It was shown that complex carbon sources would be used as efficient electron donors for sulphate reduction. The integration of algal ponding into the system provides for the generation of a sustainable carbon source, odour control with the recycling of oxygen-rich water onto the top of the facultative pond, secondary treatment of the anaerobic digester overflow, and the neutralisation of the incoming acidic effluents and removal of heavy metals.

Integration of the individual unit operations, the feasibility of which has been provisionally demonstrated in this study, into a continuous process train is being investigated in follow-up studies.

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LIST OF ABBREVIATIONS

AIPS	Algal Integrated Ponding System
AMD	Acid Mine Drainage
ATP	Adenosine Triphosphate
Chl.*a*	Chlorophyll *a*
Ci	Inorganic Carbon
COD	Chemical Oxygen Demand
DIC	Dissolved Inorganic Carbon
DWA	Department of Water Affairs
k	Division Rate Constant
Km	Affinity Constant
Ml	Megalitres
MPB	Methane Producing Bacteria
n	Number of Generations
^{o}C	Degrees Celcius
pKa	Dissociation Constant
s.d.	Standard Deviation
SRB	Sulphate Reducing Bacteria
STR	Stirred Tank Reactor
TDS	Total Dissolved Salts
tg	Generation Time
TKN	Total Kjeldahl Nitrogen
TOC	Total Organic Carbon
TR	Trench Reactor
TS	Total Solids
µ	Specific Growth Rate
UASB	Upflow Anaerobic Sludge Blanket

v/v	Volume for Volume
WRC	Water Research Commission
WSP	Waste Stabilisation Pond

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CHAPTER 1. BIOLOGICAL TREATMENT OF SULPHATE- AND METAL-RICH WASTEWATERS:AN INTRODUCTION

1.1. WATER AS A RESOURCE IN SOUTH AFRICA

Southern Africa has highly variable rainfall, with a mean annual precipitation of approximately 500 mm (Backeberg *et al*., 1996). Exposure to high solar radiation leads to large evaporative losses and it is estimated that only about 9% of the total annual rainfall results in recoverable runoff (Stoffberg *et al*., 1992). An expanding population places increasing demands on the available resources for industrialisation, urbanisation, agriculture and recreation needs. It is anticipated that the point of maximum economic utilisation of the surface and ground water resources of the country as a whole will be reached early in the next century (Odendaal, 1997).

A Council for Scientific and Industrial Research (CSIR) report in 1991 confirmed earlier studies undertaken in the 1970's indicating that the quality of many water sources in South Africa is declining, primarily as a result of salinisation, but to a lesser extent also because of eutrophication and pollution by trace metals and micro-pollutants (Neytzell-De Wilde, 1992).

The ions most commonly associated with salinity problems are calcium, magnesium, sodium, sulphate, chloride and bicarbonate (Casey *et al.*, 1996). Highly saline water may detrimentally affect animal health and performance by reducing water and feed intake. It also limits the reuse potential of the water resource due to concentration cycling.

The salinisation of river water may occur naturally by the leaching of salt-containing soils such as the Karoo shales. This can be seen in the Berg River, Western Cape, and the Great Fish, Buffalo and Sundays Rivers in the Eastern Cape. Irrigation also contributes towards the salinisation of both land and water bodies. It is estimated that approximately 18% of the irrigated area in South Africa experiences some sort of detrimental effect caused either by waterlogging and/or salt accumulation (Backeberg *et al.*, 1996).

One of the water bodies most affected by such activities is the Vaal Barrage which is a major abstraction point for the supply of Vaal River water to the Witwatersrand and other parts of the

Gauteng province. Here the total dissolved salt concentration may vary between 470 $mg.l^{-1}$ and 740 $mg.l^{-1}$, reaching 1000 $mg.l^{-1}$ at times. The Vaal Barrage was constructed in 1923 as part of the development of the Vaal River resources to improve the assurance of water supply to the area by collection and multiple cycle re-use of the resource. Many of the major wastewater treatment plants of Johannesburg, Germiston and towns on the East Rand discharge their treated effluents into tributaries of the Vaal River. After passing through wetlands, much of this flow is impounded in the Vaal Barrage. This water carries with it a heavy load of pollutants from the gold mines and urban run-off which increases the salt content of the Vaal Barrage by 400 $mg.l^{-1}$ for every cycle of reuse (DWA, 1989). Dewatering and decanting gold mines contribute annually over 400 000 tons salinity to the problem (Funke, 1990).

The Witwatersrand, which is responsible for 40% of the South Africa's Gross Domestic Product (GDP) (Statistics South Africa, 1994), is located inland, with its principal water source being the Vaal River. The demand for water in the Witwatersrand, principally from mining and industry, has long since exceeded the natural capacity of the Vaal River, with the result that there are now no less that six water transfer schemes which bring water from other rivers to supplement the Vaal River supply. However, the projected demand for water will still exceed the available supply in 2006 (Hunter, 1995). Further augmentation of the Vaal River would involve the abstraction of water from the Orange, Mzimvubi and Zambesi rivers. The political and developmental implications would be far reaching, with co-operation required between eight Southern African countries who share the Zambesi catchment area. However, the ecological implications are also substantial and may determine the feasibility of the proposed action (Lotter, 1995). It is thus evident that the Witwatersrand, particularly its industrial and commercial activity, faces physical and ecological constraints to its further growth and development.

1.2. THE GOLD MINING INDUSTRY IN SOUTH AFRICA

Gold was first discovered on the Witwatersrand in 1886 on the farm Langlaagte, 5 km west of the city of Johannesburg. This conglomeratic outcrop was found to stretch for a continuous distance of 50 km and has come to be known as the Central Rand Goldfield. Soon afterwards similar gold bearing outcrops were found in the East Rand, West Rand and around the Klerksdorp area. Further prospecting also led to the identification of the Carltonville and

Klerksdorp Goldfield, and the Welkom and the Kinross/Evander Goldfields in 1956 (Department of Mineral and Energy Affairs Report, 1995). This led to the gold-bearing reefs being exploited along a strike of 480 kms and to depths of over four kilometres, making it the largest gold mining area in the world (Funke,1990).

In the 1970's the gold mining industry underwent a period of growth due to the rise in the world gold price, with South Africa responsible for 75% of the "free world's" gold production. However, due to increased labour costs, labour and political unrest and a reduction in the gold price in the mid-1980's, the gold mining industry entered a slump. This, together with the emergence of Russia and China into the gold market led to a decline in South Africa's relative share of the gold market to a 1993 low of 27.2% (Department of Mineral and Energy Affairs, 1995).

Although the poor quality of recycled service mine water in operating mines costs the mining industry approximately R300 million annually, based on its corrosive, scaling and fouling potential (McKay *et al.*, 1991; Juby *et al.*, 1996), the problem most likely to have major ecological and economic effects is pollution from water decanting from abandoned mines. Many mines have been forced to close due to economic constraints associated with the increased depths at which ore has to be mined and a falling gold price. In 1995, there were only four mines still working underground in the Witwatersrand compared to the 39 operational mines in the 1940's (Scott, 1995). The abandonment of mines has left behind surface rock piles, sand and slime dumps and spoil heaps in stopes and haulages, all of which contain pyrite. The oxidation of this material generates acidity and dissolved salts such as calcium, sulphate, sodium and chloride at levels of up to 11 000 $mg.l^{-1}$ (Funke, 1990).

In an investigation carried out by Scott (1995) the following influences on the environment of mine water discharge were identified:

- Increase in subsurface instability and seismic activity;
- Negative impacts on the natural environment around the discharge points;
- Water quality deterioration;
- Recharge of local aquifers by mine waters;

- Negative impact on water users downstream of the discharge points;
- Water inrushes into the remaining operational mines from the filling geohydrological basins.

1.3. THE GROOTVLEI MINE:A CASE STUDY

Gold Mining in the Far East Rand began at the turn of the century, leading to the establishment of a number of mines and excavation in an underground area known as the Far East Rand Basin. In order to continue operations, mines were forced to pump water from underground to either dewater areas for new development or to keep existing works dry. Due to their different positions on the basin and the fact that the mines were interconnected, this task was not equally shared, with mines on the periphery pumping intermittently and mines operating in the deeper part of the basin, pumping large volumes continuously (Scott, 1993). With the closure of many mines in the 1960's, the lower works were allowed to flood and dewatering became the responsibility of Grootvlei, SA Lands and Exploration Gold Mining Company (Sallies) and Vlakfontein Gold Mining Company (Vlakfontein). As is shown in Figure 1.1, dolomite is intercepted by Grootvlei mine on both sides of the Blesbokspruit, and by Sallies mine which is situated west of the Blesbokspruit. Both mines are interlinked and it is also suspected that the dolomite at Grootvlei mine is recharged with water probably from the Blesbokspruit, the principal drainage route of this catchment area (Department of Minerals and Energy Affairs, 1995).

When Sallies closed in 1976, pumping was taken over by Grootvlei. With the closure of Vlakfontein in 1977, the water level in the basin was allowed to rise further, reaching 1606 metres below datum where it was maintained from 1988. Pumping at Sallies was suspended in 1991 and a new pump station was installed at Grootvlei No.3 shaft. To prevent flooding of its underground workings, Grootvlei has to pump out an estimated 24 300 $Ml.a^{-1}$ water from both mines.

The pump station discharges millions of litres of highly contaminated water into the nearby Blesbokspruit wetland on a daily basis with severe ecological implications. This water is typical acid mine drainage (AMD) with a high Total Dissolved Salts (TDS) and sulphate concentration

and contains numerous heavy metals, some in very high concentrations (Table 1.1.). The Grootvlei Mine is one of three active gold mines which, over a period of 16 months (1988/1989) contributed 9% to the flow, 48% to the TDS due to salinity and 60% to the sulphate load of the Klip River, which is the main contributor to the salt load of the Vaal Barrage (Rand Water Board, 1988; 1989) (Figure 1.2).

In 1995 the government became aware of the environmental hazard caused by the pumping into the Blesbokspruit, but allowed it to continue in the interests of keeping the operation economically viable. The alternative would have been to cease pumping, leading to flooding of the underground workings, which in turn would cause the mine to close down, with the loss of 6000 jobs as well R300 million income to mining and state. In early 1996, when the extent of pollution in the wetlands became obvious, the minister of Water Affairs ordered the mine to switch off pumps draining the mine. The environmental threat had also spread beyond the Blesbokspruit, with neighbouring farmlands and the Marievale and Daggafontein Bird Sanctuaries being threatened. These are downstream of the Blesbokspruit and are registered

"Wetlands of International Significance" under the Ramsar Convention. After a period of 2 weeks it was agreed that pumping could continue but only on condition that a permanent water treatment plant be installed to remove the harmful iron oxide deposits from the water.

Scott's report (1995) has noted that water flowing into the mining areas in the northern parts of the mine basin has a head of 20 metres over the lowest shafts connected to the basin. Thus when pumping finally stops it is likely that water will decant from those lowest shafts. The lowest shaft, which has the greatest potential to decant is the Nigel (Southgo) No. 3 shaft, located in the valley of a tributary of the Blesbokspruit, north of the central business district of the town Nigel. The expected outflow from this shaft would be in the region of 33Ml.day^{-1}. The decanting of such large volumes of water in close proximity to an urban settlement will have great

implications, financially, socially and ecologically.

Table.1.1. Water quality analyses of mine water from Grootvlei Proprietary Mines, Ltd.

Water Quality Parameter	Guideline for aquatic life	Measured
Temperature (^oC)	-	21
pH	6.5-9.0	5.95
Dissolved Oxygen ($mg.l^{-1}$)	-	5.20
Electrical Conductivity(µS/cm)	-	387
Total Dissolved Solids ($mg.l^{-1}$)	-	3788
Alkalinity ($mg.l^{-1}$)	>20	245
Total Hardness ($mg.l^{-1}$)	-	1700
Ca-Hardness ($mg.l^{-1}$)	-	980
Mg-Hardness ($mg.l^{-1}$)	-	720
Ammonia-N ($mg.l^{-1}$)	0.016-4.5	5.4
Nitrate-N ($mg.l^{-1}$)	-	2.4
Orthophosphate-P ($mg.l^{-l}$)	0.1	<0.05
Sulphate ($mg.l^{-l}$)	Med:1400	1831
Fluoride ($mg.l^{-1}$)	1.5	1.7
Chloride ($mg.l^{-1}$)	50-400	250
Iron ($\mu g.l^{-1}$)	200-1000	210000
Copper ($\mu g.l^{-1}$)	5-200	25
Nickel ($\mu g.l^{-1}$)	25-50	762
Lead ($\mu g.l^{-1}$)	20-100	340
Zinc ($\mu g.l^{-1}$)	30-100	40
Manganese ($\mu g.l^{-1}$)	100-1000	7060
Chromium ($\mu g.l^{-1}$)	10-100	150
Cadmium ($\mu g.l^{-1}$)	0.1-30	28
Sodium ($mg.l^{-1}$)	Med:500	258
Potassium ($mg.l^{-1}$)	Med:50	18.2

1.4. ACID MINE DRAINAGE

Acid mine drainage (AMD) results from the oxidation of pyritic iron sulphide to sulphate and Fe^{2+}. Under acidic conditions the process proceeds by either of two reactions (Stumm and Morgan, 1981):

$$FeS_2 + {}^7/_2\ O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \text{ ----------1.}$$

$$Fe^{2+} + ¼\ O_2 + H^+ \rightarrow Fe^{3+} + ½\ H_2O \text{ ----------2.}$$

The major inorganic oxidation reaction follows equation 1. At a pH of 4.5 the abiotic oxidation of Fe^{2+} slows down (Funke, 1990), but at pH values below 3.5 *Thiobacillus ferooxidans*, an acidic chemolithotroph, increases the rate of oxidation of the Fe^{2+} ion to the Fe^{3+} ion (Obereke and Stevens, 1991). The Fe^{3+} ion is then hydrolysed by water to form the insoluble precipitate ferrihydrite and acidity by the following reaction:

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+ \text{----------3.}$$

In addition to reacting directly with oxygen, pyrite may also be oxidised by dissolved ferric iron to produce additional Fe^{2+} and acidity as shown in equation 4.

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \text{----------4.}$$

Once the acid generating reactions have begun, oxygen is only necessary for the microbially catalysed oxidation of ferrous to ferric iron. Pyrite will continue to be oxidised, in the absence of oxygen, by ferric iron. At this stage the process becomes largely unstoppable (Brierly and Brierly, 1997).

Acidic mine drainage does not only occur from the mine itself but also from rock dumps and tailings areas. These two sources contain a high concentration of sulphides and/or sulphosalts which are associated with most ore and coal bodies. During mining, pyrite and marcasite are left on the ground surface. Exposure to oxygen and moisture results in oxidation of FeS_2 and the release of acids into the water (Funke, 1990; Johnson, 1995). Acid mine drainage may also result from slimes (pulps containing very finely ground minerals from the ore) which are contained in slimes dams, as the possibility of a wall being breached and the slimes flowing over mining areas, farmlands, roads and into watercourses exists (Querol, 1999). Mine drainage pollutants, such as iron and hydroxides, may also arise from the neutralisation of iron sulphate and are precipitated in river beds. The drainage of water from underground mining works located below the surface drainage levels also contributes to mine drainage pollution (Henzen and Pieterse, 1978).

1.5. TREATMENT PROCESSES FOR ACID MINE DRAINAGE EFFLUENT

A number of chemical processes are available for the treatment of acidic mine drainage. The most conventional of these is chemical precipitation using either hydroxide, carbonate or sulphide treatment, or a combination of these. The most commonly used method is hydroxide precipitation as it is simple and inexpensive and addresses both the metal and the acidity problem associated with mine drainage effluent. Those effluents resulting from the mining of arsenopyrite ores can be treated either by ferric sulphate or ferric chloride precipitation, although none of these are employed in South Africa at present (Pulles *et al.*, 1995).

Other chemical methods utilised for mine water remediation include coagulation and flocculation (Saha, 1993), flotation, ion-exchange, complexation and sequestration, electrochemical reactions, evaporation and distillation and adsorption using activated carbon (Peters and Ku, 1985; Volesky, 1987). A number of membrane processes are also available for the treatment of wastewater containing heavy metals (Peters and Ku, 1985; Kim 1984; Neytzell-de Wilde, 1992). Electrodialysis reversal (EDR) and tubular reverse osmosis (TRO) have been shown to be technically feasible for desalting non-scaling mine waters with high salinity (Juby, 1992; Juby and Pulles, 1990). However, the majority of mine service water tends to be, or becomes, sodium sulphate scaling water, leading to scale formation on the membrane surface. Recently the SPARRO (Slurry Precipitation and Recycle Reverse Osmosis) process has been developed to overcome scaling of the membrane and to reduce operation costs (Juby *et al.*, 1996).

These chemical processes tend to be expensive as they require the installation of a plant with agitated reactors, precipitators, clarifiers and thickeners (Gazea *et al.*, 1996). They are also inefficient especially when the metals are dissolved in large volumes of water and have relatively low concentrations. Thus biological methods for effluent treatment have become an area of increasing focus.

1.5.1. Biological treatment processes

One of the more popular extensive treatment processes is the constructed wetland. This process is considered to be passive as it relies on naturally occurring geochemical and biological processes (Gazea *et al.*, 1996) such as ammonification, denitrification, methanogenesis and the

reduction of iron and sulphur (Kalin *et al.,* 1991). Over 400 wetlands have been constructed for the treatment of AMD and the approach is very popular in the Eastern United States (Johnson, 1995), with artificial wetlands treating coal mine drainage (Hammack *et al.,* 1994) and effluent from sewage works, mines and industries (De Wet *et al.*, 1990; Mitsch and Wise, 1998). An advantage of wetlands is that they are inexpensive to construct and maintain in comparison to conventional chemical treatment plants (Johnson, 1995). However, recently the sustainability and stability of these systems under conditions of nutrient limitation has come under question. They also use large tracts of land, which in certain circumstances may not be feasible.

Kuyucak and St-Germain (1994) investigated a number of alternative processes, which may be used in conjunction with constructed wetlands, for the addition of alkalinity to acidic mine waters. Anoxic Limestone Drains, which are used as a pre-treatment step, consist of an excavated seepage interception trench which is filled with limestone and covered with plastic and clay to keep air out. ALD's produce alkalinity at a lower cost than do compost wetlands, but their use is limited to mine waters with Fe^{3+} concentrations of less than 2 mg.l $^{-1}$, a net acidity lower than 300 mg.l $^{-1}$ and $CaCO_3$ and dissolved oxygen less that 1 mg.l $^{-1}$ (Gazea *et al.*, 1996). Other alternatives include Lime Organic Mixture, which utilises a bed of limestone and manure to remove metals and increase alkalinity and Biotrenches which utilise nutrients such as straw, sawdust or woodshavings to establish microbial populations which increase the pH.

Microorganisms are known to play an important role in the solubilisation, accumulation, transport and deposition of metals in the environment (Colleran, 1997). Their ability to accumulate metals from dilute solutions, and thereby concentrate them, has the potential for exploitation, with the remediation of metal contaminated waters and the possible recovery of valuable metals as the final objectives. A number of fungi (Muraleedharan and Venkobachar, 1990; Kapoor and Viraraghavan, 1998), bacteria (Komori *et al.*, 1990; Thompson and Watling, 1987) and yeasts (Brady *et al.*, 1994; Rapaport and Muter, 1995) have been investigated for their ability to accumulate heavy metals. The bioremoval capabilities of microalga has also been extensively studied (Ziminik, 1988; Kuyucak and Volesky, 1989; Holan *et al.,* 1993; Volesky and Prasetyo, 1994), and some commercial applications have been initiated (Greene and Bedell, 1990). Constructed microbial mats, which utilise the metal accumulating ability of cyanobacteria

coupled to nutrient removal by bacteria have shown promising results in field-site studies (Bender *et al.*, 1995; Phillips *et al.*, 1995). Systems utilising immobilised biomass such as Sphagnum peat moss (Hammack and Edenborn, 1992; Spinti *et al.*, 1995), *Rhizopus arrhizus* (Tsezos *et al.*, 1989), *Penicillium sp.* (Duran *et al.*, 1997), and a range of micro-algae (Holan and Volesky, 1994; Harris and Ramelow, 1990; Mahan and Holcombe, 1992) have also been investigated. However, their long-term use and regeneration ability appears to be limited. Liehr *et al.* (1994) evaluated metal precipitation inside algal biofilms. Micro-alga are not unique in their capability to remove heavy metals but they do offer advantages over other biological materials in some conceptual bioremoval process schemes. Micro-alga use light as an energy source thus facilitating the maintenance of metabolism in the absence of organic carbon sources (Goldman, 1979), an electron acceptor of bacteria and fungi. Micro-algae cultures can also be cultivated in large open ponds (Richmond and Preiss, 1980; Chaumont, 1993; Oswald, 1991) or in large-scale laboratory culture, providing a reliable and consistent supply of biomass for such studies and eventual scale-up (Wilde and Benemann, 1993).

Although much work has been done elucidating the mechanisms involved in metal uptake and internalisation (Ting *et al.*, 1995; Schenk *et al.*, 1988), the transfer of this technology to practical scale is still in its infancy. One of the few large-scale applications was developed by Gerhardt and Oswald (1990a) and Gerhardt *et al*. (1994) for the removal of selenium and nitrate from agricultural drainage water using both micro-algae and anaerobic bacteria. Algae were grown in high-rate oxidation ponds and removed NO_3^- from the water. The micro-algae and drainage water were then transferred to anoxic units where denitrifying and selenate reducing bacteria, fed on the micro-algae, further reduced the NO_3^- levels. Although the soluble selenium concentrations only decreased slightly, it was shown that selenate was reduced to selenite and other reduced forms. Unused micro-algae were fermented to methane in digesters.

Pollutants such as acid mine drainage are rich in sulphate. Studies have shown that when these pollutants reach aquatic environments, sulphate reduction in anoxic zones increases (Schindler *et al.*, 1980). This serves to remove sulphate from the overlying water, generates alkalinity and precipitates metals as sulphides. Lawrence and McCarty (1965) had also shown that the presence or addition of sulphide to a digester containing heavy metals results in the formation of metal

sulphides which are insoluble salts. These observations led to the suggestion that biological sulphate reduction may have a role to play in mitigating these forms of pollution (Tuttle *et al.*, 1969).

1.5.2. Biological Sulphate Reduction

1.5.2.1. Ecology and Physiology

Sulphate reducing bacteria (SRB) are a large and diverse physiological group of anaerobic bacteria which are solely defined by their ability to utilise inorganic sulphate in an ATP-requiring reaction as one of their terminal electron acceptors (Peck and Lissolo, 1988). Assimilatory sulphate reduction is purely a biosynthetic process in which sulphate is reduced to sulphide before incorporation into amino acids (Gibson, 1990). Dissimilatory sulphate reducing bacteria on the other hand utilise sulphate as an electron acceptor in the oxidation of the energy substrate and produce sulphide (Hansen, 1988). This takes place via the following reaction :

$$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO^{3-} \text{----------} 5.$$

The sulphide may be converted to H_2S or HS^-, depending on the surrounding redox environment.

The pathway for dissimilatory sulphate reduction is shown in Figure 1.3. The initial step of biological sulphate reduction involves the transport of exogenous sulphate across the bacterial cell membrane into the cell (Cypionka, 1987). Once inside the cell, sulphate dissimilation proceeds via the action of ATP sulphurylase. Sulphate combines with ATP to produce the highly activated molecule adenosine phosphosulphate (APS), and pyrophosphate (PPi) which may be subsequently cleaved to yield inorganic phosphate. APS is rapidly converted to sulphite (SO_3^-) by the cytoplasmic enzyme APS reductase (Gibson, 1990). Sulphite, in turn may be reduced via a number of intermediates to form the sulphide ion. The physiological electron donors for these reactions have not been conclusively identified (Peck and Lissolo, 1988). Candidates which have been considered include cytochrome C_3 and ferredoxin.

The physiology and growth of these bacteria has been studied and well documented (Hansen, 1988; Peck and Lissolo, 1988; Gibson, 1990). Initial research concentrated on two groups of SRB: the genus *Desulfotomaculum*, which are characterised as being spore-forming straight or curved rods, and the genus *Desulfovibrio* which are non-spore forming vibrios capable of utilising sulphate as terminal electron acceptor together with hydrogen during growth on a limited number of organic substrates (Liu and Peck, 1981). The basic difference between the bioenergetics of sulphate reduction between these two genera is the enzymes involved in the removal of pyrophosphate formed during sulphate activation by ATP sulfurylase (Liu and Peck, 1981). Attempts were made to determine their preferred carbon source when grown in pure culture. However, data obtained in the field tended to conflict with laboratory data, suggesting the presence of previously unknown strains. Since then numerous new strains have been isolated and characterised. This has led to the reclassification of the bacteria and the addition of numerous genera. These include *Desulfobacter, Desulfonema, Desulfobacterium, Desulfomicrobium, Desulfococcus, Desulfobulbus, Desulfomonas, Desulfosarcina, Thermodesulfobacterium* and *Archaeoglobus* (Gibson, 1990).

The ecology, physiology, enzymology and bioenergetics of sulphate reducing bacteria have been discussed in a number of reviews (Widdel, 1988; Peck and Lissolo, 1988; Gibson, 1990), as well as their role in anaerobic corrosion (Hamilton, 1985). Sulphate reducing bacteria are found in a number of different environments: anoxic estuarine sediment (Compeau and Bartha, 1987), acid mine water (Herlihy and Mills, 1985; Herlihy *et al.*, 1987), saline water (Banat *et al.*, 1981), freshwater and generally in all soils (Watanabe and Furusaka, 1980). The temperature range at which they grow is also diverse and thermophilic sulphate reducing bacteria have been isolated at temperatures greater than 60^{o}C in deep aquifers (Olson *et al.*, 1981). Hastings and Emerson (1988) found that sulphate reducers were also able to grow in anoxic surroundings in the water column of the Cariaco Trench, either in reducing micro niches or because of the tolerance of some SRB to oxygen.

The ability of SRB to break down compounds or to convert metals which are highly toxic is also well known. Strains which are able to methylate mercury have been isolated from anoxic estuarine sediments (Compeau and Bartha, 1987) while another species of SRB, *Desulfuromonas acetoxidans* has been found to conserve energy by coupling the complete oxidation of organic compounds to the reduction of Fe (III) or Mn (IV) (Roden and Lovley, 1993). Lovley and Phillips (1992) investigated the possibility of SRB contributing to U (VI) reduction in sedimentary environments. Results indicated that the organism may be useful for the recovery of uranium from contaminated waters and wastewaters. Strains which are capable of degrading recalcitrant pollutants such as phenol, catechol, benzoate, cresol and vanillate and of growing in both saline and freshwater conditions have also been isolated (Kuever *et al.*, 1993). SRB are well known for their role in oil formation souring where they accumulate as biofilms on reservoir porous media and use short-chain organic acids obtained from oil partitioning as energy sources (Chen *et al.*, 1994).

Numerous substrates have been reported as energy sources for SRB, some of which are recorded in Table 1.2. SRB do not degrade polysaccharides, proteins or lipids, but depend on the activity of fermentative bacteria for the supply of energy sources (Hansen, 1988). Most energy substrates are typical fermentation products, monomers of cell polymers or other cell components. With the exception of two strains, none of the SRB can utilise simple sugars as energy substrates. One of

the most important substrates for SRB is hydrogen. This is especially important to the *Desulfovibrio* species, as their high affinity for hydrogen is considered to be the reason they are able to out-compete hydrogenotrophic methanogens in sulphate-sufficient environments. A number of *Desulfomataculum* species are also able to grow with hydrogen and sulphate as the sole source of energy (Klemps *et al.*, 1985).

Table 1.2. Compounds that can be used as energy substrates by sulphate-reducing bacteria (from Gibson, 1990).

Inorganic	hydrogen, carbon monoxide
Monocarboxylic acids	formate, acetate, propionate, butyrate, isobutyrate, 2- and 3-methylbutyrate, higher fatty acids up to C_{18}, pyruvate, lactate
Dicarboxylic acids	succinate, fumarate, malate, oxalate, maleinate, glutarate, pimelate
Alcohols	methanol, ethanol, propanal, butanol, ethylene, glycols (mono-, di-, tri- and tetra-), 1,2- and 1,3-propanediol, glycerol
Amino Acids	glycine, serine, cysteine, threonine, valine, leucine, isoleucine, aspartate, glutamate, phenylalanine.
Miscellaneous	choline, furfural, oxamate, fructose, benzoate, 2-, 3- and 4-OH-benzoate, cyclohexanecarboxylate, hippurate, nicotinic acid, indole, anthranilate, quinoline, phenol, p-cresol, catechol, resorcinol, hydroquinone, protocatechuate, phloroglucinol, pyrogallol, 4-OH-phenyl-acetate, 3-phenylpropionate, 2-aminobenzoate, dihydroxyacetone.

Further requirements for SRB include a nitrogen source. The majority of SRB utilise ammonium ions, but the dissimilatory reduction of nitrate and nitrite may also provide a nitrogen source. Nitrate has been shown to inhibit sulphide production by increasing the redox potential (Jenneman *et al.*, 1986). It has also been reported that some SRB are able to fix nitrogen (Widdel and Pfennig, 1984). Other nutrients which are important are phosphorous and iron.

1.5.2.2. Advantages of sulphate reduction over other biological treatment processes

The utilisation of biological sulphate reduction for the removal of heavy metals and sulphate from solution has received much attention and has been reviewed by Peters and Ku (1985). It has been determined that metal separation and settling rates obtained with hydroxide precipitation are lower than those obtained with sulphide precipitation, and that most metal sulphides have low solubilities even at acidic pH's (Singh, 1992; Hammack *et al.*, 1994). Metal sludges containing Cu, Pb, Ni, Cd and Zn are three times less susceptible to leaching at pH 5 than an equivalent metal hydroxide sludge (Whang *et al.*, 1982). Sulphides are also highly reactive with metals, even in the presence of complexing agents (Bhattacharya *et al.*, 1979), thus allowing for a low retention time in the bioreactor (Peters and Ku, 1985). The low solubility of the metal sulphides, coupled with the high reactivity of sulphides with metals, leads to improved metal removal efficiency (Whang *et al.*, 1982). The metal sulphides have also been shown to be more compact and have faster settling velocities than metal hydroxides, thus exhibiting better thickening and dewatering characteristics than hydroxide sludges (Whang *et al.*, 1982; Peters and Ku, 1985; Kuyucak and St-Germain, 1994).

The hydrogen sulphide and hydrogen carbonate formed during sulphate reduction equilibrate into a mixture of H_2S, HS^-, S^{2-}, CO_2, HCO_3^- and CO_3^{2-}. If sufficient sulphate reduction takes place, this mixture will buffer the solution pH to a particular value (pH 6-7), but this differs depending on the specific quantities and types of organic end products (Dvorak *et al.*, 1992). It has also been shown that sulphides play a significant role in preventing the toxicity of most heavy metals in anaerobic treatment (Oleszkiewicz and Sharma, 1990). Hexavalent chromium, which is highly toxic, can be reduced to the trivalent form during sulphide treatment and subsequently precipitated as a hydroxide using lime or caustic soda (Lanouette, 1977).

1.5.2.3. Treatment processes utilising biological sulphate reduction

Laboratory scale tests have shown promising results for the treatment of acid mine drainage by biological sulphate reduction. Numerous two-stage processes combining anaerobic biological sulphate reduction with an aerobic step have been developed at laboratory-scale (Maree and Hill, 1989; Du Preez *et al.*, 1992). Cork and Cusanovich (1979) developed a variation in which the

conversion of hydrogen sulphide to sulphur takes place in a second stage reactor. Hammack *et al.* (1994) in turn combined a limestone neutralisation step prior to sulphate reduction. Anaerobic packed bed reactors (Maree *et al.*, 1987; Hammack and Edenborn, 1992), stirred tank reactors (Maree and Hill, 1989), soil columns utilising *Clostridium* and *Desulfovibrio* bacteria (Kauffman *et al.*, 1986) and biofilms (Rivera, 1983) have also been studied, as has the selective removal of heavy metals from effluents by sulphide precipitation and pH manipulation (Hammack *et al.*, 1993; Hammack *et al.*, 1994).

A number of complex organic carbon sources have been considered as energy sources for biological sulphate reduction. One of the initial carbon sources to be considered was sewage sludge (Butlin *et al.*, 1956), with a system for the production of sulphur from hydrogen sulphide being developed by Burgess and Wood (1961). Since then numerous waste carbon sources have been investigated in an attempt to develop an economically feasible process for the treatment of large volumes of effluent. These include mushroom compost (Hammack and Edenborn, 1992; Dvorak *et al.*, 1992), straw and hay (Béchard *et al.*, 1993), alfalfa (Béchard *et al.*, 1994), cattle waste (Ueki *et al.*, 1988; Ueki *et al.*, 1991) and whey (Christensen *et al.*, 1996). A more conventional carbon source, molasses, has also been used in a two-stage process (Reis *et al.* 1988, Maree and Hill, 1989), while Du Preez *et al*. (1992) and Van Houten *et al.* (1996) have reported on the use of synthesis gas (gas mixtures of $H_2/CO/CO_2$) as an energy source. The use of complex organic carbon sources for the stimulation of biological sulphate reduction thus appears to be an option.

Limited full scale applications for the treatment of acid mine drainage have been developed. However, Shell Research Ltd. and Paques Co. have reported the development of a sulphate reducing bacterial process which involves the *in situ* generation of hydrogen sulphide by using simple carbon sources such as ethanol, and the precipitation of heavy metal sulphides (Barnes *et al.*, 1991; Martin, 1991). Full scale plants of this description are operational at a zinc refinery in The Netherlands and at a copper mine in Utah, United States of America (De Vegt *et al.*, 1997). In 1995 the full-scale treatment of an open pit mine in Finland commenced. In this case the mine shaft was used as the reaction vessel and liquid manure and press-juice as the carbon sources (Riekkola-Vanhanen and Mustikkamäki, 1997).

1.6. PONDING SYSTEMS FOR ACID MINE DRAINAGE TREATMENT

Despite the availability of numerous biological processes for the treatment of acid mine drainage, the implementation of these for large volume discharges, in the $Ml.day^{-1}$ range, has been limited by the requirements of reactor design able to handle such flows.

The development of Waste Stabilisation Pond (WSP) technology over the past 40 years has led to numerous applications in the wastewater treatment field (Mara and Do Monte, 1987; Mara *et al.*, 1996). They have been recognised as one of the most efficient and cost effective means of wastewater treatment in areas where land is inexpensive and climatic conditions are favourable (Rose *et al.*, 1996). One of the early systems was developed in Cape Town, South Africa in 1956 (Abbott, 1963) and since then South Africa has played a leading role in the development of this technology. Rose *et al*. (1996; 1998a) reported on the treatment of tannery effluent in an Algal Integrated Ponding System (AIPS) of the type developed by Oswald (1990). Efficient sulphate reduction was observed in the anaerobic compartment of the facultative pond, with the production of substantial quantities of sulphide which was reoxidised in a Spirulina-based algal high rate oxidation pond (HRAP). The removal of heavy metals from the tannery effluent in the anaerobic compartment was also shown to be substantial, with almost complete removal of metals such as cadmium, chromium, lead and zinc being reported (Rose *et al.*, 1998a). This work led to the consideration of algal ponding as a possible system for the treatment of acid mine drainage wastewaters. In the envisaged system, the waste stabilisation pond would serve as a biological reactor, with the reduction of sulphate and fermentation of the organic source taking place in the anaerobic compartment of the facultative pond.

A conceptual model system for using the WSP concept for AMD treatment was proposed and the integration of the various unit operations in use in algal ponding systems is shown in Figure 1.4. This involves the passage of the metal- and sulphate-rich acid wastewater, together with an organic feed, into the anaerobic compartment in the base of the facultative pond (1). The introduction of oxygen into the system is prevented by the facultative pond which surrounds and covers the anaerobic pit. A High Rate Algal Pond (HRAP) provides secondary treatment for the overflow from the facultative pond (2), utilising the oxygen released by the micro-algae to oxidise organics and residual sulphide. The odour problem normally associated with

sulphidogenic systems is controlled by the recycle of oxygenated water and micro-algal[1] biomass, harvested from the HRAP (3), onto the top of the facultative pond. With the exception of the work reported by Rose *et al*. (1996, 1998a), very little indication is available on how such a proposed system might work.

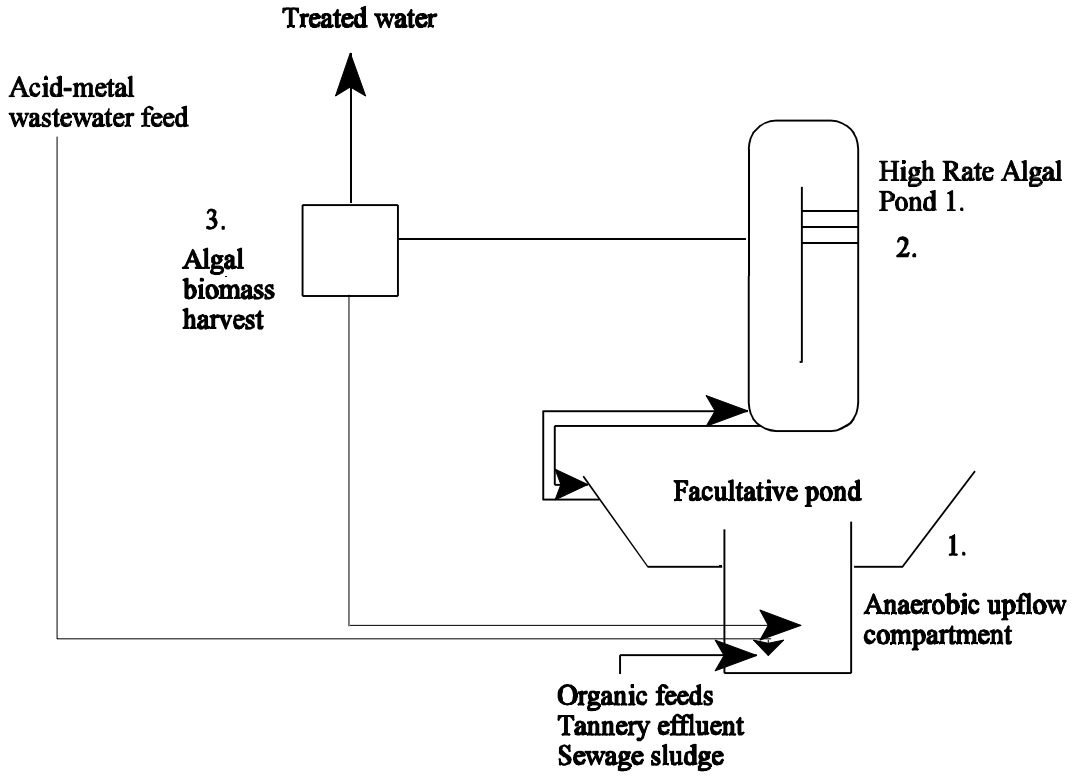


Figure 1.4. Schematic diagram of the integrated process proposed for the treatment of acid mine drainage effluent.

1.7. RESEARCH HYPOTHESIS

The study reported here was undertaken to evaluate the hypothesis that Algal Integrated Ponding Systems provide an appropriate reactor design for the treatment of acid mine drainage effluent.

[1]The term micro-algae as used in this report refers to both eukaryotic algae and cyanobacteria, unless otherwise stated.

1.8. RESEARCH OBJECTIVES

In order to establish an experimental demonstration for the application of the Algal Integrated Ponding System (AIPS) proposed above for the treatment of AMD, a number of questions needed to be answered. These included the following:

1. Can the anaerobic digestion of complex carbon sources such as tannery wastewater be linked to sulphate reduction in this type of digester?
2. What efficiency of sulphate reduction may be anticipated in such systems?
3. Can algal ponding technology be integrated into the effective operation of ponding systems for treatment of AMD?
4. Would algal biomass generated in the system provide an appropriate source of organic carbon for sulphate reduction?
5. Would effective neutralisation of acidity and heavy metal precipitation be achieved in such systems?

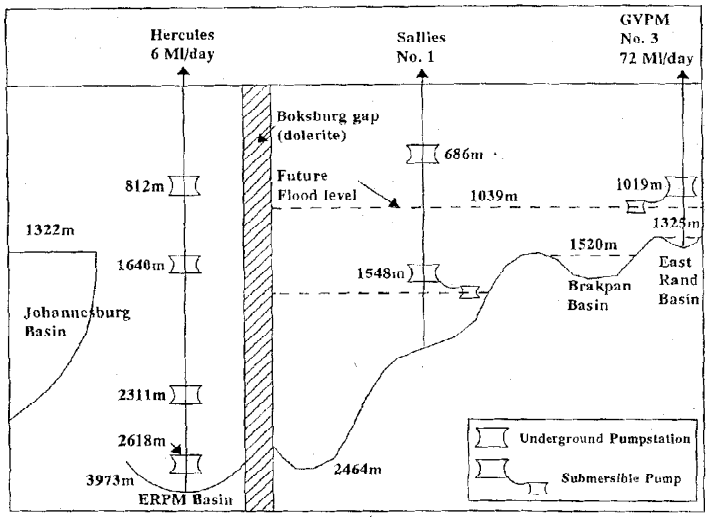


Figure 1.1. Underground water situation on the East Rand with reference to Grootvlei and Sallies mines. (Department of Mineral and Energy Affairs Report, 1995). (GVPM = Grootvlei Proprietary Mine). The elevation marks refer to depth below ground level.

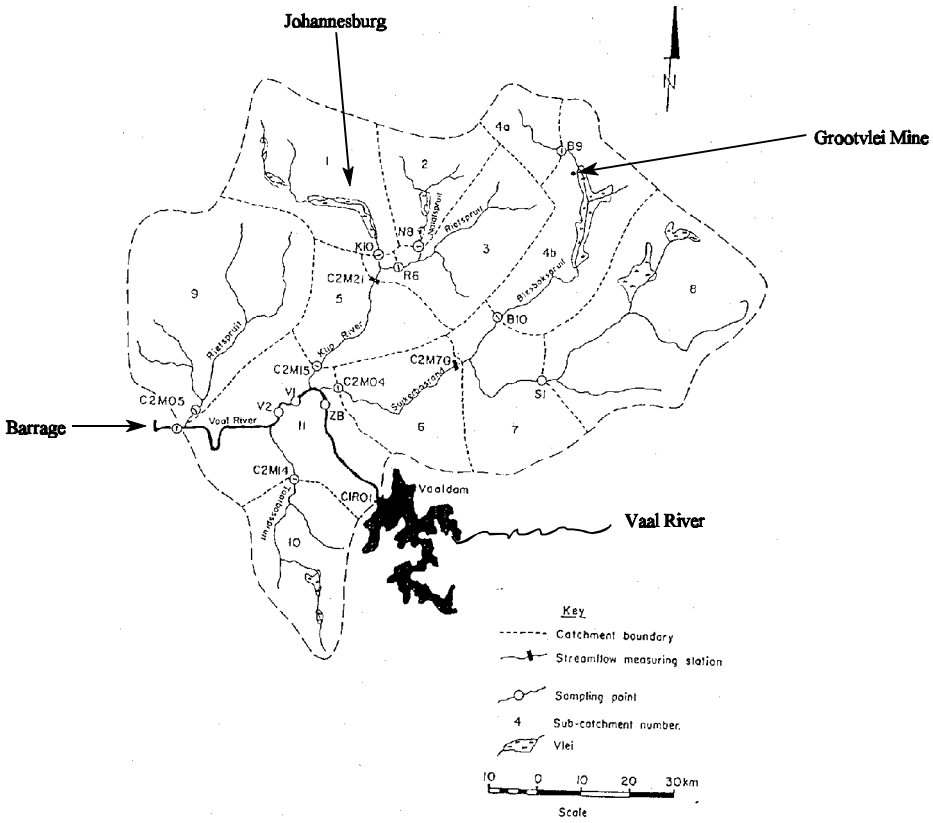


Figure 1.2. Map of Vaal Barrage catchment. (Department of Mineral and Energy Affairs Report, 1995).

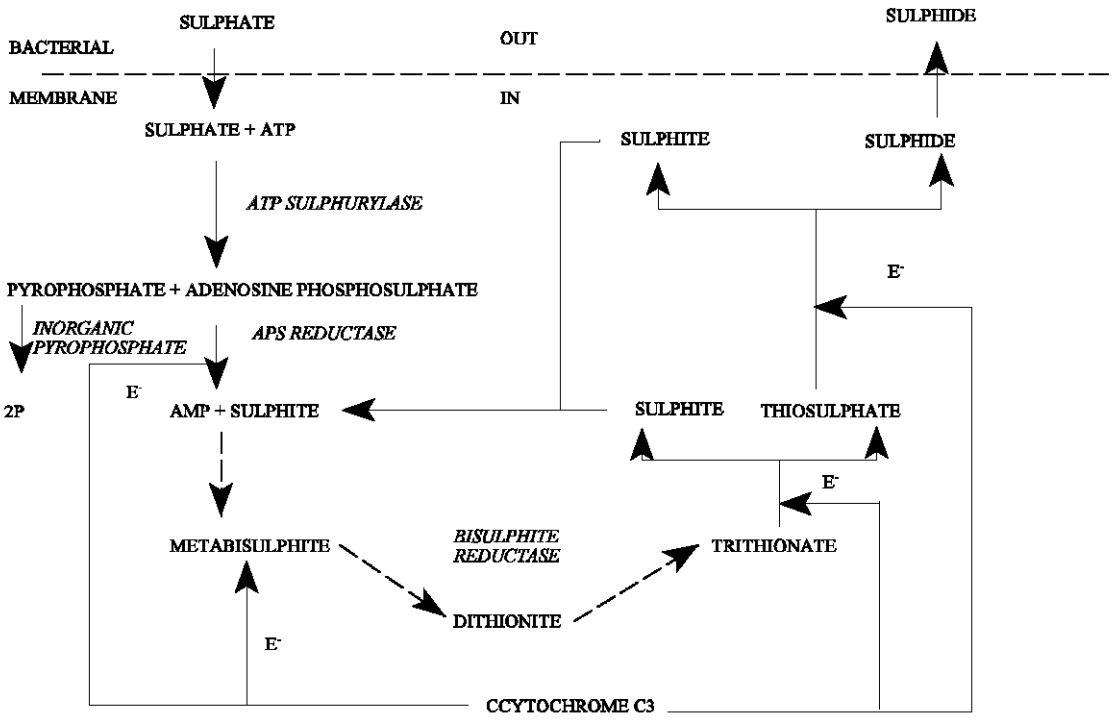


Figure 1.3. The sulphite regeneration pathway of dissimilatory sulphate reduction (E^-= electron). (From Gibson, 1990).

CHAPTER 2. TANNERY EFFLUENT AS A CARBON SOURCE FOR BIOLOGICAL SULPHATE REDUCTION

2.1. INTRODUCTION

Numerous studies have been reported in the literature on the use of simple carbon sources for biological sulphate reduction (benzoate - Li *et al.*, 1996; propionate - Uberoi and Bhattacharya, 1995; butyrate - Mizuno *et al.*, 1994; acetate -Yoda *et al.*, 1987; producer gas - Maree *et al.*, 1987; and ethanol - Barnes *et al.*, 1991). However, where economic constraints have a determining role in the choice of carbon sources, the use of available waste carbon becomes feasible. The numerous waste carbon sources which have been evaluated for active sulphate reduction were discussed in Chapter 1. However, most of this research has been undertaken at the laboratory scale or in extensive systems such as compost beds and open pit reactors (Dvorak *et al.*, 1992; Kalin *et al.*, 1991; Kuyucak and St-Germain, 1994; Bechard *et al.*, 1994).

Rose *et al.* (1996; 1998a) reported on the treatment of tannery effluent in waste stabilisation ponds. Rose *et al.* (1996) also demonstrated a link between tannery effluent and biological sulphate reduction, which raised the possibility of the use of tannery effluent as a carbon source for biological sulphate reduction in the anaerobic compartment of a WSP. Tannery effluent is characterised by a high Chemical Oxygen Demand (COD) originating from the hides as well as from chemicals added during the tanning process (Fig. 2.1). The effluent may also contain high levels of salts such as sulphate, chloride, chromium and sulphide (Genschow *et al.*, 1996).

The critical question which needed to be addressed was whether or not the rates of sulphate reduction necessary for sulphate biodesalination and metal removal from acid mine drainage effluent could be obtained in the anaerobic compartment of the WSP. The opportunity arose to build a pilot plant on site at a tannery producing automotive leather. Two anaerobic reactor types occur in the WSP system shown in Figure 1.4. The pit is analogous to an upflow anaerobic sludge blanket (UASB) while the facultative pond is comparable to an unstirred plug-flow reactor such as a trench digester (TR). The construction of the UASB and TR, as well as a conventional continuously stirred tank reactor, allowed the comparison of reduction rates and thereby to establish a provisional feasibility for the use of pond reactors in the biological treatment of acid

mine drainage.

2.2. RESEARCH OBJECTIVES

1. To evaluate the use of tannery effluent as a carbon source for the biological treatment of acid mine drainage effluent by sulphate reduction.
2. To evaluate two digester configurations, the designs of which are based on integral parts of the ponding process.
3. To compare biological sulphate reduction efficiency in the two reactors with a conventional continuously stirred anaerobic digester.

2.3. MATERIALS AND METHODS

2.3.1. The Tannery

Seeton Leathers (Nigel, South Africa) is a tannery producing leather for the automotive industry. During full production the factory processes between 1000 and 1200 local hides daily as well as imported wet-blue and wet-white hides. Of these hides 350 to 500 are green hides, thus requiring pickling and resulting in the generation of saline effluents. Effluent from the different areas of the tannery is combined in a mixing tank (Fig. 2.1) from where it is transferred to a Silflo unit for sulphide oxidation, organic precipitation and flocculation. The sludge generated in this process is allowed to dry in drying beds after which it is removed to a landfill site. The final effluent is discharged to drain and to the municipal sewage treatment works.

2.3.2. Pilot Plant

The performance of an upflow anaerobic sludge bed reactor (UASB), a stirred tank reactor (STR) with a volume of 1.5 m^3 (Fig. 2.2) and a trench reactor (TR) with a volume of 2 m^3 (Fig. 2.3) were compared on site at the tannery. Effluent was fed to the digesters from the mixing tank, receiving wastewater streams from all parts of the factory, by submersible pumps (Rule 360). A submersible pump (Rule 500) in the mixing feed tank served to keep the feed mixed. The rate at which the reactors were fed was under the control of ST203 220V timers. The chemical composition of the wastewater varied from day to day and an average composition is shown in Table 2.1. The effluent fed to the digesters from the feed tank was composed of tannery effluent diluted 50% with tap water.

2.3.3. Experimental

The reactors were operated in continuous mode with a retention time of 4 days. The UASB and STR's were fed at a rate of 375 l.day $^{-1}$ and the TR at a rate of 400 l.day $^{-1}$. The UASB was operated as an upflow system for a period of 33 days, with the overflow going to drain. Following this, it was operated as a STR by mixing with a recirculation pump and with the overflow passing into a 450 litre settling cone. Sludge from the settling cone was recycled, returning bacterial biomass to the reactor. The temperature of the digesters was not controlled.

2.3.4. Analysis

Sulphate, sulphide and total settleable solids were measured according to Standard Methods (APHA, 1989). COD was measured using the Merck Spectroquant 1800 system. The pH was measured using a Cyberscan 2500 pH meter.

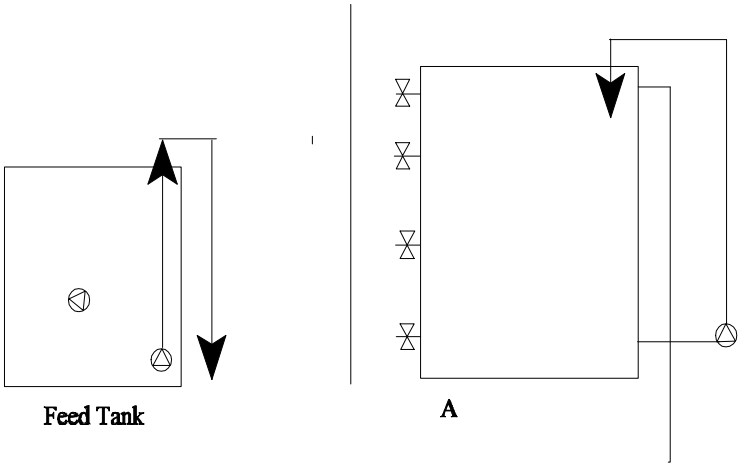

Feed Tank
A

Table 2.1. Representative analysis of the tannery effluent used in this study

Water Quality Parameter	Measured
pH	7.5
Conductivity (µS)	1850
Chemical Oxygen Demand (COD) ($mg.l^{-1}$)	5324
Permanganate Value (PV) ($mg.l^{-1}$)	1194
Suspended Solids (SS) ($mg.l^{-1}$)	5664
Total Dissolved Inorganic Solids (TDIS) ($mg.l^{-1}$)	2914
Total Dissolved Solids (TDS) ($mg.l^{-1}$)	9760
Total Kjedahl Nitrogen (TKN) ($mg.l^{-1}$)	969
Ammonia ($mg.l^{-1}$)	567
Alkalinity - Bicarbonate ($mg.l^{-1}$)	1080
- Carbonate ($mg.l^{-1}$)	120
Sodium ($mg.l^{-1}$)	2010
Chlorine ($mg.l^{-1}$)	3180
Sulphate ($mg.l^{-1}$)	3190
Sulphide ($mg.l^{-1}$)	13 540
Chromium ($mg.l^{-1}$)	49
Boron ($mg.l^{-1}$)	<1
Phosphate ($mg.l^{-1}$)	<1
Iron ($mg.l^{-1}$)	<1

2.4. RESULTS AND DISCUSSION

Figures 2.4 and 2.5 show the performance of the UASB up to day 33 and thereafter for the STR, following the reactor's conversion to completely mixed operation. Figures 2.6 and 2.7 show the performance of the TR. Organic loading rates were not constant during operation due to fluctuations in the composition of the wastewater stream derived from the tannery. The UASB was operated at an organic loading rate of 0.4-1 g $COD.l^{-1}.day^{-1}$, the STR at 0.2-1g $COD.l^{-1}.day^{-1}$ and the TR at 0.15–0.7 g $COD.l^{-1}.day^{-1}$. The three different reactor configurations were compared in terms of substrate utilisation and removal efficiency rate as well as sulphate reducing activity. In this case sulphate removal refers to the difference in sulphate levels between the effluent and outflow and thus takes into account sulphate reduction as well as sulphide re-oxidation.

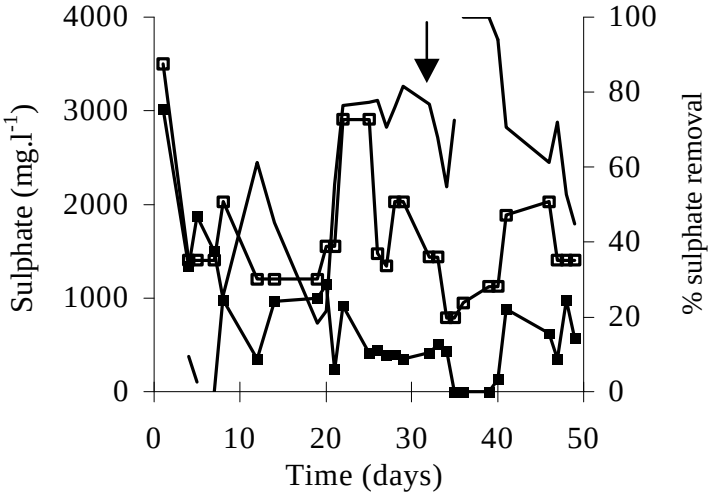


Figure 2.4. Sulphate reduction in tannery effluent in the UASB (days 1 to 33) and in the STR (day 33 to 50). Effluent sulphate (**R**), outflow sulphate (**P**), percentage sulphate removal (—). The arrow indicates where the digester switched from a UASB to a STR.

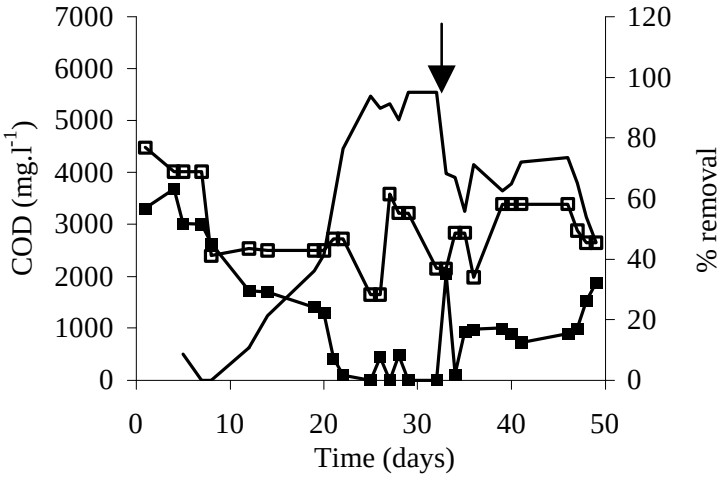


Figure 2.5. Tannery effluent COD removal in the UASB (days 1 to 33) and the STR (days 34 to 50). Effluent COD (**R**), outflow COD (**P**), percentage COD removal (—). The arrow indicates where the digester switched from a UASB to a STR.

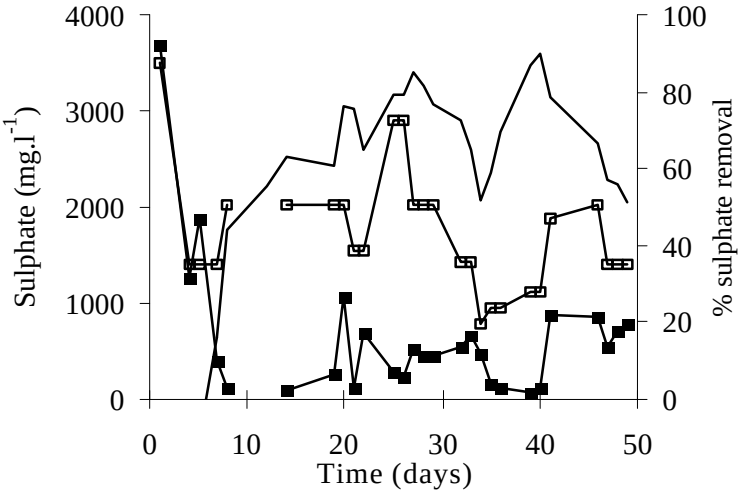
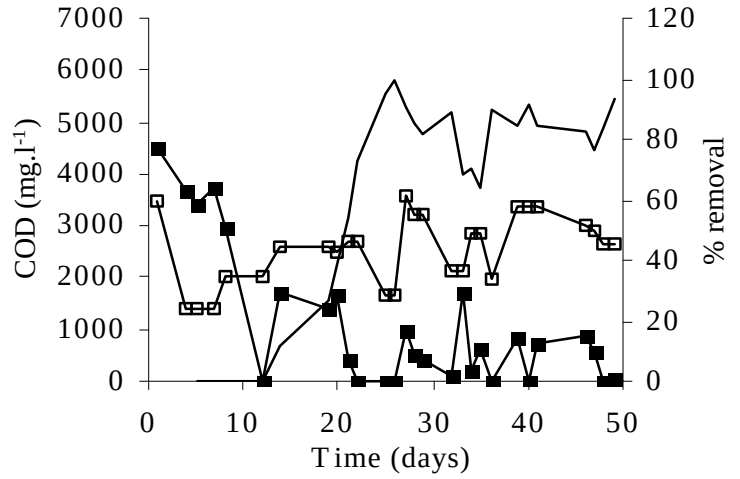


Figure 2.6. Sulphate reduction in tannery effluent in the TR. Effluent sulphate (**R**), outflow sulphate (**P**), percentage sulphate removal (—).



Figure

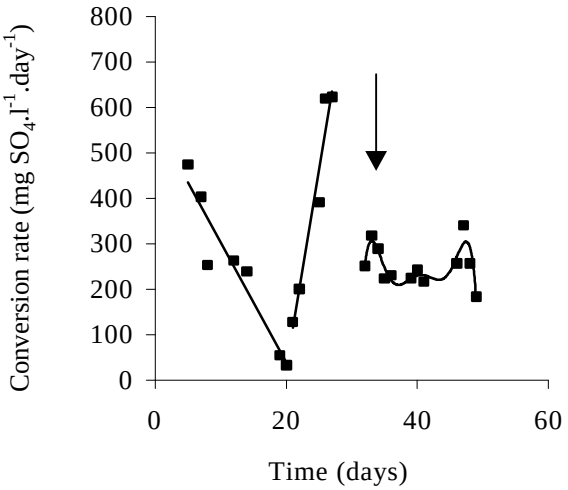


Figure 2.8. Sulphate reduction rate as a function of time for the UASB (day 1 to 33) and the STR (day 34 to 58). The arrow indicates where the digester switched from a UASB to a STR.

The rate of COD removal for the UASB (Fig 2.9) on the other hand, as well as COD removal efficiency increased from the time of commissioning of the reactor from 0 mg COD.l^{-1}.day^{-1} to 500 mg COD.l^{-1}.day^{-1}.

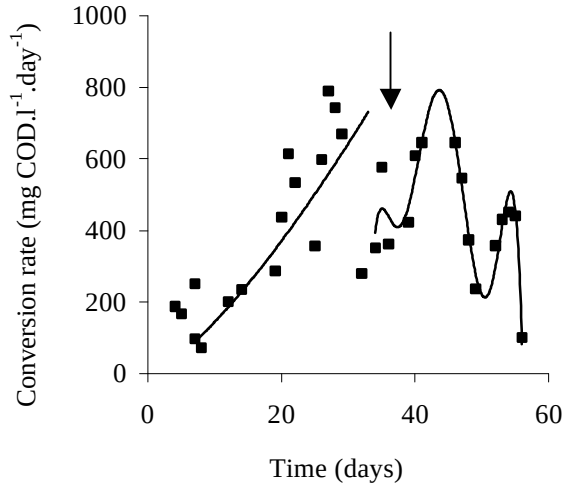


Figure 2.9. COD conversion rate as a function of time for the UASB (day 1 to 33) and the STR (day 34 to 58). The arrow indicates where digester switched from a UASB to a STR.

Change in operation mode from an upflow system to a continuously stirred system had a pronounced negative effect on sulphate removal, with rates decreasing to 250 mg $SO_4.l^{-1}.day^{-1}$. However, as can be seen in Figure 2.6, sulphate removal efficiency increased with an almost 100% removal of sulphates, before dropping to 78% removal. COD removal efficiency, as well as the rate of removal, was also affected by the change in operation mode, with removals dropping from 95% to 75% (Fig. 2.5). A possible reason for this may be the disturbance of the sludge bed leading to washout of bacterial cells participating in COD reduction. SRB use a variety of fermentative end products produced by fermenters and acetogens which are present in sulphidogenic reactors (Zhang and Noike, 1994). In the case of tannery effluent these would be the hydrolysis products of protein containing materials such as keratins and collagen, as well as lipids and fats. Lipids would be partially split up by enzymatic hydrolysis into long and then short-chain fatty acids, leading to acetate. Proteins are hydrolysed into soluble organics and amino acids, and then into acetate through the intermediate, pyruvic acid (Carre *et al.*, 1983). Although Isa *et al.* (1986a) found that acetate alone was not a good substrate for SRB, it was reported to be the major electron donor for sulphate reduction in marine and brackish water (Thauer, 1982).

As can be seen in Figures 2.10 and 2.11, settleable solids were absent in the overflows of the TR and the UASB up to day 33. Total solids in the overflow decreased at a rate of 135.7 mg $TS.l^{-1}.day^{-1}$ and 132.09 mg $TS.l^{-1}.day^{-1}$ respectively. However, there was a measurable increase in total and settleable solids in the outflow of the STR, with levels of up to 50 ml $SS.l^{-1}$ being recorded (Fig. 2.11).

The rate of sulphate conversion in the TR increased steadily over the first 30 days of operation to reach levels ranging between 400 and 500 mg $SO_4.l^{-1}.day^{-1}$. However, as can be seen in Figure 2.12, the rate declined in the final 30 days of operation to approximately 200 mg $SO_4.l^{-1}.day^{-1}$. Average removal efficiencies of 72.12% (s.d. ±11.72) were obtained. COD removal rates also increased with time, reaching 500 mg $COD.l^{-1}.day^{-1}$ (Fig. 2.13). Removal efficiencies were comparative to levels obtained in the UASB. Sulphate removals substantially higher than those reported by Genschow *et al.* (1996) for a two-stage biological sulphate reducing process for the treatment of tannery effluent were recorded in the UASB and TR digester.

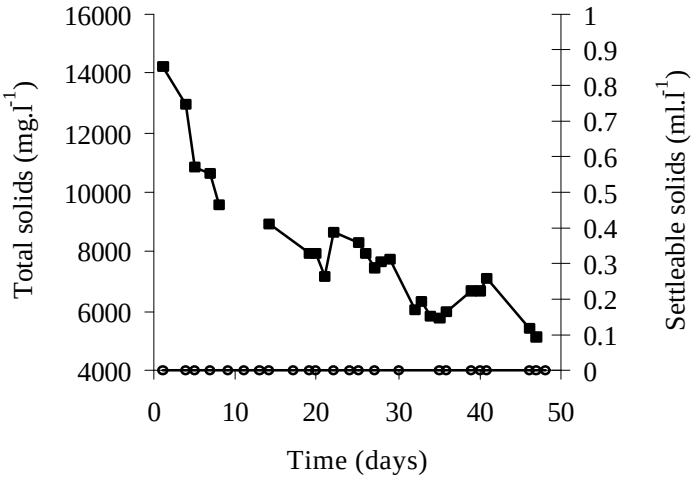


Figure 2.10. Total (**P**) and settleable (**N**) solids in the overflow from the TR.

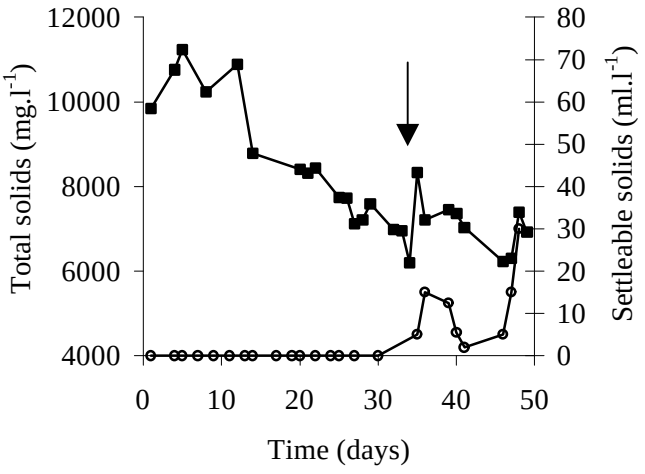


Figure 2.11. Total (**P**) and settleable (**N**) solids in the overflow from the UASB (days 1 to 33) and the STR (day 34 to 50). The arrow indicates where digester operation was switched from a UASB to a STR.

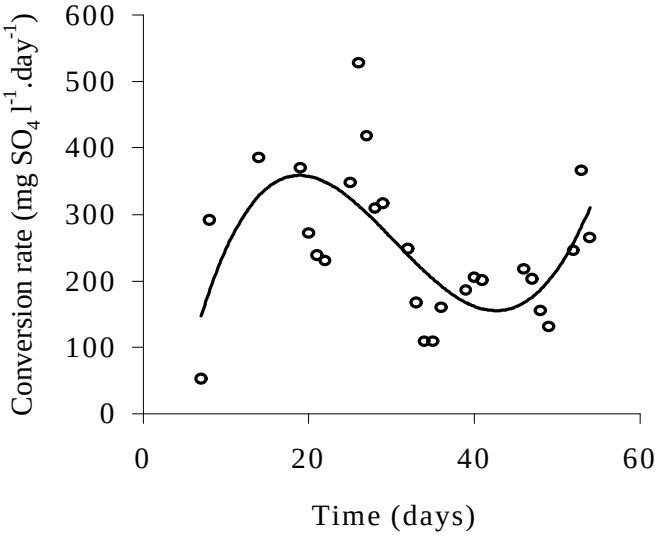


Figure 2.12. Sulphate reduction rate as a function of time in the TR.

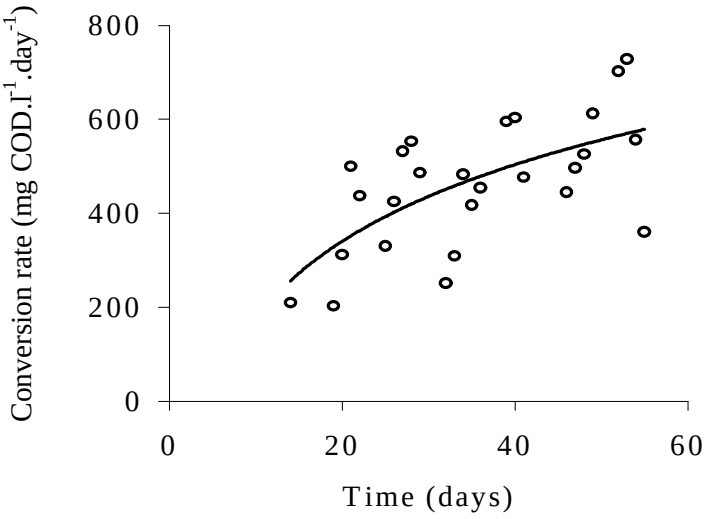


Figure 2.13. COD conversion rate as a function of time in the TR.

Although the TR and UASB digester are comparable in terms of rates of substrate utilisation and COD and SO_4 removal efficiency, the time taken for them to attain these levels differs and may be important in choice of digester design. The rate of microbial population establishment within the reactors was assessed in terms of the increase in COD and sulphate removal efficiency.

Comparatively the microbial population appeared to establish itself quickest in the TR, with sulphate removal efficiency increasing at a rate of 11.7%.day $^{-1}$, compared to 5.54%.day $^{-1}$ and 6.25%.day $^{-1}$ in the UASB and STR respectively. Similar results were obtained for COD removal, with efficiency increasing in the TR at a rate of 11.8%.day^{-1}, compared to 4.6%.day $^{-1}$ in the UASB. Differences in design of the reactors play a major role in determining removal efficiencies as well as the composition of bacterial populations within each one. However, as transport within the UASB and TR does not differ greatly, a further factor, such as biomass washout, could be in operation.

No methane production was observed which would suggest the inhibition of methanogenesis. There are two types of inhibitions on methanogenesis: the first due to competition for substrate with SRB and the second a direct inhibitory effect on the cell functions by soluble sulphides (Lawrence and McCarty, 1966; Shin *et al.*, 1996). Neutrally charged H_2S is considered the toxic component (Oleszkiewicz *et al.*, 1989) due to its ease of transport across the cell membrane without the requirement of an active transport mechanism (Tursman and Cork, 1989). Oleskiewicz *et al.* (1989) found that the maintenance of a high pH (7.7-7.9) allowed for the tolerance of much higher concentrations of sulphide. The pH of the trench digester ranged between 7.9 and 8.2, and was higher than that of the UASB and STR which ranged between 7.4-7.9 and 7.6-8.0 respectively (Fig. 2.14).

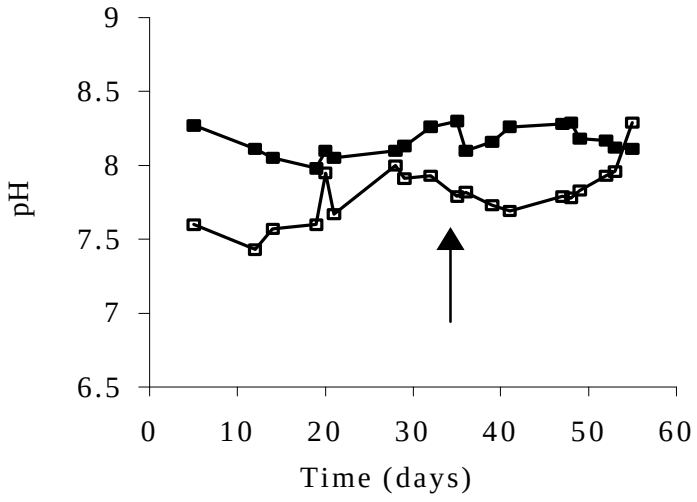


Figure 2.14. The pH of the outflow from the UASB (day 1 to 33) and the STR (day 33 to 55) (**R**) and the TR (**P**). The arrow indicates where digester operation was switched from a UASB to a STR.

Total sulphide levels were highest in the STR (1700 mg $S.l^{-1}$) (Fig. 2.15), followed by the TR (1500 $mg.l^{-1}$) (Fig. 2.16) and the UASB (1300 mg $S.l^{-1}$). The rate of sulphide production mirrored these results: 80.1 mg $S.l^{-1}.day^{-1}$ in the STR, 49.09 mg $S.l^{-1}.day^{-1}$ in the TR and 33.2 mg $S.l^{-1}.day^{-1}$ in the UASB. The concentration of H_2S was calculated from the soluble sulphide concentration and pH of the solution (Oleszkiewicz *et al.*, 1989; APHA, 1989). The percentage of H_2S in the TR was calculated to range from 6.5-7 % of the soluble sulphide concentration, that of the USBR 6.9-7.7 % and the STR 6.5-7.4 %. These values, as shown in Figures 2.15 and 2.16, are lower than have been reported to be toxic to MPB: 90-250 $mg.l^{-1}$ (Koster *et al.*, 1986) and over 1000 $mg.l^{-1}$ (Isa *et al.*, 1986a). This suggests that the repression of methanogenesis was not entirely due to sulphide toxicity, but also due in part to outcompetition by SRB.

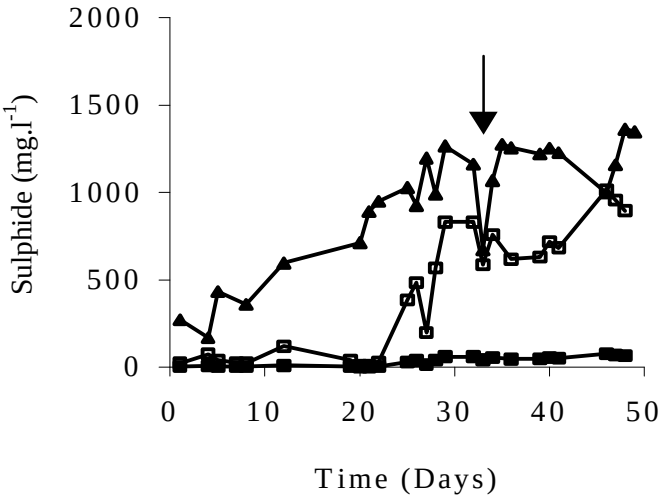


Figure 2.15. Total (>), dissolved (R) and free (P) sulphide in the outflow from the UASB (day 1 to 33) and the STR (day 34 to 50). The arrow indicates where digester operation was switched from UASB to STR.

Hydrogen and acetate are key precursors to methane formation and may also serve as electron donors for sulphate reduction (McCartney and Oleszkiewicz, 1993). The SRB have a higher affinity (Km) value for hydrogen and acetate relative to the MPB (Isa *et al.*, 1986b). This allows them to maintain the concentration of these substrates at levels below which MPB can utilise them, provided the sulphate levels exceed the threshold for SRB dominance (Ingvorsen *et al.*, 1984). In the presence of abundant substrate, this interspecies competition is reduced allowing SRB and MPB to concurrently metabolise available substrates (Vroblesky *et al.*, 1996). Li *et al.*

(1996) found that at higher COD:S ratios, 88.7% of the COD was converted to methane. However, at a COD:S ratio of 0.75, the majority of electron flow is directed towards the SRB. The ratio of COD to SO_4 in the effluent entering the TR ranged between 0.57 and 3.9 with an average of 1.68 (s.d.±0.73). That entering the UASB ranged between 0.57 and 1.68, with an average of 1.78 (s.d.±0.69). However, no relationship was found between COD removal efficiency and the COD:SO_4 ratio of the effluent in any of the reactors.

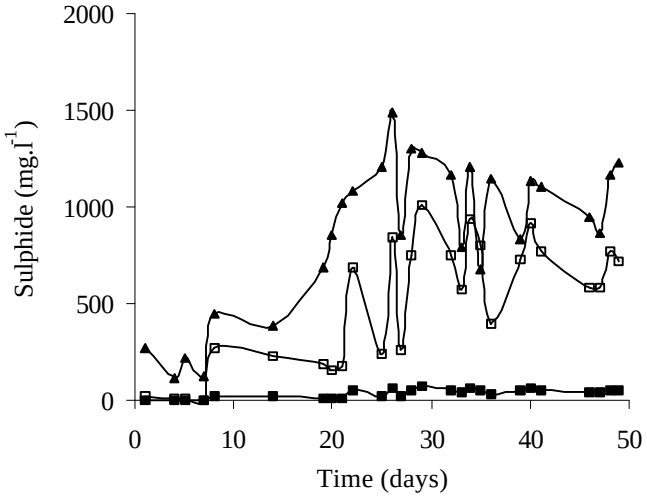


Figure 2.16. Total (>), dissolved (R) and free (P) sulphide in the outflow from the TR.

However, a correlation was observed in the UASB reactor and STR between the amount of SO_4 removed and the pH of the reactor, with higher removals correlating to a higher pH in the reactor (Fig. 2.17).

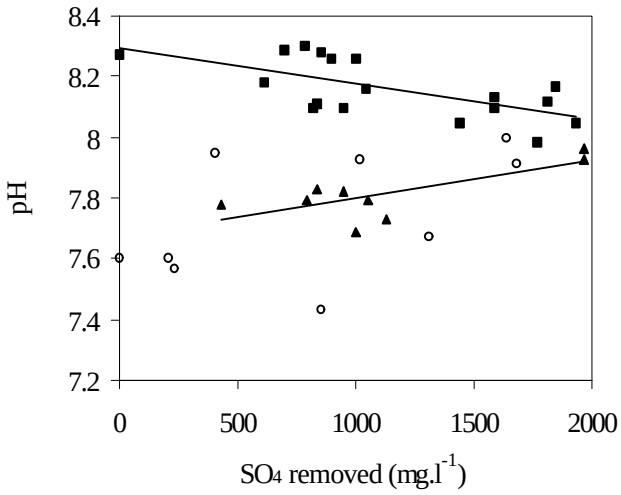


Figure 2.17. Relationship between sulphate removal and pH of the digesters. TR (P), UASB (N), STR (>).

This trend was not found in the TR. White and Gadd (1996), researching a mixed culture of SRB treating acid leachate, reported similar results. Visser *et al.*(1996) found that the optimal growth of acetotrophic SRB's was at pH 8.5-9, but that growth is only slightly reduced at pH 7. The net reduction of 1 mol SO_4 produces 2 mol H_2CO_3 (Loewenthal *et al.*, 1986) due to the conversion of organic carbon to carbonates (Kuyucak and St Germain, 1994). Thus the pH of the medium should tend to rise. However, if for example long chain fatty acids serve as electron donors, these increases in pH may be compensated for (Widdel, 1988). Alkalinity generation may also not be directly coupled to SO_4 reduction (Kuyucak and St-Germain, 1994).

2.5. CONCLUSION

Organic and sulphate loading rates varied with time. However, on average removal of between 60-80% was obtained in all three digesters, at total sulphide feed levels of up to 1800 $mg.l^{-1}$. The rates of removal were difficult to calculate accurately due to fluctuations in the effluent as well as the instability of the system. The system would most likely have reached stability if it had run for a longer period of time. However, due to time constraints associated with working on site, this was not possible. High levels of sulphide were obtained, and although not measured, alkalinity was generated from the reduction of sulphate. Both of these products have a role to play in the removal of heavy metals from the acid mine drainage effluent and their use in this application will be discussed in subsequent chapters. As the pH in the digesters was high (pH >7.4), very little sulphide was in the form of H_2S. Thus the odour problem usually associated with sulphidogenic digesters was absent. Better results were obtained in the TR and UASB compared to the STR, providing some encouragement for the use of the facultative pond anaerobic pit as a sulphate reducing bioreactor.

This study demonstrated that tannery effluent can be used as a carbon source for biological sulphate reduction. The blending of tannery effluent with acid mine drainage effluent not only negates the need to provide a carbon source for biological treatment, but would allow for the simultaneous treatment of both acid mine drainage and tannery effluent. However, the proximity of a tannery to a mine's AMD source and the amount of tannery effluent available would determine the feasibility of its use as a carbon source for high volume treatments. The supplementation with other carbon sources, such as algal biomass, sewage sludge or other organic

wastes, together with the tannery effluent, could be necessary for treatment of large volumes of AMD effluent.

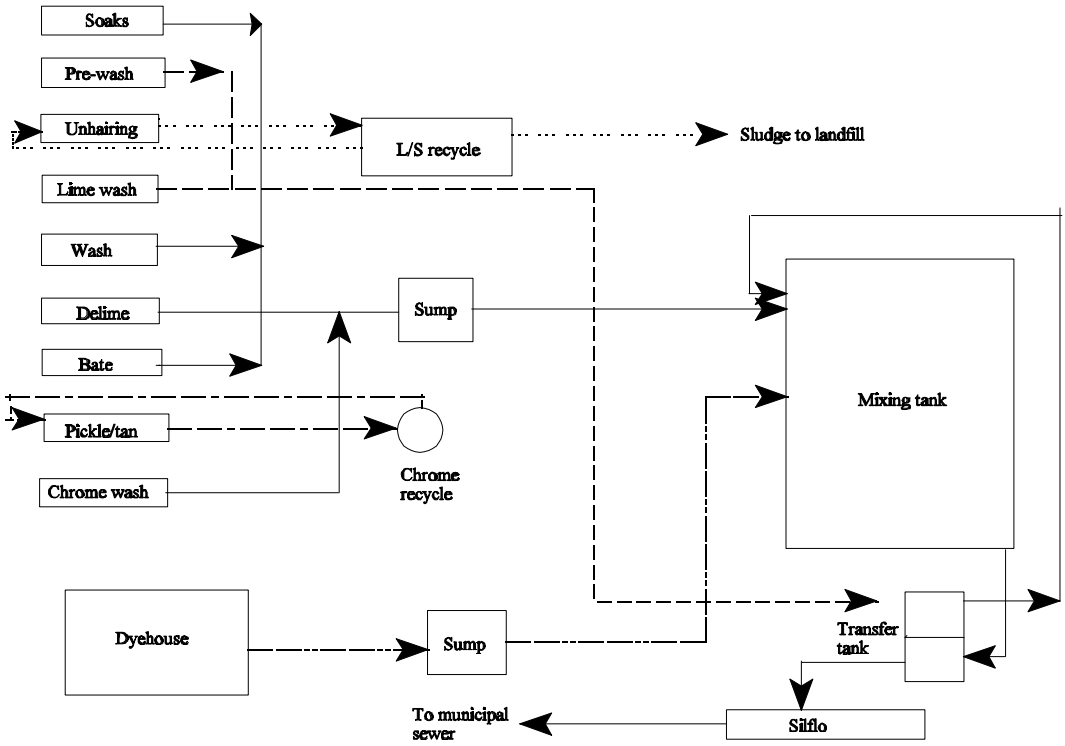


Figure 2.1. Schematic diagram of the different tanning processes which contribute to the effluent as well as the treatment facilities in operation at the tannery. (…) lime/sulphide recycle, (– –) sulphide aeration, (– -) chrome pickle recycle, (– - -) dyehouse effluent, (—) wet blue effluent.

CHAPTER 3. THE USE OF MICRO-ALGAL BIOMASS AS A CARBON SOURCE FOR BIOLOGICAL SULPHATE REDUCING SYSTEMS.

3.1. INTRODUCTION

The previous chapter demonstrated the potential for tannery effluent to be used as a carbon source for biological sulphate reduction. However, organic rich effluent derived from industrial activity may not be available in sufficient quantities or in remote and isolated areas. Thus the need arose to identify a self-sustaining carbon source whose availability is not limited by industrial activity secondary to the mining process. In 1956 Oswald first noted that the settling of micro-algae in high rate oxidation ponds led to active fermentation with the release of toxic substances such as hydrogen sulphide into the water (Oswald *et al*., 1963). Sulphide generation in natural systems due to the degradation of micro-algal biomass has also been reported by Brierley and Brierley (1983). It is against this background that micro-algal biomass was considered as a carbon source for biological sulphate reduction.

Integrated algal ponding systems developed by Oswald (1991) have been applied in South Africa under different environmental conditions and on a number of effluents (Rose *et al.*, 1996). Dunn (1998) reported the growth of *Spirulina* spp. in facultative ponds fed tannery effluent. Average productivity of 7478 mg $C.m^{-2}.day^{-1}$ was reported, which compared well to the 8-12 g $C.m^{-2}.day^{-1}$ cited for outdoor culture basins by Fox (1983). This translates into an estimated biomass production of 109 $tons.annum^{-1}$ from a 197 000 m^3 ponding system (Dunn, 1998). Micro-algal biomass has been used as an energy source for the biodegradation of manganese dioxide (Hart and Madgwick, 1987) and the bacterial reduction of nitrate, selenate and selenite (Gerhardt and Oswald, 1990a). However, its suggested use in engineered sulphate reducing systems appears to be novel.

The obvious advantage of using algal water treatment systems and recovered micro-algal biomass as a carbon source for sulphate reduction is the potential for the remediation of two effluents using photosynthetic energy as the driving force.

The study reported here investigated the use of waste grown micro-algal biomass as a carbon

source for sulphate reduction.

3.2. RESEARCH OBJECTIVES

1. To establish the feasibility of using micro-algal biomass as the sole carbon source for biological sulphate reduction.
2. To determine the rate of sulphate reduction using different concentrations of micro-algal biomass.
3. To determine the distribution of substrate and products in a micro-algal biomass fed laboratory-scale UASB reactor.

3.3. MATERIALS AND METHODS

3.3.1. Reactor operation

A bench scale anaerobic upflow reactor with a volume of 6 litres and a height of 40 cm was used to study the growth of a mixed culture of SRB (Fig.3.1). The reactor was seeded with sludge from a methanogenic reactor treating raw sewage. The reactor was initially fed on a weekly basis with lactate medium until the presence of a population of SRB was demonstrated by the production of sulphide. Thereafter it was fed on a continuous basis using a Watson Marlow peristaltic pump, at a rate of 3000 ml.day^{-1} with media of the following composition (g.l $^{-1}$): NH_4Cl 0.5; K_2HPO_4 1.0; $MgSO_4.7H_2O$ 0.2; $CaCl_2.2H_2O$ 0.1; $FeSO_4.7H_2O$ 0.1; Na_2SO_4 0.5. Dried *Spirulina* spp. biomass was used as the organic carbon source and fed to the reactor at concentrations of 4, 8 and 10 g.l^{-1}, giving organic loading rates of 4.502 (±0.586), 3.388 (±0.454) and 2.453 (±0.225) g COD.l^{-1}.day $^{-1}$ respectively. Samples were removed and analysed for sulphate, total and dissolved sulphide, Chemical Oxygen Demand (COD) and Adenosine Triphosphate (ATP).

3.3.2. Analysis

Sulphate was measured turbidometrically and sulphides by the methylene blue method according to Standard Methods (APHA, 1989). COD was measured using the Merck Spectroquant system. ATP was extracted into Tris buffer and assayed using a Bio-Orbit Luminometer. Metals were assayed on a Varian Techtronic Atomic Absorption Spectrophotometer. The percentage dissolved sulphide which was in the free sulphide form was calculated from the equation

according to Oleskiewicz *et al*. (1989):

$$H_2S = [1+1.02 *10^{-(pH-pKa)}]^{-1} \text{----------6.}$$

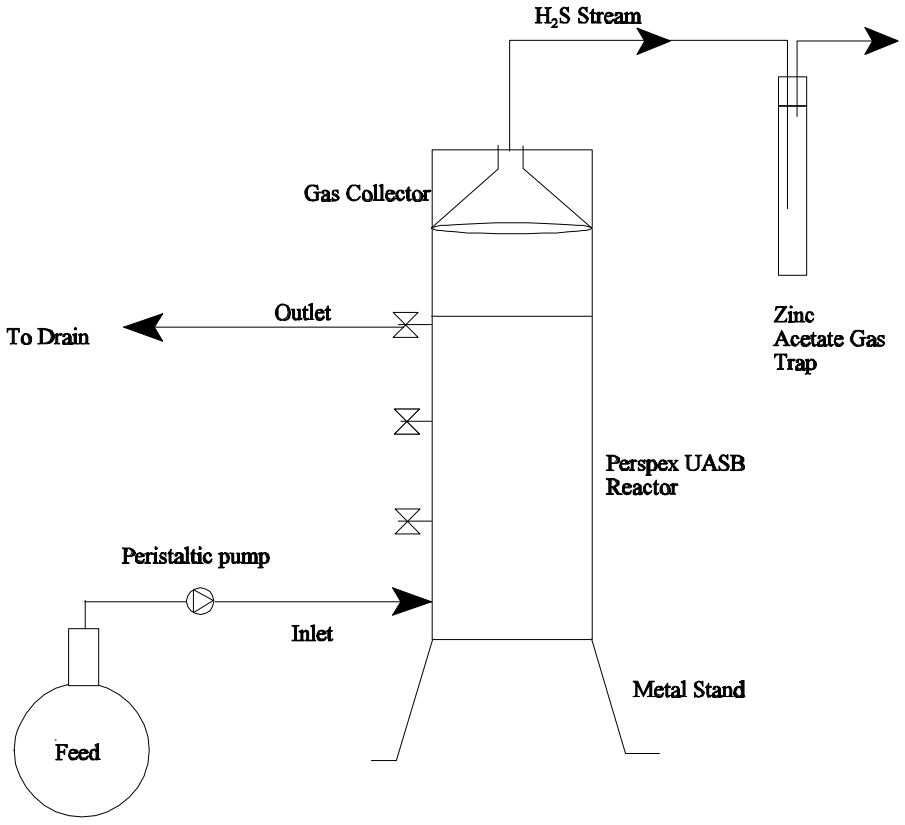


Figure 3.1. Schematic diagram of the reactor used in this study.

3.4. RESULTS AND DISCUSSION

The effect of the COD:SO_4 ratio on substrate consumption and removal efficiencies was assessed by varying the organic carbon content of the media. Li *et al.* (1996) have shown that the ratio of organic feed to sulphate feed is important in controlling the relative growth of SRB and MPB, which in turn determines the measure of sulphate reduction and COD. The reactor studies reported here showed quite similar COD removal efficiencies: 32.42 % (±17.377), 38.57% (±18.04) and 34.48% (±15.31), irrespective of the influent COD:SO_4 ratio, which was 8.1, 11.2 and 15.0 respectively. However, as can be seen in Figure 3.2, the rates of COD removal did differ with influent COD:SO_4 ratio.

The rate increased from 0 to 2500 mg COD $l^{-1}.day^{-1}$ within a 10 day period in the reactor fed medium containing 10 $g.l^{-1}$ micro-algal biomass and a COD:SO_4 ratio of 15. This compares to an increase from zero to 2000 mg $COD.l^{-1}.day^{-1}$ over a similar time period in the reactor fed medium containing 8 $g.l^{-1}$ micro-algal biomass and a COD:SO_4 ratio of 11.2. However, in the reactor fed medium containing 4 $g.l^{-1}$ micro-algal biomass and a COD:SO_4 ratio of 8.1, the rate of COD utilisation actually decreased over time. This suggests that the predominant COD utilising process is less active at lower COD:SO_4 ratios where one would expect SRB to be more active.

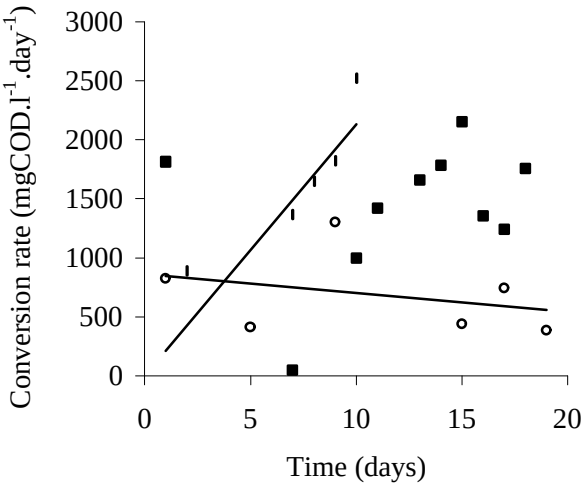


Figure 3.2. COD conversion rate as a function of time for reactors fed medium with a COD:SO_4 ratio of 8.1 (**N**), 11.2 (**P**) and 15 (♦).

This observation is supported by the decrease in sulphate removal efficiency seen with an increase in the COD:SO_4 ratio of the influent media (Fig. 3.3). The highest removal, 90.3% (±10.036), was measured in the reactor fed medium with an influent COD:SO_4 ratio of 8.1, followed by 74.52% (±17.0) and 70.76% (±17.2) in the reactors fed medium with an influent COD:SO_4 ratio of 11.2 and 15 respectively. This conversion rate is high compared to results obtained using simple organic carbon sources, which suggests a COD:SO_4 influent ratio of between 0.66 and 2 to obtain above 80% SO_4 conversion (Table 3.1). However, results obtained using settled sewage sludge as a carbon source showed that a COD:SO_4 ratio of at least 2 is required for efficient SO_4 reduction as not all carbon present is readily degradable (results not shown).

Sulphate utilisation rates (Fig. 3.4.) were highest in the reactor fed medium with a COD:SO_4 ratio of 8.1 (4 $g.l^{-1}$ algal biomass). However, in the reactor fed medium with a COD:SO_4 ratio of 11.2, the rates actually declined.

Table 3.1. Effect of COD:SO_4 ratio on sulphate reduction.

COD:SO_4 ratio	% SO_4 reduction	Substrate	Reference
1.5	67%	butyrate	Mizuno and Noike, 1994
0.66	90%	acetate	Bhattacharya *et al.*, 1996
2.0	90%	glutamic acid	Choi and Rim, 1991
0.44	60%	propionate	Uberoi and Bhattacharya, 1995
1.37	95%	propionate	Uberoi and Bhattacharya, 1995

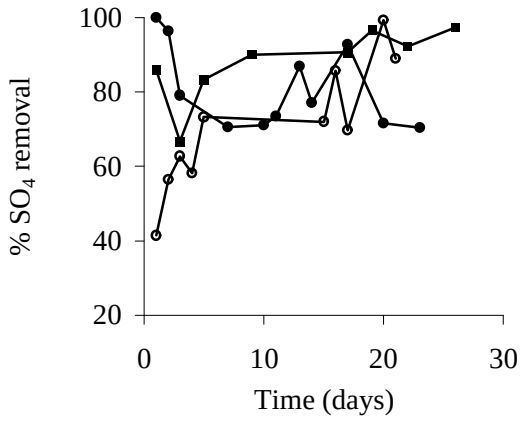


Figure 3.3. Percentage sulphate removal over time in reactors fed medium with a COD:SO_4 ratio of 8.1 (**N**), 11.2 (**P**) and 15 (**P**).

Table 3.2. compares the rate of increase of COD and sulphate utilisation between the three reactors. As was expected, the COD utilisation rate increased the fastest in the reactor fed medium with a COD:SO_4 ratio of 15. However, when it comes to the sulphate utilisation rates, even though the reactor fed media with a COD:SO_4 ratio of 8.1 had the highest rate, the rate at which the reactor fed medium with a COD:SO_4 ratio of 15 utilised SO_4 increased more quickly, and thus over a period of time would be able to catch up to the other reactor.

Table 3.2. Rate of increase in COD and sulphate utilisation.

Feed COD:SO_4 ratio	SO_4 utilisation rate (mg $SO_4.l^{-1}.day^{-1}$)	COD utilisation rate (mg $COD.l^{-1}.day^{-1}$)
8.1	3.0178	-15.8
11.2	-3.625	118.74
15	5.82	213.02

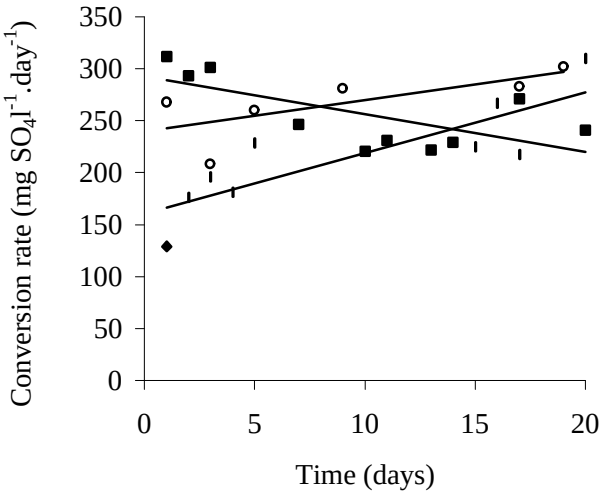


Figure 3.4. Sulphate reduction rate as a function of time for reactors fed medium with a COD:SO_4 ratio of 8.1 (**N**), 11.2 (**P**) and 15 (♦).

If the partitioning of substrate electrons (in terms of COD) is examined, only between 11% and 14% of total COD removal in the reactors fed 10 $g.l^{-1}$ and 8 $g.l^{-1}$ algal biomass can be attributed to use in SO_4 reduction. This is in comparison to the reactor fed 4 $g.l^{-1}$ algal biomass, where 31% of COD removal can be attributed to use in SO_4 reduction. The COD removal achieved at the higher COD:SO_4 ratio is thus predominantly due to processes other than sulphate reduction, possibly methane production. However, even at the lower COD:SO_4 ratio the SRB did not totally outcompete the MPB. Numerous reports have suggested that at higher organic loading rates and in SO_4 limiting conditions, MPB are able to overcome the competitive advantage of SRB (Isa *et al.,* 1986b; Choi and Rim, 1991; Bhattacharya *et al.,* 1996).

Within the reactors, there is a spatial distribution of organics, substrate and product (Fig. 3.6). The occurrence of these zones of differing COD:SO_4 ratio within the reactors may cause the

compositional microbial species to change. The organics, sulphide and intracellular ATP are concentrated at the base of the reactor closest to the inlet. Chen *et al.* (1994) also found an accumulation of biomass in the initial part of the column where the substrate concentration was the highest. This may be a result of higher levels of nutrients in this area promoting bacterial growth, thus also explaining the high sulphide levels in this area. It can also be seen from the increase in sulphate and decrease in sulphide at the outlet that sulphide oxidation is taking place.

As can be seen in Table 3.3, the majority of sulphate reduction occurs at the base of the reactor, with very little decrease in sulphate levels between the base and outlet. Thus one could assume that the SRB activity is concentrated in the lower region of the reactor. The COD levels decreased by between 80 to 90% between the base of the reactor and the outlet. Levels also fluctuated with time, but in general did not show a significant increase. Thus even though the reduction in COD can be attributed to the settling of the heavier organics in the base of the reactor, it would appear that there is an active breakdown of the organics by the microbial consortia present. However, unlike sulphate reduction which appears to be limited to the base of the reactor, COD reduction takes place throughout the length of the reactor. The high concentration of COD in the outflow and base of the reactor, suggests that the system is not carbon limited, thus allowing for the non-competitive growth of MPB, as was observed by Choi and Rim (1991).

Table 3.3. Percentage of total sulphate removed up the column.

	Feed COD:SO_4 ratio		
Distance up reactor (cm)	8.1	11.2	15
3	93.6	64.4	96.3
15	1.3	7.02	0
28	2.8	10.6	0.34

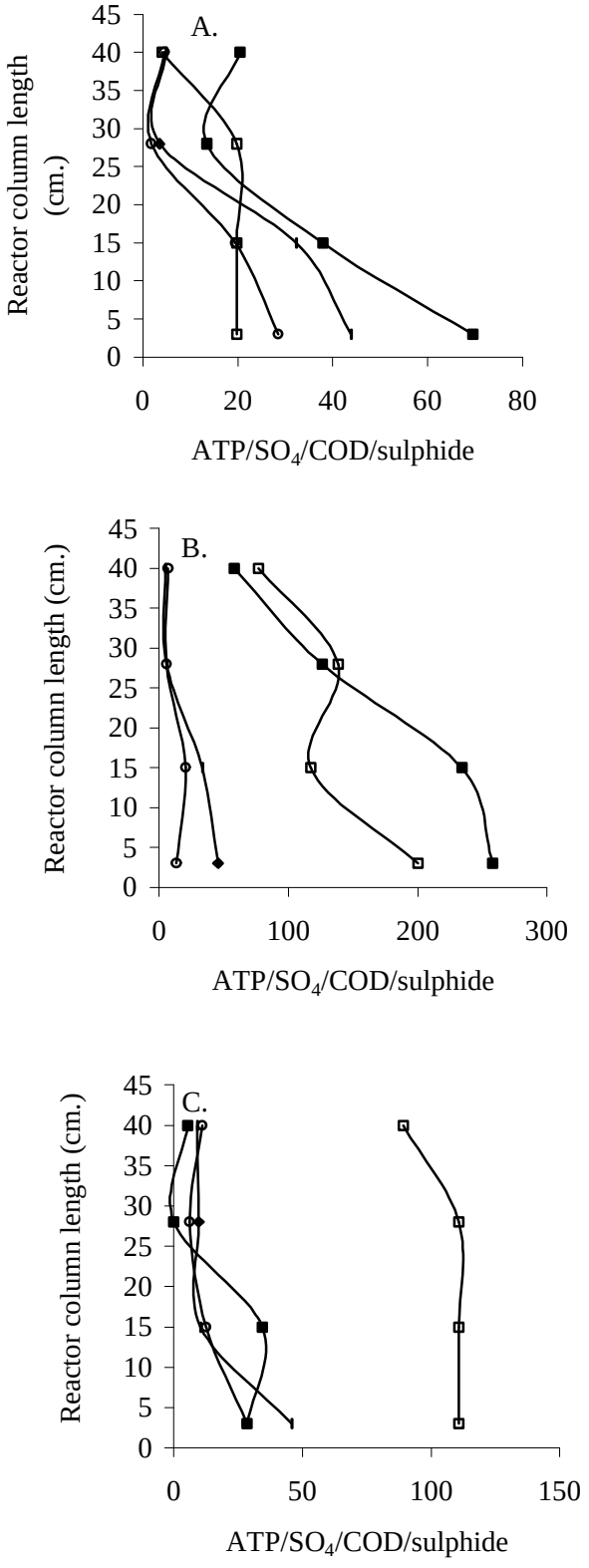


Figure 3.5. ATP (**N**), SO_4 (**P**), COD (♦) and total sulphide (**R**) profile up the column of the reactor fed medium with a COD:SO_4 ratio of A) 8.1, B) 11.2 and C)15 at steady state. Units for measured variables are as follows: SO_4= $mg.l^{-1}$, ATP = µmoles, COD = $x10^{2}$ $mg.l^{-1}$, sulphide = $mg.l^{-1}$.

Fallon and Brock (1979) concluded that the decomposition of blue-green algal biomass is a rapid process that can occur under both aerobic and anaerobic conditions. Even though the loss of chlorophyll under anaerobic conditions is considered delayed or completely inhibited (Fallon and Brock, 1979), Gunnison and Alexander (1975) showed that the cell walls of a number of species of cyanobacteria were amongst the least resistant to decomposition of a number of phototrophic organisms tested. Uziel (1978) found the fermentability of *Spirulina maxima* to be significantly higher than that of other microscopic algae. The chemical composition of *Spirulina* spp. biomass has been documented and it is known that 50% to 70% of its dry weight is composed of protein. Lipids also constitute a fraction of the dry weight, with numerous values reported: 16.6% (Tornabene *et al.*, 1985), 11% (Hudson and Karis, 1974) and even as low as 5% (Switzer, 1980). The biomass is also said to contain a number of carbohydrates such as glucose, levulose, heptose, sucrose, glycerol and several polyols (Quillet, 1975). The microbial population involved in the degradation of the algal biomass is composed of a wide consortia of bacteria: fermenters, acetogens, MPB and SRB. The proteins are degraded to organic acids, amines, CO_2 and ammonia (Almasi and Pescod, 1996).

The COD:SO_4 ratio and ATP profiles also follow each other, with high intracellular ATP levels corresponding to high COD:SO_4 ratios. These fluctuations with time up the reactor column length, as well as the cyclical pattern of the ATP and COD levels (Fig. 3.6) in the outflow, may be as a result of variations in substrate utilisation and product formation taking place in the base of the reactor. As a result the medium which is passed up the column length of the reactor will also have differing substrate levels, affecting the population dynamics up the reactor. During substrate overload the influx of substrate leads to the rapid growth of the hydrolytic and acid-forming bacteria which would metabolize it. More carbon, in a form which can be utilised by the MPB and SRB, becomes available. This results in the growth of the MPB and SRB, with a decrease in the COD and SO_4 levels. However, at a stage the MPB and SRB become inhibited due to the build-up of reduced metabolites. As a result there is a decrease in COD and SO_4 removal with a subsequent increase in COD and SO_4 levels in the outflow. As was shown earlier, only a small percentage of COD removal can be attributed to SO_4 removal. So the inhibition of the SRB would not have as great an effect on COD utilisation as would the inhibition of the MPB. One would also expect the ATP levels to decrease, as the microbial population declines.

However, in the reactor fed medium with a COD:SO_4 ratio, of 11.2 the opposite is initially shown, with high levels of ATP associated with high COD and SO_4 levels.

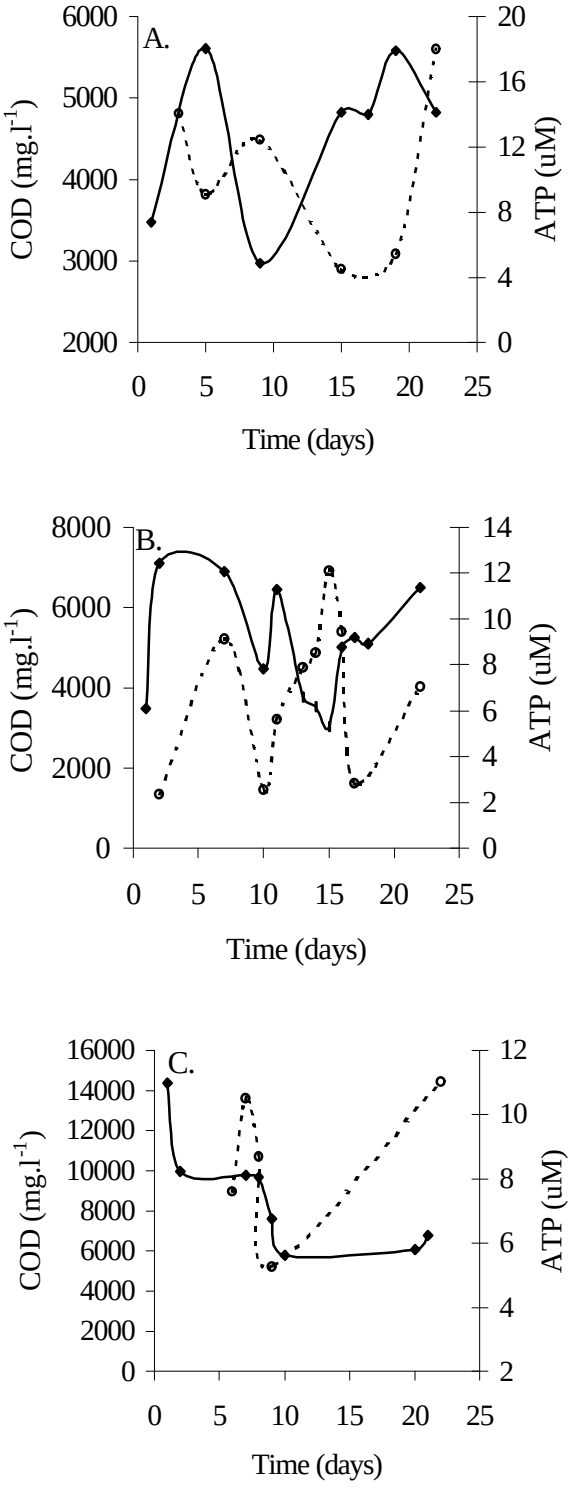


Figure 3.6. COD(♦) and ATP(N) in the outflow from reactors fed medium with a COD:SO_4 ratio of A) 8.1, B) 11.2 and C) 15.

These uncharacteristically high ATP levels may be as a result of an increase in the intracellular ATP concentration of the MPB and SRB as their maintenance energy increases in response to the toxicity. However, the levels may also be attributed to the growth of a population of bacteria which was not previously present, either because they were out competed by the MPB or because the substrate which they grow on was not present in high enough concentrations.

A number of end-products of the digestion process are known to be toxic to the populations involved. It has been demonstrated that an accumulation of acetate can lead to the inhibition of the methanogens. The acetogens, however, are able to continue producing acetate until they become inhibited by a build up of hydrogen, an end-product of their metabolism (Dinopolou *et al*., 1988). The influence of ammonia on anaerobic digestion has also been investigated as digestion failures are often accompanied by high ammonia levels (De Baere *et al*., 1984). Considering the high protein content of the algal biomass used in this study (70%), as well as the amount of biomass used, one would expect a large amount of ammonia production. However, as ammonia and acetate were not measured in this study, these suggestions are speculative and warrant further investigation.

The sulphide levels in the overflow also fluctuate over time, though more so in the reactor fed 8 $g.l^{-1}$ algal biomass (Fig. 3.8). Although sulphides have been found to be toxic to both SRB (Reis *et al.*,1992) and MPB (Isa *et al.*, 1986a), this does not appear to be the case in the digesters used in this study. High levels of sulphide in the overflow from the digester correspond to high ATP levels. The chemical equilibrium of sulphide species and thus its toxicity is pH dependent (Okabe *et al.*, 1995) and in most cases H_2S or molecular hydrogen sulphide appears to be the toxic component (Kroiss and Wabnegg, 1983; Speece, 1983; Parkin *et al.*, 1990) due to the ease with which it can pass through the cell membrane.

Sulphide levels were highest in the reactor fed 8 $g.l^{-1}$ algal biomass and lowest in that fed 4 $g.l^{-1}$. These levels compare favourably to those obtained from using compost (Hammack and Edenborn, 1992). The pH of the reactors was approximately 7.4, at which only about 13% of the dissolved sulphide is in the free sulphide form thus being below the levels which are considered toxic to MPB.

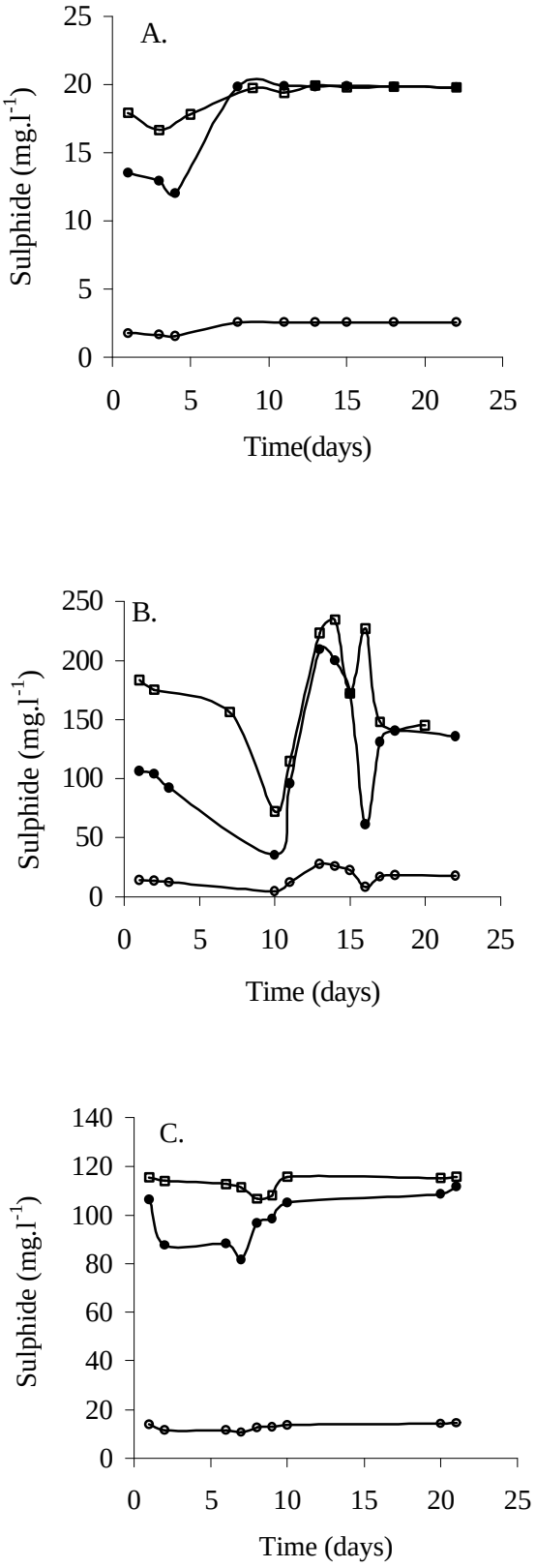


Figure 3.7. Total (R), dissolved (•) and free (N) sulphide concentration in the outflow from reactors fed medium with a COD:SO_4 concentration of A) 8.1, B) 11.2 and C)15.

Substantially lower total sulphide levels were measured than were anticipated from the amount of sulphate reduced. The visual observation of white sulphur in the outflow indicated that at least a part of the sulphide was oxidised to sulphur, thus accounting for part of the discrepancy.

3.5. CONCLUSION

This study has demonstrated that micro-algal biomass can serve as a carbon source for biological sulphate reduction. However, the load ratio is an important factor in the operation of the system. The results indicate that at lower organic loading rates, sulphate reduction is more efficient, with on average 150 mg $SO_{4.}$ g^{-1} algal biomass.day^{-1} being reduced. However, considering that not all of the algal biomass was broken down and, of that component which was digested, only 31% was used by SRB, this level could be further reduced. This in turn would also decrease the methanogenic population and may render the sulphate reducing process more efficient.

The results obtained also furnished an insight into the operation of an algal biomass-fed UASB reactor and stressed the importance of biomass hydrolysis in the process. SO_4 reduction was most prevalent in the base of the reactors, with less occurring as the effluent moved up the column length of the reactor. COD reduction, on the other hand, took place throughout the length of the reactor. The bacterial populations involved in the breakdown of the algal biomass are obviously varied and fluctuate over time. In order to optimise the process, the breakdown products need to be identified as they appear to be exerting a toxic effect on the SRB.

Despite the absence of process optimization studies it my be concluded that the growth of algal biomass as a component product of wastewater treatment operations, including tannery effluent, may be used to generate a sustainable carbon source for sulphate reducing systems. In terms of SO_4 removal efficiency, tannery effluent and micro-algal biomass provide organic carbon sources of comparable efficiency.

CHAPTER 4. PRODUCTION OF EXTRACELLULAR ORGANIC CARBON BY MICRO-ALGAL BIOMASS

4.1. INTRODUCTION

The successful production of large quantities of micro-algal biomass in ponds treating wastewater has previously been described (Dunn, 1998). The micro-algal biomass produced in these systems can in turn be used as a carbon source for biological sulphate reduction, as was reported in Chapter 3. Micro-algae are, however, also known to produce large quantities of photosynthate (Giordani *et al.*, 1994; Chrost, 1981; Davis, 1993), with levels of up to 50% cell weight.day^{-1} reportedly released to their external environment. The ability of bacterioplankton to utilise and transform this photosynthate has also been reported (Wright, 1975; Williams *et al.*, 1976; Smith and Higgins, 1978; Larson and Hagstrom, 1979; Faust and Chrost, 1981). The question thus arose as to whether photosynthate production could be manipulated to serve as an additional sustainable carbon source associated with micro-algal biomass recovery in a waste stabilisation pond system.

The physiological mechanism behind the release of the photosynthate is still not well understood and controversy exists around whether or not healthy cells excrete organic matter (Sharp, 1977; Mague *et al.*, 1980; Surek, 1983; Veira and Mykelstad, 1986; Bjornsen, 1988). However, it is considered to be a stress response mechanism. The quantity of micro-algal photosynthate released differs with species, light intensity, bicarbonate ion concentration, senescence, population density, exposure to darkness, salinity and nutrient supply (Watt, 1969; Kaplan and Bott, 1982). Very few hypersaline stress responses have been studied, with the majority of work carried out on freshwater systems.

A number of problems relating to biological activity arose at a solar evaporation ponding operation located at Sua Pan, Botswana. These included excess production of organic compounds which led to the discolouration of the final product (Rose, 1998b). The water column of the ponds was found to be dominated by *Dunaliella viridis* and *Dunaliella salina*, which have been identified as the primary producer in highly saline lakes and the crystallising ponds of commercial solar salt works. The opportunity arose to evaluate the growth of micro-algae and

photosynthate release in alkaline brine from these solar evaporation ponds and compare it to the growth of *Spirulina* spp. in a defined mineral medium. This enabled the evaluation of extracellular organic carbon production under a number of extreme stress conditions of light, temperature and salinity. It was anticipated that effective manipulation of the stress response in the micro-algae to increase photosynthate production would provide a useful model for linkage to SRB treatment systems.

4.2. RESEARCH OBJECTIVES

1. To investigate the growth of *Dunaliella* under conditions of physiological stress.
2. To establish the conditions under which cultures of *Dunaliella* produce extracellular organic carbon.
3. To establish the conditions under which cultures of *Spirulina* spp. produce extracellular organic carbon.

4.3. MATERIALS AND METHODS

4.3.1. Algal culture maintenance and growth media

A mixed culture of *D. salina* and *D. viridis*, which will be referred to as *Dunaliella* in this study, was isolated from brine samples obtained from solar evaporation ponds at Sua Pan, Botswana. The culture was maintained in BAAM medium (Ben-Amotz and Avron, 1983) of the following composition (in grams per litre): NaCl, 87.66; $NaHCO_3$, 4.2; KNO_3, 0.51; $MgSO_4$, 1.23; $CaCl_{l2}$, 0.044; KH_2PO_4, 0.027. The salinity was measured and adjusted to 12 % with NaCl.

All experimentation in this study relating to work with *Spirulina* spp. refers to the use of a mixed culture of effluent-adapted *Spirulina* spp. isolated from waste stabilisation ponds treating tannery effluent in Wellington, South Africa. The culture was maintained in Zarrouk's medium (Zarrouk, 1966) of the following composition (in grams per litre): NaCl, 1.00; $MgSO_4.7H_2O$, 0.20; $CaCl_2$, 0.04; $FeSO_4.7H_2O$, 0.01; EDTA, 0.08; K_2HPO_4, 0.5; $NaNO_3$, 2.5; K_2SO_4, 1.0; $NaHCO_3$, 16.8.

4.3.2. Experimental treatments

Dunaliella cultures in the logarithmic phase of growth were centrifuged (4420 x g for 15 minutes at 20 ^{o}C) using a Beckman J2-21 centrifuge and the pellet washed with 2 volumes of alkaline brine with a salinity of 12%. The composition of this is shown in Table 4.1. The pellet was resuspended in either 200 ml alkaline brine or 200 ml nutrient deficient media of the following composition (grams per litre): Na_2CO_3, 22.6; $NaHCO_3$, 6.93; NaCl, 123.1; KCl, 2.0. Flasks were placed in water baths to maintain the micro-algal cultures at 30^{o}C, 35^{o}C and 38^{o}C. *Spirulina* spp. cultures in the logarithmic phase of growth were harvested by filtration through a nylon gauze with a pore size of 100 µm. The cultures were washed and resuspended in 200 ml Zarrouk's medium. Flasks were placed in water baths to maintain the micro-algal cultures at 32^{o}C, 35^{o}C and 38^{o}C.

Experiments were carried out under a light regime of eighteen-hour light, six hour dark. High pressure sodium lamps were used for illumination of the cultures at 400 µEinsteins.m^{-2}.s^{-1} (high light conditions). Cold white fluorescent lamps were used for illumination of the cultures at 50 µEinstein.m^{-2}.s^{-1} (low light conditions).

4.3.3. Analysis

Cell counts were carried out using a Neubauer haemocytometer. Chlorophyll *a* (Chl.*a*) was extracted into 100% acetone and determined spectrophotometrically on a Shimadzu UV Visible Recording Spectrophotometer according to the method of Lichtenthaler (1987). Phosphates were analysed using the sulphuric acid/perchloric acid method according to Standard Methods (APHA, 1989). The Total Kjeldahl Nitrogen (TKN) of the supernatant was determined according Standard Methods (APHA, 1989). The Total Organic Carbon (TOC) content of the media was determined on a Dorhmann 18O Total Organic Carbon Analyser.

4.4. RESULTS AND DISCUSSION

4.4.1. *Dunaliella*

The net biomass productivity of an algal culture is considered to be directly correlated to the net rate of CO_2 fixation and the rate of respiration. These two metabolic activities are highly dependent on temperature, while CO_2 fixation or O_2 evolution is also light dependent (Torzillo

and Vonshak, 1994). An assessment of the effect of light and temperature on the growth of *Dunaliella* in alkaline brine was undertaken and the results are shown in Table 4.2.

Table 4.1. Composition of alkaline brine.

Water Quality Parameter	Measured
pH	9.7
Alkalinity ($g.l^{-1}$)	14.7
Chlorides ($g.l^{-1}$)	99.2
Nitrates ($g.l^{-1}$)	0.028
Sulphates ($g.l^{-1}$)	19.7
Carbonates ($g.l^{-1}$)	7.3
Bicarbonates ($g.l^{-1}$)	2.8
Silicates ($g.l^{-1}$)	0.1
Sodium ($g.l^{-1}$)	14.1
Potassium ($g.l^{-1}$)	1.4
Calcium ($g.l^{-1}$)	0.000282
Magnesium ($g.l^{-1}$)	0.000595

Table 4.2. *Dunaliella* growth, as determined according to Middelbeek *et al*. (1992), in alkaline brine under high and low light conditions.

Low light	38ºC	35ºC	30ºC
n[a]	-3.30	2.51	4.04
tg[b]	-1.20	3.20	0.99
k[c]	-0.83	0.31	1.01
μ[d]	-0.58	0.21	0.70
High light	**38ºC**	**35ºC**	**30ºC**
n[a]	-2.07	-1.99	2.61
tg[b]	-2.89	-4.02	1.53
k[c]	-0.35	-0.35	0.65
μ[d]	-0.24	-0.17	0.45

[a] Number of generations
[b]Generation time (day^{-1})
[c]Division rate constant (day^{-1})
[d]Specific growth rate (day^{-1})

As can be seen, the algae had higher specific growth rates under low light conditions. This

corresponds with Loeblich's (1982) report that *Dunaliella* is able to tolerate high light intensities but that chlorophyll production is inhibited under these conditions. The major effect of light is manifested at the photosynthetic level. The inhibition or inactivation of photoautotrophic growth by supra-optimal intensities of light is well known (Richmond, 1986b). The initial site of damage caused by photoinhibition is located in Photosystem II (PSII) and is thought to be caused by the sensitivity of ribulose biphosphate (RuBP) carboxylase to inactivation by blue wavelength light (Vonshak *et al.*, 1996). Temperature also played a role in determining growth, with rates decreasing as the temperature increased from 30ºC, in the control culture, to 35 ºC and 38 ºC. Ben-Amotz and Avron (1982) also found the highest specific growth rate of *Dunaliella* at 30ºC, with growth rates decreasing if the temperature increased or decreased. Temperature has been shown to affect the rates of cellular reactions, nature of metabolism, nutritional requirements and the composition of the biomass (Richmond, 1986b).

The time course of algal growth is illustrated in Figure 4.1. Most cultures, except those grown at 38ºC, entered an initial decline phase followed by an exponential growth phase. This exponential phase was biphasic and characterised by an initial rapid increase in cell number followed by a much slower exponential or pre-stationary phase. The length of these phases varied with growth conditions. A similar phenomenon has been reported by Fogg (1966) who thought it could be caused by an imbalance between intra- and extracellular metabolites during subculturing. However, Ginzburg (1987) found that at low NaCl concentrations the cells entered logarithmic growth phase immediately after sub-culturing. However, at higher concentrations, such as those used in this study, there was an initial period of slow growth. The interaction of temperature and light is demonstrated in results from the cultures grown at 35ºC. Both cultures showed lower specific growth rates than those grown at 30ºC under low light. However, whereas those under low light were able to grow after an initial lag phase, those under high light were not.

It was found that extracellular organic carbon production is initiated at the onset of the inoculation and continues throughout the life cycle of the micro-algal culture (Fig. 4.2.). This is important in terms of practical application as it negates the need for manipulation of the micro-algal biomass to maintain a growth state amenable to organic carbon production. However, in the case of the micro-algae grown at 38ºC, it also indicates that the organic carbon measured in

the media arose from the lysis of cells and thus was not a product of active photosynthesis.

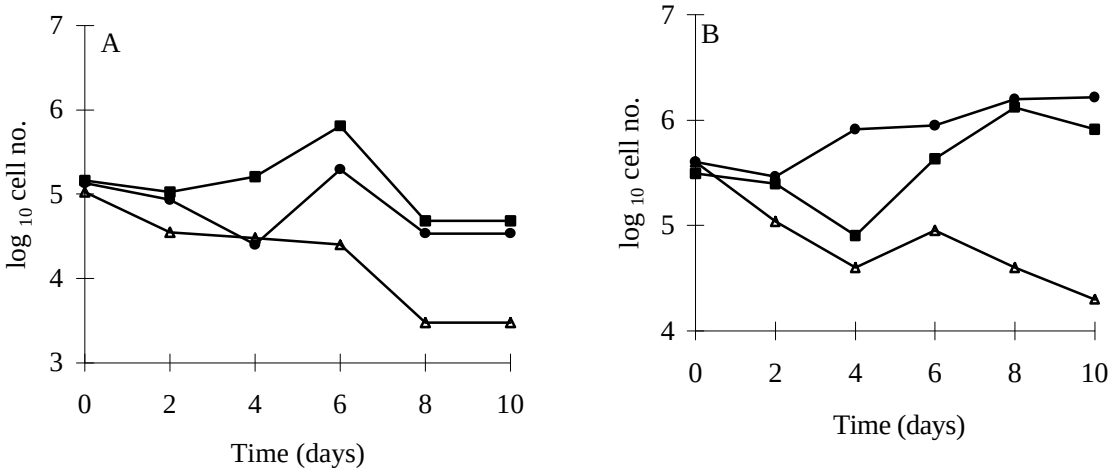


Figure 4.1. Growth of a mixed culture of *Dunaliella* in alkaline brine under A) high light and B) low light conditions at 30ºC (■), 35ºC (•) and 38ºC (▵).

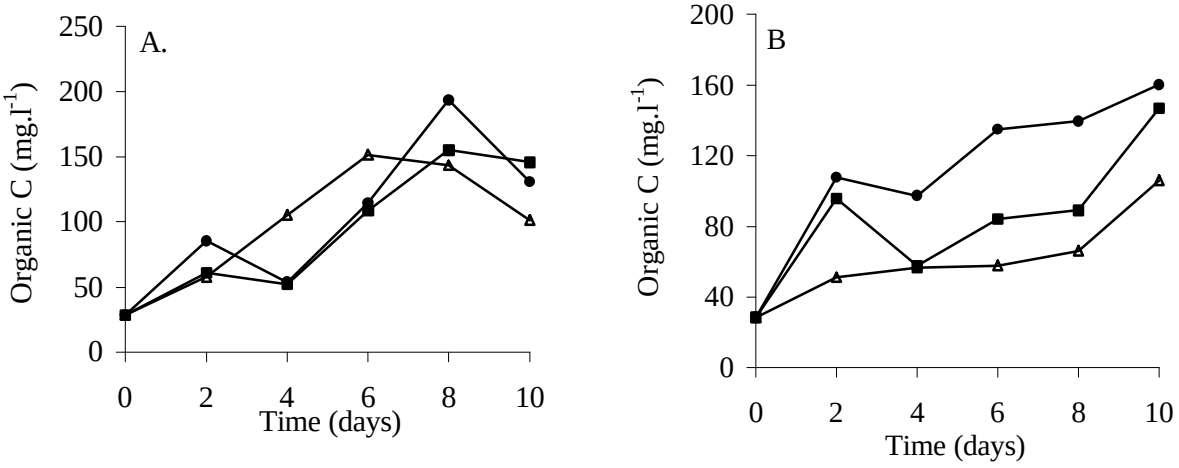


Figure 4.2. Organic carbon excretion as a function of time by a mixed culture of *Dunaliella* grown in alkaline brine under A) high light and B) low light at 30ºC (■), 35ºC (•) and 38ºC (▵).

However, of more importance is the quantity of extracellular organic carbon excreted per cell or per unit of chlorophyll. This, together with the previous results, will indicate whether or not a two-stage process is required where the growth and stress manipulation stages are separated. There was a strong association between the specific rates of extracellular organic carbon excretion, in terms of cell number, chlorophyll *a* concentration and light intensity. TOC and

carbohydrate excretion was a hundred fold higher under high light than under low light when related to chlorophyll *a* (Fig. 4.3). However, when related to cell number, the yields are only slightly higher under high light (Fig. 4.4). Numerous researchers have reported similar results (Konopka and Schnur, 1980; Groeger and Kimmel, 1989; Feuillade *et al.*, 1992).

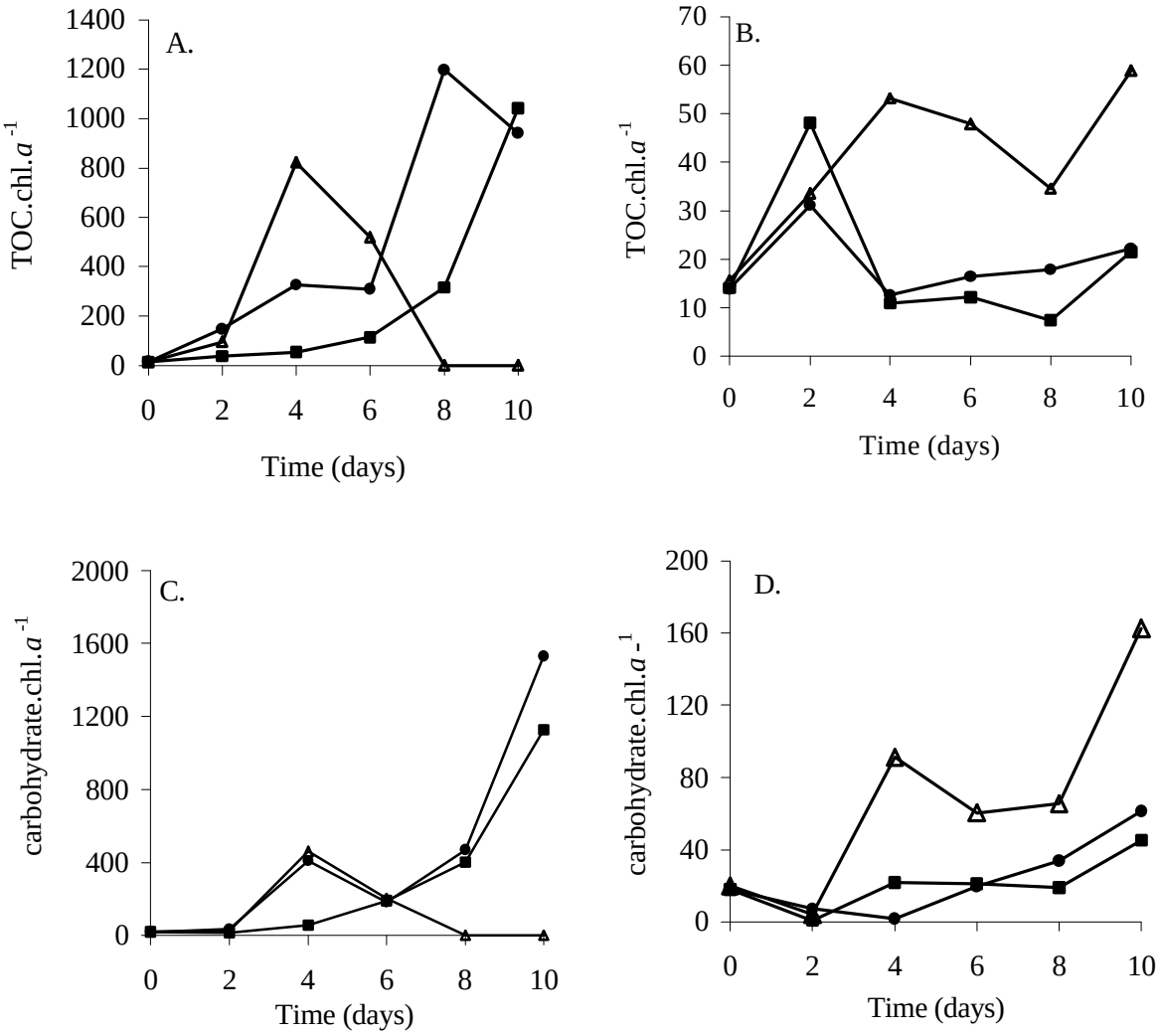


Figure 4.3. Specific carbohydrate and TOC excretion in terms of chlorophyll *a* content of a mixed culture of *Dunaliella* grown in alkaline brine under A & C) high and B & D) low light at 30°C (**P**), 35°C (•)and 38°C (▵).

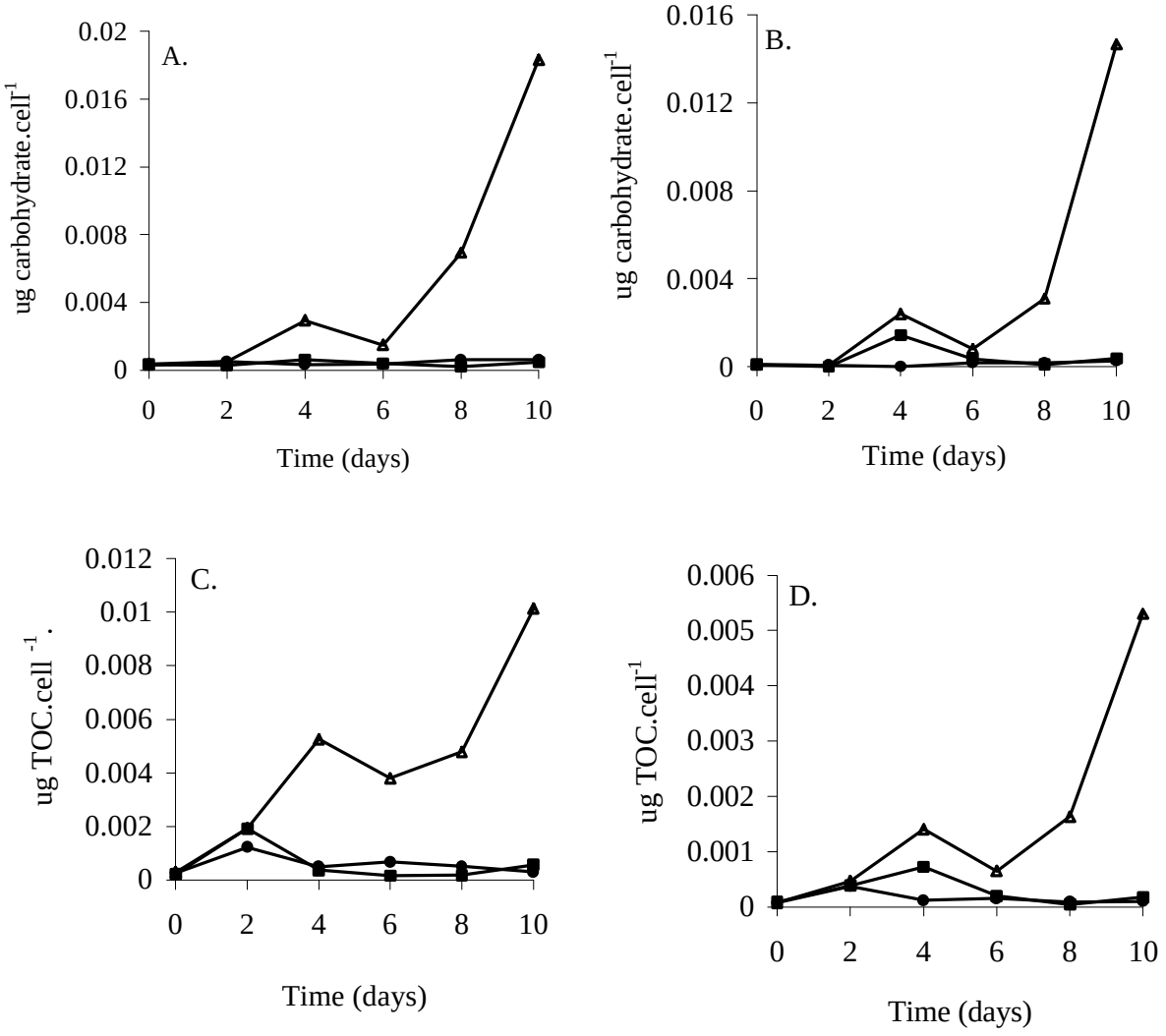


Figure 4.4. Specific TOC and carbohydrate excretion in terms of the cell number of a mixed culture of *Dunaliella* grown in inlet well brine under A & C)high light and B & D)low light at 30ºC (■), 35ºC (•) and 38ºC (▵).

The incorporation of carbon into polysaccharides instead of proteins is thought to occur when the rate of carbon fixation exceeds the maximum rate of incorporation of at least one essential nutrient required for protein synthesis. This also occurs when protein synthesis is low due to two or more nutrients being present in sub-optimal amounts (van Rijn and Shilo, 1986), conditions which arise under high light irradiance. Low light intensities on the other hand limit the rate of CO_2 fixation and ATP production, so that the organic carbon and energy produced do not greatly

exceed what can be assimilated to protein. Mague *et al.* (1980) however, proposed that during active photosynthesis the soluble intracellular pool is saturated with newly synthesised organic compounds which are readily incorporated into protein and polysaccharide. As long as there is an oversupply of these molecules, some pass out of the cell into the surrounding medium. When the photosynthetic rate decreases, the synthesis of proteins is maintained at the expense of polysaccharide production as they are broken down to supply cellular metabolism through the soluble intracellular pool.

The organic carbon yield also appears to be highest in the cultures grown at 38ºC. However, it must be remembered that the cultures at 38ºC had lower cell numbers and thus chlorophyll *a* content, due to their inability to grow under these conditions. Little is known about the effect of temperature on algal release of organic carbon, but it is known to affect enzymatically driven carbon fixation reactions of photosynthesis (Kaplan and Bott, 1982).

The TKN content of the media was assayed over the length of the experiments so as to assess the production of any organic nitrogenous compounds which would enhance the nutritional quality of the extracellular organic carbon for use in anaerobic digestion. Cultures growing at 38°C show an increase in the TKN content of the media over the final four days of the experiment (Fig. 4.5).

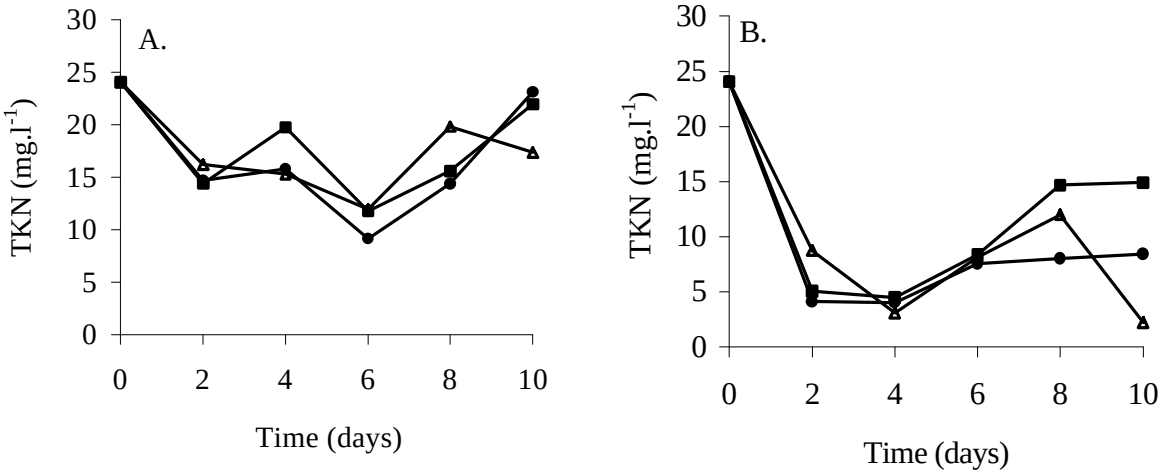


Figure 4.5. Total Kjeldahl Nitrogen content of media as a function of time from a mixed culture of *Dunaliella* grown under A) high and B) low light at 30ºC (■), 35ºC (•) and 38ºC (▵).

It has been established that several species of algae release peptide nitrogen into the culture (Fogg and Westlake, 1955). The ability of stationary phase marine phytoplankton to photo-assimilate carbon at relatively high rates and simultaneously excrete most of the assimilated carbon as well as large quantities of nitrogen containing compounds when the nitrogen source was plentiful has been reported by Hellebust (1965).

Phosphate levels in the media were monitored and shown to remain relatively constant under high light conditions, with an increase being noted towards the end of the experiment (Fig. 4.6A). However, under low light conditions levels decreased (Fig. 4.6B).

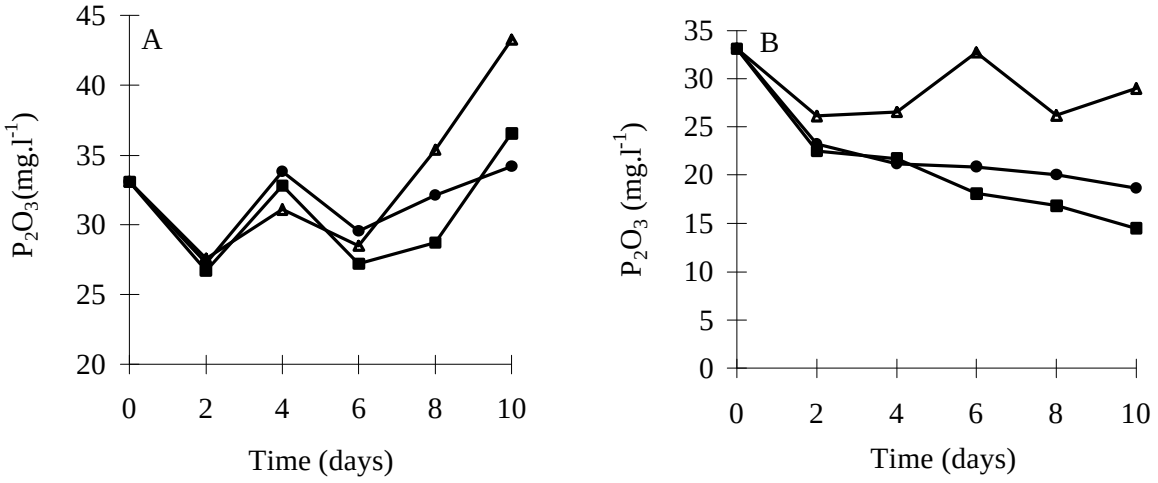


Figure 4.6. Phosphate content of medium during growth of a mixed culture of *Dunaliella* under A) high and B) low light at 30ºC (**P**), 35ºC (•)and 38ºC (▵)

A knowledge of phosphate levels in the medium is important for two reasons. Firstly phosphate depletion during growth will cause the cells to enter stationary phase, thus limiting the potential algal crop available for organic carbon production. It is also important to know the fate of phosphate especially if the algal pond is to be used as a secondary treatment process.

The effect of increasing salinity on the growth of the micro-algae under low light at 35ºC and organic carbon excretion is show in Figure 4.7. The lowest growth was found at the highest salinity. Ben-Amotz and Avron (1983) also showed that at high salinities low yields of *Dunaliella*

biomass are obtained. It has been suggested that this is due to a reduction in the solubility of gases such as O_2 and CO_2 and thus their availability at high salt concentrations. However, Pirt (1975) attributed it to an increase in maintenance energy required to maintain ion concentrations within the cell at values differing from those outside, with less energy being available for cell growth. Salinity has also been said to influence growth by effecting the activity of proteins or transport systems (Gimmler *et al.*, 1984). Thus salinity could be used as a factor for the manipulation of organic carbon production. Giordano *et al.* (1994) also showed that an increase in salinity, from 1.5 M to 3.0 M, enhanced DOC release in *Dunaliella.*

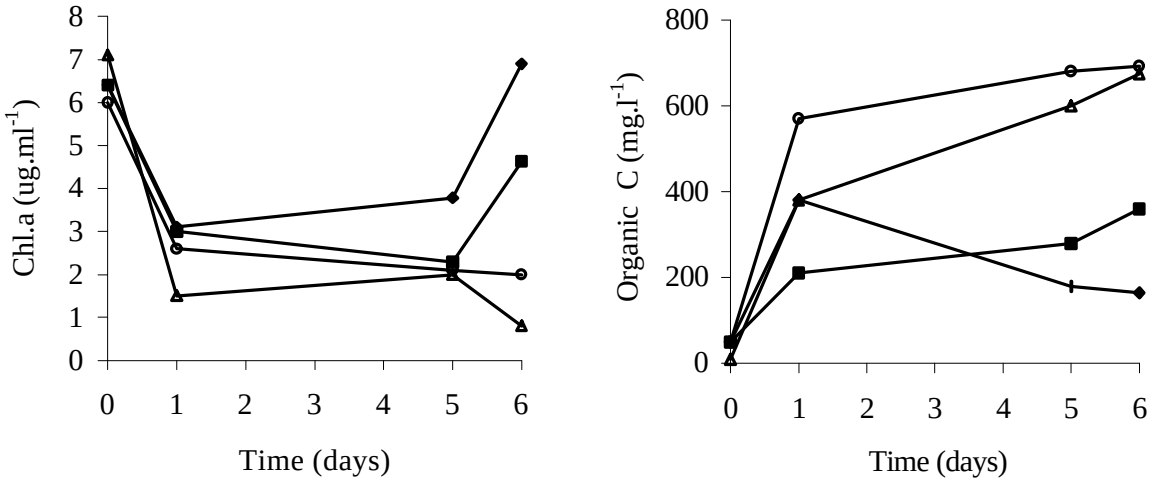


Figure 4.7. Growth of a mixed culture of *Dunaliella* under low light at 35°C and organic carbon production at 3% (♦), 6% (**P**), 9% (**N**) and 27% (△) salinity.

The nitrogen and phosphate level in the alkaline brine used in this study are relatively high and theoretically sufficient to sustain the active growth of *Dunaliella* cells. However, nutrient deficient effluents do exist and it is thus important to investigate growth and organic carbon production under these conditions. Nutrient deficiency is known to play a role in DOC excretion, with N_2 depletion causing an increase in the amount of dissolved extracellular carbohydrate per cell (Vieira and Myklestad, 1986; Thomas *et al.*, 1984). Growth is also affected by nutrient deficiency. Kroen and Rayburn (1984) found that cells suspended in phosphate deficient media continued growth, but in nitrate deficient media they did not divide and entered stationary phase.

Experiments using nitrate and phosphate deficient media showed similar results, with a decline in growth being accompanied by an increase in TOC synthesis (Fig. 4.8). Under these nutrient deficient conditions the content of photosynthetic pigments is reported to decrease and photosynthesis is reduced (Fogg, 1975). Lupi *et al.* (1994) found that cells also degrade nitrogen containing macromolecules thereby reducing cellular nitrogen, leading to the accumulation of carbon reserve compounds such as polysaccharides. Phosphate limitation has also been shown to enhance carbohydrate synthesis, but less than nitrogen deficiency (De Philippis *et al.*, 1993). These results are also representative of what happens when the cells move from logarithmic growth to stationary and decline phase, confirming numerous previous reports of maximum organic carbon production in the stationary phase of growth. However, that the highest production occurred in those cultures with the most pronounced decline phase suggests that it is not a logical consequence of photosynthesis, but possibly due to leakage from the cells undergoing senescence.

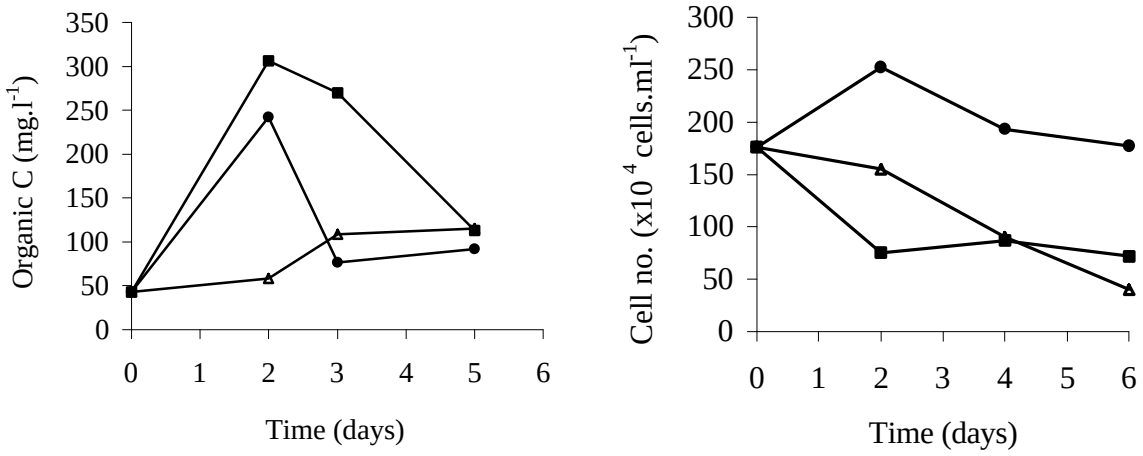


Figure 4.8. Growth of a mixed culture of *Dunaliella* in nutrient deficient medium and organic carbon production as a function of time in cultures grown under high light at 30ºC (■), 35ºC (•) and 38ºC (▵).

4.4.2. *Spirulina* spp.

The growth of *Spirulina* spp. at 32ºC, 35ºC and 38ºC under low light was assessed. As can be seen in Figure 4.9, the highest levels of chlorophyll *a* were recorded at 38ºC. *Spirulina* is

mesophilic with the optimal temperature considered to be between 35 to 40ºC (Zarrouk, 1966). However, it has also been reported that 40ºC is injurious to the alga (Richmond, 1986a). Table 4.3. also shows that the highest specific growth rate is at 38ºC, followed by 32ºC and 35ºC.

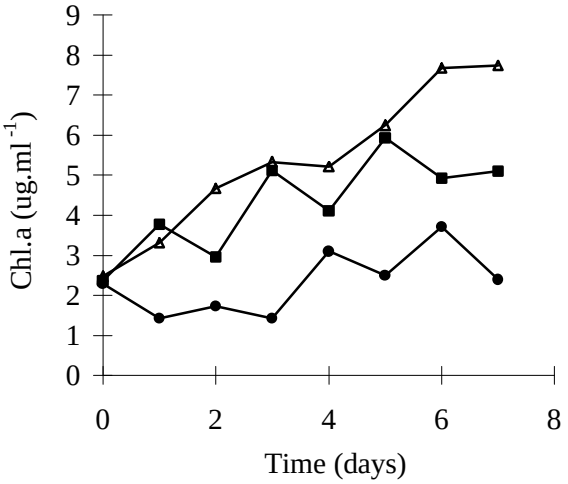


Figure 4.9. Growth of *Spirulina* spp. in Zarrouk's medium as a function of time at 32ºC (■), 35ºC (•) and 38ºC (▵).

Table 4.3. *Spirulina* spp. growth in Zarrouk's medium under low light at different temperatures.

	38ºC	35ºC	32ºC
n[a]	1.64	0.74	1.10
tg[b]	4.26	8.06	6.33
k[c]	0.23	0.124	0.16
μ[d]	0.16	0.08	0.11

[a] Number of generations
[b]Generation time (day^{-1})
[c]Division rate constant (day^{-1})
[d]Specific growth rate (day^{-1})

The production of extracellular organic matter was monitored over a period of 7 days and the results are seen in Figure 4.10. The highest levels were produced at 32ºC and 38ºC. Organic carbon production appears to follow the growth pattern of the algae thus indicating that it is a

product of actively photosynthesising cells, and not a remnant of cell lysis.

The specific rate of organic production, in terms of chlorophyll *a* also indicates maximum excretion at 32ºC, with very similar amounts being produced at 35ºC and 38ºC (Fig. 4.11).

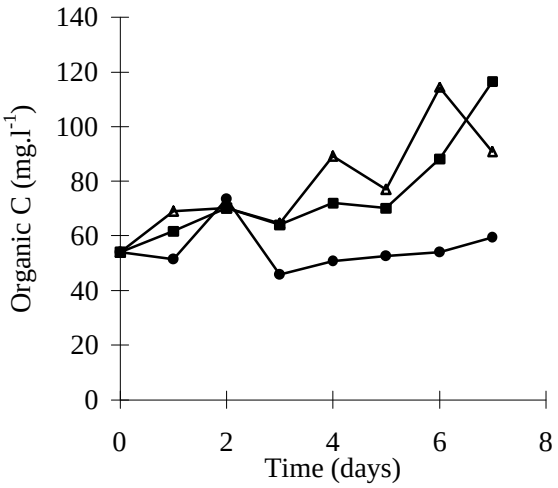


Figure 4.10. Organic carbon excretion as a function of time by a culture of *Spirulina* spp. in Zarrouk's medium at 32ºC (■), 35 ºC (•) and 38ºC(▵).

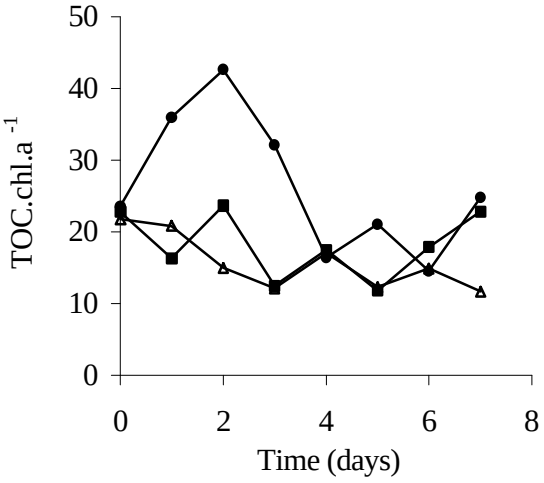


Figure 4.11. Specific organic carbon excretion in terms of chlorophyll *a* in *Spirulina* spp. grown in Zarrouk's medium under low light at 32ºC (■), 35ºC (•) and 38ºC (▵).

When grown under high light conditions, irrespective of the temperature, the cells immediately enter a logarithmic growth phase, which continues for a period of 5 days after which they enter decline phase. It is during this logarithmic phase of growth that the extracellular organic matter is excreted (Fig. 4.12). The optimal light intensity for the growth of *Spirulina* spp. has been found to be between 20 and 30 klx. (Ogawa and Terui, 1972).

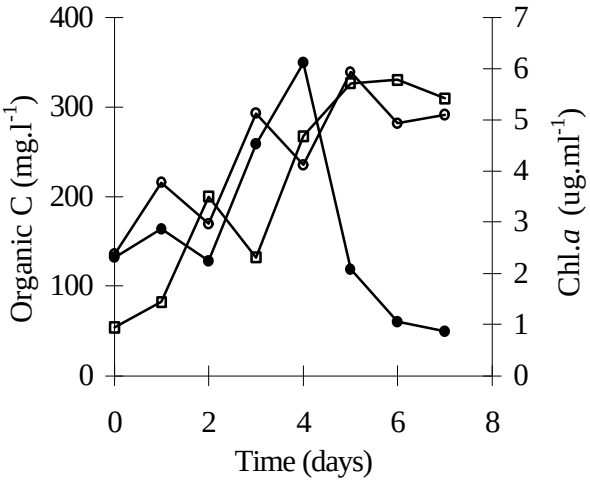


Figure 4.12. Chlorophyll *a* (♦) content of a culture of *Spirulina* spp. grown under high light at 35°C as compared to a control culture (**N**) grown at 32°C under normal light. The organic carbon content of the medium as also shown (**R**).

4.5. CONCLUSION

The effects of light and temperature on the growth of the micro-algae *Dunaliella* and *Spirulina* spp. have been demonstrated to be significant, with *Dunaliella* preferring lower temperature and *Spirulina* higher temperature levels. This in turn also affects the production and release of organic compounds into the growth environment. The growth of micro-algae in high salinity effluent for the production of biomass ideally requires a situation in which the highest growth rates can be obtained. However, this does not necessarily relate to high levels of organic carbon production. As has been seen, exposure of *Dunaliella* to high temperatures led to the release of large quantities of organic carbon, but also to the death of the cells, thus not being feasible for use in a continuous system. However, when grown at 30°C, the algae were able to produce biomass as well as extrude organic matter, more so under conditions of high light. This is in comparison

to *Spirulina* which grew best at 38°C and produced large quantities of organic carbon under low light conditions. Salinity on its own functions as a stress mechanism, decreasing growth but increasing organic carbon production.

4.6. INTEGRATED OPERATION

These results have a number of practical implications, especially for the integration of algal ponding into the sulphate reducing wastewater treatment system proposed in Chapter 1. The options demonstrated in the studies reported above could be integrated into the process described in Figure 4.13. Micro-algal biomass produced in a HRAP (2) treating overflow from the facultative pond (1) could either be harvested and fed to the anaerobic pit as a carbon source, or it could be passed into a separate HRAP (4) where it may be stressed to enhance the generation of additional organic carbon in the form of stress induced photosynthate release. The organic carbon-rich water and algal biomass could then be passed into the anaerobic pit of the facultative pond to serve as carbon source for biological sulphate reduction.

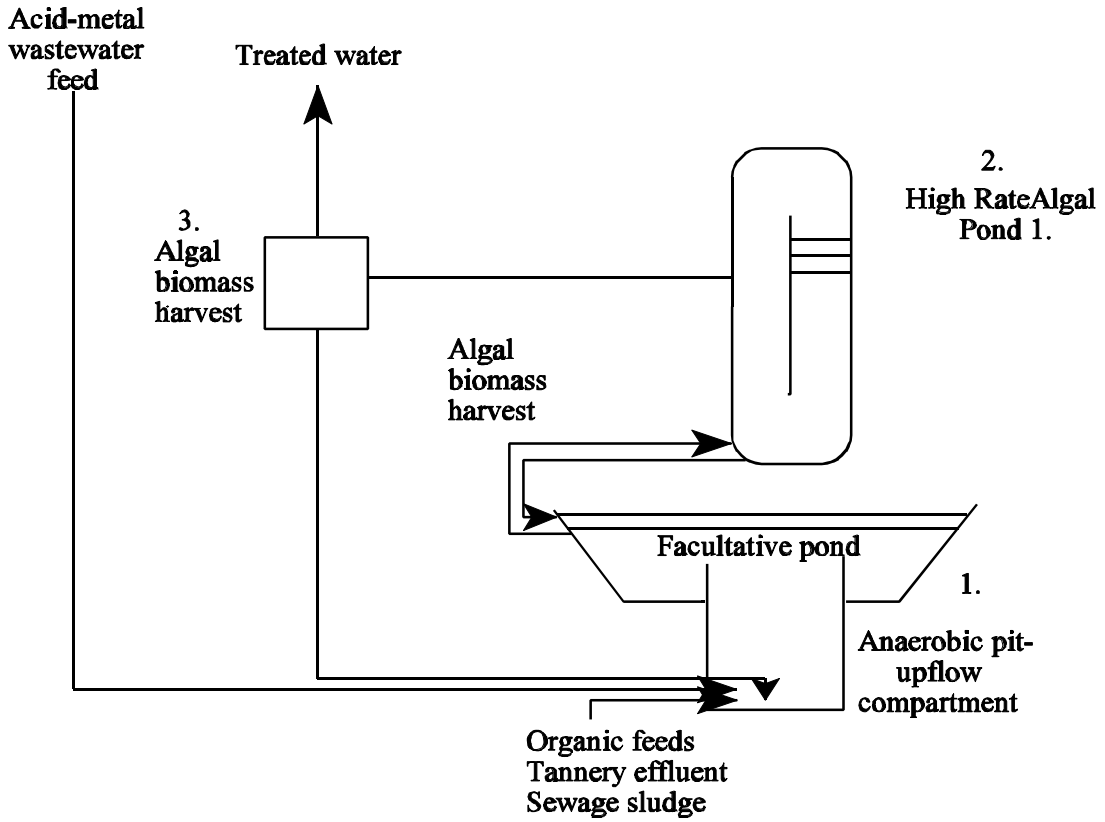


Figure 4.13. Schematic diagram of an integrated process for the treatment of acid mine drainage effluent, with the incorporation of a stress pond to enhance the organic carbon content of the micro-algal biomass.

While the results reported above established provisional feasibility for the system outlined in Figure 4.13, it is necessary to demonstrate the effect of a number of additional factors which impact on the use of the system for AMD treatment. These include the effects of sulphide concentration on the growth of the micro-algae, the capacity of the system to neutralise low pH solutions and also to effect an efficient precipitation of the heavy metals contained in these wastewaters.

CHAPTER 5. THE EFFECT OF SULPHIDE ON THE GROWTH OF *Spirulina* spp. AND *Dunaliella salina*

5.1. INTRODUCTION

The potential for algae to be utilised in biological systems for the treatment of industrial and municipal effluents has been recognised, with full-scale treatment plants in operation around the world (Oswald, 1991; Rose *et al.*, 1996). In the system proposed in Chapter 1 for the treatment of AMD, sulphate reduction takes place in the anaerobic pit of the facultative pond, generating quantities of sulphide. The residual sulphide-containing overflow from the facultative pond is fed, after metal precipitation, into a HRAP, which serves as a secondary treatment step for the oxidation of organics. An overlay of algal-rich water on the surface of the facultative pond is returned from the HRAP and serves to control odours and to affect the oxidation of escaping sulphide gas. It is thus of importance to determine the effects of sulphide on algal growth and productivity.

The growth of a number of species of cyanobacteria under reducing conditions has been recorded (Stewart and Pearson, 1970; Garlick *et al.*, 1977; Jorgensen *et al.*, 1986). They are known to occur naturally in anaerobic environments rich in sulphide (Jorgensen et *al.*, 1986), where they may form mats (Anderson *et al.*, 1987; Bender *et al.*, 1995), and are characterised by high primary productivity and rapid recycling of organic matter (Jorgensen *et al.*, 1986). The ability of the cyanobacteria *Oscillatoria limnetica* to use sulphide as an electron donor for the photo assimilation of CO_2 is well documented (Cohen *et al.*, 1975, Garlick *et al.*, 1977; Oren and Padan, 1978). However, this is not a common adaptation amongst all cyanobacteria (Cohen, 1984) and sulphide is known to exert a toxic effect on non-adapted cyanobacteria (Oren and Shilo, 1979) by inhibiting the electron transport chain (Cohen *et al.*, 1986). The species of *Spirulina* used in this study was isolated from WSP treating tannery effluent and thus had been exposed to high sulphide levels over a period of time. It was also of interest to assess the tolerance of this cyanobacteria to sulphide to ascertain whether or not it was an adapted sulphidophilic strain.

The tolerance of *Dunaliella salina* to sulphide was also monitored since these micro-algae

dominate in high salinity WSP's, a situation which will occur in the treatment of concentrated AMD solutions and other high salinity effluents. A change in the predominant micro-algal population from *Spirulina* to *Dunaliella* has also previously been noted across ponding cascades treating tannery effluent (Dunn, 1998). Therefore it is important to assess the effect of residual sulphide levels that *Dunaliella* populations are likely to encounter in the latter stages of these systems, on their growth.

5.2. RESEARCH OBJECTIVES

1. To assess the toxicity of sulphide to *Spirulina* spp. in both batch and continuous culture.
2. To assess the potential for acclimatising *Spirulina* spp. to sulphide.
3. To determine the maximum sulphide concentration that *Spirulina* spp. can tolerate.
4. To assess the toxicity of sulphide to *D. salina* in continuous culture.

5.3. MATERIALS AND METHODS

5.3.1. Algal culture and maintenance

A culture of *Spirulina* spp. was maintained at 27°C in Zarrouk's medium under a 18hr light, 6 hour dark cycle. Illumination was supplied by cold white fluorescent lights.

A culture of *D. salina* (var. bardawil Teod,) was obtained from the Culture Collection of Algae and Protozoa (CCAP 19/30). The culture was maintained at 27°C in BAAM medium under a 18 hour light, 6 hour dark cycle. Illumination was supplied by cold white fluorescent lights.

5.3.2. Experimental

5.3.2.1. Organic-rich medium

Spirulina spp. cells in the logarithmic phase of growth were harvested by filtration through a nylon mesh with a pore size of 100 µm. They were resuspended in 150 ml Zarrouk's medium supplemented with 66% v/v, 33%v/v and 14%v/v sulphide-rich overflow from a sulphate reducing UASB reactor treating tannery effluent. A representative analysis of this overflow, which is referred to here as organic-rich medium, can be seen in Figure 5.1. Samples were removed daily and analysed for chlorophyll *a*, as an indicator of micro-algal growth. Control cultures were

grown in Zarrouk's medium.

A 50 litre laboratory-scale HRAP containing Zarrouk's medium was inoculated with a culture of *Spirulina* spp. The HRAP was operated on a continuous basis with organic rich medium being fed into the algal pond daily. Samples were removed and analysed for sulphide and chlorophyll *a*. A control culture was grown in, and fed an equivalent volume of Zarrouk's medium daily.

Table 5.1. Representative analysis of overflow from the sulphate reducing UASB reactor treating tannery effluent.

Water Quality Parameter	Measured
pH	7.95
Phosphate ($mg.l^{-1}$)	36.42
Nitrate ($mg.l^{-1}$)	16.18
Ammonia ($mg.l^{-1}$)	0.57
Chemical Oxygen Demand (COD) ($mg.l^{-1}$)	2237
Sulphate ($mg.l^{-1}$)	471
Sulphide ($mg.l^{-1}$)	1029

5.3.2.2. Defined medium

Spirulina spp. cells in the logarithmic phase of growth were harvested by filtration through a nylon mesh with a pore size of 100 µm. They were resuspended in 150 ml Zarrouk's medium supplemented with sodium sulphide. Samples were removed daily and analysed for chlorophyll *a*. Control cultures were grown in Zarrouk's medium without sulphide.

Spirulina spp. cells in the logarithmic phase of growth were harvested by filtration through a nylon mesh with a pore size of 100 µm. They were resuspended in 100 ml Zarrouk's medium supplemented with increasing concentrations of sodium sulphide. *D. salina* cells in the logarithmic phase of growth were centrifuged at 4420g x 10 minutes. The pellets were resuspended in 100 ml BAAM medium supplemented with increasing concentrations of sodium sulphide. The cultures were operated on a continuous basis and fed either sulphide- supplemented Zarrouk's or BAAM medium at rates of 10%, 20% and 30% $v/v.day^{-1}$. Samples were removed and analysed

for sulphide and chlorophyll *a*. Control cultures were grown in Zarrouk's medium and fed an equivalent volume of Zarrouk's medium without sulphide daily.

5.3.4. Analysis

Chlorophyll was extracted into acetone and quantified according to Lichtenhaler (1987). Sulphide was analysed by the Methylene Blue method according to Standard Methods (APHA, 1989).

5.4. RESULTS AND DISCUSSION

5.4.1. *Spirulina* spp.

Initial experiments were carried out to determine if *Spirulina* spp. could grow on sulphide-rich organic medium. As can be seen in Figure 5.1, the chlorophyll *a* content of the alga after a 6 day period was highest in the cultures with the highest initial concentration of organic rich medium. A plot of specific growth rate (μ), as determined according to Middelbeek *et al*. (1992), versus sulphide concentration, shows an increase in μ with increasing sulphide levels (Fig. 5.2) This indicates that the organic-rich medium gives the algal cells a competitive advantage over those grown in defined medium, even in the presence of sulphide.

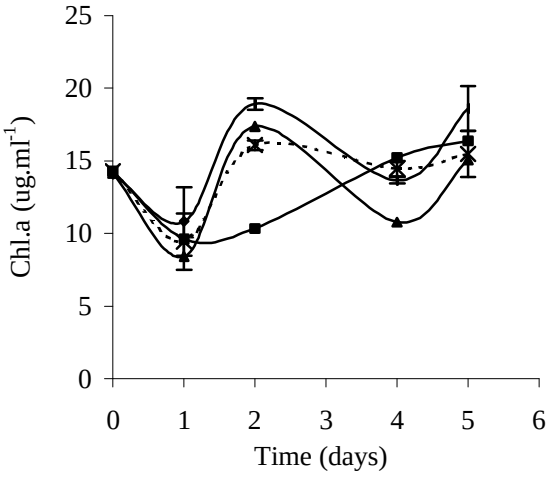


Figure 5.1. Chlorophyll *a* profile of *Spirulina* spp. grown in Zarrouk's medium supplemented with sulphide-rich organic medium so as to give a final sulphide concentration of 2.3 $mg.l^{-1}$ (**P**), 0.96 $mg.l^{-1}$ (▲) and 0.616 $mg.l^{-1}$ (♦) as compared to the control (×) grown in Zarrouk's medium without sulphide.

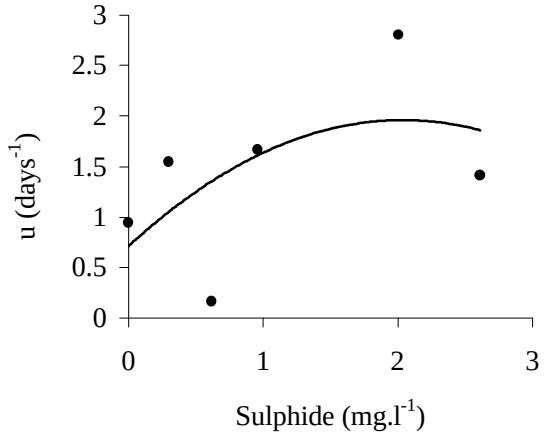


Figure 5.2. Specific growth rate (μ) of *Spirulina* spp. cultures grown in Zarrouk's medium supplemented with sulphide-rich organic medium.

Following the above results it was necessary to assess the growth of *Spirulina* spp. in defined medium supplemented with sulphide in order to ascertain the effect of sulphide on algal growth without the interference of organics. The sulphide concentration in the organic-rich medium was relatively low as the experiment was carried out using overflow liquor from a sulphate reducing digester which was in the initial phase of commissioning. It was thus decided to increase sulphide in the defined medium experiment to determine the level at which sulphide begins to have an effect on the growth rate.

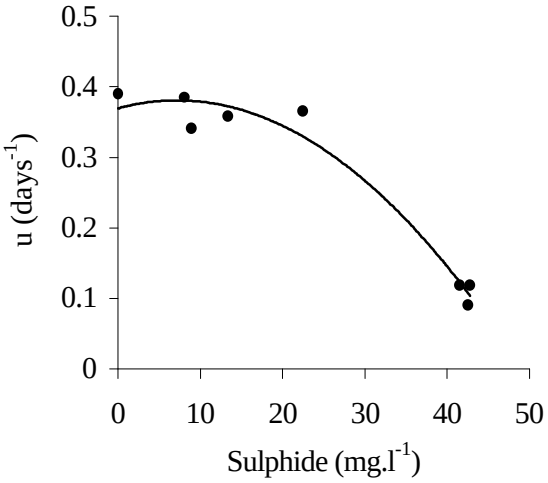


Figure 5.3. Specific growth rate (μ) of *Spirulina* spp. cultures grown in Zarrouk's medium supplemented with sulphide.

The growth rate of *Spirulina* grown in batch culture in Zarrouk's medium supplemented with Na_2S remained relatively constant at μ=0.34-0.4 day^{-1} until a sulphide concentration of 25 $mg.l^{-1}$ had been reached, after which growth declined (Fig. 5.3).

It was observed that the sulphide levels in the batch cultures declined over a 24 hour period. A continuous experiment was undertaken to monitor the growth of *Spirulina* spp. cultures over a period of 4 days in an environment with a constant level of sulphide. The results can be seen in Figure 5.4 A to F.

At daily adjusted sulphide levels between 95 $mg.l^{-1}$ and 190 $mg.l^{-1}$ the growth of the experimental and control cultures was comparable. At sulphide concentrations above 190 $mg.l^{-1}$, there is a marked decline in the chlorophyll *a* content of the experimental cultures, compared to the controls, with the cultures immediately entering decline phase. The rate of decline increases with increasing sulphide levels and does not appear to be affected by the dilution rate.

An important factor to note is the oxidation of sulphide which occurs between addition of the sulphide and sampling of the experiment for sulphide analysis, a period of approximately 10 minutes. During this period, the level of sulphide drops considerably, but more so the higher the levels of sulphide added. The sulphide level also declines during the day, with the residual level after 24 hours varying according to the initial sulphide levels. The highest residual level after a 24 hour period was recorded in the culture with the highest initial sulphide concentration. These experiments were conducted at pH 9-10. At these pHs very little of the sulphide is in the gaseous form. Thus the majority of sulphide loss must be due to the operation of oxidation mechanisms.

However, the chlorophyll *a* levels of the experimental cultures also decline as the residual sulphide concentration increases after 24 hours. This is also more noticeable at initial sulphide concentrations higher than 190 $mg.l^{-1}$. This indicates the presence of a biological component and possibly the operation of a sulphide detoxification mechanism. However, this requires further investigation and is the subject of follow-up studies.

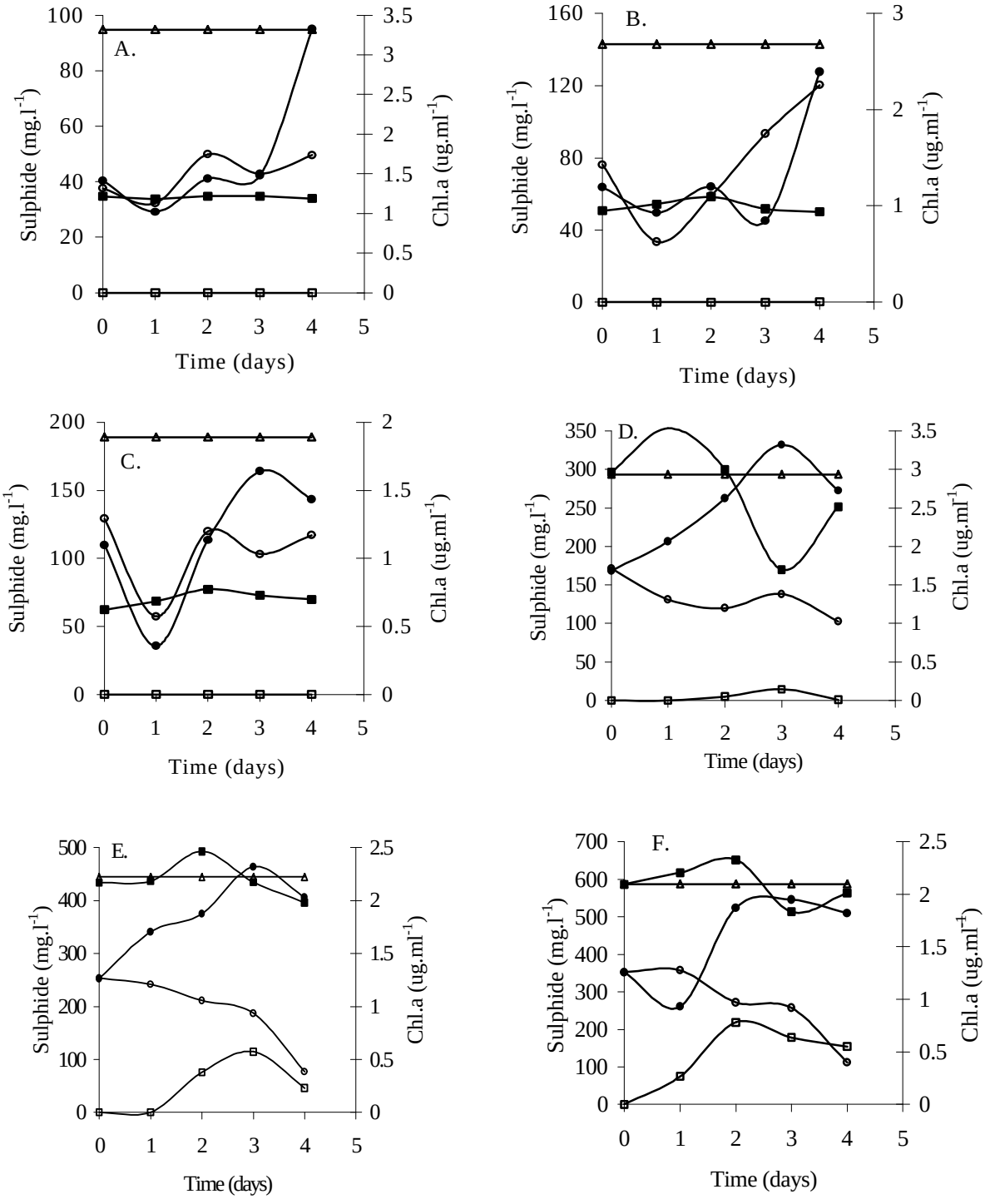


Figure 5.4. Chlorophyll *a* profile of *Spirulina* spp. grown in defined medium supplemented daily with A) 95 mg.l^{-1}, B) 145 mg.l^{-1}, C) 190 mg.l^{-1}, D) 295 mg.l^{-1}, E) 445 mg.l^{-1} and F) 590 mg.l^{-1} sulphide. Chlorophyll *a* of experimental cultures (**N**), chlorophyll *a* of control cultures (•), actual sulphide added (△), residual sulphide concentration 10 minutes after feeding (**P**), residual sulphide concentration 24 hours after feeding (**R**).

A plot of the specific growth rate (μ) against sulphide concentration shows the control cultures to have higher specific growth rates compared to the experimental, irrespective of the sulphide concentration (Fig. 5.5). When the sulphide concentration reached 190 $mg.l^{-1}$, the growth rate of the experimental cultures began to decline, reaching zero at a sulphide concentration of 290 $mg.l^{-1}$.

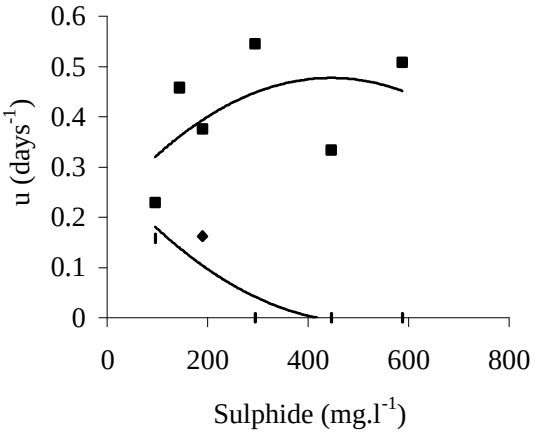


Figure 5.5. Specific growth rate (μ) of *Spirulina* spp. cultures fed defined medium supplemented with sulphide daily (♦) and control cultures fed defined medium only (**P**).

From a practical point of view it is necessary to determine if *Spirulina* spp. can be acclimated to higher levels of sulphide. An experiment was set up in which a culture of *Spirulina* spp. was fed Zarrouk's medium supplemented with sulphide. The level of sulphide was increased over a period of days. The cultures were operated as a batch-fed continuous system, with a set volume of medium and cells being removed every 24 hours and replaced with fresh medium supplemented with sulphide. The feed rate of the cultures was also varied to select for a fast-growing strain able to tolerate high sulphide levels.

In cultures fed at a rate of 20% $v/v.day^{-1}$, the sulphide concentration in the medium increased to 450 $mg.l^{-1}$ (Fig. 5.6A). There was very little difference between the chlorophyll *a* levels of the control and experimental cultures.

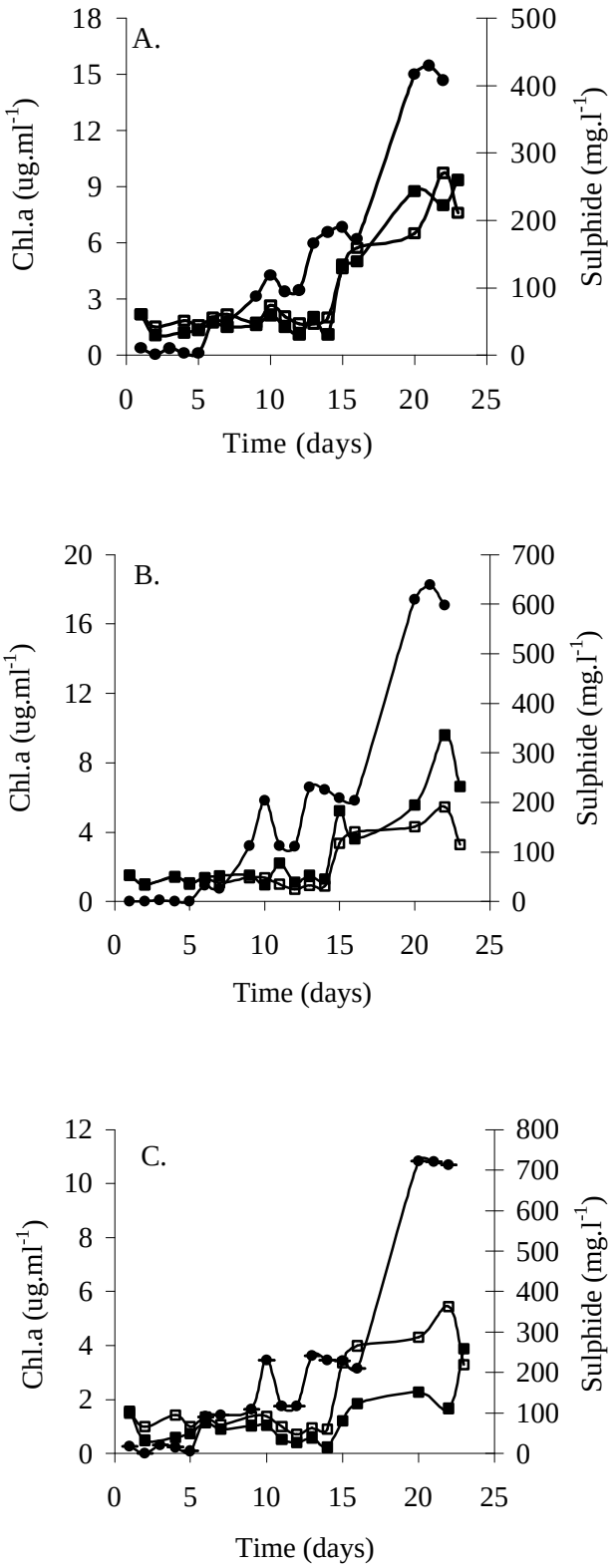


Figure 5.6. Chlorophyll *a* profile of *Spirulina* spp. cultures fed defined medium supplemented with sulphide daily at a rate of A) 20% v/v.day^{-1}, B) 30% v/v.day^{-1} and C) 40% v/v.day^{-1} (**R**) compared to the control cultures fed unsupplemented medium (**P**). The sulphide concentrations (•) were increased to acclimatise the micro-algae to the sulphide.

At a feed rate of 30% v/v.day^{-1}, the chlorophyll *a* level of the control was slightly higher than that of the experimental culture (Fig. 5.6B). However, in the cultures fed at a rate of 40%v/v.day^{-1}, the experimental cultures were able to out compete the control cultures (Fig. 5.6C).

It must be noted that the chlorophyll *a* levels of both the experimental and control cultures remained constant up to day 15, after which they increased. This would indicate that up until day 15 washout conditions applied to non-adapted cells, leading to the selection of a population of adapted cells with faster growth rates, which came into predominance from day 15 onwards. In Figure 5.6C it is also evident that a population of cells which were both tolerant to sulphide and fast growing was selected for at a feed rate of 40% v/v.day^{-1}, as the experimental cultures were able to out compete the control cultures, in terms of chlorophyll *a* levels.

Aspects of the above experiment were scaled up to ascertain the effect of adding organic-rich sulphide medium to a micro-algal culture over a long period. The daily addition of sulphide-rich digester overflow liquor leads to a decline in the chlorophyll *a* content of the experimental culture compared to the control (Fig. 5.7). However, after initial decline the chlorophyll *a* levels are relatively constant irrespective of the increase in sulphide to 150 mg.l^{-1}, which has been shown to be toxic to cyanobacteria. The micro-algae are also not able to completely oxidise the sulphide, with a steady accumulation in the background levels occurring.

The decline in chlorophyll *a* levels of *Spirulina* spp. exposed to sulphide seen in these experiments, indicates that the photosynthetic process is vulnerable to sulphide. High sulphide concentrations have previously been shown to have an adverse effect on micro-algal growth and can limit speciation (Pinheiro *et al.*,1987; Mara and Pearson, 1986). Sulphide is a known inhibitor of the metabolic pathway components of numerous organisms (Castenholtz, 1976). It acts as an inhibitor of catalases, peroxidases, succinic dehydrogenase, carbonic anhydrase, cytochrome oxidase and others, and also tends to combine with the iron of hemes (Evans, 1967), thus inhibiting the electron transport chain (Cohen *et al.*, 1986). Cohen *et al.* (1975) found that sulphide levels of around 9.6 mg.l^{-1} inhibited Photosystem 1 driven photo assimilation reactions.

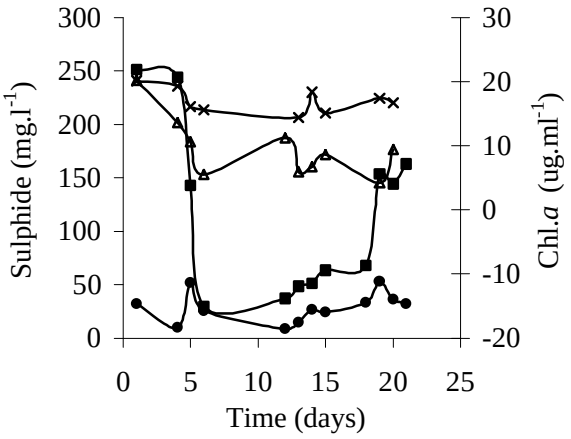


Figure 5.7. Chlorophyll *a* profile of *Spirulina* spp. fed organic-rich medium daily (△) as compared to the control culture fed defined medium daily (×). The residual sulphide concentration 30 minutes after feeding (**P**) and 24 hours after feeding (•) are shown.

The reduction in pigment content due to the presence of sulphide leads to considerable conservation of energy that may be directed toward the biosynthesis of other cell constituents (Wyman and Fay, 1987) or towards overcoming toxicity.

Water soluble H_2S, which is in a pH-dependent equilibrium with sulphide, is considered to be the toxic component. The undissociated form of H_2S is able to penetrate passively into the cell, following diffusion laws, across both the cytoplasmic and chloroplast membranes (Howsley and Pearson, 1979). It is responsible for sulphide toxicity to both Photosystem I (PS-I) and Photosystem II (PS-II). The ionized species of HS^- and S^{2-}, on the other hand, require active transport to penetrate the cell, as has been indicated by Howsley and Pearson (1979). This would explain why Almasi and Pescod (1996) found sulphide toxicity to algal cells to be greater at high temperatures and low pH. At the pH's utilised in these experiments (pH 8.5-10), H_2S would only constitute between 0.19% and 2% of the dissolved sulphide concentration, as determined from the equation of Oleskiewicz *et al*. (1989). Inhibition of the cultures, as indicated by a decline in chlorophyll *a*, fed Na_2S supplemented Zarrouk's on a daily basis, occurred at H_2S levels of 3.8 $mg.l^{-1}$ and above. However, in cultures which were acclimated to sulphide these levels increased to 14 $mg.l^{-1}$ H_2S, and in the continuous culture fed digester overflow, 5 $mg.l^{-1}$ H_2S. Gyure *et al.*

(1987) found that the photosynthesis of an algal population in the zone of H_2S production in an acid strip mine lake was severely inhibited by H_2S concentrations of 0.19 $mg.l^{-1}$. *Anacystis nidulans,* on the other hand, exhibited complete inhibition at H_2S levels of 1.9 $mg.l^{-1}$, while Abeliovich (1980) found that 0.48 $mg.l^{-1}$ hydrogen sulphide led to a decrease in photoassimilation of CO_2 by pond algae. However, similar levels of total sulphide (between 1.15 $mg.l^{-1}$ and 3.20 $mg.l^{-1}$) were found to be inhibitory to photosynthesis in three cyanobacterial isolates at pH 8 (Howsley and Pearson,1979). Thus the level and form of sulphide which is toxic appears to vary between micro-algal and cyanobacterial species.

Cyanobacteria are oxygenic phototrophs which exhibit four different types of adaptions to sulphide based on the differential toxicity of sulphide to PS-II and PS-I, and the ability to carry out anoxygenic photosynthesis (Cohen *et al.,* 1986). In anoxygenic photosynthesis sulphide replaces water as the ultimate electron donor (Howsley and Pearson, 1979), with the production of sulphur. This involves PS I activity only and thus no oxygen is evolved (Cohen *et al.,* 1975). Although the ability to carry out anoxygenic photosynthesis has been demonstrated in a range of cyanobacteria, very little information is available for *Spirulina*. However, *Spirulina labyrinthioformis* is known to exhibits anoxygenic photosynthesis *in situ* at a sulphide concentration of 1mM (Castenholtz, 1976). Sulphur was absent from the medium in these experiments, suggesting that anoxygenic photosynthesis was not part of the mechanism involved in coping with the sulphide stress. Cyanobacteria carrying out anoxygenic photosynthesis require a period of adaption, during which no photosynthetic activity occurs, before sulphide can be utilised (Oren and Padan, 1978). Thus it would be advantageous for them to be able to carry out oxygenic photosynthesis in the presence of sulphides. This would not only give them a competitive advantage, but the additional oxygen produced would allow for the oxidative removal of sulphide from the environment (Howsley and Pearson, 1979). A species of *Spirulina* growing in a hot spring has been found to evolve oxygen at sulphide concentrations in the range of 0.9-1.9 $mg.l^{-1}$ (Castenholtz, 1976).

5.4.2. *Dunaliella*

Figure 5.8 show plots of chlorophyll *a* as a function of time for *D. salina* cultures fed sulphide supplemented BAAM media on a daily basis.

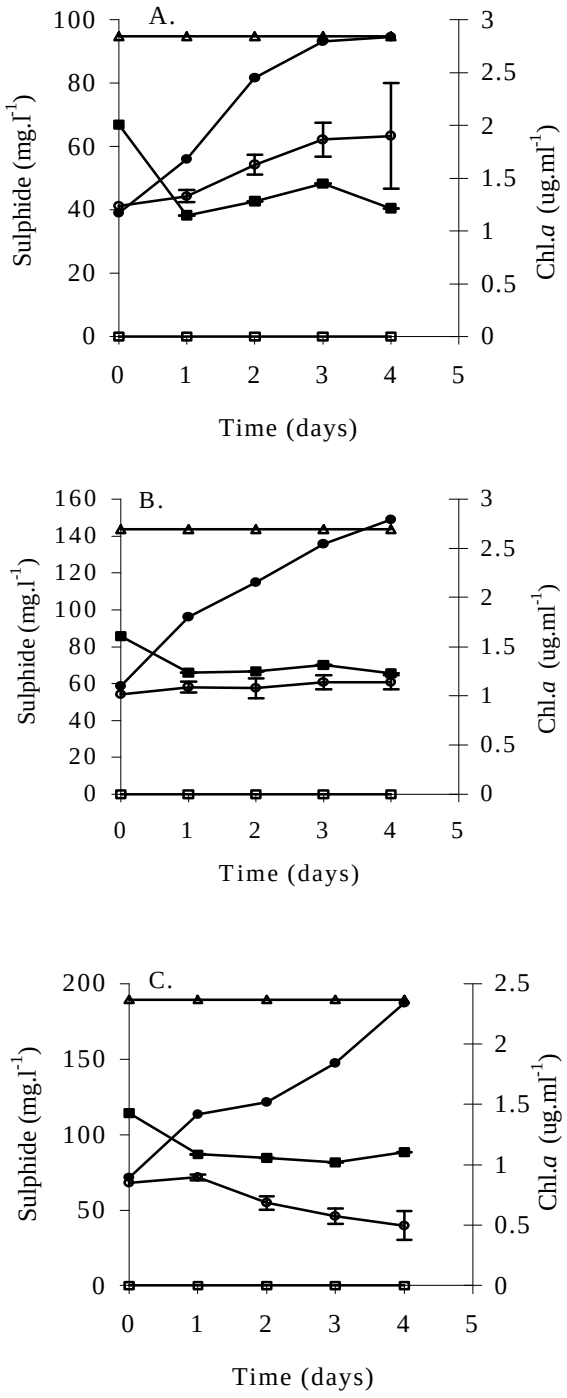


Figure 5.8. Chlorophyll *a* profile of *D. salina* grown in BAAM medium supplemented daily with A) 95 mg.l^{-1}, B) 145 mg.l $^{-1}$ and C) 190 mg.l^{-1} sulphide. Chlorophyll *a* of experimental cultures (**N**), chlorophyll *a* of control cultures (•), actual sulphide added (△), residual sulphide concentration 10 minutes after feeding (**P**), residual sulphide concentration 24 hours after feeding (**R**).

At a sulphide concentrations below 95 mg.l^{-1}, the experimental cultures are able to grow, although their chlorophyll *a* levels are much lower than that of the control cultures. At a sulphide concentration of 145 mg.l^{-1}, the culture stops growing and remains in stationary phase. As the sulphide concentration increases to 190 mg.l^{-1}, the cells enter decline phase after a period of 1 day. This is in contrast to the *Spirulina* spp. cultures where the chlorophyll *a* levels of the experimental culture were the same as that of the control up to a sulphide concentration of 190 mg.l^{-1}. Thus it would appear that the *Spirulina* spp. used in this study is more tolerant to sulphide than *D. salina*. A plot of the specific growth rate (μ) against sulphide concentration shows the control cultures to have much higher growth rates that the experimental (Fig. 5.9).

Higher levels of sulphide would most likely not occur in ponds in the latter stages of treatment, thus negating the need to assay the effect of higher sulphide levels on *D. salina* growth.

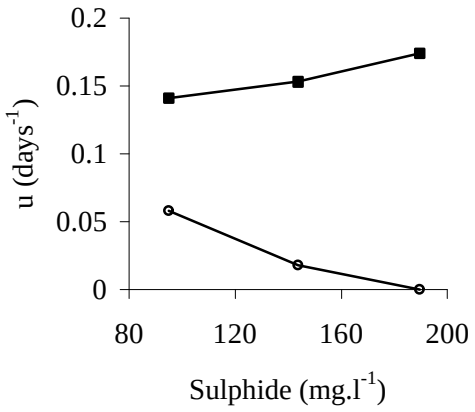


Figure 5.9. Specific growth rate (μ) of cultures of *D. salina* fed BAAM medium supplemented with different concentrations of sulphide (**N**) as compared to control cultures (**P**) fed BAAM medium without sulphide.

5.5. CONCLUSION

These results show that *Spirulina* spp. is able to adapt to high levels of sulphide over a period of time. This is very important if one considers the concentration of sulphide that would be produced in facultative ponds treating AMD. Rose *et al.* (1996) reported effluent sulphide levels

of 1065 mg.l^{-1} entering a ponding system treating tannery effluent. Rose *et al*. (1998a) measured sulphide levels of up to 1800 mg.l $^{-1}$ from the outflow of an anaerobic digester treating tannery effluent. *Spirulina* spp. also appears to play a role in reducing sulphide levels in it surrounding environment. The mechanism involved is under further investigation.

D. salina is less resistant to sulphide than *Spirulina* spp., which may primarily be due to the lack of a cell wall thus making it easier for the hydrogen sulphide to pass into the cell. With the manipulation of sulphide levels in the algal ponds, possibly the control of the residence time, it is possible to utilise algal systems for the secondary treatment of sulphide-rich digester overflow. However, the products of sulphide oxidation may need to be determined.

CHAPTER 6. THE ALGAL MEDIATED ELEVATION OF pH

6.1 INTRODUCTION

The role of micro-algae in algal wastewater treatment ponds is primarily to facilitate the removal or breakdown of soluble Biological Oxygen Demand (BOD). This is facilitated by the release of oxygen by the micro-algae and is termed photosynthetic oxygenation (Oswald, 1988). Oswald (1988) reported levels of oxygen production up to 540 $kg.ha^{-1}.day^{-1}$. In the Advanced Integrated Algal-Bacterial Systems developed by Oswald (1990), the micro-algae also have a role to play in odour control, with highly oxygenated water from a HRAP being recycled onto the top of the facultative pond to oxidise malodorous compounds and residual sulphide. Chapter 5 shows that micro-algae are able to survive and grow in the presence of sulphide and thus have a role to play in the sulphide oxidising function.

Micro-algae have a further ability, which up until now has not been utilised in wastewater treatment. Cyanobacteria and micro-algae possess mechanisms for actively acquiring inorganic carbon from the external media which leads to the elevation of the pH of the surrounding environment (Borowitzka, 1982). This mechanism elevates the CO_2 concentration around the active site of the primary photosynthetic carboxylating enzyme, ribulose biphosphate (Rubisco) (Badger and Price, 1992), to levels higher than can be obtained by simple diffusion, thus enabling micro-algae to utilise both CO_2 and HCO_3^- as the substrates for transport and to grow at very low CO_2 concentrations (Ramazanov *et al.*, 1996). In the case of cyanobacteria, in alkaline conditions it is HCO_3^- transport that largely serves to support the supply of CO_2 for photosynthesis (Miller *et al.*, 1990; Reinhold *et al.*, 1991) (Fig. 6.1). Micro-algae are more complicated as the active uptake of CO_2 and HCO_3^- may occur at both the plasma membrane and chloroplast envelope (Badger and Price, 1992). The increase in the pH of the media seems to be due to the rapid conversion of extracellular HCO_3^- to CO_2 and OH^- either by carbonic anhydrase (CA) or a carbonic anhydrase like moiety (Shiraiwa *et al.*, 1993). When the CO_2 is rapidly removed, the OH^- ion is left in solution where it combines with H^+ ions, to reduce acidity (Loewenthal, pers. comm.).

Thus algae may be used as a source of alkalinity in addition to that generated by sulphate reducing

bacteria and can play a role in the neutralisation of acid mine drainage. This is important as sulphate reducing bacteria prefer an environment around pH 7 and are usually inhibited at pH values lower than 6 or higher than 9 (Widdel, 1988) and thus form a crucial part of the integrated process.

The micro-algae chosen as the mediator of the process must have the ability to survive in the effluent, thereby making it a viable and continuous process. Thus it was of importance to consider not only the alkalising function[1] of the micro-algae, but also their growth in the effluent concerned, and its effect on productivity.

6.2. RESEARCH OBJECTIVES

1. To assess the ability of a range of micro-algal species to elevate the pH of defined media and industrial effluents.
2. To assess the ability of a range of micro-algae to grow in industrial effluents.

6.3. MATERIALS AND METHODS

6.3.1. Micro-algal cultures

The following micro-algae were assessed for their ability to elevate the pH of their surrounding environment: *Dunaliella salina*, *Spirulina* spp. and *Anacystis* spp. The culture of *Anacystis* was isolated from acid mine drainage effluent from an abandoned coal mine. Axenic stock cultures of *Anacystis* and *D. salina* were grown in 1% w/v NaCl BAAM and BAAM respectively. A stock culture of *Spirulina* spp. was grown in Zarrouk's medium. The cultures were grown at 27^{o}C and maintained under a 16hr light 8hr dark cycle, illuminated by a cold white fluorescent light.

6.3.2. Experimental

Cultures of *D. salina* and *Anacystis* in the logarithmic phase of growth were harvested by centrifugation, while cultures of *Spirulina* spp. were harvested by filtration through a nylon mesh

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1. Alkalising function refers to the ability of the algae to elevate the pH of their surrounding environment by the conversion of HCO_3^- in solution to OH^- and CO_2, and does not involve the production of net alkalinity.

with a pore size of 100 µm and resuspended in the media of interest. The pH of the media was adjusted using either NaOH or HCl and the pH measured at time intervals. The effect of the external carbonic anhydrase inhibitor acetozolamide was assayed for by resuspending a culture of *Spirulina* spp. in AMD containing 100 µM acetozolamide. The pH was measured at time intervals.

6.3.3. Analysis

The pH was measured on a Cyberscan 1500 pH meter. Inorganic carbon was analysed on a Dohrmann 180 Total Organic Carbon Analyser. Chlorophyll was extracted into acetone and quantified according to the method of Lichtenhaler (1987).

6.4. RESULTS AND DISCUSSION

6.4.1. *Spirulina* spp.

Initial experiments were carried out to determine whether *Spirulina* spp. could elevate the pH of raw AMD effluent. Low CO_2 grown [2] *Spirulina* spp. algal cells were suspended in acid mine drainage effluent from Grootvlei mine, with an initial pH of 3.1. and illuminated. Analysis of the effluent indicated the absence of inorganic carbon (Ci). No biological alkalisation response was observed, irrespective of whether or not the pH was artificially elevated to 3.5 or 4.5 with NaOH (Figure 6.1). Similar results were obtained for high CO_2 grown[3] *Spirulina* spp. cells (results not shown).

However, with the addition of a small amount of bicarbonate an elevation in pH was observed (Fig. 6.2).

A control in which bicarbonate was added to the acid mine drainage effluent without algae was performed in order to assess the effect of purely bicarbonate addition on the pH of the effluent. As can be seen in Figure 6.3., an increase in pH with the addition of a bicarbonate solution was

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[2]. The term low CO_2 grown cells refers to growth in medium rich in bicarbonate.

[3]. The term high CO_2 grown cells refers to growth in medium deficient in inorganic carbon other than CO_2.

observed. However, the pH elevation observed in the presence of the algae was greater than can be attributed to purely the presence of bicarbonate ions.

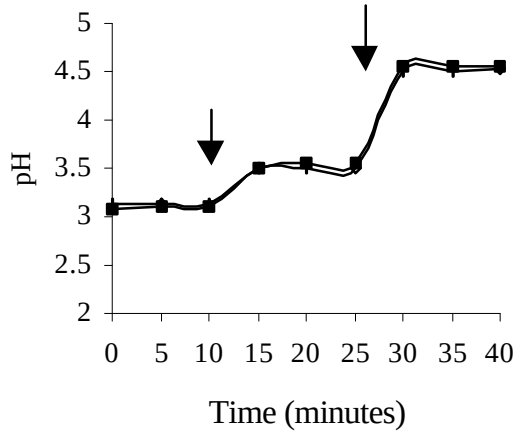

Figure 6.1. A pH profile of *Spirulina* spp. in AMD effluent under illumination (■) and in the dark (◊). The arrows indicate the points at which the pH was artificially elevated by the addition of NaOH.

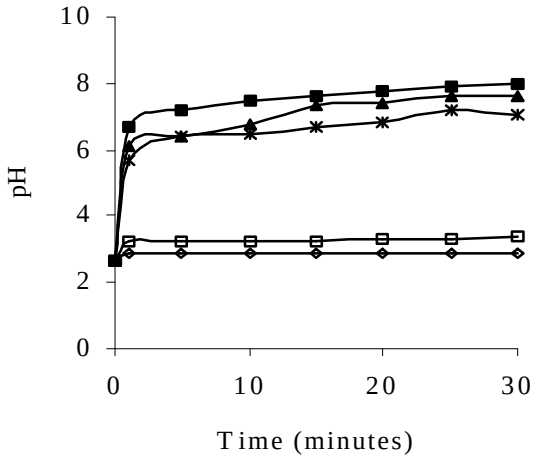

Figure 6.2. A pH profile of *Spirulina* spp. in AMD effluent after addition of 500 µmoles (■), 250 µmoles (▲), 150 µmoles (×), 100 µmoles (□) and 50 µmoles (◊) $NaHCO_3$ to the effluent.

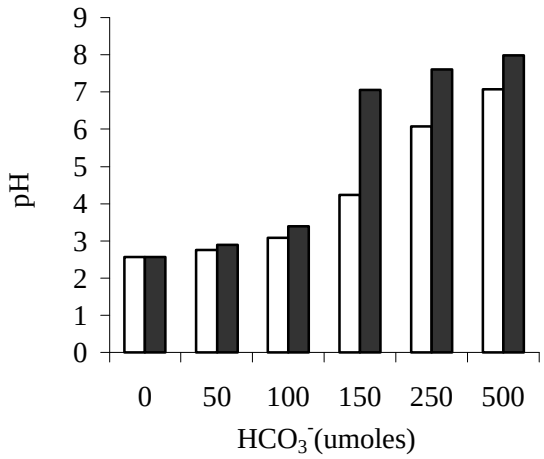

Figure 6.3. Elevation of pH of AMD effluent by the addition of increasing concentrations of $NaHCO_3$ in the presence (**O**) and absence (**G**) of *Spirulina* spp.

From this it can be seen that a minimum initial bicarbonate level is required for the algal- mediated elevation of pH to occur, with this value ranging between 100-150 µmoles HCO_3^-.

The supply of CO_2 to phytoplankton cells is limited by molecular diffusion through the unstirred layer surrounding cells in aqueous media and the rates of uncatalysed dehydration of bicarbonate (Riebesell *et al.*, 1993). The carbonic species together with the OH^- and H^+ ions of the water exist in a state of dynamic equilibrium described by the following reactions:

$CO_2 + H_2O \rightleftarrows H_2CO_3$ ---------- 1.

$H_2CO_3 \rightleftarrows H^+ + HCO_3^-$ ---------- 2.

$HCO_3^- \rightleftarrows H^+ + CO_3^{2-}$ ---------- 3.

$H_2O \rightleftarrows H + OH^-$ ---------- 4.

As can be seen in Figure 6.4, pH is a major determinant of the relative concentrations of these species in the water and therefore affects the availability of carbon for micro-algal photosynthesis (Azov, 1982).

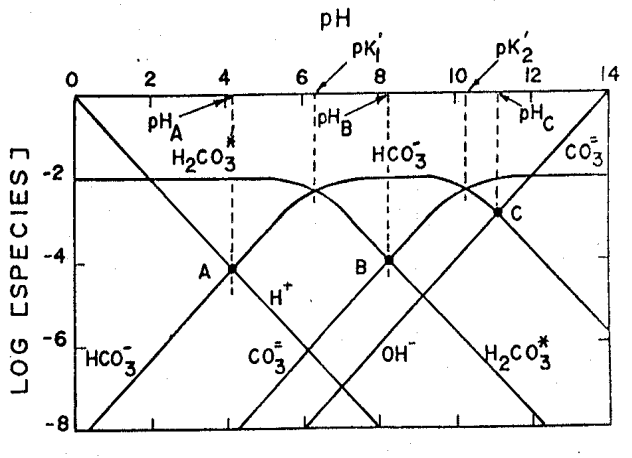

Figure 6.4. Distribution of carbonic species with pH.

At pH1, virtually all dissolved inorganic carbon (DIC) is in the form of CO_2 or H_2CO_3, whereas HCO_3^- is completely absent (Geib *et al.*, 1996). In the case of the acid mine drainage effluent used in this study, no DIC is present and thus the cyanobacteria are dependent on atmospheric CO_2 to supply their Ci needs. CO_2 exchange between a water body and a gas phase (air) depends on the difference in partial pressure of CO_2 between the water and air and mixing conditions in the water body. In waters with a high level of eutrophication there are large diurnal fluctuations in pH due to photosynthesis and respiration by algae and other plants. Thus a driving force is established for CO_2 absorption from the air during the daylight. Under low Ci conditions, active transport of CO_2 across the cell membrane occurs, with no need for the presence of an external CA and therefore no elevation of the pH.

Spirulina spp. are a micro-algae whose habitat is characterised by high alkalinity and pH. In order for *Spirulina* spp. to be used in a biological treatment system for the elevation of pH, it must be able to grow in the effluent. To assess this we suspended micro-algal cells in acid mine drainage effluent at different pH's and monitored the change in pH of the effluent (Fig. 6.5) and growth of the micro-algae (Fig. 6.6) over a period of days. It was decided to use a pH of 3, 4 and 5 as pH 4 is the cross-over point for a decrease in H^+ concentration and an increase in HCO_3^- concentration (Fig. 6.4), while pH 3 and 5 lie on either side of it.

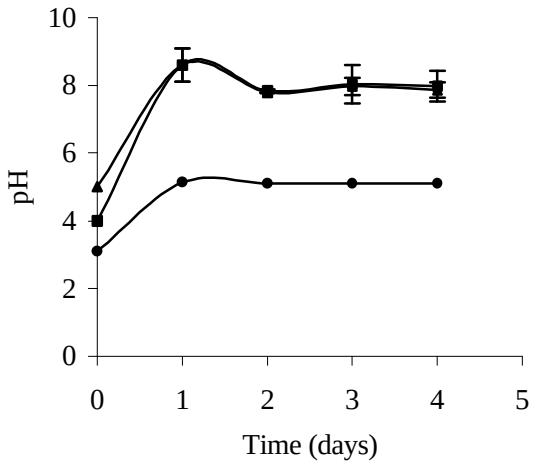

Figure 6.5. Elevation of pH of AMD by *Spirulina* spp. from an initial pH of 3 (•), 4 (■) and 5(▲).

The micro-algae suspended in acid mine drainage with a pH of 3 were unable to grow and rapidly entered decline phase. However, they were able to elevate the pH of the effluent to 5. Those placed into acid mine drainage at pH of 4 and 5 managed to overcome any inhibitory effects and were able to grow, showing a characteristic log phase and decline phase of growth (Fig. 6.6) They were also able to elevate the pH of the effluent to pH 8 and above. The initial increase in pH occurred in a matter of seconds and appears to be related to the biomass concentration, expressed here as µg chl.*a*.ml^{-1} of culture, with higher chlorophyll *a* levels corresponding to larger pH changes (Fig. 6.7).

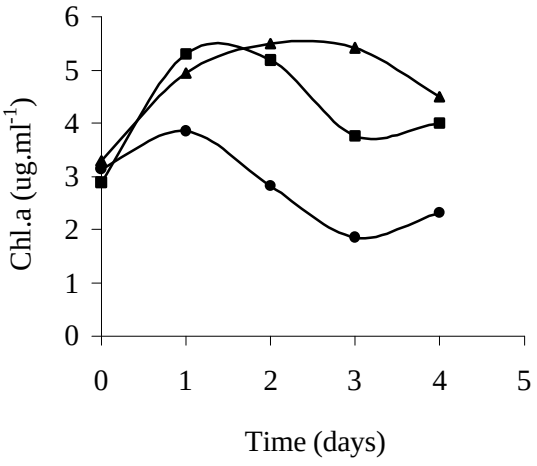

Figure 6.6. Chlorophyll *a* profile of *Spirulina* spp. grown in AMD with an initial pH of 3 (•), 4 (■) and 5 (▲).

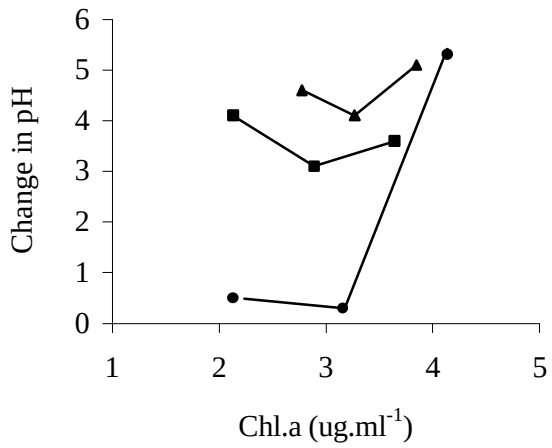

Figure 6.7. Change in pH of AMD from an initial pH of 3 (•), 4 (**P**) and 5 (▲) in relation to the chlorophyll *a* concentration of cultures of *Spirulina* spp.

Assays were carried out to measure the effect of the CA inhibitor acetozolamide (AZ) on the elevation of the pH of acid mine water. The *in vivo* effects of CA inhibitors depend on their lipid solubility (Maren, 1984). AZ is not very lipid soluble and is unable to penetrate the plasma membrane of intact cells and thus only inhibits the external carbonic anhydrase. As can be seen in Figure 6.8, cultures in the presence of AZ were able to elevate the pH, but to a lesser extent than those in the absence of AZ. This may be due to the AZ levels not being high enough to mediate complete inhibition of CA or it may indicate that not all of the pH elevation can be attributed to CA.

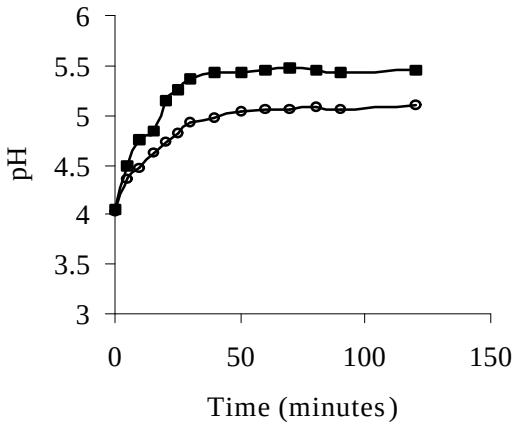

Figure 6.8. Elevation of pH of AMD effluent by *Spirulina* spp. in the presence (**N**) and absence (**P**) of acetozolamide.

Up until now it has been shown that *Spirulina* spp. has the ability to elevate the pH of acid mine drainage effluents. However, the potential use of this biological alkalisation function for the treatment of other acidic effluents also needed to be determined. The ability of *Spirulina* spp. to elevate the pH of an effluent from a zinc refinery was thus assayed. No biological alkalisation function was observed at pH 2.5. The addition to the effluent of $NaHCO_3$ and macro-nutrients such as KNO_3, H_3PO_4 and K_2SO_4, which are required for micro-algal growth, also did not lead to an elevation in pH (Fig. 6.9). However, when the effluent was neutralised, the algae were able to increase the pH to values above 10 (Fig. 6.10).

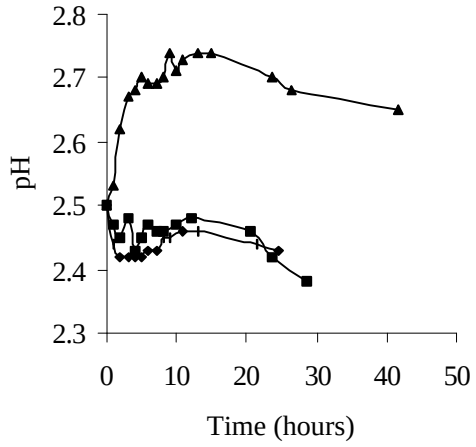

Figure 6.9. Elevation of pH of zinc refinery effluent supplemented with HCO_3 (**P**), macro-nutrients (▲) and with no supplementation (♦) by *Spirulina* spp.

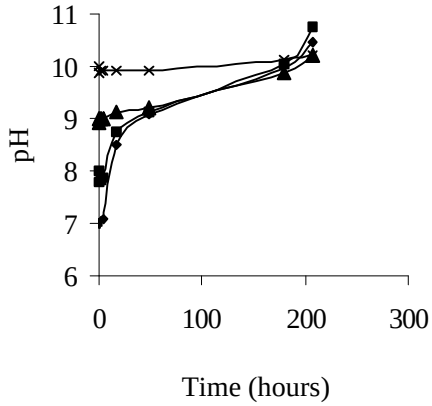

Figure 6.10. Elevation of pH of zinc refinery effluent from an initial pH of 7 (♦), 8 (**P**), 9 (▲) and 10 (×) by *Spirulina* spp.

6.4.2. *Dunaliella*

It was noted in laboratory cultures that the alga *D. salina* was able to elevate the pH of its surrounding media from 8.5 to above 10. The question arose as to the lowest initial pH at which the biological alkalisation function will operate. Cultures of *D. salina* were placed in BAAM medium, the pH of which had been artificially reduced to 4, 5 and 6. As can be seen in Figure 6.11, at an initial pH of 4 the micro-algae are able to elevate the pH only one pH point to 5. However, when the initial pH is 5 or 6, the micro-algae is able to further increase it to almost 9.

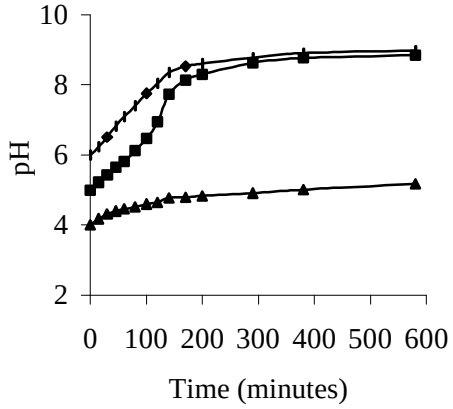

Figure 6.11. Elevation of pH of BAAM medium from an initial pH of 4 (▲), 5 (P) and 6 (♦) by *D. salina*.

Dunaliella spp. grows in an extremely wide range of environments. They are strict photoautotrophs and require inorganic carbon for survival. The pH of the surrounding medium affects many processes associated with algal growth and metabolism, including the availability of CO_2 for photosynthesis and the availability and uptake of ions (Borowitzka and Borowitzka, 1988). Most *Dunaliella* have been shown to tolerate a wide range of pH values. *D. salina* has been shown to tolerate a pH in the range of 5.5 to 10.0 (Baas-Becking, 1930). However, the growth of *Dunaliella* in acid mine drainage effluent has not been reported.

As can be seen in Figure 6.12, the chlorophyll *a* levels of *D. salina* exposed to acid mine drainage, supplemented with NaCl, at pH 3.5, 4 and 5 , decline over a period of days. Unlike the *Spirulina* spp. which were able to survive and grow, *D. salina* immediately enters decline phase. Although

effective elevation of pH was achieved within the first day of the experiment, the pH levels had declined to their initial levels after 3 days (Fig. 6.13).

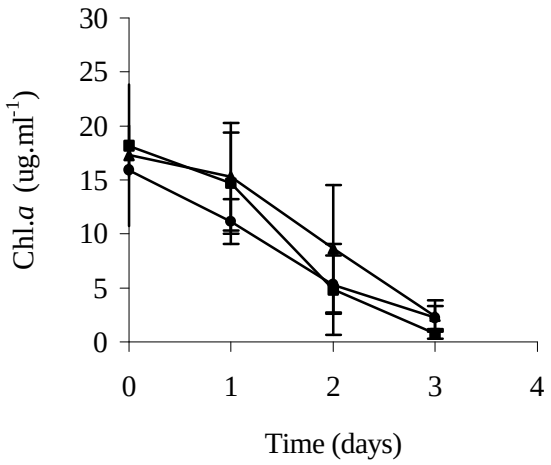

Figure 6.12. Chlorophyll *a* profile of *D. salina* grown in AMD with an initial pH of 3.5 (•), 4 (▪) and 5 (▲).

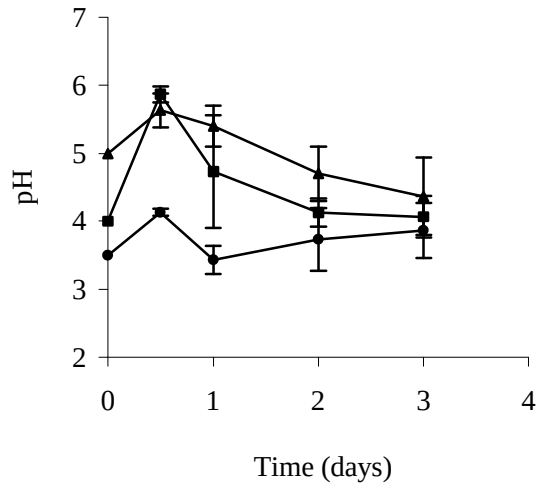

Figure 6.13. Elevation of pH of AMD from an initial pH of 3.5 (•), 4 (▪) and 5 (▲) by *D. salina*.

The initial increase in pH also seems to be related to the initial chlorophyll *a* concentration of the micro-algal population (Fig. 6.14)

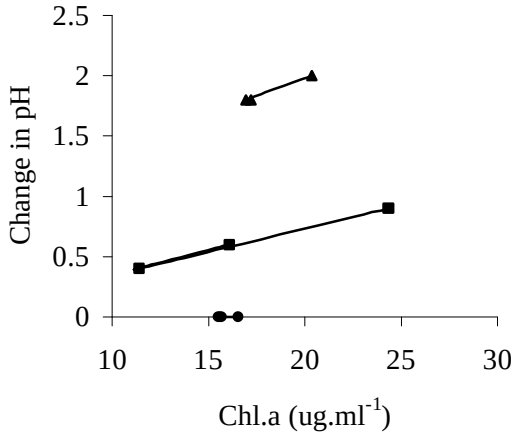

Figure 6.14. Change in pH of AMD from an initial pH of 3.4 (•), 4 (P) and 5 (▲) in relation to the initial chlorophyll *a* concentration of a culture of *D. salina.*

D. salina was also assessed for its ability to elevate the pH of a zinc refinery effluent. As was the case with *Spirulina* spp., even when $NaHCO_3$ and macro-nutrients such as NH_4Cl, KNO_3 and H_2PO_4, which are required for micro-algal growth, were added to the effluent, the micro-alga was unable to elevate the pH (Fig. 6.14).

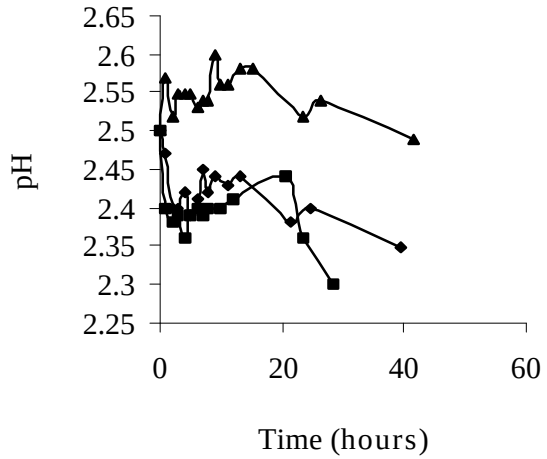

Figure 6.15. Elevation of pH of zinc refinery effluent by *D. salina* after supplementation with HCO_3^-(P), macro-nutrients (▲) and no supplementation (♦).

6.4.3. *Anacystis*

Spirulina spp. and *D. salina* are not endemic to acidic metal-rich wastewaters. It was thus necessary to determine if an acidophilic micro-alga would demonstrate the same biological alkalisation function as these micro-algae.

The ability of a strain of *Anacystis*, which was isolated from AMD wastewaters, to elevate the pH of 1% NaCl BAAM medium was assessed. As can be seen in Figure 6.15, when the initial pH of the media is below 4, no biological elevation of pH is observed. However, at an initial pH of 4 and above, biological pH elevation occurs.

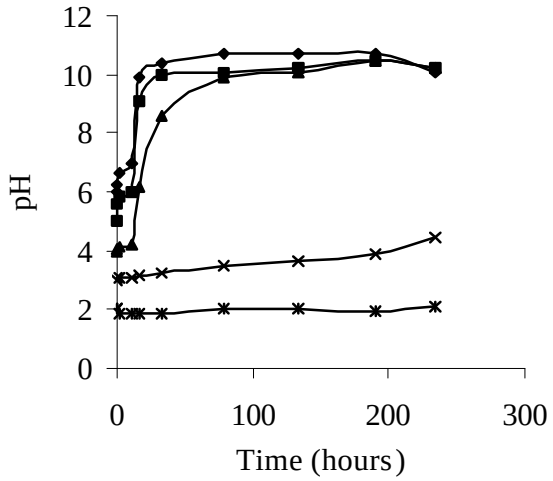

Figure 6.16. Elevation of pH of BAAM from an initial pH of 2 (✳), 3 (×), 4 (▲), 5 (P) and 7 (♦) by a species of *Anacystis*.

At pH 3 and 4 BAAM has a Ci content of approximately 54 and 72 mg.l^{-1} C respectively (Fig. 6.18). At the high salinity of BAAM medium, 8 %, the activity of the carbonic species is reduced and the pKa moved to the left of the pH scale ie. more HCO_3 present at low pH's in saline waters than freshwaters. Thus one would expect more HCO_3^- present in the BAAM compared to the 1% NaCl BAAM at the same pH.

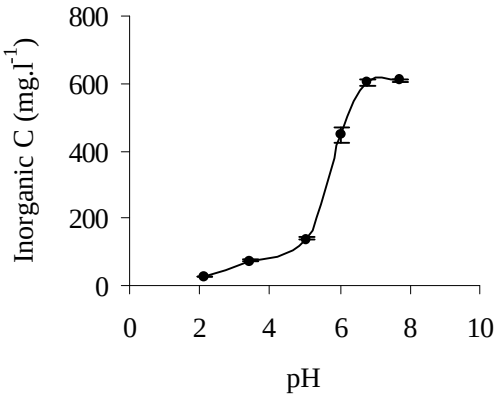

Figure 6.17. Inorganic carbon content of BAAM medium at different pH's.

However, *D. salina* was only able to elevate the pH of BAAM when it had an initial pH of 5 or above as compared to *Anacystis,* which was able to elevate it from pH 4. This would tend to indicate that *Anacystis* was better adapted to utilising HCO_3^- from the surrounding medium and was also more tolerant of low pH values. *D. salina* is know to have a pH optimum of 8 or above, whereas the *Anacystis* species isolated from the mine water was obviously more adapted to acidic conditions. This can be seen by its ability to grow in acid mine drainage effluent (Fig. 6.18).

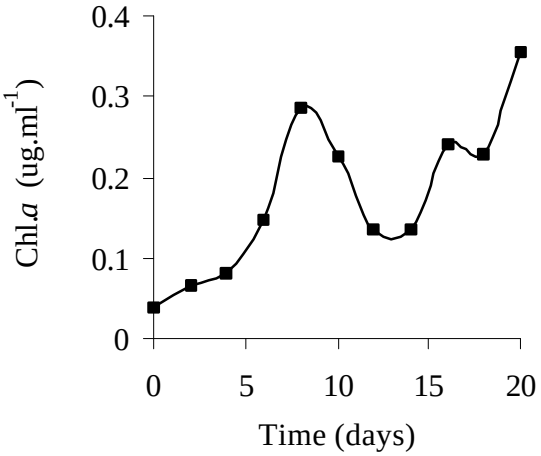

Figure 6.18. Chlorophyll *a* profile of *Anacystis* spp. in AMD effluent.

However, *Anacystis* was also not successful in elevating the pH of a zinc refinery effluent (Fig. 6.19) irrespective of the addition of HCO_3^- or macro-nutrients necessary for micro-algal growth. This would suggest either severe metal inhibition or that HCO_3^- utilisation is not a serious factor.

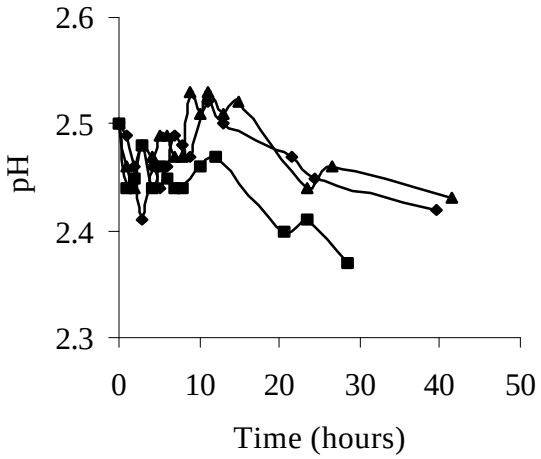

Figure 6.19. Elevation of pH of zinc refinery effluent by *Anacystis* spp. after supplementation with HCO_3^- (▲), macro-nutrients (♦), and no supplementation (**P**).

6.5. CONCLUSION

Spirulina spp. is able to elevate the pH of acid mine drainage effluent and at the same time sustain growth. However, this requires an initial amount of bicarbonate ions to be present in solution. This is important in terms of practical application, as AMD itself does not contain any bicarbonate and it would be expensive to supplement the effluent chemically. Two options thus exist for the integration of this alkalising function into the process.

1. The bicarbonate-rich overflow from the facultative pond, together with the metal-rich acid mine water, can be fed into a HRAP, with the bicarbonate ions functioning in pH elevation.
2. The alkaline-rich overflow from the HRAP can be blended with the metal-rich acid mine water thus neutralising it as well as contributing to controlled metal removal.

D. salina on the other hand was not able to carry out this function and thus does not appear to be a candidate for the remediation of acidic effluents. However, the micro-alga *Anacystis* was able to elevate the pH of BAAM medium and is able to grow in acid mine drainage effluents, thus

making it a candidate for AMD remediation. However, the toxicity of metals present in the effluent needs to be established.

CHAPTER 7. REMOVAL OF HEAVY METALS IN INTEGRATED POND OPERATIONS

7.1. INTRODUCTION

In the previous chapters methods for the desalination and neutralisation of AMD and sulphate-rich wastewaters have been addressed. However, AMD also contains a wide range of heavy metals, some of which may be valuable and others which are toxic to the environment and thus need to be removed and/or recovered. A number of biological approaches do exist for the treatment of metal-rich effluents and these have been discussed in Chapter 1. The use of microbes for the removal and accumulation of heavy metals has been well documented with the emphasis being on the use of naturally abundant or easily propagated biosorbents to replace more expensive ion exchangers. More recently the use of sulphide generated from biological sulphate reduction has also come under focus.

In the sulphate reducing anaerobic digesters examined in this study, metal sulphide precipitation took place *in situ*. This is not ideal as the recovery of heavy metals from the metal-sulphide sludge is difficult. These metal sludges also build-up in the reactor, thus necessitating periodic drainage and desludging. The metals may also be toxic to microbial processes occurring within the reactor. The ideal situation would thus be for the metals to be precipitated out of solution prior to the AMD entering the anaerobic digester.

A number of ways were considered to effect this removal, the primary one being the blending of the sulphide-rich digester overflow liquor with the AMD before it enters the anaerobic digester. However, the integration of algal ponding into the system opened up further options, including the transfer of the AMD into a HRAP prior to discharge to the anaerobic digester, thus allowing for the removal of the metals from solution by alkaline precipitation or binding to the algal biomass. The HRAP may also be used as a final polishing step for the removal of small quantities of metals from the overflow of the facultative pond.

In order to assess the feasibility of integrating algal ponds with a biological sulphate reducing reactor to mediate metal removal from AMD, a number of factors needed to be investigated.

These included the efficiency of metal removal obtained by combining sulphide-rich digester overflow with AMD, as well as the effect the heavy metals would have on micro-algal growth.

7.2. RESEARCH OBJECTIVES

1. To investigate metal sulphide precipitation using sulphide-rich overflow liquor from biological sulphate reducing digesters fed a range of complex organic carbon sources.
2. To investigate the removal of heavy metals from solution by binding to micro-algal cells or by precipitation.
2. To investigate metal toxicity to micro-algal biomass.

7.3. MATERIALS AND METHODS

7.3.1. Algal cultures for metal binding studies

A culture of *D. salina*, obtained from the CCAP culture collection, and *Spirulina* spp. were maintained in BAAM and Zarrouk's medium respectively.

7.3.2. Algal cultures for polysaccharides production.

A culture of *Spirulina* spp. was grown at 38°C under low light conditions in Zarrouk's medium. A mixed culture of *Dunaliella,* isolated from solar evaporation ponds in Botswana, was grown in BAAM medium at 38°C under low light. The culture media were assayed for the presence of organic carbon. These conditions are referred to as stress conditions throughout the text.

7.3.3. Metal binding studies:algal biomass

Biomass from a culture of *D. salina* was harvested by centrifugation at 4420 g x 10 minutes. *Spirulina* spp. biomass was harvested by filtration through a nylon gauze with a 100 µm pore size. Cultures were washed in PIPES buffer so as to remove any metal ions which may have been attached to the algal cell, and re-harvested. The respective micro-algal biomasses were then suspended in aqueous solutions of Cu^{2+}, Pb^{2+}, $Cr_2O_7^{2-}$ and Se^{2-} as metal salts ($CuSO_4.5H_2O$, $Pb(CH_3COO)_2.3H_2O$, $K_2Cr_2O_7$ and SeO_2) in conical flasks. The conical flasks were sealed with cotton wool bungs and incubated overnight in an orbital shaker at 80 rpm at 22°C. A sample was removed and filtered through a 0.45 µm MSI nylon membrane filter. The filter was acid digested and assayed for metals. The micro-algae were then harvested and re-suspended in fresh

metal solution to assess the effect of metal concentration on removal. The micro-algae were filtered and the filter assayed for metals. Experiments were performed in triplicate. The time period for metal removal was assessed by sampling at 15 minute intervals for the first 60 minutes and thereafter every 30 minutes.

The removal of zinc from a zinc refinery wastewater was also tested by the same procedure. The average concentrations of metals in the zinc effluent were as follows ($mg.l^{-1}$): Al, 1.46; Cd, 0.717; Co, 0.4; Cr, 2.24; Cu, 0.103; Fe, 1.8; K, 12.77; Mg, 335.4; Mn, 123.8; Nd, 166.7; Ni, 4.11; Pb, 4.987; Zn, 4.03; Ag, 0.08 and Se, 34.34.

7.3.4. Metal binding studies:extracellular organic matter

The mixed culture of *Dunaliella* was harvested from the medium by centrifugation at 5000 rpm x 20 minutes. The supernatant was retained and analysed for Total Organic Carbon (TOC). *Spirulina* spp. was harvested from the medium by filtration through a nylon gauze with a pore size of 100 µm. The supernatant was analysed for TOC. A suitable aliquot of $CuSO_4.7H_2O$ and $FeSO_4.7H_2O$ was added to a 24 ml volume sample of the supernatant. The pH of the supernatant was artificially lowered to pH 7, 5 and 3 with HCl prior to addition of the metal solutions. After a 24 hour period, the samples were centrifuged and the supernatant analysed for metals and TOC.

7.3.5. Toxicity studies

The toxicity of the metals to *D. salina* and *Spirulina* spp. was assessed by adding an aliquot of the metal stock solution to the micro-algal culture and monitoring the chlorophyll *a* content of the cells over a period of 15 days.

7.3.6. Sulphide precipitation studies

Sulphide-rich liquor from a digester treating tannery effluent (Chapter 2) was mixed with AMD, the composition of which is shown in Table 1.2, and a synthetic AMD of the following composition ($g.l^{-1}$): Na_2SO_4, 0.356; K_2SO_4, 0.049; $FeSO_4.7H_2O$, 1.045; $CaSO_4.2H_2O$, 1.61; $(NH_4)_2SO_4$, 0.198. The AMD was also mixed with Na_2S solutions of different concentrations. Sulphide-rich liquor from the digester fed algal biomass as carbon source (Chapter 3) was mixed

with metal solutions of known concentration. After a period of 10 hours, the samples were filtered through a nylon membrane filter and the supernatant analysed for metals.

7.3.7. Analysis

Chlorophyll was extracted into acetone and quantified according to Lichtenhaler (1987). Dry weight determinations were performed by filtering aliquots of the micro-algal culture through a pre-weighed GF/A filter and drying overnight in an oven at 30°C. Micro-algal biomass samples were prepared for metals analysis by digestion in 200 µl 25% HNO_3. Samples were made up to a volume of 4 mls. Metals were analysed on a Varian Atomic Absorption Spectrophotometer. Sulphides were measured by the Methylene Blue method according to Standard Methods (APHA, 1989). Total Organic Carbon (TOC) was analysed on a Dohrmann 180 Total Organic Carbon Analyser.

7.4. RESULTS AND DISCUSSION

7.4.1. Metal removal by sulphide precipitation

The sulphide-rich liquors from sulphate reducing digesters fed tannery effluent and algal biomass, were assessed for their metal removal capacity. Comparisons were made with a synthetic sulphide solution.

7.4.1.1. Tannery-fed digester liquors

The sulphide-rich digester overflow was mixed with a synthetic mine water solution and the amount of metal removed determined. It can be seen that levels of removal far in excess of that which can be attributed to stoichiometric sulphide precipitation were obtained (Fig. 7.1).

Similar results were obtained when effluent from Grootvlei Proprietary Mines (Ltd.), the composition of which can be seen in Table 1.1, was mixed with sulphide-rich liquor from the outflow of the tank digester. The percentage removal of iron from the effluent and the binding stoichiometry are shown in Table 7.1.

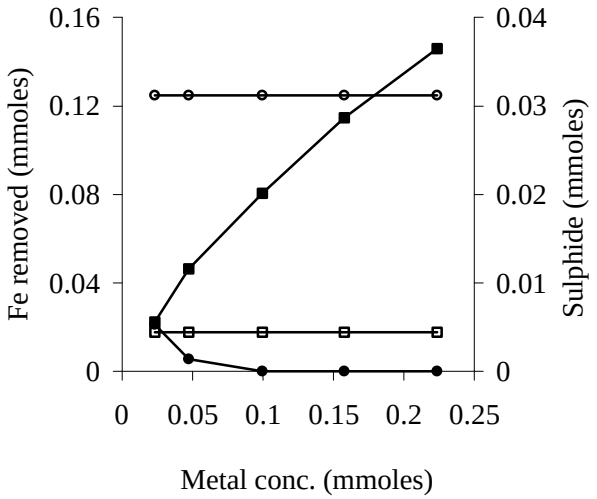

Figure 7.1. Iron removal from a synthetic mine water solution with the addition of sulphide-rich liquor from a sulphate reducing reactor treating tannery effluent. Dissolved sulphide before addition of iron (**N**), dissolved sulphide after addition of iron (•), anticipated stoichiometric removal (**R**), total removal obtained in the experiment (**P**).

Table 7.1. Dissolved sulphide concentration of liquor from tannery fed digester and iron concentrations in the AMD before and after addition of the sulphide-rich digester liquors.

% Digester liquor added (v/v)	Initial Fe (mmoles)	Initial sulphide (mmoles)	Final Fe (mmoles)	Fe removed (mmoles)	% removal	Ratio initial sulphide:Fe
75	0.012	0.43	0	0.012	100	34.84
50	0.028	0.28	0	0.028	100	10.14
25	0.058	0.10	0	0.058	100	1.71
10	0.096	0.05	0.008	0.087	91	0.59

The pH of the final solution, after addition of the digester overflow, was buffered to pH 6 by the bicarbonates present in the digester overflow liquor.

In the presence of hydrogen sulphide, insoluble ferric minerals are reduced to the ferrous state. These then react immediately with further hydrogen sulphide to form a ferrous sulphide precipitate (Widdle, 1988). This occurs via the following reactions:

$$2Fe^{3+} + H_2S \rightarrow S^0 + 2Fe^{2+} + 2H^+$$
$$2Fe^{2+} + H_2S \rightarrow FeS + 2H^+$$

A metallic precipitate is formed when the product of the concentration of free metal ions and sulphide ions exceeds the solubility product (Ksp), which for iron is 4.2×10^{-17} (Moeller *et al*., 1989).

In Table 7.1 it can be seen that in most cases the sulphide levels exceeded those needed for stoichiometric iron sulphide precipitation. This would account for the high levels of iron removal obtained, as when sulphide levels did not exceed those needed for stoichiometric metal removal, such as in the 10%v/v solution, complete removal was not obtained. However, removal exceeded levels anticipated from stoichiometric metal sulphide precipitation by a factor of 1.6 ie. 92% removal obtained compared to the 59% removal expected.

7.4.1.2. Algal biomass-fed digester liquors

The sulphide-rich overflow from a digester fed algal biomass was mixed with synthetic solutions of zinc, copper and iron. Synthetic metal solutions were used instead of AMD as the distance over which the AMD would have to be transported to the bioreactors was great, and would have resulted in the oxidation of the iron in the AMD and its subsequent precipitation out of solution.

The percentage removals obtained are shown in Figure 7.2 . Iron removal was the highest, with between 96 and 100% removal being obtained for all levels of iron (Fig.7.2A). The percentage metal removed also increased with increasing metal levels. The opposite trend was found with copper (Fig.7.2B) and zinc (Fig.7.2C), although copper removal was much more efficient than that of zinc.

The actual amount of metal removed was also much higher for iron than for copper and zinc (Fig. 7.3). In all three cases, the amount of metal removed increased with increasing initial metal levels. The levels of metal removal obtained were also far in excess of those expected purely from stoichiometric sulphide precipitation, with achieved removals being between 400 and 3000 times more than anticipated values, depending on the initial metal levels.

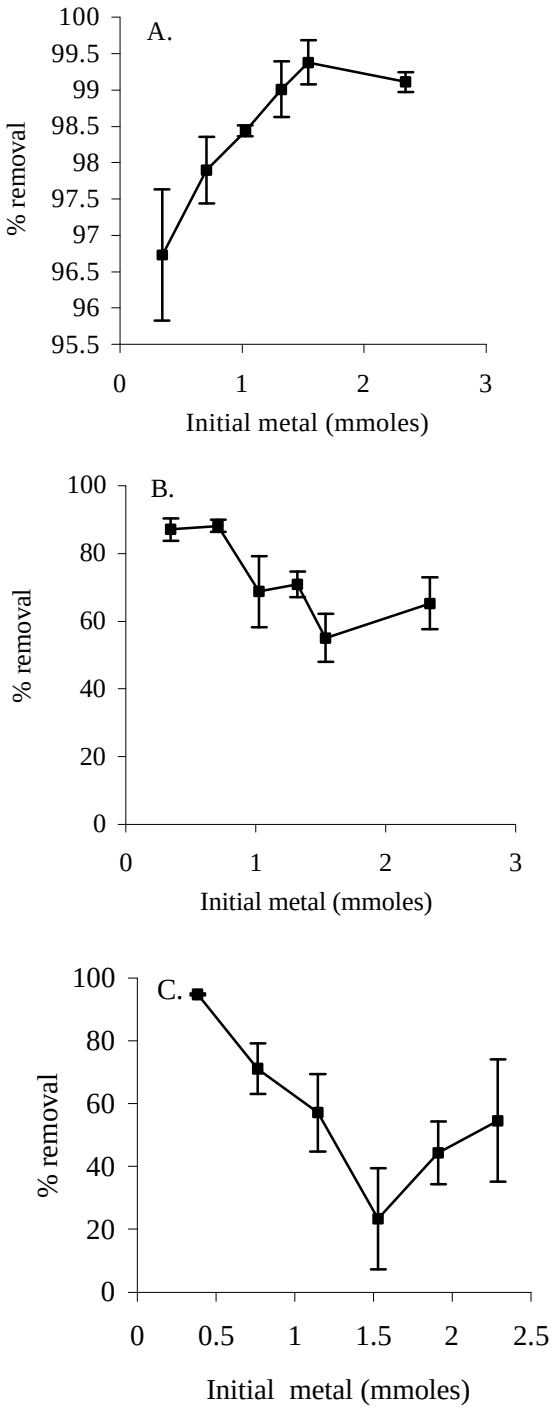

Figure 7.2. Percentage removal of A) iron, b) copper and C) zinc from solution by the addition of sulphide-rich liquor from a sulphate reducing reactor fed algal biomass.

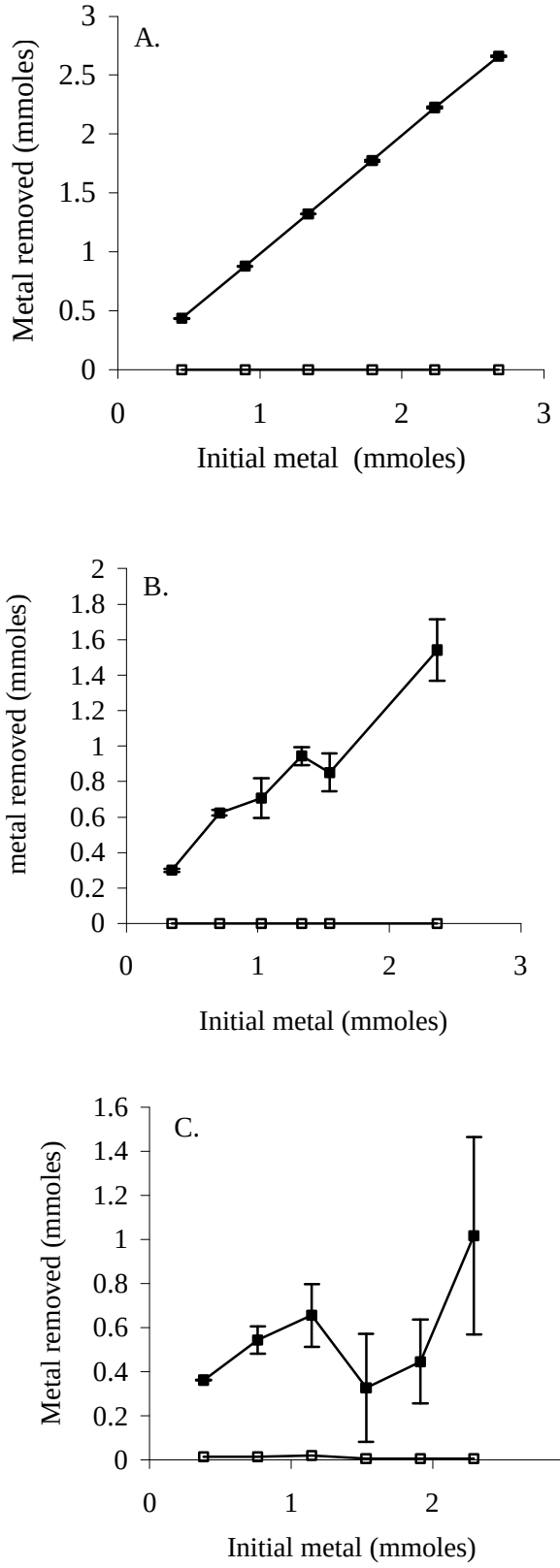

Figure 7.3. Total removal of A) iron, B) copper and C) zinc from solution by the addition of sulphide-rich liquor from a sulphate reducing reactor fed algal biomass.

All of the above results indicate that a factor other than metal sulphide formation is playing a role in metal removal. Two mechanisms have been identified as being responsible for heavy metal removal under anaerobic conditions. One is the interaction of the metal with hydrogen sulphide/sulphide system and the other the interaction with the carbon dioxide/carbonate system (Rivera, 1983). For every 1 mol of sulphate reduced biologically, 2 moles of HCO_3^- are produced, which combine with protons. This occurs via the following reactions:

$$SO_4^{2-} + 2CH_2O \rightarrow H^+ + HS^- + 2HCO_3^-$$ -------- (Machemer and Wildeman, 1992)

. $$HCO_3^- + H^+ \rightarrow H_2CO_3$$ ---------- (Singh, 1992)

7.4.1.3. Synthetic sulphide solution

The addition of a pure solution of sodium sulphide to the mine water yields similar results to that obtained with the liquor from the digester treating tannery effluent, with approximately 1.5 times more metal removal than expected (Figure 7.4), thus suggesting that alkalinity generated during sulphate reduction is not responsible for the excess removal. Machemer and Wildeman (1992) also found that pH was not as important as sulphide precipitation in removing metals from solution. It is thought that this is due to the reducing conditions of the system and high metal solubilities (Stumm and Morgan, 1981).

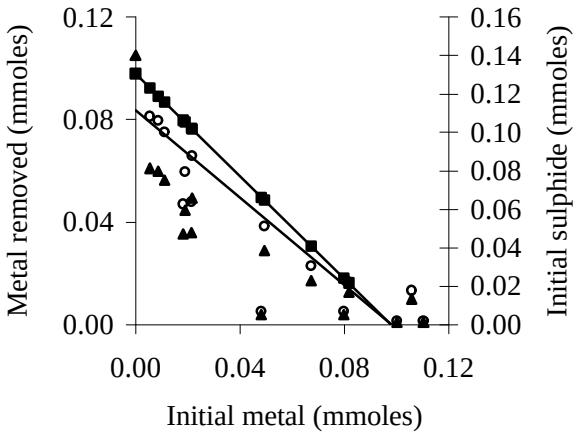

Figure 7.4. Iron removal from AMD by addition of a synthetic sulphide solution. Total metal removal obtained in the experiment (**P**), anticipated stoichiometric metal removal (**N**), initial sulphide levels before addition of AMD(▲).

Other factors playing a role may be co-complexation with inorganic ions present or with organic matter. Watson *et al.* (1995) also reported on the magnetic properties of FeS and its ability to accumulate metals from solution. However, Rivera (1983) suggested that biosorption to biosolids may also play an important role. This is important in both reactors, but more so in the digester fed algal biomass as not all algal biomass was digested. Thus the overflow used in this study contained large quantities of algal cell components with sites available for metal binding. However, this is purely speculative and the role that these algal cell components played in metal removal requires further investigation. This suggests that their might be an advantage in utilising the digester overflow liquor instead of a pure stream of hydrogen sulphide for metal removal, as it would allow one to harness the metal removal ability of more than just the sulphide, thus leading to higher removal efficiencies.

7 .4.2. *Metal removal by algal biomass*

A number of metals other than iron are present in AMD. Some of these, such as dichromate, do not readily form sulphide complexes. Oxyanions such as selenium also do not form metal sulphide complexes, and thus an alternative method of removal is required.

D. salina and *Spirulina* spp. in BAAM and Zarrouk's medium respectively, were spiked with CrO_7^{2-} and Se^{2-} ions, as well as Cu^{2+} and Pb^{2+} ions as an example of two metals which do readily form metal sulphides, and the amount of metal or oxyanion associated with the algal biomass determined. The algal biomass was consecutively exposed to the metal or oxyanion solution to determine the re-use potential of the algal biomass.

The total percentage removal, after a single exposure of the algal biomass to the metal or oxyanion solution, is shown in Table 7.2. In terms of percentage metal removal, *D. salina* was more efficient at the lower Cu^{2+} levels than *Spirulina* spp., with 91.3% removal as compared to 63.8% removal by *Spirulina* spp. (Table 7.2). However, at the higher Cu^{2+} levels similar removal efficiencies were obtained by both species of micro-algae.

It can be seen in Figure 7.5 and 7.6, that *Spirulina* spp. removed more Cu^{2+} per µg chlorophyll *a* than did *D. salina*.

Table 7.2. Percentage metal removal from solution by algal biomass after being exposed once to the metal or oxyanion solution. The actual amount of metal (mmoles) removed is shown in brackets.

Metal	Initial levels. (mmoles)	% removal	
		Dunaliella	*Spirulina*
Cu^{2+}	0.6	91.3 (0.55)	63.8 (0.38)
Cu^{2+}	6.00	30.5 (1.83)	28.3(1.70)
Pb^{2+}	0.03	38.0 (0.011)	35.0(0.01)
Pb^{2+}	0.3	10.0 (0.03)	0(0)
$Cr_2O_7^{2-}$	0.6	4.26 (0.26)	4.78(0.03)
$Cr_2O_7^{2-}$	6.0	39.25 (2.36)	56.6(3.4)
Se^{2-}	0.09	2.7 (0.002)	1.77(0.001)
Se^{2-}	0.9	30.0(0.27)	7.77(0.07)

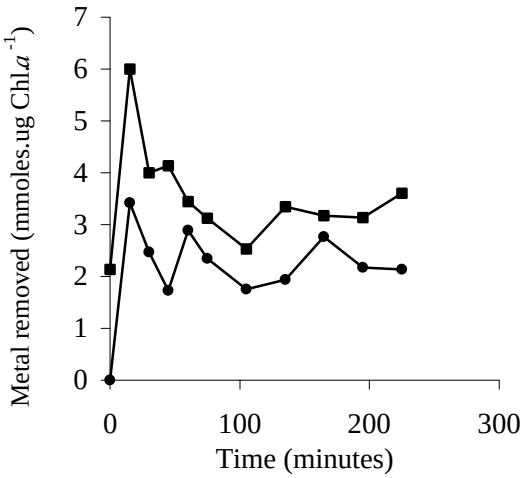

Figure 7.5. Total removal of copper from solution by *Spirulina* spp. during consecutive exposure to solutions containing 6 mmoles Cu^{2+}. 1st exposure (•), 2nd exposure (**P**).

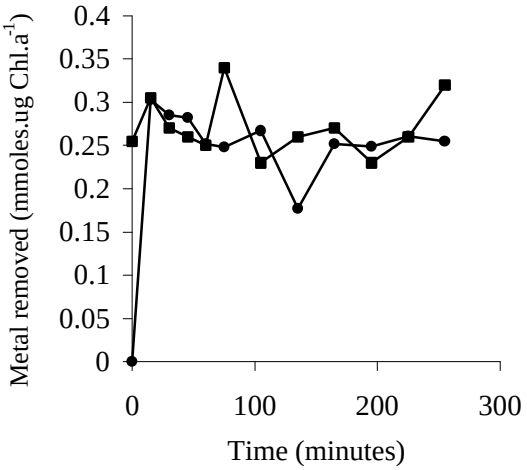

Figure 7.6. Total removal of copper from solution by *D. salina* during consecutive exposure to solutions containing 6 mmoles Cu^{2+}. 1st exposure (•), 2nd exposure (**P**).

Thus it would appear that the removal of copper is concentration dependant, with more metal being removed at a higher external metal ion level. Literature states that at low metal levels the mass of metal ions accumulated is directly proportional to the amount of the metal ion in solution (Ting *et al.*, 1989; Ting *et al.*, 1991). This is further supported by an increase in the amount of Cu^{2+} removed by *Spirulina* spp. after a second exposure to a 6 mmole Cu^{2+} solution (Figure 7.5). However, no significant increase in the amount of Cu^{2+} associated with the algal biomass was observed in *D. salina* after a second exposure to a 6 mmole Cu^{2+} solution (Figure 7.6).

The opposite result was obtained with lead, with the amount of Pb^{2+} ions associated with *D. salina* biomass increasing after the second exposure (Fig. 7.7). However, when exposing *Spirulina* spp. for the second time, the amount of biomass-associated Pb^{2+} remained about the same (Fig. 7.8). There was also little difference between the amount of Pb^{2+} removed at high and low levels by *Spirulina*, thus indicating that the Pb^{2+} removal process, be it precipitation or binding to the cell wall, has a threshold limit. In relation to binding this can be explained in terms of a fixed cell biomass offering a finite number of binding sites, which upon reaching saturation, do not allow for any further binding. On the other hand, more Pb^{2+} was removed

when the biomass was exposed to the 0.3 mmole lead solution than the 0.03 mmole solution (Table 7.2).

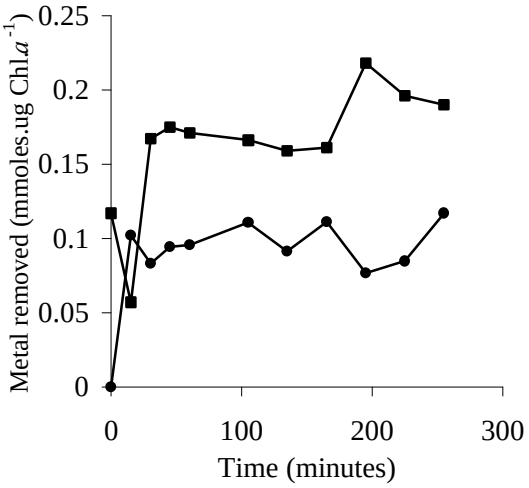

Figure 7.7. Total removal of lead from solution by *D. salina* during consecutive exposure to solutions containing 0.3 mmoles Pb^{2+}. 1st exposure (•), 2nd exposure (**P**).

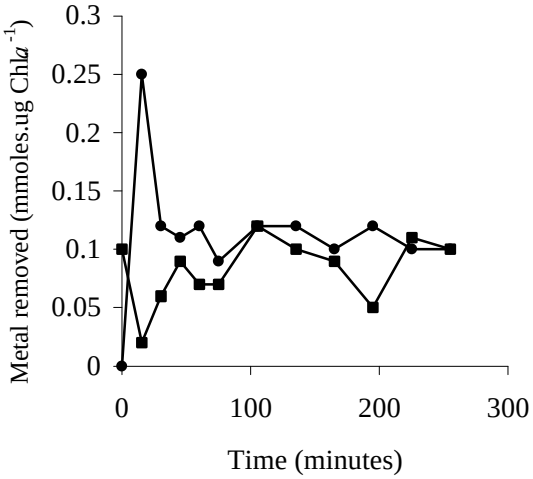

Figure 7.8. Total removal of lead from solution by *Spirulina* spp. during consecutive exposure to solutions containing 0.3 mmoles Pb^{2+}. 1st exposure (•), 2nd exposure (**P**).

Chromium removal appears to be concentration dependant, with more metal being removed from the 6.0 mmole solution than the 0.6 mmole solution (Table 7.2). However, in the case of *D. salina*, there is little difference between the amount of biomass-associated $Cr_2O_7^{2-}$ after the first and second exposure (Fig. 7.9) to a 6 mmole $Cr_2O_7^{2-}$ solution.

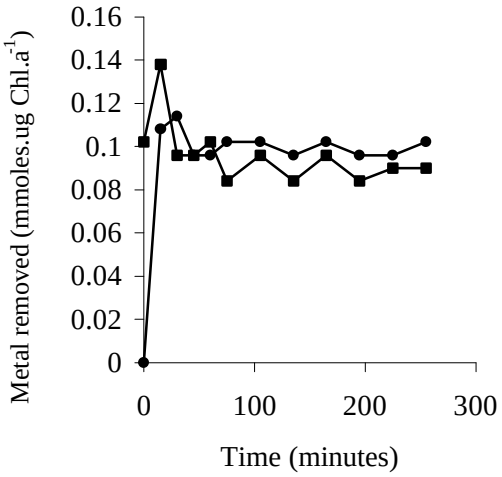

Figure 7.9. Total removal of dichromate from solution by *D. salina* during consecutive exposure to solutions containing 6.0 mmoles $Cr_2O_7^{2-}$. 1st exposure (•), 2nd exposure (P).

In *Spirulina* spp. however, the levels of biomass associated $Cr_2O_7^{2-}$ do tend to increase after the second exposure (Fig. 7.10).

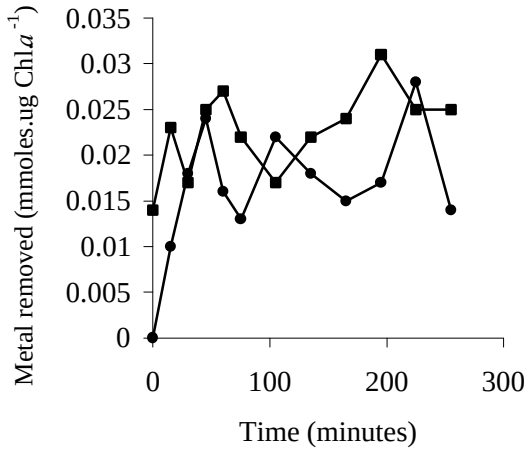

Figure 7.10. Total removal of dichromate from solution by *Spirulina* spp. during consecutive exposure to solutions containing 6.0 mmoles $Cr_2O_7^{2-}$. 1st exposure (•), 2nd exposure (P).

Cr (VI) ions were obtained from diluting $K_2Cr_2O_7$ such that the dichromate (Cr_2O_7) would carry an overall charge of -2. This would not allow for binding with the anionic groups on the algal cell surface. Thus it can be speculated that the majority of uptake can be attributed to an active transport across the cell membrane, related to the metabolic activities of the cells, or due to metal precipitation. Saraiva and Frazier (1975) were unable to demonstrate any active mode of radio labelled chromium absorption, with only 1.4% of the total radioactivity being detected in the algal cells. However, chromate (VI) can easily cross cell membranes as the phosphate-sulphate carrier in the cell membrane also transports chromate anions (Gauglhofer, 1990).

D. salina was found to remove more selenium than *Spirulina* spp. (Table.7.2). There was not much increase in the amount of biomass-associated selenium between the first and second exposure to a 0.9 mmole Se^{2-} solution (Fig. 7.11). Selenium is also in the anionic form and thus will not bind to the cell wall. However, from Table 7.2, it can be see that more selenium was removed from the 0.9 mmole Se^{2-} solution than from the 0.09 mmole solution. Gerhardt and Oswald (1990b) reported that no direct algal uptake of selenium occurs.

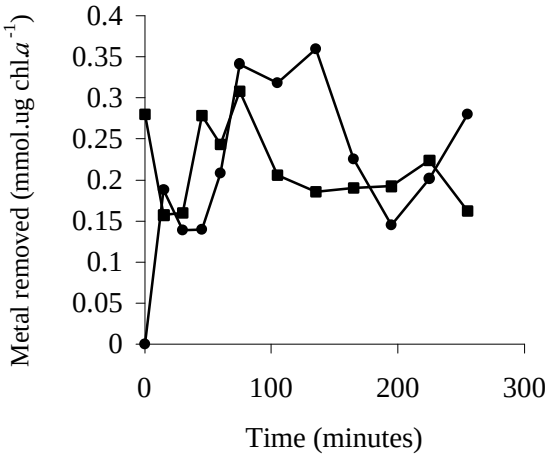

Figure 7.11. Removal of selenium from solution by *D. salina* during consecutive exposure to solutions containing 0.9 mmoles Se^{2-}. 1st exposure (•), 2nd exposure (P).

Similar results were obtained with *Spirulina* spp. biomass (Fig. 7.12).

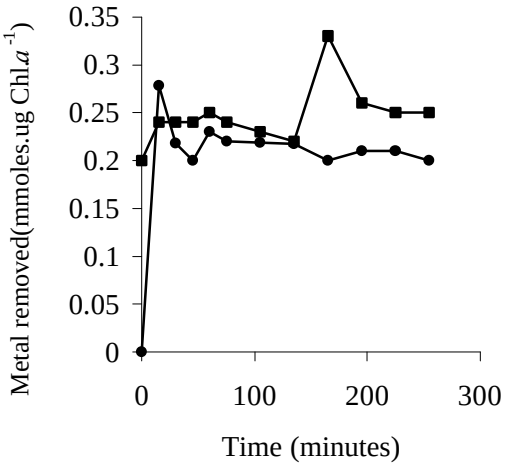

Figure 7.12. Total removal of selenium by *Spirulina* spp. during consecutive exposure to solutions containing 0.9 mmole Se^{2-}. 1st exposure (•), 2nd exposure (**P**).

7.4.3. Metal toxicity effects

The toxicity of copper and its effect on photosynthesis was assessed by growing both *D. salina* and *Spirulina* spp. in medium containing 0.6 mmoles and 6 mmoles Cu^{2+}. As can be seen in Figure 7.13 and 7.14, *Spirulina* spp. is more tolerant to 6 mmoles Cu^{2+} than *D. salina*, with cultures able to sustain growth. However, in all cases the chlorophyll *a* levels were much lower than that of the control cultures.

Examination of the *D. salina* cells under the microscope after exposure to a 6 mmole copper showed that the cells had lysed (Fig. 7.15). This in turn may explain why no increase in biomass-associated Cu^{2+} was noted after a second exposure of *D. salina* biomass to a 6.0 mmole Cu^{2+} solution. Copper is know to have toxic effects on algae and $CuSO_4$ is used as an algicide due to its ability to break down the cell function (De Haan *et al*., 1981). This toxicity is mainly due to the excessive binding of copper to the cells, resulting in their increased permeability and a resultant release of potassium. Copper has also been shown to be more toxic intracellularly than other heavy metals (Davies, 1983) as it binds to metabolically active sites in the micro-algal cells

(Overnell, 1975; Davies, 1983). Copper toxicity is also known to interfere with pigment biosynthesis as well as inhibiting the photosynthetic electron transport chain (Baron *et al*., 1995).

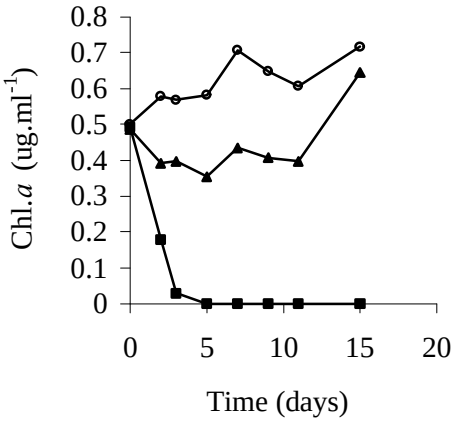

Figure 7.13. Growth of *D.salina* in the presence of 6.0 mmoles (**P**) and 0.6 mmoles (▲) Cu^{2+} in relation to a control (**N**) which did not contain Cu.

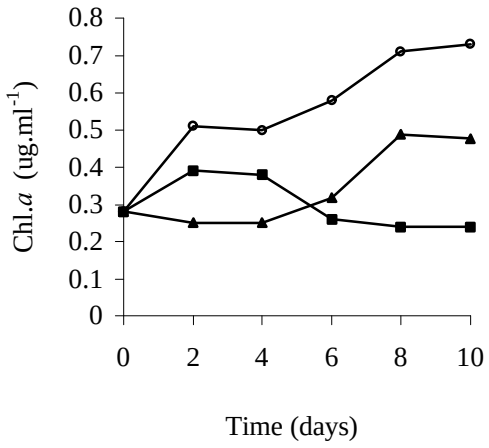

Figure 7.14. Growth of *Spirulina* spp. in the presence of 6 mmoles (**P**) and 0.6 mmoles (▲) Cu^{2+} in relation to a control (**N**) which did not contain Cu.

Figure 7.15. Light microscope image of *D. salina* A) before exposure to copper and B) after exposure to a solution containing 6.0 mmoles Cu^{2+} (40 x magnification).

Lead did not appear to have an effect on the growth of *D. salina* (Fig. 7.16) or *Spirulina* spp. (Fig. 7.17), with there being little difference between the chlorophyll *a* content of the experimental and control cultures.

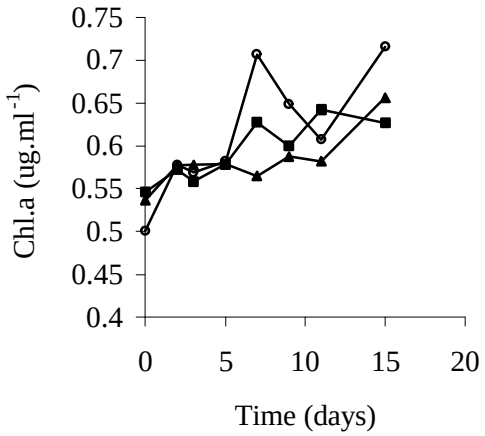

Figure 7.16. Growth of *D. salina* in the presence of 0.3 mmoles (**P**) and 0.03 mmoles (▲) Pb^{2+} in comparison to a control (**"**) grown in the absence of Pb^{2+}.

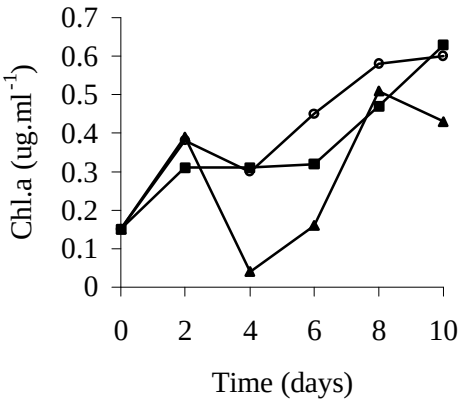

Figure 7.17. Growth of *Spirulina* spp. in the presence of 0.3 mmoles (**P**) and 0.03 mmoles (▲) Pb^{2+} in comparison to a control (**N**) grown in the absence of Pb^{2+}.

As stated by Pace *et al.* (1977) the effect of lead is more pronounced towards the end of the logarithmic phase of growth. Davies (1983) also showed that the chlorophyll content of populations of phytoplankton was the same in the presence of lead as in its absence, as did Overnell (1975) for *Dunaliella tertiolecta*. However, Hollibaugh *et al.* (1980) found that lead caused a decrease in chlorophyll production at a concentration of 1 µM. This is true for *Spirulina* spp., with a marked difference between the chlorophyll *a* content of the control and experimental cultures. However, it is interesting to note that the cultures exposed to 0.3 mmole Pb^{2+} had higher chlorophyll *a* levels than those exposed to 0.03 mmole Pb^{2+} (Fig. 7.17). Lead in the acetate form has previously been found to be stimulatory to cultures of *Chlamydomonas* (Hutchinson, 1973).

Chromium also appears to be very toxic to both *D. salina* (Fig. 7.18) and *Spirulina* spp. (Fig. 7.19) , with the chlorophyll *a* levels of the experimental cultures being much lower than that of the control cultures. However, in comparison to *Spirulina* spp., *D. salina* appears to show more tolerance to the lower concentration of dichromate.

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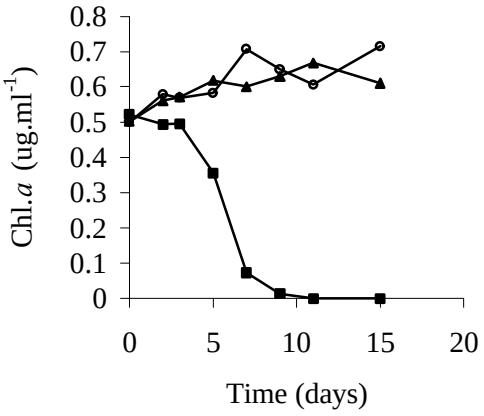

Figure 7.18. Growth of *D. salina* in the presence of 6 mmoles (**P**) and 0.6 mmoles (▲) $Cr_2O_7^{2-}$ in comparison to a control (**N**) grown in the absence of $Cr_2O_7^{2-}$.

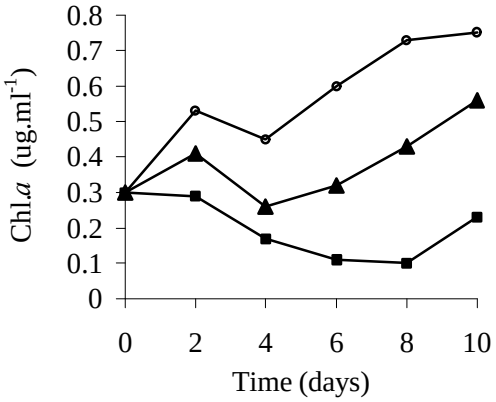

Figure 7.19. Growth of *Spirulina* spp. in the presence of 6 mmoles (**P**) and 0.6 mmoles (▲) $Cr_2O_7^{2-}$ in comparison to a control (**N**) grown in the absence of $Cr_2O_7^{2-}$

Selenium also appears to have a slightly toxic effect on both *D. salina* (Fig. 7.20) and *Spirulina* spp. (Fig. 7.21), although more apparent in *D. salina*.

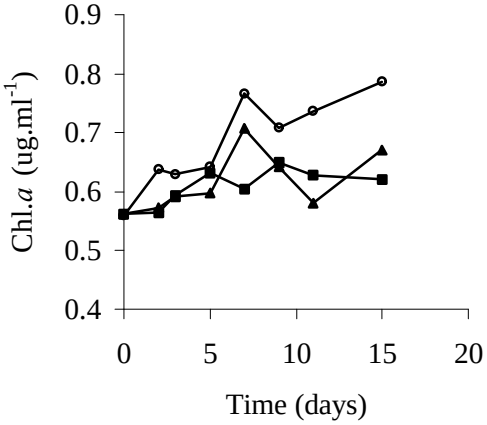

Figure 7.20. Growth of *D. salina* in the presence of 0.9 mmoles (**P**) and 0.09 mmoles (▲) Se^{2-} as compared to a control (**N**) grown in the absence of Se^{2-}.

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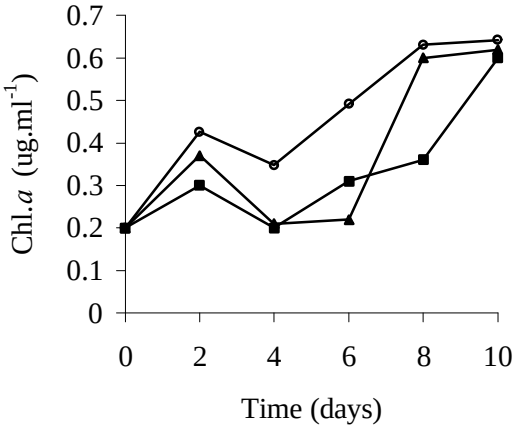

Figure 7.21. Growth of *Spirulina* spp. in the presence of 0.9 mmoles (**P**) and 0.09 mmole (▲) Se^{2-} as compared to a control (**N**) grown in the absence of Se^{2-}.

Gerhardt and Oswald (1990a,b) reported the growth of *Dunaliella* in a high rate oxidation pond in the presence of 30 µg.l^{-1} selenium, while Gerhardt *et al*. (1994) reported that selenium levels of 330 µg.l^{-1} did not inhibit algal growth. However, the levels used in this study were much higher. Shrift (1961) found that selenomethionine interfered with cell division of the alga *Chlorella* leading to the formation of giant cells. A natural selenium cycle has also been well documented with uptake of selenium by microorganisms and plants and subsequent transformation into various inorganic and organic compounds (Schrift, 1973; Besser *et al*., 1989).

7.4.4. *Metal removal by binding to extracellular organic matter*

Considering the toxicity demonstrated by the metals such as copper and chromium to the growth of the algae, the use of a continuous direct contact system in which the AMD is fed into a HRAP, may not be feasible, especially if these metals occur in high concentrations. It is against this background that the metal binding potential of the extracellular organic matter produced by the micro-algae and discussed in Chapter 4, was investigated. This would allow separation of the growth of the micro-algae from metal removal as purely the organic carbon-rich medium would be in contact with the metal-rich effluent.

The organic carbon rich media used in these metal removal studies was produced by *D. salina* and

Spirulina spp. under conditions of high temperature and low light. Light microscope studies of the algal cells revealed the presence of gelatinous-like matter surrounding cells, and in some cases holding the cells together (Fig. 7.22 and 7.23). Staining with Ruthenium Red, a cationic dye, showed this matter to be acidic in nature and thus amenable for metal binding.

Figure 7.22. Light micrographs of A) *Spirulina* spp. grown under non-stress conditions B) *Spirulina* spp. grown under stress conditions. The extracellular organic matter is stained pink. (10 x magnification)

Figure 7.23. Light micrographs of *D. salina.* A) *D. salina* grown under normal conditions B) *D. salina* grown under stress conditions. The extracellular organic matter is stained pink. (40 x magnification)

Work done by Giordano *et al.* (1994) showed the presence of a cationic periplasmic coat surrounding cells of *D. salina* grown under conditions of low CO_2 or in the presence of NH_4^+. Huntsman (1972) also found that 27% of the organic carbon excreted by senescent cells of *Dunaliella tertiolecta* was cationic in composition. However, very little work has been done to

characterise the organic component or evaluate its potential for metal removal. A water soluble extracellular polysaccharide produced by *Spirulina platensis,* calcium spirulan (Ca-SP) has also been shown to be sulphated and thus may be useful for metal removal.

Figure 7.24. shows the amount of copper removed from solution by addition of extracellular organic carbon produced by *D. salina* grown under stress conditions. Under the conditions used in this experiment, metal removal by binding to the organic matter decreases with increasing pH. The amount of copper removed by chemical precipitation in turn increases with increasing pH.

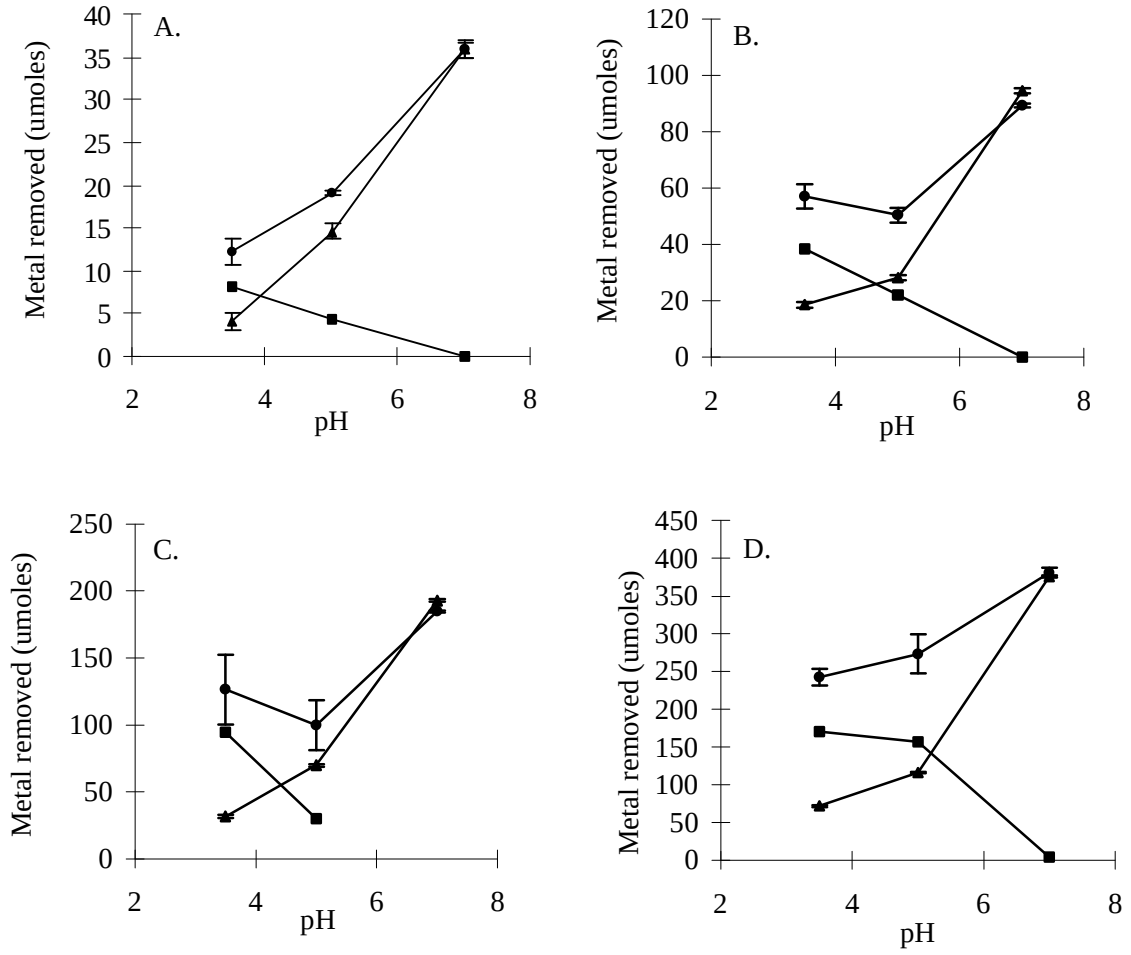

Figure 7.24. Removal of copper from solutions containing A) 40 µmoles B) 100 µmoles, C) 200 µmoles and D) 500 µmoles Cu^{2+} at a pH of 3.5, 5 and 7. Total removal obtained in the experiment (•), removal due to precipitation (▲), removal due to binding to organic matter produced by *D. salina* (**P**).

The percentage Cu^{2+} removal obtained ranged between 20 and 50%, with higher removal efficiency obtained at the higher initial metal concentrations (Fig. 7.28). There is also a linear relationship between pH and percentage removal, especially at the lower copper concentrations.

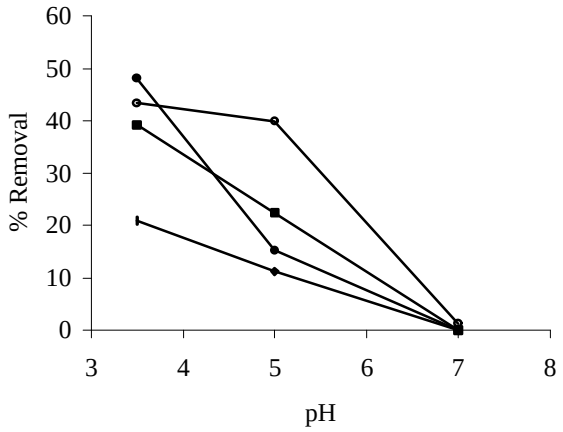

Figure 7.25. Percentage Cu^{2+} removal from solutions containing 40 µmoles (♦), 100 µmoles (**P**), 200 µmoles (•) and 395 µmoles (**"**) Cu^{2+} by binding to extracellular organic matter produced by *D. salina* under stress conditions.

The relationship between total Fe^{2+} removal, removal due to chemical precipitation and removal due to binding to extracellular organic matter is shown in Figure 7.26. As was the case with copper, chemical precipitation increases as the pH increases.

The percentage removal of iron by binding to extracellular organic matter produced by *D. salina*, on the other hand, does not show a linear relationship, with the highest percentage removal being obtained at pH5. There is also an inverse relationship between percentage removal and initial metal concentration, with the highest removal obtained at the lowest concentration Fe^{2+} solutions (Fig. 7.27).

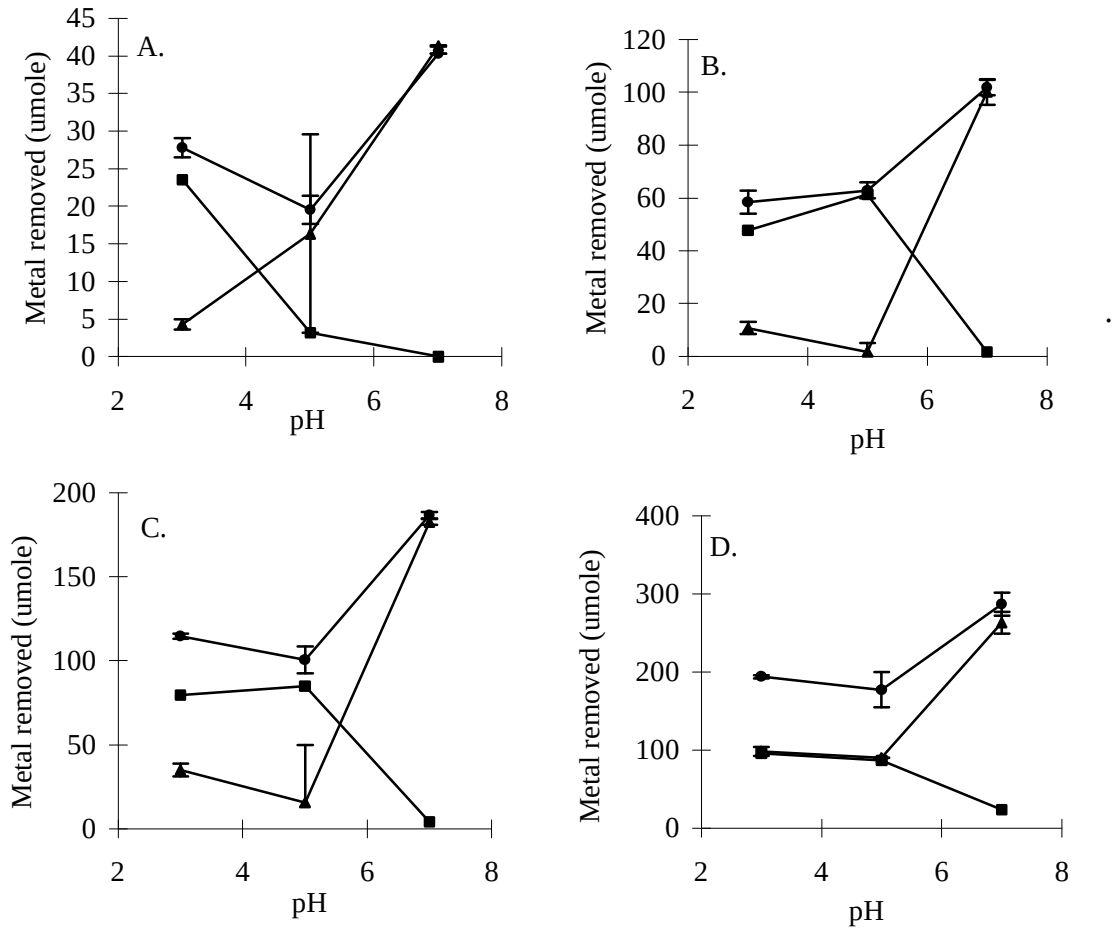

Figure 7.26. Removal of ferrous iron from solutions containing A) 45 µmoles, B) 112 µmoles, C) 225 µmoles and D) 450 µmoles Fe^{2+} at a pH of 3.5, 5 and 7. Total removal obtained in the experiment (•), removal due to precipitation (▲), removal due to binding to organic matter produced by *D. salina (*P).

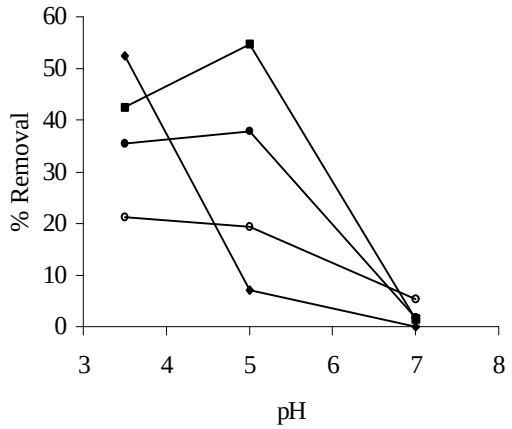

Figure 7.27. Percentage Fe^{2+} removal from solutions containing 45 µmoles (**"**), 112 µmoles (P), 225 µmoles (•) and 450 µmoles (♦) Fe^{2+} by binding to extracellular organic matter produced by *D. salina* under stress conditions.

The removal of copper by binding to organic matter produced by *Spirulina* spp. shows similar results to that obtained with *D. salina* organic matter. The percentage removal of copper ranges from 0 to 50% with the lowest removals at the highest pH (Fig. 7.28). The highest removal by binding to organic matter was also shown to occur in the metal solution with the highest concentration.

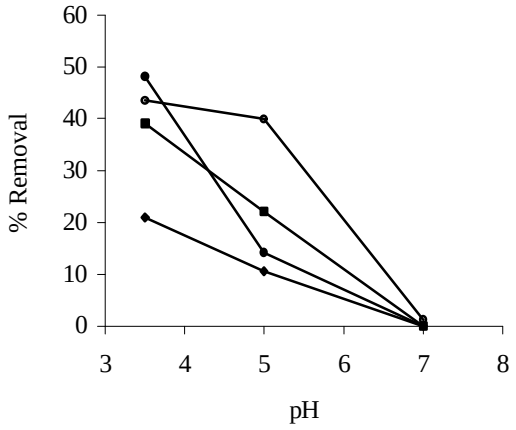

Figure 7.28. Percentage Cu^{2+} removal from solutions containing 40 µmoles (♦), 100 µmoles (**P**), 200 µmoles (•) and 395 µmoles (**"**) Cu^{2+} by binding to extracellular organic matter produced by *Spirulina* spp. under stress conditions.

Iron removal by binding to organic carbon produced by *Spirulina* spp. did not show similar results (Fig. 7.29).

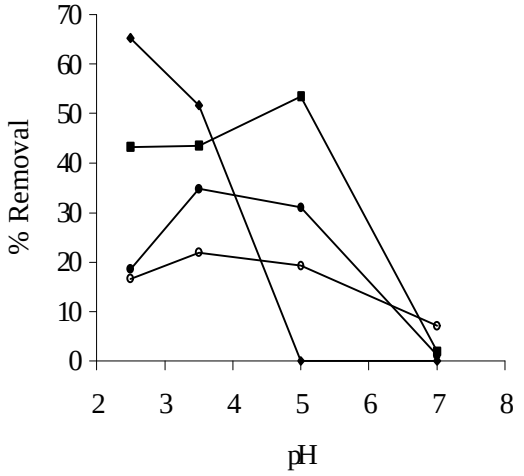

Figure 7.29. Percentage Fe^{2+} removal from solutions containing 45 µmoles (♦), 112 µmoles (**P**), 225 µmoles (•) and 450 µmoles (**N**) Fe^{2+} by binding to extracellular organic matter produced by *Spirulina* spp. under stress conditions.

At the lowest Fe^{2+} levels, most removal was shown at a pH of 2.5. At pH 5 or above, no removal by binding was shown. At the higher Fe concentrations the highest removal was obtained between pH 3 and 5, with no removal by binding occurring at pH7 (Fig. 7.29).

The relationship between total copper and iron removed and removal due to binding and precipitation are shown in Figures 7.30 and 7.31 respectively

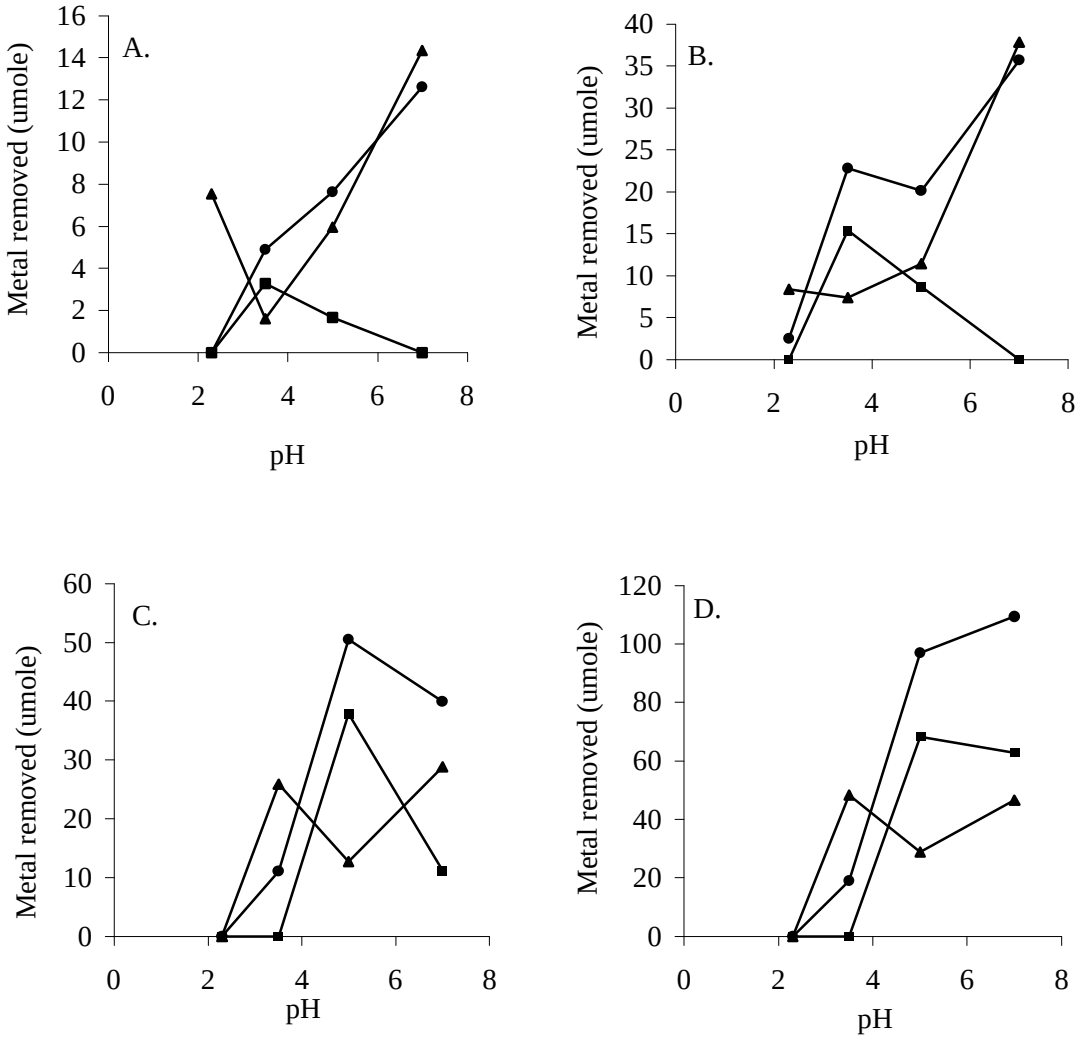

Figure 7.30. Removal of copper from solutions containing A) 16 µmoles, B) 40 µmoles, C) 80 µmoles and D) 160 µmoles Cu^{2+} at a pH of 2.5, 3.5, and 7. Total removal obtained in the experiment (•), removal due to precipitation (▲), removal due to binding to organic matter produced by *Spirulina* spp. (**P**).

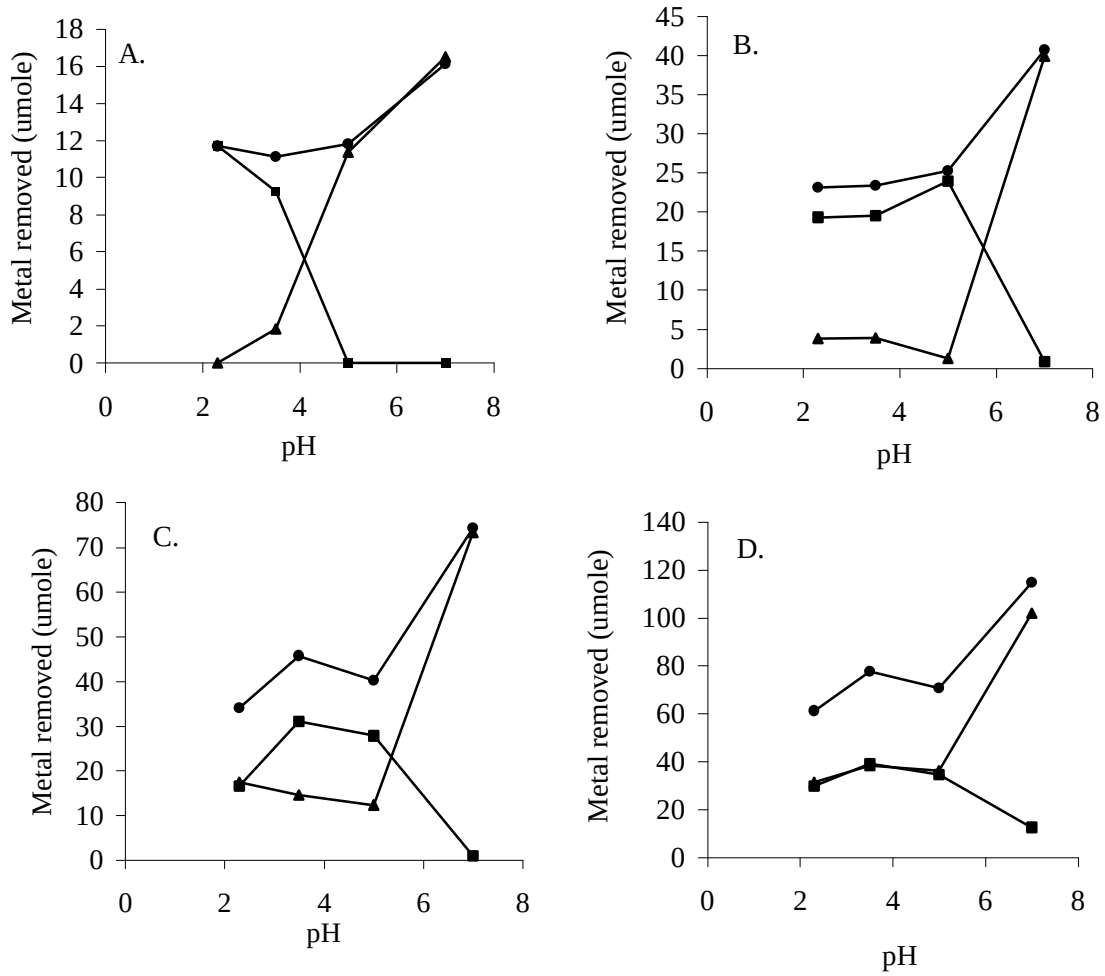

Figure 7.31. Removal of iron from solutions containing A) 18 µmoles, B) 45 µmoles, C) 90 µmoles and d) 180 µmoles Fe^{2+} at pH 3.5, 5 and 7. Total removal obtained in the experiment (•), removal due to precipitation (▲), removal due to binding to organic matter produced by *Spirulina* spp. (**P**).

The binding of metals to organics has also been found to be pH dependent by other researchers (Farrah and Pickering, 1978). An increase in pH was found to increase the uptake of Cu, Pb, Zn and Cd by humic acid (Beveridge and Pickering, 1980) and cellulose (Farrah and Pickering, 1978).

The pH of the media will affect the speciation of the metal ion in solution. This can be seen in Figure 7.32. For example, in seawater at pH 8.5, Cu occurs as $Cu(OH)_2$, whereas in acidic lake

waters, it may occur as a divalent cation. As discussed in Chapter 6, conditions in high rate oxidation ponds will lead to the formation of hydroxide and carbonate ions, which together with the extracellular organic matter have the potential for metal removal by precipitation. The solubility of certain metal oxides and hydroxides is shown in Figure 7.32.

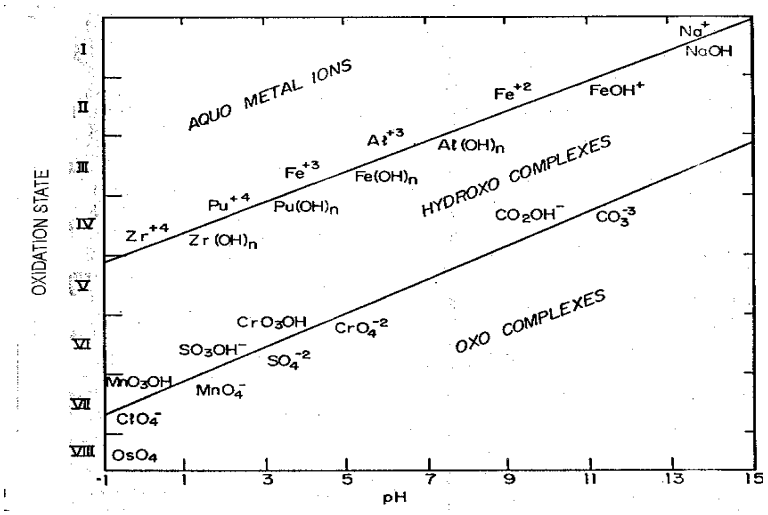

Figure 7.32. Predominant pH range for the occurrence of aquo, hydroxo, hydroxo-oxo and oxo complexes for various oxidation states (From Stumm and Morgan, 1981).

The limiting stability relations for iron is shown in Figure 7.33. This shows that at a pH below 3.5, Fe exists either as Fe^{2+} or Fe^0. Whereas at pH 5 and above, $FeCO_3$ and Fe $(OH)_3$ tend to form thus accounting for the higher removal attributed to binding to organic matter obtained at low pH's. Hydroxide ions have also been found to often have a stronger affinity for Fe^{3+} than organic or inorganic bases (Stumm and Morgan, 1981).

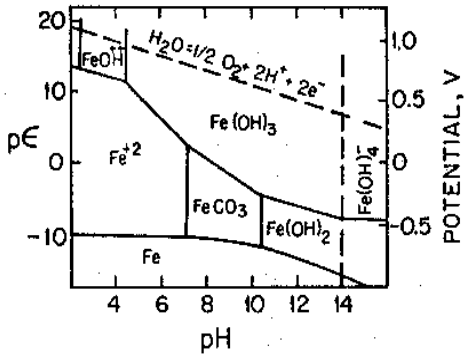

Figure 7.33. pϵ-pH diagram for iron (From Stumm and Morgan, 1981).

The level of organic carbon in solution was measured before and after addition of the metal solution. As expected it was found that the metal removal and organic carbon removal correlated. The higher the organic carbon removal, the higher the metal removal. Examples of this are shown in Figures 7.34 and 7.35. Irrespective of the metal concentration in solution, complete removal of the organic carbon from solution was not obtained. This suggests that not all of the organic carbon produced was acidic in nature and thus does not bind cations.

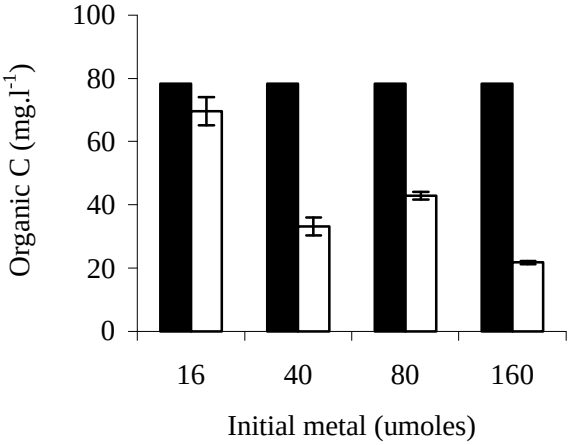

Figure 7.34. Levels of organic carbon in solution at pH 5 before (**O**) and after (**G**) addition of varying amounts of Cu^{2+} ions. The organic carbon was produced by *Spirulina* spp. grown under stress conditions.

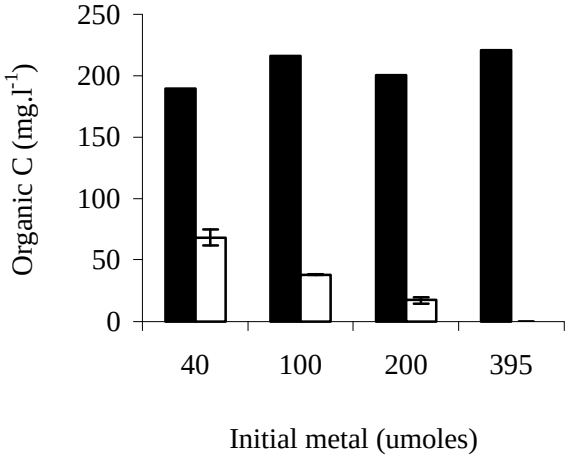

Figure 7.35. Levels of organic carbon in solution at pH 5 before (**O**) and after (**G**) addition varying amounts of Cu^{2+} ions. The organic carbon was produced by *D. salina* grown under stress conditions.

7.5 CONCLUSION

This study has demonstrated the operation of a number of metal removal mechanisms operating in micro-algal systems, each of which can be integrated into a system for the remediation of metal-rich wastewaters. The use of sulphide-rich liquors from sulphate reducing anaerobic digesters to precipitate heavy metals from solution showed positive results, with metal removal being obtained in excess of that anticipated from stoichiometric sulphide precipitation. This was especially true when sulphide-rich liquor from a sulphate reducing digester fed micro-algal biomass was utilised, with between 400 and 3000 times more removal being achieved than anticipated.

The removal of metals by micro-algal biomass, although not excellent, is quite capable of dealing with small amounts of metal ions, such as those in the overflow from the facultative pond. Metal removal was less effective when anions such as dichromate, and oxyanions such as selenium were used. However, unlike copper and dichromate which proved to be highly toxic to both species of micro-algae, selenium and lead did not have much effect on the growth of *D. salina* and *Spirulina* spp., as shown by an increase in their chlorophyll *a* levels. The toxicity of the metals to algal growth demonstrated here could be a problem, especially in the operation of a continuous system.

Metal removal by binding to extracellular organic carbon produced by algae grown under stress conditions was shown to be very successful, especially at acidic pH values. Mechanisms for the removal of heavy metals from acidic effluents have not previously been reported, thus making this very important especially in cases where the pH of acidic effluents cannot be elevated by biological means.

The incorporation of the metal removal mechanisms into the algal integrated ponding system for the treatment of AMD proposed in Chapter 1 could be used in a number of ways:

1. The sulphide-rich anaerobic digester liquors can be recycled and blended with the incoming AMD, to initiate neutralisation and metal precipitation outside of the anaerobic digester, in a controlled environment.

2. Where very large volumes of wastewater need to be treated, metal precipitation can take place in the anaerobic digester.
3. Metal removal can take place in a HRAP, with removal due to both binding to micro-algal cells as well as extracellular polymeric substances produced by the micro-algal biomass. The micro-algal biomass and extracellular polymeric substances, together with the bound metals, can be fed to the anaerobic digester, serving as a carbon source for biological sulphate reduction. The dissociation of the metal from the micro-algal biomass and polymeric substances will take place in the anaerobic reactor, with the subsequent formation of metal sulphides *in situ*. The HRAP can also serve as a final polishing step for the overflow from the facultative pond.
4. The carbonate-rich alkaline water produced as a result of the pH elevation of acidic effluents, can be used to neutralise the incoming AMD, thereby precipitating heavy metals prior to its discharge into an anaerobic digester.

CHAPTER 8. GENERAL CONCLUSION

Previous studies on the use of algal integrated ponding systems for the treatment of tannery effluents, and the high levels of sulphate reduction and metal precipitation observed, had suggested that these systems could be used for the treatment of metal- and sulphate-rich wastewaters. In order to demonstrate that such a system could operate as an integration of several distinct unit operations, a number of functional parameters needed to be evaluated. Studies were undertaken as laboratory and pilot scale investigations.

1. The use of complex carbon sources as energy for biological sulphate reduction

Both tannery effluent and waste grown algal biomass were evaluated and shown to function as carbon sources, with efficient rates of sulphate reduction recorded for both. The manipulation of algal growth to enhance the organic carbon content of the algal biomass was also investigated. Light, temperature and salinity were found to have a large role to play, with *Dunaliella* and *Spirulina* spp. requiring different environmental conditions to induce organic carbon production. The practical implication of these results was discussed in Chapter 4, with the integration into the system of an algal pond for growth and stress manipulation.

2. The role of algal ponding in the treatment system

The integration of algal ponding into the system was considered in terms of the secondary treatment of the overflow from that facultative pond, as well as the recycle of oxygen rich water and algal biomass onto the top of the facultative pond. In order for this to be viable, the algae need to be able to grow in the presence of sulphide. This was evaluated in Chapter 5 and shows *Spirulina* spp. to be a sulphidophilic species of alga, capable of tolerating up to 300 $mg.l^{-1}$ of sulphide. *D.salina* on the other hand was less tolerant to high sulphide levels but proved competent in surviving sulphide levels reflective of those occurring in the latter stages of treatment, where *D.salina* would predominate. A further function of algal ponding in the treatment of AMD is related to the ability of algae to elevate the pH of their surrounding environment. This mechanism, which is related to their ability to grow under conditions of low CO_2, was found to be operational in both *D.salina* and *Spirulina* spp., as well as in species of *Anacystis* isolated from drainage from an abandoned coal mine. It is dependent on an initial

amount of bicarbonate ions being present and does not function at pH values below which the predominant inorganic carbon source is CO_2. The ability of algae to successfully elevate the pH of AMD serves as both a neutralising function as well as contributing to the removal of heavy metals.

3. Metal removal in the integrated system

The removal of metals from the effluent was found to be a function of both sulphide precipitation and algal mediated removal. Use of the sulphide-rich liquors from the anaerobic digesters fed with algal biomass and tannery effluent for metal precipitation proved very successful, with metal removal obtained in excess of that expected from stoichiometric sulphide precipitation. This was more pronounced when liquor from the digester fed algal biomass was used, possibly due to binding to organic matter present.

Metal removal due to binding to algal biomass, although not extremely successful at high concentrations, showed that it could cope with low metal concentrations, possibly in a secondary treatment step to remove residual levels of metal after passage of the effluent through the sulphide precipitation step. A factor which could play a role in determining the use of algae in metal removal functions, is the toxicity of the metal ion to the algae. An investigation into this revealed that both copper and dichromate ions are very toxic to the growth of *D.salina* and *Spirulina* spp., with lead and selenium less so. This is important for the operation of a continuous treatment system. A way of overcoming this toxicity would be to prevent direct contact between the algal biomass and the metal-rich effluent. The use of the extracellular organic carbon matter produced by algal cells under stress conditions has a role to play in this respect. Results indicate that metal binding to this organic matter was very effective at low pH, but less so under alkaline conditions where hydroxide and carbonate precipitation outcompete it. This is extremely important as a biological method for metal removal from effluents at acidic pH has not previously been reported and will negate the need to elevate the pH of the effluent unnecessarily.

It is against this background that certain amendments to the system proposed in Chapter 1 may be advanced. The various aspects of the ponding approach to AMD treatment may be combined

to produce the Integrated Algal Sulphate Reducing Ponding Process for Acidic and Metal Wastewater (ASPAM). This process (see Figure 8.1) could allow for the treatment of a wide range of AMD and other metal and sulphate containing wastes under different circumstances.

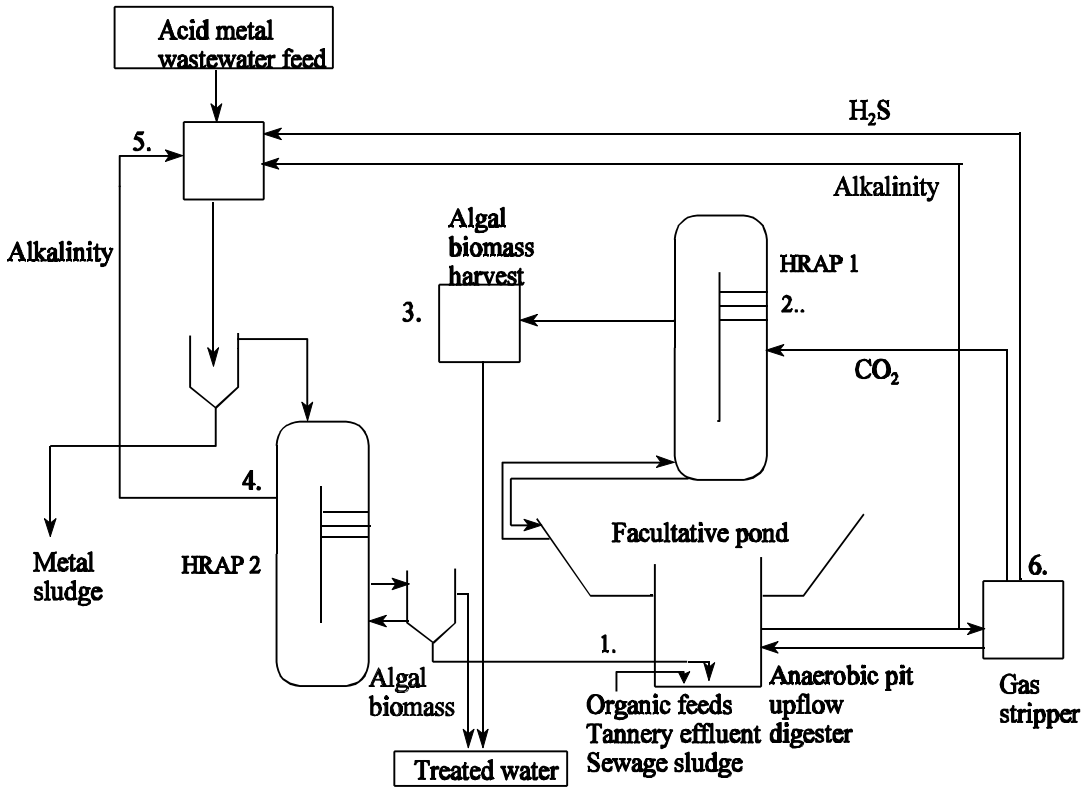

Figure 8.1. Flow diagram of the integrated unit operations of the sulphate reducing high rate algal ponding process (ASPAM) for the treatment of acid and metal pollution in mine drainage water. 1= Facultative pond with anaerobic compartment; 2= High Rate Algal Pond1; 3= Algal biomass harvester; 4= High Rate Algal Pond 2; 5= pretreatment precipitator; 6= gas stripping operation.

This system permits the separate neutralisation and precipitation of metals in the incoming flow prior to it reaching the anaerobic compartment, thus allowing for the controlled recovery of the metal precipitates. This precipitation and neutralisation can either be algal mediated, or in cases where high levels of sulphide are produced, the overflow liquor or gas (6) can be passed forward to the precipitation step (5). This offers the potential for the fine control of the selective precipitation of metal sulphide/carbonate/hydroxide complexes. This would enable the partial separation and refinement of incoming components suggesting the use of the process in the

remediation of a range of metal wastewaters in addition to AMD. The overflow liquor from the facultative pond can serve as an inorganic carbon source for the growth of algal biomass (2). The algal biomass produced in this way has two functions: firstly it can be used as overlay on top of the facultative pond thus preventing odour nuisance and secondly it can be harvested and used as an inoculum for HRAP2 (3). HRAP 2 serves to neutralise the incoming mine water while at the same time providing algal biomass for use as a carbon source for biological sulphate reduction (4). Other carbon sources may also be used, including tannery effluent and sewage sludge (1). Furthermore, CO_2 produced in the anaerobic digester can be stripped and fed into the HRAP thus preventing the pH rising to levels inhibitory to algal growth.

The studies reported here were designed to demonstrate the feasible operation of individual unit operations of the process. However, successful operation of a full-scale AIPS system treating tannery effluent needs some confidence to proceed to scale-up evaluation of the integrated process. Several aspects elucidated in this study are being further researched at Rhodes University and themselves may lead to further processes. These aspects include the following:

1. The elucidation of hydrolysis mechanisms operating in digesters has come to be recognised as important to the optimisation of the process, especially when complex organic carbon sources are utilised, and is the new subject of a separate doctoral study.

2. The assessment of sewage sludge as a carbon source for biological sulphate reduction is presently being carried out at a pilot plant operation located at the Grootvlei Mine, Springs, South Africa, and has formed the basis for a separate doctoral study.

3. Laboratory scale studies to investigate sulphate reduction rates utilising settled sewage sludge as a carbon source are being carried out at Rhodes University. The enzymes involved in the hydrolysis and fermentation process are being elucidated, as well as metal removal efficiency using the overflow from the digesters.

4. The adaptation of this process for application to the treatment of a zinc refinery wastewater is being investigated, with the emphasis on the selective recovery of certain precious

metals.

5. The utilisation of the alkalising function of algae for the treatment of numerous industrial effluents is also under investigation. The elucidation of the chemistry of the both the alkalisation and metal precipitation function of algal systems, as well as metal sulphide formation, is also being further researched.

6. The oxidation of excess sulphide produced in such systems with the production of sulphur as a by-product, is being researched.

The study reported here has attempted to demonstrate the utility of the waste stabilisation ponding process, providing an established reactor technology for the treatment of large volume AMD flows, and the feasibility of linking co-disposal of organic waste streams with AMD effluent treatment. The proposed ASPAM process is apparently the first reported use of waste stabilisation ponding technology for the treatment of AMD.

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WASTE STABILIZATION POND TREATMENT OF TANNERY WASTEWATER: OPERATION, PERFORMANCE AND MICROBIAL ECOLOGY

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INTRODUCTION

Waste Stabilisation Ponds (WSP) have been constructed for the treatment of tannery wastewaters (Rowswell *et al*., 1984; Pearson, 1996), and have been operated either as zero discharge terminal evaporation systems or for the partial treatment of effluent before its release. Despite the strategic significance of the technology in this application, few studies are reported in the literature which deal, in any detail, with the tannery wastewater WSP application (Shuttleworth, 1978; Rowswell *et al*., 1984; Rose *et al*., 1996). Torres *et al* (1997) have noted that a lack of understanding of the internal hydraulic performance of the WSP accounts, in part, for malfunction in these systems. Pescod (1996) has drawn attention to the complex interaction of factors operating in the WSP and Middlebrooks (1987) has noted the critical need for fundamental studies on the complex physical and biological factors operating in these systems in order to derive the simplyfying assumptions required for more reliable mathematical modelling. A three-year study of a WSP treating tannery wastewaters was undertaken and results reported here detail characteristic aspects of the microbial ecology of the system. Principal factors influencing the operation of the treatment process were identified and an attempt was made to describe how these affect the performance of the tannery WSP.

MATERIALS AND METHODS

A subjective grab sampling protocol was used to draw the samples across the WSP cascade from Ponds A to 11. An objective sampling protocol was followed for the vertical sampling of pond strata with samples being drawn at various depths from fixed points on a grid drawn on the pond site plan, regardless of upwelling, microalgal rafting and other circumstances prevailing on the pond's surface at the time of sampling. Analytical procedures followed A.P.H.A. Standard Methods (1989). Measurements of photosynthetic productivity were also made at the grid points noted above using the [^{14}C]-sodium bicarbonate CO_2 fixation method described in A.P.H.A. Standard Methods and modified by Oren (1992). Sample measurement was undertaken in a Beckman LS3150T scintillation counter. Salinity was measured using an Atago salinity refractometer. Bacterial identification was based on descriptions in Bergey's Manual of Determinative Bacteriology (Stanley *et al*., 1989). Eucaryotic algae and cyanobacteria (referred to collectively as microalgae in this text) were identified using type strains from the Culture Collection for Algae and Protozoa (CCAP). Chlorophyll *a* was measured according to the method of Lichtenthaler (1987). Photosynthetic bacterial pigments were extracted into 100% acetone and absorbance measured in a Shimadzu spectrophotometer. Biogas was collected through a tube attached to an inverted cone located above the floor of Pond A and gas analysed in a Chrompakgas chromatograph.

SYSTEM OPERATION

The WSP system operated by the Mossop-Western Leathers Co in Wellington, Western Province South Africa, and commissioned in 1964, is a 13.6 ha cascade of 15 ponds with a total capacity of 197 000m³ (Fig.1). Daily production averages 1500 hides processed to wet blue leather with an effluent load to the ponds of about 460 m².day. The raw effluent is segregated in the tannery into alkaline lime-sulphide and acidic tanning liquor streams and passes through pre-treatment including sulphide oxidation, blending and aeration followed by flocculant assisted settling of a portion of the solids fraction. Effluent enters the WSP at Pond A which, with surface aerators, operates as a Primary Facultative Pond, flows through B to Pond 5 (which are functionally anaerobic) where it is pumped from a collection sump to Pond C and then flows to Pond 10. Pond 11 serves as a sink for final concentrates and storm-water runoff from the site and Ponds C to 11 operate as Facultative Ponds. The cascade functions as a zero-discharge process in an area where the hot, dry summers of a Mediterranean climate enable the operation of a positive evaporation balance in the ponds. While the study documented here relates to the investigation of the Wellington plant only, the broad trends which are described were also noted in separate observations of a tannery WSP operated by East Cape Tanning Co. in Uitenhage, Eastern Province, at Okapuko in Namibia and in a number of tannery ponds observed elsewhere.

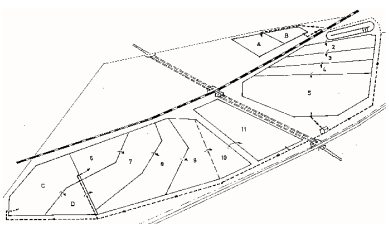

Figure 1. Plan diagram of the Waste stabilisation Pond tannery wastewater treatment operation at mossop-Western leathers Tannery in Wellington showing the effluent treatment flow path from Pond A through Pond 5 and from the collection sump to Pond 11.

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WATER COLUMN IN ANAEROBIC PONDS

Table.1. Analysis of anaerobic pond water column. Samples were drawn at three levels through anaerobic ponds at each of the seasons over the three year study period and averaged results reported.

	Surface	0.75m	1.5m
PH	8.63	8.64	8.66
Dissolved Oxygen (mg.l^{-1})	< 0.01	< 0.01	< 0.01
Chemical Oxygen Demand (mg.l^{-1})	760	841	1074
Sulphide as Na_2S (mg.l^{-1})	20	25	30
Sulphate as SO_4 (mg.l^{-1})	759	719	667
Ammonia as NH_4 (mg.l^{-1})	54	61	65

In addition to the subjective grab sampling programme, the results of which are reported above, a programme of water column sampling, at fixed stations on a pre-determined grid in each pond was also carried out over the 3 years of the study. These results are summarised in Table 1. The surface of Pond A is mechanically aerated with an oxypause at 0.5m depth providing two clearly defined compartments. It operates effectively as a Primary Facultative Pond. Effluent inflow to the base of the pond (4m depth) is subjected to active anaerobic digestion. The loading rate of around 1500kg.ha^{-1}.day^{-1} is more than twice the recommended rate for anaerobic ponds, and the approximately 45% reduction in COD effected in this unit is somewhat less than the 60-70% reduction reported by Oswald et al. (1994) for fermentation pits incorporated into Advanced Primary Facultative Ponds and treating domestic sewage. Sulphide/sulphate, ammonia and COD levels show a modest increase with depth and thereby indicating that, while some oxygen is available at the surface, DO is close to zero, and more stringent anaerobiosis prevails at the lower levels of these ponds. An average 85% COD reduction is achieved through this stage of the system which is consistent for anaerobic pond operation reported by Pescod (1996).

MICROBIAL ECOLOGY

Well-defined, and rather dramatic, colour changes occur across the pond cascade which also correlate quite closely with rising salinity, DO and pH levels (Figure 2). The brown/grey colour of the effluent and Pond A contents changes to a bright pink/dark purple in the anaerobic Ponds 1 to 5. These ponds are dominated by large populations of purple sulphur bacteria, various *Chromatium* spp., and purple non-sulphur bacteria including *Rhodobacterium* spp. Green sulphur bacteria of *Chlorobium* spp. occur in smaller numbers with the occasional observation of *Dunaliella salina* and *Dunaliella viridis*, depending on season. Bacterial photosynthesis and lithotrophic metabolism result in the oxidation of sulphide at the pond surface which manifests as a film of white elemental sulphur which, at times, may blow into thick windrows against the pond levees. Sulphide oxidation results in the pronounced elevation of sulphate levels through Pond 5.In Pond 6 the colour changes abruptly to dark green due to the blooming , at times, of near monocultures, of the cyanobacterium *Spirulina* sp. (see Figure 3). As the salinity rises through Ponds 9 and 10 numbers of *Spirulina* decrease and small mixed blooms of the halophilic chlorophytes *Dunaliella salina* and *Dunaliella viridis* occur. Photosynthetic oxygen production, and the elevation of DO and alkalinity levels commencing Pond 6, results in sharply reduced sulphide and ammonia levels and also a noticeable reduction in odour release.

Figure 2. Aerial photograph of the Waste Stabilisation Pond tannery operation at Mossop-Western Leathers Tannery in Wellington showing the change in colour across the pond cascade.

Figure 3. Photograph of rafts of the cyanobacteria *Spirulina* spp. forming in Pond 6.

PHOTOSYNTHETIC ACTIVITY OF POND SYSTEM

The photosynthetic productivity of the ponding system was estimated on the basis of carbon fixation values recorded in ponds over the various seasons and are reported in Table 4. While mid-summer fixation rates approach 15 g C.m^{-2}.day^{-1}, which are in the upper range reported for fully optimised sewage High Rate Algal Ponds (Oswald, 1996), winter values fall to about 4 g C.m^{-2}.day^{-1}, providing an annual average production of around 7.5 g C.m^{-2}.day^{-1} across the 4ha of the Facultative Ponds.

It is thus apparent from the above that total *Spirulina* biomass generated through the facultative component of the system may reach a level of about 110 ton.year^{-1}. This translates into an estimated oxygen production in the system of around 165 tons O_2.year^{-1} or a solar energy conversion equivalent to mechanical oxygenation of 165 000kW hrs (based on values provided by Oswald, 1988b).

CONCLUSION

The observation of substantial and sustained blooms of *Spirulina* in the tannery WSP, and the relatively high productivity values which have been reported, was a surprising outcome of the study. Subsequent observations of other tannery WSP systems indicate that the occurance of *Spirulina* at a specific point along the chemical, physical and biological gradients, which establish across these systems, is a particular and characteristic feature of the tanning WSP environment. The authors have not found this phenomenon previously documented or explained. The growth of *Spirulina* clearly determines the aerobic status of the latter ponds and the stratification and aerobic capping provides particularly successful and stable odour control in the facultative ponds. The development of an Algal Biotechnology approach to the treatment of tannery effluent also offers an opportunity to linearise the zero-discharge WSP treatment process by providing a mechanism for the final removal of nutrients and dissolved solids from the system.

Table 2. Average *Spirulina* productivity in facultative ponds measured as CO2 fixation rates (mg C.m^{-1}.day^{-1}) over the mid-summer to mid winter period February to June.

February	March	April	May	June	Average
12585	7730	7220	6505	4250	7478

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FACTORS CONTROLLING GROWTH AND PERFORMANCE OF *SPIRULINA SPP.* IN THE OPERATION OF TANNERY WASTE STABILISATION PONDS

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INTRODUCTION

Well-defined, and rather dramatic, colour changes occur across the pond cascade which also correlate quite closely with rising salinity, DO and pH levels (Figure 2). The brown/grey colour of the effluent and Pond A contents changes to a bright pink/dark purple in the anaerobic Ponds 1 to 5. These ponds are dominated by large populations of purple sulphur bacteria, various *Chromatium* spp., and purple non-sulphur bacteria including *Rhodobacterium* spp. Green sulphur bacteria of *Chlorobium* spp. occur in smaller numbers with the occasional observation of *Dunaliella salina* and *Dunaliella viridis*, depending on season. Bacterial photosynthesis and lithotrophic metabolism result in the oxidation of sulphide at the pond surface which manifests as a film of white elemental sulphur which, at times, may blow into thick windrows against the pond levees. Sulphide oxidation results in the pronounced elevation of sulphate levels through Pond 5.In Pond 6 the colour changes abruptly to dark green due to the blooming , at times, of near monocultures, of the cyanobacterium *Spirulina* sp. (see Figure 3). As the salinity rises through Ponds 9 and 10 numbers of *Spirulina* decrease and small mixed blooms of the halophilic chlorophytes *Dunaliella salina* and *Dunaliella viridis* occur. Photosynthetic oxygen production, and the elevation of DO and alkalinity levels commencing Pond 6, results in sharply reduced sulphide and ammonia levels and also a noticeable reduction in odour release.

MATERIALS AND METHODS

A 15 pond WSP cascade (13.5 ha) operated by Mossop-Western Leathers Co. in Wellington, South Africa served as the basis for this study. Analysis of pond contents is outlined in Table 1 and Figure 1 shows the characteristic distribution pattern of Spirulina across the system. Analytical procedures followed A.P.H.A. Standard Methods (1989). Salinity was measured using an Atago salinity refractometer. Chlorophyll a was measured according to the method of Lichtenthaler (1987). Measurements of photosynthetic productivity used the $[^{14}C]$-sodium bicarbonate CO_2 fixation method described in A.P.H.A. Standard Methods and modified by Oren (1992) and sample measurement was undertaken in a Beckman LS3150T scintillation counter.

In the study of organic nutrition colony forming units were determined for each flask at the commencement of each experiment to reduce or eliminate the possible role of bacterial mineralisation of the carbon source and hence release of labelled CO_2. $[^{14}C]$-glycine (3.81 GBq. mmol^{-1}) and D-$[^{14}C]$-glucose (10.9 GBq.mmol^{-1}) (Amersham), were added to the washed cultures containing either a concentration range of unlabelled glycine and glucose (Merck) respectively. One set of cultures contained no added glucose and glycine. Flasks were incubated at 25 °C in a constant environment room. Control cultures for each treatment, were exposed to continuous illumination at 158 mol.m^{-2}.s^{-1}, while the other set was kept in total darkness. At time intervals of 5 minutes, 3 hours and 6 hours 1 ml of culture medium was removed, filter washed with sterile defined medium (x3) and the cells transferred to aqueous scintillant (Packard). Culture supernatant was likewise transferred in 100 ml aliquots to aqueous scintillant and counted in a Beckman LS3150T scintillation counter and results adjusted to disintegration per minute (dpm) values. The results reflect a triplicate mean. Zarrouk's inorganic mineral medium was used as the defined medium control (Richmond, 1986).

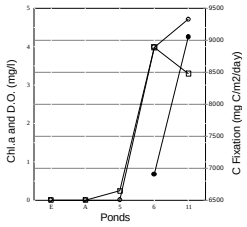

Figure 1. Distribution of microalgal biomass across the waste stabilisation pond cascade as indicated by chlorophyll a and dissolved oxygen levels, and carbon fixation rates (open square = Chl.*a*, open circle = dissolved oxygen, closed square = carbon fixation rate).

NUTRIENT STATUS

The nutrient status of the tannery effluent feed to the WSP and it's capacity to sustain *Spirulina* growth through the phases of pond treatment was evaluated on the basis of the Algal Biomass Potential (ABP) method described by Oswald (1988).

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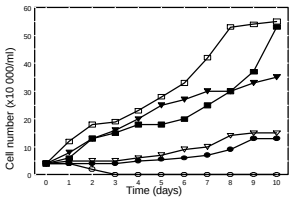

Figure 2. Growth of *Spirulina* in a dilution range of tannery effluent and in Zarrouks defined mineral medium (closed square = control in defined medium, open square = 5% effluent, closed triangle = 10% effluent, open triangle = 20% effluent, closed circle = 50% effluent, open circle = 100% effluent).

TOXICITY

The possibility that factors toxic to *Spirulina* growth may limit the organism's occurrence earlier in the pond cascade was evaluated in flask culture studies of a tannery effluent dilution series inoculated with the WSP *Spirulina* isolate. While the results of this study (illustrated in Figure 2) confirm previous observations of the complete inhibition of *Spirulina* growth in undiluted effluent, the effect is concentration related with the 50% and 20% dilutions of pre-treated effluent showing some growth, but only following a lag period of 4 to 5 days. Growth could not be sustained at all in untreated raw effluent. However, below the 20% dilution of pre-treated effluent a growth stimulation effect is observed with higher cell yields produced in the effluent dilution compared to the Zarrouk's defined medium controls. In this case no lag phase was observed.

Table 1. Analysis of effluent feed and ponded effluent at various points across the Wellington waste stabilisation ponding system.

	Effluent	Pond A	Pond 5	Pond 6	Pond 11
Total alkalinity as $CaCO_3$ (mg.l^{-1})	525	-	1246	1615	585
Dissolved Oxygen (mg.l^{-1})	< 0.005	< 0.005	< 0.005	3.98	4.71
Chemical Oxygen Demand (mg.l^{-1})	3173	1722	522	450	1677
Sulphide as Na_2S (mg.l^{-1})	1192	500	28	< 1	6
Sulphate as SO (mg.l^{-1})	364	< 1	715	1365	943
Ammonia as NH_3 (mg.l^{-1})	925	764	119	1	30
Phosphate as PO_4 (mg.l^{-1})	15	7	7	7	12
pH	7.40	8.30	8.60	9.20	9.50

POND GRADIENTS

It has been reported that a number of well defined physical, chemical and nutrient gradients establish across the tannery WSP cascade and that these could influence the observed patterns of Spirulina growth (Rose & Dunn, 1998a). These included the interaction of salinity and pH, which rise together through the system, and the concentrations of bicarbonate and phosphate and also bicarbonate and ammonia, which change with respect to one another over the course of the ponding process. The results are reported in Figures 3 and 4. Figure 3 shows that while optimum growth occurs at lower salinity levels and at pH values between 9.0 and 9.5, growth is, nevertheless, sustained at salinities up to 28 g.l^{-1} and at pH 11. The results reported in Figure 4 show an up to 5-fold bicarbonate stimulation of growth in the effluent medium demonstrating a quite severe carbon limitation which had been indicated by the APB calculation. In these studies ammonia toxicity was found to completely inhibit growth between 40-60 mg.l^{-1} without bicarbonate addition, and between 60-80 mg.l^{-1} ammonia at the bicarbonate-induced elevated growth rates.

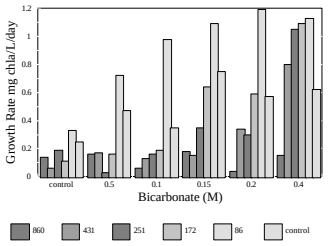

Figure 4. A matrix study of *Spirulina* growth performance in tannery effluent comparing increasing concentrations of bicarbonate and phosphate (pattern bars=phosphate mg/l)

CONCLUSIONS

1.Nutritional limitation does not appear to play a substantial role in the failure of microalgae to establish themselves in the early ponds of the tannery WSP cascade.

2. Uptake of organic compounds present in the effluent may play some role in the growth stimulatory effect observed.

3. The results of the toxicity experiments show that the presence of high levels of ammonia and sulphide in the tannery effluent act as inhibitors of Spirulina growth, and are directly involved in suppressing microalgal performance in the early stages of the system

4.The addition of bicarbonate extends the range of ammonia concentrations at which Spirulina will continue to grow.

5.The results of the flask studies indicate the possibility that Spirulina could be successfully cultivated in an HRAP paddle raceway-type application fed with pretreated tannery effluent at an appropriate loading rate,

6.A surprising finding was the enhanced growth rates and biomass yields observed for Spirulina grown in the tannery effluent medium compared to defined inorganic medium

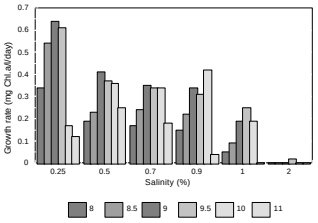

Figure 3. A matrix study of *Spirulina* growth performance in tannery effluent comparing the effects of increasing pH and salinity values. (pattern bars = pH)

ORGANIC NUTRITION

Studies of radio-labelled nutrient uptake were undertaken to determine a possible role for organic nutrition in the enhancement of *Spirulina* growth observed in the lower dilutions of tannery effluent. Figure 5 A and B report the incubation of washed cultures of the WSP Spirulina isolate, in Zarrouk's defined medium, together with $[^{14}C]$-glucose in both the absence and presence of light. The results suggest that the uptake and internalisation of the organic compound by the organism occurs during its growth in tannery effluent.

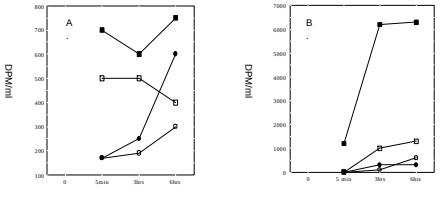

Figure 5.Uptake of D-[14C]-glucose by *Spirulina* spp. incubated in the A)dark and B) light in defined medium without the addition of unlabelled glucose and also together with the addition of increasing concentrations of unlabelled glucose (closed square = unlabelled glucose not added, open square= addition of 1mg/l unlabelled glucose, closed circle = addition of 10mg/l unlabelled glucose, open circle = addition of 50mg/l unlabelled glucose)

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