Towards a Sustainable Bioprocess for the Remediation of Acid Mine Drainage

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Abstract

Acid mine drainage is of growing concern for both developing and developed economies. Thus there is increasing pressure to develop alternative remediation strategies. Biological sulphidogenic mechanisms have long since been studied but, very few have been implemented on a large scale. Limitations are due to the inability to acquire a suitable, low cost, environmentally friendly, renewable carbon source. The present study investigated the use of an algae biomass generated by the HRAOP of an IAPS as a carbon source for the EBRU 00AB/06 SRB consortium. The algae biomass and consortium were utilized together to remediate simulated AMD. Remediation involved decreasing the sulphate and metal concentrations in solution and decreasing the acidity of a simulated AMD. Experiments were carried out to investigate the capability of the EBRU 00AB/06 SRB consortium for sulphate reduction and sulphide generation. The consortium produced colonies when grown under anaerobic conditions in Petri dishes containing modified lactate SRB medium. The SRB consortium reduced the sulphate concentration of modified Postgates medium B and generated sulphide. Further analysis of the EBRU 00AB/06 SRB consortium revealed that the consortium was minimally impacted at pH 5 and by sulphate and iron at 3 $g.L^{-1}$ and 0.5 $g.L^{-1}$ respectively. The EBRU 00AB/06 SRB consortium was exposed to Actinomycin D and Ethidium Bromide to determine whether transcription and translation of proteins was required for sulphate reduction. Results indicated that sulphide generation and sulphate reduction were inducible. Analysis of the algae biomass used in this study revealed the empirical formula $C_{1.0}H_{1.91}N_{0.084}S_{0.003}O_{0.36}$ indicating a carbon source rich in the nutrients required to sustain microbial development. Light microscopy revealed that algae cell walls and in particular those of *Pediastrum* were susceptible to acid hydrolysis. Dinitrosalicylic acid, Nile red, Bradford and Ninhydrin assays were used to determine the reducing sugar, lipid, protein and amino acid content respectively, of the mixed algae biomass. Results showed that upon exposure of the biomass to simulated AMD at pH 1 and pH 3, the concentration of reducing sugars and amino acids in solution increased. Whereas levels of lipids remained unchanged while the protein concentration decreased, indicating that, upon exposure of algae biomass to AMD, simulated or otherwise, cells ruptured, proteins were hydrolyzed and polysaccharides were broken down to sugars which are immediately available for SRB utilization. Exposure of biomass to simulated AMD revealed further that the presence of algae biomass increased the pH of simulated AMD (pH 3) to pH 7.67 after 4 d. Likewise, the pH

of simulated AMD at 1 increased to 1.77 after 2 d while pH of the neutral control increased to 8.1 after 4 d. A direct comparison between lactate and algae biomass revealed 94 % sulphate removal after 23 d in the presence of algae biomass while 82 % sulphate removal was measured in the presence of lactate. Thus the EBRU 00AB/06 SRB consortium successfully utilized algae biomass for sulphate reduction and sulphide generation. In another experiment to establish if the consortium could remediate simulated AMD (pH 5) containing 0.5 $g.L^{-1}$ iron and 3 $g.L^{-1}$ sulphate while utilizing an algae biomass as the carbon source no residual iron was detected after 14 d and by day 23, an 89.07 % reduction in sulphate was measured. The results of this investigation are discussed in terms of utilizing a readily available and renewable biomass in the form of microalgae produced in HRAOPs as an effective carbon source in the SRB catalysed remediation of AMD.

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"Determine that the thing can and shall be done, and then we shall find the way."

~Abraham Lincoln~

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List of Abbreviations

Acetyl-CoA	Acetyl Coenzyme A
AMD	Acid Mine Drainage
ANOVA	Analysis of Variance
APS	Adenosine PhosphoSulphate
ARD	Acid Rock Drainage
ASPAM	Algae Sulphate Reducing Ponding Process for Acid and Metal waste water
ATP	Adenosine TriPhosphate
BSA	Bovine Serum Albumin
CE	Controlled Environment
COD	Chemical Oxygen Demand
DMS	DiMethylSulfide
DMSO	DiMethyl SulfOxide
DMSP	DiMethylSulfonioPropionate
DOC	Dissolved Oxygen Content
DWAF	Department of Water and Forestry
EBRU	Environmental Biotechnology Research Institute Rhodes University
HPLC	High Performance Liquid Chromatography
HRAOP	High Rate Algae Oxidation Pond
IAPS	Integrated Algae Pond System
iCP-OES	Inductively Coupled Plasma Optical Emmission Spectrometer
mRNA	messenger RiboNucleic Acid
PFP	Primary Facultative Pond
RSBR	Recycling Sludge Bed Reactor
SD	Standard Deviation

SRB	Sulphate Reducing Bacteria
TCA cycle	Tri Carboxylic Acid cycle
v/v	Volume/Volume
w/v	Weight/Volume
WRC	Water Research Commission

Chapter 1: Literature Review

1.1 Introduction

Mining is an integral sector of most developing economies. It is a highly lucrative industry that has been in existence for centuries (Edwards *et al.*, 2000). Tabular conglomerates containing gold located in the Witwatersrand Supergroup in Johannesburg have been extensively mined since 1886, resulting in the generation of copious amounts of Acid Mine Drainage (AMD). Continuous exploitation of mining resources degrades large areas of land, water bodies and forest cover due to increased acidity as well as sulphate and metal concentrations. These conditions physically and chemically disrupt normal plant and animal cell function (Gordon and Robinson, 1995; Geller *et al.*, 1998; Kalin *et al.*, 2006; Das *et al.*, 2009; Gadd, 2009). Present evidence in South Africa suggests human and environmental demands for water will exceed available supplies by 2025 (Seckler *et al.*, 1999). Currently an estimated 5 000 ML of AMD is generated annually, while water is supplied at R 3 000 ML^{-1} (Odendaal, 2001; Gadd, 2009; Dlamini, 2010). Thus successful reclamation of AMD and removal of its toxic components could significantly contribute to the future recharge of South African water reserves.

Acid mine drainage (AMD) is an anthropogenically generated waste which can have detrimental impacts to the environment (Hallberg, 2009). When metal sulphides such as chalcopyrite ($CuFeS_2$), sphalerite (ZnS) and pyrite (FeS_2) are exposed to air and water, generation of sulphuric acid (H_2SO_4) and mobilization of metals occur (Cohen, 2006; Garcia *et al.*, 2008; Martins *et al.*, 2009). Metals in AMD are site specific and include Fe, Ni, Cd, Ag, Cu, As, Al, Mn, Pb, Co, Zn and many others (Bhattacharya *et al.*, 1996; Fauville *et al.*, 2004; Gitari *et al.*, 2008). Acid mine drainage predominantly arises as a by-product of mining, leachate of coal stacks, handling facilities, washeries and coal waste tips (Gordon and Robinson, 1995; Gitari *et al.*, 2008). Acid waters also arise as a consequence of construction of buildings and roads through the exposure of sulphide containing ores to water and oxygen (Zhang *et al.*, 2011). The abovementioned process also occurs as a result of biogenic sulphuric acid in sewer systems and on construction sites. The resulting acid causes corrosion and deterioration of buildings and pipelines (De Belie, 2010). In nature it develops as part of weathering or as a consequence of

volcanic eruptions. Under natural conditions it is termed Acid Rock Drainage (ARD) (Bhattacharya *et al.*, 1996; Geller *et al.*, 1998).

Remediation of AMD is possible but may be restricted by funding and availability of low cost sustainable remediation strategies (Boshoff *et al*., 2004a). Conventional treatment technologies include lime and carbonate neutralization and ion exchange (Cath *et al*., 2006). Liming and ion exchange treatments while rapid are expensive and generate secondary wastes requiring further treatment and disposal (Kurniawan *et al.*, 2006). Constructed wetlands have been traditionally utilized as biological mechanisms for remediation of waste waters including AMD (Lens *et al.*, 1995). Sulphate Reducing Bacteria (SRB) consortia are available for use as biocatalysts to clean acid waste water streams. As obligate anaerobes, SRBs are maintained under closed anoxic conditions. Sulphate reducing bacteria remediation involves sulphate removal, metal precipitation and increased water pH due to hydrogen sulphide formation and the generation of bicarbonates from oxidized carbon sources (Gibson, 1990; Colleran *et al.*, 1995; Chou *et al.*, 2008; Tang *et al.*, 2009).

Limitations to implementing SRB remediation technologies have included restrictions in the range of function of SRBs and the lack of suitable carbon sources. The rate limiting step for sulphate reduction is determined by the complexity of the organic feedstock as well as the efficiency of hydrolytic enzymes of members of the SRB consortium (Blokker *et al.*, 1998; Bond *et al.*, 2000; Blokker *et al.*, 2002). Sulphate reducing bacteria need a carbon source in order to provide the electrons required for sulphate reduction (Holland *et al*., 1987).

Acid mine drainage is an effluent containing elevated sulphates usually >1000 $mg.L^{-1}$ (Gitari *et al.*, 2008) with a pH range from 1 to 3, and an extreme example is the effluent from the Richmond Mine (California, USA). This effluent stream had a record low pH of pH -3 in the year 2000 (Hallberg, 2009). Metal and salt concentrations in excess are also common in AMD. Metal concentrations vary in ability to impact the environment. For example toxicity of the metal is dependent on type of metal and organism affected. Bacterial toxicity ranges from a few $mg.L^{-1}$ to well above 100 $mg.L^{-1}$ and some toxicity values are: Cu, 50 $mg.L^{-1}$; Zn, 110 $mg.L^{-1}$; and Fe, 500 $mg.L^{-1}$ (Martins *et al.*, 2009). A yellow precipitate termed 'yellow boy' is the universal indicator of the presence of AMD. It is caused by oxidation of iron (Fe^{2+}) to form iron hydroxide ($Fe(OH)_3$) as shown by Equation 1.1. The precipitate increases turbidity of water bodies thereby

disrupting aquatic photosynthetic activity (Gordon and Robinson, 1995; Geller *et al.*, 1998; Kalin *et al.*, 2006; Das *et al.*, 2009).

$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+$ …….Equation 1.1

1.2 **Current Status of Acid Mine Drainage Remediation**

By 2001, 23 000 km of streams had been compromised by AMD in America. A typical abandoned mine was discharging an average of 3 000 $ML.yr^{-1}$ untreated AMD (Matlock *et al.*, 2002). The Witwatersrand and Goldfields Mines have the potential to generate 350 $ML.d^{-1}$ AMD (Dlamini, 2010) and as a consequence mining companies in South Africa have been challenged to clean effluent to acceptable standards prior to release, that is, rehabilitation to a level that is not environmentally detrimental. As might be expected mechanisms adopted to treat AMD very much depend on the final water quality desired and a list of currently available methods is presented in Table 1.1 (Kurniawan *et al.*, 2006). Conventional procedures for handling AMD depend on mining activity, geographical location and the prevailing regulations. Therefore, depending on mine proximity to the coast companies may build pipelines to channel AMD offshore. For example, Lihir Gold Mine (Papua, New Guinea) has been practising this form of disposal since 1997 (Brewer *et al.*, 2007). Between 1902 and 1974 Britannia Copper Zinc Mine (British Columbia, Canada) released over 4×10^7 tonnes of tailings into Howe Sound, which is located off the Strait of Georgia (British Columbia, Canada). This practice has since been terminated (Burd *et al.*, 2008).The logic behind disposal at sea is that AMD will become so dilute, it will no longer cause significant damage to receiving aquatic environments (Gordon and Robinson, 1995; Gaydardjiev *et al.*, 1996). Conventional AMD treatment technologies include precipitation of metals with calcium and sodium hydroxide, but the resulting metal hydroxides are problematic as they contribute to toxic sludge that requires further treatment. The benefit is that valuable minerals can be recovered from the system relatively easily (Eccles, 1995). Conventional ion exchange, solvent extraction, activated carbon adsorption, cementation, reverse osmosis and evaporation are all strategies that are not viable for the remediation of large volumes of AMD, especially if a pre-treatment step is required to concentrate the influent (Gaydardjiev *et al.*, 1996). These technologies are neither economically viable nor sustainable. Sludge is still a by-product of these treatments and disposal is expensive, slow and long term environmental

implications such as water and land pollution have to be taken into consideration (Matlock *et al.*, 2002; Kurniawan *et al.*, 2006). Many countries employ rigorous regulations requiring a concerted effort to remediate all industrial wastes generated. It would appear however, that in the absence of a financial incentive there is no desire either by the mining houses or government to remediate AMD in South Africa prior to discharge. In addition, many South African researchers are reluctant to publish data regarding volumes of AMD generated by mines requiring remedial action. It therefore becomes difficult to accurately assess the level of remediation required. Since 2006, AngloCoal (Witbank, South Africa) constructed a reverse osmosis plant which is utilized to remediate AMD. Reverse osmosis generates potable water and a recalcitrant sludge. This sludge is used in the production of gypsum bricks which provides revenue depending on community interest and demand. Thus the plant not only produces potable water for reuse but also building materials contributing to community development and employment creation (Holtzhausen, 2006).

1.3 **Biological Acid Mine Drainage Treatment Technologies**

Biological treatments are reliant on activity of bacteria, fungi and plants for AMD remediation (Younger *et al.*, 1997). Biological remediation involves the exploitation and manipulation of biological entities and their ability to utilize naturally occuring processes. Advantages include reduced capital input and lower chemical and labour costs, and they tend to be sustainable. These processes are designed to have minimal detrimental impact to the environment and include examples such as phytoremediation, constructed wetlands, adsorption and bioaccumulation.

Phytoremediation utilizes plants such as *Sorghum bicolor* (sorghum) which accumulate metals from the environment into their cells (Burken, 2003). Greger *et al.* (1999) reported that *Salix viminalis* (willow) utilizing phytoextraction was able to accumulate metals such as Cd and Zn stabilizing them within its biomass. Phytostabilization employs the secretion of compounds that render toxic substances inactive. Mendez and Maier (2008) found promising results regarding the use of plants in the remediation of mine tailings. Identification of plants native to the affected area, which are also drought, salt and metal resistant, with the ability to restrict shoot metal accumulation, has limited the development of the techonology. Alevarenga *et al*. (2009) found *Lolium perenne* (perennial rye grass), was able to phytostabilize Cu, Pb and Zn in mine soils. A disadvantage is that phytoremedial processes are slower than conventional physicochemical

methods at removing metals from polluted systems. There is limited or no regeneration of plant biomass once contaminated by large amounts of metals and chemicals (Ahalya *et al.*, 2006). Phytoremediation is separated into six distinct processes as outlined in Table 1.2.

Table 1.1: Some conventional physicochemical waste water remediation mechanisms (Cath *et al.*, 2006; Kurniawan *et al.*, 2006).

Technology and Process Description
Chemical Precipitation Addition of lime/peroxide for metal removal • Large quantities of reagents are required • Expensive
Ligands (Benzandiamidoethanethiol) Bind to metals and eliminate production of secondary wastes • A stable metal-ligand precipitate is generated that is insoluble in organic and aqueous solvents as well as pH range pH 0 to14 • Precipitate is recalcitrant in the environment
Coagulation and Flocculation Deposition and aggregation of metals using pH adjustment • Saturated waste water required • Zn and Mn are decreased to 5 $mg.L^{-1}$
Flotation Dispersed air flotation, dissolved air flotation, vacuum air flotation, electroflotation, biological flotation Separation of solids and liquids using bubble attachment • 98.6 % heavy metal removal from solution • Inexpensive and effective
Membrane Filtration Ultrafiltration, nanofiltration, reverse osmosis, forward osmosis Removal of solids in solution based on size, surface electrical charges, pressure and osmotic gradient • Membranes clog • Membranes are expensive to replace (ideally replaced every 5 years)
Electochemical Treatment Electrodialysis ➢ Electrochemical potential applied to an ion exchange membrane that ionized particles move through Electrochemical Precipitation ➢ Electrochemical potential causes precipitation • Expensive • Concentrated waste water required (>2 000 $mg.L^{-1}$) • Sludge produced
Adsorption Binding by physical and chemical interactions from solution to a solid surface • Contaminated solid surface requiring further treatment before disposal. This process may be expensive

Table 1.2: Examples of phytoremediation processes (Greger and Landberg, 1999; Burken, 2003; Mendez and Maier, 2008; Alvarenga *et al.*, 2009; Komárek *et al.*, 2010; Zhao *et al.*, 2010).

Process and Description
Phytoextraction Metals are taken up by plants and concentrated in cellular biomass
Phytostabilization Plants secrete compounds that bind to metals and decrease their mobility as well as reactivity
Phytostimulation Plants secrete compounds stimulating bacterial proliferation in rhizosphere resulting in increased microbial activity and degradation of contaminants
Phytotransformation Plants metabolize contaminants resulting in inactivation due to degradation or immobilization
Phytovolatilization Plants convert contaminants into volatile less environmentally detrimental form and release
Rhizofiltration Plants filter contaminants using their micro-roots resulting in entrapment of contaminants

Constructed wetlands are an elaborate type of phytoremediation. These areas are designed to resemble bogs and/or marshes, with an aerobic top and anaerobic bottom layer (Goldsborough and Robinson, 1996). Plants specifically introduced into these systems take up contaminating metals with nutrients. Reeds and other plants in constructed wetlands generate oxygen via photosynthesis. Oxygen is utilized by aerobic heterotrophic organisms. Surface inhabiting aerobic bacteria and bottom dwelling anaerobic bacteria break down organic compounds to produce bicarbonates. Low pH associated with AMD is neutralized and metals are further removed from the system by precipitation due to increased alkalinity (Gaydardjiev *et al.*, 1996). The aerobic layer encourages hazardous 'yellow boy' formation. However, due to system stability, it settles at the base of the wetland forming sediment (Goldsborough and Robinson, 1996). Advantages of constructed wetlands include low cost, sustainability and after a period of acclimatization substantial metal removal (Gaydardjiev *et al.*, 1996). Disadvantages include the large areas of land required for construction, lengthy time to attain optimal activity and eventual sludge disposal (Gaydardjiev *et al.*, 1996).

Bioaccumulation is the gradual build up of substances within cellular material (Srinath *et al*., 2002). Chemicals may be actively or passively taken up by an organism and, over an extended period transported to specific organelles resulting in substance accumulation. When chemical accumulation occurs at a rate faster than removal by cells, death occurs (Lengke and Southam,

2006). Processes associated with bioaccumulation include breakdown of compounds into elements, localization of metals within specific organelles, metallothionein binding (cysteine rich metal binding) protein, particulate metal accumulation, extracellular precipitation and complex formation (Srinath *et al.*, 2002). Metals are subsequently released into surrounding environments upon organism senescence and death. While biosorption is a process utilizing cell surfaces to bind metals (Srinath *et al*., 2002). Algae, bacteria and fungi retain good metal binding capacities, both alive and dead (Eccles, 1995). This may be conducted via a metabolic or physicochemical pathway. It is advantageous due to low cost, high efficiency, low toxic sludge production and in some cases valuable metals have been recovered from cellular biomass (Ahalya *et al.*, 2006)**.**

The Water Research Commission (WRC, South Africa) funded the development of feasible biotechnological concepts, strategies and technologies for waste water remediation (Rose, 2002). The IAPS was one of the processes investigated (Van Hille *et al.*, 1999; Boshoff *et al*., 2004a). Utilization of IAPS and its algae components in the High Rate Algae Oxidation Pond (HRAOP) in waste water treatment is in fact a variation of phytoremediation. Initially waste water is pumped into a Primary Facultative Pond (PFP) (Oswald *et al.*, 1957). The pond is constructed to provide an aerobic and an anaerobic layer which function in sequence. Anaerobic bacteria break down suspended, dissolved organics and remove sulphates from solution. Nutrients are made available to the aerobic layer of the pond in which a flourishing algae community develops. Photosynthesis by algae reduces and scrubs odours and increases alkalinity due to reduction of sulphate and the production of bicarbonates (Van Hille *et al.*, 1999). Metals precipitate and the resulting effluent gravitates into an HRAOP. Algae are kept in motion using paddlewheel rotation to increase surface area exposure, enhance nutrient uptake and oxygenate the water (Oswald, 1991). Further polishing may be conducted in subsequent HRAOPs depending on the water quality desired prior to discharge. Algae are subsequently separated from the water; and can be used for other purposes including biofertilizers, animal feed supplements, biofuel, and as a feedstock for fermentation (Boshoff *et al*., 2004a). The system's success at pilot scale led to subsequent modifications to allow treatment of domestic, industrial and agricultural waste water streams (Van Hille *et al.*, 1999). An example of such a modification is the Algae Sulphate reducing Pond process for Acid and Metal waste water treatment (ASPAM) (Rose *et al*., 2002). This bioprocess was specifically geared to treatment of industrial effluent utilizing SRBs. Tannery effluent, pure culture *Spirulina*, and sewage sludge were all investigated as potential

carbon sources for this bioprocess. All were found to be effective carbon sources which led to the development of the Rhodes BioSURE Process® (Rose, 2002).

A major limitation with the use of organic carbon substrates is site of production relative to proximity for use in the treatment of AMD. Transporting sewage and tannery effluent would be an expensive endeavour while cultivating pure *Spirulina* would be labour intensive and highly specialized. The Rhodes BioSURE Process® is distinct from the ASPAM process and the IAPS waste water treatment process due to a Recycling Sludge Bed Reactor (RSBR), which utilizes components of the anaerobic food chain (Van Hille *et al*., 1999; Whittington-Jones, 2000). Whittington-Jones (2000) established that up to 30 % volatile fatty acids were hydrolyzed from primary sewage sludge which served as the electron donor in biosulphidogenesis. Hydrogen sulphide generated in the process reacted with metals in waste water to form metal sulphide precipitates (Williams, 2001). Effluent was polished using the IAPS via the HRAOPs, resulting in water suitable for release into a standard municipal waste water treatment plant (Rose *et al.*, 1996; Whiteley *et al.*, 2002). However, the need for an alternative treatment mechanism arose due to an inability to generate a cheap, renewable carbon source on site to drive the remediaton of mine waste waters.

1.4 **Biology and Biotechnology of Sulphate Reducing Bacteria**

Sulphate reducing bacteria are anaerobic microorganisms capable of converting sulphate rich effluents produced by volcanoes and mining into sulphides. This is executed while utilizing simple organic compounds which provide the energy for metabolism (Jorgensen, 1982; Moosa *et al.*, 2005). The pathway is termed dissimilatory metabolism (Jorgensen, 1982; Pfennig and Widdel, 1982). Sulphate reducing bacteria include the groups *Desulfovibrio, Desulfomicrobium, Desulfobacter* and *Desulfotomaculum* (Luptakova and Kusnierova, 2005; Martins *et al.*, 2009). Sulphate reducing bacteria are found in anoxic and temperature variant environments (Jorgensen, 1982).

Sulphate in AMD passively moves into SRBs where it acts as a terminal electron accepter in the cytoplasm. Hamilton (1998) noted that sulphite, thiosulphate and nitrates could serve as alternative electron accepters but not as terminal electron acceptors (Colleran *et al.*, 1995; Hamilton, 1998; Costa *et al.*, 2009). Once inside the cell, only sulphate acts as a terminal electron acceptor after its conversion to Adenosine PhosphoSulphate (APS) (Gibson, 1990;

Gibson *et al.*, 1993). ATP sulphurylase catalyzes the combination of sulphate with ATP to form APS. Adenosine phosphosulphate is very reactive and readily forms sulphite (SO_3^-). This process is catalyzed by the cytoplasmic enzyme APS reductase (Holland *et al*., 1987). Sulphite is further reduced to form sulphide. Bisulphite reductase, desulphoviridin and desulphorubidin are cytosolic enzymes that have been isolated from *Desulfovibrio* capable of reducing sulphite to sulphide. Sulphite forms various intermediates which include metabisulphite ($S_2O_5^2$), dithionite ($S_2O_4^{2-}$), trithionate ($S_3O_6^{2-}$) and thiosulphate ($S_2O_3^{2-}$). Isolation of the enzyme APS reductase from *Desulfobulbus, Desulfobacter, Desulfococcus* and *Desulfosarcina* strains indicated that the mechanisms of sulphate reduction was fundamentally similar within SRB groups (Holland *et al.,* 1987; Gibson, 1990).

One mole sulphide produced is directly proportional to one mole sulphate reduced as depicted in Equation 1.2 (Gibson, 1990). Eight electrons extracted from simple organic compounds are donated to sulphates within a system as illustrated in Figure 1.1; eight protons are subsequently released as hydrogen gas into the surrounding environment for use in interspecies hydrogen transfer (Holland *et al*., 1987). Bicarbonates formed by completely oxidizing organic compounds as shown in Equation 1.4, neutralize excess protons in solution generating alkalinity (Jorgensen, 1982; Jalali and Baldwin, 2000). Sulphate in sulphuric acid is transformed to hydrogen sulphide, subsequently improving the pH of the system by removing protons from solution. Formation of metal sulphides in Equation 1.3 results in precipitation of metals (Bhagat *et al.*, 2004). Sulphate reducing bacteria can be classified into two distinct groups, those able to completely oxidize simple organic carbon sources and those carrying out incomplete oxidation (O'Flaherty *et al.*, 1998).

$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^-$ ……....Equation 1.2

$Metal + H_2S \rightarrow MetalS^{2-} + 2H^+$ ……Equation 1.3

$HCO_3^- + H^+ \rightarrow H_2O + CO_2$ ……..……...……..Equation 1.4

Many organic substrates have been tested as electron donors for SRBs. These include sewage sludge, tannery waste, leaf mulch, wood chips, animal manure, vegetable compost, sawdust, mushroom compost, whey, *Spirulina*, *Chlorella*, agricultural and food processing wastes and low molecular weight synthetic compounds such as acetate, lactate, propionate, oxalate, methanol, ethanol, glycerol and glucose (Boshoff *et al.*, 2004a; Boshoff *et al.*, 2004b). Polluted water

containing high volumes of sulphate i.e. 3300 $mg.L^{-1}$ and five different carbon sources specifically sewage sludge, leaf mulch, wood chips, sheep manure and sawdust resulted in 100 % sulphate removal 20 days after treatment with SRBs (Jorgensen, 1982; Liamleam and Annachhatre, 2007). Volatile fatty acids such as acetate and butyrate, as well as fatty acids like lactate, pyruvate and malate are also good carbon sources for SRBs (Whittington-Jones, 2000; Whiteley *et al.*, 2002). Alcohols such as ethanol and propanol are also efficient. Hydrogen gas also serves as an energy source for SRBs. At times longer chain sugars and longer chain fatty acids can be utilized as carbon sources. Some SRBs have been reported to be able to metabolize methane to produce carbon dioxide and water (Liamleam and Annachhatre, 2007). In freshwater sedimentary environments where the carbon content exceeds the sulphate content, methanogens are responsible for using acetate and hydrogen (Girguis *et al*., 2005). However, it has been observed that SRBs have higher affinity for acetate and hydrogen and can outcompete methanogens when sulphate is present. Sulphate reducing bacteria also produce hydrogen sulphide which is toxic to methanogens even at low concentrations (Sorensen *et al.*, 1981; Girguis *et al.*, 2005).

1.5 Carbon and Energy Sources for Sulphate Reducing Bacteria

Baars (1930) isolated, cultured and cultivated the SRB culture *Desulfovibrio rubentschickii* reporting that these SRBs were able to degrade acetate to carbon dioxide. However, Selwyn and Postgate (1959), were unable to isolate a species of SRB that could degrade acetate specifically to carbon dioxide. These authors concluded that the original culture isolated by Baars, was contaminated and/or contained commensal bacteria. Jorgensen and Fenchel (1974) reported that marine systems required degradation of acetate for complete breakdown of organic compounds. Lactate, malate and pyruvate were preferred carbon sources in the bench scale research. Alcohols such as ethanol, propanol and butanol were alternatively utilized as carbon sources. Lactate via pyruvate was converted to acetate and carbon dioxide. Oxidation of carbon sources was incomplete; resulting in acetate formation. *Desulfotomaculum acetoxidans* was later isolated from ruminant gastrointestinal tracts and observed to completely oxidize acetate to carbon dioxide at an optimum growth temperature of 37 ˚C. These bacteria are able to utilize end products of metabolism as their energy source for growth and development (Gibson, 1990).

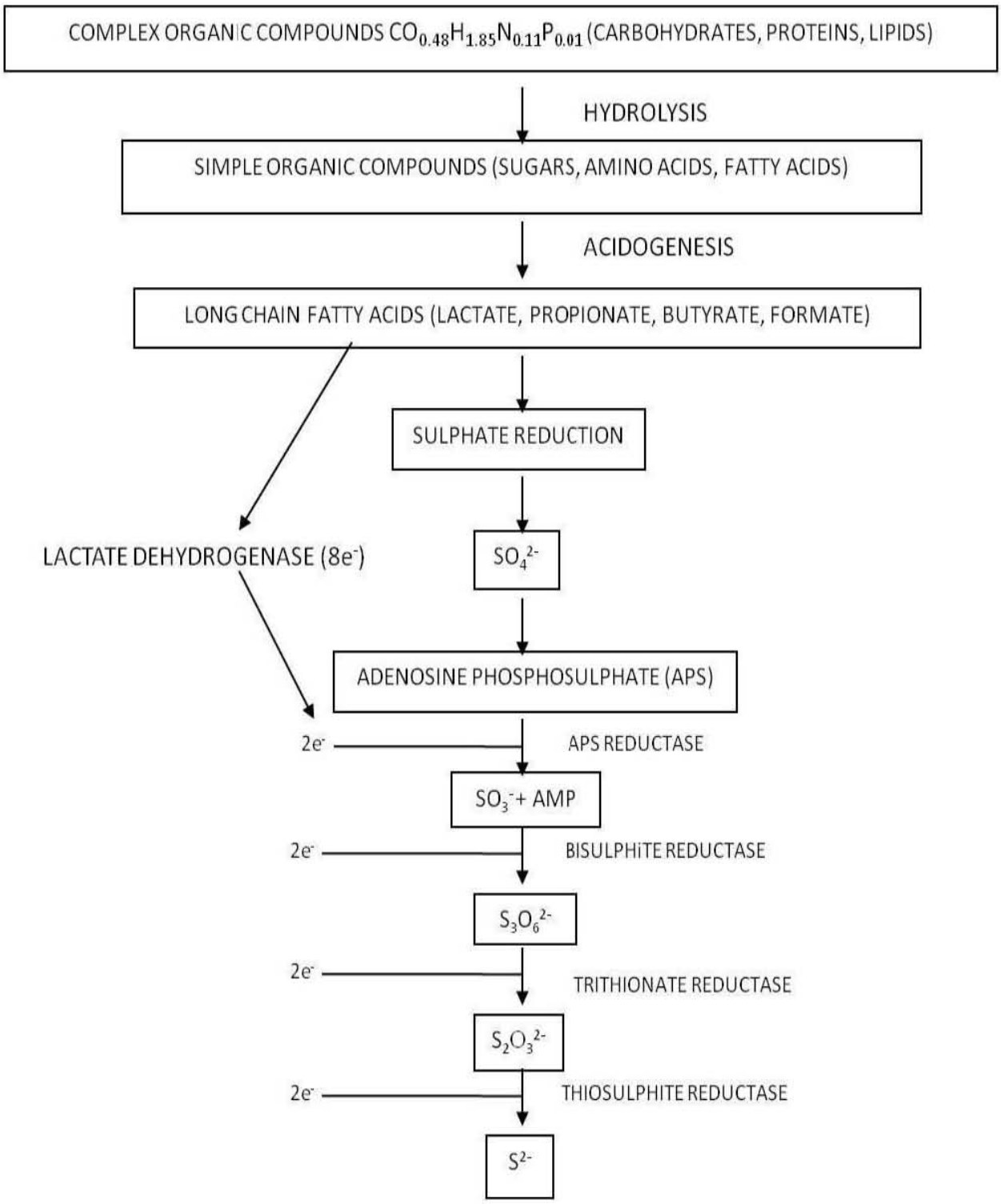

Figure 1.1: Metabolic steps and the substrates involved in the anaerobic degradation of complex organic carbon substrates (Holland *et* al., 1987; Grobbelaar *et al.*, 1990; Whittington-Jones, 2000; Molwantwa, 2002; Appels *et al.*, 2008)

Microalgae biomass with the empirical formula shown in Equation 1.5 is an ideal carbon source as illustrated in Figure 1.1 if it can be generated on site. Mine waste dumps are highly toxic and nutrient deficient and if algae were to be cultivated on site a nutritional supplement would be required. Microalgae could serve both as a useful carbon source and act as a reservoir for essential minerals like N, P, K, Mg and Zn required by SRBs. Availability of cheap and effective carbon sources has limited the large scale implementation of sulphidogenic treatment strategies for industrial waste waters (Boshoff *et al.*, 2004a). Nutrient composition of algae varies according to growth conditions and abiotic and biotic factors impacting algae, for example temperature variations can result in increased or decreased protein production or lipid content (Boshoff *et al*., 2004a). However, according to Grobbelaar *et al*. (1990) the general algae formula is:

$CO_{0.48}H_{1.85}N_{0.11}P_{0.01}$ ……Equation 1.5

Sulphate reducing bacteria demonstrate the ability to catalyze cleavage of the carbon-carbon bond in acetate using acetyl coenzyme A (acetyl-CoA). Therefore acetate oxidizing SRBs can be further separated into two groups based on their use of the TriCarboxylic Acid (TCA) cycle or acetyl-CoA pathway intermediates. Ferroxidans, flavodoxins, metaquinones, rubredoxins and cytochromes b and c are proton and electron carriers in SRBs. In *Desulfovibrio desulfuricans*, cytochrome *c3* acts as an electron carrier for hydrogenase. Ferredoxins and flavodoxin are electron carriers for pyruvate dehydrogenase and sulphite reductase; cytochrome *c553* is an electron carrier for lactate and formate dehydrogenase, while high molecular weight cytochrome *c3* and rubredoxin are electron carriers in lactate metabolism. In *Desulfovibrio* lactate is fermented to acetate and carbon dioxide in the cytoplasm of the cell (Jorgensen, 1982). The enzyme hydrogenase located in the cytoplasm of the bacterial cell converts electrons released from sulphuric acid to hydrogen. This hydrogen then diffuses across the membrane where it is oxidized by periplasmic hydrogenase. It is this hydrogen oxidation that produces protons and electrons, for ATP synthesis and reduction of sulphate with coincidental production of energy (Jorgensen, 1982; Gibson, 1990; Tang *et al.*, 2009).

1.6 **Conclusion**

Industrial activities such as coal mining produce effluents rich in sulphates and metals. Sulphates increase acidity and are a major cause of the high salt content of the industrial effluent. If this

waste water is untreated it is not fit for reuse downstream, or for industrial or agricultural purposes due to the presence of heavy metals, low pH and high sulphates (García *et al.*, 2001). The problem with high concentrations of sulphates is biogenic hydrogen sulphide production which is highly reactive, corrosive and toxic under aerobic conditions. These negative effects are pH dependent as unionized sulphide is able to move freely through cell membranes (Colleran *et al.*, 1995). Other concerns for biological reduction of sulphates are odour and safety, thus the hydrogen sulphide gas will be captured through its reaction with the reactive metals in the waste water to prevent the abovementioned concerns (Lloyd *et al.*, 2001). In an anaerobic continuous flow system, SRBs remove up to 11 % dissolved metals and 90 % sulphates initially found in AMD (Elliott *et al.*, 1998). The bacterial consortium contains microorganisms which ensure optimal SRB development (Jorgensen, 1982; Gibson, 1990). Use of SRBs in remediation of AMD is a recognized and established process (Gibson, 1990; Boshoff *et al*., 2004a; Boshoff *et al*., 2004b), but has not been implemented in large scale treatment of AMD (Van Hille *et al.*, 1999; Rose, 2002; Boshoff *et al.*, 2004a). This is due to lack of suitable carbon sources to effectively drive the system. Many developing countries are economically dependent on mining and while bioremediation does not itself generate sufficient income, development of simple, cheap and sustainable technologies for treatment of areas affected by AMD can benefit these countries in the long term indirectly improving quality of life.

1.7 **Aims and Objectives**

In view of the information presented in the preceding literature review and the current concerns regarding the quantity of AMD requiring treatment in South Africa, it seemed pertinent to investigate the use of a passive waste water treatment technology involving SRBs to remediate AMD. This was achieved by initially establishing the viability of an algae biomass as a substrate to support growth and activity of SRBs, and then by demonstrating that SRBs in the presence of an algae biomass effectively reduce the sulphate and metal content of AMD indirectly decreasing the acidity of the water.

Chaper 2: Some Characteristics of the EBRU 00AB/06 Sulphate Reducing Bacteria Consortium

2.1 Introduction

Sulphate reduction and sulphide generation are established sulphate reducing bactaria (SRB) traits and serve as indicators of the activity of SRBs within a consortium (Zagury *et al.*, 2006; Meyer and Kuever, 2007; Tang *et al.*, 2009; Ward *et al.*, 2009). Sulphate reducing bacteria include the groups *Desulfovibrio, Desulfomicrobium, Desulfobulbus, Desulfosarcina, Desulfobacter* and *Desulfotomaculum* (Luptakova and Kusnierova, 2005; Martins *et al.*, 2009). They may be isolated from gastrointestinal tracts, marine, limnic and terrestrial locations such as soil and creek sediments; domestic effluent; animal manure, as well as from industrial and mining waste waters (Jorgensen, 1982; Bhagat *et al.*, 2004; Tang *et al.*, 2009). Sulphate reducing bacteria may potentially be manipulated to generate copious amounts of enzymes thus decreasing the cellular biomass required to remediate acid mine drainage (AMD). Enzyme induction occurs when the substrate becomes available in the system (Todar, 2004). Sulphate therefore induces the production of enzymes responsible for its breakdown (Figure 2.1). These enzymes include adenosine phosphosulphate (APS) reductase, bisulphite reductase, trithionate reductase and thiosulphite reductase, resulting in the reduction of sulphate and the generation of volatile hydrogen sulphide (Holland *et al.*, 1987). However, there are other enzymes perpetually present within the cell for normal cell function.

Once formed as shown in Figure 2.1, enzymes only become inactive in substrate absence (Dubos, 1940; Willquist and van Niel, 2010). If the production of these enzymes can be controlled, it can prove vital in improving the applicability as well as the efficiency of an SRB catalyzed AMD remediation strategy. Induction of transcription would result in increased mRNA, which would consequently cause increased translation, resulting in higher levels of these enzymes in turn improving enzyme activity (Soper, 1997; Todar, 2004).

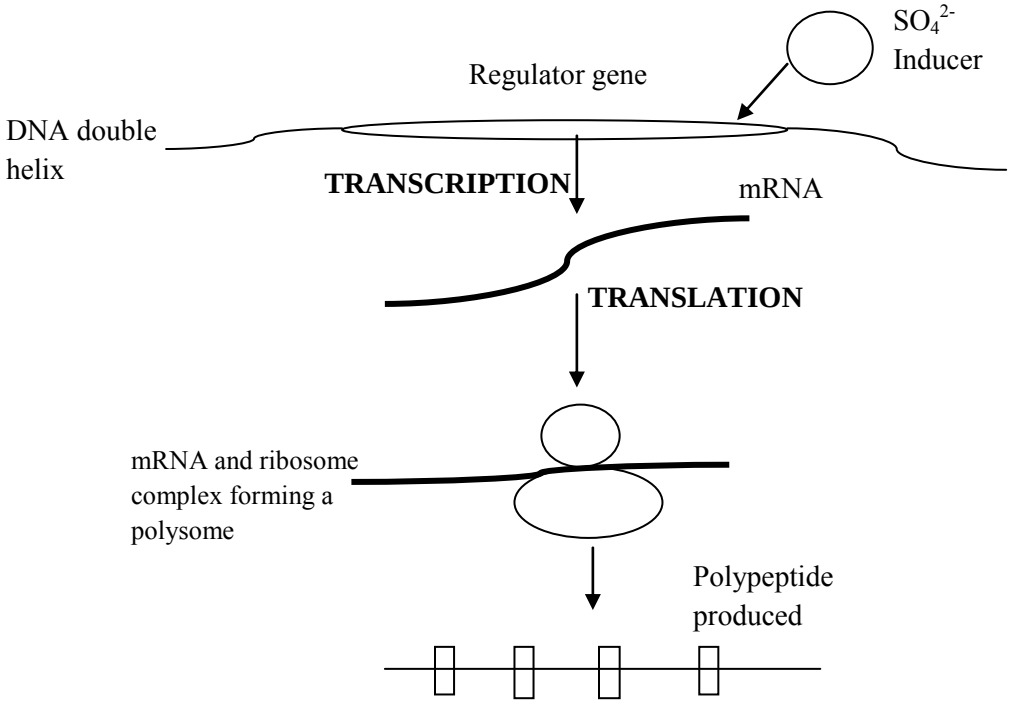

Figure 2.1: Simplified summary of major structures and processes involved in protein synthesis in a cell (Soper, 1997)

Sulphate reduction in SRBs is a highly specific reaction which occurs along a single pathway within the cell (Gibson, 1990). This dissimilatory metabolic reaction is governed by reductase enzymes as illustrated in Figure 2.2 (Holland *et al*., 1987). The pathway is termed dissimilatory metabolism because SRBs do not significantly assimilate the elemental sulphur produced into their cell biomass (Jorgensen, 1982; Pfennig and Widdel, 1982).

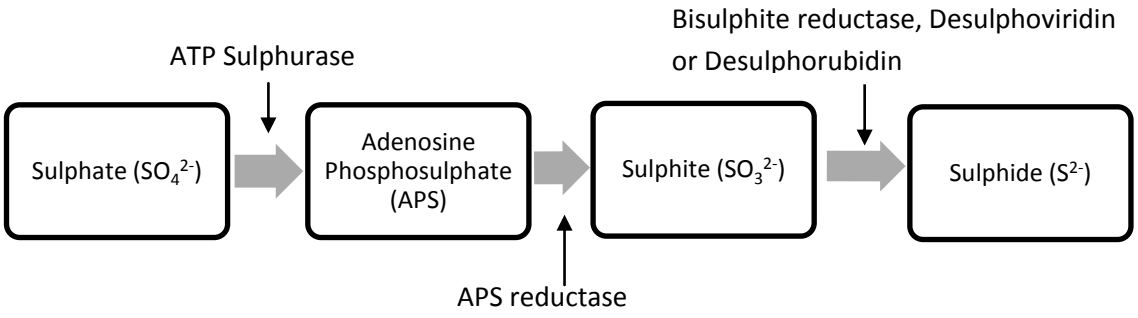

Figure 2.2: Possible sulphate reduction pathway in SRBs (Holland *et al*., 1987; Gibson, 1990; Hamilton, 1998)

Acid mine drainage varies in composition depending on the origin (Van Hille *et al*., 1999). As a result the EBRU 00AB/06 SRB consortium must be relatively tolerant to the many and varied effluent qualities that might introduced into the system. It therefore became vital to ascertain if

the EBRU 00AB/06 SRBs were able to withstand extreme conditions including low pH, high sulphates and high iron. These are toxic to many organisms at moderate levels however SRBs in consortia have been reported to withstand pH 3 (Mutambanengwe, 2006; Botes, 2010), sulphate >3 000 $mg.L^{-1}$ (Elliot *et al*., 1998) and iron concentrations >100 $mg.L^{-1}$(Martins *et al*., 2009). Sulphate reducing bacteria can become acclimatized to suboptimal conditions where they maintain minimal activity or eventually become dormant (Bhagat *et al.*, 2004).Thus it is vital to ascertain the applicability of an SRB consortium in the remediation of AMD. Inhibition of transcription through Actinomycin D binding to the DNA complex prevents the action of RNA polymerase (Tipper, and Strominger, 1965) and results in mRNA already present in the cell being translated to generate polypeptides however production of new mRNA sequences is significantly reduced (Lehninger, 1973; Borthwick *et al.*, 2005). The activity of Actinomycin D may therefore be utilized to determine the inducibility of SRB functionality through established SRB traits. Traits such as sulphate reduction and sulphide generation serve as indicators of the viability of the SRB consortium in the remediation of AMD (Kaksonen *et al*., 2006). Ethidium bromide binds to the DNA molecule and inhibits the initiation of mRNA synthesis thereby impeding tranlation. At higher concentrations it inhibits the chain elongation of mRNA already formed in the cell, thus directly inhibiting translation (Richardson, 1973). Inhibition of translation will demonstrate whether the enzymes that are directly responsible for sulphate reduction and sulphide generation are ever present in the cell or are induced by the present of the sulphate ion upon entry into the SRB cell.

The aim of the experiments described in this chapter was to further characterize the EBRU 00AB/06 SRB consortium and to separately determine its limits of tolerance to supra elevated sulphate, iron (Fe^{2+}) and extremely low pH. Furthermore it was of interest to elucidate aspects of the inducibility of the sulphate reduction function by examining the effect of inhibitors of transcription and translation on the sulphate reduction process.

2.2 Materials and Methods

2.2.1 Source of the EBRU 00AB/06 SRB Consortium

The SRB consortium utilized in this study was previously isolated from AMD (Grootvlei mine, South Africa), abattoirs and from the Institute for Environmental Biotechnology Rhodes University BioSURE® process (Grahamstown, South Africa).

2.2.2 Preparation of the Sulphate Reducing Bacteria Stock Culture

The SRB consortium stock culture EBRU 00AB/06 was cultivated anaerobically in modified Postgates medium B (Postgate, 1984) and maintained in a 10 L fermenter with a 10 % zinc acetate trap for total sulphide capture. Anaerobic conditions were imposed by purging the fermenter head space with N_2:CO_2 (80:20, %) (Afrox, South Africa) through a sealed nitrogen injection port. The head space contains gases generated by the metabolic activity of the anaerobic bacteria within the consortium. This gas is displaced into the zinc acetate trap where the hydrogen sulphide reacts with zinc acetate to generate zinc sulphide. Zinc suplhide is utilized to account for the total sulphide generated by the SRBs. The stock culture fermenter was established in a controlled environment at 30 ˚C in darkness after which the fermenter was placed on a Labcon desktop shaker at 120 rpm. Addition of fresh modified Postgates medium B was repeated regularly to ensure growth and maintenance of the stock culture. This action coincided with sampling intervals and volumes extracted (w/v).

2.2.3 Scanning Electron Microscopy

To confirm the presence of SRBs in the EBRU 00AB/06 SRB consortium, 50 µL stock culture was transferred onto modified lactate SRB medium (Atlas, 1993) agar plates using the sterile technique and placed in an Anaerocult A® in a jar (Merck, South Africa). The anaerocult A® culture is a rack with a resin that creates anaerobic conditions within the jar by absorbing all the oxygen in the system. The system takes about 5 minutes to become completely anaerobic. Anaerobicity was confirmed by the use of an indicator dye (resazurin). Resazurin turns pink in the presence of oxygen. Thus the SRBs were only exposed to oxygen for 5 minutes maximum which did not impact their viability. Modified lactate SRB medium (Atlas, 1993) is selective for bacteria able to reduce sulphate due to its high sulphate concentration >2 $g.L^{-1}$. Plates were incubated in darkness for 48 h at 30 ˚C in a controlled environment. Colonies were not placed in fresh broth prior to subculturing in order to maintain the viability of the anaerobic bacteria. Thus, immediately after incubation colonies were aseptically picked using streaking wire and streaked onto fresh sterile lactate SRB medium plates and allowed to grow for 48 h at 30 ˚C in darkness (Atlas, 1993). Colonies that grew and were amenable to conditions on the agar were extracted from the agar plates in approximately 5 mm squares using a sterile razor. Samples then underwent alcohol dehydration using the following ethanol gradient 30 %, 50 %, 70 %, 80 %, 90 % and 100 % (v/v) overnight after which critical point drying was conducted for 2 h. Samples

were then mounted on 12 mm stubs using double sided graphite tape and gold coated in a Balzars Union Sputtering Device (Balzers Union, Liechtenstein). Specimens were viewed using a Tescan Vega LMU (Tescan, USA) scanning electron microscope.

2.2.4 Viability of the EBRU 00AB/06 Consortium

An aliquot of the EBRU 00AB/06 SRB consortium stock was cultivated anaerobically in modified Postgates medium B and stored in a 1 L fermenter with a 10 % zinc acetate trap for total sulphide capture. The inoculum made up 10 % of the final 1 L volume in the fermenter.The carbon source was 6 mL sodium lactate (60 % solution, w/v). A sealed nitrogen injection port was used to purge fermenters using N_2:CO_2 (80:20 %) (Afrox, South Africa) prior to and 5 min after sampling to maintain anoxic conditions. A 60 mL syringe was used to extract 20 mL from a sealed fermenter sample port. Fermenters were kept in a controlled environment at 30 ˚C in darkness on a Labcon desktop shaker at 120 rpm and monitored. Samples were extracted from medium in the fermenters and analysed immediately in order to prevent the inaccurate quantification of volatile hydrogen sulphide gas. Analysis of the medium was conducted until a minimum sulphate level was detected. Biogenic sulphide served as the indicator of the viability of the EBRU 00AB/06 SRB consortium.

2.2.5 Effect of Inhibitors on Transcription and Translation

Actinomycin D an inhibitor of transcription and Ethidium Bromide an inhibitor of translation were obtained from Merck, South Africa and 200 µg of each was dissolved in modified Postgates medium B and after cooling applied to preequilibrated fermenters containing the SRB inoculum. The final volume in each fermenter was 1 L. A 6 mL sodium lactate (60 % solution w/v) solution served as the carbon source. Fermenters were maintained as mentioned above. Samples were extracted from fermenters as stated above and analysed immediately. Analysis of the medium was conducted until a minimum sulphate concentration level was detected. Sulphate and sulphide concentrations were monitored to determine metabolic activity of the EBRU 00AB/06 SRB consortium.

2.2.6 Quantification of Sulphate Concentration

Sulphate reduction is an indicator of SRB metabolic activity therefore, quantification of residual sample sulphate throughout the experiment was crucial. The high performance liquid chromatography (HPLC) system used to quantify sulphate consisted of a model 600 Waters

HPLC pump and model 432 Waters conductivity detector (Waters, South Africa) and was fitted with an IC-Pak ^{TM} anion 50 × 4.6 mm column (Waters, South Africa). All samples were ten fold diluted using milli-Q water prior to filtration using Watman Number 2 filter paper followed by a Waters Sep-Pak ® light C_{18} cartridge. A Waters ^{TM} 717 plus autosampler automatically injected an aliquot of the prepared samples onto the HPLC column which was eluted at a flow rate of 1 $mL.min^{-1}$using a mobile phase comprising of the borate gluconate solution described in Appendix A. A 100 $mg.L^{-1}$ sodium sulphate standard was prepared in order to establish retention time of the sulphate peak and to calibrate the method to allow for the accurate determination of the sulphate concentration within the sample. Thus the standard concentration enabled determination of sulphate concentrations in the samples by peak integration.

2.2.7 Determination of Sulphide Generation

To avoid sulphide loss due to its volatility, samples were analyzed immediately to ensure accuracy. Hydrogen sulphide is generated by the metabolic activity of the SRBs and the more hydrogen sulphide generated the greater the activity of the SRBs. Samples of 25 µL from both the medium in the fermenter and the zinc acetate trap were added to separate test tubes containing milli-Q water to a final volume of 5 mL. Sulphide concentrations were determined using Spectroquant® Nova 60 (Merck, South Africa). The test kit provided a colorimetric reaction where sulphide present in the sample reacted with dimethyl-p-phenylene diamine and iron (Fe^{3+}) to form methelene blue which is detected by a Photometer Nova 60 A Spectroquant® (Merck, South Africa).

2.2.8 Effect of Low pH and High Concentrations of Sulphate and Iron

In order to ascertain the effect of extreme conditions on EBRU 00AB/06 SRB consortium sulphate reduction and sulphide production activity, the SRB consortium was exposed to elevated sulphate, metals and low pH in 1 L fermenters containing modified Postgates medium B (Postgate, 1984). Fermenters contained sodium sulphate, which was used to give final sulphate concentrations of 3, 9, 15, 21, 27, 33 and 39 $g.L^{-1}$. This was done to determine the effect of elevated sulphate on the SRBs. Then to determine the effect of pH on the SRB consortium, the pH of the medium in the fermenters was adjusted to pH 1, 3 and 5 using 5 M nitric acid. Finally to determine effect of iron (Fe) on the activity of the consortium, fermenters containing iron (II) sulphate at iron concentrations of 0.5, 1 and 1.5 $g.L^{-1}$ were set up. In all cases except with regard

to the determination of the effect of sulphate concentration on the SRBs, the sulphate concentration was maintained at 3 $g.L^{-1}$. The EBRU 00AB/06 SRB consortium stock culture was used to inoculate each 1 L fermenter with 100 mL inoculum. Over a 30 d period, a 60 mL syringe was used to extract 20 mL from sealed sample ports on each fermenter on day 0, 10, 20 and 30 in triplicate. Samples were analysed for changes in pH, sulphide and sulphate concentration. Anaerobic conditions were maintained as stated above. Additionally to determine the effect of iron on the activity of the EBRU 00AB/06 SRB consortium and in turn the effect of the activity of the consortium on the concentration of iron in the medium, an iCAP 6000 series Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) (Thermo Electron Corporation, Cheshire, United Kingdom) was used to quantify iron in solution. Samples from the fermenters were filtered through Watman Number 2 filter paper and then diluted one hundred fold using milli-Q water after which samples were injected into the ICP-OES machine. Prior to each determination a standard curve was generated using standard iron solution (1.19781.0100) (Merck, South Africa) to obtain an equation to determine the actual concentration of iron within the samples as shown in Appendix A.

2.3 **Results**

2.3.1 Sulphidogenic Activity by the EBRU 00AB/06 SRB Consortium

Lactate SRB medium agar plates were utilized to select specifically for bacteria able to sustain themselves in the presence of elevated sulphate. The results of subculturing indicated that the EBRU 00AB/06 SRB consortium contained anaerobic bacterial cells capable of growth on lactate SRB medium agar (Figure 2.3).

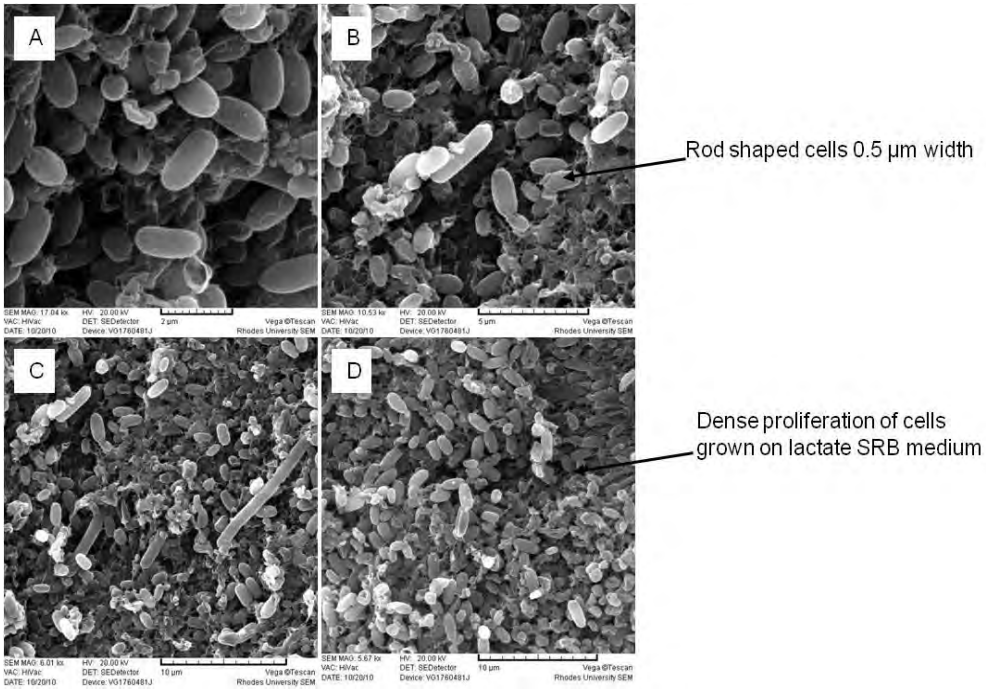

Figure 2.3: Micrographs of anaerobic bacterial cells present in the EBRU 00AB/06 SRB consortium stock culture grown on lactate SRB medium agar plates. A: Cell width 0.5 µm B: Elongated cell in presence of uniform rod shaped cells C: Cell 10 µm length width of 0.5 µm in a cluster of other rod shaped cells D: Cluster of rod-shaped cells.

The EBRU 00AB/06 SRB consortium was analyzed for sulphate reduction and sulphide generation capabilities and the results are shown in Figure 2.4. An experimental fermenter containing modified Postgates medium B was used to culture the SRB consortium. Sulphate reduction and sulphide generation were monitored as described in the Materials and Methods and revealed that a decline in sulphate concentration was associated with a concomitant increase in sulphide, indicative of SRB metabolic activity.

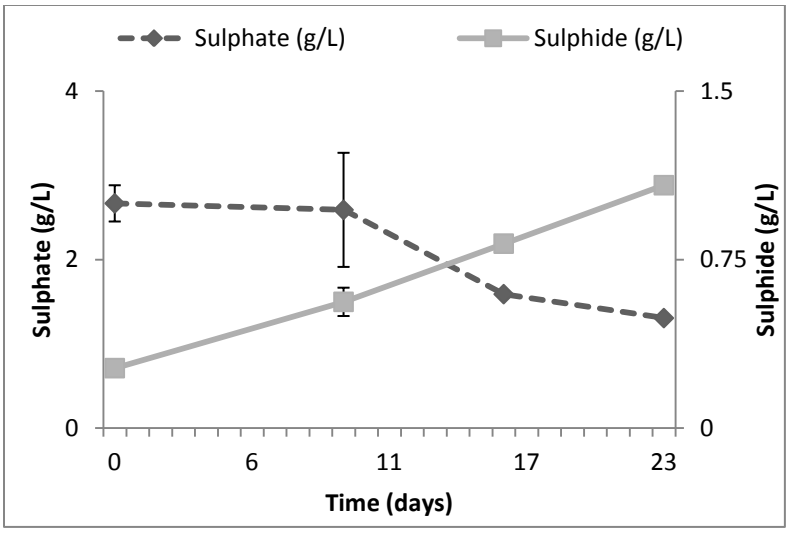

Figure 2.4: Sulphate reduction and sulphide production due to the activity of the EBRU 00AB/06 SRB consortium. Data points are the mean of 3 replicates ±SD. Experimental error may have contributed to the dramatic standard deviation observed on d 10 in the sulphate slope, leading to an inaccurate correlation to the sulphide incline.

2.3.2 Effect of Inhibitors of Transcription and Translation

To examine the inducibility of the enzyme system responsible for sulphate reduction in the EBRU 00AB/06 SRB consortium, these bacteria were exposed to medium with or without either Actinomycin D (200 $\mu g.L^{-1}$) or Ethidium Bromide (200 $\mu g.L^{-1}$). The rate of sulphate removal and of sulphide production by the SRB consortium under these conditions was monitored as described above (Section 2.3.1) and the results are presented in Figure 2.5. As might be expected, the lactate fed SRB consortium displayed optimal activity relative to both the Ethidium Bromide and Actinomycin D containing cultures. Induction of sulphate reduction was observed in cultures exposed to either Actinomycin D or Ethidium Bromide. However the presence of the Actinomycin D appeared to cause greater inhibition of the rate sulphate reduction by the SRB consortium relative to Ethidium Bromide. The presence of Ethidium Bromide in the medium impaired the sulphate reduction and sulphide generation activity of the SRB consortium relative to the activity in the control. The control contained only lactate. However, contact with sulphate in the medium, resulted in consistent sulphate reduction within both the control and the experiment. Thereby indicating the consortium's activity was not completely inhibited by the presence of Ethidium Bromide. Sulphide generation was significantly impacted by the presence of the inhibitors and a protracted delay in activity was apparent. The lactate fed culture displayed the ideal level of sulphide production relative to the the cultures treated with Actinomycin D and

Ethidium Bromide. Cultures containing the inhibitors showed minimal levels of sulphide generation.

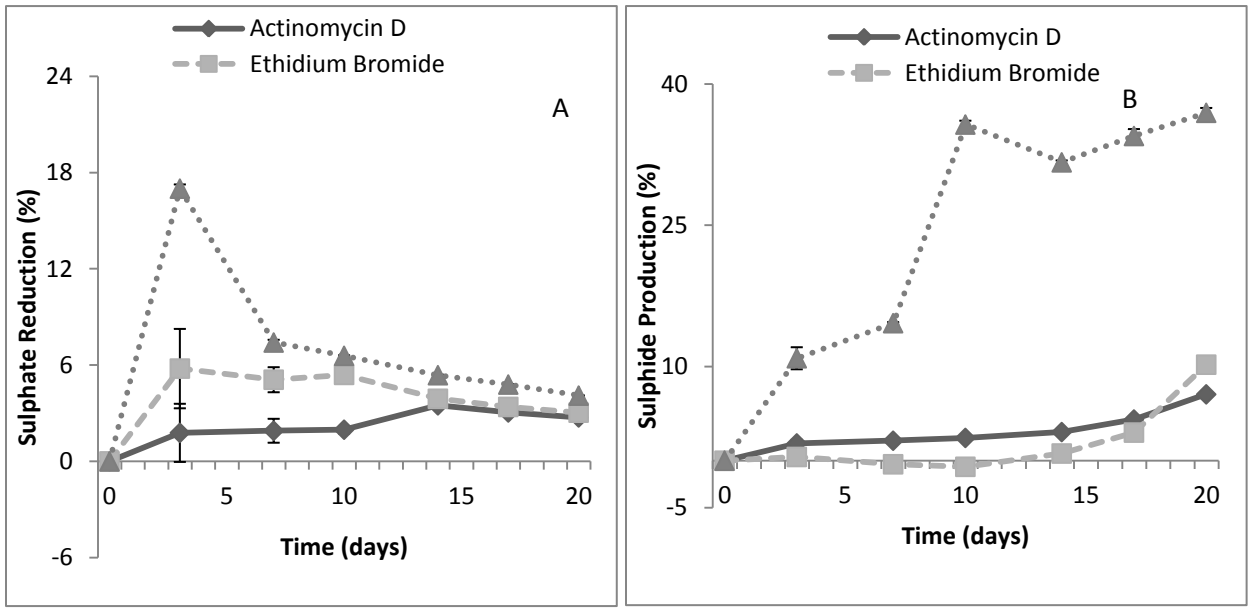

Figure 2.5**:** Percentage change in sulphate concentration and percentage increase in sulphide production by the EBRU 00AB/06 SRB consortium in the presence and absence of Actinomycin D and Ethidium Bromide. Data are the mean of 3 replicates ± SD. The pH was also monitored however the results are not displayed as the pH remained within a range of pH 7.5 to 8.6.

2.3.3 Effect of Supra Elevated Acid, Sulphate and Iron on Sulphate Reduction Activity by the EBRU 00AB/06 SRB Consortium

The EBRU 00AB/06 SRB consortium was exposed to elevated sulphate, metal ions and pH within the culture medium in order to establish its limits of tolerance. Sulphate reduction and sulphide generation were observed in all the media regardless of the sulphate concentration (Figure 2.6). However, when sulphate concentrations exceeded 3 $g.L^{-1}$, sulphate reduction and sulphide production were significantly reduced. Figure 2.6 shows that supra elevated sulphates had a negative impact on SRB activity. The consortium in the medium containing 3 $g.L^{-1}$ sulphate displayed the highest sulphate reduction and sulphide generation activity. Hydrogen sulphide is volatile, thus, its detection over 30 d within all the treatments served as an indication of the activity of SRBs. Therefore basal levels of hydrogen sulphide were detected throughout the study.

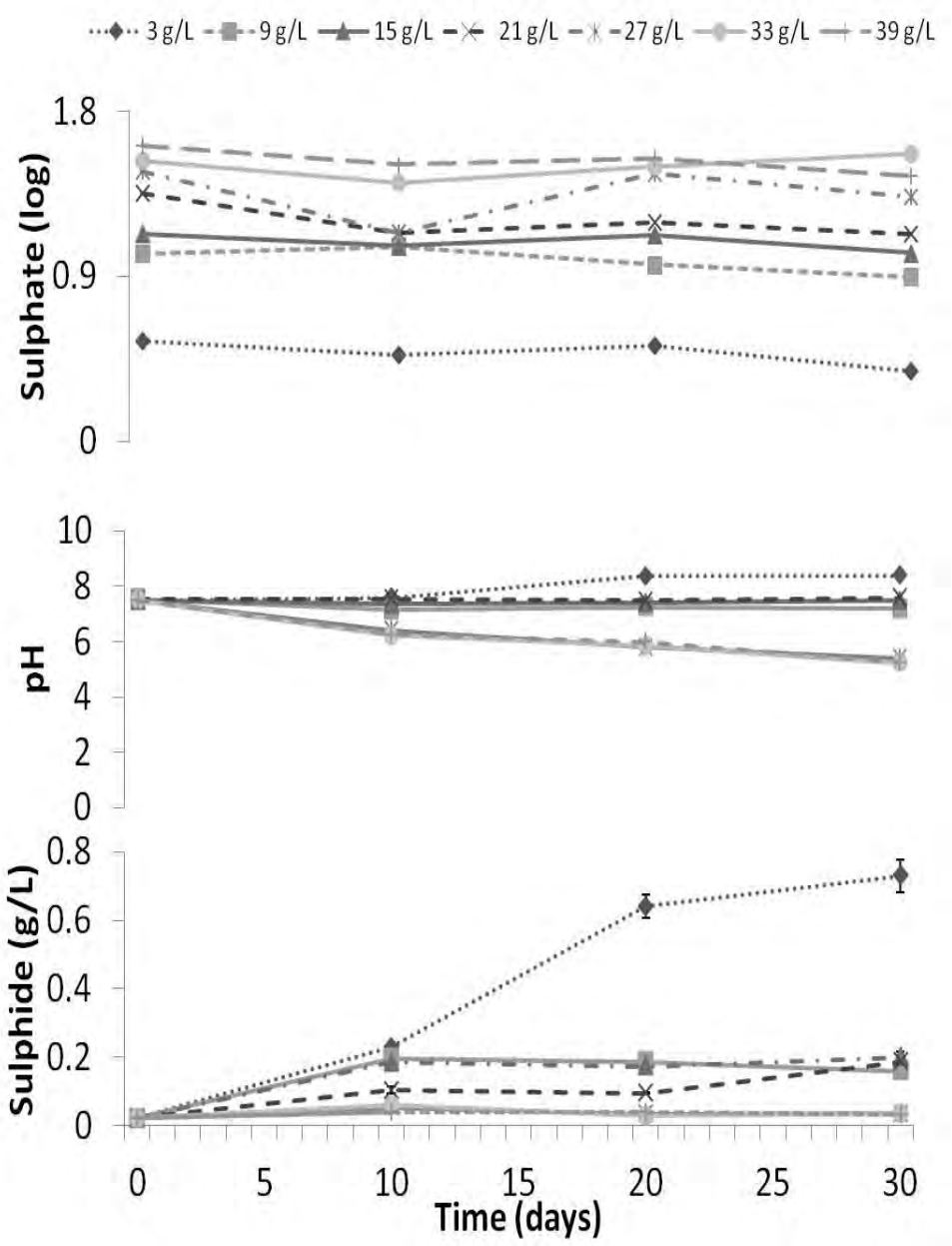

Figure 2.6: Effect of elevated sulphate concentration on sulphate reduction and sulphide production by the EBRU 00AB/06 SRB consortium and the consequent change in pH of the medium, the data points represent the mean of 3 replicates ± SD.

The starting pH of the medium determined the level of SRB activity which was observed to be significantly hindered at pH 3 and lower (Figure 2.7). The SRB activity improved at pH 5 and sulphate reduction and sulphide generation occurred. As a result, the pH of the medium increased from pH 5 to 8.6 and remained above pH 8. Though not significant, it should be noted that sulphate reduction occurred in all cultures despite the unfavourable starting pH of the medium.

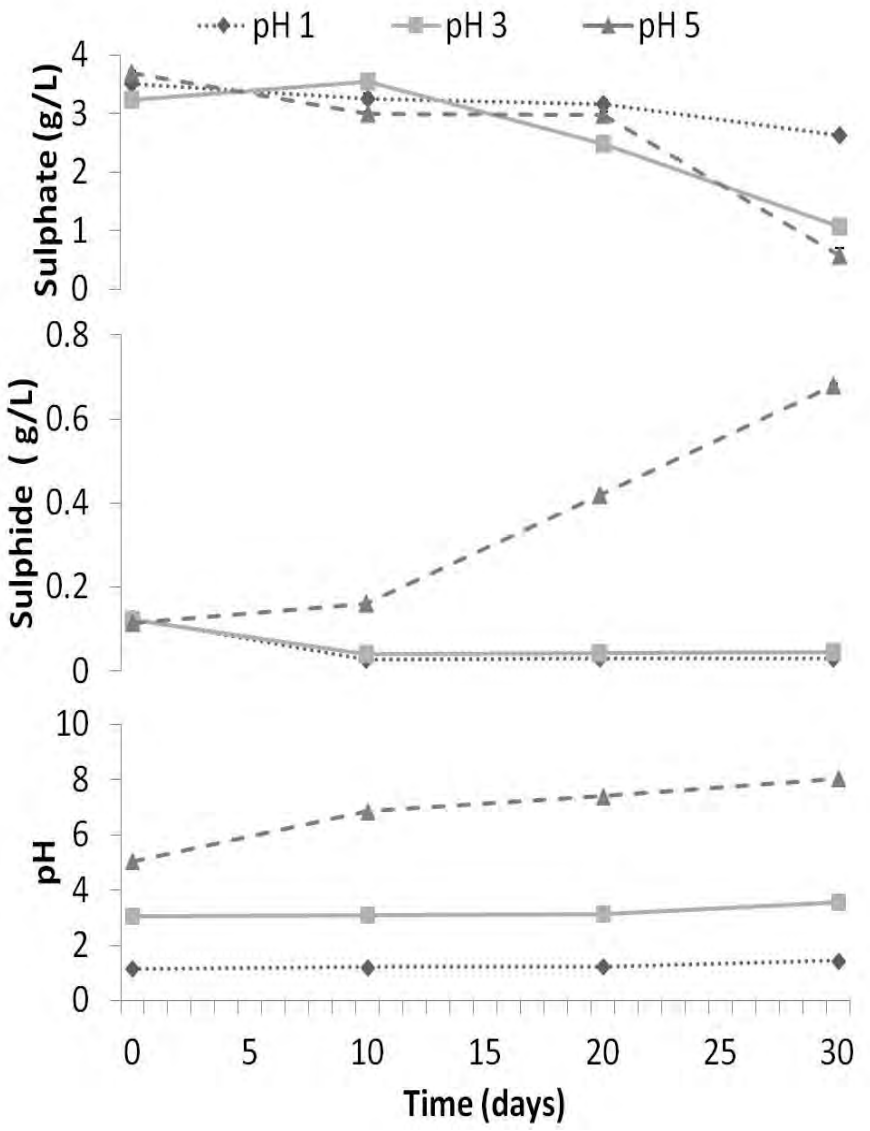

Figure 2.7: Effect of pH 1, pH 3 or pH 5 on sulphate reduction and sulphide production activity by the EBRU 00AB/06 SRB consortium and the associated change in pH of the medium. Data points are the mean of 3 replicates ±SD.

Elevated iron concentration was used to determine the effect of metals in solution on the activity of the EBRU 00AB/06 SRB consortium. The EBRU 00AB/06 SRB consortium was exposed to 0.5, 1 and 1.5 $g.L^{-1}$ iron. Sulphate reduction and sulphide generation were significantly inhibited in cultures containing 1 and 1.5 $g.L^{-1}$ iron (Figure 2.8). However, when exposed to 0.5 $g.L^{-1}$ iron the SRBs were able to generate sulphide and remove sulphates from solution. The pH of the medium also increased to above 8, which was not observed in the medium containing iron

exceeding 0.5 $g.L^{-1}$. It was however noted that all the cultures were able to remove approximately 80 % of the starting iron concentration from solution.

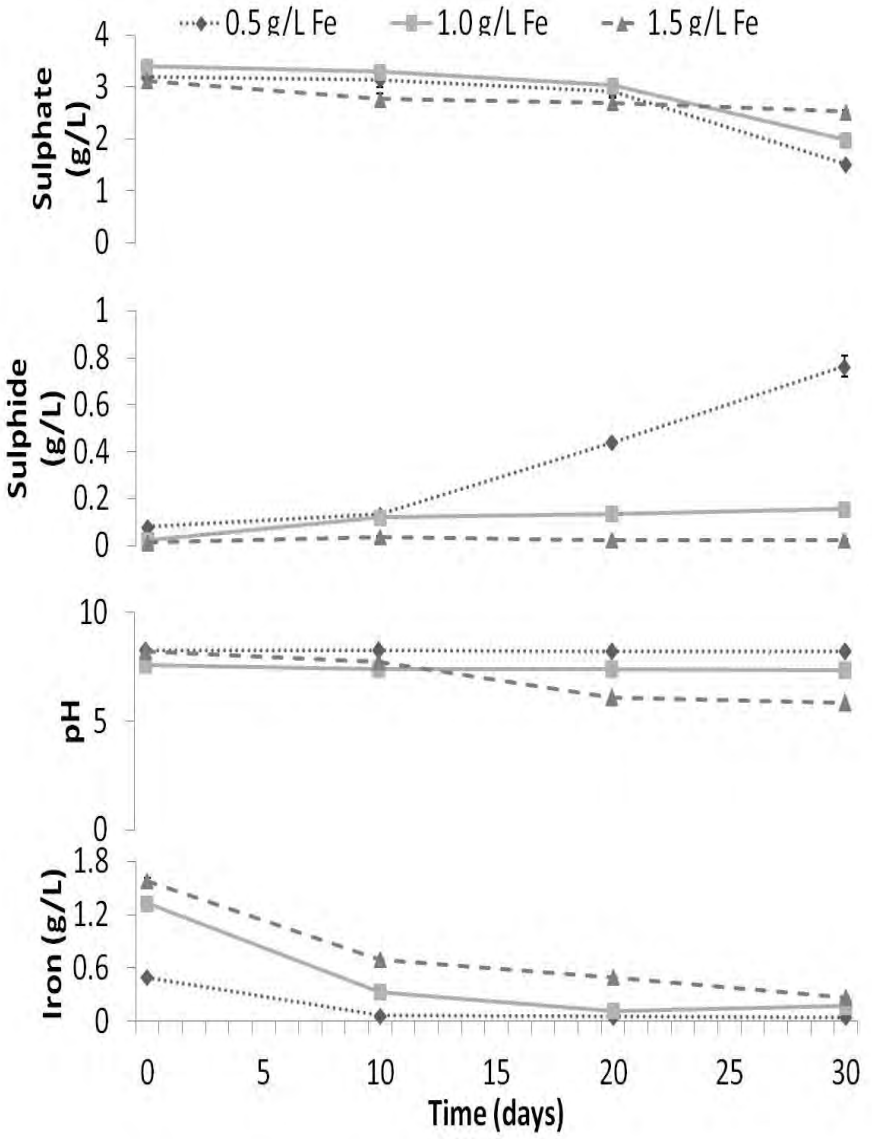

Figure 2.8: Change in sulphate, sulphide, iron and pH in the presence of elevated Fe^{2+}due to the activity of the EBRU 00AB/06 SRB consortium. Data points are the mean of 3 replicates ±SD.

2.4 **Discussion**

The EBRU 00AB/06 SRB consortium was able to reduce sulphate and generate sulphide. Sulphate reduction appeared to be substrate inducible. In addition, the EBRU 00AB/06 SRB consortium, when exposed to elevated sulphate, iron and reduced pH levels, to determine the limits of tolerance of the consortium, was able to efficiently reduce sulphates and generate

sulphides at levels generally associated with SRBs in consortium. Other characteristics of SRBs which include the ability to grow under anaerobic conditions, use sulphate as the terminal electron accepter, produce of hydrogen sulphide and an average width of 0.5 µm for the bacterial cells (Gibson *et al.*, 1993; Beech and Campbell, 2008; Tang *et al.*, 2009) were observed but not further investigated in this study.

Holland *et al*. (1987) reported that sulphate reduction is a specialized process requiring particular enzymes to carry out the conversion of this compound to sulphide. The control culture displayed optimal sulphate reduction and sulphide generation while this activity was impeded in the media containing either Actinomycin D or Ethidium Bromide. The production and activation of enzymes such as APS sulphurylase, APS reductase and bisulphite reductase occur in the presence of their respective substrates (Holland *et al*., 1987). Sulphate reduction by the EBRU 00AB/06 SRB consortium occurred at a rate faster than sulphide production in media containing the inhibitors. Actinomycin D impedes all synthesis of mRNA as well as the activity of RNA already present in the cell (Hurwitz *et* al., 1962). Abercrombie *et al*. (1973) reported that the rate of enzymatic reaction is directly proportional to the concentration of enzymes present within the cell. Thus it was noted that the presence of Actinomycin D and Ethidium Bromide impeded the enzymatic action for the terminal conversion reaction affecting the formation of hydrogen sulphide. Actinomycin D and Ethidium Bromide may have caused the accumulation of the intermediates of the sulphate reduction chain which include adenosine phosphosulphate (APS), trithionate ($S_3O_6^{2-}$) and thiosulphite ($S_2O_3^{2-}$) (Holland *et al*., 1987). However Pfennig and Widdel (1981) observed equal levels of activity from *Desulfobacter postgatei* cultures exposed to sulphite or sulphate as electron accepters while elevated activity was noted in cultures exposed to thiosulphate as the electon accepter. Thus though sulphate reduction and sulphide generation by the EBRU 00AB/06 SRB consortium may by inducible, sulphide generation was markedly negatively impacted by the presence of the inhibitors. Consequently the inhibition of sulphide production may have been as a result of the complete disruption of production of the enzymes responsible for the sulphate reduction chain, implying that these enzymes are produced in response to the presence of their substrates within the cell. Dubos (1940) noted that increased enzyme activity did not necessarily mean increased enzyme production, but rather activation of enzymes already present in the cell due to the presence of their substrate (Dubos, 1940; Babu and Aravind, 2006).

Sulphate is able to passively diffuse into the SRB cells and in elevated concentrations it can cause disruption of enzymatic functions resulting in inactivity of the cell or cell death (Gibson, 1990). At concentrations exceeding 3.1 $g.L^{-1}$ sulphates become toxic to some SRBs (Elliott *et al.*, 1998; Martins *et al.*, 2009). Elliot *et al*. (1998) reported a decline in the pH of sulphidogenic systems as an inability of the SRB consortium to reduce sulphate and generate hydrogen sulphide. Enzymatic reactions are generally pH dependent, thus enzymes will remain optimally functional within a limited pH range (Palmer, 1981). Protons present in AMD may inhibit enzyme activity by binding to and disrupting many sites located on the enzyme (Leskovac, 2003). Thus it is vital to measure and control the pH of a biologically catalysed system (Palmer, 1981; Leskovac, 2003). The EBRU 00AB/06 SRB consortium displayed limited activity in terms of sulphide generation and minimal sulphate reduction in the presence of pH 3 and sulphate concentrations exceeding 3 $g.L^{-1}$. The EBRU 00AB/06 SRB consortium was however able to sustain itself at pH 5. Gibson (1990) noted that a minimum pH of 5 was suitable for development of SRBs. At pH 5 metals are however, soluble and available to negatively impact normal cellular functionality in many microorganisms (Bell *et al.*, 2001). Iron concentrations exceeding 0.5 $g.L^{-1}$ inhibited EBRU 00AB/06 SRB activity. Metals impede functional groups on enzyme active sites inhibiting their activity. Metals further disrupt the composition of cellular membrane (Ford *et al*., 2006). Sulphide generation is vital for the precipitation of excess metals out of solution. Once out of solution, due to sulphide precipitation, metal ions do not impact metabolic activity of SRBs or other cells (Matlock *et al.*, 2002; Bhagat *et al.*, 2004). Metal sulphide precipitates are highly stable above pH 7 (Lloyd *et al.*, 2001). Thus with increased precipitation of metals, there will be improved activity of SRBs.

2.5 **Conclusion**

In summary the EBRU 00AB/06 SRB consortium contains bacteria capable of carrying out sulphate reduction. Sulphate reducing bacteria activity appears to be inducible and the sulphate reduction pathway was more sensitive to inhibitors of transcription than translation. The EBRU 00AB/06 SRB consortium appears therefore to offer a potential alternative to conventional physicochemical treatment mechanisms as the activity of the bacteria reduced the sulphate and iron concentrations of the medium and increased its basicity.

Chapter 3: Use of a Mixed Algae Biomass Generated by an Integrated Algae Pond System as a Carbon Source

3.1 Introduction

Integrated algae pond systems (IAPS) typically produce a biomass comprising largely of microalgae, bacteria and some fungi. This biomass is used to treat or polish domestic and other waste water streams. Most domestic waste water streams contain elevated quantities of N and P and K which are essential for growth and consequently high biomass, including nutrient rich algae development (Boshoff *et al.*, 2004a; Masojídek and Torzillo, 2008). Boshoff *et al*., (2004b) were able to demonstrate that a sulphate reducing bacteria (SRB) consortium related to the EBRU 00AB/06 SRB consortium could utilize tannery effluent as well as pure culture *Spirulina* as a carbon source in the remediation of sulphate bearing waste water streams. However the study did not examine the potential of a mixed algae biomass generated by an IAPS treating domestic waste water as a carbon source. Thus this work is an extension of the initial study which established that SRBs in consortium were able to use various carbon sources including pure *Spirulina* (Boshoff *et al.*, 2004a). Dominant algae species in typical waste water treatment systems include *Scenedesmus* sp., *Micractinium* sp., *Chlorella* sp. and *Pediastrum* sp. (Rose *et al*., 2002). Algae population dynamics and biochemical composition vary depending on conditions such as light intensity, nutrient availability, pH, culture agitation and temperature (Van Hille *et al.*, 1999; Stucki *et al.*, 2009).

Algae cell walls are highly robust and resistant to physical damage (Gudin and Chaumont, 1991), with *Scenedesmus* sp. and *Chlorella* sp. being renowned for resistance to bacterial hydrolysis (Tang *et al.*, 2009). Blokker *et al.* (1998) demonstrated the presence of algaenans in cell walls of algae such as *Scenedesmus obliquus, S. communis, S. quadricauda, Pediastrum boryanum, Chlorella vulgaris* and *Nannochloropsis* sp. Algaenans are long chain mono- and di-unsaturated ω-hydroxy fatty acid biopolymers that cannot be degraded by enzymes found in microorganisms, thus restricting access of SRBs to contents of the algae biomass generated by an IAPS (Blokker *et al.*, 1998). Ultrasonication is a method that completely ruptures cell walls releasing cell contents (Janczyk *et al.*, 2007). Sialve *et al*. (2009) observed ultrasonication to improve the digestibility of *Chlorella vulgaris* within rat guts. Sodium hydroxide at 150 ˚C and pH 11 increased the rate of breakdown of *Spirulina maxima,* which was observed to be an effective

carbon source for rats (Sialve *et al.*, 2009). Commercially, companies such as OriginOil have established energy intensive processes to culture, harvest and essentially crack algae cell walls to release cell contents (OriginOil, 2011). OriginOil use sonication to break algae for release of oils and lipids for biodiesel production. The energy balance is restored by harvesting products such as oil, biomass and biogenic hydrogen from the system for renewable energy production, thus these companies remain cost competitive and environmentally friendly (OriginOil, 2011). Biological remediation techniques have to be designed to be simple and sustainable. Thus consideration of low cost and improved efficiency will result in large scale implementation. Acid mine drainage (AMD) could be an ideal pre-treatment step for algae cell rupture prior to introduction into a sulphidogenic fermenter (Figure 3.1).

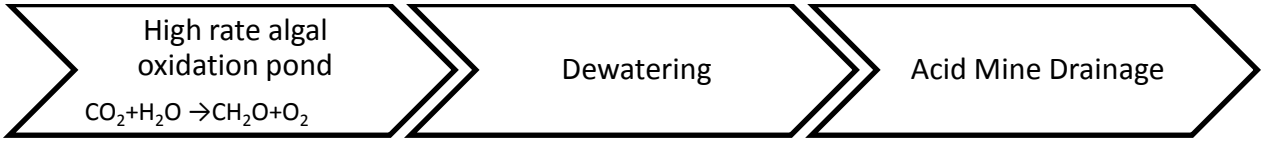

Figure 3.1: Modified representation of the hydrolysis of an algae biomass by AMD.

The solution containing an algae biomass mixture contains bicarbonates which react with the acid component of AMD (Boshoff *et al.*, 2004a; Sialve *et al.*, 2009). The reaction shown in Equation 3.1 is energy intense and exothermic. Corrosion potentially occurs resulting in the degradation of the algae cells thereby releasing cell contents for immediate use by the SRBs (Sen and Johnson, 1999). The reaction of sodium bicarbonate from the algae biomass and sulphuric acid from AMD generates salt, water, carbon dioxide as shown below:

$2NaHCO_3 + H_2SO_4 \rightarrow Na_2SO_4 + 2H_2CO_3 \rightarrow Na_2SO_4 + 2H_2O + 2CO_2$ ……..Equation 3.1

The activity rates of SRBs in AMD remediation significantly improve if substrates are immediately available to the SRBs (Zhao *et al.*, 2010). Hence, the objective of the current study was to determine the biochemical composition of an algae biomass generated by the IAPS, the efficiency of hydrolysis of algae biomass by acid waters and the sulphide contribution if any of algae biomass due to presence of sulphur containing amino acids and other compounds.

3.2 Materials and Methods

3.2.1 Production of the Algae Biomass

The study described herein was carried out at the pilot IAPS plant (33°19'07" South, 26°33'25" East) located adjacent to the Grahamstown Waste Water Treatment Plant (Grahamstown, South Africa). The IAPS used is composed of a fermentation pit (anaerobic digester), a primary facultative pond (PFP) and a high rate algae oxidation pond (HRAOP) that is 500 m^2. The fermentation pit is an anaerobic digester where methanogenesis dominates. The particulate matter found in the raw sewage settles out of suspension and is anaerobically digested (Van Hille *et al.*, 1999). The very slow 80 $m^3.d^{-1}$ velocity of the influent of raw sewage allows for solids to settle out of solution forming a sludge blanket. The capacity of the fermentation pit is 225 m^3 and has a depth of 6 m. Sewage is rich in nutrients such as nitrates and phosphates ranging in concentrations from 15 to 30 $mg.L^{-1}$ (Rose *et al.*, 2002). The CO_2 generated by anaerobic digestion provides energy for photosynthesis in the aerobic layer of the PFP (Van Hille *et al.*, 1999).

Figure 3.2: The paddlewheel mixed HRAOPs from which the algae biomass was obtained.

The PFP which is 30 m in diameter acts as a buffer system between the fermentation pit and the HRAOP. It has an anaerobic bottom layer and an aerobic top layer, ranging from a few cm to approximately 2 m in depth (Boshoff *et al.*, 2004a). The aerobic top layer of the PFP is responsible for scrubbing odour causing compounds from the effluent (Van Hille *et al.*, 1999). The HRAOP is a paddlewheel mixed raceway, 30 cm in depth with a retention time ranging between 3-5 d and increases the dissolved oxygen content (DOC) (Van Hille *et al.*, 1999; Boshoff *et al.*, 2004a). Controlled eutrophication takes place in the HRAOP generating up to 150 $tons.ha^{-1}.yr^{-1}$ renewable mixed algae biomass (Oswald, 1988; Boshoff *et al.*, 2004b).

Nutrients from the effluent are taken up by microalgae and heterotrophic bacteria. The HRAOP reduces the chemical components of waste water. For example, removal of Chemical Oxygen Demand (COD) by 78 %, ammonia by 94 % and phosphates by 92 %. The algae form flocs that sediment out of the suspension and 80 % of the biomass is removed by floc formation (Van Hille *et al.*, 1999; Wells, 2005). The effluent is alkaline ranging between pH 9.5 to 10 which destroys 100 % of anaerobic pathogens including *E. coli* (Boshoff *et al.*, 2004b). A detailed schematic of the system is presented in Appendix B.

3.2.2 Sampling of the Algae Biomass Feedstock

The algae biomass was collected from the HRAOP located immediately after the PFP. Ten litres IAPS effluent settled overnight using a settling cone and supernatant decanted, the resulting concentrated algae slurry was transferred to 2 L settling funnels. Supernatant was decanted and 100 mL algae slurry was transferred to 250 mL Erlenmeyer flasks, frozen, and subsequently placed in a freeze dryer (Vir-Tis Benchtop SLC) until a powdered biomass was obtained. This biomass was stored at - 20 ˚C until analyses were undertaken.

3.2.3 Organic Analysis of the Algae Biomass

3.2.3.1 Organic Elemental Analysis

Organic elemental analysis was conducted by placing 1 to 2 mg algae biomass onto a sample boat which was then inserted into the combustion zone of an Elementar vario MICRO CUBE Bioanalytical system for 10 s. The combustion zone is where analyte gases N_2, H_2O, CO_2 and SO_2 formed. Helium served as the carrier gas which moved the gases from the combustion zone at 1150 ˚C to the reduction zone at 850 ˚C. Helium served to separate the analyte gases sequentially according to temperature. The above procedure was conducted to determine the C, N, S and H content of the algae biomass.

3.2.3.2 Determination of Ash and Colloid Content

Ash (salt) and colloid content were determined by placing 1 g algae biomass in a muffle furnace (Carbolite, Labotech, Sheffield, England) at 600 ˚C for 4 h after which the sample was weighed then dissolved in 20 mL distilled water. The resultant mixture was filtered through Watman Number 2 filter paper and the residue dried and weighed to determine ash and colloid content.

3.2.4 Treatment of the Algae Biomass with Sulphuric Acid (Simulated AMD)

Various substrates specifically glucose, fructose, sucrose, lactate, cellulose, starch, peanut oil, Bovine Serum Albumin (BSA), a cysteine/glutamate mix and algae biomass were exposed to treatments of simulated AMD solutions prepared using 5 M sulphuric acid in distilled water at pH 1 and 3. Distilled water (pH 7) served as the control. Erlenmeyer flasks, each containing 0.5 g of a substrate and 50 mL acidic water either at pH 1 or 3 and water at pH 7 were prepared in triplicate to a final concentration of 10 $mg.L^{-1}$. Flasks were covered with aluminium foil to allow for gas escape and to prevent entry of debris and placed on a Labcon desktop shaker at 120 rpm in a controlled environment at 30 ˚C in darkness. Flasks were left to acclimatize for 1 hour prior to initial sampling on d 0 after which samples were collected at 24 hour intervals on days 1, 2 and 4. Contents of the Erlenmeyer flasks were evenly mixed and using a 10 mL syringe, 5 mL samples were collected. The samples were centrifuged at 7 840 g for 5 min using an Eppendorf Centrifuge 5810 R (Drücken, Germany) and stored at 4 ˚C until analyses were conducted.

3.2.5 Light Microscopy

A 10 µL aliquot of unfiltered algae biomass sample was collected from each Erlenmeyer flask containing simulated AMD at pH 1, and 3 and water at pH 7 respectively. The sample was placed on a glass slide to which a cover slip was applied. Algae biomass did not require staining and could be viewed immediately. Additionally iodine solution was utilized to determine if the starch component of the cell contents was leached out of the cells as opposed to bleached post exposure to simulated AMD. Thus to determine the effect of acidity on the integrity of algae cell walls light microscopic analyses were carried out and micrographs captured using a Carl Ziess Axiostar Plus light microscope (Magnification x 400) equipped with a Canon Powershot G6 digital camera.

3.2.6 Biochemical Analyses

3.2.6.1 Reducing Sugars

Determination of reducing sugar concentration was conducted utilizing 2 mL of simulated AMD treated biomass. The sample was collected in a test tube and centrifuged at 5 600 g for 5 min using an Eppendorf Benchtop Centrifuge 5810 R (Drücken, Germany) to remove debris. Supernatant (1.5 mL) was collected. A 10-fold serial dilution was prepared, and then 1.5 mL 1 % dinitrosalicylic acid solution which contained dinitrosalicylic acid (10 $g.L^{-1}$), phenol (2 $g.L^{-1}$),

sodium sulphate (0.5 $g.L^{-1}$) and sodium hydroxide (10 $g.L^{-1}$) dissolved in 1 L distilled water, was added. The tube was capped and heated at 90 ˚C for 10 min until a red brown colour developed. A 1 mL aliquot of Rochelle salt solution (40 g potassium sodium tartrate dissolved in 100 mL distilled water) was added to stabilize the resulting colour. The solution was allowed to cool for 15 min at room temperature. Samples (250 µL) were placed in microtitre plate wells and the absorbance was measured using a Multiskan spectrophotometer at 660 nm. A standard curve was prepared using dilutions of D-glucose (100 $mg.L^{-1}$) to determine concentration of reducing sugars in the samples.

3.2.6.2 Lipids

The Nile red assay was performed according to Miller (1959) to determine the sample lipid concentration. A 50 µL aliquot of 50 $\mu g.mL^{-1}$ Nile Red in 50 % DMSO solution (1:1 DMSO: absolute ethanol) was added to 200 µL supernatant from the Erlenmeyer flasks containing algae biomass treated with simulated AMD. The microtitre plate was enclosed in aluminium foil and heated to 90 ˚C in a water bath for 7 min (Miller, 1959). The sample was allowed to cool for 15 min at room temperature and then placed in a Fluoroskan fluorescence scanner and fluorescence measured at 544 nm (excitation) 620 nm (emission). Peanut oil at a concentration of 100 $mg.L^{-1}$ was used to prepare a standard curve which was in turn used to determine the lipid content in samples from the Erlenmeyer flasks.

3.2.6.3 Proteins

The Bradford assay was carried out according to Bradford (1976) to determine the protein concentration liberated after treatment of algae biomass with simulated AMD. A 20 ul aliquot of sample supernatant was placed in a test tube to which 1 ml Bradford reagent (Appendix B) was added. The solution was then mixed thoroughly for 10 sec using a vortex. After 2 min the absorbance was measured using an Aquamate spectrophotometer at 595 nm. A standard curve was prepared using dilutions of 100 mg. mL^{-1} BSA to determine the protein concentration in the samples.

3.2.6.4 Amino Acids

The Ninhydrin assay was used to detect amino acids released from algae biomass post exposure to simulated AMD. The assay was carried out according to Hwang and Ederer (1975). A 1 mL sample was placed in a test tube, to which 3 mL milli-Q water was added, after which 1 mL

Ninhydrin reagent was added. The mixtures were then placed on a vortex for 10 s. The test tubes were then sealed using aluminium foil and placed in a boiling water bath for 15 min. The test tubes were then cooled and 1 mL 50 % ethanol was added to each test tube to stabilize the colour produced by each mixture and mixed thoroughly on a vortex. The absorbance was then measured using an Aquamate spectrophotometer at 570 nm. A standard curve was generated using a 0.05 M monosodium glutamate solution diluted a hundred fold, to determine the amino acid content of the samples.

3.2.7 Analysis of the Mixed Algae Biomass as a Source of Sulphide Contamination

The EBRU 00AB/06 SRB stock culture maintained in modified Postgates medium B (Postgate, 1984) was used to inoculate the experimental fermenters. Fermenters were set up in duplicate, 2 control fermenters containing 6 mL sodium lactate (60 % solution w/v). Two positive control fermenters containing 6 g algae biomass and 2 fermenters containing a mixture of 6 mL sodium lactate (60 % solution w/v) with 12 $mg.L^{-1}$ sulphur containing amino acids commonly found in algae biomass. After cooling to approximately 40 ˚C, dithiothreitol (3 mg), glutathione (3 mg), cysteine (3 mg) and methionine (3 mg) were added to make up the sulphur containing compound medium. A 200 mL stock culture EBRU 00AB/06 SRB consortium was utilized to inoculate each fermenter. Anaerobic conditions were established by purging the fermenters with N_2:CO_2 (80: 20 %) (Afrox, South Africa) 5 min prior to and post set up. The experiment was carried out in a controlled environment at 30 ˚C in darkness after which fermenters were placed on a Labcon desktop shaker at 120 rpm. A 60 mL syringe was used for extracting 20 mL samples from a sealed sample port located on the fermenter. Sampling was carried out twice a week until a constant minimum sulphate concentration level was detected.

3.2.8 Sulphate and Sulphide Quantification

Sulphate and sulphide determination were conducted as described in Chapter 2.

3.2.9 Statistical Analyses

Statistica version 9.0 was utilized for the analysis of the data that was generated. T-tests and Repeated Measure Analysis of Variance (ANOVA) with 2 way effects were used to analyse the data collected during the experiments. The Repeated Measure ANOVA was selected for the analyses, as the measurements that were taken were recorded repeatedly and were also treatment

dependent. The level of significance adopted throughout the study was 5 %. Due to the small sample size the samples were analysed according to their assumed normal distribution.

3.3 **Results**

In order to determine the suitability of the algae biomass as a potential substrate for sulphate remediation, organic elemental analysis of the biomass was carried out. The results show the C percentage to be 55.2 % of the algae biomass. The empirical formula of the biomass was determined to be $C_{1.0}H_{1.91}N_{0.084}S_{0.003}O_{0.36}$ by atomic absorbstion and ashing. Molar mass was utilized to determine the empirical formula of the algae biomass from the percentage mass elucidated by atomic absorbtion. This biomass contains nutrients such as C, N and S and therefore has the potential to serve as a renewable substrate for the biological remediation of sulphate bearing waste water streams (Table 3.1).

Table 3.1: Biochemical composition of the biomass generated by the IAPS (% dry weight), data are the mean of 3 replicates ±SD.

C	**H**	**N**	**O**	**S**	**Ash**	**Colloids**	**Unknown**
55.2 ±0.29	8.8 ±0.17	5.4 ±0.12	26.5±0.20	0.5 ±0.05	8.3 ±1.00	19.3 ±0.50	14 ±0.71

The iodine solution test revealed that after 1 h *Pediastrum* cells exposed to simulated AMD (pH 7) stained blue black indicating the presence of starch. However cells exposed to simulated AMD (pH 1) stained orange brown indicating that the starch component had leached out of the cells or been hydrolysed (Figure 3.3). *Pediastrum* cells were further selected to show changes in cell wall integrity due to exposure simulated AMD hydrolysis and the results are shown in the light micrographs presented in Figure 3.4. Simulated AMD appeared to cause leaching out of the cell contents within 1 h.

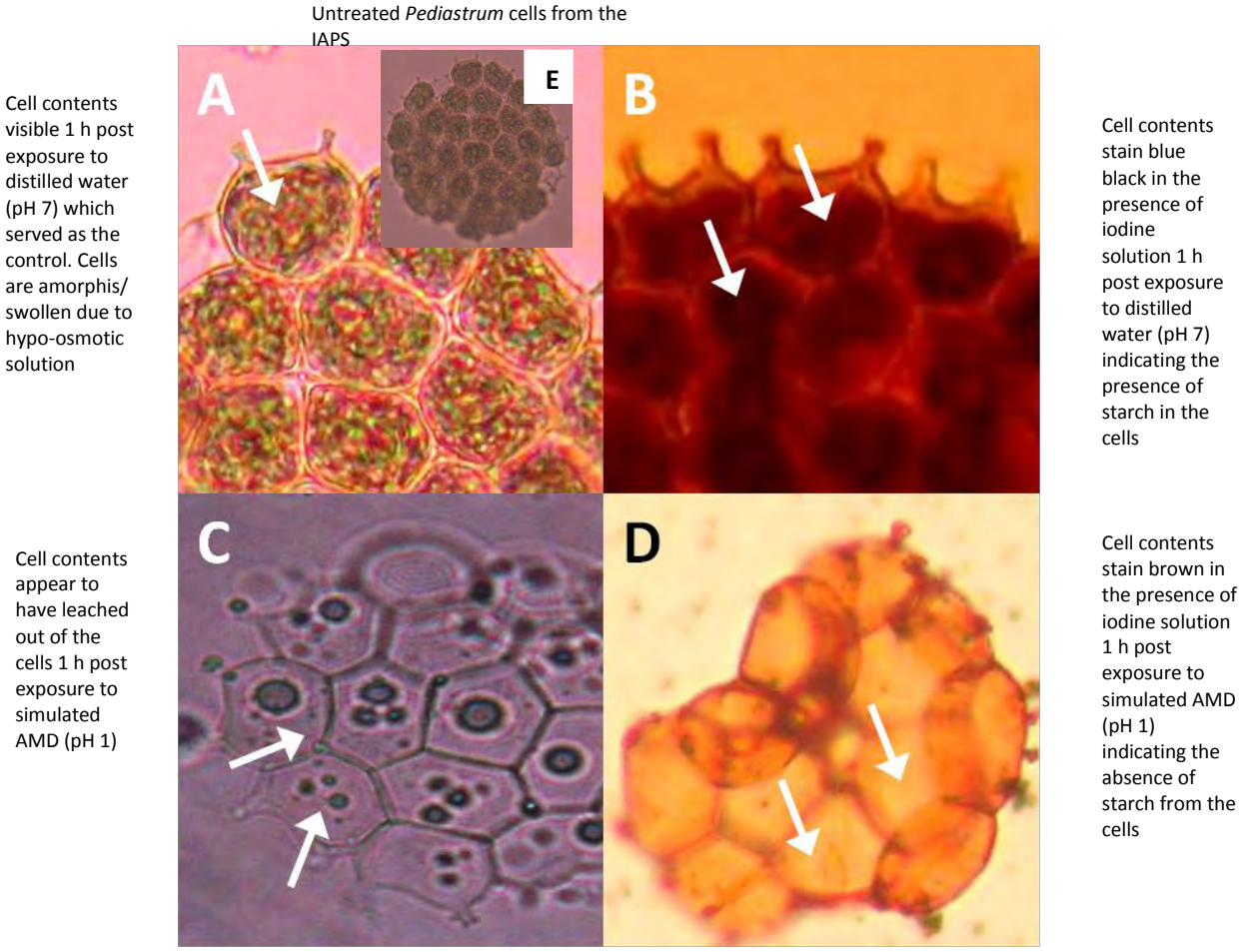

Figure 3.3: Light micrographs showing the presence or absence of starch in algae cells post exposure to simulated AMD (pH 7) and (pH 1).

3.3.1 Effect of Synthetic AMD on Algae Biomass, Peanut Oil, D-Glucose, BSA and a Cysteine/Glutamate Mix

Exposure of the algae biomass to acid waters served to release cell contents into the supernatant. The following results are the reducing sugar, lipid, protein and amino acid concentrations detected in the supernatant post exposure of the algae biomass to simulated AMD. Hydrolysis of starch generates compounds such as reducing sugars which have the potential to serve as substrates for bacterial growth and proliferation. High concentrations of reducing sugars were released by the algae biomass and detected in the supernatant. Lipids were also released from the algae biomass however they require further hydrolysis to form fatty acids and glycerol to serve as potential carbon sources. Simulated AMD did not appear to significantly impact the lipid

concentration detected (Figure 3.5). Results of the supernatant analyses utilizing the Bradford assay revealed a decline in the protein over 4 d. The greatest protein concentration was observed on d 0 post exposure to simulated AMD. The Ninhydrin analysis displayed a gradual incline in the amino acids detected in the supernatant of the algae biomass post exposure to simulated AMD (pH 3) due to the hydrolysis of proteins however a rapid decline was observed at pH 1 (Figure 3.5).

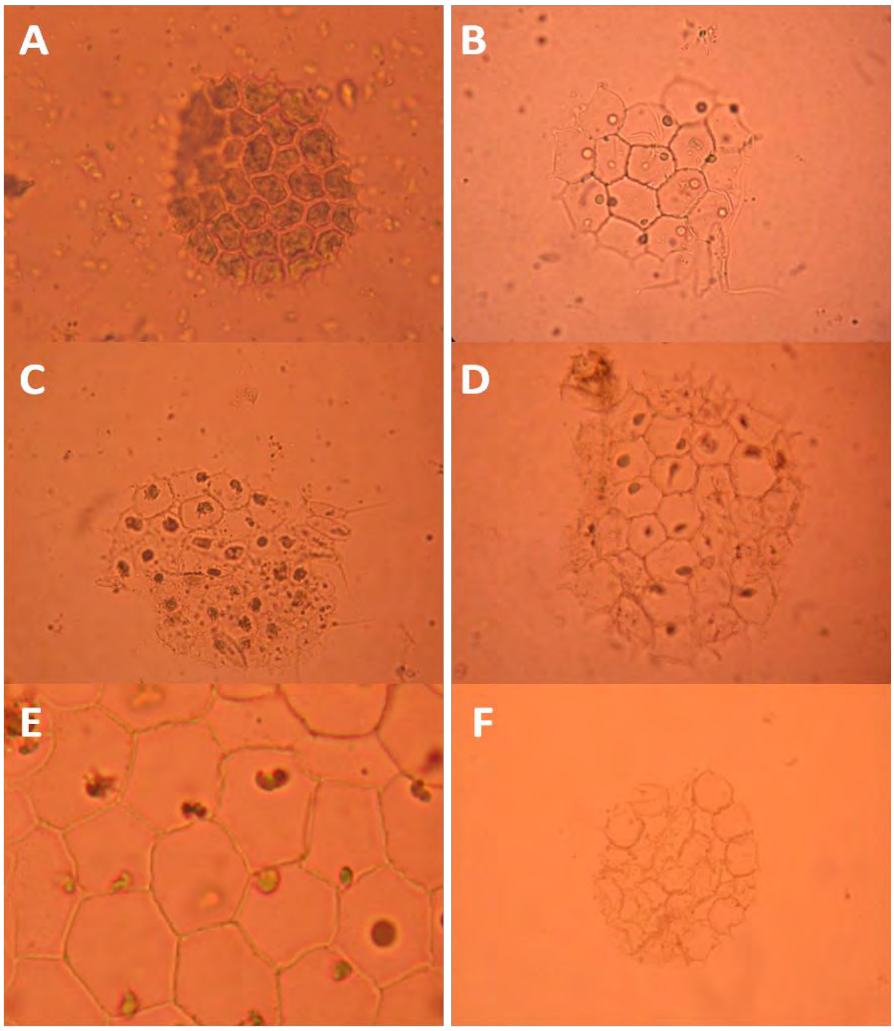

Figure 3.4: Light micrographs depicting the effect of sulphuric acid at pH 1 and pH 3 on the cell integrity of *Pediastrum* sp. (approximately 90 µm diameter). Leaching out of the cell contents was observed by d 0 (Magnification ×400). A: Resuspended *Pediastrum* specimen at pH 7 on d 0, B: *Pediastrum* specimen post exposure to pH 1 on d 0, C: *Pediastrum* cell wall deterioration 24 h post exposure to sulphuric acid pH 1, D: *Pediastrum* cell wall integrity 48 h post exposure to sulphuric acid pH 1, E: *Pediastrum* cell wall integrity 96 h post exposure to sulphuric acid pH 3 and F: *Pediastrum* specimen 96 h post exposure to sulphuric acid pH 1.

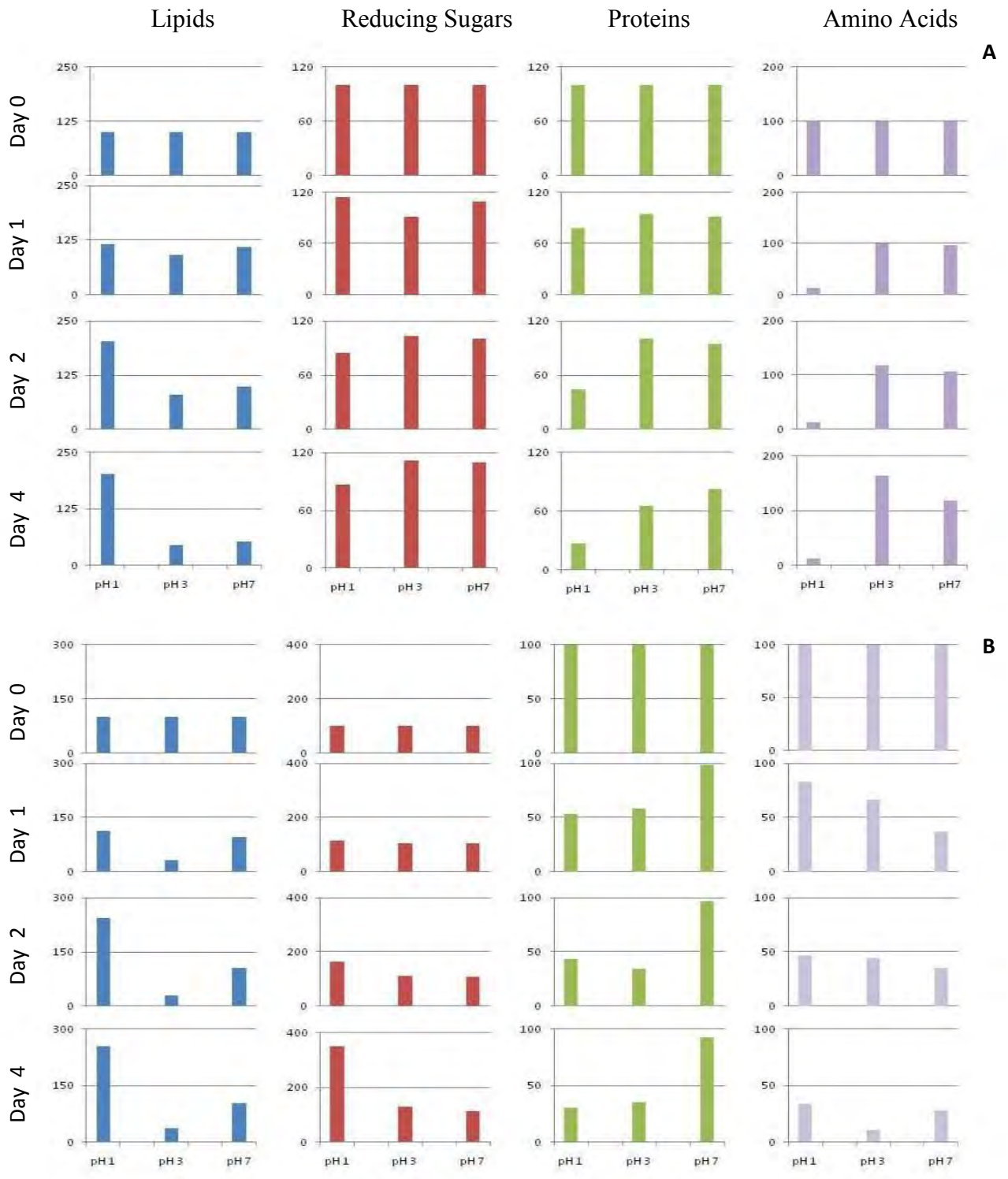

Figure 3.5: Total reducing sugar, protein, amino acid and lipid content detected upon exposure of a mixed algae biomass from an IAPS to acidic waters pH 1 and 3 and pH 7 (control). A: Algae biomass, B: Starch, BSA, peanut oil and a cysteine/glutamate mix served as controls for reducing sugars, proteins, lipids and amino acids respectively. Each bar is the mean of 3 replicates.

The results presented in Table 3.2 indicated that at a concentration of 10 $g.L^{-1}$ algae biomass significantly increased the basicity of simulated AMD (pH 3) to pH 7.67 while simulated AMD (pH 1) improved to pH 1.77. Over the course of the study an apparent concentration effect was observed where the algae biomass at the lower concentration of 1 $g.L^{-1}$ also increased the alkalinity of Postgates medium but to a lesser extent.

Table 3.2: The effect of various substrates on the pH of acidic waters.

Treatment	Substrate	Day 0	Day 1	Day 2	Day 4
AMD pH 1	AMD pH1	1.04	1.44	1.51	1.43
	Glucose	1	1.48	1.51	1.49
	Lactate	1.5	1.87	1.96	1.89
	Sucrose	1	1.45	1.48	1.49
	Starch	1	1.46	1.45	1.47
	Cellulose	1	1.44	1.49	1.51
	Peanut oil	1	1.47	1.49	1.51
	Algae biomass	1.5	1.68	1.77	1.75
AMD pH 3	AMD pH3	2.94	3.29	3.33	3.38
	Glucose	3.1	3.1	3.37	3.37
	Lactate	3.5	4.41	4.66	4.67
	Sucrose	3.2	3.28	3.32	3.3
	Starch	3.3	3.31	3.35	3.34
	Cellulose	3.2	3.29	3.33	3.31
	Peanut oil	3.2	3.31	3.32	3.29
	Algae biomass	4	6.88	7.1	7.67
AMD pH 7 (Control)	Distilled water	7.15	7.03	7.15	7.15
	Glucose	3.45	7.16	7.24	7.24
	Lactate	3.2	4.86	4.86	4.86
	Sucrose	7	7	7	6.75
	Starch	7	7.05	7.08	7.11
	Cellulose	7	6.97	7.07	7.3
	Peanut oil	7	7.1	7.05	7.1
	Algae biomass	7	6.86	7.01	8.1

Algae biomass contains reduced sulphur bearing compounds such as cysteine, methionine, glutathione and dithiothreitol. These compounds have the potential to release reduced sulphur into the system upon hydrolysis by the EBRU 00AB/06 SRB consortium or AMD. Thereby, resulting in, elevated sulphide detection, thus rendering measurement of sulphide (as the final product in sulphate remediation) artifactual. Sulphate reduction and sulphide generation by the EBRU 00AB/06 SRB consortium were observed in medium containing various substrates (Figure 3.6). Sulphide concentrations detected were statistically similar, repeated measure ANOVA analysis of the data resulted in no significant differences (p-value ≥ 0.08). Thus the

biomass generated by the IAPS in comparison to the control (lactate) did not appear to contribute significantly to the sulphide concentrations detected in the medium.The algae biomass fed cultures were also operating at a level comparable to the media containing the control indicating that the biomass did not negatively impact sulphate reduction and sulphide generation by the EBRU 00AB/06 SRB consortium. Table 3.3 shows that the SRB consortium maintained the pH of the medium between pH 7.1 and 8.65.

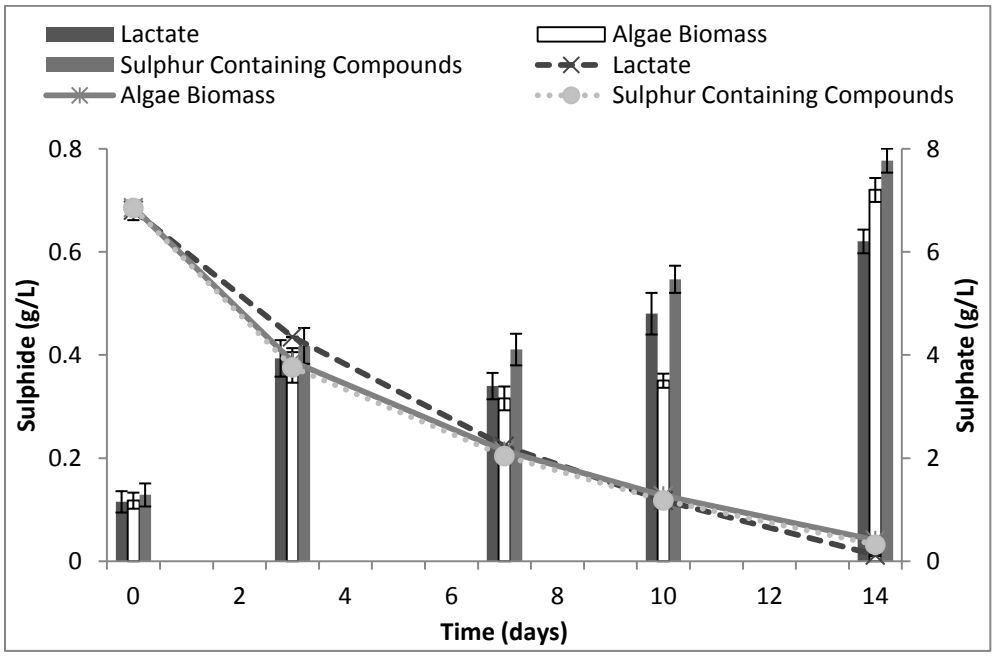

Figure 3.6: Sulphate reduction and sulphide generation by the EBRU 00AB/06 SRB consortium in the presence of lactate, algae biomass or lactate with sulphur containing compounds in the form of methionine, dithiothreitol, cysteine and glutathione. Data points are the means of 3 replicates ±SD.

Table 3.3: The effect of lactate, algae biomass or lactate with sulphur containing compounds on culture pH.

Substrate	**Day 0**	**Day 3**	**Day 7**	**Day 10**	**Day 14**
Lactate	7.11	8.29	8.13	7.78	8.05
Algae biomass	7.15	8.01	8.3	8.03	8.09
Sulphur compounds	7.47	8.63	8.65	8.23	7.92

3.4 **Discussion**

Biochemical analysis of the algae biomass upon conversion of percentage dry weight to molar mass resulted in the empirical formula $C_{1.0}H_{1.91}N_{0.084}S_{0.003}O_{0.36}$. The biomass is relatively complex, thus in order to render the components available, pre-treatment was required. Algae are composed of rigid cell walls which restrict access to cell contents and may prove to be a weakness in the overall bioprocess design. On a lab scale ultrasonication, French presses and freeze thawing are common place for algae cell rupture. However, commercially these techniques are not viable. Treatment of the algae biomass with simulated AMD caused efficient cell rupture and leaching of carbon substrates, indicating that acid hydrolysis could be introduced into the bioprocess design. Reducing sugars proved more abundant than lipids, proteins and amino acids in the algae biomass during the study. As expected the protein concentration decreased with prolonged exposure of the algae biomass to simulated AMD pH 3 while the amino acid concentration increased slightly. Addition of the algae biomass at a concentration 10 $g.L^{-1}$ in turn increased the pH of the simulated AMD. Further analysis of the algae biomass was carried out to ascertain the contribution of reduced sulphur containing compounds in algae biomass to biogenic sulphide detected post EBRU 00AB/06 SRB consortium hydrolysis. The results indicated a statistically insignificant contribution to sulphide detected.

Emission of dimethyl sulphide (DMS) into the atmosphere is of concern as it is oxidized to sulphuric and methanesulfonic acids. These compounds have a high affinity for water molecules in the atmosphere resulting in condensation, directly influencing cloud formation (Taylor and Gilchrist, 1991; Stefels, 2000). Marine micro and macroalgae and the coastal grass *Spartina alterniflora* sp. have been identified as the major contributors of biogenic sulphur emissions. These organisms spontaneously generate dimethylsulphoniopropionate (DMSP) in response to osmotic, oxidative and UV light stress (Jonhston *et al.,* 2007). Taylor and Gilchrist (1991) demonstrated that bacterial cleavage of DMSP formed DMS and/or methanethiol (CH_3SH). As DMS is responsible for 50 % of biogenic sulphur detected in the atmosphere (Taylor and Gilchrist, 1991), it has been suggested that its formation may directly influence global climatic patterns (global warming). Currently no data is available regarding potential DMSP production in algae generally found in fresh water and waste water treatment systems and Stefels (2000) noted that DMSP production is mainly confined to marine micro and macroalgae. However both marine and fresh water algae contain sulphur bearing compounds in the form of cysteine,

methionine and glutathione (Fogg, 1953; Stefels, 2000). Boshoff *et al.* (2004a) reported that the biomass of *Spirulina* sp. contained up to 70 % protein and that *Spirulina* was available for utilization by microorganisms such as SRBs. Thus it seemed pertinent to determine the reduced sulphur contribution of the algae biomass post EBRU 00AB/06 SRB consortium hydrolysis. The algae biomass contained 0.5 % sulphur. Biochemical analysis revealed that the sulphur content of algae biomass was not enough to bias results obtained from use of algae biomass as a carbon source in a sulphidogenic fermenter neither did it affect the activity of the EBRU 00AB/06 SRB consortium. The biogenic hydrogen sulphide was completely captured within the closed sulphidogenic system, thus it did not have a detrimental impact on the environment.

The algae biomass ($C_{1.0}H_{1.91}N_{0.084}S_{0.003}O_{0.36}$) contains nutrients such as C, N and S essential for microorganism development. It is similar to *Spirulina platensis* $C_{1.0}H_{1.71}O_{0.48}N_{0.19}S_{0.005}$ (Stucki *et al.*, 2009) and the general microalgae equation $C_{1.0}O_{0.48}H_{1.85}N_{0.11}P_{0.01}$ (Grobbelaar *et al.*, 1990) which has been successfully utilized as feedstock for digesters and as nutrient supplements in dairy farming respectively. Algae biomass is proteinaceous, carbon rich and rich in nutrients vital for development of anaerobic bacteria (Oswald, 1988; Wilkie and Mulbry, 2002).

The algae biomass was extracted from the HRAOP. Alkalinity in this system can increase to pH 9.5 due to microalgae photosynthetic activity (Rose *et al*., 2002). Rose *et al.* (2002) reported that in the alkaline environment of the HRAOPs both bicarbonates and hydroxyl ions are present in the system and in the biomass, thereby accounting for the improved pH of the simulated AMD. Arica *et al.* (2005) ruptured *Chlamydomonas reinhardtii* cells with heating and acid treatment pH 5.5. The current study demonstrated that pre-treatment of the algae biomass from the IAPS with only simulated AMD at pH 1 and 3 shortened the initial rate limiting step which is the conversion of complex organics such as starch and protein to simpler organic compounds. Fogg (1953) reported that algae such as *Chlorella* and *Scenedesmus* generate copious amounts of carbohydrates which are utilized as the principle storage compounds in the algae biomass. Reducing sugars and lipids were detected in the medium upon exposure of the algae biomass to AMD. Reducing sugars such as glucose, sucrose and heptose which are among the breakdown products of the algae biomass (Boshoff *et al*., 2004a) are immediately bioavailable for utilization by SRBs as well as other members of an SRB consortium (Gibson, 1990; Van Hille *et al*., 1999; Molwantwa, 2002). Lipids however require further hydrolysis to form fatty acids prior to

utilization by the SRBs. As SRBs are only able to use simple organic compounds as substrates in their metabolic activity the immediate presence of reducing sugars in the medium upon algae cell rupture demonstrates that the algae biomass is a suitable carbon source for biogenic sulphidogenesis. Thus simulated AMD improved the accessibility of substrates for the SRBs, while the algae biomass increased the alkalinity of the AMD for SRB activity.

3.5 Conclusion

In conclusion, this investigation has demonstrated that algae biomass contains minimal reduced sulphur containing compounds and that hydrolysis did not increase the sulphide concentrations detected due to the activity of the SRB consortium. A mixed algae biomass is a renewable, cost competitive and sustainable energy source that can virtually be established anywhere. The main advantage of algae biomass as a carbon source is that algae can double in 24 h under optimal conditions. Algae also contain essential nutrients such as N, P, Fe and Si which can potentially aid in growth and development of SRBs and other microorganisms. Considerable literature is available for the remediation of domestic waste waters using HRAOPs (Nurdogan and Oswald, 1995). However there is no literature on the potential use of a nutrient rich biomass generated by the HRAOPs of the IAPS as a feedstock in the remediation of an industrial effluent such as AMD.

Chapter 4: A Mixed Algae Biomass as a Carbon Source for Sulphate Reducing Bacteria in the Remediation of Acid Mine Drainage

4.1 Introduction

Suphate reducing bacteria (SRB) are potentially useful in the anaerobic treatment of high sulphate containing waste water streams. Cost and transport of large volumes of a simple energy sources have hampered development of this type of technology (Costa *et al.*, 2009). Electron donors that have been studied include mushroom compost, straw, sewage, manure, wood chips, sawdust, sugar, tannery effluent and hay (Boshoff *et al.*, 2004a; Boshoff *et al.*, 2004b; Costa *et al.*, 2009). Shimizu *et al.* (1993) and San Pedro *et al.* (1994) reported that lipids and cellulose degrade at 0.76 and 0.52 $mg.L^{-1}.d^{-1}$ respectively, while proteins and carbohydrates break down at a rate of 1.2 $mg.L^{-1}.d^{-1}$ under anaerobic conditions. Sulphate reducing bacteria can utilize the breakdown products which include reducing sugars and volatile fatty acids as carbon sources as in Equation 4.1 (Boshoff *et al.*, 2004a). The complete oxidation of a carbon source results in elevated alkalinity and increased hydrogen sulphide concentrations as in Equation 4.1 to 4.3 (Nevatalo *et al.*, 2010). Sulphate reducing bacteria metabolize ethanol, lactate and acetate in the following reactions:

$CH_3CH_2OH + ½SO_4^{2-} \rightarrow CH_3COO^- + ½HS^- + ½H^+ + H_2O$……..…...……..Equation 4.1

$CH_3CHOHCOO + ½SO_4^{2-} \rightarrow CH_3COO + HCO_3 + ½HS + ½H^+$……..…...……Equation 4.2

$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$……...……..Equation 4.3

Biogenic hydrogen sulphide and bicarbonates shown in Equations 4.2 and 4.3 cause dissolved metals to precipitate out of solution and the alkalinity generated neutralizes acidity of acid mine drainage (AMD) as depicted by Equations 4.3 and 4.4 (Bayrakdar *et al.*, 2009; Oyekola *et al.*, 2009).

$HCO_3^- + HS^- + Me^{2+} \rightarrow H_2O + CO_2 + MeS$……..……Equation 4.4

The EBRU 00AB/06 SRB consortium used in this study was viable and in the presence of lactate was able to reduce sulphate and generate hydrogen sulphide. Thus it became necessary to establish if the EBRU 00AB/06 SRB consortium could utilize an alternative carbon source in the form of an algae biomass generated by the integrated algae pond system (IAPS). Once the algae

biomass had been confirmed as a suitable alternative, it would be pertinent to establish if it could potentially be used as the energy source in the remediation of simulated AMD. Chapter 2 showed that EBRU 00AB/06 SRB consortium was able to tolerate 3 $g.L^{-1}$ sulphate, 0.5 $g.L^{-1}$ iron (Fe^{2+}) and minimum pH 5. Thus the aim of the experiments described in this chapter was to establish if the EBRU 00AB/06 SRB consortium was capable of remediating simulated AMD containing 3 $g.L^{-1}$ sulphate, 0.5 $g.L^{-1}$ Fe^{2+} and pH 5 in the presence of a biomass generated by the IAPS.

4.2 Methods and Materials

4.2.1 Determination of the Algae biomass as a Carbon Source for SRBs

The EBRU 00AB/06 SRB consortium stock culture was used to inoculate the experimental fermenters, 2 control fermenters each containing 6 mL sodium lactate (60 % solution w/v) and 2 positive control fermenters each containing 6 g algae biomass. A 200 mL stock culture was used to inoculate each experimental fermenter and control. Fermenter set up, maintenance and sampling were conducted as mentioned in Chapters 2 and 3.

4.2.2 Evaluation of an Algae Biomass as an Energy Source for SRB Catalyzed Remediation of AMD

Simulated AMD was prepared containing 3 $g.L^{-1}$ sulphate, 0.5 $g.L^{-1}$ Fe^{2+} at pH 5 to give a final volume of 1 L. The pH was adjusted using 5 M nitric acid so as not to intefer with the final sulphate concentration. This preparation was then mixed with 6 g algae biomass prior to introduction into the 1 L fermenter. Only 800 mL of simulated AMD/algae biomass solution was used to seed the fermenter. The experiment was set up in duplicate. Fermenter maintenance and analysis were carried out as previously describe in Chapters 2 and 3. The fermenters were then sealed and analyzed until the sulphate concentration reached a minimum level.

4.2.3 Biochemical Analyses

Determination of sulphate, sulphide and metal ion content was conducted as in Chapters 2 and 3.

4.2.4 Statistical Analyses

Statistica version 9.0 was utilized for the analysis of the data generated as in Chapter 3.

4.3 **Results**

The EBRU 00AB/06 SRB consortium was shown in Chapter 2 to be capable of sulphate reduction and sulphide generation when lactate was supplied as the sole carbon source. The aim of this study was therefore to ascertain if SRBs could effectively utilize an algae biomass produced by an IAPS as a carbon source and the results are shown in Figure 4.1. This algae biomass is a complex and as yet uninvestigated potential carbon source. A student T-test was used to determine the level of significance between the lactate fed control and the algae biomass fed cultures for sulphate reduction (p-value $\geq$0.98) and sulphide generation (p-value $\geq$0.89) confirming that there was no significant difference between the activity of the SRBs exposed to either algae biomass or lactate. This observation confirmed that algae biomass could serve as an alternative carbon source in biosulphidogenesis by the EBRU 00AB/06 SRB consortium.

After establishing that the EBRU 00AB/06 SRB consortium was capable of using a carbon source generated by the IAPS, the consortium was exposed to simulated AMD. Simulated AMD at pH 5 contained 3 $g.L^{-1}$ sulphate, 0.5 $g.L^{-1}$ iron. The simulated AMD was then mixed with the algae biomass and inoculated with EBRU 00AB/06 SRB consortium. The results in Figure 4.2 show that Fe^{2+} could not be detected in the medium after 14 d presumably due to precipitation of the metal as a result of hydrogen sulphide generation. A black precipitate, presumably iron sulphide, was observed to accumulate in the fermenter. Sulphate reduction and sulphide generation as shown in Figure 4.2 (R^2-value= 0.685) and (R^2-value = 0.954) respectively occurred as previously observed in Figure 4.1 (R^2-value = 0.907) and (R^2-value =0.963) respectively indicating that the presence of simulated AMD did not appear to impact activity of the EBRU 00AB/06 SRB consortium. The consortium was thus able to remediate simulated AMD through normal SRB activity.

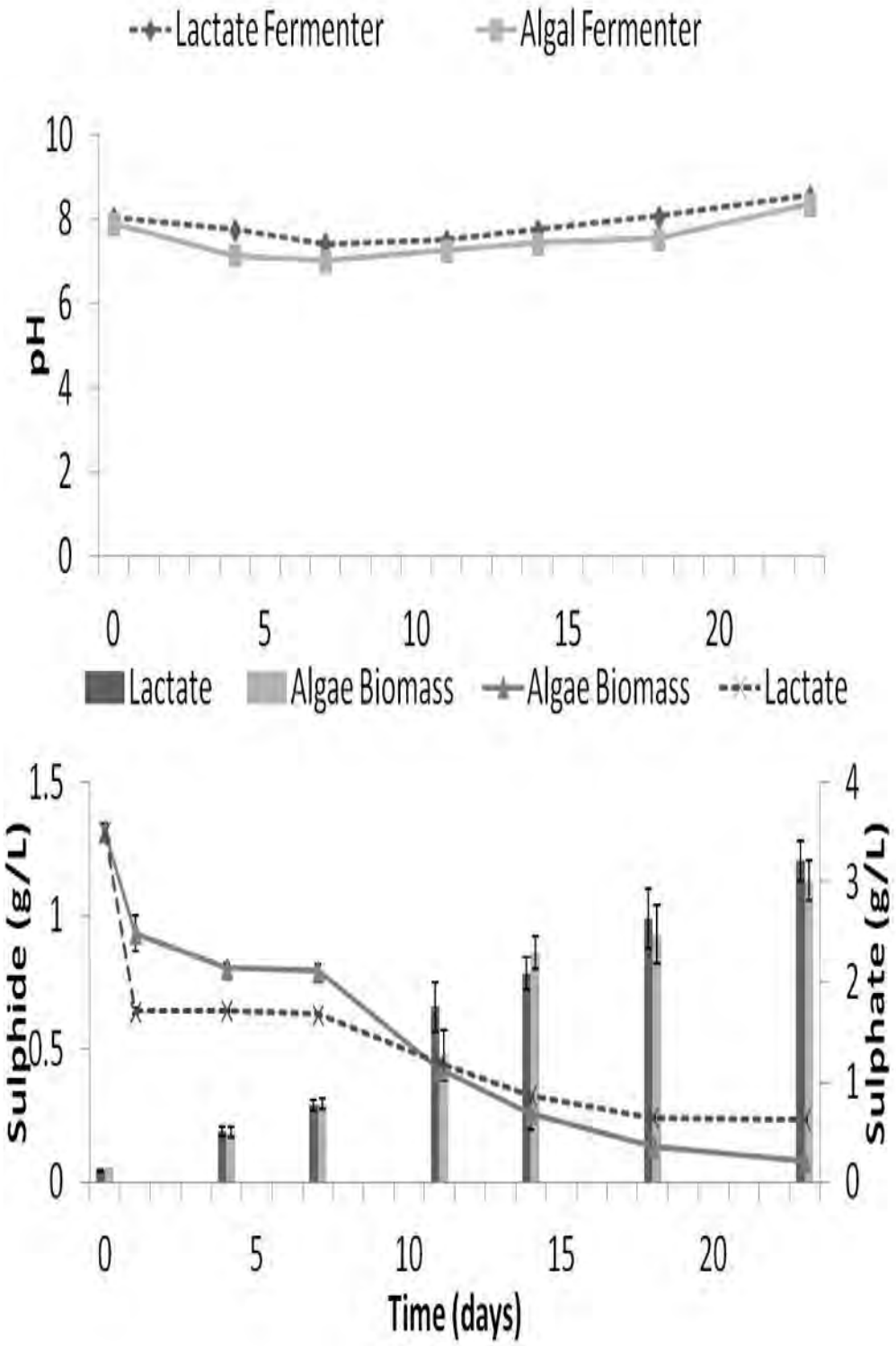

Figure 4.1: Sulphate reduction and sulphide generation by the EBRU 00AB/06 SRB consortium in batch fermenters fed with lactate (control) or an IAPS algae biomass. The pH ranged between 7.0 and 8.6. Data points are the mean of 3 replicates ±SD.

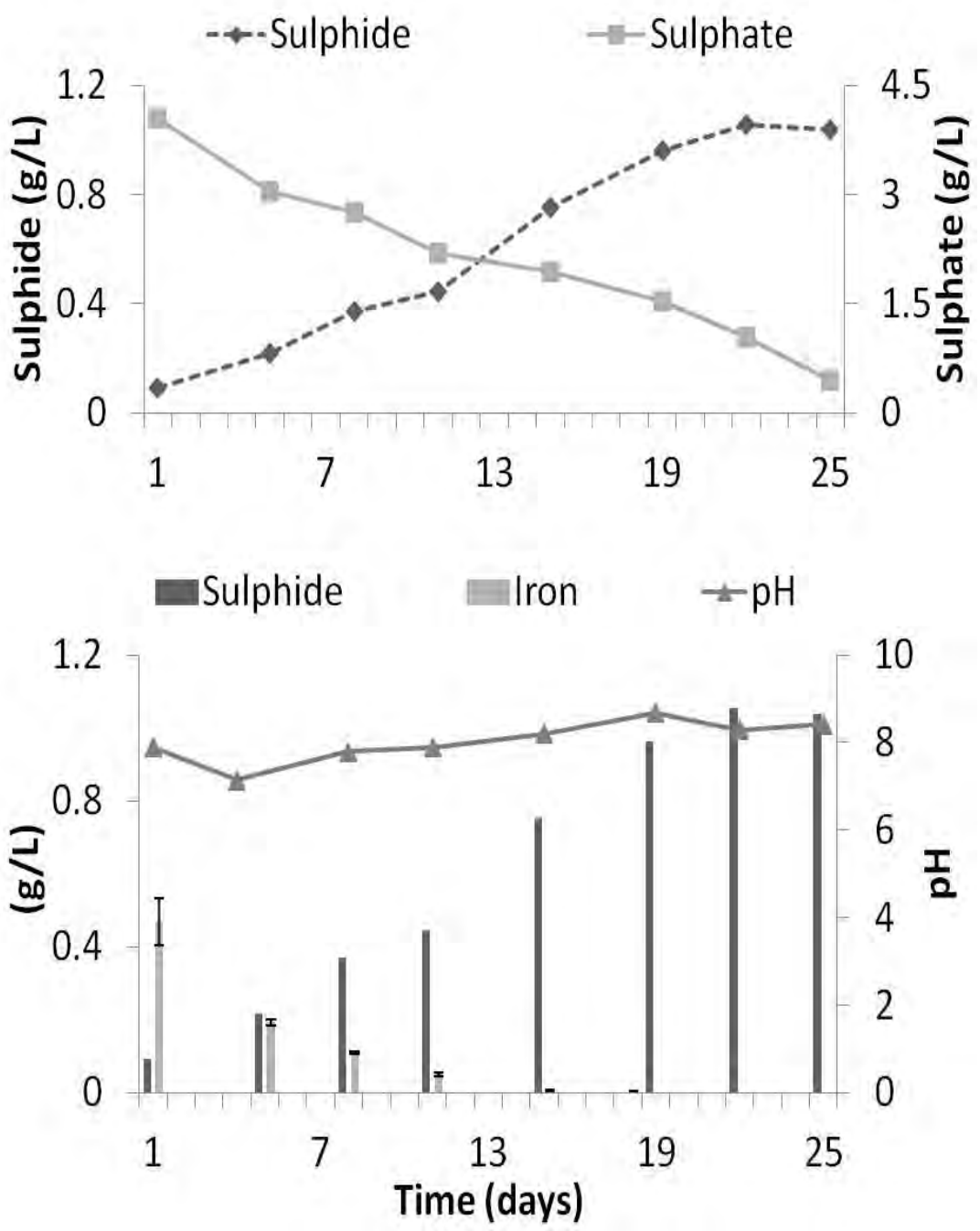

Figure 4.2: Simulated AMD remediation through iron precipitation, sulphate reduction, sulphide production and change in pH due to the metabolic activity of the EBRU 00AB/06 SRB consortium utilizing an algae biomass as a carbon source. Data points are the mean of 3 replicates ±SD.

4.4 **Discussion**

Upon exposure of the EBRU 00AB/06 SRB consortium to simulated AMD, the consortium was able to sustain itself utilizing the algae biomass as a carbon source. A comparison between the

lactate fed culture which served as the control and the algae biomass fed culture revealed that the SRBs displayed similar levels of activity. A 51 % reduction in sulphate in the lactate fed culture was observed while the algae biomass fed culture showed a 29 % decline in sulphate within 24 h. The algae biomass fed culture initially operated at a slower rate than the lactate fed culture presumably due to the time needed to hydrolyse the biomass and release bioavailable substrates. Complex carbon sources require break down prior to use by the SRBs (Schmidtova and Baldwin, 2011). However gradually the algae biomass fed culture outperformed the lactate fed culture. When the algae cells are ruptured compounds such as reducing sugars and fatty acids are immediately available for use by SRBs (Mehta and Vaidya, 1978; Sialve *et al.*, 2009), resulting in improved activity of SRBs in the algae biomass fed culture in comparison to the lactate fed culture. The study revealed algae biomass fed culture reduced the sulphate concentration by 94 % after 23 d, while the lactate fed culture reduced the sulphate concentration 82 % implying that algae biomass was a better carbon source than the lactate control. Boshoff *et al.* (2004a) observed 80 % sulphate removal from solution when utilizing *Spirulina* as the sole carbon source. Thus the algae biomass generated by an IAPS could potentially serve as a carbon source for SRBs, as the SRBs were operating at relatively the same level regardless of carbon source.

Liamleam and Annachhatre (2007) and Das *et al.* (2009) reported that pH increases in sulphidogenic systems are due to biogenic sulphide and substrate hydrolysis respectively. Acid hydrolysis of the algae biomass may have caused the increased the pH of the medium. The pH of the AMD/algae biomass mix increased from pH 7.9 to 8.41. Sulphide generated between d 0 and d 7 occurred at a rate of 0.053 $g.L^{-1}.d^{-1}$. Sulphide detection indicated efficient SRB metabolic activity (Postgate, 1978; Angell and Urbanic, 2000). Iron settled out of solution at a rate of 0.033 $g.L^{-1}.d^{-1}$. On d 14 a 98.7 % removal of Fe^{2+} from the solution was observed. Precipitation of Fe^{2+} due to the biogenic sulphide FeS (Dutta *et al.*, 2011) and improved pH of the system to above 7 due to the presence of algae biomass, may be the reasons iron could not be detected beyond d 14 by ICP-OES. Sulphide generation continued to increase steadily until d 21 after which there was no further increase (Figure 4.2). The investigation showed that the EBRU 00AB/06 SRB consortium was able to efficiently utilize a mixed algae biomass to reduce sulphate and generate sulphide. While utilizing the algae biomass as a carbon source the EBRU 00AB/06 SRB consortium was also able to remediate simulated AMD, removing 100 % of the dissolved Fe^{2+} from the water, removing 89.07 % of the sulphates originally in the medium and

finally increasing the pH of the water from an initial pH 5 to pH 8.41 after 23 d. Sulphide was stabilized either as an iron sulphide precipitate in the fermenter or as zinc sulphide in the zinc trap, thus it did not have a detrimental impact on the environment in the form of odour and corrosion.

4.5 **Conclusion**

The EBRU 00AB/06 SRB consortium was able to use the algae biomass produced by the IAPS as a carbon source in the reduction of sulphate. The EBRU 00AB/06 SRB consortium while utilizing algae biomass as a carbon source was able to remediate simulated AMD:

- The algae biomass increased the pH of the simulated AMD from pH 5 to 7.05 prior to introduction to the EBRU 00AB/06 SRB consortium
- The starting pH of the medium rose to 7.9 due to the residual alkaline nature of the consortium from the hydrogen sulphide generated
- The iron concentration in solution decreased to 0 % after 14 d
- An 89.07 % reduction in sulphate was observed after a period of 23 d, the rate of reduction of sulphate was concomitant with sulphide generated.

Thus the algae biomass from an IAPS can serve as an alternative carbon source for the remediation of synthetic AMD.

Chapter 5: General Discussion and Conclusion

The South African environment is threatened by acid mine drainage (AMD). The conundrum with AMD has reached desperate proportions. Upon liquidation of mines such as Pamodzi Gold's Grootvlei Mine (Grootvlei, South Africa), lack of funds for continued pumping of mine water as well as remediation of contaminated effluent, resulted in the mine discharging untreated AMD into the environment. This act had far reaching environmental implications. The discharge was so contaminated that it turned the water in the wetland adjacent to the mine orange due to "yellow boy" formation (Naidoo, 2009). Abandoned mines in the Witswatersrand region (Gauteng, South Africa) have reportedly been discharging an estimated 15 $ML.d^{-1}$ untreated AMD into rivers since 2008 (Naidoo, 2009). Contaminated waters have been flowing into rivers such as Tweelopiespruit, which runs directly through the Krugersdorp Nature Reserve. This incidentally is the location of the 3.5 million year old world heritage site, The Cradle of Humanity (Theunissen, 2008). Expansions of the Chromium and Platinum mines in Limpopo and Mpumalanga provinces (South Africa) and consequently deterioration of the Blesbokspruit and Klip rivers, which flow into the Vaal River, are cause for major concern, as aquatic biodiversity has been significantly impacted and reduced (Naidoo, 2009). Animals such as the *Crocodylus niloticus* (Nile crocodile) have decreased in number in the recent past within South Africa. Determinants have been the high heavy metal content of donor systems and dead fish ingested. In an attempt to circumvent the effects of high metal content, organisms accumulate heavy metals in their systems, resulting in toxic shock followed by death. This has resulted in a decrease in species diversity in Lake Loskop (Mpumalanga, South Africa) (Oberholster *et al.*, 2010).

South Africa is a semi arid country with restricted water reserves (Tutu *et al.*, 2008). Muller (1999) reported that South Africa had an estimated 12 million people without reasonable access to potable water. With the advent of AMD, resolving this issue is becoming increasingly difficult (Theunissen, 2008). The United Nations Environmental Programme estimated that one third of the world population is already living under water stress (Rawlings and Johnson, 2007). And the population in Gauteng was estimated to increase to 20 million by 2025 resulting in increased water demand (Bhagwan *et al.*, 2008). Water stress arises due to population growth, economic development as well as urgency to supply water to people without access (Muller, 1999). In

recent years Polokwane and Cape Town both declared emergency water restrictions due to severely limited water supplies (Bhagwan *et al.*, 2008). This development places further emphasis on reclamation efforts of contaminated water for future water supplies. Since the implementation of the National Water Act in 1998, all water in South Africa has been state regulated. By 2005, AMD generation necessitated the Department of Water and Forestry's (DWAF) declaration that companies were required by law to remediate their contaminated effluent in a sustainable manner (Theunissen, 2008). Of significant concern is that the South African economy is heavily reliant on mining for economic development. As a result, there is continuous mineral resource exploration, discovery and extraction. Thus non-profit mines are continuously abandoned and new mines are opened. There is employment opportunity and significant economic development during mine productivity. These mines, both productive and abandoned, generate AMD, requiring treatment.

The area where the Witswatersrand basin (Gauteng, South Africa) is located has been extensively mined for over a century (Edwards *et al.*, 2000).The Witswatersrand Basin (Gauteng, South Africa) is vast and comprises of four basins, the Far Western Basin, Western Basin, Central Basin and Eastern Basin (Theunissen, 2008). Initially the Western basin was of concern as it was flooding at a rate of 20 $ML.d^{-1}$ (Theunissen, 2008; Naidoo, 2009). The Western basin required continuous pumping due to the rate at which mines were becoming non-operational in that area. The problem however escalated, due to discontinuation of mining all accross the region, all four basins are now affected and are generating AMD on a continuous basis (Naidoo, 2009). By 2008, there was concern for the rate at which underground AMD was rising (Theunissen, 2008). However, by 2010, AMD had reached the surface and reports of car tyres and pipelines being corroded due to AMD in the Gauteng province (South Africa) were common place (De Lange, 2010). Acid mine drainage occurs naturally at pH range between pH 3.5 and pH 6.5 in the Witwatersrand region (Johannesburg, South Africa), and Bell *et al.* (2001) reported AMD between pH 2.6 to pH 3.5 at Middelburg Colliery (Witbank, South Africa) (Bell *et al.*, 2001; Naicker *et al.*, 2003; Tutu *et al.*, 2008). Conventional waste water remediation strategies were initially employed but were exposed as unsustainable when the government had to bear the remedial costs. Remedial activities such as liming, flocculation and oxygenation implemented by Pamodzi Gold's Grootvlei Mine (Grootvlei, South Africa) were neither cost efficient nor sustainable. The mine was utilizing 25-30 $tons.d^{-1}$ lime at a cost of R 1 095.ton^{-1} in order to

stabilize the iron present in the contaminated water. These remediation efforts had to be substantially subsidised by the state after Pamodzi Gold's Grootvlei Mine was unable to continue processing their contaminated effluent and had begun releasing waste water into the environment (Naidoo, 2009). Sustainability of the processes utilized is of vital concern due to the costs involved and impact on the environment. Chemical precipitation, ligands, coagulation and membrane filtration are all conventional physicochemical waste water remediation techniques. These processes are highly efficient and rapid (Eccles, 1995). However, with the quantities of AMD currently requiring remedial intervention in South Africa, these processes become costly and unmanageable (Tutu *et al.*, 2008). Resultant products such as toxic recalcitrant sludge require further financial input for treatment and disposal. Additional costs incurred are not feasible for companies especially if the mine has been abandoned due to a lack of productivity (Bell *et al.*, 2001). Thus the emphasis on sustainable biological remediation techniques such as constructed wetlands, the integrated algae pond system (IAPS) and the algae sulphate reducing pond process for acid and metal waste water treatment (ASPAM). These processes are highly efficient in the remediation of waste waters (Rose *et al.,* 2002), however due to the lack of a bioavailable, cost competitive carbon source, the application of these processes is limited.

The IAPS and the ASPAM bioprocesses require a relatively high chemical oxygen demand (COD) for the anaerobic digester component to function. These technologies also utilize HRAOPs to treat effluent generated by the anaerobic digester using photosynthetic energy. The HRAOPs treat the water by removing nitrates and phosphates, causing alkalinization and removing metals through normal microalgae functionality. Both the IAPS and the ASPAM processes however, require a constant carbon supply in the form of domestic waste water or tannery effluent (Rose *et al.,* 2002). The IAPS and ASPAM technologies were already generating copious amounts of nutrient rich algae biomass which was simply being discarded. This research identified the potential use of the wasted algae biomass as a carbon source for a sulphidogenic fermenter, where COD is not required to be high, thereby aiding in the remediation of waters such as AMD. The subsequent investigation demonstrated the capability of the algae biomass generated by the HRAOPs of an IAPS to serve as a carbon source in the sulphidogenic remediation of AMD. Anaerobic suphidogenic metabolism of biodegradable organic compounds within a closed anoxic environment decreases the release of green house gases such as carbon dioxide, hydrogen sulphide and methane into the biosphere. Oyekola *et al.*

(2009) noted that anaerobic consortia hydrolyse insoluble organic polymers such as carbohydrates in sequence until terminal utilization by sulphate reducing bacteria (SRB). Acidogenesis results in the breakdown of carbohydrates and lipids to volatile fatty acids, ammonia, carbon dioxide and hydrogen sulfide. After this process, acetogenesis takes place where acetic acid, carbon dioxide (25-50 %) and hydrogen (1 %) are produced. Finally methanogenesis and sulphidogenesis convert products of previous stages to methane (50-75 %), hydrogen sulphide, carbon dioxide and water. Fermentation of complex compounds like carbohydrates and lipids through the anaerobic food chain produces sugars and volatile fatty acids, which aid the reduction of sulphate by SRBs to sulphide (Zhao *et al.*, 2008; Zhao *et al.*, 2010).

The outcomes of Chapters 3 and 4 demonstrated the use of the algae biomass as a carbon source for sulphidogenic fermentation. Many other carbon sources have been tested for utilization by SRBs in consortia for the remediation of polluted waste waters. These include molasses, domestic sewage, whey, tannery wastes and animal manure (Boshoff *et al.*, 2004a; Boshoff *et al.*, 2004b). Present research has showed the activity of the EBRU 00AB/06 SRB consortium to be slightly higher in medium containing an algae biomass produced by an IAPS than in medium containing lactate only. Teclu *et al.* (2009) observed that while molasses was a potential carbon source for SRB activity, in comparison to a lactate containing medium, the level of SRB activity was significantly reduced.

Biochemical and elemental analysis of the algae biomass in Chapter 3 showed the algae composition to be as expected. Algae biomass in Chapter 3 contained proteins, amino acids, reducing sugars and lipids. Macromolecules such as lipids are not immediately available for use by SRBs however hydrolysis yields glycerol and fatty acids. Algae biomass is easily cultivated, harvested and nutrient rich, containing N, P, K, Fe and Si essential for the growth and development of microorganisms (Boshoff *et al.*, 2004a; Chisti, 2007; Lv *et al.*, 2011). Irisarri *et al.* (2007) successfully used an algae biomass as a slow release biofertilizer in rice cultivation while Boshoff *et al.* (2004a) demonstrated the remediation of acid waste waters by an SRB consortium utilizing pure *Spirulina*. The photosynthetic activity of the algae biomass could also serve to remove carbon dioxide from the environment and the standing algae biomass would serve as a carbon sink (Huntley *et. al*., 2007). Photosynthesis would negate the levels of carbon

dioxide generated by the anaerobic degradation of the algae biomass resulting in a near neutral technology with possible carbon accreditation. Botes *et al.* (2010) recently established a novel SRB consortium utilizing glycerol as the carbon source. The SRB consortium was able to counteract corrosive properties of low pH AMD. The consortium thrived at pH 3 and improved the pH of the system to above pH 6. A secondary fermenter was employed to capture hydrogen sulphide generated by the system using reactive iron forming iron sulphides, further remediating AMD (Botes *et al.*, 2010). The current study demonstrated a similar improvement in the pH of the AMD through the mixing of the algae biomass with the AMD prior to introduction into a sulphidogenic fermenter. After which a further increment towards alkalinity of the AMD was observed once this mixture was exposed to the activity of the EBRU 00AB/06 SRB consortium.

Results of the present study also demonstrate that the EBRU 00AB/06 SRB consortium is capable of efficient utilization of a mixed algae biomass as a carbon source. The algae biomass can be generated on site on a continuous basis and in copious amounts within the paddlewheel mixed high rate algae oxidation pond (HRAOP) provided nutrients such as N, P and K are made available to the algae. Utilization of an appropriate carbon source containing other essential minerals such as N, P and K in the remediation of AMD is the ideal approach to overcoming nutrient limitations associated with areas where AMD occurs. Based on the findings of the study and information in literature a novel process for the remediation of AMD is proposed and the details are presented in Figure 5.1. An IAPS could be constructed adjacent to the site of AMD occurrence. This IAPS would not only serve to supply a biomass but also remediate domestic effluent generated by the mine on site. The algae biomass generated would be harvested from the HRAOP after which, the algae biomass would be mixed with the AMD in a mixing tank. The mixing tank is the site of increased alkalinity of AMD and the rupture of the algae cells. It should be noted that a paddlewheel mixed HRAOP can be established as a separate entity from the IAPS and fertilizers containing N, K and P could generate nutrient rich algae communities that would provide an energy source as well as essential mineral supply for the SRB consortium. The AMD/algae biomass mixture would then be transferred into an anaerobic sulphidogenic fermenter containing an SRB consortium. The SRB consortium would reduce the sulphate and iron concentrations of the influent, while further decreasing the acidity of the water.

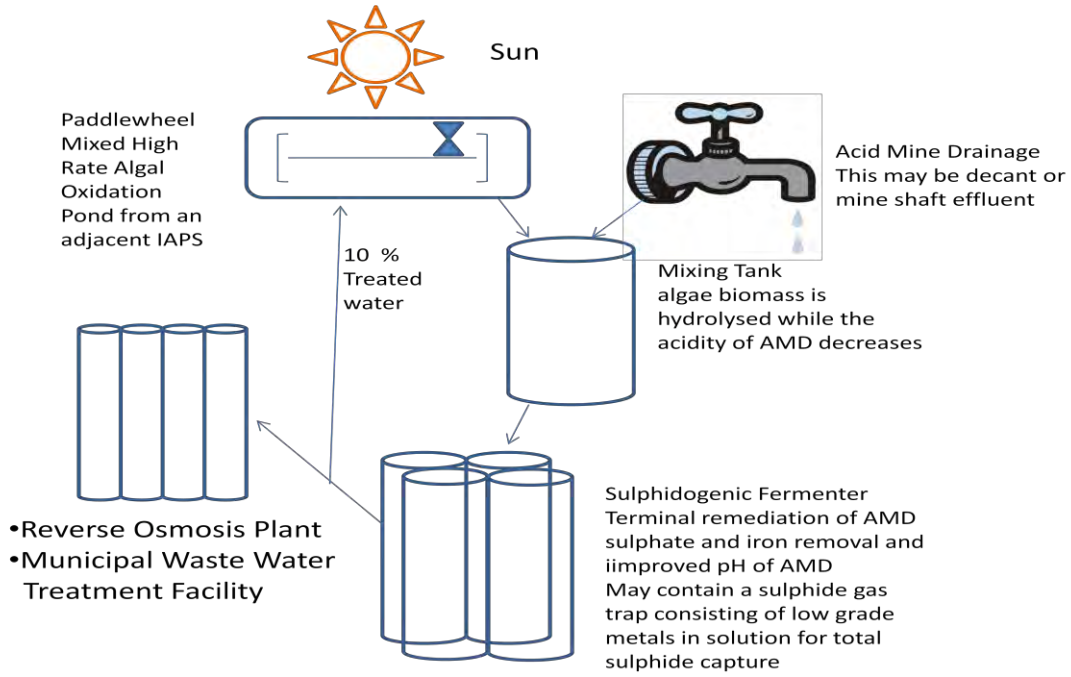

Figure 5.1: A proposed integrated sulphidogenic process for the remediation of AMD by the EBRU 00AB/06 SRB consortium utilizing an algae biomass generated by an IAPS.

The biological strategy investigated, does not generate a toxic recalcitrant sludge. McCauley *et al.* (2009) reported that metal sulphide precipitates generated during biological sulphate reduction are highly stable and form sediment at the base of the reactor. However excess sulphide gas generated by the system still needs to be eliminated. Equation 5.1 demonstrates a potential avenue for stabilization of the sulphide gas utilizing iron (Botes, 2010).

$H_2S + Fe^{2+} \rightarrow FeS + H_2$…….Equation 5.1

One mole of sulphate remediated from AMD generates one mole of sulphide gas. Therefore the sulphate concentration of AMD can be utilized to determine the potential concentration of hydrogen sulphide generated by the sulphidogenic system. Sulphide, a highly reactive gas may be displaced by the continuous gas production of the SRB consortium into a sulphide trap established adjacent to the sulphidogenic fermenter to ensure total sulphide capture. Low cost divalent cations in solution such as Fe^{2+}, Ca^{2+}, Cu^{2+} and Mg^{2+} (Bhagat *et al.*, 2004), easily react with hydrogen sulphide to generate low grade sulphide precipitates, thereby serving as a potentially permanent sulphide sink. These sulphide precipitates do not interfere with the functionality of the SRBs or other microorganisms.

Sulphate reducing bacteria are a promising avenue for remediation of AMD due to abundance, ease of cultivation and storage for extended periods (Oberholster *et al.*, 2010). Exposure of the consortium to elevated sulphate showed that the SRB consortium could withstand and treat sulphate concentrations exceeding 3000 $mg.L^{-1}$. High sulphate containing waste water streams generally contain sulphate concentrations of between 2000 to 3100 $mg.L^{-1}$ (Naicker *et al.*, 2003). Iron in the form of 'yellow boy' is the universal indicator of AMD (Akcil and Koldas, 2006). Accordingly it was vital to determine the concentration at which iron would inhibit activity of SRBs. An iron concentration >0.5 $g.L^{-1}$ was inhibitory; this concentration is relatively high, unless the waste water stream is excessively contaminated. Thus, the reaction of sulphide a highly corrosive, reactive and volatile gas (Chou *et al.*, 2008) within the sulphidogenic system with the metals in solution to form sulphide precipitates, which would capture this gas as well as increase pH of treated effluent. The resultant metal sulphide precipitate is stable and does not affect the activity of the SRB consortium (Botes *et al.*, 2010; Zhao *et al.*, 2010). Zhang *et al.* (2009) reported that the continuous occurrence of metal sulphide precipitation generated a stable sludge that could be harvested and from which valuable minerals recovered. Low grade reactive metals could be utilized to stabilize sulphide generated by the extraction of the valuable minerals thereby preserving the sulphur sink. Other AMD physicochemical treatment mechanisms such as membrane filtration and ion exchange systems generate a concentrated toxic sludge composed metal hydroxides, sulphates and phosphates that are recalcitrant, environmentally detrimental and require costly disposal (Wei *et al.*, 2003). Approximately 150 $tons.ha^{-1}.yr^{-1}$ algae biomass is generated by the IAPS implemented in Grahamstown, this waste biomass can potentially remediate 124 $tons.ha^{-1}.yr^{-1}$ AMD. Thus the use of a biological catalyst such as the EBRU 00AB/06 SRB consortium utilizing an algae biomass to remediate AMD is a viable, environmentally friendly, cost effective and malleable process.

Recommendations for future work would be to molecularly identify components of the EBRU 00AB/06 SRB consortium to accurately ascertain parameters within which the components can function. Then to transfer the entire process into a continuous upflow packed bed fermenter. Sulphate reducing bacteria have been reported to work optimally when immobilized (Gibson, 1990; Gibson *et al.*, 1993; Tang *et al.*, 2009). The packed bed fermenter would run as a continuous flow system and would be able to account for flushing out of the components of the SRB consortium from the fermenter. The packed bed fermenter would also determine the extent

to which the consortium is able to degrade the algae biomass. An inability to efficiently degrade algae cells could result in clogging of the fermenter. Finally the upflow packed bed fermenter would serve as a preliminary analysis prior to pilot scale operation consequently establishing a process where an immobilized SRB consortium is able to function in the presence of an AMD/algae biomass mixture, without the inoculum washing out of the continuous upflow packed bed fermenter. A pilot plant may then be constructed. In the case of a two part fermenter a section could be utilized for sulphidogenesis and another for methanogenesis. Methanogenesis would decrease the chemical oxygen demand (COD) dramatically by generating methane which could eventually be used in energy production. While the sulphidogenic section would generate sulphide and remove dissolved minerals, remove sulphates and improve the pH of the system. Methanogenesis followed by sulphidogenesis could also act as a pre-treatment step prior to introduction of a waste water stream into a reverse osmosis plant. AMD remediation will decrease the salt concentration of the water prior to introduction into a reverse osmosis plant. This would in turn decrease membrane clogging and production of a recalcitrant sludge. The membrane life span would be longer and minerals could easily be harvested appropriately at a later stage from the sulphidogenic fermenter.

Sulphate reducing bacteria (SRB) are a widely researched group of microorganisms (Postgates 1984; Holland *et al*., 1987; Gibson, 1990; Van Hille, 1999; Boshoff *et al*., 2004b). The cost effective carbon source in the form of the algae biomass proposed by this study was efficiently utilized by the EBRU 00AB/06 SRB consortium in the remediation of AMD. A biological mechanism for remediation of AMD is optimal due to its efficiency, low input and labour costs, low maintenance, ability to be set up virtually anywhere, concentration of valuable minerals and decreased sludge (Azimi and Horan, 1991; Zhao *et al.*, 2010). The EBRU 00AB/06 SRB consortium displayed that it is a viable mechanism for the remediation of simulated AMD when utilizing the algae biomass. Thus more needs to be done in terms of the implementation of such SRB mediated technologies.

Acid mine drainage (AMD) is an ever increasing concern for the South African government and public, and will continue to be at the forefront of the environmental agenda until viable remediation strategies can be utilized. The legacy of mining in South Africa has had disastrous implications for the environment. These implications are however reversible, thus it becomes

vital to engineer sustainable strategies, generated specifically for problems facing the South African environment. All components of the bioprocess investigated in this research are of South African origin and as stated in the findings of the research able to deal with the problem of AMD within South Africa. Access to a clean water supply is a right not a privilege. Therefore it is vital to implement sustainable remediation strategies such as the one demonstrated in this investigation. In conclusion, the potential of bioremediation is limitless and the present study demonstrates how an idea and a vision with perseverance and planning can internally resolve one of South Africa's many water pollution concerns while utilizing indigenous resources.

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Appendices

Appendix A

A1 Preparation of Modified Postgates Medium B (Postgate, 1984)

Solution A

6 mL Sodium lactate (60 % solution w/v)

4.5 g Sodium sulphate

1.0 g Ammonium chloride

1.0 g Yeast extract

0.5 g Potassium hydrophosphate

1.0 g Sodium citrate dihydrate

0.06 g Calcium chloride hexahydrate

0.06 g Magnesium sulphate heptahydrate

The above reagents were dissolved in 990 mL distilled water.

Solution B

1.0 g Ascorbic acid

1.0 g Sodium thioglycolate

The above reagents were dissolved in 100 mL distilled water.

The pH of both solutions was adjusted to between pH 7.0 – pH 7.5 with 5 M sodium hydroxide and 5 M nitric acid. Solutions were autoclaved separately, after which 10 mL solution B was added to solution A to make 1 L and mixed thoroughly.

Laboratory Scale Sulphidogenic Fermenter

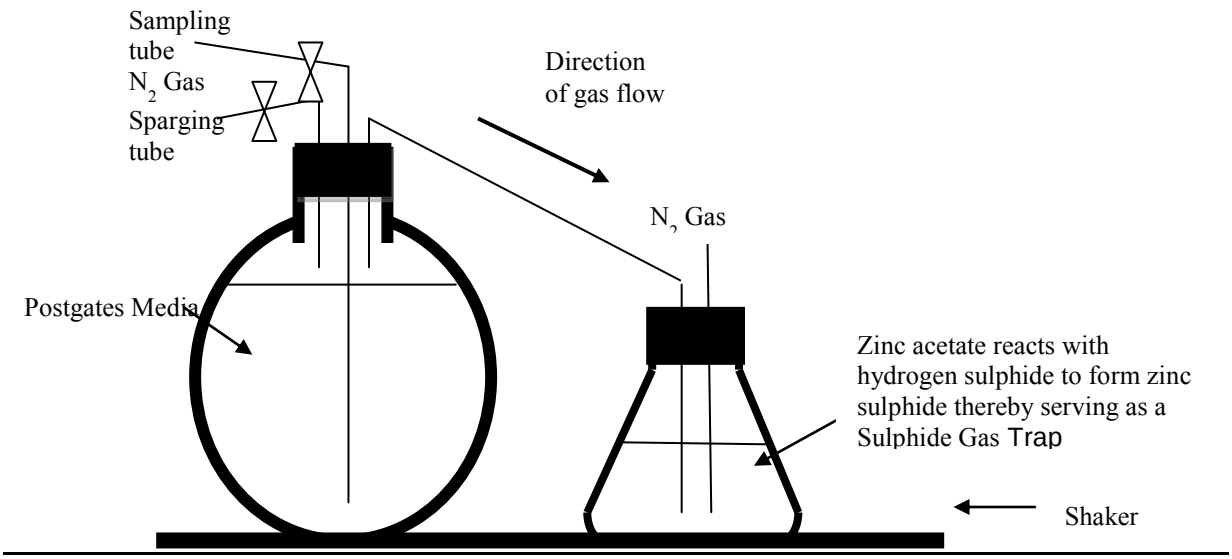

Figure A.1: Design of the sulphidogenic fermenter for culturing an SRB consortium

A2 Preparation of Modified Lactate SRB Medium (Atlas, 1993)

3.5 mL Sodium lactate (60 % solution w/v)

0.5 g Magnesium sulphate heptahydrate

0.5 g Ammonium chloride

1.0 g Calcium sulphate dihydrate

1.0 g Yeast extract

0.3 g Potassium hydrophosphate

0.1 g Sodium thioglycolate

0.2 g Ascorbic acid

0.5 g Iron (II) sulphate heptahydrate

0.01 g Resazurin

0.1 g Calcium chloride dihydrate

Components were dissolved in 1 L distilled water prior to addition of 15 g nutrient agar to the medium. Medium was then autoclaved for 15 minutes at 121 ˚C. After cooling to approximately 45 ˚C plates were poured using the sterile technique and left to solidify.

A3 Preparation of Concentrate Borate Gluconate Buffer for the HPLC

9.6 g Calcium gluconate is dissolved in 500 mL milli-Q water.

7.2 g Lithium hydroxide was added

25.5 g Boric acid was added

100 mL Glycerol

This was then made up to the 1 L mark with milli-Q water and stored in the fridge at 4 ˚C.

A4 Preparation of Mobile Phase Borate Gluconate Buffer

20 mL Concentrate

120 mL Acetonitrile

Made up to 1 L with milli-Q water

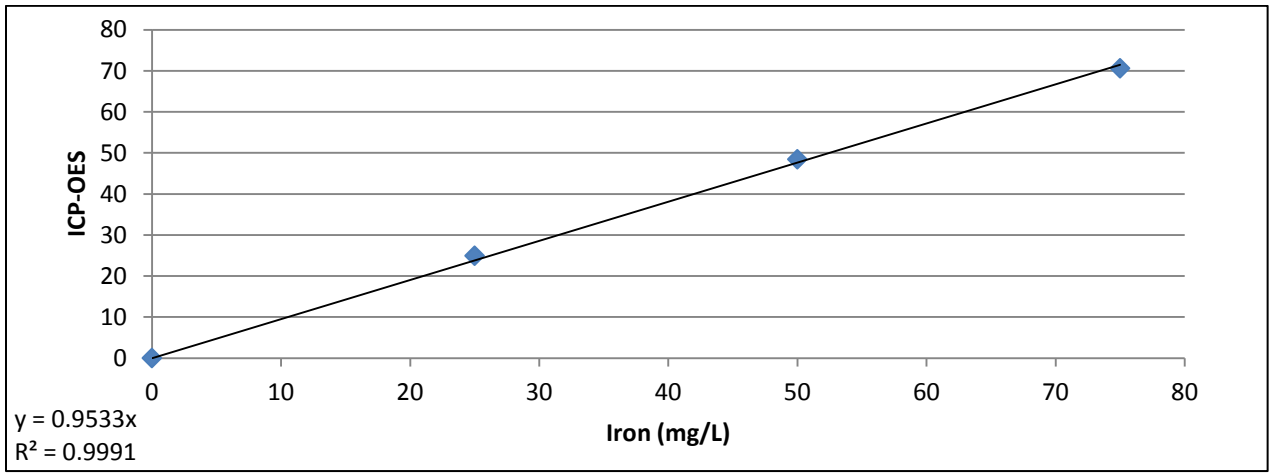

Figure A.2: Graph of increasing iron concentrations measured by ICP-OES utilized as the standard curve.

Appendix B

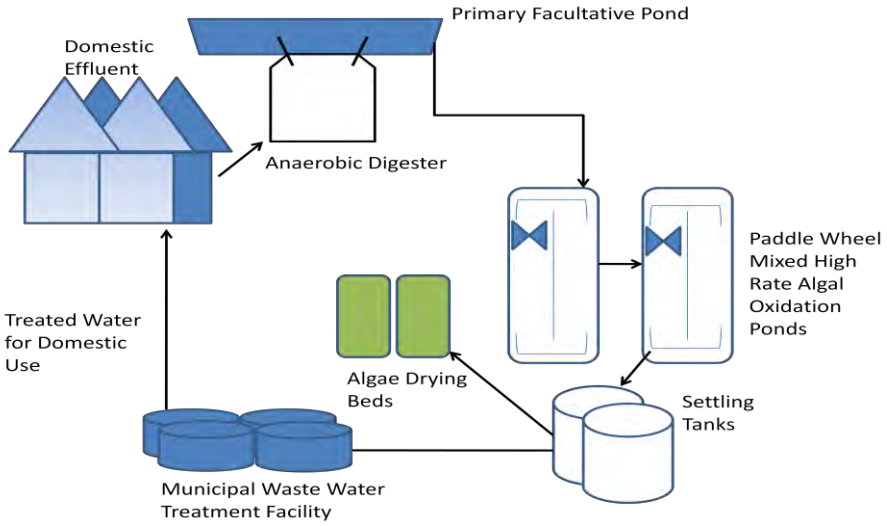

Figure B.1: A schematic of the integrated algae pond system (IAPS) at the Institute for Environmental Biotechnology Rhodes University (Grahamstown) utilized for the treatment of domestic waste water.

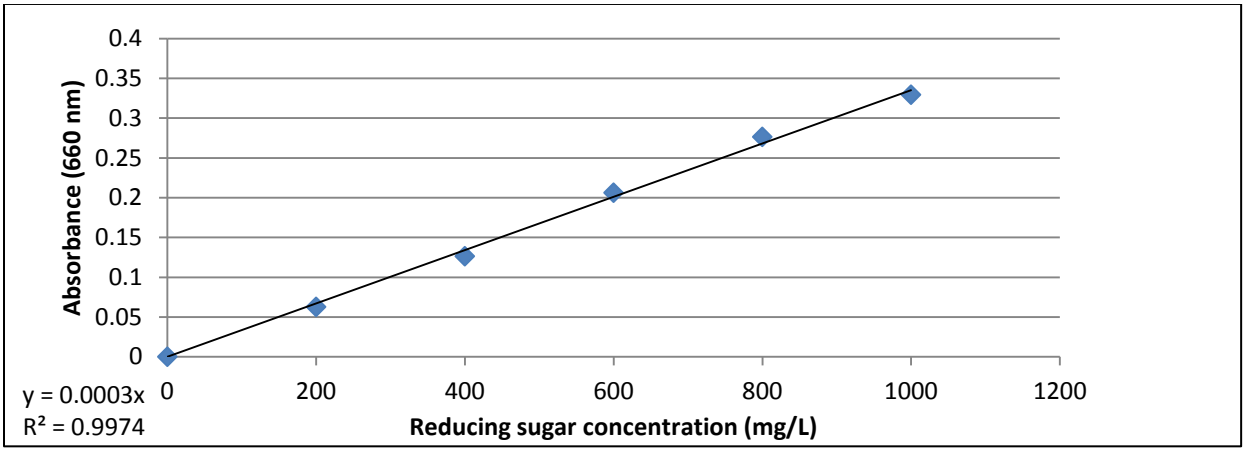

Figure B.2: Graph of the increasing concentrations of D-glucose at 660 nm, the resultant curve was used to interpolate unknown reducing sugar concentrations in the samples.

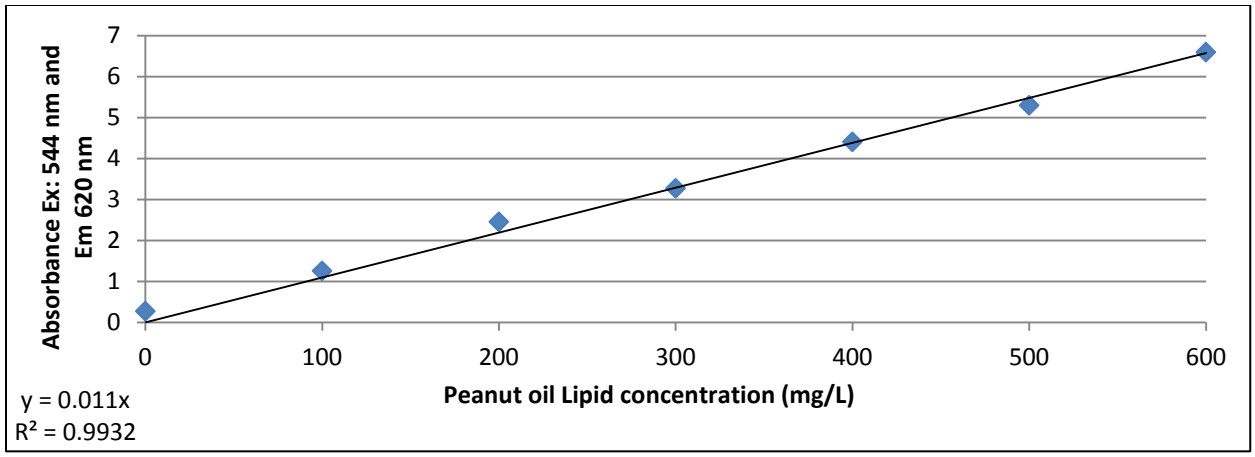

Figure B.3: Graph of increasing concentrations of peanut oil determined using the Nile Red assay, the curve was used to interpolate unknown lipid concentrations in the samples.

B1 Preparation of Iodine Solution to Determine the Presence of Starch

0.5 g Iodine

1.0 g Potassium iodide

Dissolve the above reagents in 100 mL warm distilled water. Test the solution using pure starch as the control. Apply a droplet of iodine solution to the starch control; if a blue black colour develops starch is present however an orange colour brown indicates the absence of starch.

B2 Preparation of the Bradford Reagent to Establish Protein Concentration

100 mg Coomassie Brilliant Blue G-250

50 mL 95 % Ethanol

100 mL 85 % (w/v) Phosphoric acid

Dissolve completely in milli-Q water to give a final volume of 1 L then filter through Watman Number 1 filter paper, prior to use.

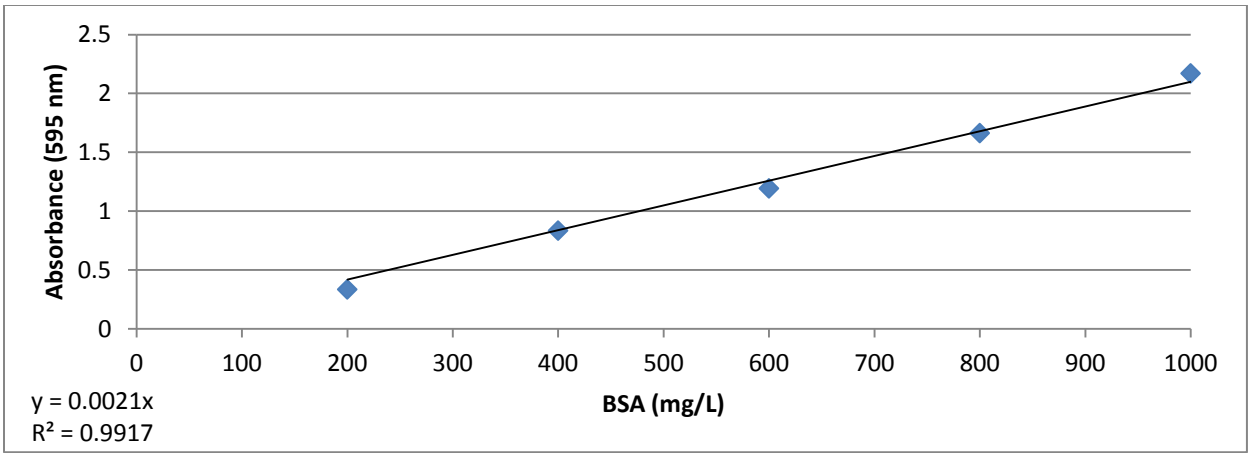

Figure B.4: Graph of increasing concentrations of BSA determined using the Bradford assay, the curve was used to interpolate unknown protein concentrations in the samples.

B3 Preparation of the 4 % (w/v) Ninhydrin Reagent to Determine Amino Acid Content

4 g Ninhydrin

Dissolve in 100 mL acetone.

B4 Preparation of the 0.05 M Monosodium Glutamate Stock Solution

935.7 mg Monosodium glutamate

Dissolve the above reagent in 100 mL distilled water.

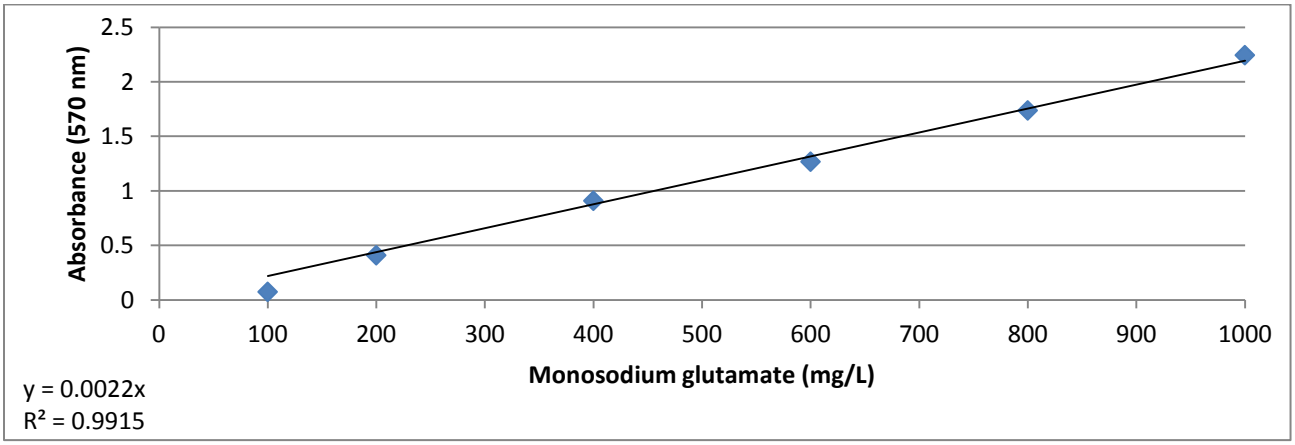

Figure B.5: Graph of increasing concentrations of an amino acid determined using the Ninhydrin assay, the curve was used to interpolate unknown amino acid concentrations in the samples.