DEVELOPMENT OF A

FLUIDISED-BED BIOREACTOR SYSTEM

FOR THE TREATMENT OF ACID MINE DRAINAGE,

USING SULPHATE REDUCING BACTERIA

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ABSTRACT

Dissimilatory sulphate reduction, brought about by the action of sulphate reducing bacteria (SRB) was used in the treatment of acid mine drainage (AMD) in a fluidised bed bioreactor. Biologically produced hydrogen sulphide and bicarbonate ions, by SRB, facilitated the precipitation of heavy metals and the generation of alkalinity in the synthetic acid mine water, respectively. The SRB that had been selected were able to utilize acetate as the sole carbon source and were capable of growing in the bioreactors at low pHs, facilitating an increase in the influent pH from 2.75-7.0 to 5.4-7.8, after a 24-hour hydraulic retention time (HRT). The precipitation efficiencies for Fe, Mn, Zn, Cu, Cr and Al after a HRT of 24 h as metal sulphides ranged between 84- 99% for influent pH values of between 4 and 7, and above 54% for influent pH values between 2.75 and 4. Microbial metabolic activity decreased with decreasing influent pH. This was inferred from the decreasing differences in chemical oxygen demand (COD) depletion rate over a 24 h HRT, as influent acidity levels approached pH 2.75. Molecular studies, using PCR-DGGE analysis on the microbial consortium in the bioreactor, revealed the presence of at least 8 different bacterial species in the consortium. Attempts at sequencing these bands yielded inconclusive results, with the bands showing sequence homology to a large number of previously uncultured and undescribed bacteria. Scanning electron microscopy confirmed the presence of bacteria of different morphology, as well as the presence of biofilms, which account for the heavy metal and low pH tolerances that the bacteria sustained.

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DECLARATION

I declare that this research report is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

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LIST OF ABBREVIATIONS AND UNITS

AMD	Acid Mine Drainage
APS	Adenosine phosphosulphate
ATP	Adenosine triphosphate
C	Carbon
cm	Centimetre
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxy ribose nucleic acid
EPS	Exopolysaccharide
g	grams
HCl	Hydrochloric acid
HDS	High density separation
L	Litre
LB	Luria-Bentini
M	Molar (moles per litre)
mg	Milligrams
ml	Millilitre
Ml	Mega litres
NaOH	Sodium hydroxide
ORP	Oxidation reduction potential
PCR	Polymerase chain reaction
psi	Pounds per square inch

rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
SRB	Sulphate reducing bacteria
TAE	Tris acetate buffer
TBE	Tris boric acid buffer
TCA	Tri-carboxylic acid
µl	Micro litre
µm	Micro meter
µM	Micro molar
ºC	Degrees Celsius

1 INTRODUCTION

1.1 Background on mining in South Africa

Gold mining in South Africa has significantly contributed to the economic development of the country. The industry started around the 1870s, and has grown extensively over the past 130 years (www.bullion.org.za). Many towns and cities have been built as a result of the mining industry, and many of the roads and infrastructure in existence have been a direct result of the attempts to serve the emerging mining industry. South Africa is ranked top in the world for identified resource production, and currently has thirty five percent of the world's gold reserves. The South African gold mining industry has been the basis of the economy for over a century, and has also been instrumental in the country's foreign exchange, contributing to the largest portion of foreign exchange (www.bullion.org.za), and will continue to play a vital role in the export earnings of South Africa, thereby impacting on business in the country. Another significant offering from the mining industry is in the employment sector. Mining activities have provided numerous jobs for South Africans with varying levels of skill (www.bullion.org.za).

With advancement of technology, mining operations were able to move deeper, in some cases to as much as four kilometres below the surface (www.bullion.org.za). However, mining at these depths was not sustainable and it soon became

uneconomical to continue mining, owing to exorbitant costs associated with ventilation, cooling, tunnelling and processing (Naicker *et al*., 2003). Associated with this increase in depth, and with the increase and expansion of mining operations, a greater amount of mining waste was produced.

1.2 Pollutions resulting from mining processes

The mining extraction process exposes many metals, sulphides, oxide minerals and ores, the major one being pyrite (FeS_2). Other conglomerates encountered in the mining process have been identified as uraninite (UO_2), brannerite ($UO_3Ti_2O_4$), arsnopyrite (FeAsS), cobaltite (CoAsS), galena (PbS), pyrrhotite (FeS), gersdofite (NiAsS) and chromite ($FeCr_2O_4$). Many metals occur primarily as sulphide ores, associated with pyrite. Metal sulphides that are embedded in the waste or processed ore are usually removed and collected together in the mine dumps, close to the extraction sites (Naicker *et al*., 2003).

The accumulation of these wastes or mining spoils has been shown to impact negatively on the environment and biodiversity. In addition, mine closure and the abandoning of mines, without the proper application measures in place to prevent the development of acid mine drainage has contributed to heavy metal pollution and acidification of surface waters. Most of these mining spoils have never been disturbed, which has resulted in their exposure over prolonged periods to oxygenated rainwater, resulting in the oxidation of the contained metals and waste. (Naicker *et al*., 2003).

The oxidation of pyrite, the most abundant of ores, leads to the acidification of the water that percolates through the dumps. This water is commonly known as acid mine drainage (AMD), and is a significant contributor to pollution of groundwater. Acid, sulphur-rich water is also a by-product of a variety of industrial operations apart from mining, and is widely regarded as one of the most serious forms of water pollution (Johnson, 1995). These waters typically contain elevated concentrations of ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron, as well as sulphates. The low pH of these waters poses a greater threat in that it accelerates mineral dissolution and makes metals and metalloids more soluble (Johnson 1995). Other heavy metals contained within these acidic waters are zinc, cadmium, cobalt, lead, nickel, aluminium, manganese and copper, all in elevated concentrations.

1.3 Pyrite oxidation

The key factors involved in acid generation are the presence of sulphide minerals - which depends on the local rock and mineralogy, water or humidity, and on the presence of an oxidating agent. The process of pyrite oxidation however, can be multi-step, and involves both an oxygen-dependant and an oxygen-independent reaction. The primary oxidant in many cases is ferric iron instead of oxygen (Evangelou, 1995). AMD can therefore be generated in a number of ways and is also dependent on the local microbiology, as will be further discussed. Using the

sulphide mineral pyrite as an example, its oxidation into dissolved sulphate, iron and hydrogen is as follows:

$$FeS_2 + O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \quad (1)$$

The modes of sulphide oxidation have been described as either direct, or indirect (Johnson, 1995). Direct oxidation follows the route of equation 1.

Ferrous iron (Fe^{2+}) is generated, as well as H^+ and SO_4^{2-}, resulting in an increase in acidity. The ferrous iron can also, depending on the oxygen concentrations, microbial activity, and pH, be oxidised to ferric iron (Fe^{3+}). Sulphur oxidising bacteria, such as the acidophilic *Acidothiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*, as well as other *Thiobacillus*-like bacteria, are instrumental in the continual generation of acidic waters in that they are capable of oxidising ferrous iron back to ferric iron, thus allowing further oxidation of pyrite (equation 3). This mechanism has been described as indirect oxidation (Johnson, 1995), and follows the equation:

$$Fe^{2+} + O_2 + H^+ \rightarrow Fe^{3+} + H_2 \quad (2)$$

The ferric iron that is generated by the sulphur oxidising bacteria can act as an oxidising agent for further pyrite oxidation, especially in the absence of molecular oxygen, according to equation 3:

$$FeS_2 + Fe^{3+} + H_2O \rightarrow Fe^{2+} + SO_4^{2-} + H^+ \qquad (3)$$

The regeneration of ferrous iron is thus vital in ensuring pyrite oxidation. Although *Acidothiobacillus ferrooxidans* has historically been the centre of research attention, owing to its easy isolation and purification, other iron-oxidising bacteria have been shown to be as, or even more abundant and possibly more important to iron oxidation than *A. ferrooxidans* (Johnson, 1995). Nevertheless, research has indicated that *A. ferrooxidans*, and other sulphur oxidising bacteria enhance the rate of sulphur oxidation above that which is achieved by chemical means, i.e., through oxidation via molecular oxygen and water (Crundwell, 2003). However, authors differ in their explanation of the mechanisms employed by these microbes to bring about pyrite oxidation.

Some authors are of the opinion that *A. ferrooxidans* plays a direct role in pyrite oxidation, a mechanism that is independent of the presence of ferrous ions. This direct mechanism follows that of equation 1, and intimate contact is maintained between the microbes and the oxidated substrate. In this mechanism, bacteria are the catalyst in the oxidation of pyrite.

It is evident that the difference between the two mechanisms by which bacteria enhance the rate of pyrite oxidation is in the role assumed by the ferric ions. The indirect mechanism assumes that pyrite is leached by ferric ions, which are regenerated from ferrous iron by bacterial action, as outlined in equation 3; while the direct mechanism provides that pyrite is leached by some biological agent, excreted by the microbes themselves (Crundwell, 2003). It has also been documented that irrespective of the proposed mechanisms utilised by the microorganisms in the oxidation of pyrite, their presence on the mineral surface shows a definite increase in the rate of leaching (Crundwell, 2003). According to equation 2, the bacterial action proposed in the generation of ferric iron consumes H^+, thus resulting in an increase in the pH at the mineral surface. This explains the increase in the leaching rate in the presence of microbes, given that leaching rate is proportional to pH (Holmes *et al*., 1999).

According to Akcil and Koldas (2006), the primary factors that determine the rate of acid generation include temperature, pH, oxygen concentration in the water and gas phase, chemical activity of ferric iron, the surface area of the exposed metal sulphide that is oxidised, and microbial activity. Other factors that promote or enhance acid generation are physical factors, such as oxygen permeability of the mine dump or the local rock. A high permeability implies greater oxygen ingress, leading to higher chemical reaction rates and ultimately increased mineral oxidation (Akcil and Koldas, 2006).

1.4 Effects of mining and AMD in South Africa

Following oxidation of the pyrite, the effects of AMD are widespread. Precipitated ferric iron, which is a common component of AMD, tends to clog the interstitial spaces of the substrate by covering the soil layer. As a result, plant life is unable to be sustained in many cases, and is subsequently destroyed. Further effects of AMD are evident from studies of surrounding aquatic habitats. Marked declines in aquatic biodiversity and ecosystems, in addition to declines in productivity have been documented. In extreme cases, AMD has been linked to the total elimination of certain fish species (Gray, 1998). Owing to the site-specific variability in the rock composition, predicting the actual effects of AMD for a particular area can be a challenge (Akcil and Koldas, 2006).

While many countries in the world are able to deal with AMD through dilution, South Africa is unable to do so since it is a water-scarce country, and therefore efforts and measures need to be in place to ensure effective treatment of waste water as well as to ensure that this contaminating water does not enter rivers and streams that serve as water supplies. One area in South Africa where acid mine water poses a great threat is the Witwatersrand area of Johannesburg. Gold fields in this area (Wits basin) span distances 350 km long and 200km wide, reaching depths of 3500 m. This area has been a considerable source of gold mining in South Africa. As a result of the lucrative mining industry in the area over the years, a plethora of shafts and adits have been left unattended underground, which have steadily filled with water. All the mines in these areas are believed to be

interconnected, with water filling each shaft in turn until it finally collects at the lowest point, all the time exposed to minerals and rocks that characterise these areas, especially pyrite (Anon, 2005). It has been reported that gold mines in the Wits basin have been contributing as much as 35% of the salt load that enters the Vaal Barrage (Anon, 2005).

At the Grootvlei Gold Mines at present, an average of 75 Ml of water is pumped out daily in order to retain access to the mining operations. This water is treated at a high-density separation (HDS) plant in order to remove iron and increase the pH to suitable levels before the water is released into the Blesbokspruit River. The HDS technique employed is effective in reducing iron levels from more than 180 $mg.l^{-1}$, to less than 1 $mg.l^{-1}$. However, the water that enters the Blesbokspruit River still retains a high level of dissolved salt concentrations, such as sulphate, calcium, magnesium, chloride and sodium, which invariably impacts on the water quality downstream (Anon, 2005). This is a major concern, since a portion downstream of this river has been declared an international RAMSAR wetland site (www.environment.gov.za). The increasingly poor water quality of the Blesbokspruit is also said to impact on the freshwater resources of the Witwatersrand area in the long term (Roychoudhury and Starke, 2006).

Heavy metals present in AMD, as well as trace elements have little degradation potential, and they are thus inclined to accumulate in sediments, resulting in intrinsic toxicity levels over time (Schulin *et al*., 1995). From these repositories,

the metals have the potential of moving along the food chain through bioaccumulation, and result in genotoxicity among living organisms (Patra *et al.*, 2004). The formation of AMD is more significant when mines are abandoned or closed, since in many active mining operations water tables are kept relatively low through pumping. When these mines are closed, water tables rise, leading to the formation of AMD-contaminated groundwater, which are often discharged into the environment.

The need for acid mine water treatment should be evident, in light of the potential damage these waters pose to sensitive eco-systems. Another important factor that needs to be kept in mind is that South Africa is water–scarce, and therefore the existing and potential contamination of our scarce water sources should warrant immediate attention.

1.5 Current strategies in place for treating AMD

There are currently a few programs in place aimed at treating the effects of AMD, as well as programs aimed at preventing excess formation of AMD. These can be classified as "source control", in which prevention of AMD is of primary concern; and "remediation", in which either biotic or abiotic strategies are employed in the treatment of AMD (Johnson and Hallberg, 2005).

Given that water and oxygen are needed for the formation of AMD, the exclusion of either of these, or both, can possibly minimise AMD production. This can be achieved through flooding and sealing of abandoned mines. In these cases, the microorganisms consume the dissolved oxygen present in the water, leaving little oxygen for AMD formation. Sealing of the mine will then prevent oxygen replenishment (Johnson and Hallberg, 2005). This approach can only be successful if all the mine openings are known and there is no danger of water or oxygen entry through any adit or other opening. Since it is very difficult to ensure that water and oxygen will never enter the sealed off mine, this approach has its limitations (Johnson and Hallberg, 2005).

Underwater storage of potentially acid producing mine water is also employed, in which contact between the minerals and dissolved oxygen is minimised. Organic material may also be used to cover the tailings in an effort to further reduce oxygen influx and prevent resuspension of tailings through waves and winds. The tailings can also be sealed off using clay, although seasonal temperature fluctuations may be problematic in some cases, depending on the properties of the sealing layer (Swanson *et al.*, 1997).

The production of environmentally friendly and stable products through the blending of acid-generating and acid-consuming material is another suggested approach (Mehling *et al.*, 1997). In the case of AMD, solid-state phosphates can be added to pyritic mine wastes in order to reduce the oxidation potential of Fe (III), by precipitating it as ferric phosphates. Soluble phosphates with hydrogen

peroxide have also been developed, in which the peroxide oxidises the pyrite, producing ferric iron, which subsequently reacts with the phosphate to produce ferric phosphates (Evangelou, 1998).

Since sulphur-oxidising bacteria play a vital role in the continual generation of AMD, approaches have been developed aimed at limiting or inhibiting their activity. Various surfactants have been tested, such as sodium dodecyl sulphate (SDS). These chemicals are usually highly toxic to this group of bacteria. The effectiveness of these biocides tends to be highly variable, and have shown to provide only short-term control, implying that repeated applications are necessary (Johnson and Hallberg, 2005). It is evident that inhibiting the formation of AMD is often not practical, and the effectiveness of this strategy depends on a number of factors. In this regard, often the only feasible manner of dealing with AMD is through treatment controls, which can be classed into different categories, depending on whether the treatment is biotic, abiotic, active or passive.

The most widely used method involves an active treatment process in which chemical-neutralising agents are added. Alkaline materials are added to raise the pH, facilitating the precipitation of the metals as metal hydroxides. An increase in pH also causes an increase in the rate of ferrous iron oxidation, resulting in an iron-rich sludge that, depending on the chemistry of the water being treated, may contain a host of other metals (Johnson and Hallberg, 2005). Neutralising agents that are being used presently include calcium oxide (lime), sodium carbonate, sodium hydroxide and calcium carbonate. When a calcium-containing neutralising

agent is used, some sulphate is removed in the form of gypsum, which is bulky and needs to be disposed of, creating a great disadvantage through high operational costs.

Alkalinity can also be generated for AMD treatment through the use of anoxic limestone drains. This method is a passive approach, in which mine water is directed to flow through a bed of limestone in a drain that excludes water or air. These anoxic limestone drains offer a lower cost alternative to chemical addition, but are not suitable for all kinds of AMD, since hydroxide precipitates tend to build up in these systems, reducing their effectiveness (Johnson and Hallberg, 2005). Also, aerated mine water is not suitable for this treatment process, and often have to be passed though anoxic ponds to reduce the dissolved oxygen concentration in order to prevent iron oxidation (Johnson and Hallberg, 2005). Studies have shown that water quality can be drastically improved when mine water is treated using both constructed wetlands and anoxic limestone drains (Kleinmann *et al.*, 1998).

Biological remediation strategies are often the preferred approach, in which biological systems are used in AMD treatment. Natural wetlands have been used extensively for the treatment of environmental contaminants and a wide array of water quality problems. Constructed wetlands are able to emulate natural wetlands, and afford the luxury of manipulation and control (Sheoran and Sheoran, 2006). In a constructed wetland, some basic processes are involved, such as uptake of nutrients by plants, degradation and oxidation of contaminants by

bacteria, particle sedimentation, etc. (Sheoran and Sheoran, 2006). Wetlands are complex ecosystems that allow interactions between the terrestrial and aquatic ecosystems, as well as a continuum of habitats from open water to marginal wetlands. Soil properties in the wetland are also variable. Wetland habitats are therefore exploited in the remediation of contaminated waters, owing to the wide array of chemical and physical properties present, as well as the fauna and microbial organisms that are instrumental in the removal of heavy metals.

1.6 Microbial based remediation

Microbial processes that are exploited in generating net alkalinity are mostly reductive processes such as methanogenesis, denitrification, and sulphate reduction. Phototrophic microorganisms can also generate alkalinity through the consumption of a weak base such as bicarbonate, and in turn producing a stronger base like hydroxyl ions (Johnson and Hallberg, 2005). Research has shown that bacterial metabolism can contribute significantly to the removal of heavy metals and to the generation of alkalinity in contaminated waters. In particular, sulphate-reducing bacteria (SRB) are able to oxidise simple organic material, while using the sulphate ion as the terminal electron acceptor, in a process that produces hydrogen sulphide (H_2S) and the bicarbonate ion (HCO_3^-) (Johnson and Hallberg, 2005; Dar *et al*., 2005). Added advantages of AMD remediation using SRB are the opportunities to recover and recycle metal sulphides present in the AMD (Boonstra *et al*., 1999). Before discussing SRB in more detail, it being the subject

of the present study, it is fitting to discuss the role of sulphur, and sulphur cycling within ecosystems.

1.7 Sulphur and the sulphur cycle

Sulphur, as part of the chemical structure of living organisms, is an essential element of the biosphere and is widespread on earth. It accounts for 0.2 to 0.7 % of the dry mass of most organisms (Anderson, 1978). The element can exist in a large number of oxidation states, the most common in the environment being the oxidation state + 6, in the form of sulphate (SO_4^{2-}). This is the most oxidised form in which sulphur exists, and is also the most stable sulphur form. Other oxidation states of sulphur do exist in localised conditions, such as in anaerobic soils, where sulphur can be found as sulphides (S^{2-}) and thiosulphates (S^{2+}) (Anderson, 1978).

Like all biological elements, sulphur is cycled in the biosphere through biogeochemical means, which is a combination of chemical and biological processes. Sulphur oxides and hydrogen sulphide are liberated into the atmosphere through the burning of fossil fuels, and through volcanic and thermal emissions, which are then deposited as sulphurous and sulphuric acid onto the earth's surface, from where they are leached into rivers and streams, eventually reaching the sea (Postgate, 1984). These are some of the geochemical agencies that facilitate sulphur turnover in the biosphere. Supplementing the chemical means are the biological processes, which ultimately balance the cycling of

sulphur. A wide variety of sulphur interconversions are achieved through oxidation and reduction reactions mediated by organisms.

Reduced forms of sulphur are oxidised by either heterotrophic organisms, or chemo- or photo- autotrophic bacteria. Chemoautotrophic bacteria aerobically oxidise reduced forms of sulphur in exergonic reactions that serve as energy sources for C assimilation. Photoautotrophic bacteria on the other hand, use sulphide as an electron donor in aerobic conditions instead of water, in a manner analogous to photosynthesis in green plants (Anderson, 1978). Heterotrophic organisms, like animals, are also able to oxidise sulphur as part of normal protein and amino acid turnover (Anderson, 1978). The reduction of sulphur on the other hand, is achieved through either assimilatory or dissimilatory reactions.

Assimilatory sulphate reduction allows for sulphate to be reduced to sulphide, which in turn is biologically incorporated into the organism as amino acids and proteins. Green plants and most aerobic bacteria are able to carry out assimilatory sulphate reduction, at the expense of metabolic energy (Anderson, 1978).

Dissimilatory sulphate reduction reactions are those in which oxidised forms of sulphur are reduced in the anaerobic respiratory process, with very little amounts of the reduced sulphur used for amino acid synthesis. The rest of the reduced sulphur is stored in the cells or is released into the environment. *Desulfovibrio* sp. and *Desulfotamaculum* sp. are among the dissimilatory sulphate reducers. Under

anaerobic conditions, these organisms use the oxidised sulphur compounds as the terminal electron acceptor in anaerobic respiration, rather than oxygen as is the case in aerobic respiration. The electron transport chains are similar in both aerobic and anaerobic respiratory processes, found in the bacterial cytoplasmic membrane (Levett, 1990).

1.8 Sulphate-reducing bacteria

The diversity, phylogeny and metabolism of SRB have constantly been re-evaluated in the literature. They were first discovered in 1895 by Beijerinck (Postgate, 1984), and since then many authors have attempted to describe and categorise these organisms, but to this day they remain a relatively poorly understood group. This difficulty in their understanding can be attributed to the obstacles encountered in their culture, as well as to the bioenergetics of their metabolism and energy sources. The existence of acetate-utilising SRB for example, was previously doubted by Postgate (1979). They were however, subsequently found to be in widespread existence. Other dogmas associated with SRB, their metabolic patterns and their ecology have also been recently renewed. The extent of the diversity between SRB has only recently been appreciated.

SRB are a diverse group of chemo-organotrophic bacteria that are characterised by their ability to utilise oxidised sulphur compounds as the terminal electron acceptor in the degradation of organic compounds, under anaerobic conditions.

The requirements for sulphur and anaerobiosis determine the environments in which these organisms are found.

The cellular physiology and carbon metabolism of SRB are dependent on the energetic limitations of the redox reactions present in their anaerobic respiration. Sulphate, for example, is only capable of acting as an electron acceptor once it has been metabolically converted to adenosine phosphosulphate (APS), an energy-costing reaction involving ATP. Also, energy is required for cellular uptake of sulphate and co-transport with either Na^+ or H^+ (Cypionka, 1995). More importantly, sulphur-based electron acceptors, as a result of the negative redox potential values from the main reductive reactions in their metabolism, have a greatly reduced energy yield, compared with that achievable from oxygen in the aerobic respiratory process (Hamilton, 1998; Baumgartner *et al*., 2006).

The metabolic patterns of many SRB have not yet been fully ascertained. Some of the difficulties have been attributed to the energy yields, as mentioned. Acetate metabolism poses a particular problem, since the coupling of acetate metabolism via the TCA cycle is apparently impossible. The redox potential of the succinate/fumarate couple of the TCA cycle in the case of acetate oxidation is an energy-requiring step, rather than an energy-generating one (Hamilton, 1998). Some mechanisms have been suggested to account for this apparent incongruence, since sulphidogenic species that use acetate as an energy and carbon source, while still maintaining a functional TCA cycle, are known. *Desulfobacter postgatei* for example, along with a few other sulphidogenic organisms are equipped with

unique mechanisms that ensure a small net energy yield from the TCA cycle (Hamilton, 1998), although these proposed mechanisms have not been fully elucidated. Other acetate-utilising sulphidogenic species are known to utilise the carbon monoxide dehydrogenase pathway (Acetyl-CoA pathway), instead of the TCA cycle (Wood *et al*., 1986, Wood and Pezacka, 1988).

SRB have long been regarded as strict anaerobes, and thus are restricted to anoxic zones of the environment. This restriction to anoxic zones has been attributed to the fact that oxygen is lethal to the activity of pyrophosphatase, the enzyme responsible for the conversion of sulphate to APS, as well as to the considerably reduced energy yield from its respiration compared to that of aerobic respiration, leading to them being out-competed in an oxic environment (Hamilton, 1998; Baumgartner *et al*., 2006). However, subsequent findings have pointed out that SRB may not be as strictly anaerobic as previously thought. Cypionka *et al*. (1985) exposed various strains of SRB to oxygen and demonstrated that these bacteria were capable of survival for long periods under oxic conditions, while still retaining their sulphate reducing capacity. It has also been shown that some SRB are even able to reduce oxygen (Cypionka *et al*., 1985). Some *Desulfovibrio* strains have demonstrated an ability to reduce oxygen at levels comparable to those seen by aerobic bacteria, although the rates usually decrease with increasing oxygen concentration (Dolla *et al*., 2006). SRB have been found to co-exist with aerobic bacteria in the oxic zones of microbial mats (Baumgartner, 2006). It is clear that the obligate anaerobe dogma associated with SRB has been challenged, and in some cases falsified in recent years, and with advancement in molecular

techniques, understanding and knowledge of the metabolism, cell physiology, diversity and ecology of SRB continues to grow.

SRB however, still demonstrate variable levels of sensitivity to oxygen. This tolerance continuum has been attributed to a number of mechanisms and enzymes present to deal with oxygen stress, as well as behavioural adaptations to address environmental stresses. The presence of superoxide dismutase (SOD), catalase, cytochrome c oxidase, and other free radical removing enzymes, display some of the molecular mechanisms available to combat reactive oxygen stress (Dolla *et al*., 2006). Another important factor conferring a measure of oxygen tolerance to SRB, involves the ability to form aggregates in the presence of oxygen (Dolla *et al*., 2006; Baumgartner *et al*., 2006). In addition to an oxygen defence mechanism, biofilms or microbial mats present many advantages to microbial systems, and often are the basis for microbial activities of economic importance.

1.9 Biofilms

Biofilms have been defined as a group of microorganisms that have irreversibly attached to a surface through a complex matrix of microbial-secreted polymers or extracellular polymeric substances (Lindsay and von Holy, 2006; Singh *et al*., 2006). That presents the microbial community with a high degree of heterogeneity owing to the many microenvironments that exist within the biofilm (Singh *et al*., 2006). It also confers on the microbial community a different phenotype, growth rate, gene expression, as well as a different set of metabolic and tolerance

properties from those of the planktonic cells (Lindsay and von Holy, 2006; Singh *et al*., 2006). These complex microbial communities allow the microbes to withstand to a certain degree, changes in pH, shear forces, nutrient deprivation, and other environmental stresses that are usually lethal to discrete cells. Biofilms also support high cell biomass, allowing optimised localised conditions within microenvironments, thus rendering these cells economically useful in many industries and bioremediation facilities (Singh *et al*., 2006). Biofilm reactors have, for example, proven worthy in the treatment of industrial and municipal wastewaters. In microbial-based heavy metal remediation, studies have shown that an increase in exopolysaccharides (EPS) corresponded to an increase in heavy metal waste. In addition, it was suggested that EPS might play a role in the entrapment of metal sulphide precipitates that form from this remediation option (White and Gadd, 2000). In the present study biofilm formation will be encouraged through the addition of a charcoal substrate. This will allow the microbial communities present to withstand the low pH of the incoming mine water and create microenvironments to deal with pollutant water, which may not be possible in their planktonic stage.

1.10 The present study

The present study utilises SRB, focussing on their ability to produce hydrogen sulphide (H_2S) and bicarbonate (HCO_3^-) as metabolic products. The H_2S produced binds to metals and renders them insoluble, or reduces their solubility, thereby forming metal precipitates, while the bicarbonate ion serves to generate alkalinity

in the water. (Luptakova and Kusnierova, 2005). The principal constituents of AMD are transformed by SRB as follows:

$$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O \text{ (catalysed by SRB)} \quad (4)$$

$$\text{Organic matter} + SO_4^{2-} \rightarrow HS^- + HCO_3^- \text{ (catalysed by SRB)} \quad (5)$$

$$Me^{2+} + HS^- \rightarrow MeS \text{ (precipitate)} + H^+ \quad (6)$$

where Me represents metals, and MeS a metal sulphide precipitate.

Many types of remediation strategies have been developed exploiting these AMD transformation strategies through the use of SRB. Studies have focussed on the removal, using the abovementioned principals (equations 4 to 6), of selected heavy metals in most cases, rather than the generation of net alkalinity in the acid mine water. Bioreactor systems have been developed using SRB for the removal and recovery of heavy metals from these waters. In addition, Elliot *et al*. (1998) have documented that SRB are capable of surviving at pH levels as low as 3.0, although at this pH the efficiency of sulphate reduction dropped drastically. However, at a pH of 3.25, those authors have shown that SRB were able to remove 38.3% of influent sulphate, as well as raise the pH of the acid mine water to 5.82. In that study (Elliot *et al*., 1998), an upflow bioreactor was used in the treatment of AMD. Similar studies have been conducted by Jong and Parry

(2003), in which acidic and sulphate-contaminated water was treated in a bench-scale upflow anaerobic packed bed reactor, using SRB. That study was aimed at the removal of heavy metals using the principals of SRB-mediated transformation, as shown in equation 6, and documented heavy metal removal efficiencies of greater that 77 % for iron, and 97.5 % for copper, zinc and nickel, over 14 days.

In the present study a fluidised bed bioreactor was employed in the treatment of AMD. Fluidised bed bioreactors are suited to processes that involve microbial flocs or consortiums. They comprise an up-flow stream of liquid, used to fluidise or suspend solids in each reactor. The superficial velocity above the settling zone should kept at a value below that which allows the solids to escape into suspension. As a result, solids sediment in the reactor, allowing them to be retained, while the liquids were able to flow out (Chisti and Moo-Young, 2002). Fluidised bed bioreactors were thus suited to the treatment being tested presently. The reactor system also contained additional clarifying vessels to allow precipitate settling.

The Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR_DGGE) technique was used to elucidate the microbial communities present in the reactors. PCR products generated through DNA extraction from each reactor were analysed using denaturing gradient gel electrophoresis (DGGE), which was then used to determine the genetic diversity of the total microbial population in each bioreactor. DGGE is a molecular fingerprinting technique that allows for the separation of the PCR-generated DNA products. Given that PCR

products from a particular reaction are of similar size, conventional agarose gel separation was not suitable for community analysis, since this yields a single band that does not facilitate description. DGGE overcomes this limitation by allowing separation of PCR-generated products based on sequence differences that result from different denaturing properties of DNA. In this technique, PCR products encounter increasing concentrations of chemical denaturants during their migration through a polyacrylamide gel. Migration of these PCR products slows down dramatically when the weaker melting domains begin to break down after reaching a threshold denaturation concentration. A pattern of bands- each band representing a different bacterial population- is generated as a function of different sequences of DNA from different bacteria. This technique allows for analyses into the identity of the microbial community members (Dar *et al*., 2005).

2 AIMS

The aims of the present study involve the development of a fluidised bed bioreactor system for the treatment of acid mine drainage using sulphate reducing bacteria, isolated and cultured from a mine dump run-off. This would be a one-step process that addresses the low pH, and the heavy metal content in acid mine waters. We hypothesised that as hydrogen ions and sulphate are consumed by the SRB, bicarbonate ions and hydrogen sulphide would be generated, allowing for an increase in pH and the precipitation of the heavy metals, respectively.

3 MATERIALS AND METHODS

3.1 Sample collection and handling:

Soil samples from which sulphate reducing bacteria (SRB) were isolated and cultured for the present study were collected from a stream exiting a mine dump on the eastern end of the Johannesburg city centre at co-ordinates of 26º 13' 05.92" S and 28º 07' 54.74" E. Soil samples were taken using a shovel at a depth of between 15 to 20 centimetres. This distance corresponded with the presence of dark or blackened soil substrate - indicative of iron reduction and hydrogen sulphide production (Elliot *et al*., 1998). In addition, the hydrogen sulphide was easily detectable by smell at this depth. Samples were extracted from both the soil on the stream banks, as well as from below the stream. The stream at the collection site showed signs of high iron levels, indicated by the yellowing of the water, typical of acid mine waters.

The dark soil substrate was collected and immediately transferred to airtight Schott® bottles (previously sterilised by autoclaving), which were then promptly sealed. This procedure had to be followed since the SRB isolated in the manner described above were anaerobic and therefore had to be maintained as such.

3.2 Culturing of SRB

SRB were cultured in a slightly modified form of Postgate's (1984) media. Medium B of Postgate's (1984) media range was chosen since this has been described as a general-purpose long-term media for SRB cultivation and growth. The media constituents are given in Appendix A. Thioglycollic acid was omitted from the described media since the inoculum was a fresh culture and in this case the thioglycollic acid may be left out (Postgate, 1984). In addition, the sodium lactate given as the carbon source in Postgate's (1984) medium B was replaced with sodium acetate.

Postgate's (1984) medium B was prepared and the pH adjusted to between 7.0 and 7.5 using 2M NaOH and 2M HCl. This was then sterilised by autoclaving at 15 psi and 121ºC for 20 minutes, prior to inoculation. In addition to a media at pH 7.0 to 7.5, three other pHs were also tested in an attempt to investigate the pH tolerance of planktonic SRB. Media was therefore also prepared at pH 5, as well as at pH 4.5, 4 and 3. In order to maintain anaerobic conditions, the culture Schott® bottles were filled right to the top with media so as to exclude air. Media was prepared in 2 litre shake flasks and transferred to 500ml Schott® bottles for the bacterial culture period. The use of shake-flasks for the culture step is not recommended since airtight bottles are necessary for these anaerobic strains (Postgate, 1984).

The media in Schott® bottles were inoculated aseptically with approximately 10g of wet soil per 500 ml media in the laminar flow cabinet, ensuring minimal exposure of the media and inoculum to air. Sterilisation techniques included flaming the mouth of the Schott® bottles before and after inoculation, as well as flaming the inoculating spatula prior to inoculation.

The culture bottles were maintained at 30ºC on a constant shaker. Positive cultures were identified by the blackening of the media, indicating the presence of iron sulphide precipitate as a result of hydrogen sulphide production (Elliot *et al*., 1998). Also, the very pungent hydrogen sulphide gas could easily be detected when the culture bottles were opened slightly.

3.3 Fluidised bed Bioreactor

3.3.1 Reactor description

The bioreactors and clarifiers used were built from clear Perspex, and connected in series. The system consisted of three bioreactors with two settling vessels or clarifiers, one after the first bioreactor and the second after the second bioreactor. This arrangement of clarifiers was expected to facilitate the removal of metal sulphide precipitate from the bioreactor effluent streams. It was expected that a large amount insoluble metal sulphides would be formed. The reactors were surrounded by water-jackets so as to regulate the temperature at which the system

operated. Each of the reactors had a volume of approximately 2.65 litres, while the clarifiers were built to hold approximately 1.85 litres each. The total volume of the entire system was approximately 11 litres. The reactors and clarifiers were connected to each other in series as shown schematically in Figure 1, with Masterflex® and Tygon® tubing. Liquid was transferred through the system using Masterflex® peristaltic pumps, with flow rates being manually adjusted.

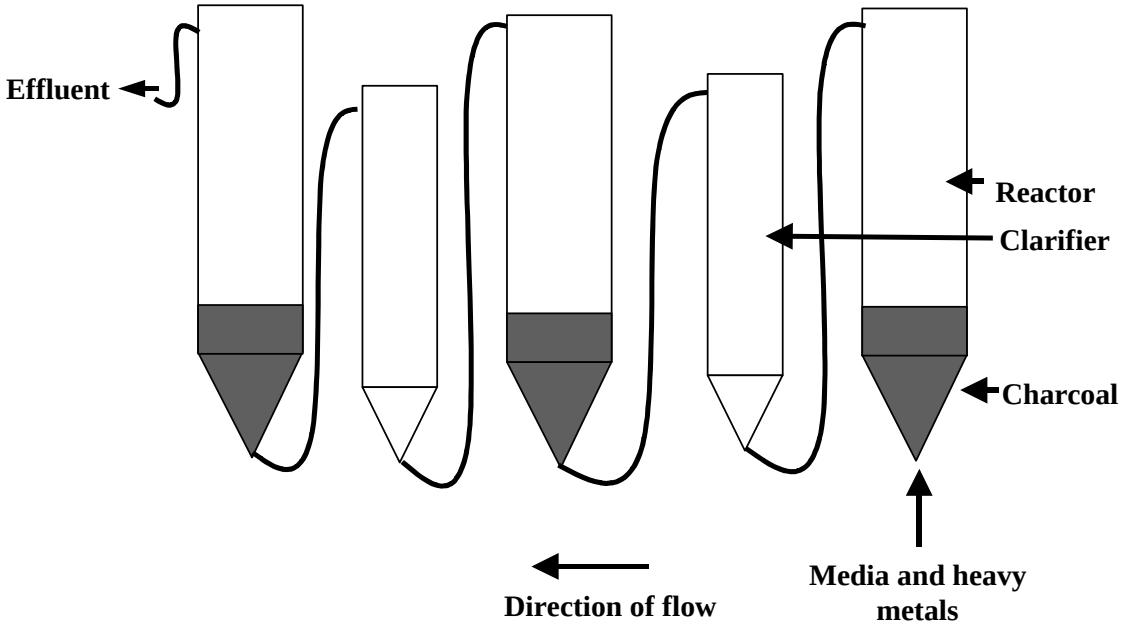


Figure 1: Schematic representation of the bioreactor arrangement used.

The reactors were filled to approximately 1/7 of their volume with charcoal as a substrate to allow bacterial attachment and subsequent biofilm formation. The temperature within the reactors were maintained at 35ºC with water heated in a water bath and circulated through the water-jackets of each reactor.

3.3.2 Reactor operation

Synthetic acid mine water (containing a range of heavy metals, at pH values ranging from 2.75 to 7) was used for the purposes of this study. Heavy metal containing water was fed into the system, together with Postgate (1984) media B, in the direction shown in Figure 1. These were introduced into the first reactor, where the initial hydrogen sulphide was formed through the action of the bacterial populations attached to the charcoal substrate, and some heavy metal precipitation occurred. The effluent overflow from the first reactor was then passed under gravity into the attached clarifier, where the aim was to allow settling of the black metal sulphide precipitate. The supernatant overflow of the first clarifier was actively pumped into the second reactor. The effluent overflow from the second bioreactor was again passively passed under gravity into the second clarifier for further precipitate settling. Finally, the supernatant from the second clarifier was pumped into the third reactor for final remediation, after which the effluent was collected into a 10 L Schott® bottle for analysis following sedimentation of the metal sulphide precipitate.

Before inoculating the reactors with the SRB consortium, the system was first made anaerobic by inoculating with *Escherichia coli* cultures grown in LB media. This *E. coli* culture was circulated through the system for one week. All ports were sealed with clamps so as to exclude any air from entering the system.

3.3.3 Control

A control experiment to test for occurrence of non-biological sulphide formation and corresponding metal precipitation was conducted prior to the inoculation of the bioreactors with bacteria. In the control experiment the synthetic mine water was passed through the system and analysed. After this exercise the bioreactors were inoculated with the cultured SRB consortium. This control had all the components of the media, as well as all the heavy metals used in the present study. The hydraulic retention times used for the control experiment were the same as those used for the rest of the study, i.e. 5, 10 and 24 hours.

3.3.4 Reactor operation and mine water treatment

Postgate (1984) medium B was prepared in 10 litre Schott® bottles. Positive bacterial cultures (see above) were poured directly into these media bottles and the reactors were subsequently inoculated with the bacterial consortium by pumping the medium and culture into the system. The reactors were first run as a batch/ recycle system for two weeks in order to allow the bacterial consortium to grow up to sufficient numbers and form biofilms. During this time fresh media was introduced into the system every second day to maintain a steady supply of nutrients and carbon.

Following the two-week recycle period, the system was switched to continuous wherein the synthetic mine water was passed directly through the system at different hydraulic retention times, and collected out of the final reactor for analysis.

The media entering the system was supplemented with a number of heavy metals, mainly in the form of metal sulphates, to simulate mine water. These included iron sulphate, copper sulphate, aluminium sulphate, zinc sulphate, manganese sulphate and chromium trioxide. The concentration of the metals used was based on those reported by Naicker *et al.* (2003). Those authors recorded high and low range metal concentrations in mine water, as a function of seasons, rainfall and water source, such as ground water, surface water and seepage water. In the present study, the high range levels reported by Naicker *et al*. (2003) were chosen as a reference point for the metal concentration that was added to the reactor system, given in Table 1. Since metal compounds were added, and not just elemental metals, the mass of the compound eventually added to the system had to exclude that of the sulphates (in the case of metal sulphates), trioxides and hydrates, where applicable. Table 1 shows the mass of the metal compounds added in total in order to get the effective heavy metal concentration. These values were determined photometrically.

Table 1: Concentrations of heavy metals used in the water treatment analysis

Metal	Approximate mass of metal compound added (mg)	Effective concentration ($mg.l^{-1}$)
Iron	2000	500
Manganese	700	200
Zinc	250	50
Copper	27	10
Chromium	270	100
Aluminium	100	10

The effect of three hydraulic retention times on the bioremediation of heavy metals and low pH was investigated. The water was retained in the system (i.e. hydraulic retention time (HRT)) for 5, 10 and 24 hours by adjusting the flow rate on the peristaltic pumps, and subsequently analysed.

3.4 Water analysis

Following each run at the different HRTs and influent pH values, the water was collected from the last reactor and analysed for changes in heavy metal concentrations and pH. In addition, the change in chemical oxygen demand (COD) was determined at the start and at the end of the 24-hour HRT. The pH

was measured using the Precisa® (Switzerland) pH 900 pH meter, calibrated using Saarchem® calibration solutions.

The heavy metal concentrations were determined using the Hanna® C99 Multiparameter Bench Photometer. The principle method for each metal tested are as follows (Hanna handbook, supplied):

- Aluminium: The reaction between aluminium and the reagent aluminon causes a reddish tint in the sample. Photometry was conducted at 525 nm.
- Chromium: The reaction between chromium (VI) and the reagent diphenylcarbohydrazine caused a purple tint in the sample. Photometry conducted at 525 nm.
- Copper: The reaction between copper and the reagent bicinchoninate causes a reduction in copper from Cu (II) to Cu (I), and a purple tint in the sample. Photometry conducted at 575 nm.
- Iron: The reaction between iron and phenanthroline causes a reduction in Fe (II) and an orange tint in the sample. Photometry conducted at 525 nm.
- Manganese: Periodate method. Reaction between manganese and the reagent causes the oxidation of manganese, giving a pink tint in the sample. Photometry conducted at 525 nm.
- Zinc: Zincon method, in which the reaction between zinc and zincon causes a chelation, resulting in an orange to dark violet tint in the sample. Photometry conducted at 575 nm.

3.5 Analysis of bacterial consortium

3.5.1 DNA extraction

Water samples were also extracted in an attempt to analyse the bacterial consortium in each reactor, through DNA analysis. Fifty ml of liquid was eluted from each reactor for this purpose. The liquid was then centrifuged for 20 minutes at 13 000 rpm to facilitate pellet formation. The supernatant was discarded. The pellet was then resuspended in 200µl of deionised water in preparation for DNA extraction, which was conducted using the Zymo Research ZR Fungal/ Bacterial DNA kit ™, supplied by Inqaba Biotechnical Industries (South Africa), according to the manufacturer's instructions. The extracted DNA, suspended in RNAse-free water was then amplified.

3.5.2 Polymerase chain reaction (PCR)

Five µl of the template DNA was used in the amplification. This was added to the PCR mix which contained 25µl PCR Master Mix (Fermentas, Switzerland), 1 µl each of 10 µM forward and reverse primer, as well as 18 µl DNAse-/ RNAse-free water to make up a final PCR volume of 50 µl. The primers used were bacterium-specific 16S rDNA forward and reverse primers P63f and P518r respectively (Dwettinck *et al*., 2001), which are based on a universally conserved region. In addition, a GC clamp of 40 base pairs was constructed into the forward primer, specifically for use on DGGE. These were constructed by Inqaba Biotechnical

Industries (Pty) Ltd, South Africa. The sequence of the forward primer was ATTACCGCGGCTGGCTGG, while that of the reverse primer, together with the GC clamp, was CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCCAGGC CTAACACATGCAAGTC.

The samples were amplified according to the procedure described in Dwettinck *et al*. (2001) who have found success using the same primers with the present procedure. Using the Applied Biosystems GeneAmp® PCR System 2700, the samples were held at 94ºC for 5 minutes. This was followed by 30 cycles as follows: denaturation at 95ºC for 1 minute, annealing at 53ºC for 1 minute, and extension at 72ºC for 2 minutes, with a final extension 72ºC for 10 minutes.

3.5.3 Agarose gel electrophoresis

In order to confirm the presence of amplified DNA fragments, the samples were electrophoresed on a 1% agarose gel in a 0.5X TBE buffer. The agarose, water and 5X TBE buffer were melted in a microwave for $1^1/_2$ to 2 minutes and left to cool to approximately 40ºC. To allow visualisation of the DNA fragments through UV illumination, 1µl of a 10 $mg.ml^{-1}$ ethidium bromide solution was then added to the agarose gel. Five µl of the PCR products were added to each lane, together with a 6X orange loading dye (Fermentas, Switzerland). The DNA ladder used was a Fermentas (Switzerland) LabAid MassRuler™ DNA Ladder, Low Range.

The bands included in this ladder were of the following sizes: 80; 100; 200; 300; 400; 500; 600; 700; 800; 900; and 1031 base pairs. The electrophoresis was run at 100V for 45 minutes in the presence of a 0.5X TBE buffer, following which viewing was conducted using the BioRad® Gel Doc transilluminator viewing system.

3.5.4 Denaturing Gradient Gel Electrophoresis (DGGE)

Bearing in mind that the DNA extracted and amplified from the reactors in the previous steps was from a bacterial consortium, DGGE using the BioRad® (California) Dcode Universal Mutation Detection System was employed to separate the collectively PCR-amplified fragments, according to the method described by Muyzer *et al*. (1993). The samples, 5µl each, were run for approximately $2^1/_2$ hours at 130V on an 8% (w/v), 10cm X16 cm polyacrylamide gel, with a denaturation gradient ranging from 40% to 65%, where 100% denaturant consists of 7 M urea and 40% formamide. A 0.5X TAE buffer was used during the electrophoresis run. Following the electrophoresis, the gel was carefully removed from the system and stained for 15 minutes and destained for another 15 minutes. The staining solution was made by adding 10µl of a 10 $mg.ml^{-1}$ ethidium bromide solution to 250 ml of 0.5X TAE buffer. Destaining was carried out using 250 ml of the 0.5X TAE buffer. The gel was then viewed using a UV transilluminator.

3.5.5 Fragment extraction from the polyacrylamide gel

The separated PCR fragments were then excised from the polyacrylamide gel under UV light using a scalpel. The bands were then transferred to the BioRad® (California) Freeze 'n Squeeze DNA extraction kit, and according to the manufacturer's instruction, the DNA was extruded from each band, and subsequently re-amplified through PCR, as described above, before being sequenced by Inqaba Biotechnical Industries (Pty) LTD (South Africa).

The DNA sequences were then tested for homology by comparing them to sequences in the Basic Local Alignment Search Tool (BLAST) server of the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/).

3.5.6 Scanning Electron Microscopy (SEM)

Approximately 50g of the charcoal substrate was extracted from the reactors and prepared for SEM in order to visualise biofilms. The samples were first fixed in 2% gluteraldehyde overnight, and then rinsed in deionised water several times. Dehydration followed in increasing concentrations of ethanol, for 10 minutes each. The concentrations used were 40%, 50%, 60%, 70%, 80%, 90%, 95% and finally in 100% ethanol overnight. Samples were then sent to the Electron Microscopy Unit at the University of the Witwatersrand, Johannesburg, where

they were subjected to critical point drying, and finally fixed and mounted on stubs for viewing. Viewing was conducted using a Joel® JSM-840 Scanning microscope.

4 RESULTS

4.1 Bacterial culture

Postgate's (1984) medium B that was used to culture the SRB is shown in Figure 2 A. This media was inoculated with a soil sample taken from a stream exiting a mine dump on the Johannesburg East Rand. Media was prepared at different initial pH values (pH 3 to 7.0) to ascertain the range of acidic pHs under which the bacteria could grow. Positive growth of SRB cultures was confirmed by the appearance of a black iron sulphide precipitate that was the result of H_2S production. Figure 2 B shows an example of a positive SRB culture.

A **B**

Figure 2: A) Postgate medium B before inoculation; B) Positive culture for SRB, indicated by black iron sulphide precipitate, formed due to H_2S production from the SRB, owing to iron sulphate in the culture medium.

The resistance of suspended or planktonic SRB to the inhibitory effects of low pHs was qualitatively determined by their ability to grow in acidic liquid cultures. Table 2 shows the time (days) taken to obtain a positive growth response when cultured in liquid media at different pH values.

Table 2: Time (days) taken for positive SRB culture for Postgate medium B at different pH values.

Medium pH	Time taken to positive culture (days)
7	4
5	15
4.5	28
4	-
3	-

Media at pH 7 displayed the quickest time to achieve a positive culture. With decreasing media pH, growth of SRB was retarded, indicated by an increased positive culture time. Growth ceased at a media pH of below 4.5, indicating the lack of ability of SRB to survive as free cells below this pH. The positive cultures were then used to inoculate the bioreactor for the AMD bioremediation study.

4.2 Fluidised Bed Bioreactor Studies

The fluidised bed bioreactor system used in the present study is shown in Figure 3. The entire system clearly shows the presence of dark metal sulphide precipitate during operation, as a result of H_2S production. A schematic representation of the reactors was shown in Figure 1.

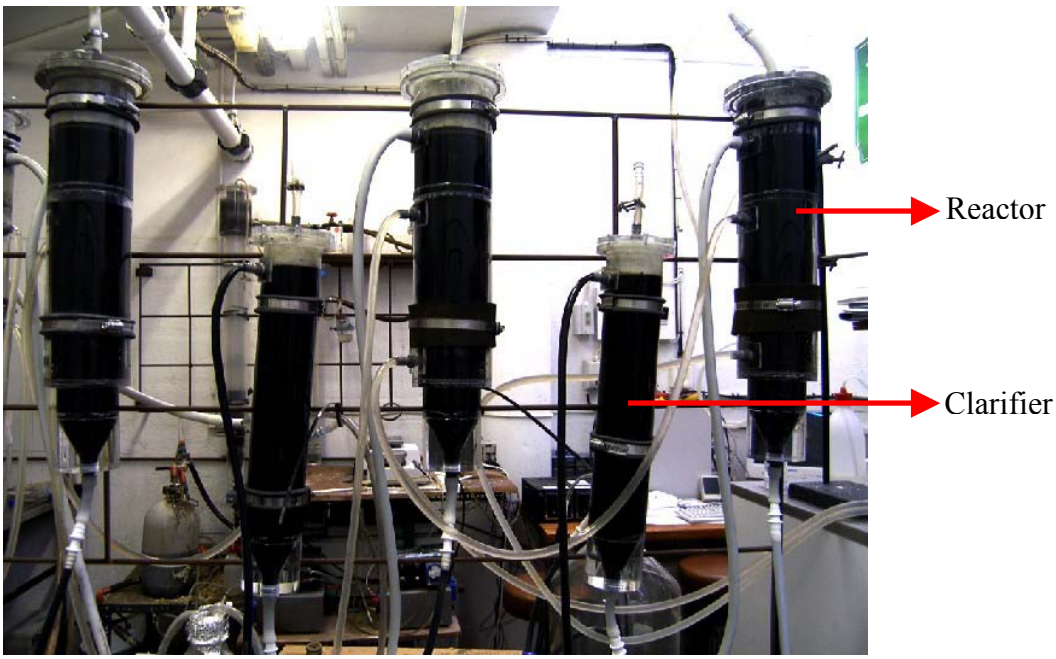


Figure 3: Bioreactor system, with clarifiers, used in the present study.

4.2.1 Control:

A control experiment was conducted in the bioreactors, with all the constituents of the synthetic mine water and the media used to feed the cells (Postgate (1984) medium B), save for any inoculum. In keeping with the HRTs used in the actual

experiments, the same times were used for the control experiment. Table 3 presents the results obtained from the control experiment. The synthetic mine water was retained in the system for 5, 10 and 24 hours. The HRT of 0 hours represents the heavy metal concentrations at the start of the study.

Table 3: Heavy metal concentrations after HRTs of 5, 10 and 24 hours in a control experiment, run in the bioreactor system with no inoculum.

HRT (hours)	Fe ($mg.l^{-1}$)	Mn ($mg.l^{-1}$)	Zn ($mg.l^{-1}$)	Cu ($mg.l^{-1}$)	Cr ($mg.l^{-1}$)	Al ($mg.l^{-1}$)
0	500	200	50	10	100	10
5	467	208	48.4	8.3	97.3	9.2
10	488	189	49.1	9.4	104.6	9.4
24	453	176	46	8.7	89.7	8.9

The change in heavy metal concentration over the increasing HRT in the control process (in the absence of SRB) was not substantial, when compared to the inoculated system. In the SRB-free control process there was only a marginal reduction in the initial heavy metal concentration. In Table 3 it can be seen that the high level of heavy metals added to the system was only slightly higher than the concentration in the effluent stream leaving the series of bioreactors. In the control process the loss of heavy metals in response to HRT did not differ greatly. It was hypothesized that the loss of heavy metals in the control process was possibly due the absorption of heavy metals onto the charcoal bed in the bioreactor. Addition of SRB inoculum resulted in large loss of soluble heavy

metals from the effluent stream relative to the control process. This was the case for all HRTs.

4.2.2 Water analysis

Following the control experiment, the reactors were inoculated with positive SRB cultures. To allow the establishment and maintenance of the SRB culture in the bioreactor system, the media plus inoculum was recycled through the bioreactor. This allowed for biofilm formation and SRB acclimatisation. After this pre-treatment the operation of the bioreactor was switched from recycle to continuous mode. The synthetic mine water was then passed continuously through the reactor at HRTs of 5, 10 and 24 hours. The effluent from the final bioreactor was collected into a 10 L Schott® bottle. After sedimentation of the insoluble metal sulphides the clear supernatant water was analysed for heavy metal concentration. Figure 4 A represents the water immediately after extraction from the reactor, while Figure 4 B represents the water following precipitate settling. The black precipitate is clearly visible at the bottom of the vessel.

A

B

Figure 4: A) Water extracted from reactor, showing black metal sulphide precipitate; B) Water following precipitate settling. Analysis was conducted on the supernatant.

4.2.3 Chemical oxygen demand

The chemical oxygen demand (COD) was determined at the start and at the end of the 24-hour HRT for each of the decreasing influent/feed pH runs. Figure 5 graphically illustrates these changes in COD.

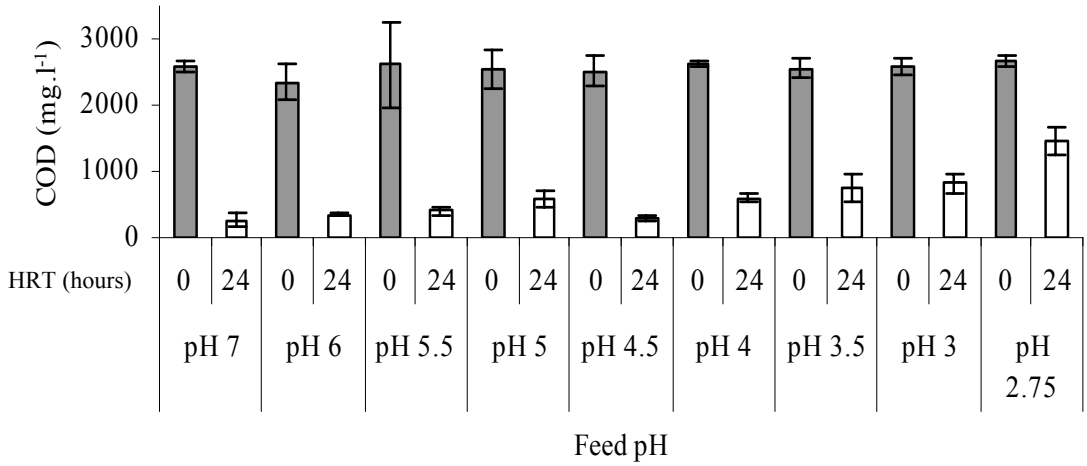


Figure 5: Change in COD over a HRT of 24 hours, as a function of the influent/feed pH. The difference between influent and effluent COD after 24 hours decreases with decreasing pH.

When comparing COD usage over a 24-hour HRT for different feed pH values (Figure 5), it can be seen that as the feed pH decreases, a decrease in the use of organic material by the contained microbes is noticed over a 24-hour HRT. This is brought about by a reduction in bacterial activity as pH decreases.

4.2.4 pH change:

In order to investigate the alkaline-generating capability, synthetic mine water at decreasing pH values was fed into the system. Figure 6 illustrates the alkaline yielding capacity of the system, as a function of decreasing feed pH values, at HRTs of 5, 10 and 24 hours.

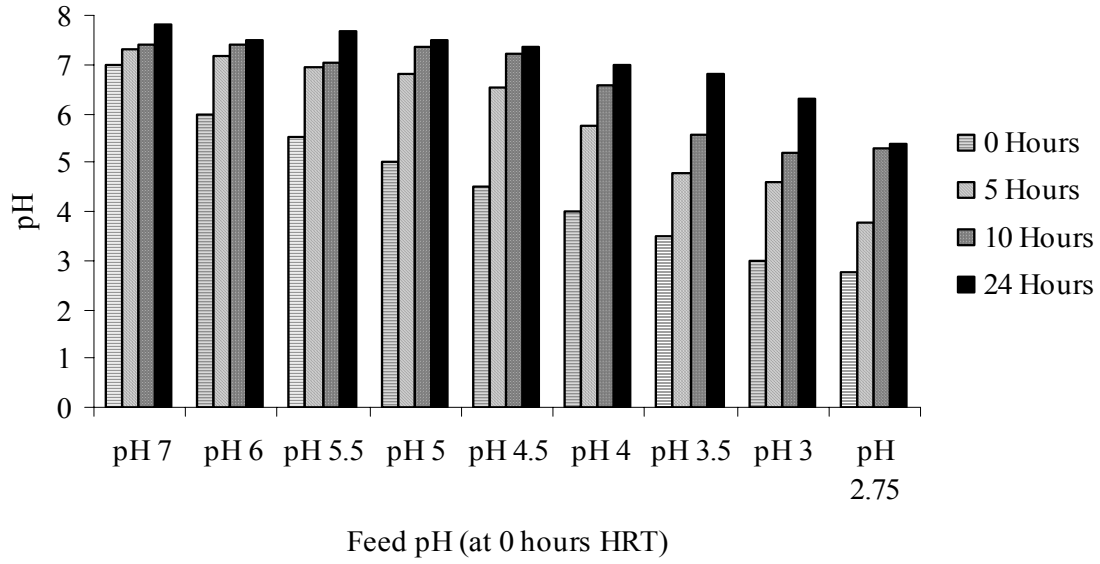


Figure 6: Alkaline generating capacity of the SRB contained within the fluidised bed bioreactor. At a given start or feed pH value, the pH was monitored over 5, 10 and 24 hour HRTs. An increase in HRT results in an increase in alkalinity. Alkalinity was generated from influent pH values as low as 2.75.

The bioreactor system was successful in raising the pH values to above 7 from a feed or influent pH as low as 4. A HRT of 24 hours allowed sufficient time for the alkalinisation of the effluent stream. At pH values between 3 and 4, the bioreactor system was capable of generating a final pH of between 6 and 7, after 24 hours; while at a feed pH of below 3, a final pH of only 5.4 was achievable after a 24-hour HRT. In all cases tested in this study the system was capable of generating alkalinity.

4.2.5 Heavy metal removal from polluted water

The heavy metals used in the present study were Fe, Mn, Zn, Cu, Cr and Al. At each decreasing feed pH value used, and with each of the HRTs (5, 10 and 24 hours), the water was analysed for effectiveness in heavy metal precipitation. Tables 4 to 12 demonstrate the ability of the bioreactor system to precipitate some heavy metals in mine water, under conditions of decreasing feed pH values.

Table 4: Heavy metal removal from water at a feed pH of 7, over increasing HRTs (standard deviations of the mean included).

HRT (hours)	Fe ($mg.l^{-1}$)	Mn ($mg.l^{-1}$)	Zn ($mg.l^{-1}$)	Cu ($mg.l^{-1}$)	Cr ($mg.l^{-1}$)	Al ($mg.l^{-1}$)
0	500	200	50	10	100	10
5	12.5 ± 6.36	10.5 ± 3.53	13.5 ± 3.53	1.3 ± 1.06	6.9 ± 3.18	3.1 ± 0.56
10	3 ± 0.84	1.8 ± 0.49	0.4 ± 0.14	0.5 ± 0.35	1.7 ± 1.84	0.4 ± 0.49
24	0.06 ± 0.06	2.5 ± 0.06	0	0.00013 ± 0.000023	0.00017 ± 0.00028	0

Table 5: Heavy metal removal from water at a feed pH of 6, over increasing HRTs (standard deviations of the mean included).

HRT (hours)	Fe ($mg.l^{-1}$)	Mn ($mg.l^{-1}$)	Zn ($mg.l^{-1}$)	Cu ($mg.l^{-1}$)	Cr ($mg.l^{-1}$)	Al ($mg.l^{-1}$)
0	500	200	50	10	100	10
5	16.5 ± 4.9	16 ± 2.82	15.5 ± 3.5	2.2 ± 1.13	4.6 ± 1.55	2.65 ± 0.77
10	5 ± 2.8	2.45 ± 0.77	0.5 ± 0.56	0.5 ± 0.56	0.75 ± 0.07	0.35 ± 0.2
24	0.19 ± 0.21	2.7 ± 1.3	0	0.00011 ± 0.00011	0.02 ± 0.04	0

Table 6: Heavy metal removal from water at a feed pH of 5.5, over increasing HRTs (with standard deviations of the mean included).

HRT (hours)	Fe ($mg.l^{-1}$)	Mn ($mg.l^{-1}$)	Zn ($mg.l^{-1}$)	Cu ($mg.l^{-1}$)	Cr ($mg.l^{-1}$)	Al ($mg.l^{-1}$)
0	500	200	50	10	100	10
5	17.5 ± 0.7	14.5 ± 7.77	13 ± 4.2	1.7 ± 1.03	9.6 ± 2.8	1.9 ± 1.9
10	7.5 ± 2.12	20.1 ± 28.14	0.9 ± 0.9	1.9 ± 0.21	1.1 ± 0.77	1.1 ± 0.6
24	0.3 ± 0.22	2.7 ± 3.8	0.4 ± 0.4	0.1 ± 0.17	0.003 ± 0.004	0.002 ± 0.003

Table 7: Heavy metal removal from water at a feed pH of 5, over increasing HRTs (with standard deviations of the mean included).

HRT (hours)	Fe ($mg.l^{-1}$)	Mn ($mg.l^{-1}$)	Zn ($mg.l^{-1}$)	Cu ($mg.l^{-1}$)	Cr ($mg.l^{-1}$)	Al ($mg.l^{-1}$)
0	500	200	50	10	100	10
5	27 ± 9.90	21 ± 12.73	14.5 ± 3.5	2.3 ± 2.16	11.1 ± 3.6	2.3 ± 1.27
10	9.5 ± 7.77	35.5 ± 10.6	1.1 ± 0.84	1.9 ± 1.41	1.1 ± 0.28	1.6 ± 1.63
24	0.4 ± 0.28	2.8 ± 2.07	0.7 ± 0.91	0.2 ± 0.16	0.06 ± 0.05	0.02 ± 0.04

Table 8: Heavy metal removal from water at a feed pH of 4.5, over increasing HRTs (with standard deviations of the mean included).

HRT (hours)	Fe ($mg.l^{-1}$)	Mn ($mg.l^{-1}$)	Zn ($mg.l^{-1}$)	Cu ($mg.l^{-1}$)	Cr ($mg.l^{-1}$)	Al ($mg.l^{-1}$)
0	500	200	50	10	100	10
5	45.9 ± 58.13	103.5 ± 38.89	27 ± 9.89	3.9 ± 1.31	17.7 ± 4.52	2.2 ± 0.56
10	14.5 ± 9.19	44 ± 29.69	20 ± 4.24	3.6 ± 3.11	3.5 ± 1.13	0.02
24	0.3 ± 0.09	6.3 ± 8.06	0.9 ± 0.96	0.2 ± 0.16	0.02 ± 0.04	0.014 ± 0.02

Table 9: Heavy metal removal from water at a feed pH of 4, over increasing HRTs (with standard deviations of the mean included).

HRT (hours)	Fe ($mg.l^{-1}$)	Mn ($mg.l^{-1}$)	Zn ($mg.l^{-1}$)	Cu ($mg.l^{-1}$)	Cr ($mg.l^{-1}$)	Al ($mg.l^{-1}$)
0	500	200	50	10	100	10
5	47.2 ± 59.17	66.5 ± 44.54	27.5 ± 12.02	2.9 ± 1.73	28.2 ± 6.43	3.3 ± 0.49
10	9 ± 1.41	65.5 ± 13.43	22.5 ± 13.43	2.6 ± 1.23	3.4 ± 2.75	0.7 ± 0.95
24	0.6 ± 0.17	30.3 ± 9.61	30.3 ± 1.38	0.4 ± 0.35	0.003 ± 0.003	0.01 ± 0.01

Table 10: Heavy metal removal from water at a feed pH of 3.5, over increasing HRTs (with standard deviations of the mean included).

HRT (hours)	Fe ($mg.l^{-1}$)	Mn ($mg.l^{-1}$)	Zn ($mg.l^{-1}$)	Cu ($mg.l^{-1}$)	Cr ($mg.l^{-1}$)	Al ($mg.l^{-1}$)
0	500	200	50	10	100	10
5	57 ± 19.79	93.5 ± 37.48	29.5 ± 9.19	4.9 ± 1.65	47.6 ± 5.37	4 ± 1.27
10	29.5 ± 12.02	64.5 ± 31.82	22.3 ± 3.18	3.9 ± 1.57	18.5 ± 7.70	2.3 ± 0.78
24	0.6 ± 0.40	57.7 ± 6.43	11.7 ± 3.05	3.2 ± 1.80	3.9 ± 2.05	2.6 ± 0.71

Table 11: Heavy metal removal from water at a feed pH of 3, over increasing HRTs (with standard deviations of the mean included).

HRT (hours)	Fe ($mg.l^{-1}$)	Mn ($mg.l^{-1}$)	Zn ($mg.l^{-1}$)	Cu ($mg.l^{-1}$)	Cr ($mg.l^{-1}$)	Al ($mg.l^{-1}$)
0	500	200	50	10	100	10
5	49 ± 26.87	125 ± 43.84	32 ± 15.56	5.1 ± 2.40	59.1 ± 10.18	4.9 ± 0.28
10	15 ± 2.83	86.5 ± 14.85	29.5 ± 3.53	3.6 ± 2.40	35.5 ± 11.67	4.1 ± 3.04
24	0.9 ± 0.89	52.3 ± 10.56	3.8 ± 3.27	4.6 ± 1.96	16.2 ± 9.87	1.3 ± 1.15

Table 12: Heavy metal removal from water at a feed pH of 2.75, over increasing HRTs (with standard deviations of the mean included).

HRT (hours)	Fe ($mg.l^{-1}$)	Mn ($mg.l^{-1}$)	Zn ($mg.l^{-1}$)	Cu ($mg.l^{-1}$)	Cr ($mg.l^{-1}$)	Al ($mg.l^{-1}$)
0	500	200	50	10	100	10
5	182.5 ± 34.65	149.5 ± 37.48	51.5 ± 6.36	6 ± 1.56	59.6 ± 0.92	5.9 ± 2.76
10	70.5 ± 10.61	100 ± 7.07	31.5 ± 3.56	5.5 ± 3.75	51.5 ± 6.36	5.7 ± 0.85
24	9.6 ± 1.52	80.7 ± 24.76	6.2 ± 2.96	4.5 ± 3.93	20.7 ± 16.00	2.2 ± 1.82

Tables 4 to 12 demonstrate the ability of the SRB contained within the fluidised bed bioreactor system to precipitate heavy metals from solution. This precipitation was still achievable at the upper ranges of the low pH values that AMD usually occurs, between 2.5 and 3.5. The amount of heavy metal precipitated increases in each case with increasing HRT. As the influent pH values decrease however, the amount of heavy metal precipitated decreases over a given HRT. This trend was consistent for each of the heavy metals used.

Iron, for example, at a concentration of 500 $mg.l^{-1}$, was reduced to 0.06 $mg.l^{-1}$ at a feed pH of 7 (Table 4), and to 9.6 $mg.l^{-1}$ at a feed pH as low as 2.75 (Table 12), each at a HRT of 24 hours. The other heavy metals used also displayed marked decreases in concentration between the feed concentration and the effluent concentrations.

4.2.6 Percentage efficiency of each heavy metal precipitation

The precipitation percentage efficiency for each heavy metal tested at every influent pH value was calculated, over a HRT of 24 hours. Figures 7 to 12 graphically represent these percentage efficiencies.

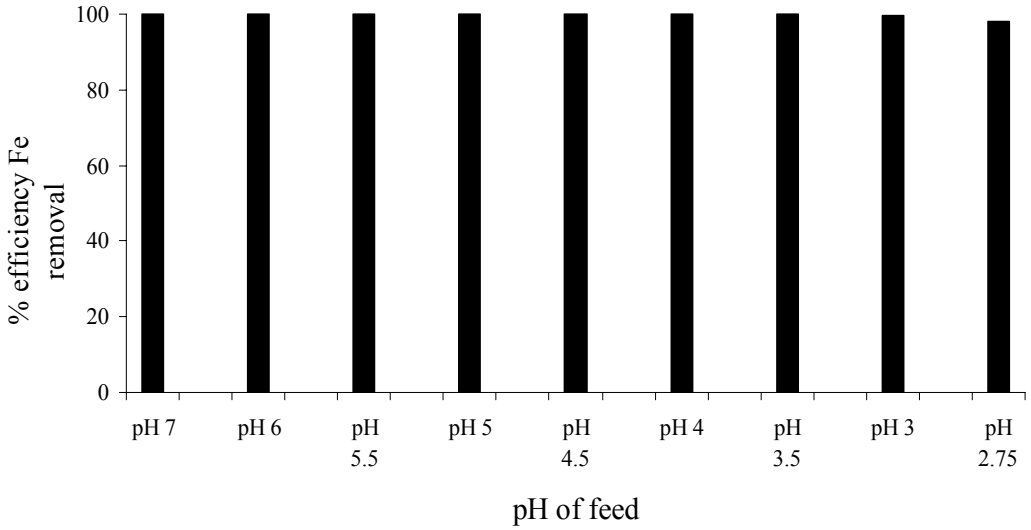


Figure 7: Percentage efficiency Fe removal at decreasing feed pH values

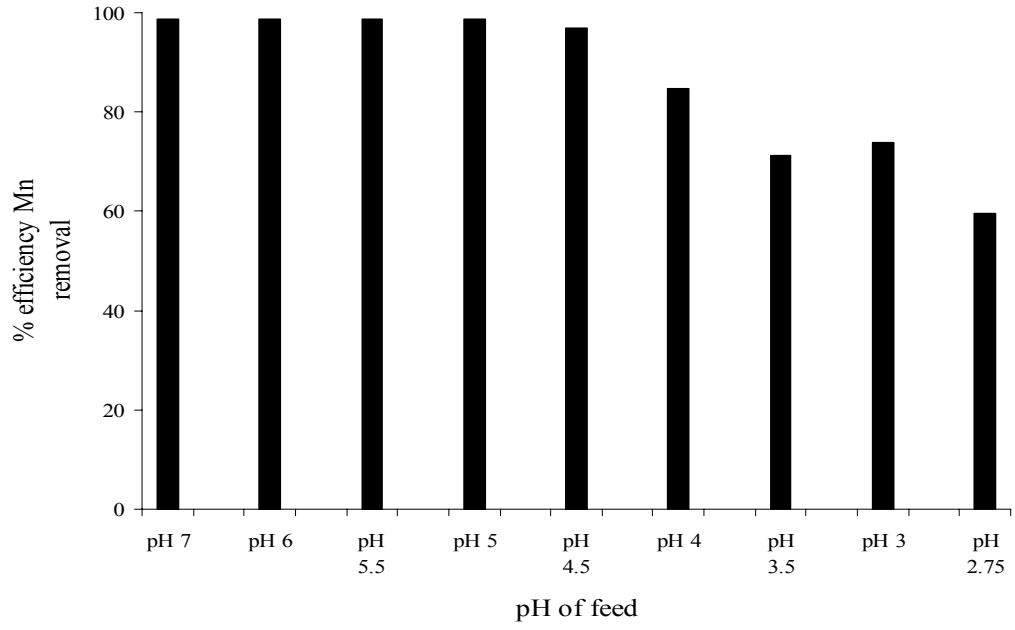


Figure 8: Percentage efficiency Mn removal at decreasing feed pH values

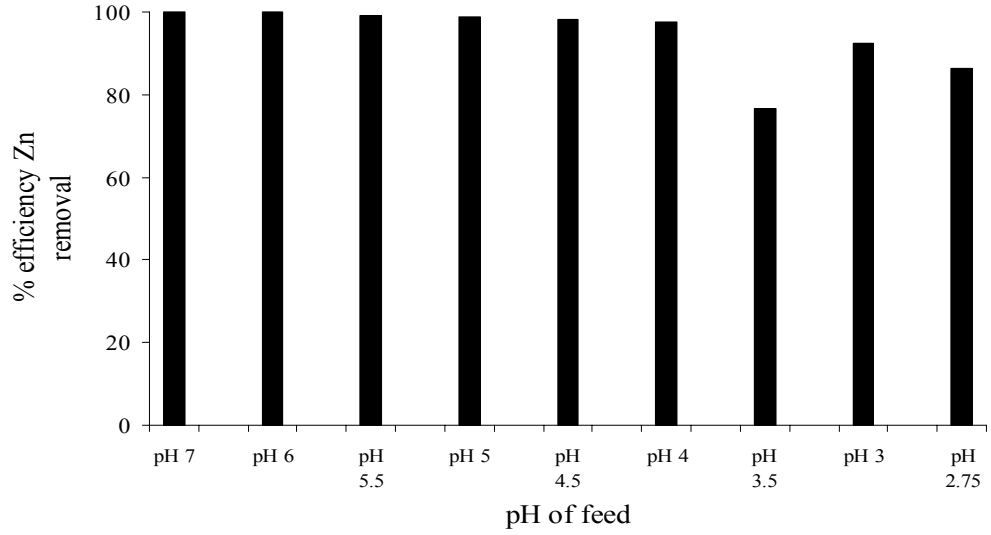


Figure 9: Percentage efficiency Zn removal at decreasing feed pH values

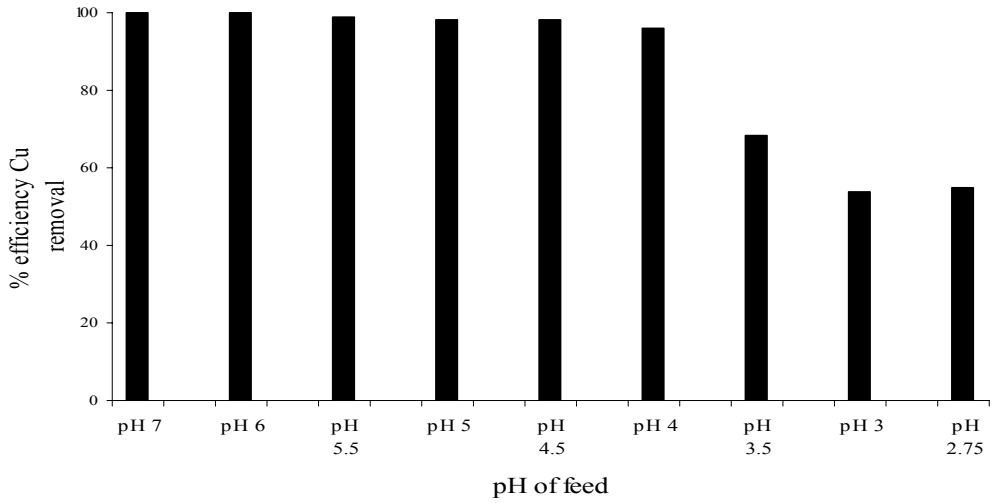


Figure 10: Percentage efficiency Cu removal at decreasing feed pH values

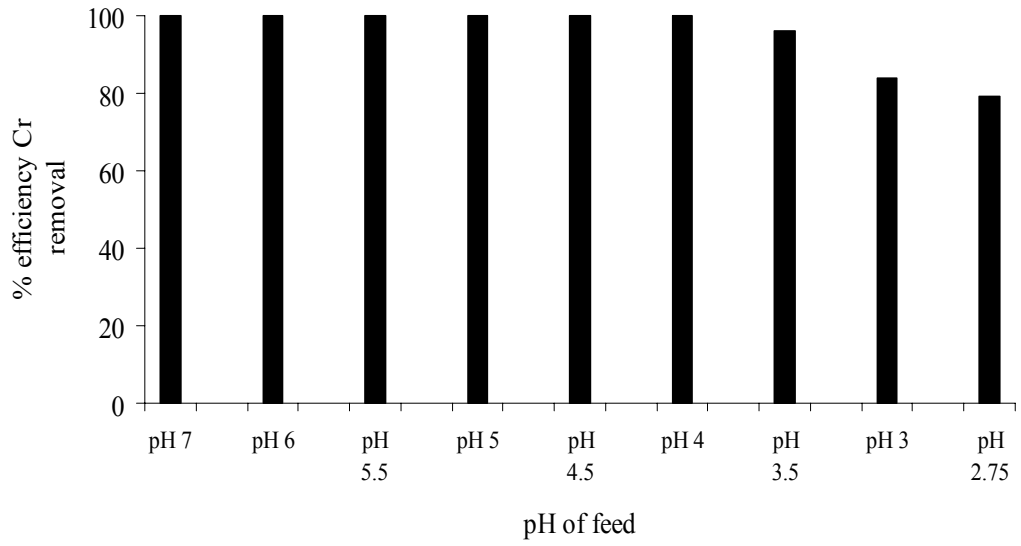


Figure 11: Percentage efficiency Cr removal at decreasing feed pH values

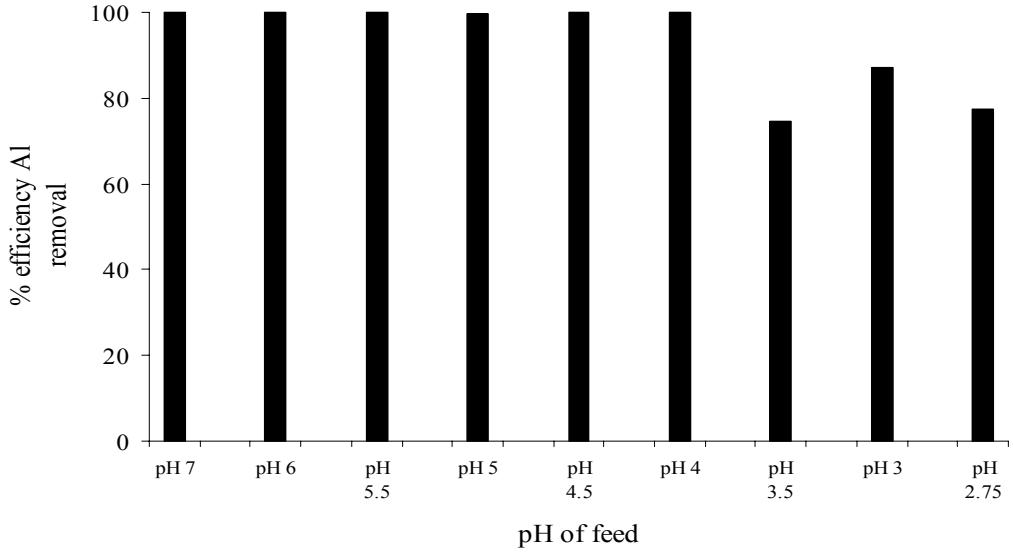


Figure 12: Percentage efficiency Al removal at decreasing feed pH values

It is seen from Figures 7 to 12 that the percentage efficiencies of the system in precipitating heavy metals decreases with decreasing feed-pH, but still remains above 50%, over a HRT of 24 hours. Almost all the heavy metals are removed at pH values above 4. Below this value the system's efficiency decreases, indicating a reduction in bacterial activity.

4.3 Analysis of bacterial consortium

Two samples were taken from each reactor for microbial community analysis. The DNA from these was extracted and amplified, then run on a 1% agarose gel to confirm the presence of amplified fragments. Figure 13 shows the agarose gel with the amplified fragments visible. The sizes of the fragments on the ladder (Lane M) are as follows: 1031, 900, 800, 700, 600, 500, 400, 300 and 200 base pairs.

The amplified fragments correspond to a size of approximately 400 to 430 base pairs. Since these fragments represent a bacterial consortium, in order to elucidate the bacterial species present, DGGE was conducted on these amplified fragments. Each of the fragments present in Figure 13 was separated in the gradient gel as represented in Figure 14.

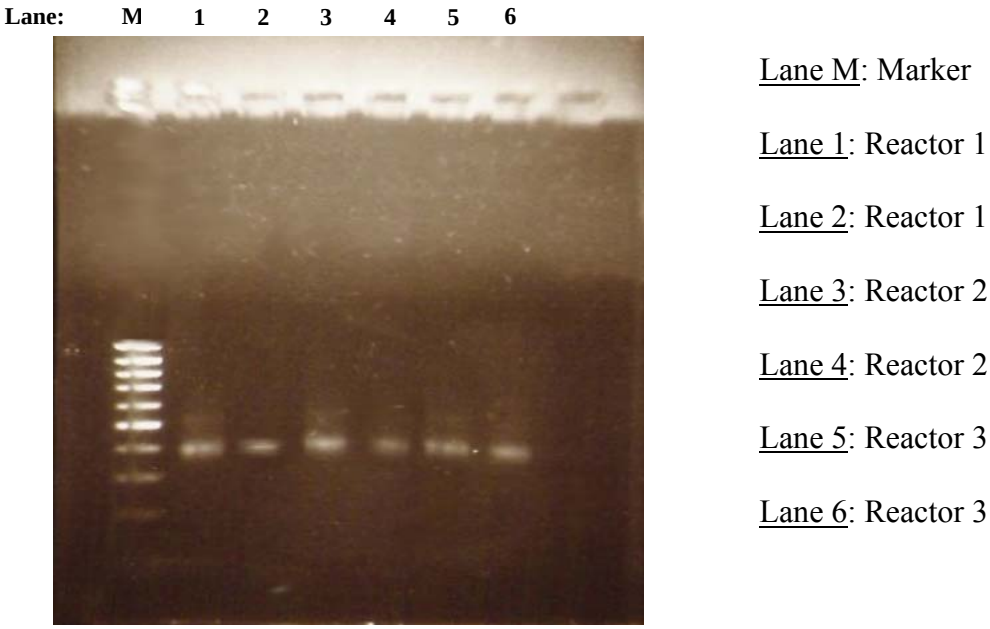


Figure 13: 1% Agarose gel electrophoresis of amplified fragments. Lane M represents the marker lane; while lanes 1 and 2 represent DNA extracted and amplified from reactor 1; 3 and 4 from reactor 2; and 5 and 6 from reactor 3.

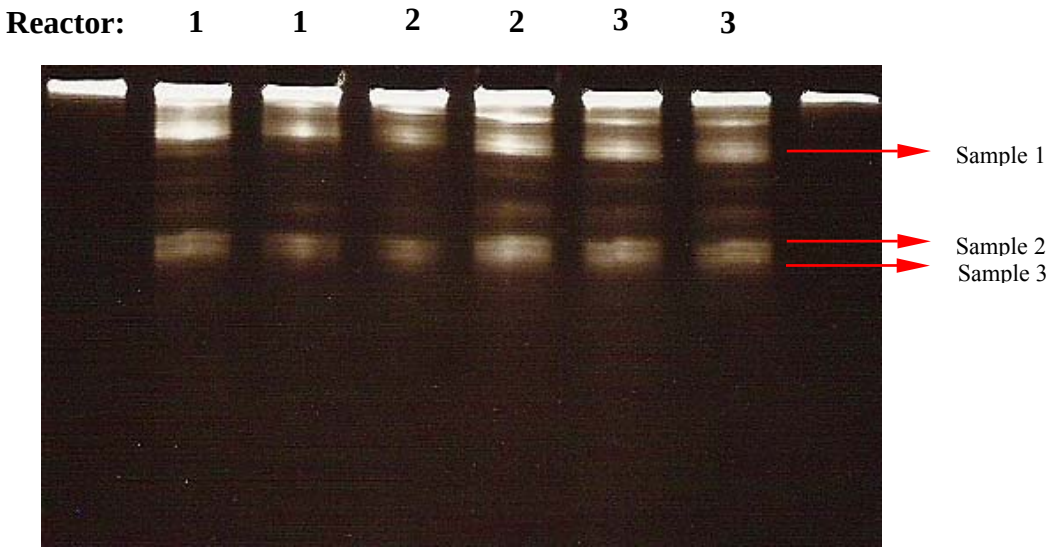


Figure 14: DGGE electrophoresis of bacterial consortiums from each reactor. The single bands from the amplification stage (Figure 13) were separated into a number of fragments, representing different bacterial species. Samples 1 to 3 indicate the bands excised for sequencing. Banding pattern is similar for each reactor's microbial community. At least 8 different species were present.

From the fragments generated in Figure 14, it is seen that the bioreactors contained at least 8 different bacterial species. However, only 3 bands (sample 1 to 3) were successfully excised and sequenced. The similar banding patterns present for each reactor consortium, indicates a similar bacterial community throughout the system. Although more bands were excised, the sequencing of these was unsuccessful. The sequencing results of the three samples represented in Figure 14 are shown in Table 13.

The results of the DGGE microbial community analysis were therefore largely inconclusive.

Table 13: BLAST search homology sequence for the three samples sequenced.

SAMPLE 1:

Accession number	Description	Max Identity	Reference
EF103571.1	Acinetobacter sp. JB54 16S ribosomal RNA gene, partial sequence	96%	Lee, S.S. and Lee, H.Y. (2006)
EF103561.1	Acinetobacter sp. JB7 16S ribosomal RNA gene, partial sequence	96%	Lee, S.S. and Lee, H.Y. (2006)
AJ534674.1	Uncultured gamma proteobacterium partial 16S rRNA gene	96%	Selena-Pobell, S.I. (2002)
DQ342794.1	Uncultured bacterium clone ADPS1_12D 16S ribosomal RNA gene	96%	Robinson, *et al*. (2005)
AY642549.1	Uncultured gamma proteobacterium clone LV57-17 16S ribosomal RNA		Lopez-Garcia *et al.* (2005)

SAMPLE 2:

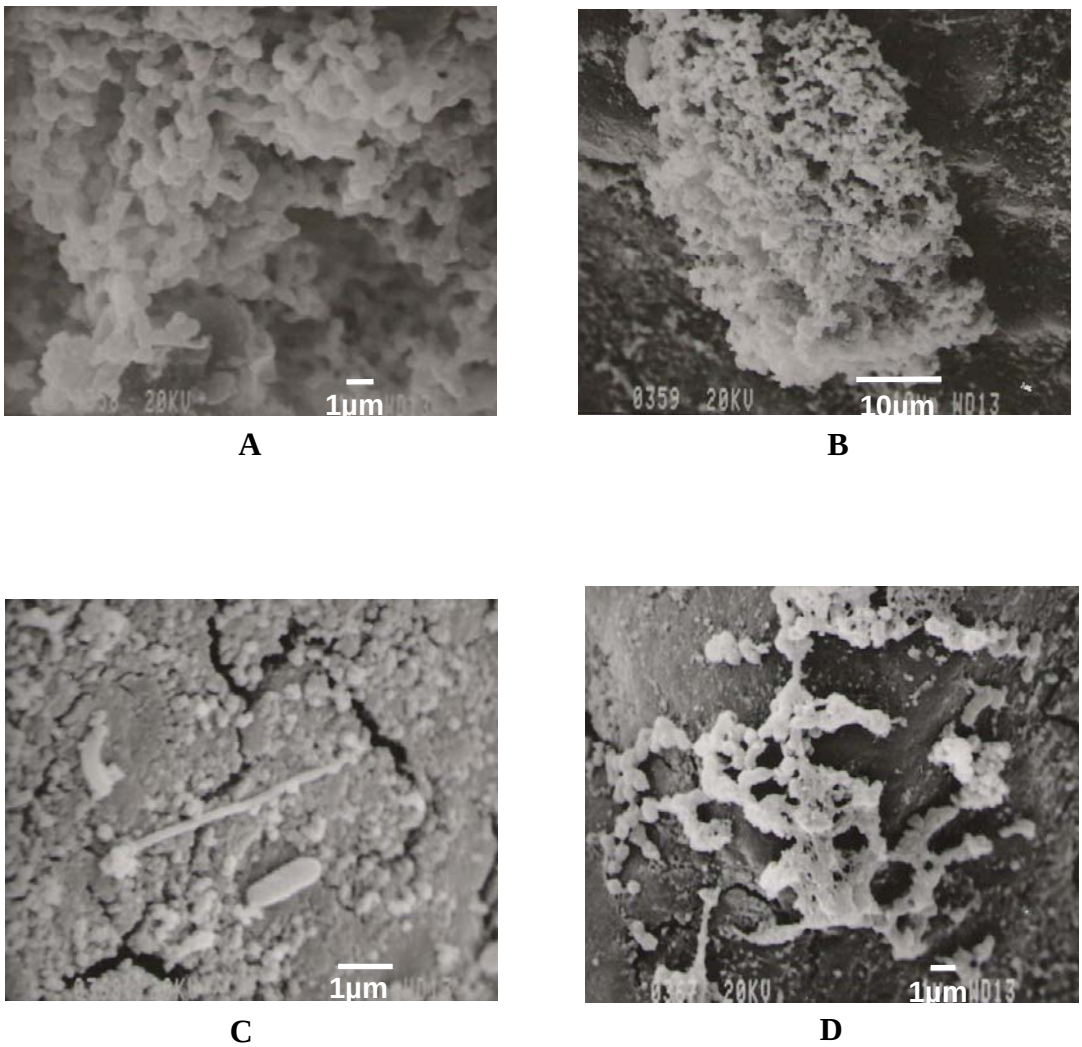
Accession number	Description	Max Identity	Reference
EF434413.1	Acinetobacter sp. VKPM45 16S ribosomal RNA gene, partial sequence	94%	Sunitha *et al*. (2007)
DQ343038.1	Uncultured bacterium clone 2G6F04 16S ribosomal RNA gene	94%	Robinson *et al*. (2005)
DQ342738.1	Uncultured bacterium clone S2-2D 16S ribosomal RNA gene	94%	Robinson *et al*. (2005)
EF149067.1	Uncultured bacterium G1Clone54 16S ribosomal RNA gene	94%	Maknojia *et al*. (2006)

SAMPLE 3:

Accession number	Description	Max Identity	Reference
AY221636.1	Acinetobacter glutaminasificans 16S ribosomal RNA gene	90%	Vanbroekhoven,K. *et al*. (2003)
DQ342500.1	Uncultured bacterium clone PSAD1_06B 16S ribosomal RNA gene	90%	Robinson *et al*. (2005)
DQ342794.1	Uncultured bacterium clone ADPS1_12D 16S ribosomal RNA gene	90%	Robinson *et al*. (2005)
AJ534674.1	Uncultured gamma proteobacterium partial 16S rRNA gene	90%	Selenska-Pobell, S.I. (2002)

4.4 Scanning electron microscopy

Samples of the charcoal substrate contained within the reactor were extracted and prepared for SEM. Figure 15 gives an example of the morphology of some of the bacterial cells, colonies and biofilms present within the system.



A **B**

C **D**

Figure 15: A) Biofilm formed and attached to the charcoal substrate by resident microbes (7000X); **B)** A bacterial colony on the surface of the charcoal (2200X); **C)** Two single cells of different morphology, a rod and a filamentous cell (14000); **D)** Small bacterial colony attached to the charcoal surface (7000X).

The bacterial consortiums within the reactors were able to form biofilms successfully, as established from Figure 15. Some single cells were also present, and clearly these were of different morphology, either rods (curved or straight) or filamentous. This demonstrates the existence of consortiums, rather than a single bacterial species. The possible presence of extracellular polymeric substances (EPS) should also not be discounted, as seen in Figure 15 D. These can only be confirmed with more analyses.

5 DISCUSSION

5.1 Bacterial culture

A modification of Postgate's (1984) medium B proved to be successful for the selection and culturing of the SRB isolated from soil samples that were waterlogged with mine dump water. The production of an iron sulphide (FeS) precipitate after inoculating the medium with the mine dump soil sample was taken as an indication of the successful growth of a culture of anaerobic SRB. The use of acetate as the carbon source instead of lactate, as prescribed by Postgate (1984), would have promoted the selection of acetate-utilising sulphidogenic species. The selection of acetate as a substitute of lactate was based on the fact that acetate is one of the main volatile fatty acids in anaerobic sewage effluents, and other anaerobic treatments (Elefsiniotis *et al*., 2004). The ability of sulphidogenic microbes to grow in the absence of the reducing agent thioglycollic acid prescribed by Postgate (1984) was an interesting finding. This suggests that the growth of the SRB in the soil sample were not inhibited in culture medium that was initially not strictly anaerobic or strongly reducing. The ability of the bacteria isolated from the soil sample to generate H_2S was confirmed by the production of the characteristic odour associated with this compound and also by the appearance of the black FeS precipitate in the medium. Hence it was concluded that a suitable sulphidogenic bacterial consortium was successfully cultured in the modified Postgate (1984) media B.

This sulphidogenic culture that had been isolated at pH 7.0 was then sub-cultured in Postgate medium B adjusted to lower pHs. The low pH experiments indicated that the planktonic forms of the bacterial consortium were tolerant to or could be adapted to pHs below 7.0. It was seen that the SRB were unable to adjust to pH values below 4.5. However, SRB in the bioreactor were able to adjust to influent pH values as low as 3. This increased pH tolerance can be attributed to the establishment of biofilms in the reactor, which protect the microbes from environmental stresses (Lindsay and von Holy, 2006), in this case a low influent pH value.

5.2 Control experiment

The control experiment demonstrated the absence of H_2S production and hence the absence of metal sulphide precipitation. This showed that in the absence of SRB heavy metal precipitation was chemically not possible when only the modified Postage (1984) medium B was recycled through the bioreactor system. The high levels of heavy metals added to the control system were the same as those added to the inoculated system. At each of the HRT, although the concentrations deviated from those added, the change was not consistent with the increasing HRT and no trend was adhered to. Therefore the deviations can be attributed to either absorption of some heavy metals by the charcoal substrate, or to experimental error in analysis, which mostly likely was a result of the dilutions necessary to facilitate analysis of the heavy metal concentrations beyond the

tolerance range of the photometer. Nevertheless, the control experiment did sufficiently demonstrate that the bioreactor system excluding inoculum was incapable of precipitating heavy metals, and no biological or chemical H_2S could be produced in the absence of SRB.

5.3 Water analysis

Since the analysis of heavy metal concentration was carried out using photometric procedures, the water extracted from the reactor had to be free from heavy metal precipitate in order to allow absorption of light and exclude scattering, in keeping with the requirements for accurate photometry. Therefore, the effluent had to undergo a precipitate settling step prior to analysis. This step was carried out at 4ºC to repress microbial activity and further H_2S and HCO_3^- production. In addition to this, it was found that carrying out this step at 4ºC allowed the precipitate to settle faster than at ambient temperature, which usually took more than 8 hours, compared to between 3 to 4 hours in the cold.

The bioreactor system included clarifiers specifically in place to allow metal sulphide precipitate to settle, thus allowing for clearer effluents. It was seen however, that the precipitate was too light to settle while the bioreactors were in operation, and so in such a system, a final undisturbed settling step was necessary, out of which the treated supernatant was collected.

The differences observed in the parameters tested presently were brought about by differences in microbial activity, as a function of the decreasing influent pH during the study.

5.4 COD

A good indication of the microbial activity as a function of the decreasing influent pH was indicated by the declining COD depletion rate with falling pH. This dynamic shows the change in COD concentration over a HRT of 24 hours. Decreasing influent pH resulted in a decreasing COD depletion rate. This was attributed to decreasing microbial activity, resulting in reduced consumption of organic substrates, as the microbes could not readily adapt to the decreasing pH, especially at low HRTs.

5.5 pH change

The bioreactor system was capable of increasing the pH of the polluted mine water through the metabolic production of HCO_3^-. Influent with initial pH values of between 4 and 7 were increased to between 7 and 7.8 after a 24 HRT; while influent with initial pH values of between 2.75 and 3.5 were increased to between 5.4 and 6.8 when the HRT was increased to 24 hours. In order to attain an effluent pH of greater than 7, a HRT of 5 hours was not sufficiently long enough, except in the case of an influent pH of 6 and above. A HRT of 10 hours was sufficient to

reach an effluent pH of 7 from feed pH values of 4.5 and above. Below a feed pH of 4.5, it was necessary to retain the synthetic mine water for at least 24 hours. Studies by Elliot *et al*. (1998), using SRB in a much smaller bioreactor of approximately 1 litre, showed an increase in pH from 4.5 to 6.1 after a 2-day HRT; and an influent pH of 3.25 was raised to 5.82 after 21 days. Kaksonen *et al*. (2006), in a study similar to the present, using a fluidised bed bioreactor, showed that pH could be increased from 2.5-3 to 7.5-8.5. In that study, the results were obtained while operating a lactate-fed bioreactor of 0.5 litres, with a HRT of 16 hours.

It was evident that with decreasing influent pH, the HRT needed to be increased in order to attain a neutral or slightly alkaline effluent pH. It should be noted however, that an influent with an initial pH of 2.75 did not readily become more alkaline with passage of the medium through the bioreactor system. It appears that long HRTs are necessary for the system to cope with extremely low influent pHs. Bacterial activity was reduced dramatically when this pH was maintained. For extremely acidic pHs, if the HRT was less than 24-hours, then bacteria were unable to generate sufficient alkalinity. The increase in pH from an influent of 2.75 could thus be in part, attributed to residual HCO_3^- in the system. However, it can be hypothesised that if the HRT were increased from 24 hours to perhaps a few days, the microbial communities in the bioreactor would have more time to adjust and therefore would be capable of treating mine waters with a pH below 2.75.

5.6 Heavy metal precipitation

The heavy metals added to the system were successfully precipitated through the action of biologically produced H_2S. Fe, Mn, Zn, Cu, Cr and Al were the heavy metals used, at concentrations based on the high range values reported (except for Al) by Naicker *et al*. (2003). A similar trend as observed for the alkaline generating efficiency, was also noticed for the heavy metal precipitation study, in that as the influent pH decreased, the amount of heavy metals precipitated decreased. This again could be explained by the reduction in bacterial activity with decreasing pH, as was demonstrated by the COD depletion rate study. The heavy metal precipitation study is represented in Tables 4 to 12.

The South African Water Quality Guidelines manuscript (Holmes, 1996) has stipulated the water tolerance ranges for a number of water uses, such as domestic, industrial, recreation, agriculture and irrigation. It was useful to compare the water quality of the effluent from the present study, in terms of heavy metal concentration, to those given in the guideline.

The tolerance levels for heavy metal concentration for domestic use were met after a 24-hour HRT for the influent pH values of 7 and 6. However, below this pH, the high levels of Fe, Mn and Zn added to the system, as a reflection of the levels typically occurring in AMD, were not sufficiently reduced to remain within these limits.

As the feed pH decreased, the effluent water quality over a 24-hour HRT decreased, and subsequently became more suited to livestock feeding, short-term irrigation and finally industry. However, with a HRT of greater than 24 hours, more heavy metals will invariably be precipitated, and thus the HRT of the influent can be tested and stipulated with reference to the desired effluent water quality.

The percentage efficiencies of heavy metal removal over a 24-hour HRT, as a function of feed pH values decreased with decreasing feed pH values. For all the influent pH values tested, the percentage efficiencies of heavy metal removal after a HRT of 24 hours, was greater than 65%. These efficiencies were all above 98% for feed pH values of 4 and above, over the same HRT. Evidently, an influent pH of 4 was the lowest pH value necessary to allow greater than 98% heavy metal precipitation efficiency in the bioreactor system used presently. Although significant precipitation did occur at feed pH values as low as 2.75, a HRT of greater than 24 hours is more desirable for treating these low pH influents.

Kaksonen *et al*. (2006), using SRB in a fluidised bed bioreactor and a HRT of 6.5 hours, were able to precipitate 600 $mg.l^{-1}$ Zn and 300$mg.l^{-1}$ Fe per day on a lactate and ethanol carbon source. Jong and Parry (2003) also had similar success in treating water with a low pH and rich in metals and sulphates. Those authors, using an upflow anaerobic packed bed reactor inoculated with a mixed population of SRB from a mine site, were able to remove 97.5% of Cu, Zn and Ni; greater than 77% As; and >82% Fe from synthetic mine water.

While it may be more informative to compare the heavy metal removal efficiencies from other studies with the present investigation, it must be remembered that different bioreactors will have different volumes and operational methods. For this reason, flow rates and HRTs will differ between studies, while possibly still attaining similar results. Due to the diversity of bioreactor assemblies and operational methods, attempts at scaling up a successful bench-scale bioreactor system often present numerous difficulties. Even in the present study, the system often behaved as a packed bed bioreactor when flow rates were reduced to allow longer HRTs.

5.7 Microbial community analysis

DNA was extracted from each reactor in order to gain some understanding of the bacterial consortium present. The DNA extraction procedure was successful, as well as the amplification process that followed. Amplification yielded products of between 400 to 430 base pairs, using the forward and reverse primers p63f and p518r respectively. Boon *et al*. (2000b) pointed out that PCR products using the abovementioned primers should yield fragments of 530 base pairs, with the GC clamp attached. However, in yet another study by Boon *et al*. (2000a) using the same forward and reverse primers as in the present study, it was reported the expected size of the amplified fragments was 474 base pairs. Repeated electrophoresis using the same primers in the present study, however yielded fragments of the same size, i.e. 400 to 430 base pairs. This difference could either

be attributed to the quality of the primers supplied, or to the detachment of the GC clamp at some stage during amplification. The purpose of the 40 base pair GC clamp was to strengthen the DNA fragments and make them robust for later use in DGGE. The amplified products, and not the fragments from the agarose gel, were then subjected to DGGE in order to separate the bacterial consortium, so as not to erroneously select non-specific bound fragments.

The DGGE process did achieve the purpose of separating the amplified fragments. The products were separated into at least 8 bands, representing at least 8 species of bacteria present in the bioreactor system. Similar banding patterns for each of the reactors implied that a similar consortium was present throughout the system. Some of the separated bands were too feint to be separated accurately, and so only 5 prominent bands were chosen from each lane. These were excised and sequenced by Inqaba Biotechnical Industries (Pty) LTD (South Africa). However, 2 of the bands could not be sequenced, and hence only 3 bands were successfully sequenced.

The close sequence similarity among the SRB in the bioreactors was evident from in that the bands are quite close together, implying minimal difference among the 16S rDNA. As a consequence of this, it was difficult to separate the bands more than that that represented in Figure 14, even after attempting to run the polyacrylamide gels for longer periods, or at reduced voltage. For this reason some bands were too close together to resolve sufficiently, and were thus

probably excised together as one, possibly accounting for the fact that 2 of the excised bands could not be sequenced.

The three bands that were sequenced, on the other hand, rendered rather inconclusive results. Sequencing identified these bacteria as either *Acinetobacter* sp., or uncultured bacterium clones. Since a bacterial consortium was present in the bioreactor during operation, it was expected that bacteria other than dissimilatory sulphate reducers would be present. However, *Acinetobacter* sp. are not known to, and many have been proved to be unable to produce H_2S (Shakibaie *et al*., 2003). Therefore it is unlikely that the *Acinetobacter* sp. were responsible for heavy metal precipitation. In addition, some *Acinetobacter* sp. are able to grow on a range of aromatic alcohols and esters as energy sources (Jones *et al*., 1999). *Acinetobacter* sp. have also been extensively used in the remediation and biodegradation of phenols and other organic compounds (Abdel-El-Haleem, 2003). *Acinetobacter* sp. are widespread in the environment, and it is therefore likely that these microbes live in a commensal relationship with SRB.

In a study by Kaksonen *et al*. (2006), in which the diversity of SRB in a bioreactor was studied, it was shown that several bacterial strains isolated from the reactors in that study were previously uncultured. Those authors go on to say that those uncultured strains could possibly represent novel species or novel genera.

Pruden *et al*. (2007) conducted a study on the effect of inoculum on the performance of sulphate reducing columns. Those authors also made use of

DGGE to ascertain the microbial community active with the columns, and also found that the columns contained a number of uncultured bacterium clones. Interestingly, in that study the proportion of SRB within the columns was relatively low compared with the proportion of *Clostridium* sp. The majority of the microbes represented cellulose- and other polysaccharide- degrading bacteria.

In many acid mine drainage treatment systems, SRB have been shown to represent a relatively low proportion of the total bacterial population present (Johnson and Hallberg, 2003; Hallberg and Johnson, 2005). This was true for the present study as well, since the effects of SRB metabolism was present in the bioreactor through dissimilatory sulphate reduction and H_2S and HCO_3^- production, but the molecular techniques employed were unable to classify the species present. The low proportion of SRB in the total bacterial population probably accounted for this.

However, it cannot be discounted that the uncultured bacterium clones possibly represent novel species, since PCR-DGGE analysis of bacterial consortiums often encounters previously uncultured anaerobic bacteria (Kaksonen *et al*., 2006; Pruden *et al.*, 2007). Further molecular analysis is warranted in this regard.

Each of the samples however, did show homology to isolates that were either pH tolerant over a large range, heavy metal tolerant, or tolerant to anoxic environments. One such example would be the 96% homology that sample 1 showed to an uncultured gamma proteobacterium clone LV57-17 (Lopez-Garcia

et al., 2005). Those authors showed that this proteobacterium was alkaline tolerant. Similarly, the uncultured gamma proteobacterium that sample 3 shared 90% homology with, was isolated from deep-well injection sites, suggesting a possible anoxic environment. Similarities can thus be drawn between the properties that the SRB exhibited in the present study with those that displayed a high degree of sequence homology to them. Bacteria that share homology with the SRB isolated in the present study also tend to share some of the properties, such as tolerance to extreme pH values, as well as to low oxygen levels.

5.8 Scanning electron microscopy

The results from the SEM study lent valuable insight into the bacterial consortiums present in the reactor. From the initial culture experiment using Postgate's (1984) media at different pHs, it was seen that planktonic SRB in liquid media were unable to survive at pH values below 4.5. However, the performance of the bacterial consortiums within the bioreactor did not reflect this pH intolerance.

It was seen that the bioreactor was able to operate effectively at influent pH values as low as 3. The presence of biofilms attached to the charcoal substrate was observed. As mentioned by Lindsay and von Holy (2006); and Singh *et al*. (2006), biofilms confer upon the cells tolerances to environmental stresses that are not present in the planktonic stage. Baumgartner *et al*. (2006) also pointed out that SRB are often found in oxic zones of microbial mats, illustrating the oxygen

tolerance that these biofilms confer. Hence, the formation of biofilms within the reactors allowed the bacterial consortiums to withstand variation in influent pH, as well as high concentrations of heavy metals.

Although the presence of at least 8 different bacterial species based on differences in the sequences of their 16S rDNA was confirmed, these microbes could not be easily detected with the scanning electron microscope. This could be attributed to the fact that most bacterial species would have been attached and confined to biofilms, hence altering their morphology and making the description of their planktonic morphology impossible.

5.9 Conclusion

The effects of AMD and other industrial water pollutants need urgent attention, especially in a water-scarce country like South Africa where mining and industrial pollutants cause widespread environmental damage to sensitive ecosystems and wetland habitats. A large portion of South Africa's revenue is generated from eco-tourism. With water pollutants entering sensitive habitats, a number of endemic and endangered wildlife become threatened. As industrial and mining operations expand in South Africa and around the world, more effective and economical measures are needed to combat resulting pollutants. As discussed previously, existing AMD treatment and remediation programs are often expensive, require removal of heavy precipitate, or are selective either in the metals they remove or in the pH range in which they are efficient.

The present study represents an economical and effective method of treating wastewater that may be rich in heavy metals, and/or have a low pH. The high efficiency of heavy metal precipitation is encouraging, and the alkalinity generation efficiency lends promise to the treatment of a range of polluting waters from many industrial processes. In a one-step operation, both the low pH and the heavy metal content can be addressed and treated, thereby saving costs associated with step-wise treatments that deal with each AMD pollution individually.

Future studies can expand on the present and explore the effectiveness of a range of carbon sources and volatile fatty acids, as well as explore the effect of different HRTs, bioreactor designs, bioreactor volumes, etc. on wastewater treatment. Another area that offers opportunities for further study is in microbial community analyses of these bioreactor systems. It was seen from the present study that not only SRB were present in the bioreactors, and hence the relationship between these microbial communities can be elucidated to develop better bioremediation systems in future.

6 APPENDICES

6.1 Appendix A: Media and solution constituents

50X TAE Buffer:

Tris base	242 g	2M
Acetic acid, glacial	57.1 g	1M
0.5M EDTA, pH 8	100 ml	50mM
dH_2O	to 1000 ml	

Denaturation solutions (for a 6% (w/v) gel):

40% Denaturation:

40% Acrylamide/Bis	15 ml
50X TAE buffer	2 ml
Formamide	16 ml
Urea	16.8 g
dH_2O	to 100 ml

60% Denaturation solution:

40% Acrylamide/Bis	15 ml
50X TAE buffer	2 ml
Formamide	26 ml
Urea	27.3 g
dH_2O	to 100 ml

1X TAE Running buffer:

50 X TAE buffer	140 ml
dH_2O	6860 ml
Total volume	7000 ml

PCR Master Mix composition:

Taq DNA polymerase	0.05 units.μl^{-1}
$MgCl_2$	4mM
dNTPs	0.4mM of each

1% Agarose gel:

Agarose	0.5 g
5X TBE buffer	10 ml
Distilled water	40 ml

Heat until agarose has dissolved

Ethidium bromide	1µl

5X TBE buffer:

Tris base	54 g
Boric acid	27.5 g
0.5M EDTA (pH 8)	20 ml
dH_2O	to 1000ml

LB media (pH 7):

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

Postgate Media B:

KH_2PO_4	0.5 g
NH_4Cl	1 g
$CaSO_4$	1 g
$MgSO_4 \cdot 7H_2O$	2 g
$FeSO_4 \cdot 7H_2O$	0.5 g
Ascorbic acid	0.1 g
Thioglycollic acid	0.1 g
Yeast extract	1 g
Sodium lactate	3.5 g

6.2 Appendix B: Sequences of 3 samples extracted from DGGE gel

Sequence of sample 1:

CGACTAGTGCTATGCACCATTGCATAAATCTTGGACGGGTGAGTATATCG

CTTAGGAATCTGCCTATTAGTGGGGGACAACATCCCGAAAGGAATGCTAA

TACCGCATACGCCCTACGGGGGAAAGCGGGGGATCTTCGGACCTTGCGCT

AATAGATGAGCCTAGAGTCGGATTAGCTAGGTGGTGGGGAAAAGGCCCAC

CAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCACACCTGGG

ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCGGTGGGGAATATTGG

ACAATGGGGGGAACCCTGATCCAGCCCGCCGCGGTAATAAGCATTTTTCT

TTTACTTTTTGTTTTTTTTTTTTGGGGGTTGCTCCGGGGACGCTGCGACG

AGCGCGCCGGAACGTTTTTGCTTGGTCTTTGTGGCTGACAATTGATGATG

ATGGGCTATATGTGCTTTGTGGGGGTGTGTTGTGTGTGCTGGGCTAAAGG

AGAGCGCAGGCGACTCGTCGTCCTCTCCCGACACACTACCCGCAGTCGTC

TTTTAGGATATATTGATGTTTTATGCTGCGATGTATCTGTCTGTGTGTGT

GTGTGTGAGTGACGTGCATGGCTGAGCTGTCTGCTGTCGCTGTTCTCATG

TCGTTTTTCTAGTTGCACGAGTGTACGACGCGCCGCCCCCCCGTCGCGTG

CGTTGCAGATGACAGGAGGAGATAGGGGGTAGCTGGTTGGTGTGTGTTGT

TTGGTGTTGTTGGCGTCTGTGAGGACGTGTCCGTGAGAGCACAACACAAC

ACTGATCCAGTGTGGCCGCTCCGTACCGCTGGACCGTGGCGCGTCTGCAT

GTCGATCTGTGTGTGAGGAAAGATAGCACTGTCACATGGCGTATGTGGGT

TTGGT

Sequence of sample 2:

TACGGGGCATTGCATCCATTGACTAAAATCTTGGACGGGTGAGTGATCAT

GCTTAGAATCTGCCTATTAGTGGGGGACAACATCCCGAAAGGAATGCTAA

TACCGCATACGCCCTACGGGAGAAAGCGGGGGATCTTCGGACCTTGCGCT

AATAGATGAGCCTAGGTCGGATTAGCTAGTTGGGGGGGTAAAGGCCCTAC

CAAGGCGACGATCTGTAGCGGGTCTGAGAAGATGATCCGCCACACTGGGA

CTGAGACGCGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGTA

CTATGGGCGGAAGCCTGATCCACGCCCGCCGCGGTAATATAGTAGTGTTT

TTTGCCCTCTTCTTTTATTTTGTGTGGGCGTGTGGCGCTGGCGCATTTAG

TACATGTATATGAGAATATGTGTTGTGCTGCTCTGCATGCATCGAACGAT

CCAAGTTATAGATAGACTAAACATCGTGTGTCGTCTGTCGCGCGTGGTGT

GTGTGGCTGTGGTGGGGCGCGCGTCGCGCGCGGGCCAGCTGCCCCGCGTG

AAGAAGGATGAAAGAGAGGTGTGTTGGACGCGCCCTGTCGCGCGTGTGGC

CGACAGAAGTTGGGGGCGGGGGTGCGTCGCGTGCTCTTAAGTTTTGTTTT

TTGTTGTGTGGTTGTTGTGAGTGGGAGAACGTGATAATGATATTTTATCG

CGTGCACGCGCACTAGACTGCCTCCTCCGTTCTTTCTCTGGTTGTAGTGG

GTGTGGTCGTCGGCGTTGGCGGGCGGGGAGAAGTGGCTGCGATGCTGTTC

GTTGCTGATGAGATCATGATACATATATCTTTTATACTACAAATGATATG

TACTTGTGGTCTCTGGTGCTCGAGCTAGCGCCACAGAGAATC

Sequence of sample 3:

GCTGAGCCTGTGCGGGACGGGTGAGTATATGCTTAGGAATCTGCCTATTA

GTGGGGGACAACATCCCGAAAAGAATGCTAATACCGCATACGCCCTACGG

GGGAAAGCGGGGGATCTTCGGACCTTGCGCTATTAGATGAGCCTAACTCG

GATTAGCTAGTTGGGGGGGTAAAGGCCCACCAAGGCGACGATCCTGACGC

GGGTCTGAGAGGATGATCCGCCCCGCTGGAACTGAGACGCGGCCCAGACT

CCTATCGGGAGGCAGCTACGTGGGGAATATTAGGACAGTGGGGGGAACCC

TGATCCGGCCCGCCGCGGTAATATTGTAGTTTTTGTAGTTGGTCCATTTT

TGGTAGAGCGGCGAGGCTGGCTTGGGGGGGTGTGGTGATGTTGACAAACA

ATCTCGTGTGTGTGTGTGTTCTCCCTCTCGCACGGCTTGTCTGCGTTCCC

CCATCGGCTGTGAGCGAGTGTGAGCTAGATTGTACATGTGTATGTGCCGT

CACTCGTGAGCTGTGGGCTGGCGCGCGCCTGAGCCACGATGCTGGTGTGC

TCTCTATATTGTGTCGTGGCCGATGTCGACGTATGCGAGTCGAGCGCTGC

TGCTGCGCGTCTCTGCGTGGTGACGACACACACGCAAGACACAGCCGCCG

CGCGTGGGTGGTCTGCGCCCGTGCTCGACGTGCGATGCGCGAGTGATGTA

TGAGGCTCGTTACGTTCTGGGGGTGGGCGGGCCCGTAAGCATACGCACTA

TGATGTGATGTGTGAGAGTAGTAAAGAGACTATCACGCTTGAGCGCATAG

CTGTCACTTGCTGTCGTTGTGTCGTCGTGCGTGGGGGGGACCGCAGCCGC

ACACCTGCGCTGCGCACTGGTCTGC

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