

MICROBIAL SULPHATE REDUCTION USING DEFINED CARBON SOURCES  
AND ARTIFICIAL ACID MINE DRAINAGE

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MICROBIAL SULPHATE REDUCTION USING DEFINED  
CARBON SOURCES AND ARTIFICIAL ACID MINE  
DRAINAGE

MASTER OF SCIENCE

In the Faculty of Biological and Agricultural Sciences

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University of Pretoria

Pretoria

South Africa

December 1997

# **MICROBIAL SULPHATE REDUCTION USING DEFINED CARBON SOURCES AND ARTIFICIAL ACID MINE DRAINAGE**

I, the undersigned, certify the thesis hereby submitted to the University of Pretoria for the degree of M.Sc. and the work contained herein is my own original work and has not previously, in its entirety or in part, been submitted at any institution for a degree.

By

**SUSANNA ELIZABETH COETSER**

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Date:

Submitted in partial fulfilment of  
the requirements for the degree

**MASTER OF SCIENCE**

In the Faculty of Biological and Agricultural Sciences

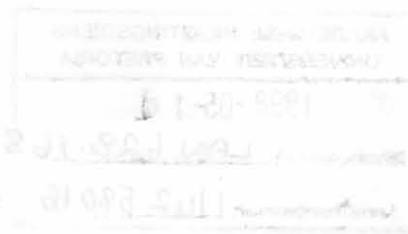
Department of Microbiology and Plant Pathology

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## MICROBIAL SULPHATE REDUCING BACTERIA IN DEFINED CARBON SOURCES AND ARTIFICIAL ACID MINE DRAINAGES

### DECLARATION

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Signature:

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Date:

27/11/1998

# **MICROBIAL SULPHATE REDUCTION USING DEFINED CARBON SOURCES AND ARTIFICIAL ACID MINE DRAINAGE**

by

**SUSANNA ELIZABETH COETSER**

**Promoter:** Prof. T.E. Cloete  
**Department:** Microbiology and Plant Pathology  
**Degree:** M.Sc. (Microbiology)

## **SUMMARY**

The production of acid mine drainage (AMD) containing high amounts of sulphate, heavy metals and low pH is of increasing concern. Due to the fact that it is highly corrosive, it results in environmental and economic problems.

The potential use of different defined carbon sources to drive sulphate reduction in artificial AMD was studied. This was done in a process for developing a standard laboratory procedure for the evaluation of carbon sources for potential use in passive treatment systems of AMD.

The conceptual model for the passive treatment of AMD accounts for major events of interest occurring within the passive treatment system. This model will assist in identifying the parameters that significantly influence the system response as well as possible causes for malfunction.



# **MIKROBIOLOGIESE SULFAAT REDUKSIE DEUR GEBRUIK TE MAAK VAN GEDEFINIEERDE KOOLSTOF BRONNE EN KUNSMATIGE SUURMYNAFLOOP**

deur

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## **OPSOMMING**

Die toenemende produksie van suurmynafloop is kommerwekend. Suurmynafloop bevat groot hoeveelhede sulfaat, swaar metale en 'n lae pH. As gevolg van die feit dat dit hoogs korrosief is, veroorsaak dit omgewing en ekonomiese probleme.

Die potensiaal om verskillende gedefinieerde koolstof bronne te gebruik vir die reduksie van sulfaat in suurmynafloop is bestudeer. Dit is gedoen deur 'n standaard laboratorium prosedure te ontwikkel vir die evaluasie van koolstof bronne vir potensiale gebruik in passiewe behandeling stelsels van suurmynafloop.

Die konseptuele model vir passiewe behandeling stelsels van suurmynafloop beskryf die belangrikste gebeure in hierdie passiewe behandeling stelsels. Die konseptuele model identifiseer die belangrikste parameters wat stelsels negatief kan beïnvloed.

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## CHAPTER 1

### INTRODUCTION

The production of effluents containing high amounts of sulphate, heavy metals and a low pH is a world-wide problem, of increasing concern. These effluents are known as acid mine drainage (AMD) and the primary source is coal mining. The metabolism of sulphur –and iron-oxidising bacteria when pyrite is exposed to atmospheric oxygen and the combination of autoxidation and microbial sulphur and iron oxidation leads to the production of large quantities of sulphuric acid (Atlas and Bartha, 1993). AMD leads to various environmental problems, killing aquatic life as well as economic problems due to the fact that it is highly corrosive.

By neutralising the effluent and removing the sulphate, AMD may be remediated.

Currently, a variety of sulphate removal technologies are available. These include:

- Desalination processes, such as reverse osmosis and ion exchange (Dill *et al.*, 1994).
- Chemical methods, using barium ions such as barium hydroxide or barium chloride (Maree and Strydom, 1985).
- Biological removal of sulphate, using sulphate reducing bacteria (SRB) (Dill *et al.*, 1994).

Chemical and physical methods, however, require a high degree of maintenance and supervision. Thus, there is an increasing demand for inexpensive, environmental friendly



technologies to remediate AMD. The use of SRB in the biological removal of sulphates is such an alternative. SRB can oxidise organic compounds like lactate or acetate, if a suitable electron donor is available (Dill *et al.*, 1994). During this process, sulphate is used as electron acceptor and reduced to sulphide.

Various experiments using different bioreactor setups and different carbon sources have been studied for potential use in the biological removal of sulphate by SRB (Tuttle *et al.*, 1969; Maree and Strydom, 1985; Du Preez *et al.*, 1991; Van Houten *et al.*, 1994).

Another method which involves SRB, is the 'passive treatment of AMD' (Batchelor, 1993). Passive treatment processes refer to those systems which utilise natural resources and processes to drive the overall treatment process of polluted or contaminated water. However, due to the absence of methods to evaluate the potential use of different undefined carbon sources, there is a lack of experience with passive treatment systems designed for the treatment of AMD.

The aims of this study were therefore:

- to develop a standard procedure for the evaluation of carbon sources for sulphate reduction in AMD;
- to evaluate different defined carbon sources for the reduction of sulphate of AMD.
- to propose a conceptual model for the passive treatment of AMD.

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## 2.1. Introduction

Manufacturing processes, particularly steel making, often result in effluents containing high amounts of sulphate and other inorganic compounds. These effluents are known as acid mine drainage (AMD) and are a major environmental problem. AMD is a highly acidic solution containing high concentrations of sulphate and iron, which can be harmful to the environment. The treatment of AMD is a major challenge for the steel industry. The most common method for treating AMD is neutralization, which involves adding a base to the effluent to raise the pH. This process is often done using lime or limestone. However, this method is expensive and can result in the formation of large amounts of sludge. Another method for treating AMD is biological sulphate reduction, which involves using bacteria to reduce sulphate to elemental sulphur. This process is more expensive than neutralization, but it can result in a more stable effluent. In this paper, we describe a new method for treating AMD using gas-lift reactors. These reactors are fed with hydrogen and carbon dioxide as energy and carbon source. The bacteria in the reactor reduce sulphate to elemental sulphur, which is then removed from the effluent. This process is more expensive than neutralization, but it can result in a more stable effluent.

By removing sulphate from the effluent, AMD may be converted to a neutral pH. Several removal technologies are available (Dall et al., 1994). These include chemical processes, such as reverse osmosis and ion exchange (Dall et al., 1994). By using chemical agents, such as barium hydroxide or barium chloride, sulphates can be precipitated out of the effluent (Mazze and Strohman, 1953). Sulphur can be recovered from gypsum by acid treatment, by sulphate reduction followed by removal of the sulphide as elemental sulphur gas, which can be chemically oxidized to elemental sulphur (Middleton and Lawrence, 1977). This process can be used to neutralize sulphuric acid wastes (Middleton and Lawrence, 1977). Considering current problems of acid mine drainage, development of new and



## Chapter 2

### LITERATURE STUDY

#### 2.1. Introduction

Manufacturing processes, primarily coal mining, often result in the production of effluents containing high amounts of sulphate and heavy metals. These effluents are known as acid mine drainage (AMD). AMD is the consequence of the metabolism of sulphur -and iron-oxidising bacteria when pyrite is exposed to atmospheric oxygen and the combination of autoxidation and microbial sulphur and iron oxidation produces large amounts of sulphuric acid (Atlas and Bartha, 1993). Due to the fact that AMD is highly corrosive, it results in economic and environmental problems.

By removing sulphate from the effluent, AMD may be prevented. Various sulphate removal technologies are available (Dill *et al.*, 1994). These include desalination processes such as reverse osmosis and ion exchange (Dill *et al.*, 1994). By using barium ions, such as barium hydroxide or barium chloride, sulphates can be chemically removed (Maree and Strydom, 1985). Sulphur can be reclaimed from gypsum (calcium sulphate) by sulphate reduction followed by removal of the sulphide as hydrogen sulphide gas, which can be chemically oxidised to elemental sulphur (Middleton and Lawrence, 1977). This process can be used to neutralise sulphuric acid wastes (Middleton and Lawrence, 1977). Considering current problems of acid mine drainage, development of microbial

sulphate reduction as a treatment process for these wastes is a desirable objective. Provided a suitable electron donor is available, SRB can oxidise organic compounds like lactate or acetate, with sulphate as electron acceptor being reduced to sulphide (Dill *et al.*, 1994):



Growth studies done with pure cultures of SRB have been limited to less complex compounds, such as short chain carboxylic and dicarboxylic acids (Middleton and Lawrence, 1977). In mixed cultures, SRB have been grown with a variety of complex substances as carbon source (Middleton and Lawrence, 1977). These included fish meal, wastewater sludge, cellulose and sawdust.

Mixed cultures of SRB increased the pH of a lactic acid-mineral salts medium, containing sulphuric acid, from 5 to 8,9 in 8 days at room temperature (Tuttle *et al.*, 1969).

Du Preez *et al.* (1991) used producer gas, consisting of a mixture of  $\text{H}_2$ ,  $\text{CO}$ , and  $\text{CO}_2$  and  $\text{N}_2$  generated from coal, as energy source for biological sulphate removal. Sulphate was reduced from 1900mg/l to less than 200mg/l during the anaerobic treatment of sulphate rich water, in a trickling filter. A maximum sulphate conversion rate of  $30\text{g/l SO}_4^{2-}/\text{d}$  was achieved by Van Houten *et al.* (1994). Gas-lift reactors fed with hydrogen and carbon dioxide as energy and carbon source were used during these studies.

Maree and Strydom (1985) studied biological sulphate removal in an upflow packed bed reactor. Good sulphate removal was achieved by providing anaerobic conditions on a solid medium and maintaining a low hydrogen sulphide concentration by recirculating the water through a photosynthetic reactor for sulphur production. Sugar, pulp mill effluent or sewage was used as energy sources. Recirculation enhanced sulphate reduction and the presence of light increased sulphur production. Therefore, under ideal conditions, SRB may be used for the remediation of AMD.

The passive treatment of AMD, involving SRB is one other method which should be considered (Batchelor, 1993). There is a lack of established local or international experience with passive treatment systems designed for the removal of sulphate. To develop efficient passive AMD treatment, methods to evaluate the potential use of different undefined carbon sources must be established and the anaerobic sulphate reduction process must be optimised. This can be done by selecting the most appropriate carbon sources, or combination of various carbon sources. The primary objective of this project was to develop a standard procedure for the evaluation of carbon sources for sulphate reduction in acid mine drainage, using defined carbon sources. The information obtained from these experiments would form the basis for studies conducted on undefined carbon sources. A conceptual model for the passive treatment of acid mine drainage will also be presented.

## 2.2. ACID MINE DRAINAGE (AMD)

### 2.2.1 Sources and problems associated with acid mine drainage

Atlas and Bartha (1993) defined acid mine drainage as the consequence of the metabolism of sulphur- and iron-oxidising bacteria when coal mining exposes pyrite to atmospheric oxygen and the combination of autoxidation and microbial sulphur and iron oxidation produces large amounts of sulphuric acid, which kills aquatic life and contaminates water. AMD renders the contaminated stream unsuitable as a water supply or for recreational use due to the fact that it is highly corrosive. This leads to many economic and environmental problems.

The discharge of industrial effluents containing a high concentration of sulphate into surface waters contributes directly to mineralization and corrosion potential of the receiving waters (Dill *et al.*, 1994). Some acid mine drainage originates from subsurface mining because of water flowing through the mine itself (Atlas and Bartha, 1993). The problem with subsurface mining is limited and easily controlled. After the coal has been removed, the areas surrounding these streams are collapsed (Atlas and Bartha, 1993). This procedure limits the amount of rock exposed to oxidative action at any one time. During strip-mining, tailings are left as porous rubble which are exposed to oxygen and percolating water (Atlas and Bartha, 1993). Due to iron and sulphur oxidation, the pH drops rapidly and prevents the establishment of vegetation or stable soil cover. The recovery of the land may take from 50 – 150 years (Atlas and Bartha, 1993).



Thus, sulphate in mine water originates from at least two sources:

- bacterial oxidation of pyrite
- the spent sulphuric acid used in metallurgical or chemical plants

(Du Preez *et al.*, 1991).

According to McGinness and Johnson (1993), the scale of the pollution problem associated with AMD is vast. It was estimated in 1963 that 3 million tons of sulphuric acid were entering the Ohio river in the form of AMD. They concluded that besides its acidity (pH of AMD may range from <2 to 4), the toxicity of AMD to most life-forms comes from its heavy metal content. Soluble iron concentrations, both as ferrous and ferric are inevitably high and other metals for example copper, lead and zinc may also be present at elevated concentrations (McGinness and Johnson, 1993). Soluble iron concentrations depend upon the geochemical nature of the material being oxidised. The dominant anionic species present is sulphate and may reach concentrations up to >40g/l (McGinness and Johnson, 1993).

The water situation in South Africa is threatened both from a supply and a quality point of view (Maree, 1988). The supply and demand curves of water in South Africa will converge before 2020 due to the fact that it is a semi-arid country with limited water resources (Maree, 1988). As a result of the growing population, the total water demand in South Africa for agriculture, housing, industrialization and mining will increase rapidly.

Sulphate significantly effects the utilisation of water (Maree, 1988). Therefore, the treatment of sulphate polluted water will contribute considerably to the prevention of pollution of South Africa's surface water.

At the moment the Municipality of Johannesburg and water authorities in the Gauteng area allow the discharge of water with higher sulphate concentrations than the acceptable levels of 200-500mg/l into sewer systems or rivers (Dill *et al.*, 1994). This is accepted due to the fact that the ratio of sulphate-rich water produced by industrial activities to surface water, is high in that region. As soon as proven technologies are available for the removal of sulphate at an acceptable cost, legislation would be enforced to prevent the discharge of waters with high sulphate concentrations into the receiving waters (Dill *et al.*, 1994).

### 2.2.2 The microbiology of acid mine drainage

*Thiobacillus ferrooxidans*, an autotrophic bacteria is responsible for the enzymatic oxidation of ferrous sulphide minerals like pyrite which are often found associated with coal in nature (Tuttle *et al.*, 1969). This oxidation process leads to an accumulation of ferric, sulphate and hydrogen ions in the drainage waters from coal mines. It was reported that hydrogen ions are responsible for the inhibitory effects of AMD on heterotrophic bacteria of neutral streams (Tuttle *et al.*, 1969).

According to McGinness and Johnson (1993), the microbiology of AMD is surprisingly complex. Acidophilic bacteria are dominant, however eukaryotes, ranging from fungi and yeasts to protozoa and rotifera, may be found. Chemolithotrophic bacteria that obtain energy from the oxidation of ferrous iron and/or reduced sulphur are the primary producers and have been shown to form the basis of an acidophilic food web *in vitro*. The numbers of iron oxidising bacteria decreased with distance from the mine, whilst neutrophilic heterotrophs increased. McGinness and Johnson (1993) also found that the highest counts of total bacteria were found in the AMD water within the mine. *Thiobacillus ferrooxidans*, was the dominant iron-oxidising bacterium at all sampling sites while *Leptospirillum ferrooxidans* accounted for between 9 and 50% of the isolates. Acidophilic heterotrophic bacteria were occasionally isolated from the downstream river, but they did not detect any iron-oxidising bacteria.

Sulphuric acid and ferric ions have a deleterious influence on the heterotrophic biota of streams that receive the mine drainage (Tuttle *et al.*, 1968). Ecological reports have indicated that  $H_2SO_4$  could cause the killing of the normal microflora of affected waters and that aciduric species, notably fungi, appeared to thrive (Tuttle *et al.*, 1968). Tuttle *et al.* (1968) studied the activity of microorganisms in acid mine water and found that acid-tolerant aerobes survived when acid entered the stream and actually increased in number to about  $2 \times 10^3$  per ml until the pH approached 3. The organisms then represented the heterotrophic aerobic microflora of the streams comprised of a mixture of mine drainage and nonacid

water. Similar microflora to that of the streams comprising of a mixture of mine drainage and nonacid water, was not found in a stream that was entirely acid drainage. It was also found that most gram-positive aerobic and anaerobic bacteria died out very rapidly in acidic water, and they comprised a very small percentage of the microbial population of the streams examined. Where mine water entered a stream, iron- and sulphur-oxidizing autotrophic bacteria were present and sulfur-oxidizing bacteria predominated over iron oxidizers.

During studies conducted on sulphate reduction in AMD, Tuttle *et al.* (1969) found that the SRB represented two different types. These were tentatively identified as a *Desulfovibrio* and a *Desulfotomaculum* species. They also isolated ten different yeasts from the ponds and examined them for sugar-fermenting capacity. This was done because of their potential production of alcohols and organic acids which could serve as nutrients for the dissimilatory SRB. Four gram-positive and seven physiological types or groups of gram-negative bacteria were also isolated.

### 2.2.3 Treatment of acid mine drainage

Designed for organic pollution, conventional water treatment techniques are ineffective for treating AMD.



### *Sealing methods*

According to Atlas and Bartha (1993) the best way to deal with the AMD problem is to prevent it at the source. Abandoned subsurface mines can be sealed off to prevent or restrict the availability of oxygen for pyrite oxidation. Prompt reclamation of the land can effectively control AMD in the case of strip mining (Atlas and Bartha, 1993). This involves spreading topsoil over the rubble and establishing a vegetation cover. This technique is also effective on mounds of mine tailings.

### *Broad-spectrum antimicrobial agents*

In theory, AMD can still be curbed if the sealing off of pyretic material from oxygen cannot be accomplished, by suppressing the activity of the iron- and sulphur-oxidising bacteria. However, broad-spectrum antimicrobial agents could be dangerous pollutants themselves and cannot be considered for this purpose (Atlas and Bartha, 1993). Anionic surfactants, benzoic acid, organic acids, alkyl benzene sulfonates, and sodium dodecyl sulphate inhibit iron- and sulphur-oxidising bacteria (Bitton, 1994). Some of these chemicals or their combinations were able to reduce acidic drainage from coal refuse under simulated field conditions. However, the application of these techniques under field condition has not been attempted. The volumes of AMD would however render bactericide treatment too expensive for consideration.

### *Desalination*

Other technologies which are available for the removal of sulphate, include sulphate removal from water by desalination processes such as reverse osmosis and ion exchange (Dill *et al.*, 1994). Chemically, sulphates can be removed by using barium ions, such as barium hydroxide or barium chloride (Maree and Strydom, 1985).

### *SRB treatment methods*

There is an increasing demand for inexpensive, environmental friendly technologies for the removal of sulphates in order to prevent the formation of AMD. SRB may be used in the biological removal of sulphates from AMD. If a suitable electron donor is available, SRB can oxidise organic compounds like lactate or acetate, with sulphate as electron acceptor being reduced to sulphide (Dill *et al.*, 1994). During this reaction, protons are consumed which lead to an increase in pH of the treated water up to a final pH of 7.0 - 7.5. Heavy metals are precipitated by produced  $H_2S$  as virtually insoluble heavy metal sulphides (Dill *et al.*, 1994).

It has been shown that sulphate can be converted quantitatively to  $H_2S$  by *Desulfovibrio desulfuricans* and further conversion to elemental sulphur can be effected by the photosynthetic bacteria *Chlorobium limicola* forma *specialis thiosulfatophilum* and *Chromatium vinosum* (Maree and Strydom, 1985). On the basis of this theory, various configurations of bioreactors were developed for the

removal of sulphate. Success was achieved by using two separate reactors for hydrogen sulphide and sulphur production, respectively (Maree and Strydom, 1985).

According to Atlas and Bartha (1993), Higgins and Burns demonstrated the feasibility of a novel treatment technique for AMD using the activity of *Desulfovibrio* and *Desulfotomaculum*. They combined mine effluent with large amounts of organic waste materials. The activity of aerobic and facultatively anaerobic cellulolytic micro-organisms lowered the redox potential and produced degradation intermediates which could be utilised by SRB. Even though this process restores neutral pH and removes the iron and sulphur from the effluent, the economic and environmental feasibility of the proposed process is yet to be explored.

Tuttle *et al.* (1969) studied the microbial dissimilatory sulphur cycle in acid mine water. They studied water carrying ferric, sulphate, and hydrogen ions produced from pyretic minerals associated with coal as a result of autotrophic bacterial metabolism. The water accumulated behind a porous dam composed of wood dust originating at a log-cutting mill. The water was enriched in organic nutrients as it seeped through the porous dam. This then supported growth and metabolism of heterotrophic bacteria in the water downstream from the dam. Dissimilatory SRB, which reduce sulphate to sulphide was included in the heterotrophic microflora within and below the sawdust dam. Black iron sulphide ( $\text{FeS}$ )

precipitate was deposited on the pond bottom as a result of the reduction of ferric to ferrous ion by sulphide. When they compared the pH of the lower pond water with that of the upper pond water, a net increase was observed. Microbial activity in the wood dust was demonstrated, and a sequence of cellulose degradation processes was inferred on the basis of sugar accumulation in mixed cultures in the laboratory, ultimately yielding fermentation products which serve as nutrients for SRB. They also found that mixed cultures which contained SRB reduced sulphate at pH 3.0 in the laboratory with sawdust as the only carbon source while pure cultures isolated from the mixed cultures did not reduce sulphate below pH 5.5. During their laboratory studies they also found that maximal sulphate reduction occurred in flasks containing partially degraded wood dust and that the rise in pH correlates with the removal of sulphate.

### *Trickling filter reactor*

Du Preez *et al.* (1991) studied the biological sulphate removal from mining effluents in a trickling filter, utilising producer gas (a mixture of  $H_2$ , CO,  $CO_2$  and  $N_2$  generated from coal) as energy source. They concluded that:

- during anaerobic treatment of sulphate rich water in a trickling filter, influent sulphate was reduced from 1900mg/l to less than 200mg/l
- both producer gas and pure carbon monoxide are viable energy sources for the biological sulphate reduction process, which can be used for the treatment of acidic mine effluents.



### *Gas lift reactors*

Biological sulphate reduction using gas-lift reactors fed with hydrogen and carbon dioxide as energy and carbon source, was studied by van Houten *et al.* (1994). It was concluded that when free  $H_2S$  concentrations are kept below 450mg/l, a maximum sulphate conversion rate of  $30g\ SO_4^{2-}/l.d$  can be achieved after only 10 days operation and that the gas-to-liquid hydrogen mass transfer capacity of the reactor determines the maximum sulphate conversion rate.

### *In-situ treatment of water*

In-situ treatment of water is another possibility to avoid the release of AMD in the environment (Dill *et al.*, 1994). In a long term study over a 18 month period, where different organic materials were mixed with dump material and flooded with AMD, an initiation of sulphate reduction in the oxygen free layers of the soil was obtained. After the addition of sugar beet waste water as additional carbon- and energy source, the sulphate reduction carried on over the complete experimental period (Dill *et al.*, 1994).

### *Upflow packed bed reactor*

Maree and Strydom (1985) studied biological sulphate removal in an upflow packed bed reactor. Sulphate removal from mine water was achieved by using either sugar, pulp mill effluent or sewage as energy sources. They optimised environmental conditions necessary for sulphate and sulphur producing bacteria. This was accomplished by providing anaerobic conditions on a solid medium and

by keeping the hydrogen sulphide concentration constantly low, by recirculating the water through a photosynthetic reactor for sulphur production. They concluded that recirculation enhanced sulphate reduction, the presence of light increased sulphur production and that 1.6g sugar, 16.7ml spent liquor from a sulphite pulp mill and 172ml raw sewage sludge are required for the removal of 1800mg sulphate.

It was showed that a three stage process (anaerobic - aerobic - anaerobic) employing upflow packed bed reactors for anaerobic treatment, and an activated sludge system for aerobic treatment, could be used for producing reusable water from mining effluents (Dill *et al.*, 1994). Sulphate was reduced from 2500mg/l to less than 500mg/l with concomitant removal of  $H_2S$ , carbonates, complexed cyanides, phenol and heavy metals. Molasses was used as energy source.

$H_2S$  produced during biological sulphate reduction can be oxidised to elemental sulphur only (and not oxidised totally to sulphate) provided that the oxygen level in the process is kept low (Dill *et al.*, 1994).

## 2.3 The microbiology of the sulphur cycle

### 2.3.1 The sulphur cycle

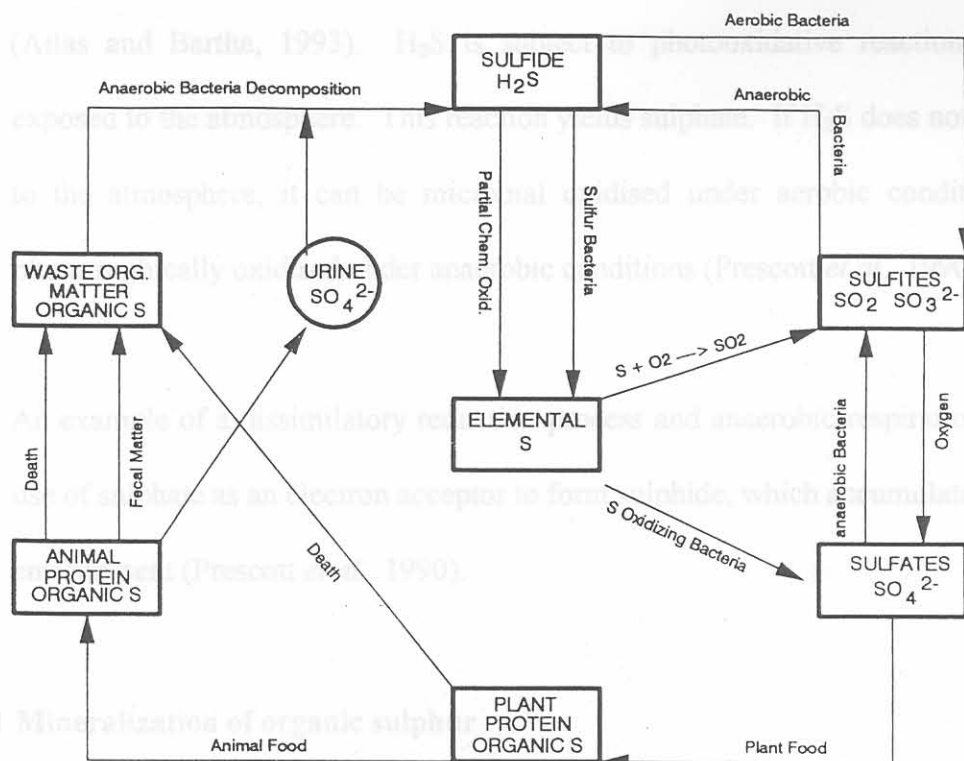


Figure 1. Simplification of the steps involved in the sulphur cycle (Bitton, 1994).

Microorganisms play an important role in the sulphur cycle (Figure 1). Sulphur is transformed by photosynthetic microorganisms by using sulphide as an electron source (Prescott *et al.*, 1990).

Sulphur can also be assimilated in the form of sulphate by plants, algae and many heterotrophic microorganisms (Prescott *et al.*, 1990). Sulphate can be reduced to the sulphide by assimilatory sulphate reduction. This reduction is necessary for

incorporation into cysteine, methionine and coenzymes in the form of sulfhydryl (-SH) groups (Prescott *et al.*, 1990). The toxicity of H<sub>2</sub>S makes the direct uptake of sulphide unfeasible for most microorganisms. The toxicity is avoided by immediately reacting the reduced sulphur with an acceptor, for example FeS (Atlas and Bartha, 1993). H<sub>2</sub>S is subject to photooxidative reactions when exposed to the atmosphere. This reaction yields sulphate. If H<sub>2</sub>S does not escape to the atmosphere, it can be microbial oxidised under aerobic conditions or phototrophically oxidised under anaerobic conditions (Prescott *et al.*, 1990).

An example of a dissimilatory reduction process and anaerobic respiration is the use of sulphate as an electron acceptor to form sulphide, which accumulates in the environment (Prescott *et al.*, 1990).

### 2.3.1.1 Mineralization of organic sulphur

Mineralization is carried out through aerobic and anaerobic pathways by several types of micro-organisms. Under aerobic conditions, sulfatase enzymes are involved in the degradation of sulphate esters to sulphate:



Sulphur-containing amino acids are degraded to inorganic sulphur compounds or to mercaptans under anaerobic conditions (Bitton, 1994).



### 2.3.1.2 Sulphur assimilation

Both oxidised and reduced forms of sulphur can be assimilated by micro-organisms (Bitton, 1994). Reduced forms such as  $H_2S$  are assimilated by anaerobic micro-organisms whereas aerobes utilise the more oxidised forms.

### 2.3.1.3 Sulphur oxidation reactions

According to Bitton (1994) several micro-organisms are involved in sulphur oxidation. He made the following conclusions:

#### 1. $H_2S$ oxidation:

- $H_2S$  is oxidised to elemental sulphur under aerobic and anaerobic conditions
- *Thiobacillus thioparus* oxidises  $S^{2-}$  to  $S^0$  under aerobic conditions.
- Photoautotrophs and a chemoautotroph, *Thiobacillus denitrificans* carry out oxidation under anaerobic conditions.

#### 2. Oxidation of elemental sulphur

- This reaction is mainly carried out by aerobic, gram-negative, non-spore-forming thiobacilli which can grow at very low pH.

#### 3. Sulphur oxidation by heterotrophs:

- In neutral and alkaline soils oxidation of sulphur by heterotrophs can occur.

### ***Sulphur -oxidizing bacteria***

Sulphur-oxidising bacteria are chemolithotrophs (Prescott *et al.*, 1990).

*Thiobacillus* is the best studied example (Prescott *et al.*, 1990). Sulphur –

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oxidising bacteria oxidises sulphur,  $H_2S$ , thiosulphate and other reduced sulphur compounds to sulphuric acid. Both oxidative phosphorylation and substrate-level phosphorylation involving adenosine-5'-phosphosulphate (APS) generate ATP (Prescott *et al.*, 1990). Sulphur-oxidising bacteria can use carbon dioxide as carbon source. However, many will grow heterotrophically when they are supplied with reduced organic carbon sources like glucose or amino acids (Prescott *et al.*, 1990). A few other species can grow aerobically as sulphur-oxidising bacteria. These bacteria carry out anaerobic respiration with molecular sulphur as an electron acceptor (Prescott *et al.*, 1990).

*Thiobacillus* can also oxidise  $H_2S$  and other reduced sulphur compounds (Atlas and Bartha, 1993). Due to their low acid tolerance, the deposit elemental sulphur rather than generating sulphuric acid by further oxidation. Other members of this genus produce sulphate from the oxidation of elemental sulphur and other inorganic sulphur compounds (Atlas and Bartha, 1993). Photosynthetic sulphur bacteria, the *Chromatiaceae*, *Ectothiorhodospiraceae*, and *Chlorobiaceae*, are capable of photoreducing carbon dioxide while oxidising  $H_2S$  to elemental sulphur (Atlas and Bartha, 1993).  $H_2S$  can be oxidised by microaerophilic bacteria for example *Beggiatoa*, *Thioploca* and *Thiotrix* (Atlas and Bartha, 1993).

Purple sulphur bacteria as well as green sulphur bacteria, also play a role in the sulphur cycle. Purple sulphur bacteria oxidize hydrogen sulphide to sulphur and deposit it internally as sulphur granules (Prescott *et al.*, 1990). Green sulphur

bacteria are also able to oxidize hydrogen sulphide to sulphur, but it is deposited outside the cell (Prescott *et al.*, 1990).

#### 2.3.1.4 Sulphate reduction

Bitton (1994) made the following statements about sulphate reduction:

##### 1. *Assimilatory sulphate reduction:*

- The anaerobic decomposition of organic matter containing sulphur amino acids such as methionine, cysteine, and cystine by proteolytic bacteria may result in H<sub>2</sub>S production.

##### 2. *Dissimilatory sulphate reduction:*

- SRB (strict anaerobes) are responsible for this reaction:



- Sulphate is used as terminal electron acceptor in the absence of oxygen and nitrite.

#### *Sulphate reducing bacteria*

SRB are an ubiquitous group of micro-organisms. They share an ability to couple the reduction of sulphate and other sulphur compounds to the oxidation of a variety of electron donors (De Bruyn, 1992). SRB are all strict anaerobes. It is insufficient to only exclude oxygen from culture medium when growing pure cultures of SRB. Redox-poising agents are generally required to maintain a redox potential of -150 to -200mV in the medium (Dasu *et al.*, 1993). Some are known to be capable of fermentative growth in the absence of sulphate, analogous to the

fermentative growth of a yeast without oxygen, but none can grow with oxygen as electron acceptor, and oxygen always inhibits their growth (Postgate, 1979). These microbes are responsible for dissimilatory sulphate reduction. During dissimilatory sulphate reduction, sulphate acts as an oxidising agent for the assimilation of organic matter. Transport of exogenous sulphate across the bacterial membrane into the cell is the initial step in the biochemical sulphate-reduction pathway (De Bruyn, 1992). Once inside the cell, sulphate dissimilation proceeds by the action of adenosine tri-phosphate (ATP) sulphurylase which combines sulphate with ATP to produce the highly activated molecule adenosine phosphosulphate (APS), as well as pyrophosphate. The cytoplasmic enzyme APS reductase rapidly converts APS to sulphite, which can further be reduced via a variety of intermediates to form the sulphide ion. Virtually all of the reduced sulphur is released into the external environment as the sulphide ion, usually substantially hydrolysed to free  $H_2S$  (Postgate, 1979).

SRB plays a significant role in anaerobic digestion of complex substrates. It was suggested that SRB:

- generate sulphides that may result in product inhibition of SRB and/or toxicity to methane producing bacteria
- change the reactor pH via generation of alkalinity in the conversion of sulphate to sulphide.
- accelerate the oxidation of organics, such as lactate, which are normally degraded at a lower rate by incomplete oxidising non-SRB



- reduce the rate of methanogenesis
- decrease the quantity of methane produced by competing for the available carbon and/or hydrogen (McCartney & Oleszkiewicz, 1991).

A further special nutritional feature of species of SRB is the ability to grow with reduced organic compounds that cannot be utilised in pure cultures of fermentative bacteria (Zehnder, 1988). These compounds include: propionate, butyrate, higher fatty acids or phenyl-substituted organic acids. SRB can exploit the reduced products as energy sources by using an external electron acceptor.

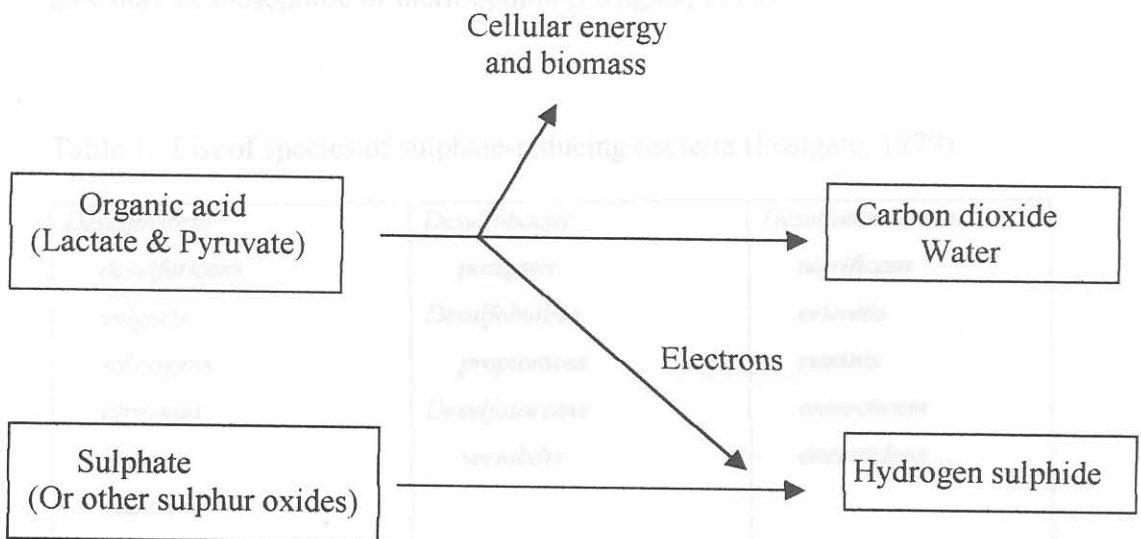


Figure 2. Generalised schematic of the physiology of sulphate reducing bacteria (Apel *et al.*, 1992).

### Classification of sulphate reducing bacteria

Accordingly to Postgate (1979), the taxonomy of SRB is in an unsatisfactory state, having become confused in the 1920s to 1940s by the prevalence of impure cultures and the use of inappropriate culture media. Three genera of SRB exists namely *Desulfovibrio*, *Desulfotomaculum* and *Desulfomonas* (Postgate, 1979). The first two genera seem to be quite unrelated to each other, but the third genus *Desulfomonas*, is very like *Desulfovibrio*.

*Desulfovibrio* is usually easier to isolate and purify (Postgate, 1979). *Desulfovibrio* is mesophilic but can be halophilic and they do not form spores. Naturally-occurring halophilic strains of *Desulfotomaculum* are not known, but they may be mesophilic or thermophilic (Postgate, 1979).

Table 1. List of species of sulphate-reducing bacteria (Postgate, 1979).

<i>Desulfovibrio</i>	<i>Desulfobacter</i>	<i>Desulfotomaculum</i>
<i>desulfuricans</i>	<i>postgatei</i>	<i>nigrificans</i>
<i>vulgaris</i>	<i>Desulfobulbus</i>	<i>orientis</i>
<i>salexigens</i>	<i>propionicus</i>	<i>ruminis</i>
<i>africanus</i>	<i>Desulfosarcina</i>	<i>antarcticum</i>
<i>gigas</i>	<i>variabilis</i>	<i>acetoxidans</i>
<i>baculatus</i>		
<i>sapovorans</i>		
<i>baarsii</i>		
<i>thermophilus</i>		

Several strains of oxygen-tolerant sulphate reducing bacteria are known (Cohen, 1993). Those resistant to high oxygen levels require hydrogen for growth. Other strains are seemingly facultative sulphate reducing bacteria, namely they may switch from heterotrophic aerobic growth to anaerobic sulphate reduction mode (Cohen, 1993). Another group of isolated sulphate reducing bacteria are oxygen sensitive when grown in axenic culture and can cope with oxygen only when grown in co-culture with thiobacilli (Cohen, 1993).

SRB classified according to oxygen sensitivity:

- Oxygen-sensitive strains capable to function under oxygen only when grown in co-culture with oxygen scavenging bacteria such as thiobacilli.
- Oxygen-insensitive sulphate reducing bacteria capable of carrying out sulphate reduction activity under oxygen by elevated hydrogenase activity, which protects the oxygen-sensitive sites in the oxygen-sensitive sites in the organism.
- Facultative sulphate reducing bacteria carrying out aerobic respiration under oxygen conditions and then shifting to sulphate reduction when exposed to anaerobic conditions. Among the metabolically related sulphur-reducing bacteria, certain types are facultatively aerobic or microaerobic; the archaeal lithoautotrophic *Acidianus* can even switch between sulphur reduction and aerobic sulphur oxidation (Cohen, 1993).

### *Ecology of sulphate reducing bacteria*

SRB are widespread and active in locations made anaerobic by microbial digestion of organic material and are present in almost all aquatic and terrestrial habitats (Prescott *et al.*, 1990). They thrive in habitats such as muds and sediments of polluted lakes and streams, sewage lagoons and digesters, as well as in waterlogged soils. They have a remarkable capacity for survival in terrestrial and aquatic environments even though they grow relatively slowly compared with a common soil or water organism (Postgate, 1979). Although these bacteria are strictly anaerobic, they have been detected in many ostensibly aerobic regions (De Bruyn, 1992).

### *Growth and isolation of sulphate reducing bacteria*

- Isolation of sulphate reducing bacteria

SRB is present in most soils and waters, but is often outnumbered by other types of microbes (Postgate, 1979). Enrichment of the SRB population is usually necessary before isolation is attempted. According to De Bruyn (1992), various media and modifications of these media are available for the detection and isolation of SRB. Sodium lactate is normally used as carbon source while ferrous salt is used as an indicator of sulphide production. These media also contain redox poisoning agents and yeast extract. A pH between 7.2 and 7.6 is required for the growth of SRB, but the optimum temperature is SRB-species dependent (De Bruyn, 1992).



- Incubation conditions

Due to the fact that SRB are strict anaerobes, handling and cultivation require techniques to effectively remove oxygen from both the medium and the gas phase in contact with the medium as well as lowering the redox potential. A negative redox potential of -100mV (Eh) is recommended for successful growth of SRB (De Bruyn, 1992). Ascorbate, cysteine hydrochloride, dithiothreitol and titanium(III)citrate can be used as reducing agent (De Bruyn, 1992). Preparation of the reducing agents must be done anaerobically because they may react with oxygen to form toxic substances. Agar plates can be incubated in the conventional manner using an anaerobic cabinet with an anaerobic atmosphere (De Bruyn, 1992).

## 2.4 Substrate utilisation of SRB

SRB may be involved in fatty acid turnover either by direct metabolism of fatty acids, or indirectly because of their importance as  $H_2$  -scavengers (Banat and Nedwell, 1982). Figure 3 gives a schematic diagram illustrating the possible substrate utilisation and metabolite formation by SRB in the presence of exogenous sulphurous electron acceptors.

When divided solely on the basis of substrate utilisation, most species of the genera *Desulfovibrio*, *Desulfotomaculum* and *Desulfomonas* can be described as predominantly “fermentative” (De Bruyn, 1992). The remaining species of the “fermentative” genera, with the exception of *Desulfovibrio baarsii* and

*Desulfotomaculum acetoxidans*, are lactate-utilising bacteria which incompletely oxidise substrates to acetate and hydrogen sulphide (De Bruyn, 1992). All incomplete substrate oxidations result in the formation of acetate. The only way that dissimilatory sulphate-reduction will be significant as a terminal oxidation process will be if acetate can be oxidised in the course of sulphate reduction (Joubert, 1987).

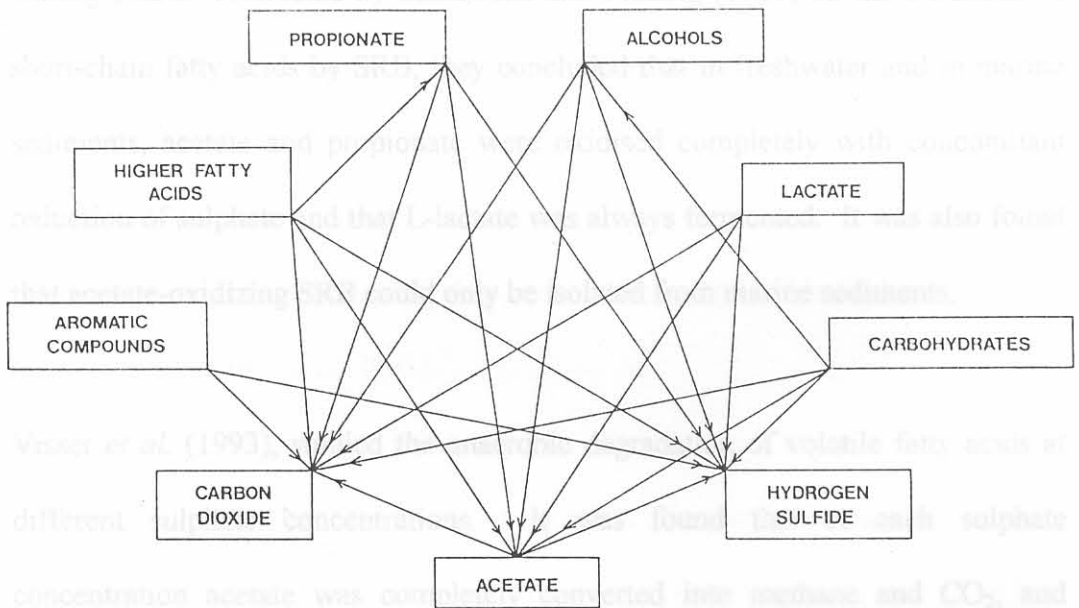


Figure 3. Metabolic formation by sulphate-reducers in the presence of exogenous sulfurous electron acceptors (Joubert, 1987).

### 2.4.1 Utilisation of acetate

The first real confirmation that the SRB were able to anaerobically oxidise acetate, was reported on the non-sporing sulphur reducing *Desulfuromonas acetoxidans* species (Joubert, 1987). *Desulfobacter* species are nutritionally very specialised sulphate reducers and show the best growth on acetate (Zehnder, 1988).

The oxidation of acetate in *Desulfobacter postgatei* was shown to occur via the citric acid cycle (Zehnder, 1988):



During studies conducted by Laanbroek and Pfennig (1981) on the oxidation of short-chain fatty acids by SRB, they concluded that in freshwater and in marine sediments, acetate and propionate were oxidised completely with concomitant reduction of sulphate and that L-lactate was always fermented. It was also found that acetate-oxidizing SRB could only be isolated from marine sediments.

Visser *et al.* (1993), studied the anaerobic degradation of volatile fatty acids at different sulphate concentrations. It was found that at each sulphate concentration acetate was completely converted into methane and  $\text{CO}_2$ , and acetotrophic SRB was not detected. Some reports show a predominance of SRB growing on acetate while a number of reports mention complete conversion of acetate by methanogens, even at high sulphate concentrations. During these

studies it was evident that acetate is mainly consumed by methanogens, thus acetotrophic methanogens can very effectively compete with acetate-degrading SRB. Acetate degradation is independent of sulphate reduction (Qatibi *et al.*, 1990).

According to Ahring and Westermann (1987), acetate, energetically, is a poor substrate. This might result in the energy required for acetate uptake at very low concentrations exceeding the energy gained from acetate metabolism, thereby limiting the acetate uptake at a certain threshold concentration. During the studies they conducted, they found when the concentration of acetate was reduced to less than 0.5 to 0.7mM, the degradation rate was concentration dependent, whereas the degradation was slow when the concentration was less than 0.15mM.

#### 2.4.2 Utilisation of lactate

Lactate is a common intermediate during anaerobic degradation of complex organic matter and can be further degraded under anaerobic conditions via several pathways (Sørensen *et al.*, 1991).

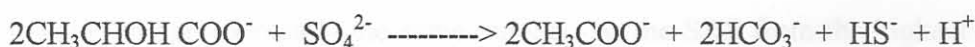
These include:

- formation of acetate, hydrogen and carbon dioxide, requiring a hydrogen-utilising methanogen as hydrogen sink
- formation of carbon dioxide or acetate by simultaneous reduction of oxidised sulphur compounds to carbon dioxide
- formation of propionate, acetate and carbon dioxide



- reduction of oxaloacetate to form acetate, formate and succinate
- fermentation to acetate, propionate and hydrogen, independent of the activity of hydrogen-utilizing bacteria (Sørensen *et al.*, 1991).

Lactate has been used as an excellent organic substrate for enrichment, isolation, and cultivation, or for determining cell numbers of *Desulfovibrio* and *Desulfotomaculum* species (Zehnder, 1988). Lactate is also oxidised by several completely oxidising sulphate reducers, while a number of incompletely fatty acid-oxidising sulphate reducers are unable to use lactate. The equations of incomplete and complete lactate oxidation are as follows (Zehnder, 1988):



$$\Delta G^{0'} = -160.1\text{kJ}$$



$$\Delta G^{0'} = -255.3\text{kJ}$$

In the presence of sulphate, lactate, is generally accepted to support growth of the SRB(Joubert, 1987). Thermodynamic feasibility studies have shown that lactate will only sustain growth when it is incompletely oxidised to acetate according to the equation (Joubert, 1987):



Oxidisation of lactate, via pyruvate by SRB has also been shown (Joubert, 1987).

Apart from acetate and carbon dioxide as major products, small amounts of

gaseous hydrogen may also be formed through the mediation of reversible hydrogenase.

In 1981, Laanbroek and Pfennig studied the anaerobic mineralization of L-lactate in the presence and in the absence of sulphate. They found that lactate was always fermented and that lactate oxidising, sulphate reducing bacteria, belonging to the species *Desulfovibrio desulfuricans*, and lactate-fermenting bacteria were found in approximately equal amounts in the sediments. The fact that fermentation of L-lactate occurred in the presence as well as in the absence of added sulphate was rather unexpected, although fermentative bacteria were isolated in approximately the same numbers as the SRB from the highest diluted agar shake tubes. Hydrogen or formate might be more important electron donors for the reduction of sulphate by *Desulfovibrio* under natural conditions and lactate may not be the primary substrate for sulphate reduction (Laanbroek and Pfennig, 1987).

Qatibi *et al.* (1990) studied the effects of sulphate on anaerobic lactate degradation by a mixed microbial culture from an anaerobic fermenter fed with wine distillery waste water. They found that without sulphate and with both sulphate and molybdate (inhibits sulphate reduction), lactate was rapidly consumed and propionate and acetate were produced; whereas with sulphate alone, only acetate accumulated. No sulphate was utilised in the presence of molybdate which indicated that lactate degradation was largely due to

fermentation. In the presence of sulphate, lactate degradation was accompanied by a concomitant consumption of the electron acceptor. It was also found that methane was not affected by the presence of sulphate, although less methane was produced in the presence of molybdate.

#### 2.4.3 Utilisation of pyruvate

Even though pyruvate is a major intermediate during lactate metabolism, utilisation of this intermediate plays a central role in regulating the fermentation products of the SRB (Joubert, 1987). Oxidation of pyruvate, both in the presence and absence of sulphate, can be carried out by most of the “fermentative” SRB. Pyruvate is dismutated mainly to acetyl phosphate, carbon dioxide and hydrogen in the absence of sulphate (Joubert, 1987).

#### 2.4.4 Utilisation of hydrogen and formate

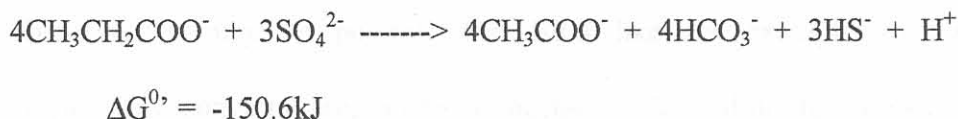
According to Zehnder (1988), incompletely oxidising sulphate reducers using lactate are usually able to grow just as well with hydrogen as electron donor and accordingly, *Desulfovibrio* strains isolated from natural sources with hydrogen were all able to grow on lactate. Many SRB using hydrogen, may grow with formate but there are a few species that only use either hydrogen or formate. The first hint from nutrition physiology that SRB could conserve energy solely by electron transport phosphorylation was the utilisation of hydrogen by *Desulfovibrio* species (Zehnder, 1988). Several completely oxidising SRB that may grow autotrophically may use hydrogen but with relatively slow growth.

#### 2.4.5 Utilisation of propionate, butyrate and higher fatty-acids

Next to acetate, propionate was shown to be quantitatively the most important product in the fermentation of organic materials by natural populations of bacteria (Widdel and Pfenning, 1982).

In the anaerobic conversion of organic matter to methane and carbon dioxide volatile fatty acids are important intermediates (Ahring and Westerman, 1987). Normally, 20 % of the total methane produced in a digester is accounted for by propionate and butyrate.

One characteristic feature of *Desulfobulbus* species is the incomplete oxidation of propionate to acetate (Zhender, 1988):



In 1993, Visser *et al.* found that at higher sulphate concentrations, oxidation of propionate by SRB became more important and only under sulphate-limiting conditions did syntrophic propionate oxidisers out-compete propionate-degradating sulphate reducers. Remarkably, syntrophic butyrate oxidisers were well able to compete with SRB for the available butyrate, even with an excess of sulphate.



Two groups of bacteria are involved in the degradation of propionate, butyrate and longer-chain fatty acids (Ahring and Westermann, 1987). These are the obligately hydrogen-producing acetogenic bacteria oxidising the acids and the methane-producing bacteria utilising the acetate and hydrogen produced.

According to Zhender (1988), *Desulfobulbus* species isolated so far are a physiologically homogeneous group, growth with lactate may be somewhat faster than with propionate, but only the latter allows selective enrichment.

Propionate is also removed under anaerobic conditions in sludge from a sulphide removing reactor (Widdel and Pfennig, 1982). No fresh water sulphur-reducing bacteria are known which are able to use propionate (Widdel and Pfennig, 1982). Therefore, it may be possible that SRB like for example *Desulfobulbus propionicus* converts propionate to acetate. According to Zehnder (1988), incomplete oxidation of propionate to acetate is a characteristic feature of *Desulfobulbus* species. Nearly all *Desulfobulbus* isolates described use ethanol, propanol and a few strains slowly degrade butyrate or 2-methylbutyrate to acetate. Methanogenic and sulphate reducing conditions lead to at least 13 possible pathways for conversion of propionate (Speece, 1996). Speece (1996), concluded that since propionate and butyrate were barely detectable in effluents at steady-state, their oxidation under high influent sulphate conditions may be completely or incompletely mediated by the fatty acid-utilising SRB. It was also shown that

the presence of SRB enhanced the degradation of propionate either through direct utilisation or through interspecies  $H_2$  transfer.

Stams *et al.* (1984) studied the pathway of propionate formation in *Desulfobulbus propionicus*. The most remarkable characteristic is the ability to form as well as to degrade propionate. In the presence of sulphate, lactate, pyruvate, ethanol, propanol and propionate, are oxidised to acetate with a concomitant reduction of sulphate to sulphide. In the absence of an external electron acceptor, lactate and pyruvate are fermented to propionate and acetate.

During studies conducted by Widdel and Pfennig in 1982, it was shown that propionate can be oxidised by SRB without dependence on syntrophic bacteria. The new type of oval to lemon shaped SRB was able to completely oxidise propionate under anaerobic conditions when grown together with acetate-oxidising species. Such commensalism was observed in the marine enrichments with propionate where in addition to the small oval propionate-utilising SRB, larger cells of the *Desulfobacter* type were observed.

In 1983, Banat and Nedwell studied the mechanisms of turnover of  $C_2$ - $C_4$  fatty acids in high-sulphate and low-sulphate anaerobic sediments. They found that the anaerobic oxidation of propionate and butyrate could be due to two possible mechanisms: fatty acids can be oxidised by proton reducing bacteria in association with a  $H_2$ -scavenger, or SRB may be capable of direct metabolism and

oxidation of propionate and butyrate in a high-sulphate sediment. The addition of 20mM molybdate to slurry of saltmarsh sediments, almost entirely eliminated propionate and butyrate turnover. This suggested that the SRB were involved in their metabolism and indeed were entirely responsible for their oxidation. Molybdate did not have any apparent effect on fatty acid turnover in freshwater sediments. The presence of a  $H_2$  atmosphere had no effect on propionate or butyrate turnover in the high sulphate saltmarsh sediments, suggesting that their oxidation in these slurries did not involve proton-reduction mechanisms, and inter-species  $H_2$  transfer, which would be inhibited by a high  $H_2$  concentration. The metabolism of propionate and butyrate metabolism must have been due to the SRB which directly metabolise fatty acids and whose metabolism would not be inhibited by hydrogen. Butyrate turnover was clearly inhibited by the  $H_2$  atmosphere in freshwater sediments. Although propionate turnover was extremely slow, the  $H_2$  atmosphere seemed to further inhibit its turnover. In low sulphate freshwater sediment, it was suggested that, propionate and butyrate oxidation involves proton-reducing fatty acid oxidising bacteria linked to  $H_2$ -scavenging bacteria, probably methanogens. In high-sulphate sediments, it seemed that direct oxidation of fatty acids by SRB predominates and fatty acid oxidation involving interspecies  $H_2$  transfer is of minor importance.

## 2.5 Competition between sulphate reducing bacteria and methanogens

Both sulphate reducers and methanogens (MPB) live under strict anaerobic conditions with similar pH and temperature ranges (Bitton, 1994). Some SRB are able to oxidise  $H_2$  like methanogens and thus may compete with methanogens for these substrates (Figure 4) (Bitton, 1994). SRB are normally dominant in natural ecosystems such as freshwater and marine sediments and also in anaerobic digesters where methanogenesis was found to be inhibited by the presence of sulphate (Isa *et al.*, 1986). The actual description of the MPB/SRB competition within a reactor is complex.

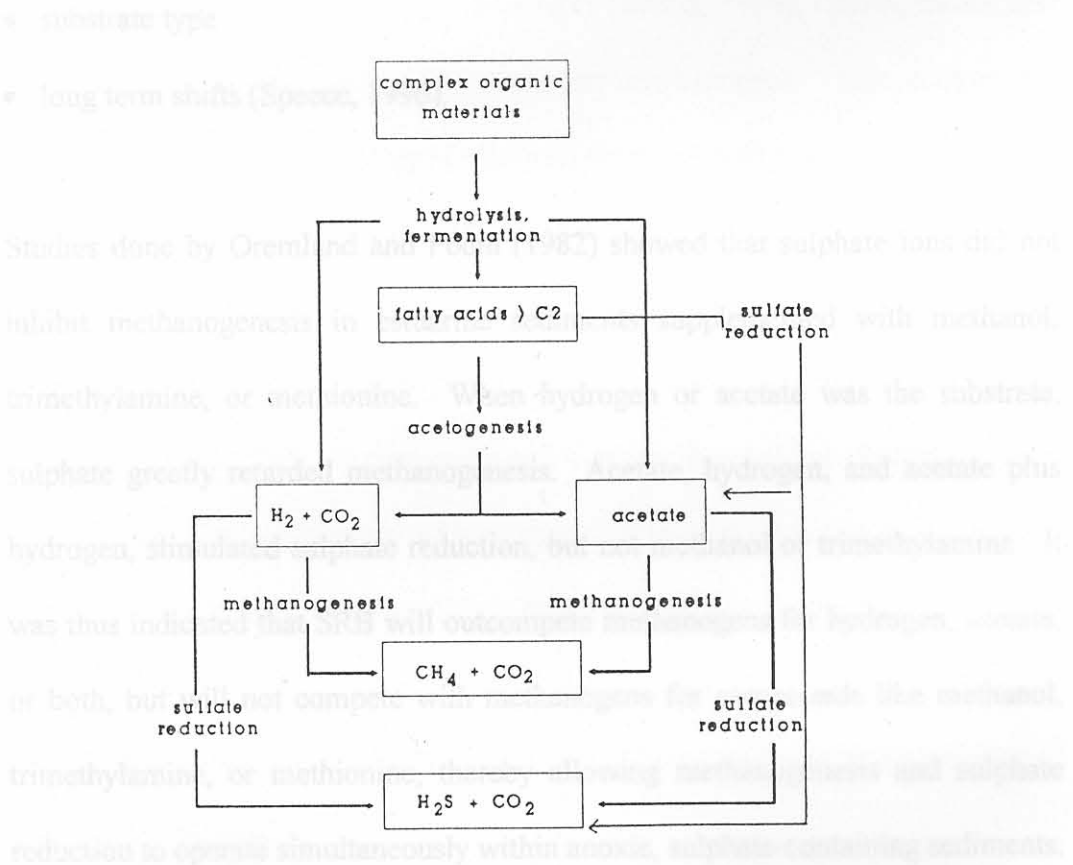


Figure 4. Substrate competition between sulphate-reducing and methanogenic or acetogenic bacteria (Bitton, 1994).



Thus, factors influencing MPB/SRB competition are:

- sulphate concentration in feed
- maximum specific utilising rate ( $k_{\max}$ )
- half velocity constant ( $k_s$ )
- free energy of the reaction
- nutrient availability
- adhesion properties
- proximity of cells (biofilms vs. dispersed cells)
- temperature
- substrate type
- long term shifts (Speece, 1996).

Studies done by Oremland and Pociu (1982) showed that sulphate ions did not inhibit methanogenesis in estuarine sediments supplemented with methanol, trimethylamine, or methionine. When hydrogen or acetate was the substrate, sulphate greatly retarded methanogenesis. Acetate, hydrogen, and acetate plus hydrogen, stimulated sulphate reduction, but not methanol or trimethylamine. It was thus indicated that SRB will outcompete methanogens for hydrogen, acetate, or both, but will not compete with methanogens for compounds like methanol, trimethylamine, or methionine, thereby allowing methanogenesis and sulphate reduction to operate simultaneously within anoxic, sulphate-containing sediments.

It was shown by kinetic studies that sulphate reducers generally have higher maximum growth rates and higher affinity for substrates (i.e. lower half-saturation constants,  $K_s$ ) than methanogens (Bitton, 1994). The half-saturation constant for hydrogen is  $6,6\mu\text{M}$  for methanogens, as compared with  $1,3\mu\text{M}$  for sulphate reducers. Similarly, the  $K_s$  values for acetate are mM and  $0,2\text{mM}$  for methanogens and sulphate reducers, respectively (Bitton, 1994). Thus, sulphate reducers may predominate over methanogens, providing that the sulphate supply is not limiting. Despite their kinetic advantages, SRB rarely predominate in anaerobic wastewater treatment. SRB have a higher affinity for acetate ( $K_s = 9,5\text{mg/l}$ ) than methanogens ( $K_s = 32,8\text{mg/l}$ ) (Bitton, 1994). Thus, under low acetate concentrations, SRB will outcompete methanogens. This competitive inhibition results in the shunting of electrons from methane generation to sulphate reduction. SRB and MPB are very competitive at COD/SO<sub>4</sub> ratios of 1,7-2,7 (Bitton, 1994). A decrease in this ratio is favourable to SRB, whereas an increase is favourable to MPB. Little is known about the competition between acetogenic bacteria (AB) and SRB for propionate and butyrate in anaerobic digestors (Visser *et al.*, 1993). For waste-water with an excess of sulphate it is assumed that SRB will out-compete MPB because of their better growth kinetic properties.

Accordingly to Speech (1996), the following has been published about acetate and SRB/MPB competition:

- Acetate a favoured substrate for MPB
- MPB predominant over SRB for acetate

- MPB generally solely present in low alkalinity anaerobic reactors
- MPB able to form a biofilm faster than SRB at higher acetate concentrations
- MPB primary acetate converter at high acetate/sulphate ratios
- MPB outcompeting at higher acetate concentrations over several months, but SRB predominant with low acetate concentrations in biofilms during the same period
- MPB using 60% COD and SRB 40% for acetate
- MPB using 93-97% of acetate substrate at COD/sulphate ratios of 1-50
- MPB acetate utilising clearly less with increasing sulphate concentrations.

In anaerobic microbial systems acetate would be completely oxidised to carbon dioxide rather than to methane and carbon dioxide in the presence of sulphate even if acetate proves to be the major energy source for the bacteria that use it in methane production (Bryant *et al.*, 1977).

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# *A novel anaerobic bioreactor for laboratory scale experiments*

(Provisional patent applied for)

## *J. L. van der Merwe*

The following is a preliminary report on the design and construction of a novel anaerobic bioreactor. Such a reactor is required for the study of strictly anaerobic microorganisms. The design of such a reactor is a complex task and the design of a reactor for laboratory scale experiments has however been reported by Zehnder (1988). The design of a reactor for laboratory scale experiments is a complex task and the design of a reactor for laboratory scale experiments has however been reported by Zehnder (1988).

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Strictly anaerobic bacteria (SRB) are strictly anaerobic microorganisms. During the growth of SRB, oxygen must be excluded from the system (Zehnder *et al.*, 1990). This is the main reason for the design of a reactor for laboratory scale experiments.

The design of a reactor for laboratory scale experiments is a complex task and the design of a reactor for laboratory scale experiments has however been reported by Zehnder (1988). The design of a reactor for laboratory scale experiments is a complex task and the design of a reactor for laboratory scale experiments has however been reported by Zehnder (1988).

## CHAPTER 3

### A novel anaerobic bioreactor for laboratory scale experiments

(Provisional patent applied for)

#### 3.1. Introduction

The study of anaerobic bacteria has intensified tremendously over the past years. Such microorganisms, in addition to being of clinical interest, have considerable ecological importance as well as potential use in industrial processes. Experimentation has however been hampered by the lack of inexpensive, readily available equipment.

Currently a variety of techniques and equipment are used for anaerobic studies. These include custom made bioreactors, anaerobic cabinets, Gaspak systems (Figure 1) and a variety of other devices. Professionally manufactured anaerobic bioreactors are prohibitively expensive and also expensive to operate.

Sulphate reducing bacteria (SRB) are strictly anaerobic microorganisms. During anaerobic culturing, all oxygen must be excluded from the system (Prescott *et al.*, 1990).

This can be accomplished by:

- Using special anaerobic media containing reducing agents such as thioglycollate or cysteine. The reducing agents will eliminate any dissolved oxygen present within the medium so that anaerobes can grow beneath the surface.

- Oxygen may also be eliminated by removing air with a vacuum pump and flushing out residual oxygen with nitrogen gas. Often  $\text{CO}_2$  as well as nitrogen is added to the chamber since many anaerobes require a small amount of  $\text{CO}_2$  for best growth (Figure 2) (Prescott *et al.*, 1990).

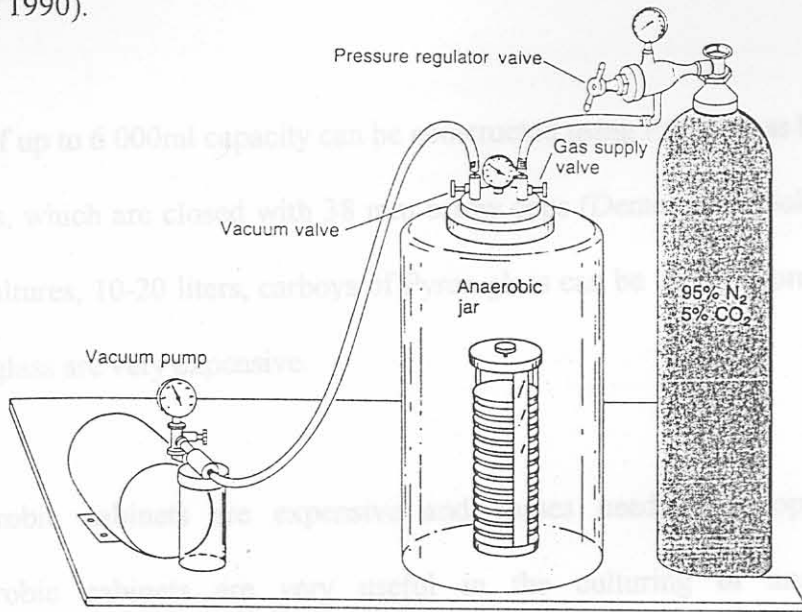


Figure 1. Vacuum and gas displacement method of anaerobic culture. The anaerobic jar is evacuated at least three times and refilled each time with nitrogen or a nitrogen-carbon-dioxide mixture (Prescott *et al.*, 1990).

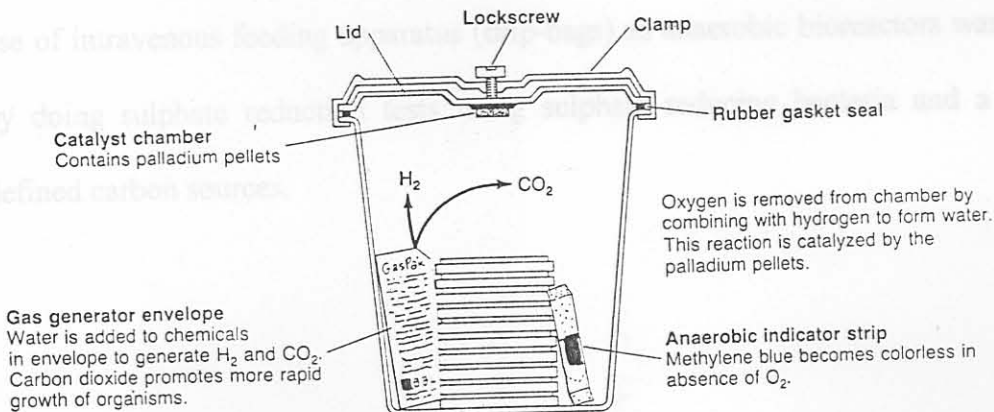


Figure 2. The GasPak anaerobic system (Prescott *et al.*, 1990).

One of the most popular ways of culturing small numbers of anaerobes is through the use of a GasPak jar (Figure 2) (Prescott *et al.*, 1990). In this procedure, the environment is made anaerobic by using hydrogen and a palladium catalyst to remove oxygen through the formation of water.

Cultivation vessels of up to 6 000ml capacity can be constructed using Pyrex glass bottles or Erlenmeyer flasks, which are closed with 38 mm screw caps (Demain and Solomon, 1986). For larger cultures, 10-20 liters, carboys of Pyrex glass can be used. Bioreactors manufactured from glass are very expensive.

Conventional anaerobic cabinets are expensive and gasses needed for operation expensive. Anaerobic cabinets are very useful in the culturing of anaerobic microorganisms but not practical for the use as anaerobic bioreactors.

From the above it is clear that there is a lack of inexpensive, anaerobic bioreactors, especially suited for training purposes and laboratory experimentation. In this study, the use of intravenous feeding apparatus (drip-bags) as anaerobic bioreactors was evaluated by doing sulphate reduction tests using sulphate reducing bacteria and a variety of defined carbon sources.

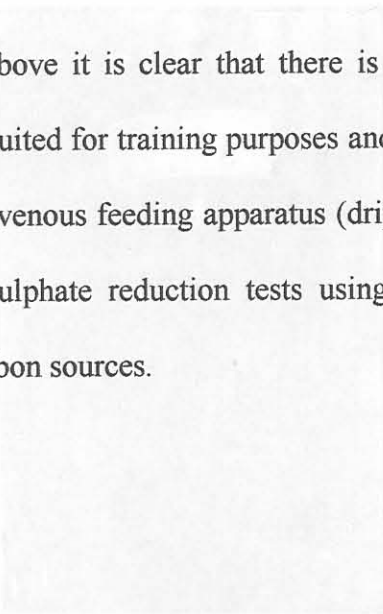


Figure 4. The anaerobic bioreactor (the expansion of the bag indicates gas production)



### 3.2 Materials and methods

#### *Fermentor design*

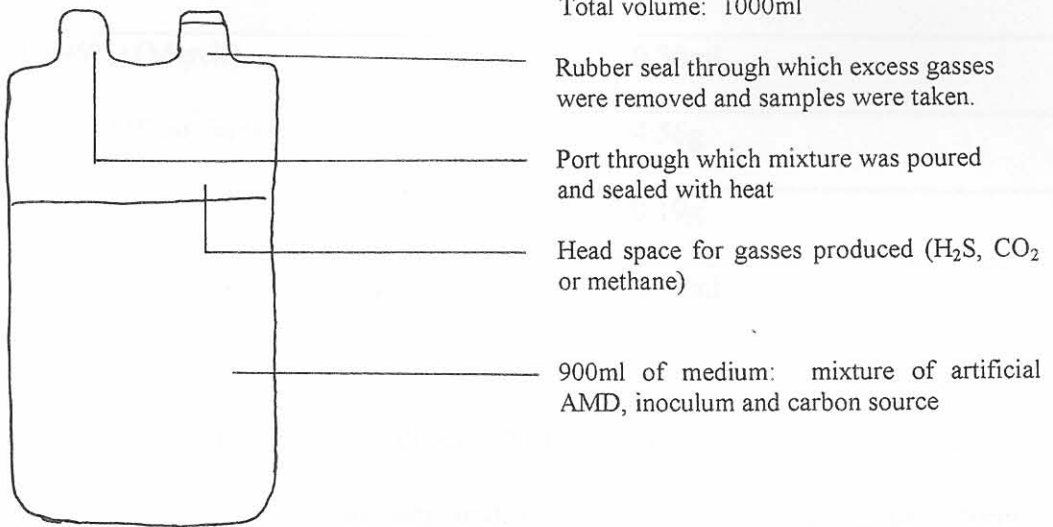


Figure 3. A schematic representation of the anaerobic bioreactor



Figure 4. The anaerobic bioreactor (the expansion of the bag indicates gas production).

### *Media composition*

Table 1. Artificial acid mine drainage (AMD) composition.

Component	Quantity
MgSO <sub>4</sub> .7H <sub>2</sub> O (Merck)	1.31g
H <sub>2</sub> SO <sub>4</sub> (96%) (Merck)	0.30ml
FeSO <sub>4</sub> .7H <sub>2</sub> O (Saarchem)	4.56g
NH <sub>4</sub> Cl (Labchem)	0.19g
H <sub>2</sub> PO <sub>4</sub> (85%)(Merck)	0.02ml

The above chemicals (Table 1) were dissolved in one litre of distilled water. The pH was adjusted to 7.2 using 10M Sodium hydroxide (Merck). By using this formula, theoretically, an average sulphate concentration of 2 500mg/L should be achieved. The SQ118 spectroquant (Merck) (kit no. 1.14791.00010) was used to determine the sulphate concentration of the AMD.

### *Standardization of the inoculum*

In order to find the most suitable concentration of inoculum (digester sludge), the following experiment was done. Mixtures of 1 litre AMD (Table 1) and 5.56ml lactic acid (92%) were prepared. The pH was adjusted to 7.2-7.5 before the digester sludge obtained from the Daspoort Water Purification Plant in Pretoria, South Africa was added. Volumes of 100ml, 200ml and 300ml of inoculum were added to the AMD and lactic acid mixtures, respectively.

### Chemical analysis

A SQ118 spectroquant (Merck) was used to determine the amount of sulphate reduction using kit no. 1.14791.0001.

### Preliminary sulphate reduction tests on AMD

A volume of 300ml inoculum was added to 1000ml of artificial AMD (Table 1). The respective carbon source was also added to this mixture (Table 2). Of this, 900ml were added to the drip-bags, respectively, which were used as bioreactors. The pH was adjusted to 7.1-7.5.

Table 2. Defined carbon sources used during this experiment

Carbon sources	Quantity per 100ml AMD
Lactic acid (92%) (Merck)	5.56ml
Acetic acid (Merck)	5.95ml
Butyric acid (BDH)	4.80ml

All experiments were done in triplicate. The sulphate concentration of the different bioreactors were monitored every 2-3d. As a control experiment, only AMD and inoculum was used with no added carbon sources.

### 3.3 Results and Discussion

#### *Media composition*

The average sulphate concentration of the artificial AMD was 2356mg/l. This resembles the sulphate concentration of AMD.

#### *Standardization of the inoculum*

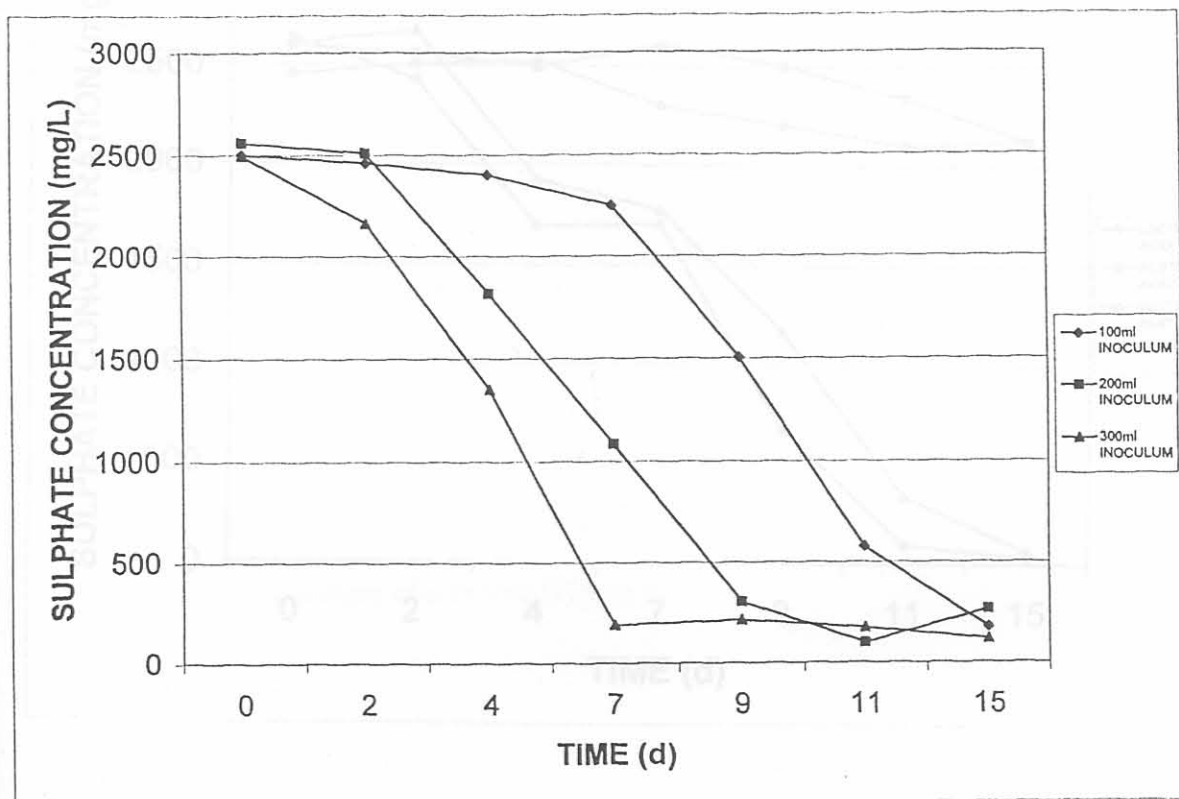


Figure 5. Sulphate reduction using 100ml, 200ml and 300ml of inoculum respectively.

Digester sludge consists mostly of fermentative bacteria and have low numbers of SRB (Zhender, 1988). The 300ml inoculum proved to be the most suitable concentration of



inoculum (Figure 5). A larger volume of inoculum would be impractical, due to the 1000ml limit of the bioreactor. However, this did not pose a problem, since effective sulphate reduction was obtained using 300ml inoculum (Figure 5).

#### Preliminary sulphate reduction tests with AMD

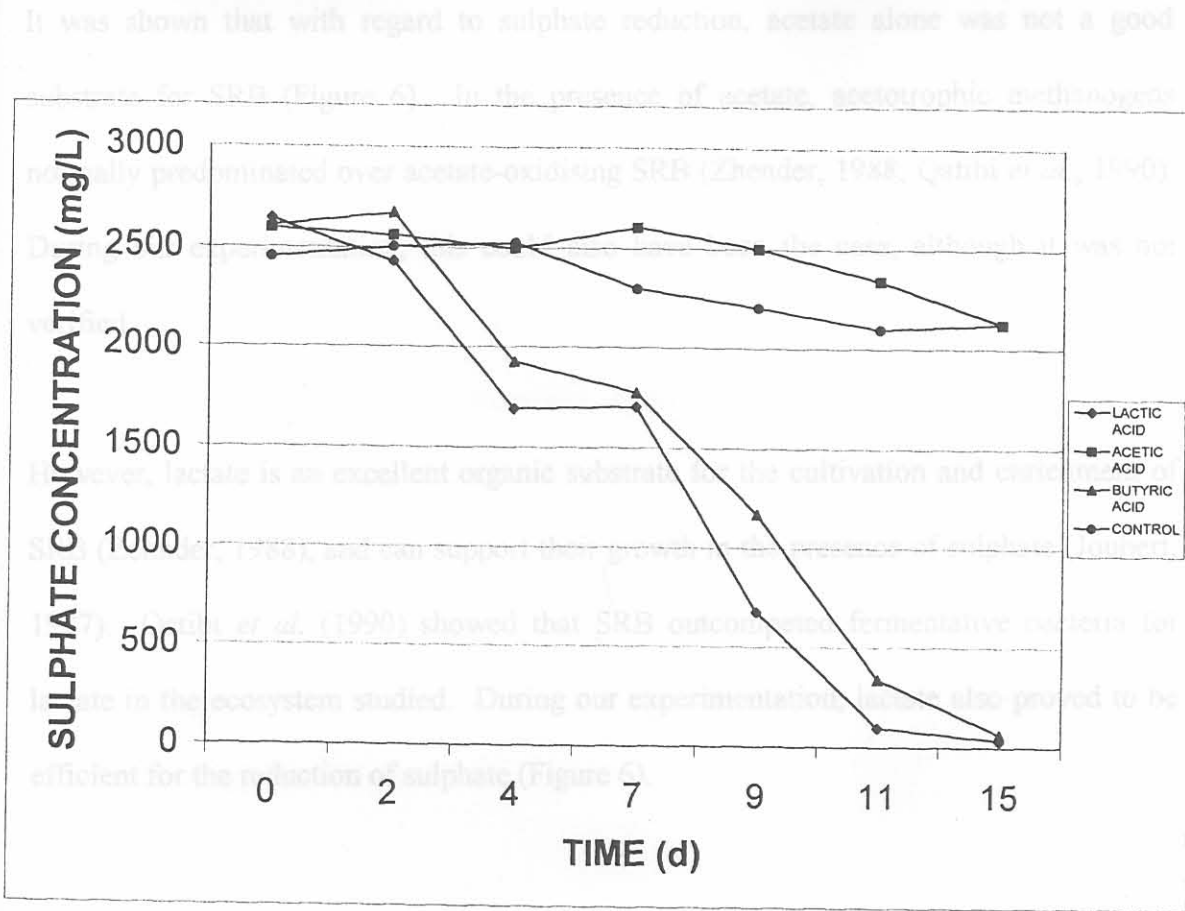


Figure 6. Preliminary sulphate reduction tests using lactic acid, acetic acid and butyric acid as carbon sources.

Preliminary sulphate reduction tests with AMD were done to see if the bioreactor was suited to be used as an anaerobic bioreactor. It was found that during the two week

period, lactic acid and butyric acid proved to be good carbon sources for the removal of sulphate while acetic acid was not efficient (Figure 6). These results confirmed studies done by other researchers (Isa *et al.*, 1986; Ahring and Westerman, 1987; Joubert, 1987; Zhender, 1988; Qatibi *et al.*, 1990; Visser *et al.*, 1993).

It was shown that with regard to sulphate reduction, acetate alone was not a good substrate for SRB (Figure 6). In the presence of acetate, acetotrophic methanogens normally predominated over acetate-oxidising SRB (Zhender, 1988; Qatibi *et al.*, 1990). During our experimentation, this could also have been the case, although it was not verified.

However, lactate is an excellent organic substrate for the cultivation and enrichment of SRB (Zehnder, 1988), and can support their growth in the presence of sulphate (Joubert, 1987). Qatibi *et al.* (1990) showed that SRB outcompeted fermentative bacteria for lactate in the ecosystem studied. During our experimentation, lactate also proved to be efficient for the reduction of sulphate (Figure 6).

### Bioreactor design

In view of the above results, the drip-bag proved to be an effective, inexpensive small-scale anaerobic bioreactor. During all of the sulphate reduction tests, gasses were produced (Figure 4). The drip-bags were air-tight and no gas leakage occurred. This was illustrated by the fact that the drip-bags expanded to their full capacity. The rubber seal provided a convenient sampling port through which gasses could be removed by syringes.

Only minimal contamination of oxygen, if any, occurred during the taking of samples. This inexpensive, easy to use anaerobic bioreactor did not require the addition of catalysts to achieve anaerobic conditions and no contamination of oxygen occurred. Therefore we concluded that the intravenous feeding apparatus could be used as an anaerobic bioreactor for various laboratory experiments.

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## CHAPTER 4

(Submitted for publication in Journal of Applied Microbiology\*)

### **Biological sulphate reduction in artificial acid mine drainage using different defined carbon sources**

\* The language and style used in this chapter are in accordance with the requirements of the Journal of Applied Microbiology

**Keywords:** Acetic acid, acid mine drainage, butyric acid, digester sludge, ethanol, lactic acid, methanol, propionic acid, pyruvate, sulphate reduction

## **Biological sulphate reduction in artificial acid mine drainage using different defined carbon sources**

*SE Coetser, TE Cloete and S Dill*

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The production of acid mine drainage (AMD) containing high amounts of sulphate, heavy metals and low pH is of increasing concern. AMD is highly corrosive and results in economic and environmental problems. The potential of different defined carbon sources to drive sulphate reduction in artificial AMD was studied. This was done in a process for developing a standard laboratory procedure for the evaluation of carbon sources for potential use in passive treatment systems of AMD. Intravenous feeding apparatus (drip-bags) were used as anaerobic bioreactors. These were filled with artificial AMD, an inoculum of anaerobic digester sludge and defined carbon sources. It was found that propionic acid, butyric acid and lactic acid were the carbon sources giving the most effective sulphate reduction, while acetic acid, pyruvate and ethanol did not result in effective sulphate reduction.

**Keywords:** Acetic acid, acid mine drainage, butyric acid, digester sludge, ethanol, lactic acid, methanol, propionic acid, pyruvate, sulphate reduction

## INTRODUCTION

Coal mining often results in the production of effluents containing high amounts of sulphate and heavy metals, generally referred to as acid mine drainage (AMD). AMD is defined as the consequence of the metabolism of sulphur- and iron-oxidising bacteria when pyrite is exposed to atmospheric oxygen and the combination of autoxidation and microbial sulphur and iron oxidation produces large amounts of sulphuric acid (Atlas and Bartha, 1993). AMD is highly corrosive resulting in economic and environmental problems (Dill *et al.*, 1994).

AMD may be remediated by neutralising the effluent and removing the sulphate. Various sulphate removal technologies are available (Dill *et al.*, 1994). These include sulphate removal from water by desalination processes, such as reverse osmosis and ion exchange (Dill *et al.*, 1994). Chemically, sulphates can be removed by using barium ions, such as barium hydroxide or barium chloride (Maree & Strydom, 1985). A high degree of maintenance and operating supervision is required with available treatment strategies. Therefore, there is an increasing demand for inexpensive, environmental friendly technologies for sulphate removal, in order to remediate AMD. One such an alternative is the use sulphate reducing bacteria (SRB) in the biological removal of sulphates from AMD (Dill *et al.*, 1994). Provided a suitable electron donor is available, SRB can oxidise organic compounds like lactate or acetate, with sulphate as electron acceptor being reduced to sulphide (Dill *et al.*, 1994).

Earlier investigations showed that mixed cultures of SRB increased the pH of a lactic acid-mineral salts medium, containing sulphuric acid, from 5 to 8,9 in 8 days at room temperature (Tuttle *et al.*, 1969). Sulphate can be quantitatively converted to  $H_2S$  by *Desulfovibrio desulfuricans* and further conversion to elemental sulphur can be effected by the photosynthetic bacteria *Chlorobium limicola* forma specialis *Thiosulfatophilum* and *Chromatium vinosum* (Maree and Strydom, 1985). Success was achieved using two separate reactors for hydrogen sulphide and sulphur production respectively.

Producer gas, consisting of a mixture of  $H_2$ , CO,  $CO_2$  and  $N_2$  generated from coal, has also been used as energy source for biological sulphate removal (Du Preez *et al.*, 1991). During the anaerobic treatment of sulphate rich water in a trickling filter, sulphate was reduced from 1900mg/L to less than 200mg/L.

Van Houten *et al.* (1994) used gas-lift reactors fed with hydrogen and carbon dioxide as energy and carbon source for the reduction of sulphate. It was concluded that when free  $H_2S$  concentrations are kept below 450mg/L, a maximum sulphate conversion rate of 30g/l  $SO_4^{2-}$ /l/d could be achieved after 10 days operation.

In-situ treatment has been used to avoid the release of AMD in the environment (Dill *et al.*, 1994). Different organic materials have been evaluated for the passive treatment of AMD. Wood dust was investigated as a potential economical carbon source for sulphate reduction (Maree and Strydom, 1985). Sulphate removal from mine water was furthermore achieved by using either sugar, pulp mill effluent or sewage as energy

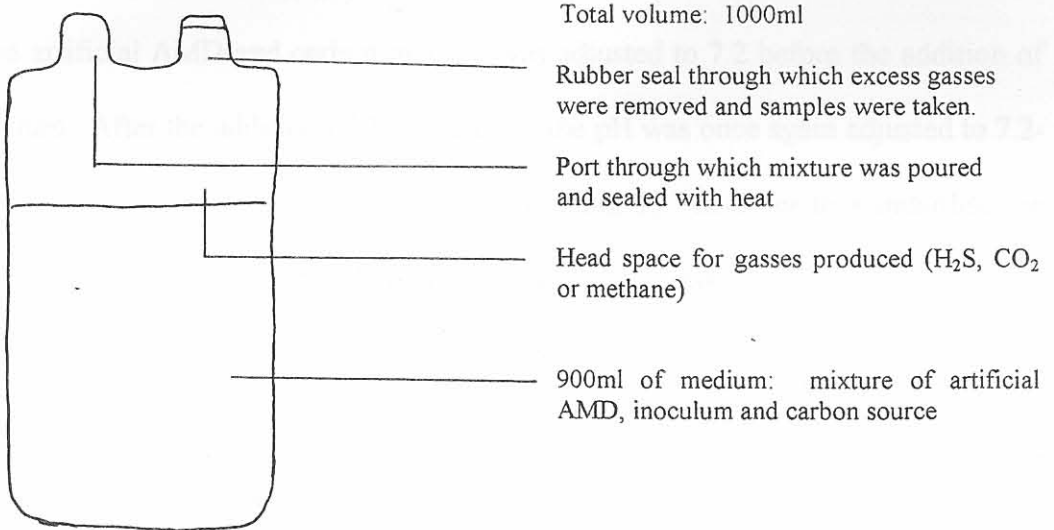


sources (Maree and Strydom, 1985). This was accomplished by providing anaerobic conditions on a solid medium and by keeping the hydrogen sulphide concentration relatively low. It was concluded that recirculation enhanced sulphate reduction, the presence of light increased sulphur production and that 1.6g sugar, 16.7ml spent liquor from a sulphite pulp mill and 172ml raw sludge was required for the removal of 1800mg sulphate. Therefore, under ideal conditions, SRB can be used for the remediation of AMD.

One other method involving SRB which should be considered, is the passive treatment of AMD (Batchelor, 1993). Nevertheless, there is a lack of local and international experience with passive treatment systems designed for the treatment of AMD. This is mainly due to the absence of methods to evaluate the potential use of different undefined carbon sources. To develop efficient passive AMD treatment, the anaerobic sulphate reduction process must be optimised. This can be done by selecting the most appropriate carbon sources, or combination of various carbon sources. The primary objective of this project was to develop a standard procedure for the evaluation of carbon sources for sulphate reduction in acid mine drainage, using defined carbon sources. The information obtained from these experiments would form the basis for evaluation studies of undefined carbon sources.

## MATERIALS AND METHODS

### Bioreactor design



**Figure 1.** A Schematic representation of the anaerobic bioreactor.



**Plate 1.** The anaerobic bioreactor (the expansion of the bag indicates gas production under anaerobic conditions).

## Inoculum

Inoculum was obtained from an anaerobic digester at Daspoort Water Purification Plant in Pretoria, South Africa. A volume of 300ml was added to 1000ml of artificial AMD (Table 1). The respective carbon source was also added to this mixture (Table 3). The pH of the artificial AMD and carbon mixture was adjusted to 7.2 before the addition of the inoculum. After the addition of the inoculum, the pH was once again adjusted to 7.2-7.5. Of this, 900ml was added to each bioreactor (Fig 1). In order to standardise the inoculum, pH, density, moisture content, temperature, alkalinity and total solids determinations were carried out on unfiltered samples, according to standard analytical procedures (APHA, 1985).

**Table 1.** Characteristics of the digester sludge used as inoculum

Parameter	Quantity
Average pH	6.93
Average temperature (°C)	15
Average alkalinity (mg/L CaCO <sub>3</sub> )	866.67
Average total solids/L (MLSS) (mg/L)	30.075
Moisture content (%)	96.26
Density	0.805
COD (mg/L)	6000
SRB (cfu)	$1.8 \times 10^{-3}$

## Artificial acid mine drainage

**Table 2.** Artificial AMD composition.

Component	Quantity
MgSO <sub>4</sub> ·7H <sub>2</sub> O (Merck)	1.31g
H <sub>2</sub> SO <sub>4</sub> (96%) (Merck)	0.30ml
FeSO <sub>4</sub> ·7H <sub>2</sub> O (Saarchem)	4.56g
NH <sub>4</sub> Cl (Labchem)	0.19g
H <sub>2</sub> PO <sub>4</sub> (85%)(Merck)	0.02ml

The above chemicals were dissolved in one litre of distilled water. The pH was adjusted to 7.2 using 10M Sodium hydroxide (Merck). This gave an average sulphate concentration of 2500mg/L, resembling an average sulphate concentration in AMD.

## Carbon sources

**Table 3.** Carbon sources used during this experiment.

Carbon sources	Quantity per 1000ml AMD
Lactic acid (Merck)	5.56ml
Acetic acid (Merck)	5.95ml
Butyric acid (BDH)	4.80ml
Propionic acid (Saarchem)	5.19ml
Pyruvic acid sodium salt (Merck)	7.64g
Methanol (Merck)	8.44ml
Ethanol (Merck)	6.05ml



The amount of carbon sources as displayed in Table 3, gave a carbon: sulphate ratio of 1:1 (w/w). Each experiment was done in triplicate.

### **Control experiments**

Control experiments were done using 900ml of a mixture of 1000ml of artificial AMD (Table1) and 300ml of inoculum. The pH was adjusted to 7.2-7.5. No extra carbon sources were added. These experiments were also done in triplicate.

### **Sampling**

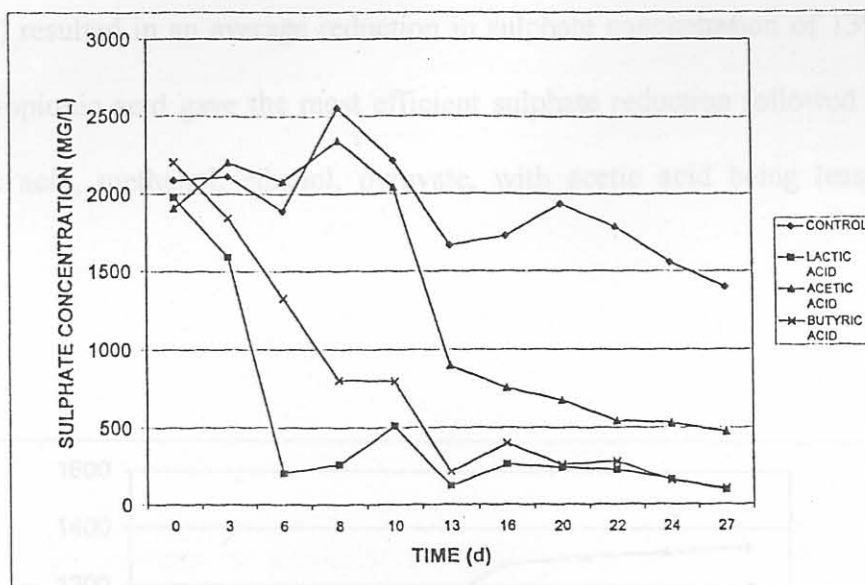
Drip-bags were shaken every day by hand to achieve a well-mixed suspension. Samples were taken from the bioreactors through a rubber seal using syringes (Promex) with a gauge of 1.00mm (Fig 1). To exclude the possibility of H<sub>2</sub>S inhibition on the biological processes, excess gasses were removed as required through the rubber ports (Fig 1).

### **Chemical analysis**

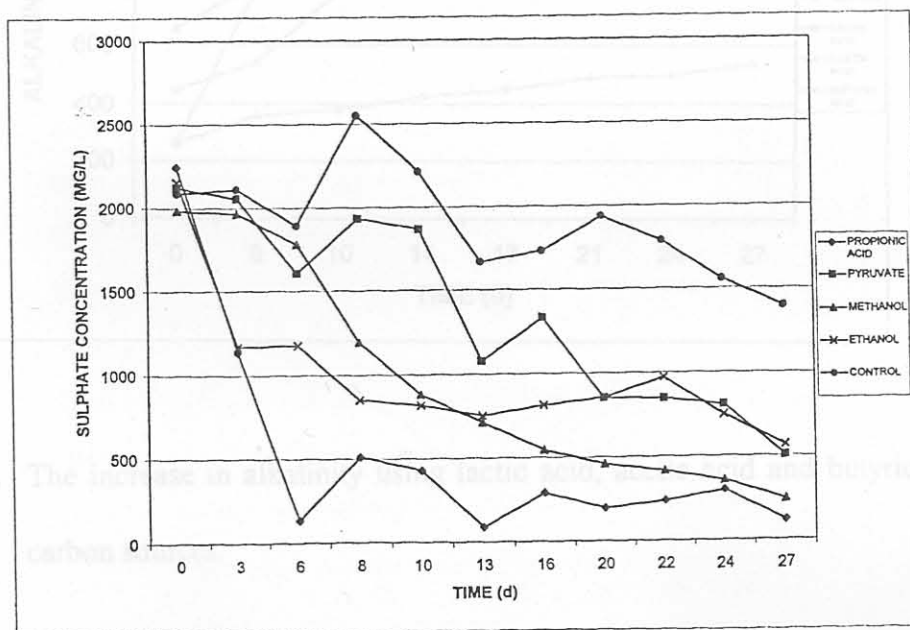
Alkalinity and pH determinations were carried out according to analytical procedures as described in Standard Methods (APHA, 1985). The pH and the alkalinity of the different bioreactors were monitored every 2-3 d. A SQ118 spectroquant (Merck) was used to determine the amount of sulphate reduction using kit no. 1.14791.0001. This was done every 2-3 d. The Chemical Oxygen Demand (COD) was monitored every 2-3 d using a SQ118 spectroquant (Merck). All analyses were carried out on unfiltered samples. Experiments were monitored over a time period of 27 d.

## RESULTS

*Sulphate reduction, COD utilization, pH and alkalinity of AMD with different defined carbon sources*

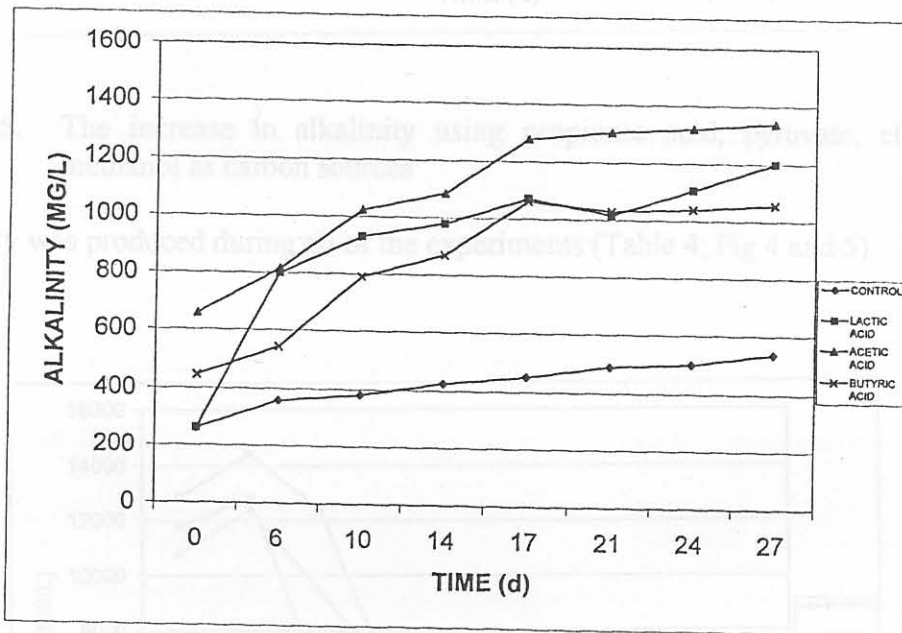


**Figure 2.** Sulphate reduction of AMD with lactic acid, acetic acid and butyric acid as carbon sources

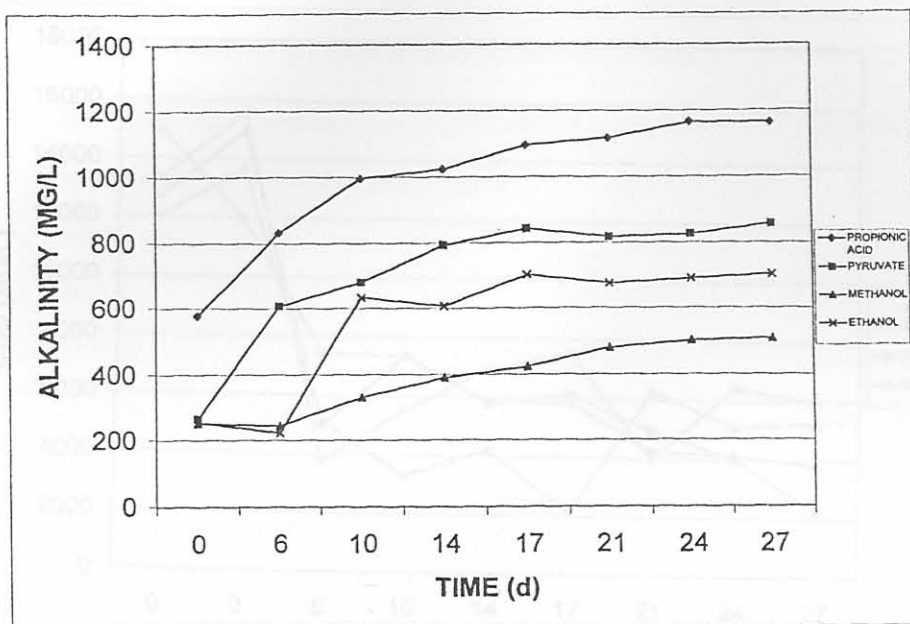


**Figure 3.** Sulphate reduction of AMD with propionic acid, pyruvate, ethanol and methanol as carbon sources.

The average initial sulphate concentration of the different experiments was 2084.25 mg/L (Table 4). After 6d propionic acid and lactic acid resulted in an average reduction in sulphate concentration of 2000mg/L (Fig 3) and 1770mg/L (Fig2), respectively. Butyric acid (Fig 3) resulted in an average reduction in sulphate concentration of 1394.33mg/L after 8d. Propionic acid gave the most efficient sulphate reduction followed by butyric acid, lactic acid, methanol, ethanol, pyruvate, with acetic acid being least effective (Table 4).

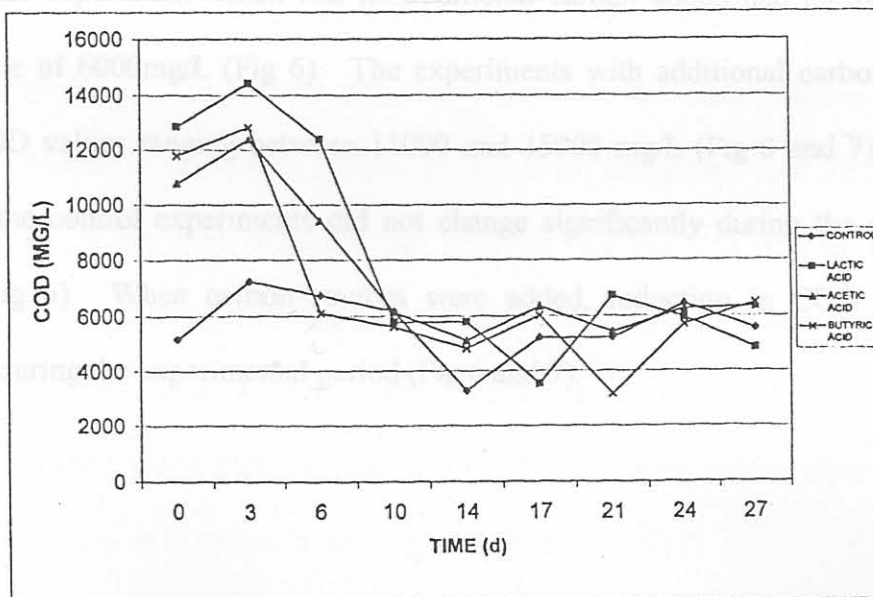


**Figure 4.** The increase in alkalinity using lactic acid, acetic acid and butyric acid as carbon sources.



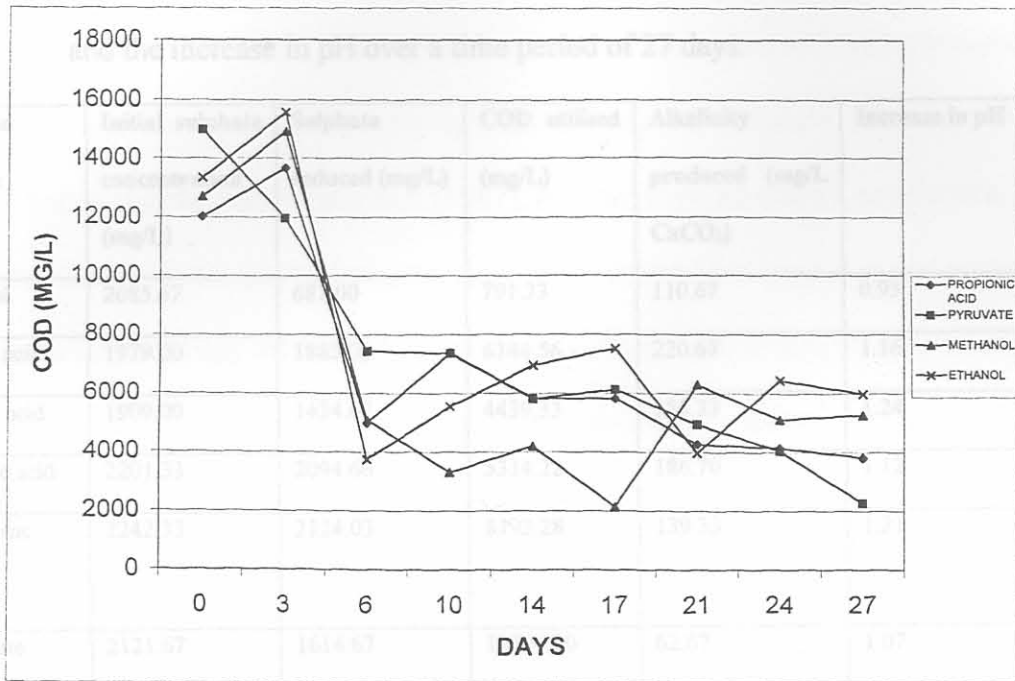
**Figure 5.** The increase in alkalinity using propionic acid, pyruvate, ethanol and methanol as carbon sources

Alkalinity was produced during all of the experiments (Table 4; Fig 4 and 5).



**Figure 6.** COD decrease using lactic acid, acetic acid and butyric acid as carbon sources





**Figure 7.** COD decrease using propionic acid, pyruvate, ethanol and methanol as carbon sources

## DISCUSSION

The control experiment which had no additional carbon added had an average initial COD value of 6000mg/L (Fig 6). The experiments with additional carbon added had initial COD values ranging between 11000 and 15000 mg/L (Fig 6 and 7). The COD value of the control experiments did not change significantly during the experimental period (Fig 6). When carbon sources were added, reduction in COD values were observed during the experimental period (Fig 6 and 7).

The results of this study indicate that *S. cerevisiae* can utilize a wide variety of carbon sources including propionate, butyrate and higher fatty acids, which other fermentative bacteria were unable to utilize in pure cultures (Zehnder, 1988).

**Table 4.** The average amount of sulphate reduced, COD utilised, alkalinity produced and the increase in pH over a time period of 27 days.

Carbon source	Initial sulphate concentration (mg/L)	Sulphate reduced (mg/L)	COD utilised (mg/L)	Alkalinity produced (mg/L $\text{CaCO}_3$ )	Increase in pH	Mg COD per mg $\text{SO}_4^{2-}$ reduced
Control	2085.67	687.00	791.33	110.67	0.93	1.15
Lactic acid	1979.00	1885.00	6144.56	220.67	1.16	3.25
Acetic acid	1909.00	1434.67	4439.33	255.33	1.24	3.09
Butyric acid	2201.33	2094.66	5314.22	186.70	1.12	2.54
Propionic acid	2242.33	2124.03	8192.28	139.33	1.21	3.86
Pyruvate	2121.67	1614.67	12706.00	62.67	1.07	7.87
Methanol	1982.67	1739.00	6391.00	116.67	1.02	3.68
Ethanol	2152.33	1585.66	7345.67	97.3	1.62	4.63

## DISCUSSION

Widdel and Pfennig (1982) indicated that some short-chain fatty acids could be directly oxidized by SRB. The sulphate reducers responsible for these processes have been isolated (Widdel *et al.*, 1977; Widdel *et al.*, 1980; Laanbroek and Pfennig, 1981).

Subsequently, it has been indicated that SRB may be involved in fatty acid turnover either by direct metabolism of fatty acids, or indirectly because of their importance as  $\text{H}_2$  –scavengers (Banat and Nedwell, 1982). SRB can utilize a wide variety of carbon sources including propionate, butyrate and higher fatty acids, which other fermentative bacteria were unable to utilize in pure cultures (Zehnder, 1988).

Isa *et al.* (1986) showed that with regard to sulphate reduction, acetate alone was not a good substrate for the SRB. Best growth on acetate was observed with *Desulfobacter* species which are nutritionally very specialised sulphate reducers (Zehnder, 1988). According to Ahring and Westerman (1987), acetate, from an energy perspective, was a poor substrate. This might be because, the energy required for acetate uptake at very low concentrations exceeds the energy gained from acetate metabolism, thereby limiting the acetate uptake at a certain threshold concentration. Some reports indicate a predominance of SRB growing on acetate, while a number of reports mention complete conversion of acetate by methanogens, even at high sulphate concentrations (Qatibi *et al.*, 1990). These contradicting reports suggest that acetate degradation might be independent of sulphate reduction (Qatibi *et al.*, 1990). Acetotrophic methanogens completely predominated over acetate-oxidising SRB on acetate (Qatibi *et al.*, 1990). Although sulphate reduction did occur during our studies, it was not as efficient as with the other carbon sources. This may be as a result of methanogenesis predominating over sulphate reduction, as was found by Qatibi *et al.* (1990).

Lactate has been used as an excellent organic substrate for enrichment, isolation, and cultivation of certain SRB species (Zehnder, 1988). In the presence of sulphate, lactate, can support the growth of SRB (Joubert, 1987). Qatibi *et al.* (1990), showed that in a bioreactor fed with wine distillery waste water, lactate was rapidly consumed with both sulphate and/or sulphate and molybdate (inhibitor of sulphate reduction). The produced propionate was strongly oxidised in the presence of sulphate. They showed that SRB outcompeted fermentative bacteria for lactate in the ecosystem studied. In our studies



lactate also proved an efficient carbon source for sulphate reduction. This is in agreement with the work of previous researchers (Joubert, 1987; Zhender, 1988; Qatibi, 1990).

Oxidation of pyruvate, both in the presence and absence of sulphate, can be carried out by most of the “fermentative” bacteria (Joubert, 1987). Anaerobic digester sludge was used as inoculum during this study, which consisted mostly out of fermentative bacteria and low numbers of SRB (Zhender, 1988). This may account for the fact that pyruvate was less effective as a carbon source for sulphate reduction compared to the other carbon sources during our studies (Table 4).

Visser *et al.* (1993) found that at higher sulphate concentrations, oxidation of propionate by SRB became more important and only under sulphate-limiting conditions did syntrophic propionate oxidisers out-compete propionate degrading sulphate reducers. Syntrophic butyrate oxidisers were able to compete with SRB for the available butyrate, even with an excess of sulphate (Visser *et al.*, 1993). According to Zehnder (1988), many *Desulfobulbus* species among the SRB, oxidise propionate. Methanogenic and sulphate reducing conditions lead to many possible pathways for conversion of propionate (Speece, 1996). Speece (1996), concluded that since propionate and butyrate were barely detectable in effluents at steady-state, their oxidation under high influent sulphate conditions may be completely or incompletely mediated by the fatty acid-utilising SRB. The presence of SRB enhanced the degradation of propionate, either through direct utilisation, or through interspecies hydrogen transfer (Speece, 1996).



Propionate and butyrate proved to be the best carbon sources for the reduction of sulphate during our study (Table 4). This confirms results obtained by other researchers (Visser *et al.*, 1993; Speece, 1996).

According to Stams *et al.* (1984), ethanol and pyruvate are oxidised to acetate with a concomitant reduction of sulphate to sulphide, in the presence of sulphate. The ability to grow on ethanol as electron donor is common among completely and incompletely oxidizing sulphate reducers (Zehnder, 1988). Pure cultures in batch enrichments with ethanol or higher alcohols as carbon sources, sometimes cease to grow after a while and produce intensely smelling organic sulphur compounds that seem to affect the SRB (Zehnder, 1988). Ethanol and pyruvate was not as sufficient as the other carbon sources tested for the reduction of sulphate (Table 4, Fig 3). This might be due to the production of organic sulphur compounds which inhibit SRB (Zehnder, 1988).

Methanol is seldomly used by SRB (Zehnder, 1988). Even if some species grew well with ethanol, they did not metabolize methanol (Zehnder, 1988). This may account for the fact that methanol was less effective as a carbon source for the reduction of sulphate during our studies (Table 4).

The limited sulphate reduction which occurred in the control experiments was ascribed to the carbon present in the digester sludge, used as inoculum. No significant change in the COD values of the control experiments occurred. This was not unexpected, due to the fact that most of the carbon in the anaerobic digester sludge was already used during

the anaerobic waste water treatment processes in the digester. When additional carbon source was added, efficient sulphate reduction occurred (Table 4).

The process of sulphate reduction is accompanied by the production of alkalinity in the form of bicarbonate ions. This reacts with  $H^+$  ions to form water and neutralisation occurs with a release of carbon dioxide (Watzlaf and Hedin, 1994). Alkalinity was produced during all our experiments (Fig 4 and 5). This confirmed the findings of other researchers.

The results observed during our studies were in agreement with published results by other workers (Joubert, 1987; Zhender, 1988; Qatibi, 1990; Visser, 1993; Speece, 1996). Therefore, we concluded that the method developed in this study, could be used as a standard laboratory procedure, for evaluating carbon sources for potential use in the passive treatment of AMD.

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## CHAPTER 5

### A conceptual model for the passive treatment of acid mine drainage

#### 5.1 Introduction

A model may be defined simply as a purposeful representation or description (often simplified) of a system of interest (Cloete and Muyima, 1997). In terms of this definition, models are widely used in Science and Engineering. For example, microbiologists and sociologists study model organisms (e.g. *E. coli*) and model communities; engineers apply models in the design of a diverse variety of systems (e.g. wastewater treatment plants). Many different types of models exist; these can be broadly categorized into (1) physical, (2) conceptual, (3) mathematical (Simeonov *et al.*, 1996), (4) steady state and (5) dynamic models (Cloete and Muyima, 1997).

The physical model is a spatial scaled representation of the system. For example, the laboratory- and pilot-scale experiments used by scientists and engineers to investigate system response and behaviour are physical models (Cloete and Muyima, 1997).

The conceptual model provides a qualitative description of the system and usually is developed from detailed observations; these models can be presented as schematic diagrams (e.g. flow diagram) or as a series of narrative statements (Cloete and Muyima, 1997).

The mathematical model provides a quantitative description of the system (Cloete and Muyima, 1997). With mathematical models the rates of the processes acting in the system and their stoichiometric interaction with the compounds are formulated mathematically. The mathematical formulations need to be incorporated in a solution procedure that takes account of the physical constraints and characteristics imposed by the system in which the processes take place, e.g. temperature, mixing conditions, etc (Cloete and Muyima, 1997). For the design and operation of biological wastewater treatment systems, it is the mathematical models that have proved most useful (Simeonov *et al.*, 1996). By providing quantitative descriptions, they allow predictions of the system response and performance to be made (Cloete and Muyima, 1997). From the predictions design and operational criteria can be identified for optimization of system performance. Also, mathematical models can serve as very powerful research tools. By evaluating model predictions, it is possible to test hypotheses on the behaviour or the wastewater treatment system (e.g. biological processes, their response to system constraints, etc) in a consistent and integrated fashion (Cloete and Muyima, 1997). This may direct attention to issues not obvious from the physical system, and lead to a deeper understanding of the fundamental behavioural patterns controlling the system response. In essence, mathematical models can provide a define framework which can direct thinking (design, operation or research). Although this usually is very advantageous, it does have some disadvantages – the framework can restrict innovative and new developments (Cloete and Muyima, 1997).



The steady state models have constant flows and loads and tend to be relatively simple (Cloete and Muyima, 1997). This simplicity makes these models useful for design; in these models complete descriptions of system parameters are not required, but rather the models are oriented to determining the more important system design parameters.

The dynamic models have varying flows and loads and accordingly include time as a parameter; the dynamic models are more complex than the steady state ones (Cloete and Muyima, 1997). The dynamic models are useful in predicting time dependent system response of an existing or proposed system; their complexity, however, requires that the system parameters have to be completely defined (Cloete and Muyima, 1997). For this reason the use of dynamic models for design is restricted. Often the steady state design and dynamic kinetic models evolve interactively: The dynamic kinetic models can provide guidance for the development of the steady state design models; they help identify the design parameters that have a major influence on the system response and help eliminate those processes that are not of major importance at steady state (Cloete and Muyima, 1997).

For the dynamic models with their greater complexity, only those parameters that appear to be of importance are considered for inclusion in the model (Cloete and Muyima, 1997). Usually information from lower levels of organization is microbiological and/or biochemical, and the more complete this information is the more reliable the model. To make use of this information, "model" organisms are identified and the known

microbiological and biochemical characteristics of the organism used (Cloete and Muyima, 1997).

These tasks cannot be completely sequentially, but rather the model tends to evolve with the tasks being undertaken interactively (Cloete and Muyima, 1997). For example, to identify processes and compounds, one needs to have some initial conceptualization of the system behaviour, i.e. a rudimentary conceptual model. As more information becomes available from model application and testing, aspects of the rudimentary model are improved as new compounds and processes are identified for inclusion, or processes already included are modified.

The final use for the model needs to be identified; if it is for design, steady state models usually are adequate, if for simulation a dynamic model is required (Cloete and Muyima, 1997). Also, the objectives of the model should be matched to the functions that are required of the biological treatment system.

From the above, it is apparent that to develop conceptual model for the passive treatment of acid mine drainage a number of tasks need to be completed including:

- Identifying the objectives for the model.
- Describing the conditions within which the model is to operate.
- Identification of the essential compounds utilized and formed.
- Identification of the processes acting on these compounds.

- Conceptualization of a mechanistic model that qualitatively describes the kinetic and stoichiometric behaviour of the processes and compounds.
- Mathematically formulation the process rates and stoichiometry.
- Setting up a solution procedure that incorporates the process rates, stoichiometry and transport terms.
- Calibration of the model and test its response against that observed experimentally (Cloete and Muyima, 1997).

Not all of the above tasks fall within the scope of this model. The objectives of this conceptual model, were to:

- Account for major events of interest occurring within the passive acid mine drainage treatment system;
- Assist in identifying the parameters that significantly influence the system response and thereby give guidance for the establishment of design criteria (this will include identification of essential compounds utilized and formed and identification of the processes acting on these compounds) and
- Assist in identifying possible causes for system malfunction or failure, and in devising remedial measures.

## 5.2 Major events of interest occurring within the system

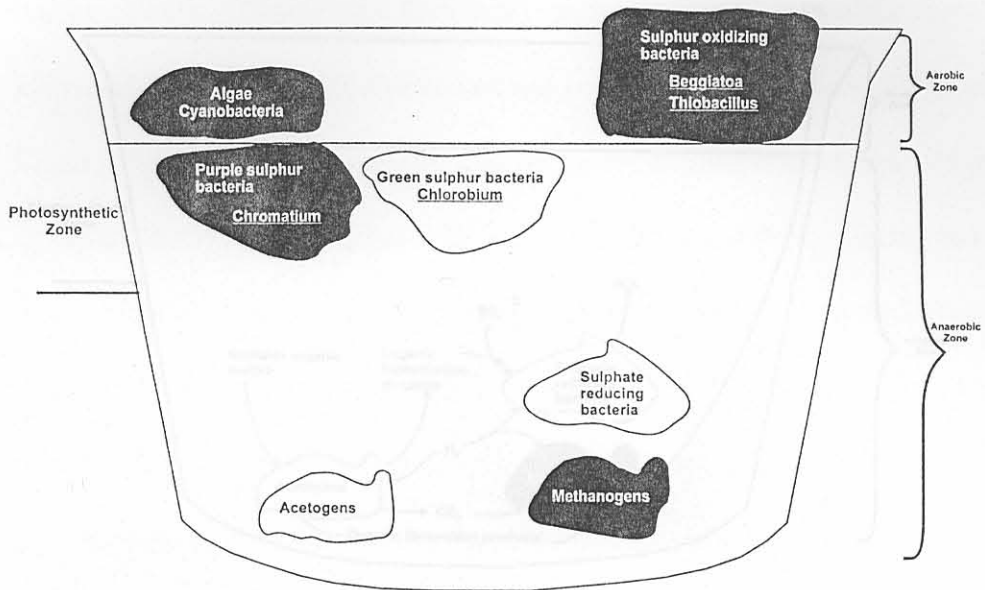


Figure 1. Organisms playing a role in the passive treatment system of acid mine drainage

The microbial consortia active in anaerobic treatment execute a complex process involving many classes of bacteria and several intermediate steps. A passive treatment system can be divided into two major zones, the aerobic zone and the anaerobic zone, with a photosynthetic zone at the top where light can penetrate. The most important organisms which play a role in the aerobic zone are algae, cyanobacteria and sulphur oxidizing bacteria e.g. *Beggiatoa* and *Thiobacillus*, while Purple sulphur bacteria e.g. *Chromatium*, Green sulphur bacteria e.g. *Chlorobium*, acetogens, sulphate reducing bacteria and methanogens are important in the anaerobic zone. Purple sulphur bacteria and Green sulphur bacteria are also photosynthetic bacteria. The role of these organisms will now be discussed in detail.



## 5.2.1 Methanogenic bacteria, acetogens and sulphate reducing bacteria

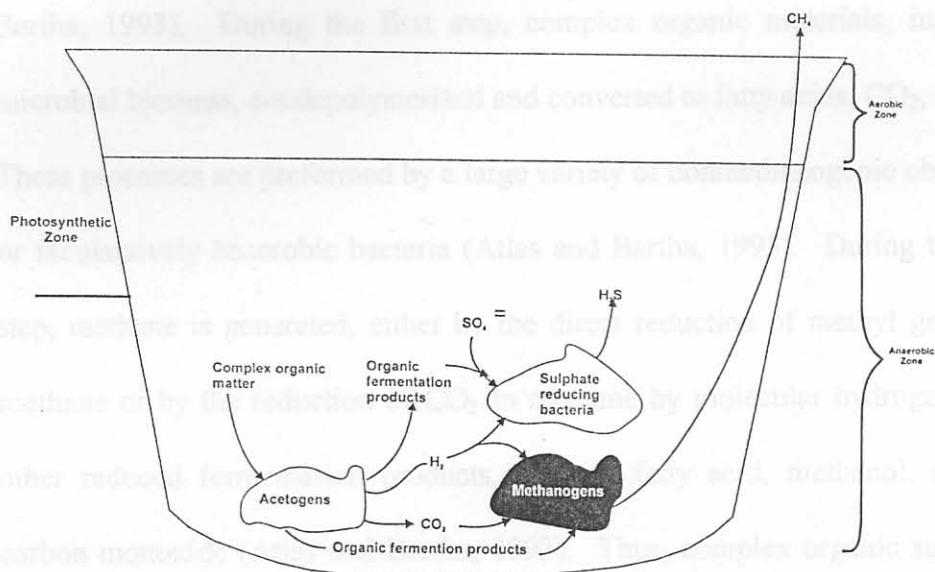


Figure 2. The role of methanogenic bacteria, acetogens and sulphate reducing bacteria in the passive treatment system of AMD.

### 5.2.1.1 Methanogens

Methanogens are methane-producing prokaryotes, a group of archaeobacteria capable of reducing carbon dioxide or low-molecular-weight fatty acids to produce methane (Atlas and Bartha, 1993). Methanogens are obligate anaerobic bacteria (Prescott *et al.*, 1990) which thrive in anaerobic environments rich in organic matter and are often of ecological importance (Prescott *et al.*, 1990). The rumen and intestinal tract of animals, freshwater and marine sediments, swamps and marshes, and anaerobic sludge digesters are only a few of these environments.

The anaerobic digestion of wastes can be considered as a two-step process, even though it really is a coupled sequence of microbiological interactions (Atlas and Bartha, 1993). During the first step, complex organic materials, including microbial biomass, are depolymerized and converted to fatty acids,  $\text{CO}_2$ , and  $\text{H}_2$ . These processes are preformed by a large variety of nonmethanogenic obligately or facultatively anaerobic bacteria (Atlas and Bartha, 1993). During the next step, methane is generated, either by the direct reduction of methyl groups to methane or by the reduction of  $\text{CO}_2$  to methane by molecular hydrogen or by other reduced fermentation products, such as fatty acid, methanol, or even carbon monoxide (Atlas and Bartha, 1993). Thus, complex organic substrates must be hydrolyzed to simpler organics after which they are fermented to volatile acids by the acidogens (Speece, 1996). Obligate hydrogen producing acetogens convert volatile acids longer than two carbons to acetate and  $\text{H}_2$  gas. The acetate and  $\text{H}_2$  are finally converted to methane by methanogens (Speece, 1996).

Hydrogen is an important precursor of methanogenesis (Cohen, 1993). Most of the so far isolated methanogens are able to utilize  $\text{H}_2$ . In methanogenic sediments and rice fields,  $\text{H}_2$  usually accounts for about 30% of methane production (Cohen, 1993).

Methanogenesis is rate-controlled (Speece, 1996).

Sulphide is an obligate nutrient requirement for anaerobic biotechnology and must be provided to insure process stability (Speece, 1996). There is an obligate requirement for sulphide by methanogens but it seems to be easily satisfied as long as there are a few mg/L of sulphide in solution (Speece, 1996). The optimal growth and the specific rate of methane production requires between 0.001 and 1.0 mg/L sulphur as S (Speece, 1996).

As an example, in one study sulphide was inadvertently omitted from the nutrient solution fed to a reactor for 2 months, and the effluent COD increased from 100 to 1900 mg/l for an influent COD of 5000 mg/L (Speece, 1996). Within a day of returning sulphide to the nutrient solution methane production nearly tripled and within a period of 2 weeks the filtered COD decreased to about 100mg/L.

In another study undertaken to research the stimulatory role of sulphate in methanogenesis, a laboratory reactor which had been metabolizing 30kg/m<sup>3</sup>-d of acetate abruptly ceased gas production within one day because the sulphide supply was inadvertently exhausted (Speece, 1996). Supplementation of 20mg/L of sulphide caused resumption of full gas production within 24 hours.

Sulphide production shunts energy in the electron donor to H<sub>2</sub>S, and therefore stoichiometrically reduces CH<sub>4</sub> production (Speece, 1996).

The severe limitation of just one trace metal can drastically alter metabolism of some MPB (Speece, 1996). Therefore it is possible that such a principle may also apply to SRB, which probably have developed specialized techniques for obtaining their trace metal requirements in the presence of sulphide (Speece, 1996).

The inhibition of acetate-utilization by methanogens is controlled by  $H_2S$  (Speece, 1996). Not all methanogens exhibit the same toxicity response to  $H_2S$ , so it is to be expected that the literature reports would vary on the response of different classes of methanogens to a given concentration of  $H_2S$  depending on which class of MPB was predominant in that particular system (Speece, 1996). At an alkaline pH the inhibitory effect of  $H_2S$  was higher than at neutral or acidic pH (Speece, 1996).

#### 5.2.1.2 Sulphate reducing bacteria

SRB are a group of organisms which share an ability to couple the reduction of sulphate and other sulphur compounds to the oxidation of a variety of electron donors (De Bruyn, 1992). Due to the fact that SRB are very strict anaerobes, it is insufficient to only exclude oxygen from culture medium when growing pure cultures (De Bruyn, 1992). In the absence of sulphate, some are known to be capable of fermentative growth, but none can grow with oxygen as electron acceptor, and oxygen always inhibits their growth (Postgate, 1979). SRB are responsible for dissimilatory sulphate reduction, where sulphate acts as an



oxidizing agent for the dissimilation of organic matter (De Bruyn, 1992). Virtually all of the reduced sulphur is released into the external environment as the sulphide ion, usually substantially hydrolysed to free  $H_2S$ . Sulphate reduction in polluted water usually ceases because the sulphate is exhausted rather than because all the organic material has been used.

The presence of SRB in anaerobic digestion systems was therefore not surprising since they are active during the mineralization of organic carbon (Joubert, 1987). If the SRB were, thus, to be used during the production of elemental sulphur, many of the intermediary process phases leading to the formation of sulphide, could be solved (Joubert, 1987). One of the major problem areas in this process is still the inability of the SRB to actively ferment many "primary substrates" such as carbohydrates (Joubert, 1987). At present it would consequently be essential to combine the metabolic activities of an acidogen with the SRB. In practice, however, it will probably be more rational to select the activities of several SRB, rather than to operate with a bacterial consortium, to attain this objectives (Joubert, 1987).

Several strains of oxygen-tolerant SRB are known (Cohen, 1993). Those resistant to high oxygen levels require hydrogen for growth (Cohen, 1993). Other strains are seemingly facultative SRB, namely they may switch from heterotrophic aerobic growth to anaerobic sulphate reduction mode (Cohen, 1993). Another

group of isolated SRB are oxygen sensitive when grown in axenic culture and can cope with oxygen only when grown in co-culture with thiobacilli (Cohen, 1993).

According to McCartney and Oleszkiewicz (1991), SRB generate sulphides that may result in product inhibition of SRB and/or toxicity to methane producing bacteria, change the reactor pH via generation of alkalinity, reduce the rate of methanogenesis and decrease the quantity of methane produced by competing for the available carbon and/or hydrogen.

The main bacterial groups which utilize carbon dioxide and sulphate as electron acceptors in the anaerobic process are the methanogens and the sulphate-reducers which also closely interact in anoxic environments (Joubert, 1987). Since these bacterial groups occupy rather similar ecosystems and are both strict anaerobes, they seem to have a somewhat competitive association (Joubert, 1987). The availability of sulphate in anaerobic environments will, to a large extent, determine the dominant terminal process (Joubert, 1987). The microbial interactions between these two groups are therefore of particular interest in explicating the complexity of the terminal degradation process and were therefore studied in anaerobic digesters and in freshwater and marine sediments (Joubert, 1987).

In exceptional cases methanogenesis, in itself, have also been observed in high-sulphate ecosystems (Joubert, 1987). However, the dominance of the sulphate-

reducers over methanogens in high-sulphate environments does not necessarily imply that sulphate-reduction will always be the dominant terminal process in anaerobic mineralizations (Joubert, 1987). Methanogenesis has been shown to dominate in deeper layers of marine sediment where sulphate is depleted or where the sediment is highly enriched with organic matter (Joubert, 1987). Recent studies have also shown that the SRB were poor competitors in comparison to methane-producing bacteria in high-rate anaerobic reactors (Joubert, 1987). The methanogens were observed to gain dominance in the anaerobic reactor even when the reactor was initially colonized mainly by sulphate-reducers (Joubert, 1987). These significant observations were to some extent ascribed to the better colonization and adherence characteristics of the methanogens to the fixed carrier matrix in the reactor (Joubert, 1987). Proposed mechanisms for the apparent inhibition of methanogenesis in high-sulphate environments are mostly unsatisfactory, but a number of fundamental aspects possibly responsible for this inhibition have been presented as likely explanations (Joubert, 1987).

Thus, under anaerobic conditions, acetogenic bacteria are responsible for the degradation of complex organic matter to organic fermentation products,  $H_2$  and  $CO_2$ . Methanogens utilize the  $CO_2$  and organic fermentation products to produce methane while SRB utilizes  $H_2$  in the presence of sulphate to produce  $H_2S$ . Therefore, SRB and methanogens compete for available  $H_2$  and carbon sources.

The alkali and alkaline earth sulphides dissociate in solution to yield free  $H_2S$  as well as  $HS^-$  and  $OH^-$  ions. Since  $H_2S$  is volatile the pH of the environment thus tends to become alkaline. Over a long period this alkalinity is neutralized by atmospheric  $CO_2$ , so that the carbonate and bicarbonate accumulate. During periods of active sulphate reduction, however, the environment tends to become alkaline unless compensating metabolic reactions leading to acid formation are taking place simultaneously, or unless the sulphide is trapped as insoluble derivatives of heavy metals.

$H_2$  is a characteristic product of anaerobic fermentations, and the presence of hydrogenase in the majority of SRB enables them to utilize  $H_2$  for sulphate reduction. Scavenging of  $H_2$  has been suggested as one interpretation of the incompatibility of sulphate-reducing and methane-producing bacteria (Prescott *et al.*, 1990).

### 5.2.1.3 Acetogens

In the case of anaerobic treatment, the slowest step is characterized by an accumulation of substrate buildup found just prior to the rate controlling step (Speece, 1996). If this form of substrate is a non-acid organic (e.g. alcohol) there may be no adverse impact on the overall consortia (Speece, 1996). The slowest growing members of the consortia often are the propionic- or acetic acid-utilizers. An accumulation of these organic acids can overwhelm the reserve bicarbonate alkalinity (Speece, 1996). Such malfunction may cause a decrease in pH which



can have a drastically adverse impact upon the entire microbial consortia (Speece, 1996). Unfortunately the greatest inhibition of a low pH may also be directed at the propionic- and acetic-utilizers themselves, compounding the problem (Speece, 1996).

It should be evident from this series reaction analogy that the anaerobic process works well as long as each subsequent class of organisms processes the organic intermediaries at least as fast as they are produced (Speece, 1996). Since microbial processes function at a rate proportional to their substrate concentration, an accumulation of substrate may result before they are able to process it as fast as it is passed on to them (Speece, 1996).

## 5.2.2 Algae and Cyanobacteria

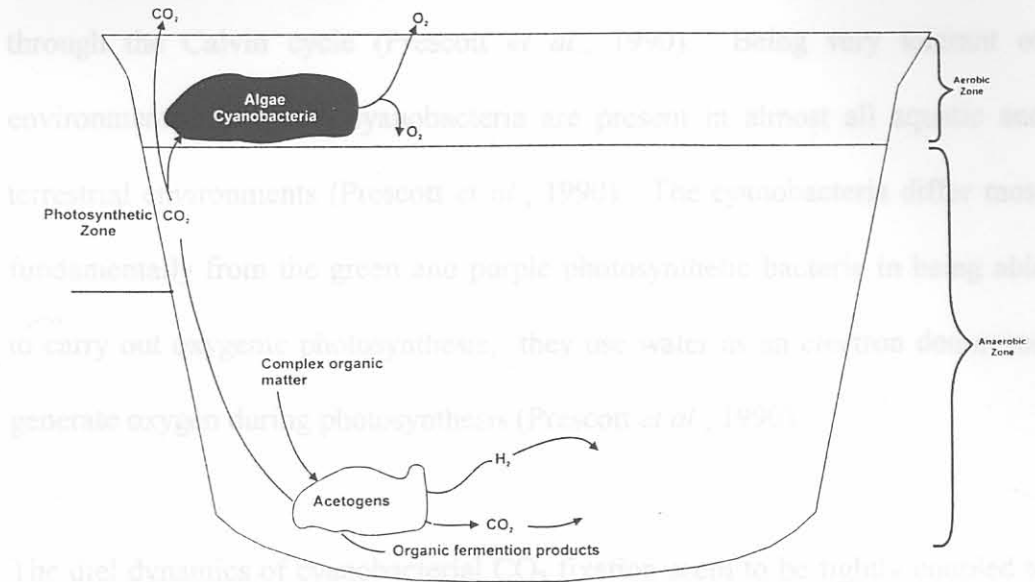


Figure 3. The role of algae and Cyanobacteria in the conceptual model of the passive treatment system of acid mine drainage.

Algae can be described as eucaryotic organisms that lack roots, stems, and leaves but have chlorophyll and other pigments for carrying out oxygen-producing photosynthesis (Prescott *et al.*, 1990). Algae can be either autotrophic or heterotrophic, but most are photoautotrophic, and require only light and  $\text{CO}_2$  as their principal source of carbon and energy sources (Prescott *et al.*, 1990). Preliminary observations indicate that sulphate

*al.*, 1990). Chemoheterotrophic algae require external organic compounds as carbon and energy sources (Prescott *et al.*, 1990).

The cyanobacteria are the largest and most diverse group of photosynthetic bacteria (Prescott *et al.*, 1990). Cyanobacteria assimilates carbon dioxide through the Calvin cycle (Prescott *et al.*, 1990). Being very tolerant of environmental extremes, cyanobacteria are present in almost all aquatic and terrestrial environments (Prescott *et al.*, 1990). The cyanobacteria differ most fundamentally from the green and purple photosynthetic bacteria in being able to carry out oxygenic photosynthesis; they use water as an electron donor and generate oxygen during photosynthesis (Prescott *et al.*, 1990).

The diel dynamics of cyanobacterial CO<sub>2</sub> fixation seem to be tightly coupled to CO<sub>2</sub> production by SRB and methane production by methanogens (Cohen, 1993).

Sulphate reducers and methanogens are believed to be strictly anaerobic bacteria and require negative redox potentials for growth (Cohen, 1993). Upon illumination during daytime, O<sub>2</sub> production is so high that O<sub>2</sub> concentration microzones overlap with those of sulphate reduction and methanogenesis (Cohen, 1993). In other words, anaerobic processes such as sulphate reduction and methanogenesis may be affected dynamically by photosynthetic O<sub>2</sub> production (Cohen, 1993). Preliminary observations indicate that sulphate

reduction and methanogenesis are at least partially operative and sometimes even highly enhanced in the presence of oxygen (Cohen, 1993).

During anaerobic conditions, both algae and cyanobacteria may use the  $\text{CO}_2$  produced by acetogens during anaerobic degradation of complex organic matter.

### 5.2.3 The role of purple sulphur bacteria, green sulphur bacteria and sulphur oxidizing bacteria

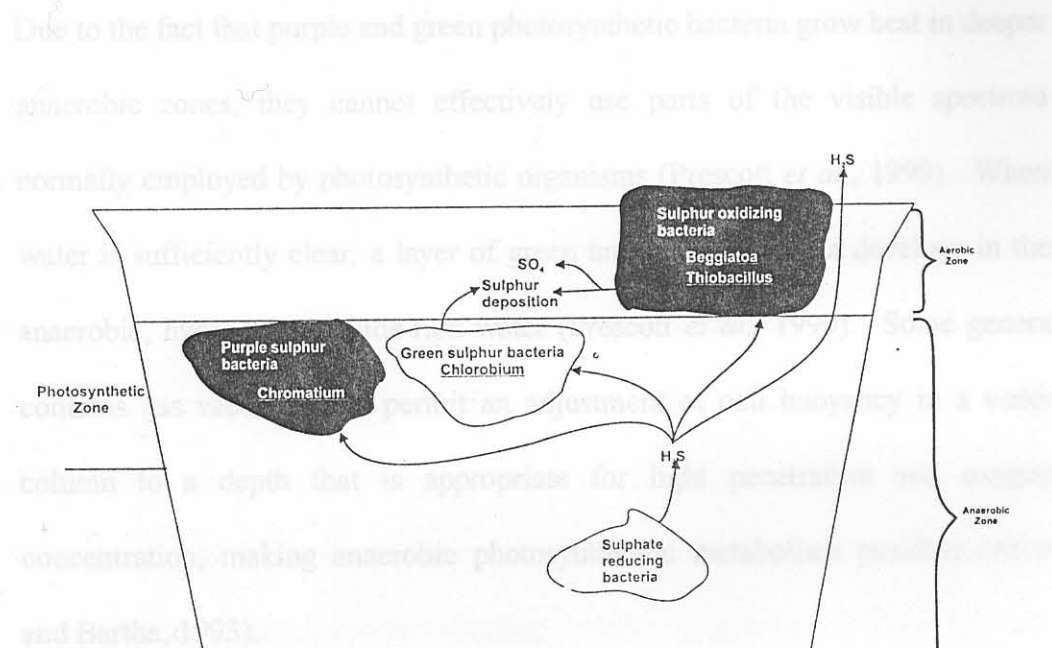


Figure 4. The role of purple- and green sulphur bacteria, and sulphur oxidizing bacteria in the conceptual model of the passive treatment system.



### 5.2.3.1 Purple sulphur bacteria

The purple sulphur bacteria are strict anaerobes and usually photolithoautotrophs (Prescott *et al.*, 1990). These bacteria oxidize hydrogen sulfide to sulphur and deposit it internally as sulphur granules (Prescott *et al.*, 1990). Typical purple sulphur bacteria are *Thiospirillum*, *Thiocapsa*, and *Cromatium* which are found in anaerobic, sulphide-rich zones of lakes (Prescott *et al.*, 1990). These bacteria produce carotenoid pigments and may appear orange-brown, red-brown, purple-red, or purple-violet (Atlas and Bartha, 1993). Due to the fact that purple and green photosynthetic bacteria grow best in deeper anaerobic zones, they cannot effectively use parts of the visible spectrum normally employed by photosynthetic organisms (Prescott *et al.*, 1990). When water is sufficiently clear, a layer of green and purple bacteria develops in the anaerobic, hydrogen sulphide-rich water (Prescott *et al.*, 1990). Some genera contains gas vacuoles that permit an adjustment of cell buoyancy in a water column to a depth that is appropriate for light penetration and oxygen concentration, making anaerobic photosynthetic metabolism possible (Atlas and Bartha, 1993).

### 5.2.3.2 Green sulphur bacteria

Green sulphur bacteria are a small group of obligately anaerobic photolithoautotrophs that use hydrogen sulphide, elemental sulphur, and hydrogen as electron sources (Prescott *et al.*, 1990). Sulphur is produced by sulphide oxidation and is deposited outside the cell (Prescott *et al.*, 1990). Green sulphur

bacteria produce green or green-brown carotenoid pigments (Atlas and Bartha, 1993). These bacteria flourish in the anaerobic, sulphide-rich zones of lakes.

### 5.2.3.3 Sulphur oxidizing bacteria

Reduced sulphur compounds are capable of supporting chemolithotrophic microbial metabolism in the presence of oxygen (Atlas and Bartha, 1993).

*Beggiatoa*, *Thioploca* and *Thiotrix*, are filamentous, microaerophilic bacteria capable of oxidizing  $H_2S$  to water and water (Atlas and Bartha, 1993). In the absence of  $H_2S$ , these sulphur globules are slowly oxidized further to sulphate.

Some species of *Thiobacillus* are also able to oxidize  $H_2S$  and other reduced sulphur compounds and, because they have a low acid tolerance, deposit elemental sulphur rather than generate sulphuric by further oxidation (Atlas and Bartha, 1993). These *Thiobacillus* and the filamentous sulphur bacteria are facultatively chemolithotrophic while other members of the genus *Thiobacillus* species produce sulphate from the oxidation of elemental sulphur and other inorganic sulphur compounds (Atlas and Bartha, 1993). *Beggiatoa* is very versatile metabolically and can oxidize hydrogen sulphide to form large intracellular sulphur grains and can subsequently oxidize the sulphur to sulphate (Prescott *et al.*, 1990).

Sulphates are common minerals in soils and water, so sulphate reduction, once established, can continue for long periods provided organic matter is available

(Postgate, 1979). The sulphide found is a chemical reducing agent, tending to preserve the anoxic environment necessary for activity of SRB (Postgate, 1979). Ecologically, zones of sulphate reduction are the foci of ecosystems called 'sulfureta', in which other sulphur bacteria grow in association with the SRB, in which coloured, photosynthetic sulphide-oxidizing bacteria grow in a zone whose depth is determined by the depth to which light penetrates (Postgate, 1979). Above that zone, dissolved sulphide and dissolved air co-exist and aerobic sulphide-oxidizing bacteria (e.g. *Beggiatoa* in fresh waters) may be found as well as thiobacilli (Postgate, 1979). These organisms oxidize reduced sulphur back to sulphate and by thus recycling sulphur they tend to perpetuate the sulfuretum: by their autotrophic and photosynthetic CO<sub>2</sub> fixation they also regenerate organic matter and help to perpetuate the system (Postgate, 1979). Since the SRB generate sulphide and the oxidizers fix CO<sub>2</sub>, the sulfuretum can act as an anaerobic primary producing system (Postgate, 1979). But it is limited by the amount available of either sulphide (for CO<sub>2</sub> fixation) or pre-formed organic matter (for H<sub>2</sub>S formation) (Postgate, 1979). These sulfureta readily become populated by appropriate bacteria and, because of the reducing action of H<sub>2</sub>S, have a considerable degree of ecological stability (Postgate, 1979).

Thus, green sulphur bacteria (e.g. *chlorobium*) and purple sulphur bacteria (e.g. *chromatium*) can utilize the H<sub>2</sub>S produced by SRB, anaerobically, while sulphur oxidizers (e.g. *thiobacillus*) can utilize the H<sub>2</sub>S aerobically. Sulphur oxidizing



bacteria produces sulphate and sulphur deposits from  $H_2S$ , while green sulphur bacteria only produces sulphur deposits.

### 5.3 Conceptual model for the passive treatment system of AMD

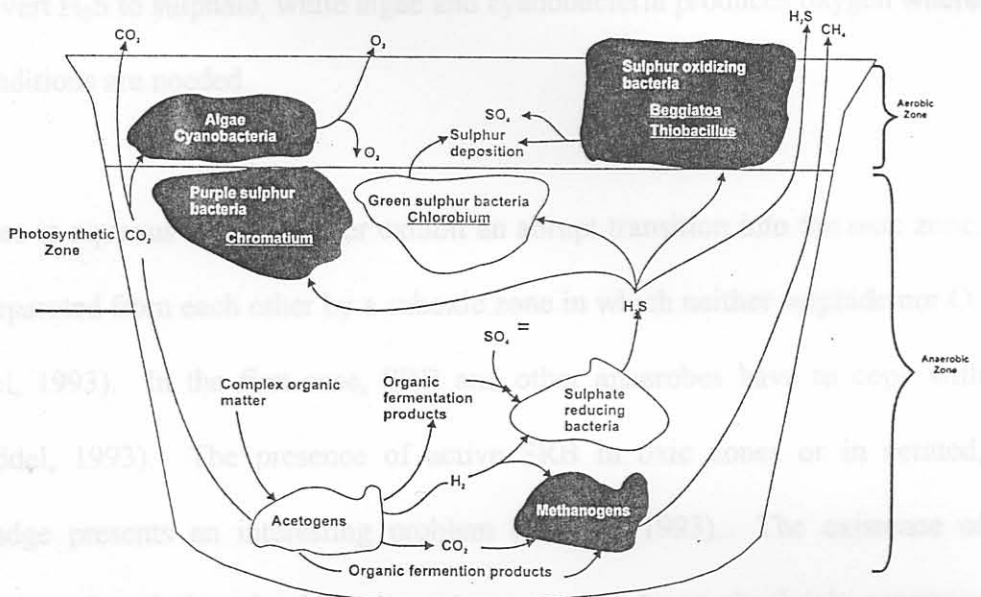


Figure 5. Conceptual model for the passive treatment system of AMD.

### 5.4 Microbiological conversions which could cause system malfunction

Due to the fact that SRB and methanogens live under strict anaerobic conditions with similar pH and temperature ranges, they are in competition with each other. In normal ecosystems such as freshwater and marine sediments and also in anaerobic digesters,



SRB are normally dominant and methanogenesis was found to be inhibited by the presence of sulphate (Isa *et al.*, 1986). However, there are several factors influencing the competition between SRB and methanogens. These include sulphate concentration in feed, maximum specific utilising rate, free energy of the reaction, nutrient availability, temperature, substrate type etc. (Speece, 1996). Methanogenesis must be inhibited during the passive treatment of AMD. Other microbial processes which need to be eliminated are that of the sulphur oxidizers, the algae and the cyanobacteria. Sulphur oxidizers convert  $H_2S$  to sulphate, while algae and cyanobacteria produces oxygen where anaerobic conditions are needed.

Reduced zones in aqueous habitats either exhibit an abrupt transition into the oxic zone, or both are separated from each other by a suboxic zone in which neither sulphide nor  $O_2$  exist (Widdel, 1993). In the first case, SRB and other anaerobes have to cope with oxygen (Widdel, 1993). The presence of active SRB in oxic zones or in aerated, activated sludge presents an interesting problem (Widdel, 1993). The existence of microniches, even though they should be important, seems to be no absolutely necessary for active sulphate reduction; in oxic layers with active sulphate reducers, microniches could not be detected by the use of microsensors (Widdel, 1993).

## 5.5 References

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## CHAPTER 6

### Conclusions

A standard procedure for the evaluation of carbon sources in acid mine drainage was developed. The results obtained during these studies confirmed work done by other researchers. Therefore we concluded that the intravenous feeding apparatus could be used as an anaerobic bioreactor for various laboratory experiments.

Sulphate reduction using different defined carbon sources in acid mine drainage will form the basis for the evaluation studies with undefined carbon sources. It was concluded when acetate is not as efficient as the other carbon sources for the reduction of sulphate. Propionate, butyrate, and lactate proved to be efficient carbon sources for the reduction of sulphate. Ethanol, pyruvate and methanol were also not efficient as carbon sources for the reduction of sulphate. These findings are in agreement with work done by other researchers.

The conceptual model for the passive treatment of acid mine drainage accounts for major events of interest occurring within the passive acid mine treatment system. This model will assist in identifying the parameters that significantly influence the system response. Possible causes for system malfunction or failure were also identified.