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):

$$8CH_3COO^- + 8SO_4^{2-} + 16H^+ \text{ -----> } 16CO_2 + 16H_2O + 8HS^-$$

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 H_2, CO, and CO_2 and 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 30g/l SO_4^{-2}/l/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 2×10^3 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 of 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 (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 sulpur cycle

2.3.1 The sulphur cycle

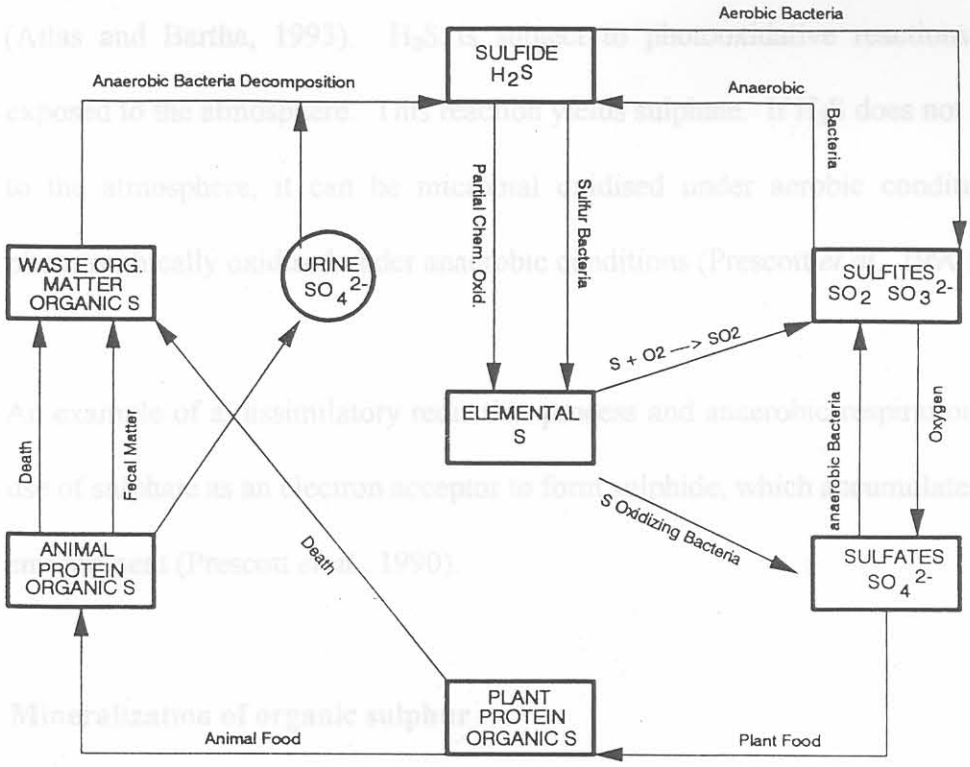

Figure 1. Simplification of the steps involved in the sulphur cycle (Bitton, 1994).

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_2S 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_2S is subject to photooxidative reactions when exposed to the atmosphere. This reaction yields sulphate. If H_2S 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:

$R\text{--}O\text{--}SO_3^- + H_2O \text{ -------> } ROH + H^+ + SO_4^{2-}$ (Bitton, 1994).

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 –

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 microaerophillic 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_2S production.
2. *Dissimilatory sulphate reduction:*
   - SRB (strict anaerobes) are responsible for this reaction:

     $SO_4^{2-} + 2H^+ \text{------} > H_2S$
   - 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 pyrophospate. 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.

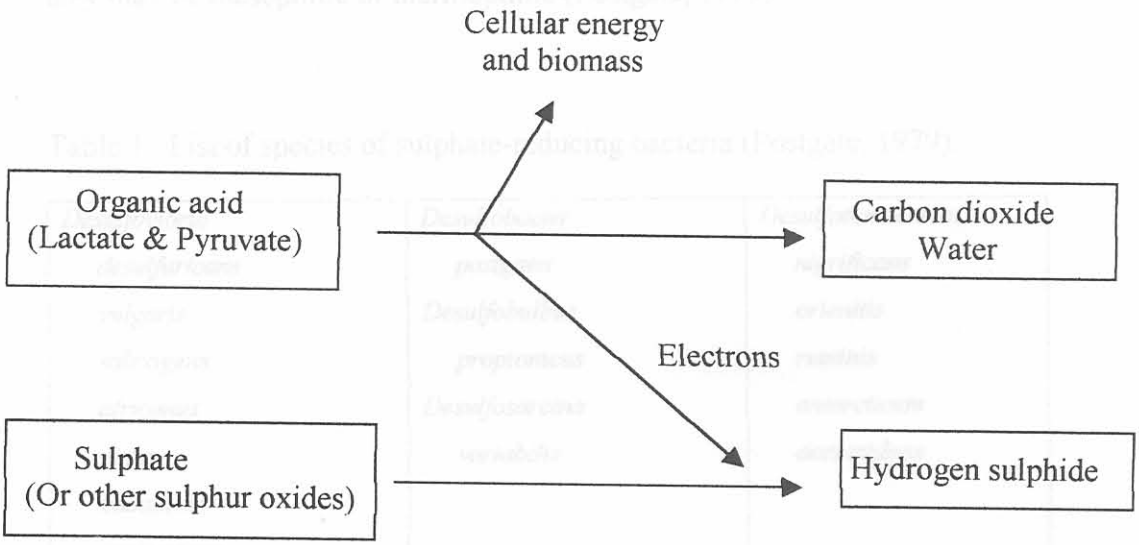

Figure 2. Generalised schematic of the physiology of sulphate reducing bacteria (Apel *et al.*, 1992).

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).

Desulfovibrio	*Desulfobacter*	*Desulfotomaculum*
desulfuricans	*postgatei*	*nigrificans*
vulgaris	*Desulfobulbus*	*orienitis*
salexigens	*propionicus*	*ruminis*
africanus	*Desulfosarcina*	*antarcticum*
gigas	*variabilis*	*acetoxidans*
baculatus		
sapovorans		
baarsii		
thermophilus		

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 poising 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).

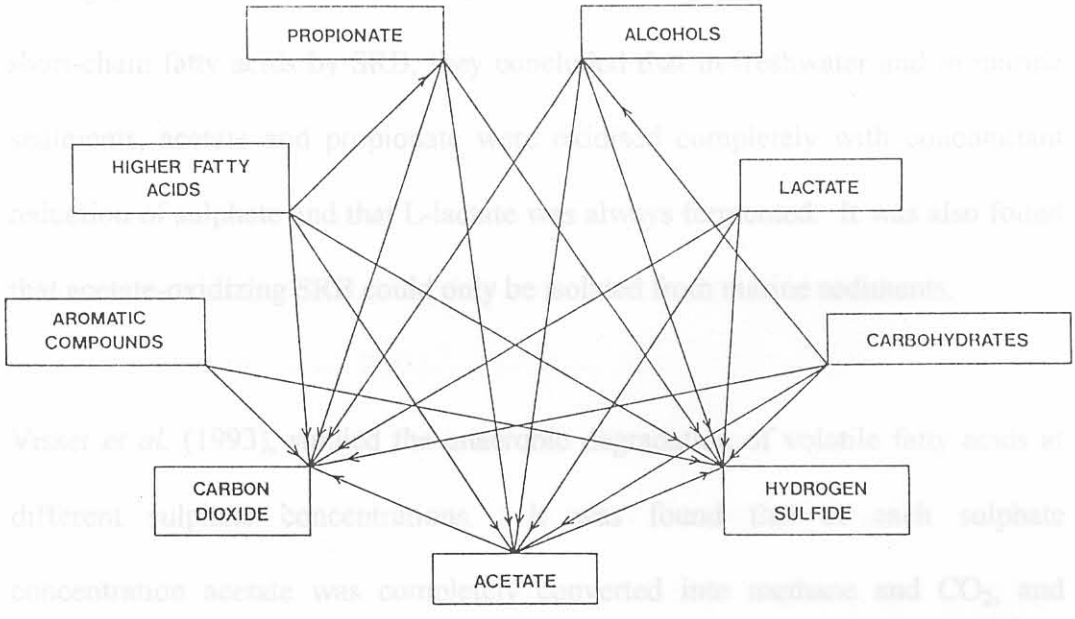

Figure 3. Metabolic formation by sulphate-reducers in the presence of exogenous sulfurous electron acceptors (Joubert, 1987).

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):

$CH_3COO^- + SO_4^{2-} \text{------>} 2HCO_3^- + HS^- \qquad \Delta G^{o'} = -47.6kJ$

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 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):

$$2CH_3CHOH\,COO^- + SO_4^{2-} \text{ ---------> } 2CH_3COO^- + 2HCO_3^- + HS^- + H^+$$

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

$$2CH_3CHOH\,COO^- + 3SO_4^{2-} \text{ --------->} 6HCO_3^- + 3HS^- + H^+$$

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

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):

$$2\ Lactate^- + SO_4^{2-} \text{ -----> } 2\ acetate^- + 2H_2O + 2CO_2 + S^{2-}$$

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 dismuted 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):

$$4CH_3CH_2COO^- + 3SO_4^{2-} \text{-------} > 4CH_3COO^- + 4HCO_3^- + 3HS^- + H^+$$

$$\Delta G^{0'} = -150.6kJ$$

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 synthrophic 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.

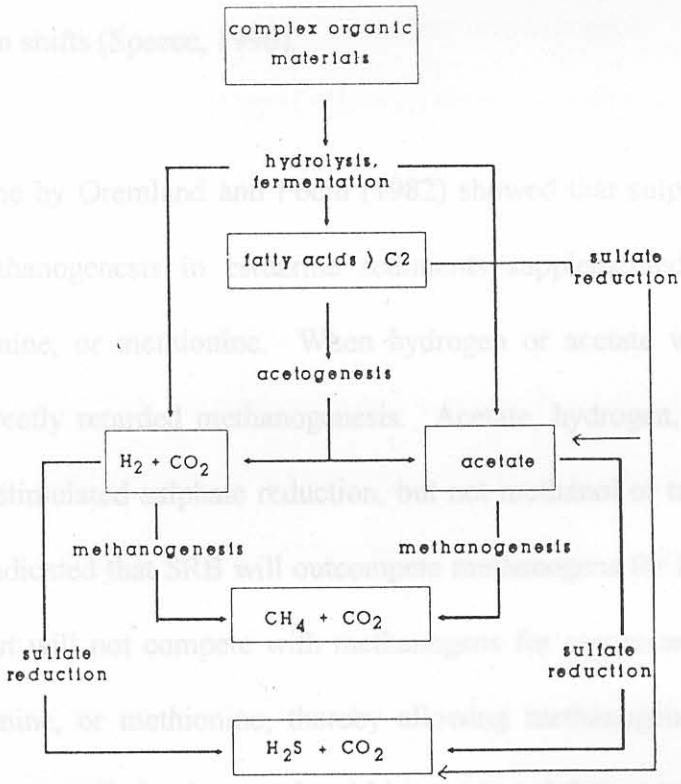

Figure 4. Substrate competition between sulphate-reducing and methanogenic or acetogenic bacteria (Bitton, 1994).

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 Pocin (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 6,6μM for methanogens, as compared with 1,3μM for sulphate reducers. Similarly, the K_s values for acetate are mM and 0,2mM 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 = 9.5mg/l) than methanogens (K_s = 32,8mg/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_4 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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