THE HYDROLYSIS OF PRIMARY SEWAGE SLUDGE UNDER BIO-SULPHIDOGENIC CONDITIONS

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Abstract

The potential for using readily available and cost-effective complex carbon sources such as primary sewage sludge for a range of environmental remediation processes, including biological sulphate reduction, biological nutrient removal and the bioremediation of acid mine drainage, has been constrained by the slow rate of solubilization and low yield of soluble products, which drive the above mentioned processes. Previous work conducted by the Environmental Biotechnology Group at Rhodes University indicated that the degradation of primary sewage sludge was enhanced under sulphate reducing conditions. This was proven in both laboratory and pilot-scale (Reciprocating Sludge Bed Reactor) systems, where the particulate matter accumulated in the sludge bed and the molecules in smaller flocs were rapidly solubilized. The current study was aimed at investigating in more detail the factors that govern the enhanced hydrolysis under sulphate reducing conditions, and to develop a descriptive model to explain the underlying mechanism involved.

The solubilization of primary sewage sludge under sulphate reducing conditions was conducted in controlled flask studies and previously reported findings of enhanced hydrolysis were confirmed. The maximum percentage solubilization obtained in this study was 31% and 63% for the methanogenic and sulphidogenic systems respectively, and this was achieved over a period of 10 days. A rate of reducing sugar production and complex molecule breakdown of 51 mg. $L^{-1}.hr^{-1}$ and 167 $mg.L^{-1}.hr^{-1}$ was observed for the methanogenic and sulphidogenic systems respectively. The flask studies revealed that during hydrolysis of primary sewage sludge under sulphidogenic conditions there was enhanced production of soluble products, specifically carbohydrates (reducing sugars) and volatile fatty acids, compared to methanogenic conditions. The rate at which these products were utilized was also found to be more rapid under sulphidogenic as compared to methanogenic conditions. A study of the distribution of volatile fatty acids indicated that acetate was utilized preferentially in the methanogenic system, and that propionate, butyrate and valerate accumulated with time. The converse was found to occur in the sulphidogenic system.

The descriptive model developed from the results of this study was based on the fact that a consortium of bacteria, composed of hydrolytic, acidogenic and acetogenic species, carries out the solubilization of complex carbon sources. Furthermore, it is essential that equilibrium between product formation and utilization is maintained, and that accumulation of soluble end

products impacts negatively on the rate of the hydrolysis step. It is therefore proposed that the relatively poor utilization of VFA and reducing sugars in the methanogenic system activates a negative feedback inhibition on the hydrolytic and/ or acidogenic step. This inhibition is reduced in the sulphidogenic system where the utilization of end products is higher.

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List of Abbreviations

ABR	Anaerobic Baffled Reactor
ALD	Anoxic Limestone Drains
AMD	Acid Mine Drainage
APS	ATP Sulphurylase
ATP	Adenosine Triphosphate
BNR	Biological Nutrient Removal
CE	Constant Environment
COD	Chemical Oxygen Demand ($mg.L^{-1}$)
CODf	Filtered COD ($mg.L^{-1}$)
CODp	Particulate COD ($mg.L^{-1}$)
CODt	Total COD ($mg.L^{-1}$)
CSIR	Centre for Scientific and Industrial Research
CSTR	Continuous Stirred Tank Reactor
DNA	Deoxyribose Nucleic Acid
EBG	Environmental Biotechnology Group
GC	Gas Chromatography
H_2S	Dihydrogen sulphide
HDS	High Density Sludge
HPLC	High Pressure Liquid Chromatography
MME	Molecular Microbial Ecology
Mo	Molybdate
MPB	Methane Producing Bacteria
RPM	Revolutions per minute
PFA	Pulverized Fuel Ash
PSS	Primary Sewage Sludge
RSBR	Reciprocating Sludge Bed Reactor
SRB	Sulphate Reducing Bacteria
TDS	Total Dissolved Solids
UASB	Upflow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acids
VSS	Volatile Suspended Solids

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Chapter 1

General Introduction

1.1 Water Problem in South Africa

Water plays an important role in all spheres of life as a major dietary constituent for our existence and, in the agricultural, chemical, medical and mining industries (National Water Act, 1998). South Africa is a relatively arid country with an average annual rainfall of 487mm compared to the world average of 860mm. Currently approximately a third of the country receives 300mm annually and this has led to imbalances between supply and demand (Basson & Van Rooyen, 2001). Much of the water that is available for reuse is in poor condition and with just over 12 000kL of available freshwater for each person per year, the country is on the threshold of the internationally used definition of 'water-stress' (National Water Act, 1998). Also, many of the largest and fastest growing water users are found at the beginning of small erratic streams rather that at the end of large reliable rivers. This limits the amount of water available in these areas. The major river systems such as the Orange, Limpopo and Komati are shared with neighbouring countries and this has led to a decrease in the amount of available water (WRC report 203/1/90). It has been estimated that South Africa will reach the point of maximum utilization of all water resources in 2025 (Odendaal, 2001).

Due to a number of mining industries concentrated in urban South Africa, mainly in the former Pretoria-Witwatersrand-Vereeniging complex, the demand for water in this area is high (Ramsingh *et al.*, 1998). The need for quality water is more severe as there is an increase in the number of people moving to the urban areas (Department of Water Affairs 1986). Therefore, the waste from human and economic activity is concentrated where it has the most severe impact. There is also a high salinity as well as total dissolved solids (TDS) introduced to the Vaal River (Department of Water Affairs and Forestry, 1987). This has a negative impact as the Vaal serves as the largest source of water in the Gauteng area, and this means that there is substantial contamination of the fresh water resource (Department of Water Affairs and Forestry, 1986; Boshoff, 1999).

The National Water Act recognizes the fact that water is a scarce commodity, which is unevenly distributed in the country and that there should be measures in place to protect the water quality if the national water resources are to be sustained (National Water Act, 1998).

1.2. Acid Mine Drainage

During mining activities oxygen and water are introduced to the deep geological environment resulting in the oxidation of reduced minerals. When reduced minerals are brought to the surface, iron disulphide (pyrite), which is ubiquitous in most metal sulphides and coal deposits, undergoes a cycle of reactions during oxidation. These result in the production of ferrous iron, sulphate, and acid. The equation that represents the pyrite oxidation according to Davison *et al.*, (1989):

$$4FeS_2\,(s) + 15O_2\,(aq) + 10H_2O \rightarrow 4FeO\,(OH) + 8SO_4^{2-}\,(aq) + 16H^+\,(aq) \qquad (1)$$

pyrite acidity

Other sulphide minerals will react with oxygen and water in a similar manner and release other heavy metals and sulphate. The presence of acidophilic sulphide-oxidizing bacteria, in particular, *Thiobaccilus ferroxidans,* which thrives at pH range 1.5-3.0, catalyzes the above equation (Banks *et al.*, 1997). The water released from waste heaps and underground is usually characterized by low pH, high salinity (sulphate) and a high concentration of heavy metals. This is often referred to as Acid Mine Drainage (AMD). If drained into the land or aquatic systems, it can lead to adverse effects on the aquatic life and the soil (Banks *et al.*, 1997; Jarvis 1999).

In South Africa**,** the gold bearing reefs on the East Rand have a shallow basin-like structure. During the mining of these reefs, water flowed into the mine. To prevent flooding of shafts, the water was pumped to the surface to be treated (Scott, 1995). Although mining has now ceased at many of the mines, AMD is still pumped to prevent uncontrolled decanting in nearby towns. Between 1988 and 1991, Grootvlei shafts 3 and 4 were pumping 110ML.d^{-1} (Scott, 1995; Grootvlei (Pty) Mines Ltd, 1997). This water is currently being treated using the high-density sludge (HDS) process that removes heavy metals. To date, the iron concentration in the water discharged into the Blesbok Spruit has been decreased to less than 1mg.L^{-1}. There are still

problems with the high salt content of the water, which is made up mainly of sulphate. The HDS process is very expensive and produces a high volume of metal-rich sludge that requires disposal (Pulles, *et al.*, 1995). An estimated 200ML.d^{-1} of mining effluent is discharged into the streams of Gauteng province, representing a sulphate load of 73 000 t.$year^{-1}$ (Maree *et al.*, 1989). As a result, remediation of AMD has become a topic of interest for many scientists. Current remediation technologies may be either physico-chemical or biological.

1.3. Physico-chemical treatment methods

Attempts to remediate AMD have focussed on reducing the metal and salt concentration as well as increasing the pH of the water in the most cost-effective way. The ideal remediation technology should generate small amounts of sludge (Gazea *et al.*, 1996), and be suitable for remediation of large volumes of water.

Traditionally, chemical treatment of AMD has involved the use of lime (calcium hydroxide ($Ca(OH)_2$) and calcium oxide (CaO)) to raise the pH. These processes normally precipitates metals as metal hydroxides and complexing the sulphate present with calcium to form gypsum ($CaSO_4$ $2H_2O$) (King *et al.*, 1975; Maree *et al.*, 1996; Elliot *et al.*, 1998). Disadvantages of this treatment method include the high cost of reagents, large volumes of sludge produced (1tonne lime: 1tonne sludge) (Bunce *et al.*, 2001), need for accurate dosing as well as the disposal of gypsum and metal hydroxides. Limestone has also been utilized as it provided 29% reduction in cost as compared to lime treatment (Henze & Pieterse, 1978; Whittington-Jones, 2000). An advantage of limestone is that it only dissolves when water pH is below 7, overcoming the dosing problem of lime (Maree *et al.*, 1996). However, the metal precipitates coat the limestone surface ultimately inhibiting further precipitation (Sazowsky *et al.*, 2000). Other chemical treatment methods include the use of a fluidized bed to prevent coating of limestone (Cole *et al.*, 2001) and anoxic limestone drains (ALD) have been used to avoid the precipitation of iron hydroxides (Hamilton *et al.*, 1997; Younger, 1998a; Johnson, 2000). Evangelou (2001) has reported the use of pyrite microencapsulation to control pyrite oxidation. Electrodes have been considered in order to drive out unfavourable chemical reactions (Bunce *et al.*, 2001). Membrane filtration systems have been considered for the treatment of AMD (Schoeman & Steyn, 2001). Most of these systems are still being proven. Limitations to chemical treatment methods include the high cost of purchasing chemicals, particularly where the mines will decant for a long time after closure. There is also a large amount of sludge produced and disposal and

maintenance can be very expensive. As a result focus has increasingly been placed on biological treatment systems.

1.4 Biological Treatment Processes

Biological treatment systems offer a number of advantages over the traditional chemical treatment methods. These include a reduced capital input, potentially lower cost reagents, reduced labour requirement and the small amount of sludge produced can be handled more easily. These treatment methods depend on the use of bacteria, fungi and plants for the remediation of AMD (Johnson 1995; Maree *et al.*, 1996; Younger *et al.*, 1997). Both passive systems utilizing wetlands and trench reactors (Robinson & Robb, 1995; Van Zyl, 1996; Johnson, 1995; Gazea *et al.*, 1996; Younger *et al.*, 1997; Banks *et al.*, 1997), and active systems utilizing specially designed bioreactors have been described (Johnson, 1995; Rose *et al.*, 1998; Boshoff, 1999).

Passive treatment systems rely on naturally occurring biological processes that require no external energy input, including ammonification, denitrification and the reduction of iron and sulphur (Boshoff, 1999). Impermeable organic covers are also used to cover sites of AMD generation in order to prevent the introduction of oxygen and water. The use of an organic cover such as compost results in anaerobic decomposition, which produces humic substances that associate with metal hydroxides and oxides to form an impermeable waterproof layer on the rock surface (Peppas *et al.*, 2000). Natural and constructed wetland treatment systems have become increasingly popular over the last two decades (Johnson, 1995). These systems are sought after as possible long-term, self-sustainable and cost-effective solution for the treatment of large volumes of water (McGiness *et al.*, 1997; Younger *et al.*, 1997). These will require a large surface area (Rose *et al.*, 1998). They can either be aerobic or anaerobic and are sometimes used in conjunction with anoxic limestone drains (ALD). Also, there is little or no day-to-day process control requirement and the system can provide environmental benefits such as diversified wildlife habitat (Edwards *et al.*, 1997). This idea was realized when AMD was passed through sawdust and an increase in pH was noted, while the concentrations of sulphate, iron and heavy metals were reduced (Tuttle *et al.*, 1969). Wetlands have a complex ecosystem which modify the water chemistry by a number of mechanisms including precipitation by reductive or oxidative processes, dilution, and increase in pH, adsorption of salts and metals as well as uptake of biomass. The presence of macrophytes and algae play an important role of

supplying the water with oxygen to be used in the oxidation of metal ions and for the use of aerobic bacteria to degrade organic waste material (Kalin *et al.*, 1991; Johnson, 2000).

Anaerobic wetlands generate alkalinity from microbial reductive processes, which lead to acid neutralization, however, the effectiveness of this treatment has been variable and unpredictable due to seasonal variations (Mays & Edwards, 2001). Following the success of a pilot-scale wetland in 1994, a full-scale anaerobic constructed wetland was constructed in 1997 in the UK, for the remediation of spoil tip drainage from the abandoned Morrison Busty Colliery, County Durham (Younger *et al.*, 1997). Waste manure and compost were used as the main substrate for the wetland. This substrate was contained within embankments constructed of compacted Pulverised Fuel Ash (PFA). During the first 12 months of operation, the wetland yielded a decrease in the iron and aluminium concentrations by 45% and 65% respectively, as well as an increase in pH. The performance of this wetland demonstrates the efficiency and cost-effectiveness of constructed wetland technology (Jarvis *et al.*, 1999). Based on the successes of the Busty Colliery wetland, a series of wetlands have been set up for the remediation of AMD in the UK. This includes the Pelenna Phase III wetland for the treatment of five minewater discharges. After 18 months in operation, Pelenna has yielded up to 80% iron removal and decrease in acidity (Edwards *et al.*, 1997). The Ynysarwed minewater wetland in South Wales has yielded similar results (Robins & Younger, 1997; Banks *et al.*, 1997; Jarvis, 1999).
There are, however, limitations to the biological wetland systems, including the need for a suitable carbon source, the availability of land and saturation of metals in the wetland which leads to decreased efficiency (Gazea *et al.*, 1996). Johnson (1995) suggested that improved control would be obtained if microbial remediation of AMD would be conducted in active biological systems involving the use of specially designed bioreactors.

1.5. Sulphate Reducing Bacteria

Sulphate reducing bacteria (SRB) are found in a wide range of anaerobic environments, particularly in the anoxic sediments of freshwater and marine systems (Marty, 1981; Johnson, 1995). These include waterlogged soils (rice fields and tidal zones), polluted environments such as sour whey digesters, spoiled foods, anaerobic purification plants, sewage plants, and subterranean water systems and oil-bearing formations. SRB are obligatory anaerobes classified primarily on the basis of their nutritional and morphological characteristics. Their classification

is supported by some chemical criteria, such as the GC content of the DNA, and the presence of special pigments (Widdel, 1988).

They function optimally at a temperature of 30^{o}C, however, they have also been reported to grow over a broad temperature range between 0^{o}C and 98^{o}C (Postgate, 1984; Radchenko & Tashirev, 1991). Their optimum pH is 7-8 but they have also been reported to go up to pH 8, although acidophilic forms have been reported, they are generally inhibited below pH 6 and above pH 9 (Heukelekian & Balmat, 1959; Widdel, 1988; Barnes *et al.*, 1995). The neutrality of pH is achieved in nature through chemical equilibrium involving precipitation and/or dissolution of carbonate and sulphide. The genera of SRB comprise of both Gram negative (*Desulfovibrio, Desulfobulbus, Desulfomaculum, Desalfobacter, Desulfosarcins, Desulfococcus*) and Gram-positive (*Desulfonema*) species (Postgate, 1984; Widdel, 1988).

The use of sulphate reducing bacteria (SRB) for the bioremediation of acidic sulphate-rich wastewater has attracted much attention in the field of biotechnology due to the fact that they share a common ability to dissimilate sulphate for energy gain. The initial step in the biological sulphate reduction pathway is the transport of exogenous sulphate across the bacterial membrane into the cell (Cypionka & Pfenning, 1986). Once inside the cell, sulphate dissimilation then proceeds by the action of ATP sulphurylase, which combines sulphate with ATP to produce the highly activated adenosine phosphosulphate (APS) molecule. APS is rapidly converted to sulphide by the cytoplasmic enzyme APS reductase and the sulphide is then released from the cell as a waste product (Gibson, 1990). The overall reaction carried out by SRB is as follows:

$$2CH_2O + SO_4^{2-} \leftrightarrow 2HCO_3^{-} + H_2S \qquad (2)$$

Under anaerobic conditions, SRB oxidize simple organic compounds using sulphate as the terminal electron acceptor to generate hydrogen sulphide and bicarbonate ions. The hydrogen sulphide reacts with heavy metals in the AMD to precipitate them from solution as metal sulphides. This can be illustrated in equation 3 below.

$$H_2S + M^{2-} \rightarrow MS\ (s) + 2H^{+} \qquad (3)$$

Where M represents metals including Cd,Fe,Pb and Zn. As a result, the bicarbonate ions react with protons to form CO_2 and water, thereby neutralising the acidity in solution. This is illustrated in the equation 4 below.

$$HCO^{3-} + H^{+} \rightarrow CO_2\,(g) + H_2O \qquad (4)$$

H_2S, being a strong reducing agent, can inhibit the growth of certain aerobic microorganisms (Widdel, 1988). It plays an important role in the natural environment in that it functions as an electron donor for the growth of sulphur bacteria (Gibson, 1990). However, it has a distinctive smell and in addition to odour nuisance it is toxic to plants, animals and humans (Postgate, 1984). The rate of sulphate reduction may be influenced by the quantity of the suitable carbon and environmental conditions under which the cells are growing such as temperature and salt concentration of the medium (Postgate, 1984; Widdel 1988).

Maree *et al.,* (1989) described a process whereby sulphate-rich water, rich in heavy metals could be treated biologically for sulphate removal without prior neutralisation with alkali such as lime but through alkalinity produced during the biological sulphate reduction process. By contacting metal-containing water with H_2S gas (stripped from the anaerobic reactor), heavy metals can be precipitated as metal sulphides. In addition the heavy metals can be precipitated through controlling the pH of the feed. This could be raised by re-circulation of a side stream of the highly buffered waste from the anaerobic reactor. The principle of selective recovery of heavy metals was also demonstrated by Hammack & Edenborn (1992), who showed that copper, zinc, and iron can be separated selectively by bubbling H_2S gas through three reactors, connected in series, at pH values 1.6, 3.8 and 6.2 respectively.

1.6. Electron donors for SRB

1.6.1 Simple electron donors

The SRB have a wide range of small molecular weight compounds that they can utilise as electron donors. Under anaerobic conditions SRB are able to oxidise a range of organic acids which are typical fermentation products or monomers from the degradation of cell polymers

such as lactate, propionate and butyrate, while methane producing bacteria (MPB) preferentially utilize acetate produced from the breakdown of other organic acids (Stams *et al.*, 1984; Widdel, 1988; Ueki *et al.*, 1992; McCartney & Oleszkiewicks, 1993; Aguilar *et al.*, 1995 and Whittington-Jones, 2000). The more commonly utilized of these energy sources include:

- Dicarboxylic acids: succinate, furamate, malate, oxalate, glutarate, etc.
- Alcohols: methanol, ethanol, butanol, propanol, glycerol, ethylene glycols, etc.
- Amino acids: glycine, serine, cystein, valine, leucine, aspartate, glutamate, etc.
- Miscellaneous: hippurate, fructose, benzoate, 2-, 3-, 4-hydroxybenzoate, nicotinic acid, indole (Parker *et al.*, 1994).

According to Foucher *et al.*, (2001), hydrogen is the simplest electron donor source that SRB can utilize and in a study they performed using hydrogen as an electron donor for SRB, the process was found to be slow due to hydrogen's low solubility in water. However, the major problem with using hydrogen is that unless there is a source of hydrogen readily available, the cost of production or transportation to the site of treatment makes hydrogen economically nonviable. Short chain fatty acids have been identified as the optimum electron donor source with lactate being the most suitable (Glombitza, 2001). These can be partially degraded to acetate, which if not utilized, gives the effluent a high COD. Acetate and lactate have been used by SRB for energy gain in the remediation of AMD.

Middleton & Laurence (1977) and Dicker & Smith (1985) determined the kinetics of microbial sulphate reduction in completely mixed reactors using acetate and lactate as electron donors and observed a sulphate reduction rate of 0.29g $SO_4.L^{-1}.d^{-1}$. Maree *et al.*, (1991) reported on pilot-plant studies on biological sulphate removal from industrial effluent using energy sources such as sugar or producer gas for the production of sulphide from sulphate and then used nitrogen gas for the stripping of the sulphide. They reached a conclusion that acetic acid and sugar are suitable substrates for the biological sulphate reduction, with the specific reduction rate of 0.05g $SO_4.L^{-1}.d^{-1}$. Acetate and ethanol are also utilized by SRB although not as efficiently as lactate (Canfield, 2001). Methanol also offers a cheaper alternative to lactate (Tsukamoto & Miller, 1999). Other studies have shown that they are even able to oxidise compounds that were previously thought to be either toxic or highly recalcitrant such as phenolic derivatives (Widdel, 1988; Whittington-Jones, 2000).

The major limitation in utilizing simple electron donor sources is the cost implication, especially when considering the large volumes of water associated with AMD treatment.

1.6.2 Complex carbon sources

In the light of the above-mentioned limitation in the use of simple electron donor, the use of more complex carbon sources, which are mostly waste products of other processes such as agriculture, paper production etc., has gained increasing attention as electron donor sources for SRB in the treatment of AMD. However, SRB have been reported to be unable to utilize large complex organic molecules such as proteins and carbohydrates, and are thus reliant on a combination of hydrolytic and acidogenic bacteria (figure 1.1) to provide a suitable electron donor source (Hansen, 1988, Ueki *et al.*, 1988; Molipane, 1999; Whittington-Jones, 2000). The mechanism by which complex molecules are hydrolyzed to simpler molecules is discussed in detail in section 1.8. Inexpensive carbon sources, which have been considered as carbon sources for SRB include algal biomass and tannery effluent (Boshoff, 1999; Rose *et al.*, 1998), cattle waste (Ueki *et al.*, 1988), molasses (Maree & Hill, 1989), among others.

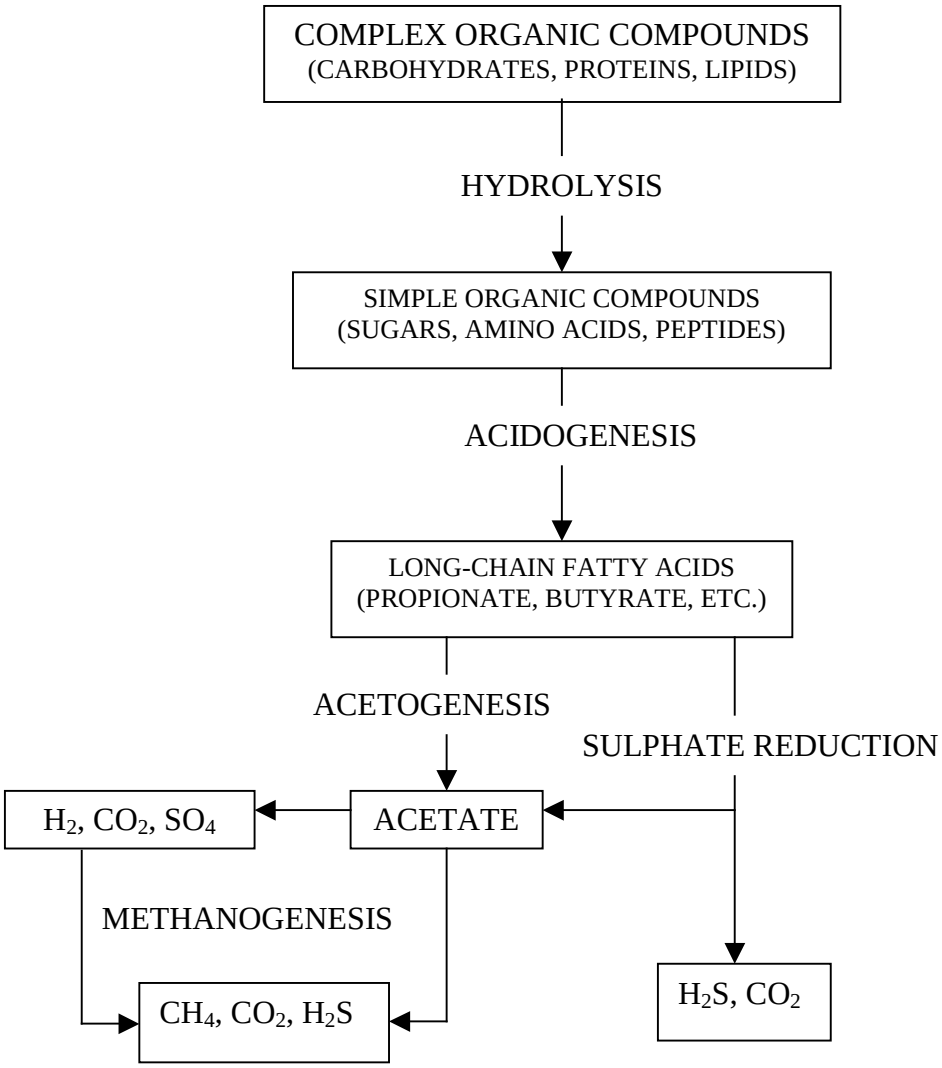


Figure 1.1 Metabolic steps and substrates involved in the anaerobic digestion of complex carbon. (Novaes, 1986; Whittington-Jones, 2000).

Substrates of varying complexity have been added to AMD in bench- and full-scale experiments to evaluate their suitability as electron donors in the bioremediation process. Christensen *et al.*, (1996) used whey (mainly lactose, proteins, ash and fat) as an electron donor in batch systems containing an influent with a sulphate concentration of 900mg.L^{-1} and at pH 4. The pH increased to 7.6 and sulphate also increased from 10mM to 60mM, which was unexpected. The sulphide produced was between 40 and 150mg.L^{-1}, showing active sulphate reduction. Mollases has also been used as an electron donor for SRB for the purpose of sulphate reduction. Upflow packed bed reactors were used by Maree *et al.,* (1986) to establish well developed microbial biofilms for sulphate removal from mine water with either sugar, pulp mill effluent or sewage sludge as

energy sources. They concluded that 1.6g spent sugar or 16.7ml of spent sulphide liquor or 172ml raw sewage sludge was necessary to remove 1800mg.L^{-1} sulphate. However, over-saturated calcium carbonate levels and unutilised carbonaceous material prevented water from being directly re-used after anaerobic treatment. To overcome this problem, Maree *et al.*, (1986) showed that a three-stage process (anaerobic-aerobic-anaerobic) employing upflow packed bed reactor and an activated sludge system for aerobic treatment could be used for producing re-usable water from mining effluents using mollasses as an electron donor. Sulphate was reduced from 2.5g.L^{-1} to less than 0.5g.L^{-1}.

There are species of SRB, which have been reported to use other types of substrates, which had not been thought of as possibilities. These include methanol degraded by an SRB species isolated from sewage sludge, which could either be *Desulfovibrio desulfuricans* or *D. vulgaris.* After growth on pyruvate, malate and furamate, ^{14}C-methanol was completely oxidized to CO_2 (Braun & Stolp, 1985). Phenol and aromatic compounds such as benzoate, phenylacetate, 2-, 4-hydroxyacetate, p-cresol, indoleantranilic acid and phenylalanine have also been shown to be used as a sole electron donor by a species of *Desulfobacterium phenolicum*. Sulphate and thiosulphate served as electron acceptors and were reduced to H_2S (Widdel & Bak, 1986). Catechol was completely oxidised to CO_2 by the sulphate reducing *Desulfobacterium catecholicum*, in a defined sulphide reduced freshwater medium. Thiosulphate and dithionate served as electron acceptors and were reduced to H_2S (Szwezyk & Pfenning, 1987).

1.7 Lignocellulose

Many industrial processes favour the use of plant biomass as an energy and raw material source due to its abundance and renewability. Lignocellulose is the most abundant of plant biomass and is composed of cellulose, hemicellulose and lignin (Pareek *et al.*, 1998). The anaerobic degradation of plant and plant-derived waste such as paper, agricultural and municipal solid waste and woody biomass has been reported in swamps and marshes, (Benner *et al.*, 1984) and landfill sites (Pareek *et al.,* 1998), with the end product being methane and carbon dioxide. When sulphate is added in sufficient quantities, the degradation is carried out under sulphate reducing conditions and methanogenesis is suppressed (Kim *et al.,* 1997). The use of lignocellulosic material as a carbon source for AMD treatment has long been recognized and various lignocellulosic materials have been tried as substrates including straw and hay (Bechard *et al.*, 1993), peat (Coleberg & Young, 1985), spent mushroom compost, wood chips and

sawdust (Chang *et al.*, 2000). Wood dust contains a range of complex organic carbon compounds such as cellulose, lignin and lignocellulose. Tuttle *et al.*, (1969) studied the removal of sulphate from solution by using the microbial reduction of sulphate to sulphide. A mixed culture of microorganisms was used and this resulted in the biological degradation of 400g.L^{-1} COD and acid mine water was biologically remediated at a rate of 72mg.L^{-1}.d^{-1} removing 590mg. L^{-1} of sulphate over the entire experimental period. The degradation products served as an electron donor for SRB. However, the actual method of breakdown of the cellulose to these products was not explained. Ueki *et al.*, (1988), reported the use of an anaerobic digester slurry of cattle waste for the reclamation of AMD. The results of this study proved cattle waste to be a good electron donor as sulphate reduction was enhanced and the pH of the water was raised from pH 4.3 to neutral.

1.8 Primary sewage sludge

Microbial reduction of sulphate to sulphide in sanitary sewerage systems is a universal maintenance problem because of the noxious odours, health hazards and corrosion mainly of concrete sewers (Nielson & Hviyved-Jacobsen, 1989). Domestic sewage in rising main sewers contains organic sulphur and sulphate, which under anaerobic conditions may be reduced by bacterial action to sulphide and other malodorous sulphur compounds (Boon & Lister, 1975). (Parker (1951), Butlin & Postgate (1953 & 1954), and the South African CSIR (1959), as cited in Boon & Lister, 1975) found that, as sewage becomes progressively more anaerobic, the production of sulphide arises initially from the action of proteolytic bacteria on proteinaceous organic sulphur compounds. Parker (1951) observed that sulphide is formed in flowing sewage, although Baumgartner (1934), Pomeroy & Bowlus (1946), and Reid & Tien-Sheng Yang (1959) suggested that most sulphide formed in bacterial slimes growing on the walls of the sewer (all cited in Boon & Lister, 1975). Nielson & Hviyved-Jacobsen (1989) have shown that the microbial reduction of sulphate takes place in the biofilm at the sewer pipe inner surface. The presence of dissolved oxygen in the sewage reduces the tendency for anaerobic conditions to develop in such slimes and results in the oxidation of any sulphide formed (Boon & Lister, 1975).

Davison *et al.*, (1989) reported the reclamation of acid water using sewage sludge. Water in an exhausted sand quarry, which had filled with acid water (pH 3) from the oxidation of pyrite, was treated with calcium hydroxide to neutralise the water (pH 8), and sewage sludge to prevent

further increase in acid. Sulphate reduction took place on the sludge bed, taking two years to remediate the AMD totally. This showed that sewage sludge had a capacity to support sulphate reduction, even though sulphate reduction takes place slowly on the sludge bed. Thus, the use of sewage sludge as an electron donor for SRB in the treatment of wastewaters has gained a lot of interest. Of the studies reported, it has proved to be an efficient and cost effective system for both AMD treatment and sewage treatment.

Over the last few years, the Environmental Biotechnology Group (EBG) at Rhodes University has studied bioremediation of AMD using sewage sludge as electron donor for SRB (Molipane, 1999; Whittington-Jones, 2000). Molipane (1999) investigated the feasibility of sewage sludge as a potential low-cost, readily available feedstock of complex particulate organic matter. This study was done in a continuous stirred tank reactor (CSTR), and primary sewage sludge (PSS) was the sole carbon source. The results of this study showed that sewage sludge could be used as an electron donor for SRB and can be added directly to AMD in an anaerobic reactor. Sulphate reduction and COD removal were 80% and 55% respectively and the pH in the reactor was increased from between 3.5 and 6.5 to 7.8. The study was scaled up to a $1m^3$ anaerobic stirred feed tank and a 400L-settling tank for recycling solids into the anaerobic reactor. There was an increase in the efficiency of sulphate reduction compared to that observed in the laboratory-scale system and this was attributed to the increased volume of the reactor where substrate diffusion was enhanced. The introduction of solids recycle led to an increase in efficiency as it provided more readily biodegradable organic particulates (Molipane, 1999). Whittington-Jones (2000) studied the effect of sulphate reduction and reactor design on the hydrolysis of primary sewage sludge (PSS). This work led to the development of the Reciprocating Sludge Bed Reactor (RSBR) concept and demonstrated its efficiency compared to the conventional anaerobic digestion systems such as the upflow packed bed reactor. The results of this study again showed that the solubilization of PSS is enhanced under sulphate reducing conditions.

1.9. Hydrolysis of complex carbon sources

Because SRB are known to rely on the products of hydrolytic bacteria such as short-chain volatile fatty acids (VFA) (Widdel, 1988), a large portion of the energy of PSS remains trapped in complex molecules until freed by hydrolytic and acidogenic bacteria. Thus, the hydrolysis of sewage sludge under anaerobic conditions without the effect of SRB is slow, and this was

thought of as the result of the high concentration of mineral oil in PSS (0.2-0.5 $g.L^{-1}$). The significant proportion of the readily available COD has been suggested to be trapped by semi-recalcitrant cell components such as cell walls (Ghosh, 1991) thus cell lysis or hydrolysis would be required before the cell components could be utilized by acidogenic bacteria.

Anaerobic digestion is usually thought of as a multi-step process in which complex organic material (protein, lipids, carbohydrates) is cleaved to smaller compounds such as sugars, amino acids and peptides. These are further cleaved during acidogenesis to short-chain acids by facultative bacteria, then to methane and carbon dioxide by methane producing bacteria (Ohron *et al.*, 1999). The first step in the decomposition of these polymers to smaller intermediates is catalyzed by enzymes secreted by microorganisms, is generally regarded as the rate-limiting step in the microbial mineralization of organic matter (Novak & Ramesh 1975, Benner *et al.*, 1984; Vavillin *et al.*, 1996; Penaud *et al.*, 1997), and is described by first order kinetics (Shimizu *et al.*, 1993). On the other hand, the processes of acidogenesis and acetogenesis are relatively rapid (San Pedro *et al.*, 1994), and their rates may be strongly influenced by environmental and operational parameters including pH, temperature, microbial mass, particle size, and product concentration (Eastman & Ferguson, 1981).

Recent studies have shown that the performance of biological nutrient removal (BNR) processes may be enhanced by the addition of the soluble products of hydrolysis of PSS (Brinch *et al.*, 1994; Skalsky & Daigger, 1995; Canzain *et al.*, 1996; Banister & Pretorius, 1998). However, their success has been limited by the fact that, optimization of PSS hydrolysis for the production of large quantities of soluble product has not been used for biological sulphate reduction. Current understanding of the microbial interactions involved in the solubilization of complex carbon sources, especially under sulphate reducing conditions, is lacking. This information is essential for the production of soluble carbon from complex substrates for driving a range of biological processes. Literature on anaerobic digestion shows that in the environment, there is a consortium of microorganisms, which individually excrete enzymes necessary for anaerobic digestion. These enzymes and therefore, microorganisms rely on each other and also work together to achieve the desired results (Zendher, 1988).

Reports on the rate of hydrolysis and yields of primary sewage sludge are limited, and comparison of published figures is complicated by the difference in the criteria used to measure these rates (Whittington-Jones, 2000). Some calculations have been based on the production of

ammonia (Henze & Mladenivski, 1991), COD and VFA concentrations (Eastman & Ferguson, 1981; Lilley *et al.*, 1990; Banister & Pretorius, 1998), biogas production (Siegrist *et al.*, 1993), as well as the removal of particulate volatile suspended solids (VSS) (Shimizu *et al.*, 1993). Calculation of the rate of hydrolysis from the production of soluble products may be misleading (Whittington-Jones, 2000) as the processes of production and removal of these products take place rapidly and the measured concentration of these products is therefore the net result of production and utilization. Also, as hydrolysis of PSS is the rate-limiting step, the process of acidogenesis and internalization are relatively fast (San Pedro *et al.*, 1994). Thus the rates calculated by this method probably describe the hydrolysis/acidogenesis step rather than hydrolysis alone. Furthermore, the rapid utilization of soluble products such as VFA could lead to underestimates of the rates and yields of the combined process. Thus the rates calculated from the removal of particulate VSS (San Pedro *et al.*, 1994) may be more accurate.

Shimizu *et al.*, (1993) reported that different molecular components of PSS are degraded at significantly different rates, where, lipids and cellulose were hydrolyzed relatively slowly, at 0.76 and 0.52.d^{-1} respectively. Proteins and carbohydrates on the other hand were both degraded at approximately 1.2.d^{-1} and less complex organics such as starch (San Pedro *et al.*, 1994) and the organics released from sonicated activated sludge (Shimizu *et al.*, 1993) exhibited higher hydrolysis rates, with values 3.28 and 1.2d^{-1} respectively. Thus the rate of hydrolysis describes the overall rate of degradation of PSS, which will be influenced by the relative concentrations of slowly and more rapidly degradable compounds. Reported yields from hydrolysis of PSS range from 5% under psychrophilic conditions (Canziani *et al.*, 1996) to around 35% at 24^{o}C (Hatziconstantinou *et al.*, 1996). Although yields are affected by environmental and operational parameters, reported yields are generally low, with the average being less than 20%.

In an investigation of the effect of sulphide on the solubilization of PSS by Whittington-Jones (2000), the presence of sulphate reduction, sulphide, alkalinity and recycling, which form an integral part of the RSBR, were found to play a significant role in the reduction of floc size as compared to the non-sulphidogenic system. A descriptive model was developed to relate this phenomenon to enhanced solubilization. A three-stage reactor proved to be substantially better than the conventional single stage reactors, with a total sulphate reduction of 59%.

In the scale up experiments undertaken at the Grootvlei Gold Mine on the East Rand Mining Basin, a 23m^3 RSBR was employed to investigate performance in terms of solubilization of PSS

in a sulphate-rich environment, as well as to characterize the spatial variation within the reactor. This consisted of three principal stages namely sludge solubilization, sulphate removal, and polishing. Supplementary sulphate reduction was carried out in the Anaerobic Baffled Reactor (ABR) and was followed by polishing in two high-rate algal ponding systems. Performance of the RSBR showed a mean removal of COD and sulphate of 57.9% and 31.4% respectively. The mean pH increased to 7 from 6.4 in the influent. The minimum percentage solubilization of 52% was achieved for PSS in the pilot-scale reactor, although this was considered to be an underestimate by Whittington-Jones (2000). This yield is significantly higher than the previously reported values for this carbon source.

The sulphide-enhanced hydrolysis model developed from this study showed that the process relies on the reduction of floc stability in the presence of sulphide as a result of increase rate of hydrolysis of lignin, carbohydrates and protein. Deflocculation and mixing, which are essential led to the exposure of inaccessible macromolecules to cleavage by hydrolytic enzymes. The configuration and operation of the reactor plays a significant role in the increased contact between particle bound enzymes and substrates, retention of biomass and enzymes, removal of soluble products and the maintenance of active sulphate reduction in close proximity to the hydrolytic reactions (Whittington-Jones, 2000).

Thus, although hydrolysis of PSS is enhanced in the presence of SRB that then use the end products of the complex carbon for energy gain. The actual breakdown mechanism has not been fully understood. A number of different techniques have been employed in an effect to understand the complex interactions involved in the solubilization of complex carbon sources.

In aquatic environments such as sediments and with sewage sludges, an advance in studies of microbial degradation of these complex carbon compounds has been achieved using ^{14}C-labelled plant preparations under aerobic conditions. Coleberg & Young (1985) did some of the initial work and examined the mineralization of lignocellulose in the anaerobic sediments of an arctic lake. They used [^{14}C-labelled] lignocellulose, and nitrogen to stimulate oxygen depleted conditions. In this study they found that 16–23% of the labeled cellulose was mineralized to $^{14}CO_2$ and $^{14}CH_4$. Their final postulation was that anaerobic cellulolytic bacteria grow preferably when there is a decline in activity of the aerobic cellulolytic population. Thus nutrient availability controls turnover. Benner *et al.,* (1984) made a contribution in this field by conducting long-term studies in the wetlands of a salt marsh, freshwater marsh and a mangrove

swamp. Over a period of 40 weeks, samples were collected and analyzed, the highest conversion obtained under anaerobic conditions was 30% of the initial ^{14}C.

Indirect methods have also been used to study the initial decomposition of the material. In these studies, the activities of extracellular enzymes in the environment were assessed by the addition of artificial substrate at saturation concentrations. These activities should be viewed only as potential values, as the concentration of the substrate was above natural levels, as its availability to the enzyme and its interactions with other polymers, is not taken into consideration (Boshker & Cappenberg, 1994).

Other studies have looked at the use of radiolabelled compounds, by studying the pool of intermediates released during initial decomposition. However, these have proved problematic as not all the intermediates formed will be freely available in radiolabelled form and the concentration of free bio-available intermediates might be hard to assess (Henrichs, 1986). Thus the actual degradation of complex carbon to yield the necessary soluble products to be utilized by SRB, in order to drive the process of sulphate reduction and subsequently to remediate AMD, has not been fully identified.

The use of inhibitor studies has proved to be an insightful option for explaining the hydrolysis/acidogenesis, as well as the acetogenesis, processes within complex systems in isolation, and also for the determination of the rates and yields for each. In 1995 Boschker *et al.*, used a study in which initial decomposition rates of naturally occurring polysaccharides could be measured as they occur in the natural environment or sediment. Their study applied a method of the selective inhibition of microbial carbohydrate uptake using toluene, without affecting the extracellular hydrolysis of the polysaccharides in the samples under study. The hydrolysis products would then accumulate in the reactors, and these were monitored over time using high performance liquid chromatography (HPLC).

Sodium molybdate is known to inhibit sulphate reduction and H_2S production (Banat *et al.*, 1981; Postgate, 1984; Clancy *et al.*, 1992). Being stoichiometrically similar to sulphate, molybdate may compete in biochemical reactions such as membrane transport (Peck, 1959). Molybdate can act as an inhibitor of ATP sulphurylase, the key enzyme in the pathway of sulphate reduction (Ranade *et al.*, 1999). Work has been reported using batch digestion of

synthetic distillery waste (Karhadkar *et al.*, 1987). Wu *et al.*, (1991) and Ranade *et al.*, (1999) have shown that sodium molybdate inhibits SRB without any significant inhibition to fermentative bacteria. Thus inhibition method can therefore be useful to study the acidogenesis and acetogenesis processes, by comparing the rates of VFA uptake in the hydrolysis of PSS (Ranade *et al.*, 1999).

Inhibitor studies can therefore provide further insight into the mechanisms of enhanced hydrolysis of complex carbon sources such as PSS as it would be possible to follow accumulation/production of intermediates formed during the solubilization process. Based on their findings, it would be possible to study the process of acidogenesis and acetogenesis in isolation. This would further enable the rates of production to be determined.

1.10 Research hypothesis

Based on the above, it is proposed that the presence of active biological sulphate reduction, results in the enhanced hydrolysis of primary sewage sludge and a subsequent increase in the rate of production of soluble products. Enhanced hydrolysis is related to the ability of SRB to utilize a greater range of soluble products than MPB, and potential negative feedback effects on the hydrolytic and acidogenic steps, associated with soluble product accumulation in methanogenic systems, are reduced.

1.11 Research objectives

A number of objectives were identified in order to investigate the above hypothesis:

- To investigate comparative rates of carbon utilization when PSS is used as an electron donor for SRB or MPB;
- To compare the relative rates of PSS hydrolysis and the yield of soluble products such as carbohydrates and VFA under comparable methanogenic and sulphideogenic conditions;
- To investigate the rates of utilization by SRB and MPB of the soluble products of PSS hydrolysis;
- To investigate the distribution of carbohydrates under both methanogenic and sulphidogenic conditions respectively;

- To develop a descriptive model based on the processes involved in the enhanced biological hydrolysis of PSS, clearly describing the steps involved and the role that is played by different populations in the microbial consortia applied.

Chapter 2

The Enhanced Hydrolysis of Primary Sewage Sludge under Bio-Sulphidogenic Conditions

2.1 Introduction

Success in the use of organic substrates for a range of biological processes, such as sulphate reduction, biological nutrient removal (BNR) and the bioremediation of acidic wastewater, has been limited by their cost and availability. In South Africa, current disposal methods of sewage have proved to be expensive and it has considerable potential of being used as a successful electron donor by SRB (Molipane, 1999). A major limitation is the inefficient hydrolysis of the complex substrate and thus there is a need for understanding and subsequently controlling the hydrolysis process (Whittington-Jones, 2000). Operational parameters such as mixing (Banister &Pretorius, 1998), temperature and pH (Banerjee *et al.,* 1998), and reactor design (Skalsky & Daigger, 1995) have been shown to influence the rates and yields of the hydrolysis process. These factors need to be considered when optimizing the yield of soluble product from a complex carbon source.

Molipane (1999) investigated the use of PSS as a carbon source for sulphate reduction and demonstrated the removal of sulphate (85%) and COD (65%) in a $1m^3$ stirred tank reactor. These results together with insights into the mechanism of enhanced hydrolysis led to the development of the Recirculating Sludge Bed reactor (RSBR) (Whittington-Jones, 2000). This reactor is reported to have advantages over conventional reactors such as the anaerobic baffled reactor (Barber & Stuckey, 1999) and the upflow anaerobic sludge blanket (UASB) (Lens *et al.,* 1998), as it is relatively inexpensive to construct and operate. The results of this study yielded a total sulphate reduction of 59% and COD removal of 67% and the conclusions drawn were that:

- Sulphate reduction has a positive effect on the solubilization of PSS
- The development of the RSBR could lead to improved COD removal and sulphate reduction.

In terms of the hypothesis proposed in Chapter 1, this study aimed to investigate the use of PSS as an electron donor for biological sulphate reduction in a more controlled environment, and to confirm the results obtained by Molipane (1999) and Whittington-Jones (2000). Preliminary studies into the effect of sulphate reduction on two organic fractions, namely volatile fatty acids (VFA) and reducing sugars, will be conducted to assess the viability of using these parameters to gain a greater understanding of the mechanism underlying hydrolysis.

2.2. Methods

2.2.1. Experimental procedure

A 5L-covered conical flask fitted with a zinc acetate trap for sulphide (Figure 2.1) was used to generate sulphate-reducing biomass for the study. The carbon source, PSS, was obtained from the Grahamstown Municipal Works and was sieved through a 2mm mesh before being diluted with analytical grade Na_2SO_4 (sulphate-rich water) to yield a total sulphate and COD concentration of 2000mg.L^{-1} which was adopted from previous studies (Whittington-Jones 2000; Molipane 1999) respectively. A seed of SRB from an existing sulphidogenic bioreactor that had been running for three months was used to inoculate the cell generator. A control methanogenic reactor was also set up in a similar manner to the above without sulphate-rich water or the SRB seed to generate methanogenic biomass. These reactors were kept in a constant environment (CE) room at 25^{o}C and shaken on a Labcon desktop shaker at 100rpm. For experimental preparations 500ml conical flasks were set up in triplicate and kept under the same environmental and operational conditions as the cell generator using fresh PSS as a carbon source. These were seeded with 10% inoculum from the ongoing cell generators. In order to maintain anaerobic conditions in the flasks, aluminium foil was used to exclude light, syringes were used for sampling purposes and a sealed nitrogen injection port was created. The flasks were sampled every 2 days and were flushed with nitrogen for 3 minutes to expel any oxygen present before re-sealing.

In order to determine the maximum solubilization of the PSS under methanogenic and sulphidogenic systems, the flasks were analyzed until the COD concentration reached a minimum. This period was 12 days (Figure 2.2).

Figure 2.1 5L-batch reactor used as cell generator showing syringes for sampling and nitrogen flushing, with an adjoining smaller flask containing zinc acetate to trap sulphide.

2.2.2 Analytical procedures

Triplicate samples were drawn and the following analytical procedures were used.

2.2.2.1 COD

A Merck® spectroquant test kit was used to determine the total and filtered COD of the reactor. Before the analysis, all samples were acidified with 32% HCl to pH 2 as modified by Whittington-Jones (2000) in order to eliminate any sulphide interference with the assay. To measure the total COD (CODt), macerated samples were diluted 10 times. For the determination of filtered COD (CODf), samples were centrifuged in an Eppendorf centrifuge 5415D rotor F45-24-11at 3000 rpm for 5 minutes, and the supernatant was then passed through a 0.45mm nylon

filter. The COD of the filtrate was then determined. A blank was prepared using deionized water in place of the sample. Particulate COD (CODp) was calculated as the difference between CODt and CODf.

2.2.2.2 Sulphate

An HPLC anion method applying a model 510 Waters HPLC and model 430 Waters conductivity detector fitted with a Hamilton PRPX 100 150X4.1 mm column was used for the determination of sulphate concentration. Before the analysis, all samples were acidified as above then centrifuged as with COD samples. A ten-fold dilution of the sample was prepared using milli-Q water and then filtered through a 0.45mm nylon filter before passing it through two Waters sep-pak® light C18 cartridges to remove the organics. The samples were then injected into the HPLC and run at $2mL.min^{-1}$. A 100ppm Na_2SO_4 standard was prepared in order to determine the retention time of the sulphate peak as well as to standardise the accuracy of the instrument. From the sulphate standard curve, the concentrations of the samples could be determined (Appendix III, figure A.3.3).

2.2.2.3 Sulphide

A modified turbidometric sulphide determination method was employed (Du Bois *et al.*, 1956). Samples were collected into test tubes containing 100μL of a 0.1M zinc acetate solution to prevent sulphide from escaping. Samples were then centrifuged in an Eppendorf centrifuge 5415D rotor F45-24-11 at 3000rpm for 5 minutes and the supernatant was collected. Solutions A and B (Appendix I, 3.1 & 3.2) were then added to the supernatant and the absorbency was determined using a Beckman DU® UV/VIS spectrophotometer at 670nm, and the concentration was determined from the sulfide standard curve (Appendix III, figure A.3.2).

2.2.2.4 Reducing Sugars

A modified Smoggy-Nelson method (Saeman *et al.*, 1963) was employed for the determination of reducing sugars. A sample was collected into a test tube to which a Nelson solution (Appendix I, 2.1) was added. After 20 minutes of boiling at 90^oC an arsenomolybdate solution (Appendix I, 2.2) was added to the reaction tube. The reducing sugar absorbencies were

determined using a Beckman DU^{R} UV/Vis spectrophotometer at 520nm. The reducing sugar concentrations were then determined from the glucose standard curve (Appendix III, figure A.3.1).

2.2.2.5 Volatile fatty acids

A modified steam distillation and titration method (APHA Standard Methods, 1994) was used to determine the total volatile fatty acids in the reactors. See Appendix II, 5.5 for method.

2.2.2.6 Statistical analysis

Statistica version 6.0 was used for statistical analysis of the recorded data. The ANOVA method was used based on the determination of sample normality. If not normal, the data was transformed using $\log_{10}$ (concentration) or /(concentration) to give a normal distribution pattern. Due to small sample size in other cases, the data was statistically analyzed based on the assumption that data recorded followed a normal distribution pattern. The statistical data used in the text includes (p) significance value set at $p<0.05$ and amount of samples (n).

2.3 Results and Discussion

The aim of the initial study was to determine the maximum percentage removal of 2000mg.L^{-1} COD that could be achieved under sulphidogenic and methanogenic conditions, and the length of time required to achieve this. These results (Figure 2.2) provided initial evidence that the removal of COD was significantly higher (ANOVA, $p<0.001$, $n=72$) under sulphidogenic conditions. Separate experiments were then set up to examine other parameters of the system including CODp, CODf, sulphate sulphide and pH. Flask experiments were set up similar to the initial experiment.

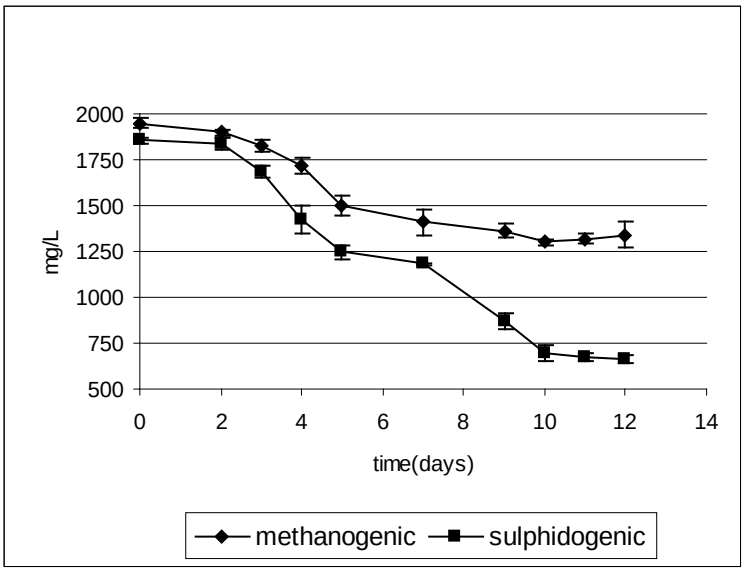


Figure 2.2 Total COD concentration showing the maximum solubilization of PSS in the methanogenic and sulphidogneic reactors. [I] indicates standard deviation.

Total COD removal of 63% and 31% was recorded for the sulphidogenic and methanogenic systems respectively over a period of 12 days. The removal of COD was slow in the first two days for both systems as reactor conditions acclimatised. An increase in COD removal was noted between days 2 and 5 and this was the highest removal over the entire experimental period for both systems with the sulphidogenic system removing 750 $mg.L^{-1}$ and the methanogenic system removing 500 $mg.L^{-1}$. This result shows that under high COD concentration, there is a high initial COD removal, which decreases afterwards. This could be attributed to the ready availability of carbon matter and this is taken up quickly. After day 5, both systems showed a decrease in the rate of COD removal, and this trend continued to day 12 in the methanogenic system. However, removal in the sulphidogenic system increased again between days 7 and 10, after which time very little removal was observed. This decrease in the removal rate after day 5 was probably a result of the more complex carbon being degraded in order to provide simple carbon matter to the system. Although COD remained in both systems after day 12, results indicate that this fraction was only very slowly degradable or refractory. The maximum percentage removals were 31% for the methanogenic system and 66% for the sulphidogenic system, with the refractory component being 69% and 34% for the methanogenic and sulphidogenic systems respectively. The rates of COD removal were $50mg.L^{-1}.d^{-1}$ for the methanogenic system and $102mg.L^{-1}.d^{-1}$ for the sulphidogenic system indicating that the rate of removal of COD is significantly higher under sulphidogenic as compared to methanogenic conditions. The trend demonstrated in this experiment confirms the preliminary findings of

Molipane (1999) and Whittington-Jones (2000), who had shown enhanced degradation of PSS under sulphate reducing conditions compared to non-sulphate reducing conditions.

Following determination of the degradation pattern outlined above, separate experiments were set up to examine CODp, CODf, sulphate, sulphide and pH. From the results of figure 2.2, it was deduced that the operational time for the experiments would be 7 days as the most COD removal in the methanogenic system took place within 7 days. The refractory COD left after day 7, took longer to degrade. This was 65% in the sulphidogenic system and 35% in the methanogenic system. To determine the methanogenic and sulphidogenic status of the systems, sulphate and sulphide were monitored and these results are reported in figures 2.3 and 2.4.

Sulphate reduction was measured in both systems (Figure 2.3). The rate of sulphate reduction from an initial concentration of 2000mg.L^{-1} was slow for the first 2 days and then increased after day 2 until day 7. According to Lens *et al.*, (1995), 2mg of COD is required to reduce 1mg of sulphate, thus the sulphidogenic system needed 1500mg of soluble COD to reduce the 750mg.L^{-1} of sulphate over the 7 day period. This therefore implies that 214mg of soluble COD was required per day to give the recorded overall percentage sulphate reduction of 39% over the 7 day period. The methanogenic system contained a minimal amount of sulphate, and this would not have affected the results of the experiment significantly.

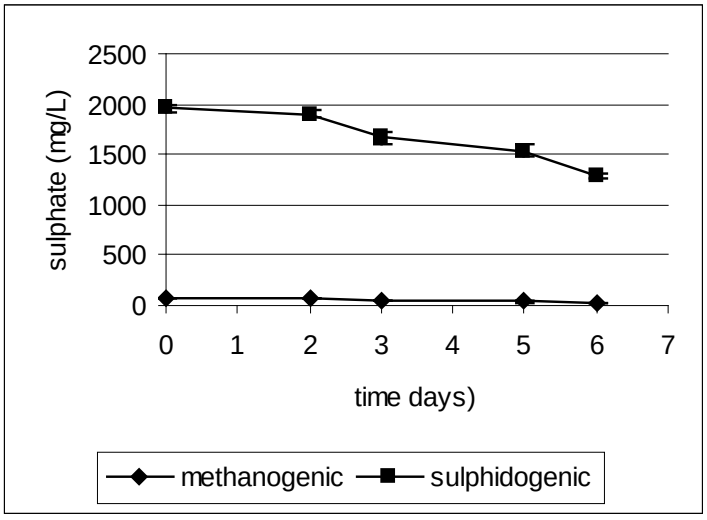


Figure 2.3 Total concentration of sulphate reduction observed in the methanogenic and sulphidogenic reactors.

The production of sulphide (Figure 2.4) also began after day 2, confirming the onset of active biological sulphate reduction. The maximum sulphide produced from sulphate reduction was 533mg.L^{-1} at day 5. The methanogenic system also showed a production of sulphide reaching the maximum concentration of 109mg.L^{-1} at day 7. This has been attributed to other bacterial activities including the reduction of sulphate that was initially present in the sewage sludge. Thus, although sulphide production did take place in the methanogenic system over the experimental period, it was low. It was assumed that the reactions within the flasks were representative of those in the methanogenic anaerobic digester.

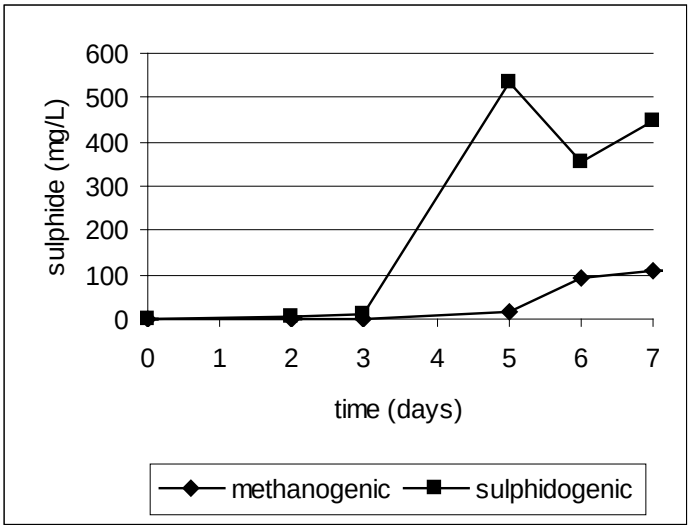


Figure 2.4 Total sulphide concentration of the methanogenic and sulphidogenic reactors.

Total COD was also measured from the same set of flasks (Figure2.5). The trend noted was similar to that observed in figure 2.3, where there was an overall significant percentage CODt removal (ANOVA $\log_{10}$ CODt, $p<0.05$, $n=36$) of 28% and 36% for the methanogenic and sulphidogenic system respectively. The rate of CODt removal was 91mg.L^{-1}.d^{-1} for the methanogenic system and 113mg.L^{-1}.d^{-1} for the sulphidogenic system. CODp and CODf were also measured for the methanogenic and sulphidogenic systems. The results are depicted in figures 2.6 and 2.7.

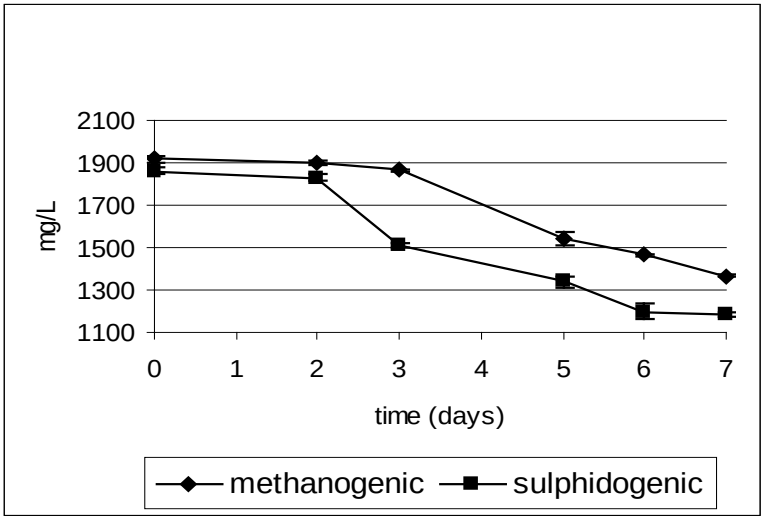


Figure 2.5 Concentrations of the methanogenic and sulphidogenic systems showing the removal of total COD from the reactors over a period of 7 days.

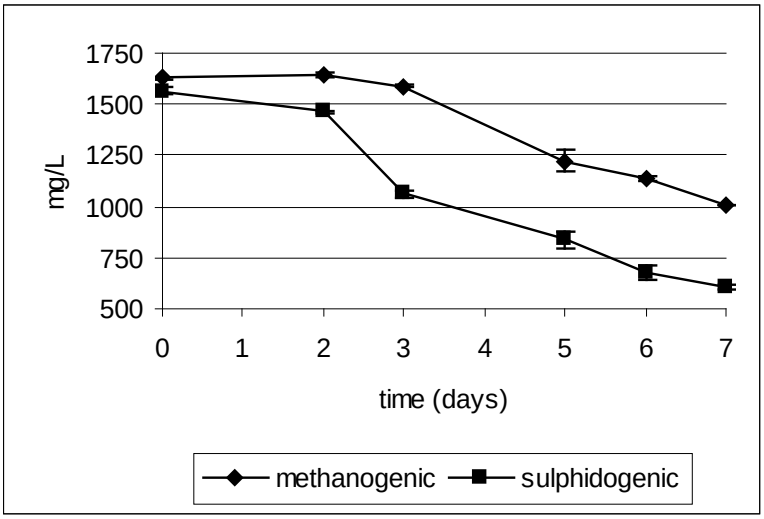


Figure 2.6 Concentrations of the methanogenic and sulphidogenic systems showing the removal of particulate COD from the reactors over the 6-day experimental period.

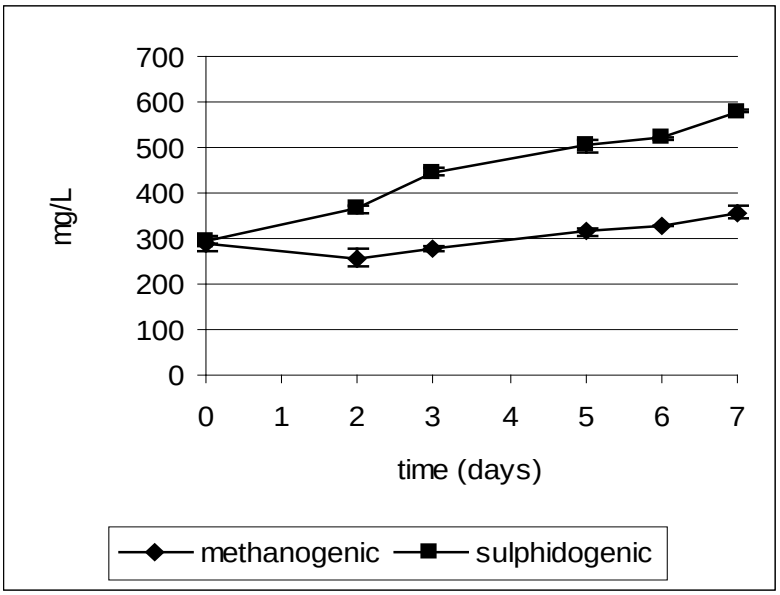


Figure 2.7 Concentrations of the methanogenic and sulphidogenic systems showing the production of filtered (soluble) COD in the systems over the 6-day experimental period.

The measurements of particulate and soluble fractions of COD are presented in figures 2.6 and 2.7. CODp removal reflects the solubilization of the more complex particulate carbon fraction in the two reactors. A similar trend to that observed in CODt removal (Figure 2.5) was shown in this experiment, where there was a significant decrease (ANOVA, $\sqrt{CODp}$, $p<0.05$, $n=36$) in CODp (Figure 2.6) in the methanogenic system (38%) and sulphidogenic system (61%). In the first 2 days, there was slow solubilization of CODp probably due to acclimation of the system, and the solubilization of more complex carbon began after 2 days. At day 7, the methanogenic and sulphidogenic systems had removed 600mg.L^{-1} and 1000mg.L^{-1} of COD respectively. These values were then translated into estimates of the rates of hydrolysis and it was found that the rate of hydrolysis under sulphidogenic conditions (137mg.L^{-1}.d^{-1}) was 64% higher than the methanogenic system (88mg.L^{-1}.d^{-1}).

When complex carbon is degraded, intermediates of smaller molecular weight are released into solution to become readily available to other bacterial populations including SRB and MPB. These soluble products appeared to accumulate in both systems over the experimental period (Figure 2.7), with the methanogenic system showing a lower increase in CODf compared to the sulphidogenic system. The overall percentage increase was 19% and 49% respectively. This result correlated with the increased solubilization of CODp (Figure 2.6). The observed increase in CODf is actually a function of the relative rates of production and utilization. Thus, the

estimated actual rate of utilization of the solubilized material was derived from the CODp and CODf results. The rate of utilization under sulphidogenic conditions (102mg.COD.L^{-1}.d^{-1}) is 74% greater than that under methanogenic conditions (76mg.COD.L^{-1}.d^{-1}).

The sulphidogenic system had an initial CODf of 294mg.L^{-1}, based on the removal of CODp solubilized (1000mg.L^{-1}) the system would yield a further 1000mg.CODf.L^{-1}. However, according to the ratio of 2COD:1SO_4^{2-} (Lens *et al.*, 1995), the system would have required 1500mg.L^{-1} of CODf over the 7-day period, instead this system only produced 1300mg.L^{-1} and there was still some CODf available at the end of the experiment. Therefore, although the figures did not balance, probably due to experimental error, the sulphidogenic system proved to be very efficient.

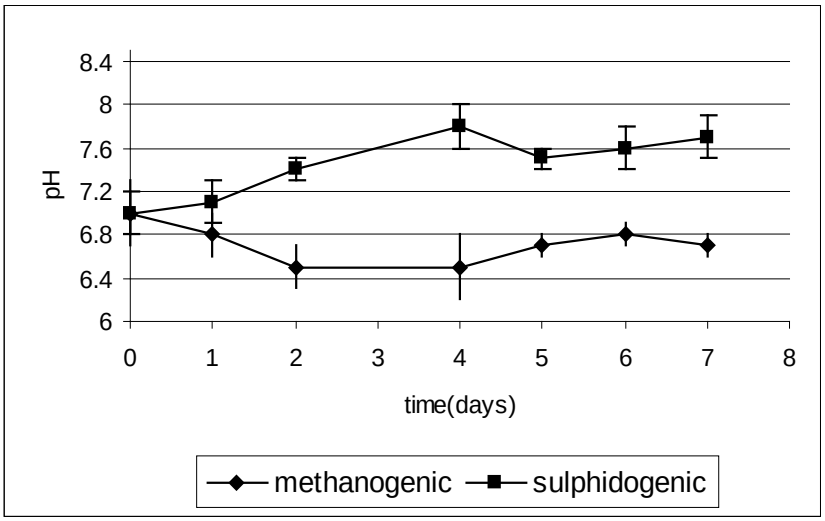


Figure 2.8 Recorded pH profile of the methanogenic and sulphidogenic systems.

Because of active biological sulphate reduction and subsequent production of bicarbonate ions, it was expected that the pH in the sulphidogenic system would be higher than in the methanogenic system (Figure 2.8).Recorded After the first day the pH of the sulphidogenic reactors increased, and remained significantly higher than in the methanogenic system for the remainder of the 7-day experimental system (ANOVA, $p<0.001$, $n=42$). There was a slight decrease noted in the sulphidogenic system after day 4, this could have resulted from the accumulation of soluble products such as VFA. However, the average pH for this system was 7.4, which is reported to be favourable for SRB (Widdel, 1988). In the methanogenic system, there was a decrease in pH until day 4. This was followed by a slight increase, which continued

until day 6. The average pH measured for the methanogenic system was 6.7 (Figure 2.8), reflecting a favourable pH for the growth of MPB (Widdel, 1988).

The soluble products of hydrolysis, particularly, reducing sugars and VFA were also monitored in this experiment. The recorded results are depicted as $mg.L.^{-1}$ of glucose for reducing sugars (Figure 2.9) and $mg.L^{-1}$ of acetic acid for VFA (Figure 2.10).

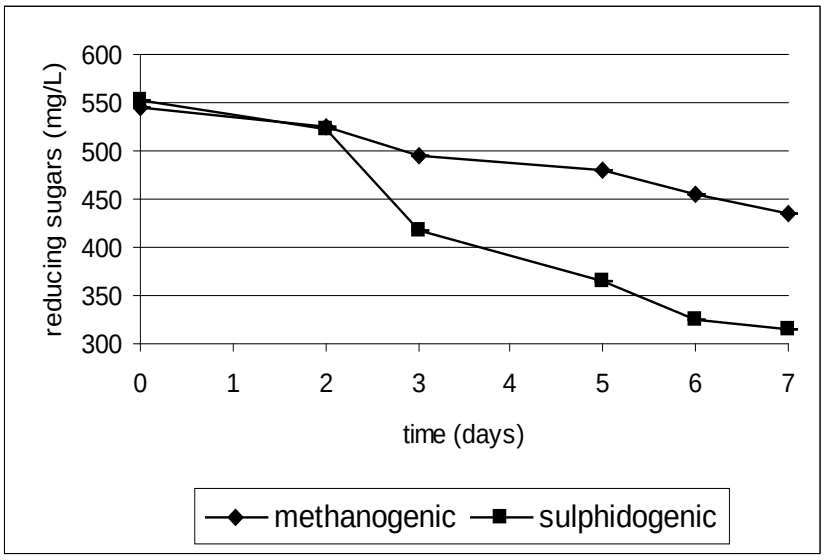


Figure 2.9 Concentrations of reducing sugars in flask studies under methanogenic and sulphidogenic conditions.

The concentration of reducing sugars was monitored over the 7-day period and was found to decrease with time. The overall percentage reducing sugar removal was 20% for the methanogenic system and 43% for the sulphidogenic system. This result indicated that there was significantly higher utilization of reducing sugars under sulphidogenic compared to methanogenic conditions (ANOVA, $p<0.05$, $n=36$). As with sulphate, sulphide and pH data, there was no significant difference in the reducing sugar profile between the two systems (ANOVA, $p>0.05$, $n=6$) for the first 48hr. After day 2, the reducing sugar concentration in the methanogenic system continued to decrease gradually until day 7, with an average rate of decrease of 16mg reducing $sugars.L^{-1}.d^{-1}$. Under sulphidogenic conditions, the decrease in reducing sugars increased rapidly between days 2 and 3 ($54mg.L^{-1}.d^{-1}$), and then slowed to give a decrease of $25.5mg.L^{-1}.d^{-1}$. Interpretation of the reducing sugar data alone is difficult as it is not possible to differentiate between the rates of production and utilization of the reducing sugars, and which one was significant in determining the rates of the observed decrease. Based

on the COD data, which indicated that the rate of hydrolysis of complex COD was greater under sulphidogenic than methanogenic conditions, it would seem likely that the greater decrease in the reducing sugars in the sulphidogenic system was due to enhanced utilization rather than reduced production. However, conclusive evidence for higher production and utilization of reducing sugars under sulphidogenic conditions could only be provided if either one of these parameters could be inhibited.

The uptake of reducing sugars by the bacteria in the anaerobic consortia result in their conversion to short-chain fatty acids or VFA. These may then be used directly by SRB and/or MPB, or converted to acetate before utilization (Figure 1.1). The change in the concentration of this organic fraction was monitored over the experimental period and the results are recorded in figure 2.10.

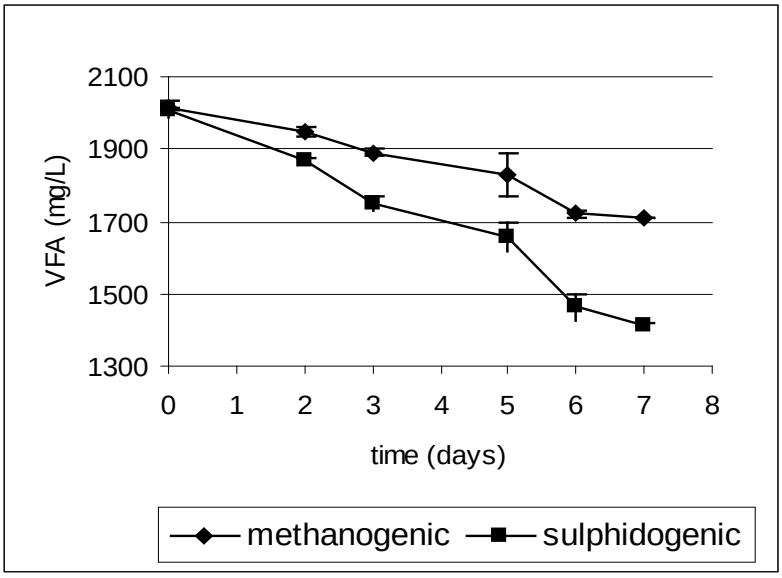


Figure 2.10 Concentrations of the methanogenic and sulphidogenic systems showing the removal of total VFA from the reactors.

The concentration of VFA showed a similar trend to the reducing sugars and decreased over time in both the methanogenic and sulphidogenic systems. However, the concentration of VFA in the sulphidogenic system was significantly lower than that of the methanogenic system (ANOVA, $p<0.001$, $n=36$). The overall VFA decrease was 15% for the methanogenic system and 30% for the sulphidogenic system. The apparently high rate of utilization in the sulphidogenic system ($86mg.VFA.L^{-1}.d^{-1}$) can be attributed to the fact that SRB have a higher

affinity for most of the other VFA with the exception of acetate. In the case of the methanogenic system the apparent utilization rate of 43mg.VFA.L^{-1}.d^{-1} was attributed to acetate utilization. However, as in the reducing sugar data, the rates calculated can not be attributed to utilization as the rate of production also play a role in the system, therefore, the conclusive rates can be calculated if the utilization or production of the VFA can be inhibited.

The above results provided further evidence to support the claims of previous workers (Molipane, 1999; Whittington-Jones, 2000) that the hydrolysis of PSS is enhanced under sulphate reducing conditions.

By monitoring the disappearance of the particulate fraction (CODp), it is possible to determine the estimates of the actual rates of hydrolysis and these were 88mg.COD.L^{-1}.d^{-1} and 137mg.COD.L^{-1}.d^{-1} for the methanogenic and sulphidogenic systems respectively. Furthermore, it is proposed that the data obtained for the sulphidogenic system suggests for the existence of three distinct types of CODp i.e. that which is rapidly hydrolyzed, that that is hydrolyzed more slowly and finally, a refractory component. The refractory component was 37% and 69% of the total COD for the methanogenic and sulphidogenic systems respectively.

It was proposed that the soluble hydrolysis products would be utilized more rapidly in a sulphidogenic compared to a methanogenic system, because of the ability of SRB to utilize a wider range of VFA and reducing sugars than MPB (Aguilar *et al.*, 1995). The concentrations of both reducing sugars and VFA decreased in the sulphidogenic system. However, without a better understanding of the relative rates of utilization and production of soluble hydrolysis products, it is impossible to say conclusively whether this is due to a high rate of utilization or low rate of production. COD data would tend to support the former, but further studies are required and would need to separate the two distinct metabolic processes in order for rates of each to be calculated.

Such experiments could include the use of selective inhibitors, which will allow for the study of each process in isolation from the rest. A study of the effect of inhibitors on reducing sugar uptake and SRB activity will enable the calculation of the rate of production of both reducing sugars and VFA, and will therefore provide accurate rates of hydrolysis. Furthermore, it will be possible to examine the effect of product accumulation on the hydrolytic step and to examine preferential utilization of soluble products by the MPB and SRB populations.

2.4 Conclusion

Based on the flask experiments reported here comparing the hydrolysis of PSS under sulphidogenic and methanogenic conditions, the following conclusions were drawn:

- The rate of hydrolysis of PSS was found to be significantly higher under sulphidogenic (142mg.COD.L^{-1}.d^{-1}) than methanogenic (85mg.COD.L^{-1}.d^{-1}) conditions;
- CODp of PSS is comprised of rapidly digested, slowly digested and refractory fractions, with the latter being approximately 37% of the total CODp. Under methanogenic conditions, the refractory portion is approximately 69%, suggesting that biosulphidogenic conditions are required for the release of certain elements of CODp;
- The decrease of reducing sugars and VFA from batch systems is higher under sulphidogenic than methanogenic conditions. However, using the current data it is not possible to conclude whether this was due to lower production or higher utilization of these organic fractions.

Chapter 3

Solubilization of Complex Carbohydrates under Sulphidogenic and Methanogenic Conditions

3.1 Introduction

Anaerobic digestion is usually thought of as a multi-step process in which complex organics are first degraded to long and short chain fatty acids by facultative bacteria and then to methane, sulphide, and carbon dioxide by methanogenic and sulphate reducing bacteria respectively (Novak & Ramesh, 1975; Eastman & Ferguson, 1981; Vavilin *et al.*, 1996). This process is best described by first-order kinetics and is the initial hydrolytic step regarded as the rate-limiting step in the digestion of complex carbon sources (Gujer & Zehnder, 1983). Eastman & Ferguson (1981) proposed that the overall hydrolysis rate constant for sewage solids represent a number of separate biochemical reactions, each having its own rate constant.

Carbohydrates (polysaccharides, hemicellulose and starch) and lignin form a significant, but variable proportion of PSS. Estimates have ranged from 25% of the settleable fraction of raw sewage (Hunter & Heukelekian, 1965) to as high as 42% of the total solids in PSS (Elefsiniotis & Oldham, 1994). Despite the variable nature of PSS, there is a large amount of lignocellulose and about 60% of the total carbohydrates in PSS may be in the form of cellulose and lignin accounting for up to 6% of the settleable solids fraction (Hunter & Heukelekian, 1965). The common source of this complex organic matter may include wood fibers from toilet paper, rags and vegetable matter (Knapp & Howell, 1978).

The abundance of lignocellulotic waste and their potential as a cheap energy source has led to continued research into improved utilization of these compounds. It has also been reported that these materials are degraded initially by extracellular hydrolases produced by a variety of different bacterial genera. Pectin and dextran can also be degraded in anaerobic digestion (King, 1986; Boschker *et al.*, 1995). Kim *et al* (1997) proposed that if one considered the wide range of organic substrates used by SRB together with the thermodynamic and kinetic aspects of sulphate reduction, waste stabilization might be enhanced by the presence of relatively high sulphate concentrations.

Although large amounts of data exist on the processes and factors that govern the decomposition of bulk sediment organic matter, information on the decomposition of specific polymers under natural conditions is still lacking (Henrichs, 1986). Several approaches have been used to study the initial decomposition of polymeric material. This has been done in order to understand the processes that govern the degradation of these polymers. The results could be used to optimize systems utilizing these polymers as electron donors and for processes such as biological nutrient removal. Some of the methods employed before include the addition of an artificial substrate at a saturating concentration to assess the activities of extracellular enzymes produced by microorganisms (Boschker & Cappenberg, 1994). Other studies have followed changes in the composition of the sediment itself (Benner *et al.,* 1990) or used radiolabelled compounds to study the turnover of the free pool of intermediates formed during the initial decomposition (Banat *et al., 1983*).

The major limitation of the estimates of hydrolysis of PSS described in chapter 1 and 2 was that it was not possible to make any accurate estimates based on changes in the concentration of soluble products because no data was available for relative rates of their utilization. The use of inhibitors to block certain steps in the decomposition process would provide an elegant method to study initial decomposition/hydrolysis by separating this step from acidogenesis and acetogenesis and/or preventing the utilization of soluble products. These studies are also able to give insight into the rates at which each step takes place and the identification of the different organic compounds produced and utilized at each step. Two such methods involve the selective inhibition of microbial carbohydrate uptake using toluene and the blocking of sulphate reduction using molybdate.

Toluene acts on simple carbon without affecting the extracellular hydrolysis of polysaccharides in the sample. It disturbs the proper functioning of the cytoplasmic membrane by increasing its permeability. As a result, transmembrane gradients like proton gradient will collapse. This, in turn, decreases ATP formation and leads to the inhibition of the active uptake systems that depend directly or indirectly on ATP or the proton gradient as an energy source. Carbohydrates will probably be taken up by active transport under natural conditions since the amounts of free extracellular pools of carbohydrates are low. Non-active uptake systems will also be affected by toluene treatment. They depend on a concentration gradient over the semi-permeable cytoplasmic membrane, which probably collapses in the presence of toluene (Boschker *et al.*, 1995). The accumulation products would then be monitored using a Simogyi-Nelson method for

the determination of reducing sugars (Saeman *et al.*, 1963). The distribution of the reducing sugars produced can be monitored using HPLC. The only source of reducing sugars is the hydrolysis of PSS. Therefore the inhibition of sugar uptake using toluene can be used to provide more information on the rates and yields at which the reducing sugars are produced during the hydrolysis of PSS. It is therefore proposed that there would be an accumulation of reducing sugars during the period of inhibition and that rate of accumulation would provide an accurate estimate of the relative rates of hydrolysis under methanogenic and sulphidogenic conditions. The addition of toluene has been reported to inhibit the uptake of reducing sugars but not to have an effect on the biological digestion of the large molecular weight carbonaceous matter such as polysaccharides in PSS (Clancy *et al.*, 1992). Thus, there should be no effect on the actual hydrolysis/ solubilization of PSS.

Clancy *et al.* (1992) did a study where molybdate was used as a selective inhibitor of sulphate reduction in fed-batch systems. This selective inhibition relied on the fact that molybdate is stoichiometrically similar to sulphate and can thus be involved in some biochemical pathways. In this study, the reduction of sulphate and subsequent production of sulphide was inhibited. This in turn inhibits the uptake of VFA by SRB. This selective inhibitor can therefore be used to inhibit the process of acidogenesis and as a result more accurate rates of reducing sugar utilization and VFA production can be calculated.

3 2 Methods

3.2.1 Inhibition studies

The two controls (methanogenic and sulphidogenic) and the two experiments to which toluene had been added (methanogenic + toluene and sulphidogenic + toluene) were set up in triplicate in 500mL flasks. Each flask was innoculated with 10% (v/v) sludge from either the methanogenic or sulphidogenic stock culture as described in chapter 2. 400mL of fresh sieved (2mm mesh) PSS was then added to each flask as the sole carbon source. Table 3.1 shows a summary of the preparation of the flasks.

Table 3.1 Experimental design to test the effect of inhibition of reducing sugar uptake on the rate of hydrolysis.

Treatment	**COD ($mg.L^{-1}$)**	**SO_4 ($mg.L^{-1}$)**	**Toluene (v/v)**	**Innoculum (v/v)**	**N**	**Total vol. (ml)**
Methanogenic (control)	2000	0	0	10%	3	500
Methanogenic (experiment)	2000	0	3%	10%	3	500
Sulphidogenic (control)	2000	2000	0	10%	3	500
Sulphidogenic (experiment)	2000	2000	3%	10%	3	500

These flasks were covered with aluminium foil to exclude light and then incubated on the Labcon desktop shaker at 100rpm in the CE room at 25^{o}C. The flasks were allowed to acclimate for 2 days before the addition of 15ml of toluene (to give a final concentration of 3% v/v) to the six experimental flasks. The remaining six flasks served as the sulphidogenic and methanogenic controls and had no toluene added to them. Samples were collected (similar to chapter 2) at hourly intervals for 8 hours (as the inhibition of toluene on the bacteria only lasts for 7-8 hours). The first sample was taken at t=0 where after toluene was immediately added to the experimental flasks.

The experimental set-up for the molybdate study was similar to that of the toluene study (table 3.1), except that sodium molybdate was added to give a final concentration of 0.2$mg.L^{-1}$. From the results obtained in Chapter 2, it was concluded that two days should be allowed after experimental set up in order to allow the bacteria to acclimate to the reactor conditions. Once the two systems have stabilized toluene was added, therefore t = 0 represents the initial time before toluene addition. Also, the fact that molybdate inhibition is irreversible, the experiment was carried out over a period of 7 days and the reactors were sampled every 2 days.

3.2.2 Analytical procedures

All analytical procedures were carried out in triplicate on each sample. COD, sulphate and sulphide and total concentration of reducing sugars were assayed as described in chapter 2. The identification of specific reducing sugars was carried out using a Beckman 110B solvent delivery module HPLC fitted with a KNAUER RI detector K-2301 and a Hamilton HC-40 Ca^{++} column. The samples were prepared as described for the sulphate analysis (chapter 2) and were analysed for Glucose, Xylose, Mannose, Galactose and Arabinose using commercially available standards (Analytical grade, Sigma).

3.3 Results and Discussion

The effect of reducing sugar uptake on sulphate reduction and sulphide production was also monitored in the two systems.

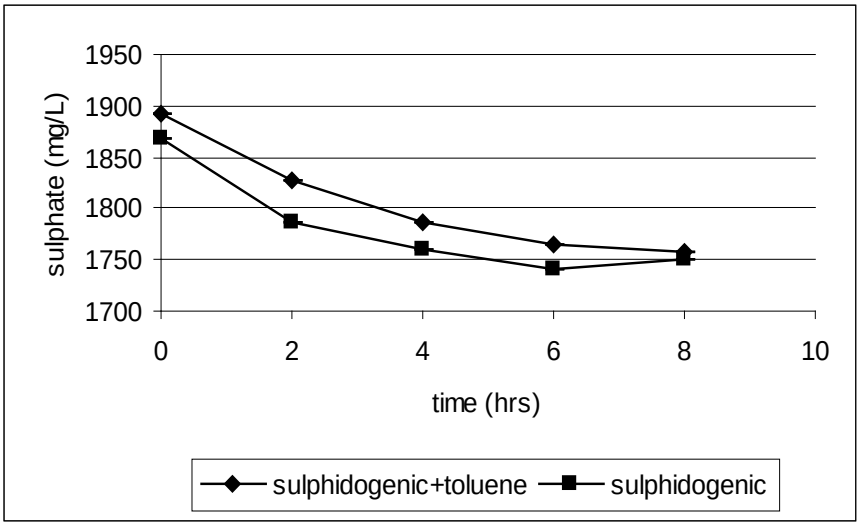


Figure 3.1 Sulphate concentrations of the control and the experimental flasks under sulphidogenic conditions.

Toluene, though affecting the transmembrane transport of reducing sugars, has been reported not to have an impact on the processes that rely on osmotic transport such as sulphate transport into the cells and as a result the process of sulphate reduction. (Boschker *et al.*, 1995). As depicted on figure 3.1, the inhibition of sugar uptake had no significant impact on the reduction of sulphate (ANOVA, $p>0.05$, $n=30$). The SRB continued to reduce sulphate over the inhibition

period with the control showing sulphate reduction of 7.3% and the sulphate reduction of the experimental system was 6.3%. This was confirmed by the observed sulphide profile (figure 3.2) where the sulphide concentrations increased by 60% and 63% for the sulphidogenic control and experimental systems respectively. The maximum sulphide produced was 100 mg. L^{-1} and 50 $mg.^{-1}$ for the sulphidogenic and the methanogenic systems over the inhibition period respectively. The methanogenic flasks also had a production of sulphide but again, the presence of toluene did not seem to have an effect as it is shown by an increase of 56% and 54.5% on the control and the experimental flasks respectively.

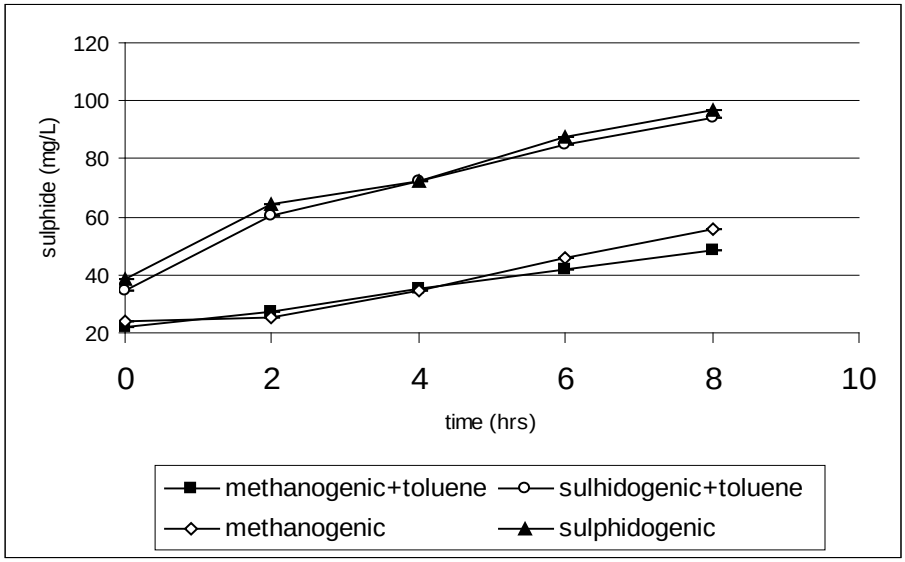


Figure 3.2 Sulphide concentrations in the controls and experimental flasks under methanogenic and sulphidogenic conditions

The observation that inhibition of reducing sugar uptake had no significant effect on biological sulphate reduction suggests that reducing sugars may not have been an important source of electron donors, or that SRB may have been able to switch to another source rapidly in the absence of reducing sugars. Alternatively, SRB may rely on acidogenic populations to produce VFA, which can then be used as electron donors. If this is the case, then the reservoir of VFA in the experimental flasks must have been sufficient to sustain sulphate reduction over the 8-hr period, even in the absence of further conversion of reducing sugars. A study of the changes in reducing sugars and VFA under the experimental conditions may provide further evidence to support these proposals.

The solubilization of PSS was analyzed in terms of CODt, CODp and CODf as in Chapter 2. During the 2-day acclimation period prior to toluene addition in the experimental flasks, the removal of CODf was higher in the sulphidogenic than the methanogenic flasks (ANOVA, $p < 0.05$, $n=12$). This removal accounted for the difference in the CODt concentration of the flasks at $t = 0$ (figure 3.3).

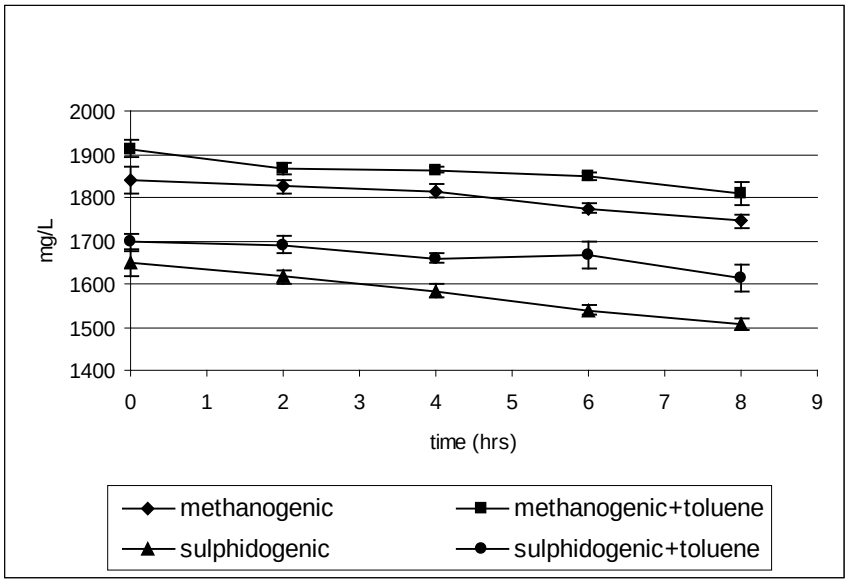


Figure 3.3 total COD concentrations for the methanogenic and sulphidognic control and, experimental systems under reducing sugar inhibition with toluene.

The inhibition of reducing sugar uptake had no significant effect on the hydrolysis of PSS under methanogenic conditions with a 5% decrease in CODt in both the control and experimental flasks over the 8 hour period (ANOVA, $p > 0.05.123$, $n=30$). The rate of CODt removal in the methanogenic flasks was $18mg.COD.L^{-1}.hr^{-1}$ and $22mg.COD.L^{-1}.hr^{-1}$ for the methanogenic experimental and control flasks respectively. Similarly the inhibition of sugar uptake did not appear to result in significant CODt removal under sulphate reducing conditions over the 8-hour period (ANOVA, $p > 0.05$, $n=30$). The overall CODt removal was 5% and 8% for the inhibited and control sulphidogenic systems respectively. The rate of CODt removal under sulphidogenic conditions was $58mg.COD.L^{-1}.d^{-1}$ and $43mg.COD.L^{-1}.d^{-1}$ for the control and experimental flasks respectively.

As with CODt, initial removal of CODp (figure 3.4) during the solubilization of PSS was higher in the sulphidogenic system as compared to the methanogenic system (ANOVA, $p<0.05$, $n=12$).

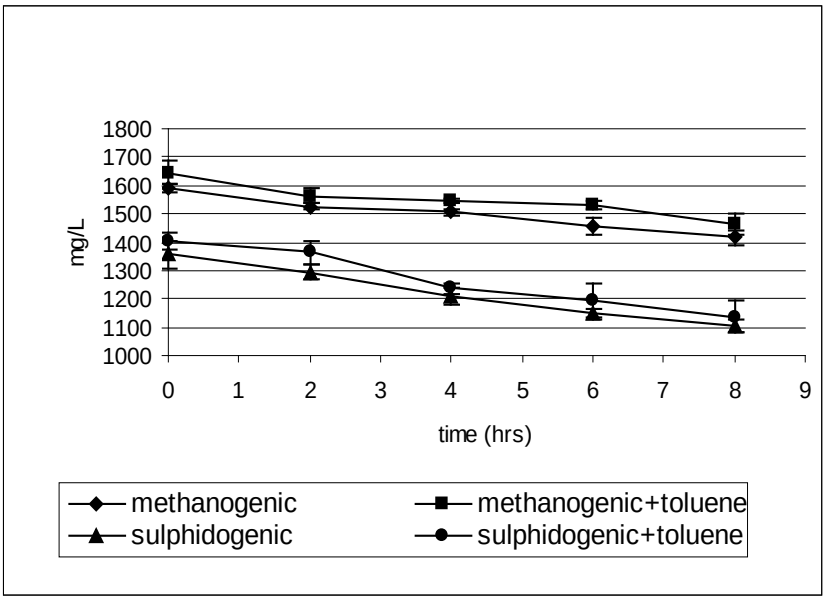


Figure 3.4 Particulate COD concentrations for the methanogenic and sulphidogenic control and, experimental systems under reducing sugar inhibition with toluene.

CODp was reduced from 2000mg.L^{-1} to 1400mg.L^{-1} in the sulphidogenic system but only to 1600mg.L^{-1} in the methanogenic system over the 2-day acclimation period. Although the concentration of CODp decreased in all 4 systems between t=0 and t=8, the rate of removal was low relative to that over the acclimation period. There was no significant difference between the methanogenic control and experimental systems (ANOVA, $p>0.05$, n=30) and for the sulphidogenic control and experimental systems (ANOVA, $p>0.05$, n=30). The rates of CODp removal were 22mg.COD.L^{-1}.hr^{-1} and 24mg.COD.L^{-1}.hr^{-1}. The overall percentage CODp decrease was 11% for both methanogenic systems and 19% for both sulphidogenic systems. These results showed that although the sulphidogenic system showed a significantly higher CODp removal rate over the methanogenic system, this was largely due to the initial removal over the accumulation period. Furthermore, the presence of toluene appeared to have had no significant effect on the removal of CODp in either of the two experimental flasks.

It was predicted that inhibition of reducing sugar uptake would either have resulted in accumulation of sugars or a shortage of VFA, and that one or both of these would have had a negative impact on the rate of hydrolysis. It is thus possible that due to the temporary nature of toluene inhibition, sugar accumulation or VFA depletion failed to reach critical levels within the 8-hr period.

While the inhibition of sugar uptake had a significant effect on the accumulation of CODf (figure 3.5) in the sulphidogenic system (ANOVA, $p<0.05$, n=30), this was not the case for the methanogenic systems (ANOVA, p>, n=30).

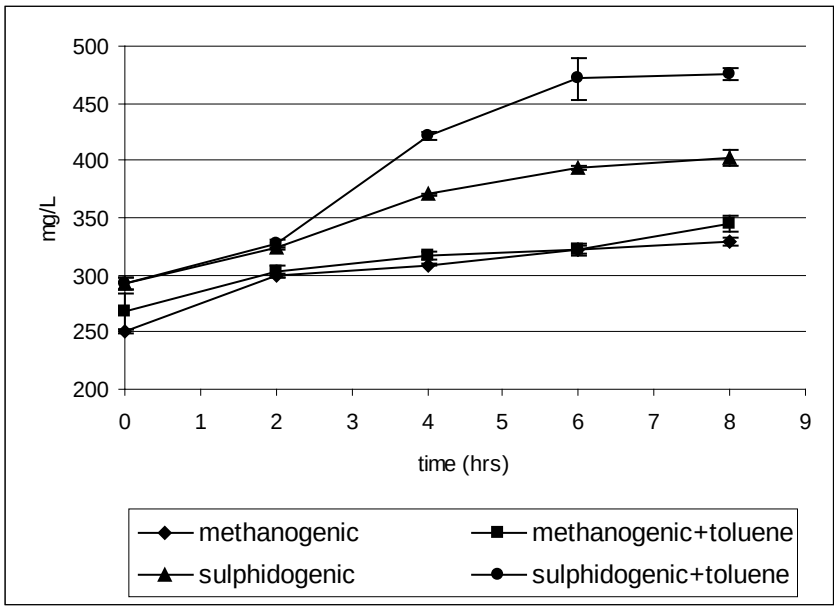


Figure 3.5 Filtered COD concentrations for the methanogenic and sulphidognic control and, experimental systems under reducing sugar inhibition with toluene.

The overall percentage increase in CODf under toluene inhibition was 22% and the decrease in the control was 24%, suggesting that the reducing sugars accumulated at a rate of 9.6 $mg.COD.L^{-1}.hr^{-1}$. The sulphidogenic system showed an overall CODf increase of 38% for the experimental and 27%decrease for the control system. This suggests that the reducing sugars were accumulating at a rate of $22mg.COD.L^{-1}.hr^{-1}$ in the sulphidogenic system compared to the methanogenic system. This difference can therefore be accounted for by the accumulation of reducing sugars under toluene inhibition, and suggest that the average rate of production of reducing in the sulphidogenic system was approximately 50% higher than in the methanogenic system. The implications of these observations are that production of reducing sugars (that would contribute to the soluble COD fraction) under methanogenic conditions is extremely low, due to a low rate of hydrolysis of complex carbohydrates. This in turn implies that the hydrolysis of more complex carbon to produce soluble COD fraction is higher under sulphidogenic than methanogenic conditions.

In chapter 2, the problems associated with attempting to obtain accurate values for hydrolysis rates from changes in CODf were discussed. Comparison of the accumulation of reducing

sugars in a control system with that in a system where the uptake of reducing sugars is inhibited, should enable the determination of an accurate quantification of the rate of reducing sugar production, and therefore, a rate of hydrolysis of the parent complex carbohydrates. The rates of hydrolysis under methanogenic and sulphidogenic conditions could be compared.

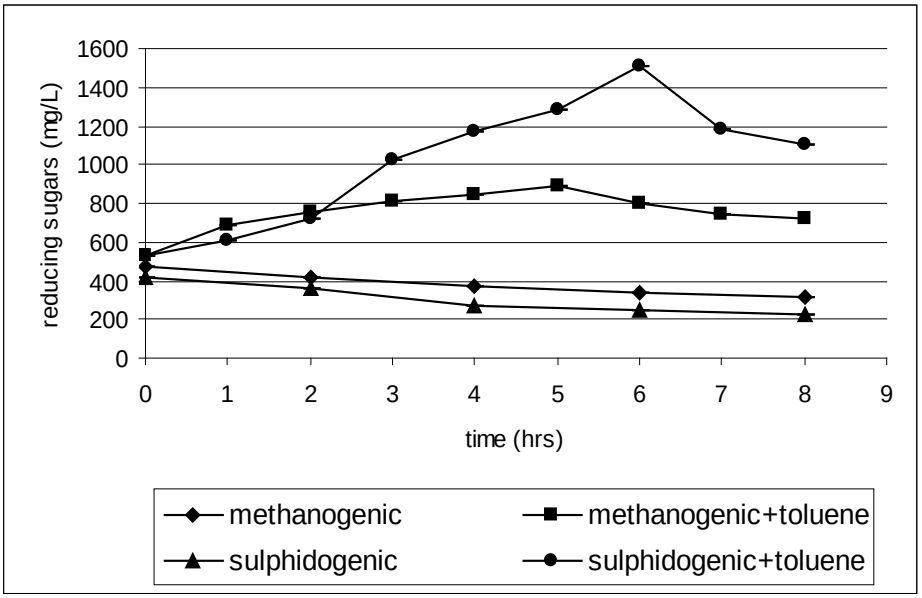


Figure 3.6 Reducing sugar concentrations of the control and experimental flasks under methanogenic and sulphidogenic conditions.

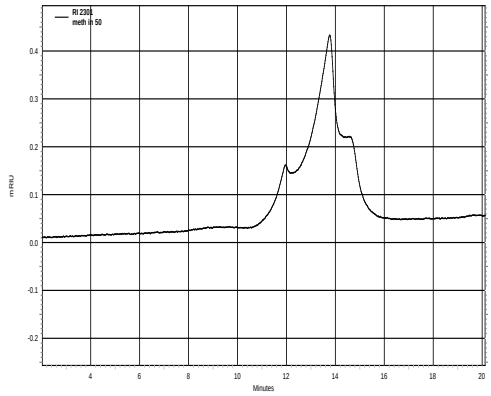
The addition of toluene to the experimental flasks had a significant impact on the uptake of reducing sugars under methanogenic and sulphidogenic conditions (ANOVA, $p<0.001$, $n=42$) (figure 3.6). There was a decrease in reducing sugar in both the control systems, while the experimental systems showed a significant increase in reducing sugars over the 6-hour period after the two-day acclimation period, after which there was a decrease for the last two hours. The controls showed a total decrease in the reducing sugars of 32% and 58% in the methanogenic and the sulphidogenic systems respectively. These values were calculated off a straight line on the graph and confirmed the results obtained in chapter 2, which indicated that there is a significantly higher utilization efficiency of reducing sugars under sulphidogenic as compared to methanogenic conditions (ANOVA, $p<0.05$, $n=30$).

The addition of toluene resulted in a significantly high accumulation of reducing sugars in the methanogenic and sulphidogenic systems, however, the sulphidogenic system showed a significantly higher accumulation and therefore production rate of reducing sugars was higher in

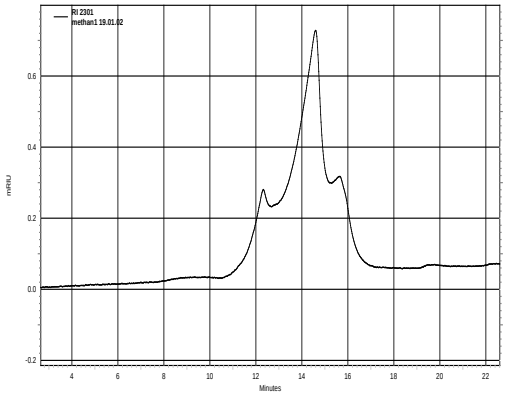
this case as compared to the methanogenic system. The overall percentage increase in reducing sugars calculated over a straight line during the inhibition period was 41% and 65% for the methanogenic (t=5) and sulphidogenic (t=6) systems respectively. The maximum rate of production for the methanogenic system was calculated (51mg.L^{-1}.hr^{-1}) and was found to be 60% less than that of the sulphidogenic system (167 mg.L^{-1}.hr^{-1}). The increase was linear over a 6-hour period after toluene addition and the last two hours showed a decrease in reducing sugar concentration in both experimental systems presumably due to the gradual removal of the inhibition.

The difference between the rate of accumulation of reducing sugars under inhibitory and non-inhibiting conditions gives the actual rate of utilization of the reducing sugars. Again, data showed that utilization of reducing sugars under sulphidogenic conditions was greater than under methanogenic conditions, although it is not possible to say whether this was due to direct or indirect usage by the SRB population. However, under both conditions, a decline in the concentration of reducing sugars over the 8 hours indicated that utilization was greater than production suggesting that the acidogenic step was not rate limiting.

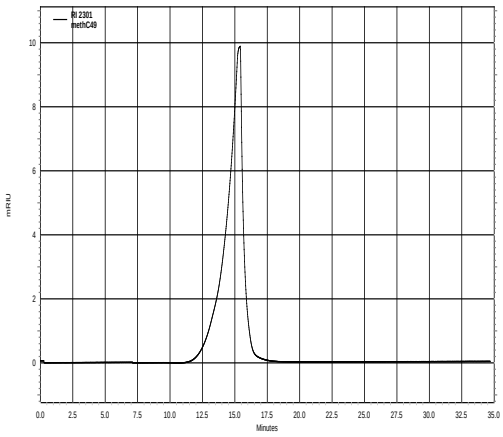
The change in the distribution of the individual reducing sugars under methanogenic and sulphidogenic conditions was monitored using HPLC. A single or mixture of very similar products (figures 3.7 and 3.8) were present in both treatments, but did not show any similarity in retention time to any of the commercial standards tested (see section 3.2.2). However, there was product accumulation in both the methanogenic and sulphidogenic systems and this served to support the fact that the eluted product, even though not identified, was a reducing sugar as the addition of toluene has an accumulating effect on reducing sugars. These results are reported in figures 3.7 (methanogenic + toluene) and 3.8 (sulphidogenic + toluene). The controls in both systems showed a chromatogram similar to that of methanogenic at t=0 and sulphidogenic at t=0, throughout the 8-hour period, thus only the HPLC chromatograms under inhibition were shown.



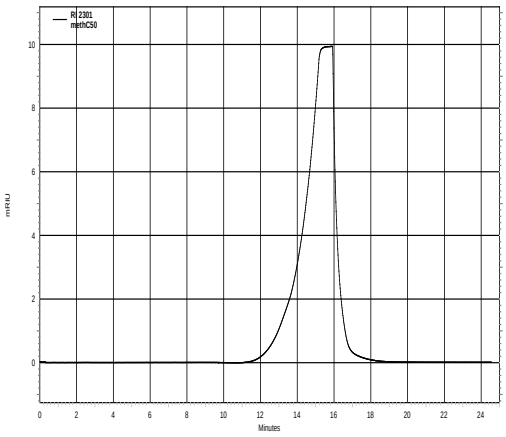
Methanogenic t=0



Methanogenic t=2hrs

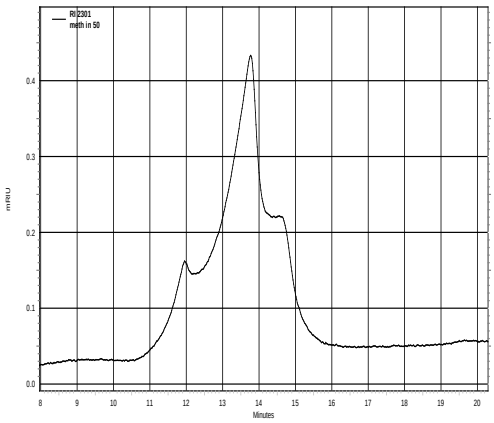


Methanogenic t=4hrs

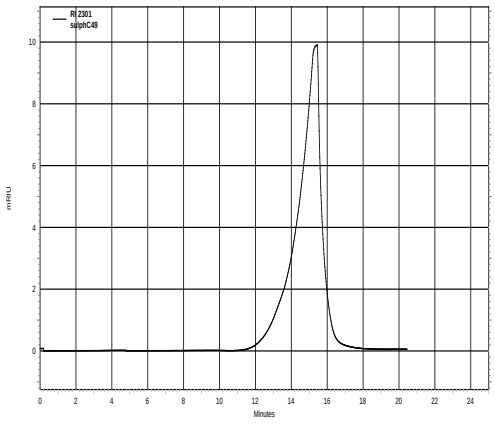


Methanogenic t=6hrs

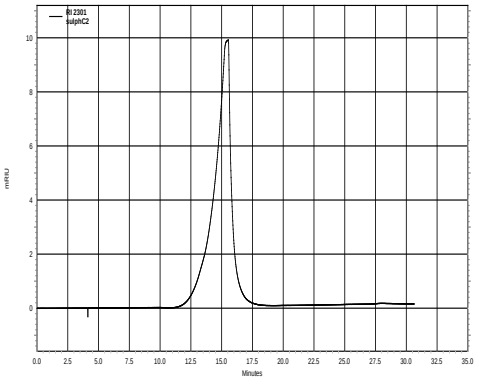
Figure 3.7 HPLC chromatograms showing product accumulation in the methanogenic system under toluene inhibition.



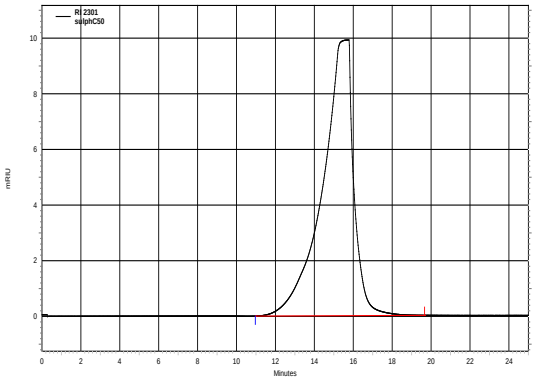
Sulphidogenic t=0



Sulphidogenic t=2hrs



Sulphidogenic t=4 hrs



sulphidogenic t=6hrs

Figure 3.8 HPLC chromatograms of the sulphidogenic system showing product accumulation.

This product, or a group of closely related products, although not identified, was believed to be some form of a sugar as it accumulated in the presence of toluene as was expected. There was more significant increase in the case of the sulphidogenic system (figure 3.8, t=2hrs) as compared to the methanogenic (figure 3.7, t=2hrs) system, which correlates with the significantly higher accumulation of total reducing sugars in the presence of toluene (figure 3.6) under sulphidogenic conditions than methanogenic conditions. The rate of increase calculated against glucose as an internal standard was 23mg.VFA.L^{-1}.hr^{-1} for the methanogenic system and 42mg.VFA.L^{-1}.hr^{-1} for the sulphidogenic system. These rates of accumulation were however, lower than those calculated from figure 3.6. This result could be attributed to the possibility that the contribution of reducing sugars to the CODf could have been small, even thought there was accumulation of reducing sugars. This would also account for the difference in the rates of production recorded for the HPLC method and the Simogyi-Nelson (figure 3.6), where toluene

could have another unexplained effect on the reducing sugars for example indirect stimulation of other organic macromolecules to produce soluble products, under sulphate reducing conditions.

The data obtained in the above study supported the observation of Whittington-Jones (2000) that a significant contribution to the phenomenon of enhanced hydrolysis of PSS under sulphate reducing conditions was due to enhanced hydrolysis of complex carbohydrates. However, it was necessary to prove that this was as a direct result of SRB population and not due to elevated sulphate concentrations. Testing of this involved monitoring the changes in the levels of reducing sugars after SRB have been inhibited using molybdate. These results are depicted in figure 3.9.

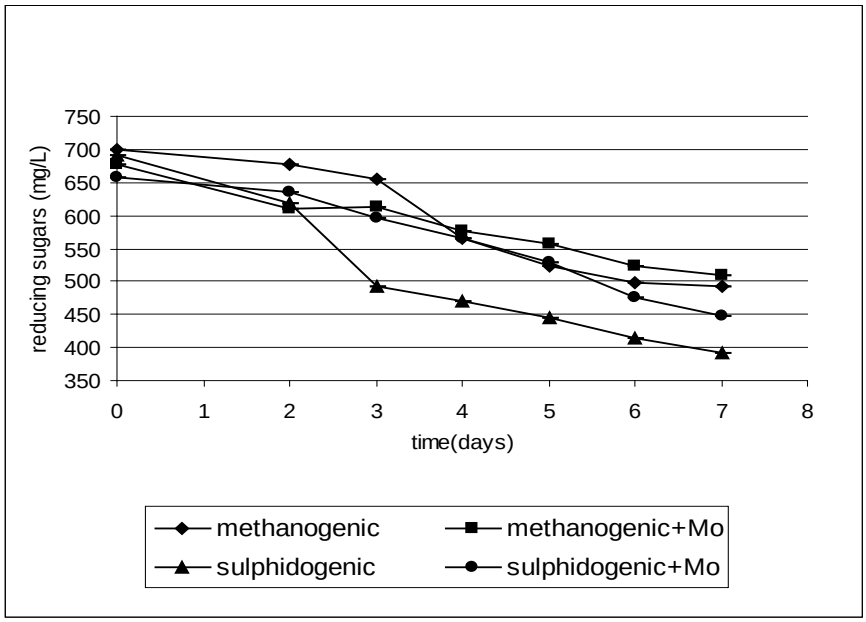


Figure 3.9 Reducing sugar concentrations of the control and experimental flasks under methanogenic and sulphidogenic conditions.

In the first 2 days of acclimation to the reactors, there was already a notable difference in reducing sugars in the two systems. The addition of molybdate on day 2 had an effect on the sulphidogenic system's ability to utilize reducing sugars. This is marked by the 29% decrease in the experimental as compared to the 37% decrease in the control system. The methanogenic control system showed a 29% decrease compared to the 16% of the experimental system. A similar decrease in the mineralization of glucose under the inhibition of sulphate reduction has been reported by Smith & Klug (1981), and this was an indication that sulphate reducers utilize an array of substrates as electron donors compared to methanogens. The sulphidogenic

experimental system exhibited a similar removal of reducing sugars as the methanogenic control system even though there was high sulphate concentration present in the system. Although there was no significant difference between the four data sets (ANOVA, $p>0.05$, $n=84$), the higher removal of reducing sugars in the uninhibited sulphidogenic system compared to the inhibited system indicated that the SRB themselves had some influence on the solubilization. The decrease in reducing sugars in all four systems again indicated that the rate of utilization was higher than the rate of production and therefore acidogenesis was not the rate–limiting step.

From the results obtained it can be concluded that the rate of hydrolysis is enhanced under sulphate reducing conditions as compared to methanogenic conditions and that this is at least partially due to the enhanced hydrolysis of complex carbohydrates. Furthermore, when sulphate reducing bacteria were inhibited, the methanogens present in the system seemed to become active and reducing sugars continued to be removed. The high sulphate concentration did not seem to affect the ability of the methanogens to dominate the system and the sulphidogenic experimental flasks seemed to have similar utilization rates to those of the methanogenic control system.

Based on the above results, toluene was successfully used to inhibit the process of acidogenesis by inhibiting the uptake of reducing sugars in both methanogenic and sulphidogenic systems. This enabled the calculation of the rates of production of reducing sugars as a product of overall PSS hydrolysis. The identification of the different sugars present under methanogenic and sulphidogenic systems was also possible but not conclusive. The use of molybdate as an inhibitor confirmed that the utilization of reducing sugars was more rapid under sulphate reducing conditions than methanogenic conditions, and that reducing sugars are utilized in the presence of high concentrations of sulphide even when SRB are inhibited.

3.4 Conclusion

The hydrolysis of PSS to produce reducing sugars was studied in isolation from the other processes involved in the degradation of complex carbon under both methanogenic and sulphidogenic conditions. The aim of using the inhibitors was to calculate actual rates of utilization without any interference soluble product production. Toluene and molybdate used as inhibitors of reducing sugar uptake and sulphate reduction respectively were successfully used in this study and the following conclusions could be drawn:

- Inhibition of reducing sugar uptake had no significant effect on CODp removal, although removal in both sulphidogenic systems was significantly higher than in the methanogenic systems (ANOVA, $p>0.05$, $n=30$);
- Production of reducing sugars under methanogenic conditions is extremely low and this suggests that the hydrolysis of complex carbohydrates is poor relative to sulphidogenic systems;
- There was no significant effect on sulphate reduction, indicating that the SRB did not use reducing sugars directly as electron donors. Because inhibition of reducing sugar uptake is for a limited period, even if the acidogenic step is impaired (i.e. the conversion of reducing sugars to VFA), there is enough VFA to sustain sulphate reduction;
- The significant effect on total reducing sugars enabled the calculation of rates of hydrolysis of complex carbohydrates. This was approximately 31% higher in the sulphidogenic than the methanogenic system;
- Acidogenesis of reducing sugars does not appear to be the rate–limiting step in the hydrolysis of PSS.

Chapter 4

The Impact of Volatile Fatty Acid Utilization by Sulphidogenic and Methanogenic Populations on the Rate of Hydrolysis of Sewage Sludge

4.1 Introduction

Anaerobic digestion of organic matter to methane and carbon dioxide is carried out by the coordinated action of various groups of microorganisms and passes through several intermediate stages. The most important of these is the formation of short chain fatty acids (Aguilar *et al.*, 1995), and relatively few studies on fermentation of pure volatile fatty acids have been reported (McCarty & Vath, 1962, Aguilar *et al.*, 1995). Acetate has been shown to be the precursor of more than 75% of the methane produced in anaerobic digesters, and its degradation has been regarded as the rate limiting step in the terminal decomposition of more complex substrate organic molecules (Lawrence & McCarty, 1969; Aguilar *et al.*, 1995). Propionate and butyrate are other important VFA, which may be further converted to acetate and hydrogen (Widdel, 1988; Stams *et al.*, 1984; Aguilar et al, 1995). Glucose has been considered the representative of soluble carbohydrates (in the anaerobic digestion) and its degradation leads to the formation of products such as propionate, butyrate, lactate or ethanol (Aguilar *et al.*, 1995).

The electron donors oxidized by SRB are always low-molecular weight compounds, which are known to be fermentation products from the anaerobic bacterial degradation of carbohydrates, proteins and other components of complex organic matter. By using an external, inorganic electron acceptor, sulphate reducers perform a net oxidation of their substrates (Widdel, 1988; Qabiti *et al.*, 1990; Espinosa *et al.*, 1995). The *in situ* activities of this group of organisms have received attention in order to determine the effects of organic amendments and metabolic inhibitors on sulphate reduction rates. These investigations showed that sulphate reduction is potentially an important process in the terminal oxidation steps in anaerobic systems (Qabiti *et al.*, 1990).

Evidence that simpler fatty acids could be quantitatively converted to methane and carbon dioxide was obtained by Sohngen (Barker, 1936a, as cited in Whittington-Jones, 2000; Torien & Hattingh, 1969), a fact, which is of primary importance in the digestion of organic wastes. He

also found out that a mixture of hydrogen and carbon dioxide could be used to produce methane. Other investigations considered the use of other organic substrates for the production methane and included the fermentation of cellulose and ethyl alcohol by MPB (Torien & Hatting, 1969). However, reports have shown that this process is not carried out by MPB alone but as discussed earlier, that there is a consortia of microorganisms that act together to carry out these processes (Figure 1.1). In order to investigate these complex interactions, researchers used a number of approaches. These included the calculation of the rates of oxidation of low-molecular weight compounds (Banister & Pretorius, 1998), quantification of intermediate products (Shimizu *et al*., 1993), and the estimation of metabolic rates and studies of competitive interactions between SRB and MPB for electron donors (Zhender, 1988, Clancy *et al.*, 1992). However, not any single study has pursued all these objectives simultaneously (Dicker & Smith, 1985).

So far in this study, it has been shown that the rate of hydrolysis is enhanced under sulphidogenic conditions and that the enhanced hydrolysis of the carbohydrate fraction contributes to the overall enhanced hydrolysis that was observed. Furthermore, the rate of utilization of reducing sugars is more rapid under sulphidogenic conditions. The mechanism underlying the enhanced hydrolysis of carbon sources under sulphidogenic conditions would seem to be complex, and involves action at more than one step in the degradation process (Figure 1.1). A number of biological processes are influenced or regulated by a possible negative feedback mechanism (McCartney & Oleszkiewicks, 1993), where accumulation of the product of a particular reaction will result in a decrease or complete inhibition of the reaction itself. Conversely, if the concentration of the end product falls below a critical level, the reaction is “switched on”.

As discussed earlier, methanogenic populations would preferentially utilize acetate over longer chain VFA, thereby resulting in accumulation of the latter until such a point that the pool of acetate is depleted or has fallen below a critical level at which it can be utilized. It is therefore proposed that the ability of SRB to preferentially use a wider range of VFA, (Qabiti *et al.*, 1990; Thile & Zeikus, 1988, as cited in Clancy *et al.*, 1992) limits the accumulation of these end products, thereby avoiding possible feedback inhibition of either the acidogenic and/or hydrolytic steps. In this way, the utilization of VFA is thought to play a critical part in the enhanced hydrolysis of PSS under sulphate reducing conditions.

In order to investigate the distribution and effects of accumulation of VFA in this study, molybdate was used to inhibit sulphate reduction. This study would serve to block one of the steps in which VFA are utilized, thereby making it possible to calculate the rate of production and utilization of this organic fraction and to monitor the impact on the rate of hydrolysis. This inhibitor is not effective against MPB and thus no direct comparison of the rates could be made.

In a study by Clancy *et al.* (1992), molybdate was proved to be an effective and selective inhibitor of sulphate reduction in fed-batch systems. In one experiment, SRB were grown in the presence of high sulphate and molybdate over a ten-day incubation period to study the sulphate reduction. The results of this experiment proved molybdate not to be inhibitory to methanogens. However, in the experiment with SRB, it proved that molybdate was inhibitory to the SRB's reduction of sulphate to sulphide. Smith & Klug (1981) reported the reduction in the mineralization of VFA such as lactate and propionate when sulphate reduction was partially inhibited using molybdate.

The aim of this study was thus to inhibit SRB activity using molybdate to provide more insight into the role of SRB and sulphate reduction in the enhanced hydrolysis of PSS and carbohydrates. It was expected that inhibition of SRB would result in an accumulation of the VFA in the inhibited sulphidogenic system and that this would have a negative effect on the rate of hydrolysis. It was also thought that once SRB activity had been inhibited, that methanogenic species would dominate and that this would result in the utilization of any accumulated acetate.

4.2 Methods

4.2.1 Experimental procedure

The toluene experiments and controls were set up as described in chapter 3 (table 3.1). For the molybdate experiments, four sets of 500ml flasks were set up as described in chapter 3. After the first 2 days of acclimation, sodium molybdate (Analytical grade, Sigma) was added to the experimental flasks at a final concentration of 0.2mg.L^{-1}. t=0 marks the point at which molybdate was added. All treatment and controls were prepared in triplicate and incubated in the 25^{o}C CE room on a Labcon desktop shaker at 100rpm. These flasks were sampled every second day over a period of 6 days, as described in chapter 2.

4.2.2 Analytical procedures

The analytical procedures performed on the samples in triplicate are described in chapter 3. These included total, filtered and particulate COD, sulphate and sulphide. Total VFA concentrations were determined using the steam distillation method (Appendix II, 5.1) as described by APHA, standard methods (1994). The extraction and GC quantification of VFA was determined according to Appendix II, 5.2.

4.3 Results and Discussion

The sulphate and sulphide concentration were monitored to evaluate the effect of molybdate on sulphate reduction and sulphide production.

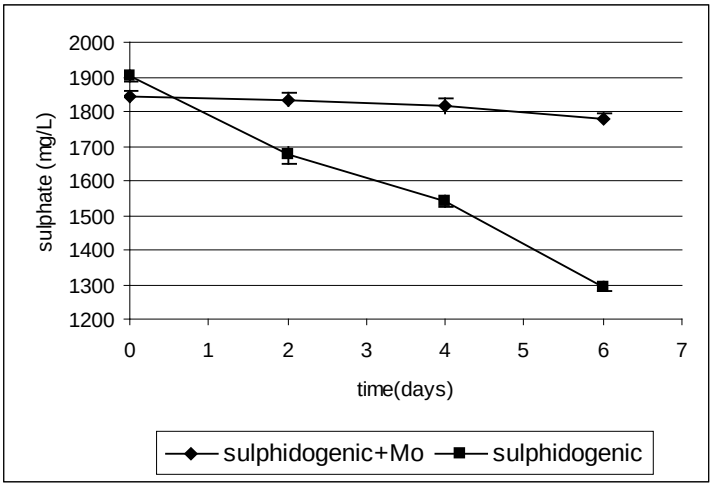


Figure 4.1 The sulphate concentrations of the sulphidogenic control and experimental flasks.

The addition of molybdate led to the successful inhibition of sulphate reduction (Figure 4.1). Sulphate reduction in the control continued until the end of the experiment and resulted in an overall reduction of 625mg.L^{-1} (32%). In the experimental system, the overall sulphate removed was 62mg.L^{-1} (3.4%). However, this did not conclusively prove that sulphate reduction was inhibited, therefore sulphide production had to be taken into consideration. The change in sulphide concentrations in the methanogenic and sulphidogenic systems is shown in Figure 4.2.

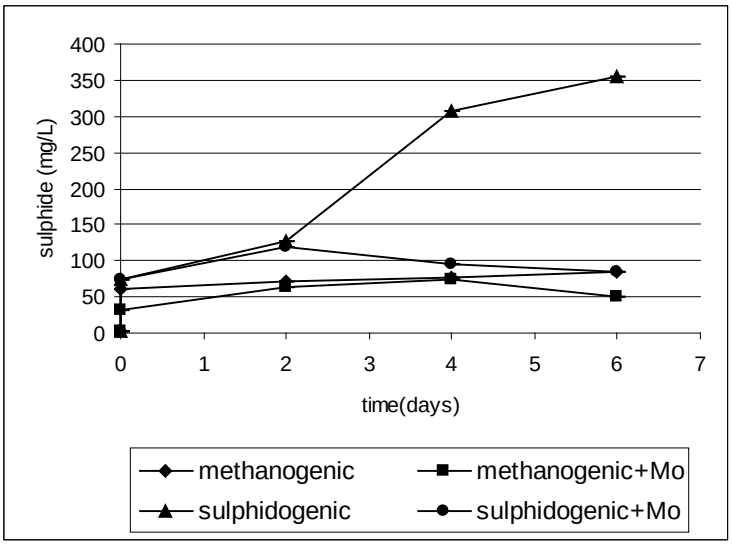


Figure 4.2 Sulphide concentrations of the methanogenic and sulphidogenic control and experimental systems. Molybdate was added to the flasks at day 0 (two days after acclimation).

In the case of the sulphidogenic experimental and control flasks, sulphide increased to reach 120mg.L^{-1} at day 2, after which the inhibited sulphidogenic system showed a decrease to 85mg.L^{-1}. The control system increased significantly to reach a maximum of 350mg.L^{-1} at day 6 (ANOVA, $p < 0.001$, n=48). The methanogenic system also showed limited sulphide production possibly due to reduction of sulphate present in PSS. This production of sulphide reached a maximum of 83mg.L^{-1} at day 6 while that in the inhibited system increased to 73mg.L^{-1} at day 4 and then decreased to 51mg.L^{-1} on day 5. The sulphide production in the inhibited sulphidogenic system was not significantly different from the methanogenic control and experimental systems (ANOVA, $p>0.05$, n=27). These results gave an indication that sulphate reduction was inhibited upon addition of molybdate but that there appeared to be a delay of 2 days before the onset of inhibition. This could be explained by the fact that the bacteria had to acclimate using molybdate as an alternative electron acceptor to sulphate.

CODt, CODp and CODf were monitored in order to monitor the effect of sulphate reduction inhibition on the hydrolysis of particulate matter. The recorded results are shown in Figures 4.3, 4.4 and 4.5.

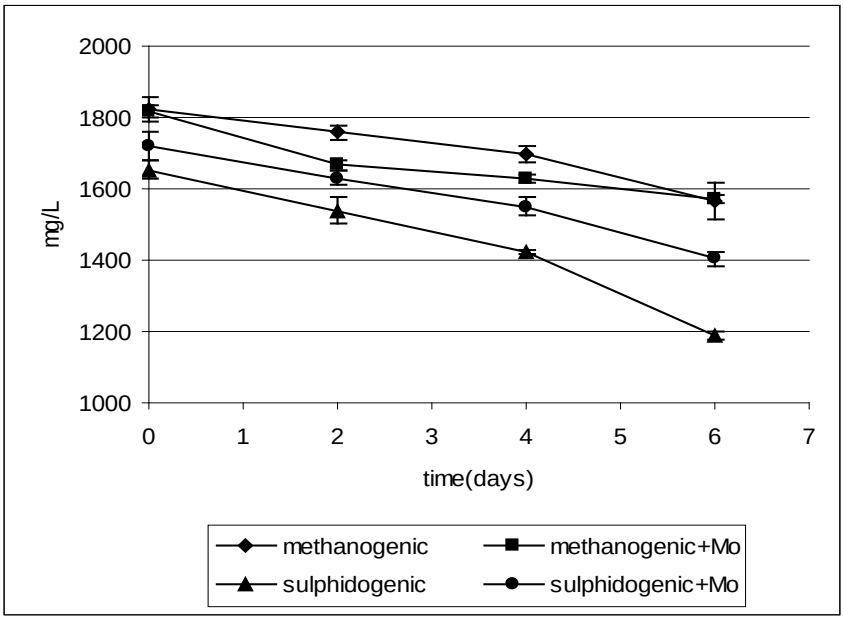


Figure 4.3 Total COD concentrations of the methanogenic and sulphidogenic controls and experimental systems.

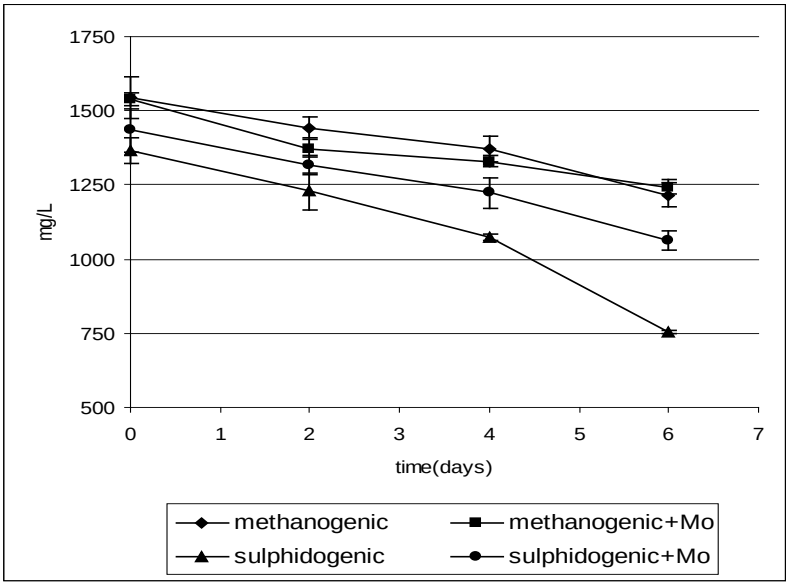


Figure 4.4 Particulate COD concentrations of the methanogenic and sulphidogenic experimental systems.

CODt and CODp decreased in the first two days of acclimation with the sulphidogenic systems showing a significantly higher decrease at t=0 as compared to the methanogenic systems (ANOVA, $p<0.05$, n=48). The CODt of the sulphidogenic systems was approximately 1700mg.L^{-1} while those of the methanogenic systems were 1800mg.L^{-1} immediately before the

addition of molybdate. Both CODt and CODp data suggests that hydrolysis in a sulphate-rich system is only enhanced if SRB are active. Once they are inhibited, the removal and rates of hydrolysis were similar to the methanogenic system. The sulphidogenic control system showed a significantly higher removal of CODt (ANOVA, $p<0.05$, $n=48$), as represented by an overall rate of $77mg.L^{-1}.d^{-1}$ (28%). The sulphidogenic system inhibited with molybdate showed a CODt removal rate of $52mg.L^{-1}.d^{-1}$(18%), which was not significantly different to the methanogenic control and inhibited systems (ANOVA, $p>0.05$, $n=36$). The rates of removal were of the methanogenic control and inhibited systems were $43mg.L^{-1}.d^{-1}$ (15%) and $41mg.L^{-1}.d^{-1}$ (14%) respectively. The filtered COD (CODf) was also monitored in the four systems, as depicted in Figure 4.5.

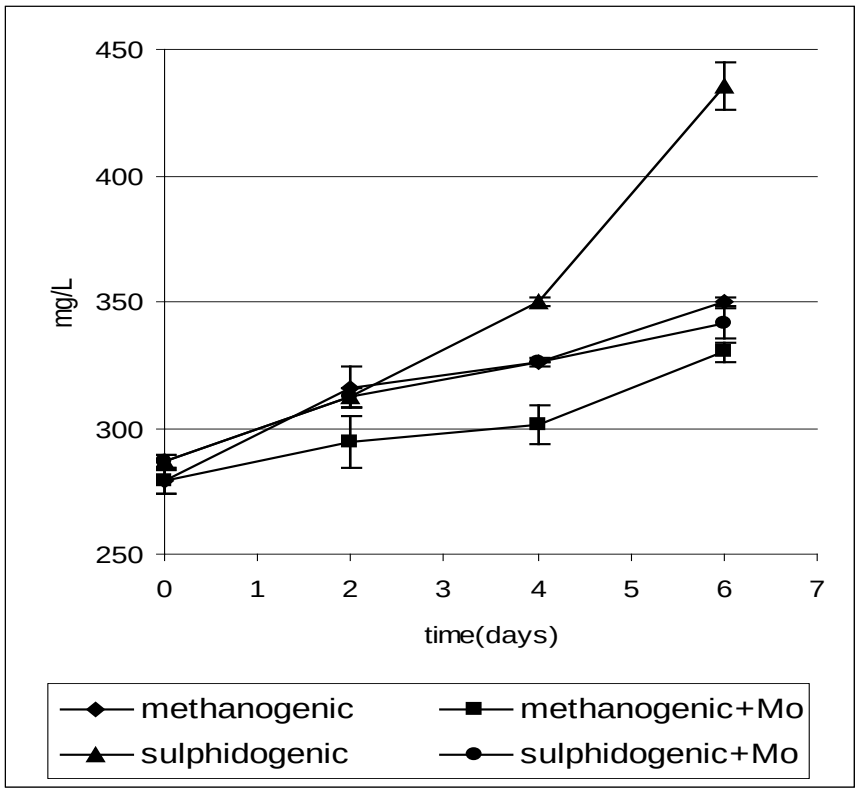


Figure 4.5 Filtered COD concentration of the methanogenic and sulphidogenic control and experimental systems.

The CODf data supports the data observed before that SRB are required for the removal of soluble products as the inhibited sulphidogenic system showed a significantly lower rate of CODf production $9mg.L^{-1}.d^{-1}$ (16%) as compared to the control system $25mg.L^{-1}.d^{-1}$(34%) (ANOVA, $p<0.05$, $n=24$). Although the CODf data alone is not enough, previous data has supported the increase in CODf under sulphidogenic conditions was due to enhanced production

of this fraction compared to other systems, rather than reduced consumption. The rates of production for the methanogenic systems were 12mg.L^{-1}.d^{-1} (20%) and 9mg.L^{-1}.d^{-1} (15%) for control and experimental systems respectively. These values were not significantly different from the rates observed in the inhibited sulphidogenic system (ANOVA, $p>0.05$, $n=36$).

pH was monitored in all 4 systems and the recorded results are shown in Figure 4.6.

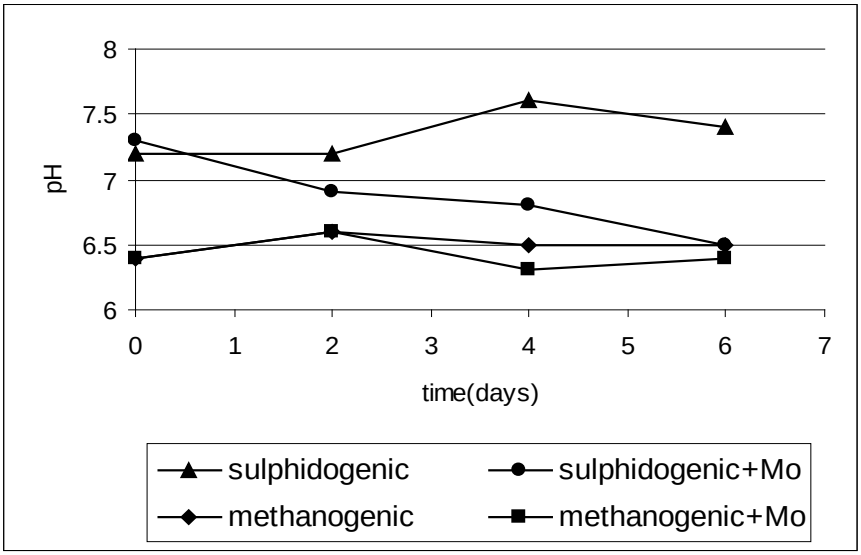


Figure 4.6 pH profiles for the methanogenic and sulphidogenic control and experimental systems.

Based on the hypothesis that utilization of VFA would be better under sulphidogenic conditions due to the ability of SRB to utilize a wider range of acids than the methanogenic system, it was predicted that inhibition of sulphate reduction would result in an increase in VFA, and a decrease in pH, relative to the control. At t=0, immediately prior to molybdate addition, the mean pH values in the sulphidogenic and methanogenic systems were 7.2 and 6.4 respectively. These values were significantly different at t=0 (ANOVA, $p<0.001$, $n=48$). The inhibition of sulphate reduction in the sulphidogenic system led to a significant decrease in pH from 7.4 at t=0 to 6.5 at t=6. This was most likely due to the accumulation of other VFA as a result of SRB inhibition and a reduction in the bicarbonate alkalinity. The mean pH obtained under molybdate inhibition in the sulphidogenic system was 6.6 and this value is in line with the pH optima for a methanogenic system. The addition of molybdate to the methanogenic systems did not have any effect on pH and the mean pH of the control and experimental systems were 6.5 and 6.4 respectively over the 6-day period. The results obtained, therefore showed that the presence of

molybdate successfully inhibited the process of sulphate reduction, and that the relatively low mean pH in the methanogenic systems may be as a result of the inability of MPB to utilize as wide a range of VFA as SRB. Thus this result further confirms the findings obtained from the COD data that the inhibited sulphidogenic system turned out to behave more like a methanogenic system.

The VFA were monitored when reducing sugar uptake was inhibited and when sulphate reduction was inhibited. The results of these experiments would give insight into the rates of VFA uptake under sulphidogenic and methanogenic conditions, and hopefully into the role of SRB in the enhanced hydrolysis of PSS. The inhibition of reducing sugar uptake was expected to result in the reduced production of VFA in the two systems. It was predicted that the removal of VFA in the two systems would continue taking place until such a point where the VFA concentrations had reached a critical level where they can not be utilized any further. The total VFA were monitored under the inhibition with toluene. The recorded results are shown in Figure 4.7.

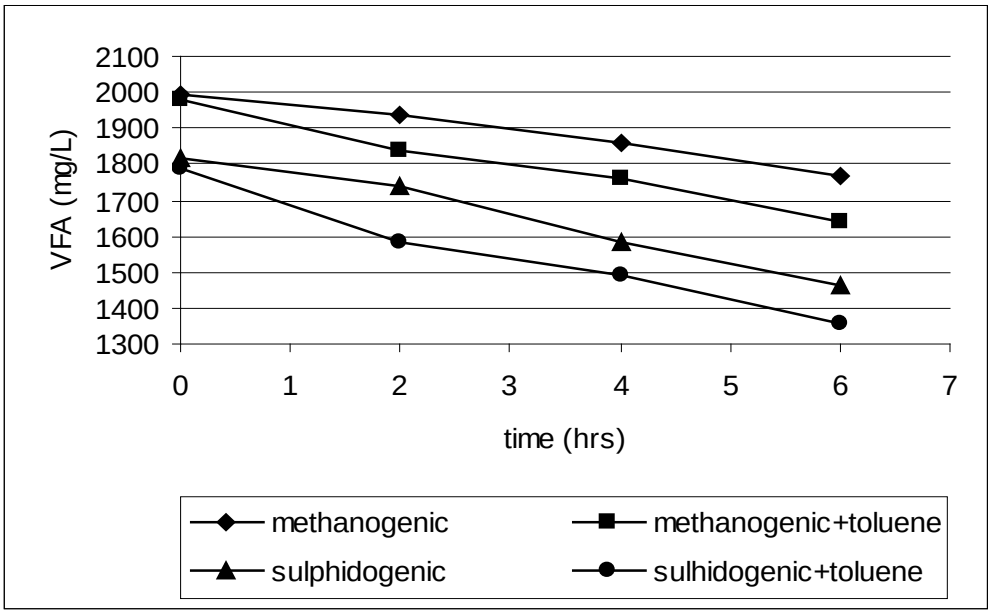


Figure 4.7 Total VFA concentrations of the methanogenic and sulphidogenic control and experimental systems, showing the effect of inhibition of reducing sugar uptake by toluene.

The two systems showed the removal of VFA over the 6-hour inhibition period, which is the maximum inhibition period before inhibition is lifted. After the 2-day acclimation period, the sulphidogenic systems showed a significantly higher removal over the methanogenic systems (ANOVA, $p<0.05$,$n=12$), the sulphidogenic systems had reached 1800mg.L^{-1} while the

methanogenic systems had reached 1980mg.L^{-1}. As discussed earlier, the change in the concentration of total VFA in all four systems over the 6-hour period is a factor of the relative rates of VFA production and utilization. After the addition of toluene to inhibit the uptake of reducing sugars, the methanogenic system showed a rate of VFA removal of 56mgVFA.L^{-1}.d (17%), which was higher but not significantly different for the two systems from 37mgVFA.L^{-1}.d^{-1} (11%) of the control system (ANOVA, $p>0.05$, $n=24$). The removal rate of the inhibited sulphidogenic system was 72mgVFA.L^{-1}.d^{-1} (24%), which was higher but not significantly different (ANOVA, $p<0.05$, $n=24$) to 60mgVFA.L^{-1}.d^{-1} (20%) of the control system. The noted decrease in total VFA concentration in all four systems (Figure 4.7) indicates that VFA production was slower than utilization even under methanogenic conditions. The lack of significant difference between the two methanogenic systems would seem to indicate that the rate of production and utilization under these conditions were relatively slow. The higher percentage removals in the sulphidogenic systems was also attributed to the sulphidogenic systems ability to use a wider range of VFA than the methanogenic system, while the lack of significant difference would seem to indicate that the rates of utilization and production were rapid under these conditions. The distribution of VFA was monitored over the 6hr period using the GC (Appendix II, 5.2) in order to investigate the VFA utilization between the methanogenic and sulphidogenic systems and when the reducing sugar uptake was inhibited. The results are shown in Figures 4.8 and 4.9.

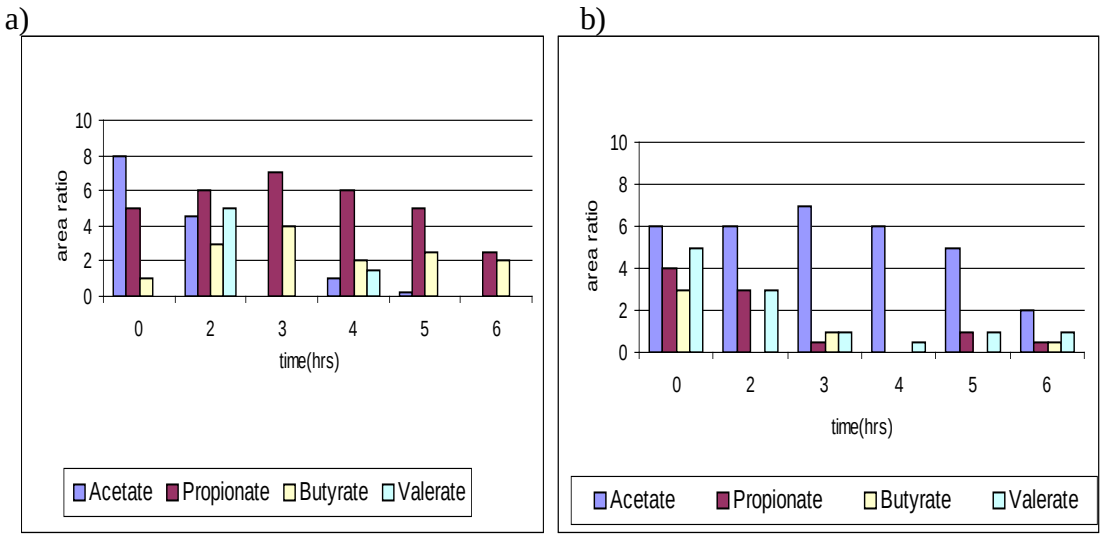


Figure 4.8 Change in the distribution of four volatile fatty acids under methanogenic (a) and sulphidogenic (b) conditions over 6 hours. (n=3, standard deviations were below 5%).

a) b)

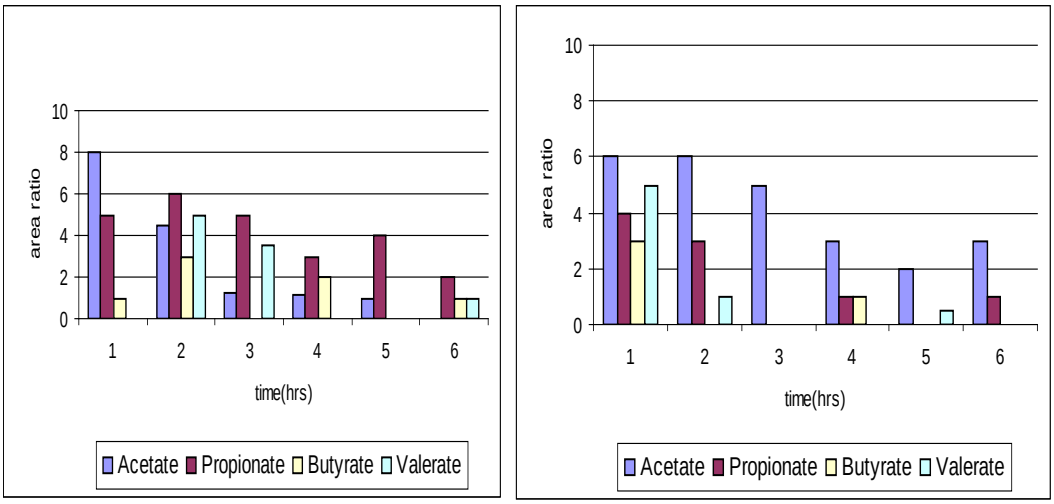


Figure 4.9 Change in the distribution of four volatile fatty acids in the methanogenic (a) and sulphidogenic (b) systems under reducing sugar uptake inhibition with toluene. (n=3, standard deviations were below 6%).

The distribution of VFA under methanogenic conditions (Figure 4.8a) shows the utilization of acetate and the accumulation of other VFA until the depletion of acetate. In the sulphidogenic system (Figure 4.8b), there is a reverse in the trend where acetate accumulates and the other VFA are utilized. These results confirm what has been stated in literature, that MPB utilize acetate preferentially over other VFA, while SRB utilize others VFA over acetate (Qabiti *et. al.*, Smith & Klug, 1981). A similar trend was noted when the uptake of reducing sugars was inhibited with toluene (Figure 4.9). The relative increase in the concentrations of propionate and butyrate at t=4 in Figure 4.9(b), and butyrate and valerate at t=6 in Figure 4.9(a) indicates that the uptake of reducing sugars is not essential for the production of VFA in either the methanogenic or sulphidogenic system.

It was predicted that the addition of molybdate to the sulphidogenic system would, either lead to the accumulation of VFA or to the decrease in the rate of VFA removal in this system. It was further predicted that there would be little or no effect in the methanogenic system, which comprises of little or no SRB population. The total concentrations and distribution of VFA under inhibition of sulphate reduction with molybdate was monitored. These results are reported in Figures 4.10 and 4.11

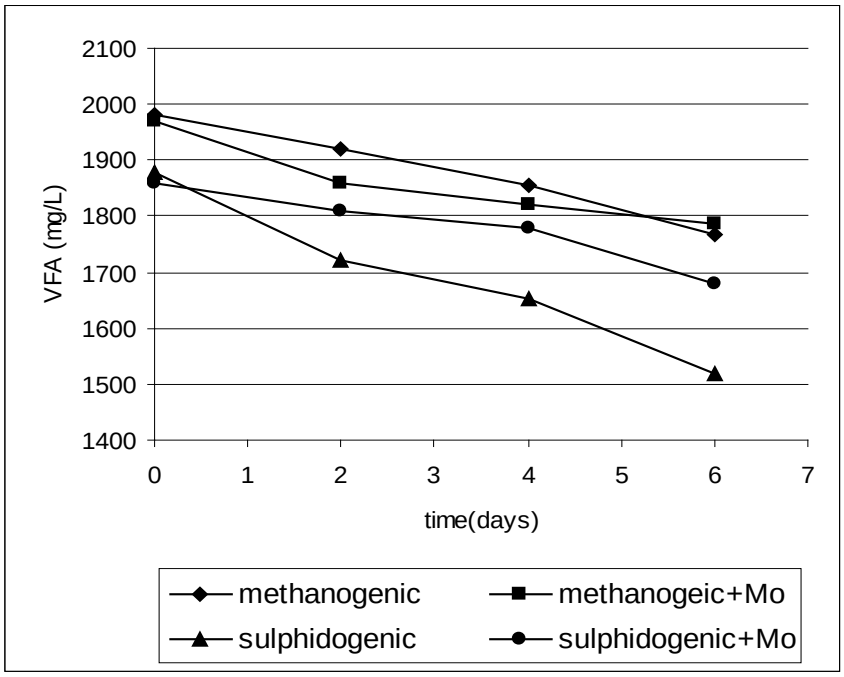


Figure 4.10 Total VFA concentrations under methanogenic and sulphidogenic conditions showing the sulphate reduction inhibition with molybdate.

During the two-day acclimation period, the uptake of VFA was significantly lower in the two methanogenic systems than the two sulphidogenic systems (ANOVA, $p<0.05$, $n=12$). This is supported by the fact that the methanogenic systems had reached 1870mg.L^{-1} while the sulphidogenic systems had reached 1880mg.L^{-1}. However, there was no significant difference in the rates of the methanogenic control and experimental systems (ANOVA, $p>0.05$, $n=24$), indicating that molybdate was selective for SRB and that they were not active in the methanogenic systems. The rates of VFA removal were 33mgVFA.L^{-1}.d^{-1} (11%) and 35mgVFA.L^{-1}.d^{-1} (10%) for the methanogenic control and experimental system respectively. The VFA removal in the sulphidogenic control was 60mgVFA.L^{-1}.d^{-1} (20%) and that of the sulphidogenic experimental system was 30mgVFA.L^{-1}.d^{-1} (10%). There was no significant difference between the inhibited sulphidogenic system and the two methanogenic systems (ANOVA, $p>0.05$,$n=36$), however the uninhibited sulphidogenic system was most significantly different to the other three systems (ANOVA, $p<0.001$, $n=48$). The concentration of the uninhibited sulphidogenic system decreased rapidly from 1870mg.L^{-1} at t=0 to 1520mg.L^{-1} at day 6. This indicates the rapid utilization of VFA, and would prevent occurrence of a negative feedback due to VFA accumulation.

Based on the pH data and the fact that SRB are effective in using a wider range of VFA, it was thought that the VFA in the methanogenic systems and in the inhibited sulphidogenic system should increase. From the recorded results in Figure 4.10, this was not the case, rather the rates of removal in the methanogenic systems and the inhibited sulphidogenic system were lower than that of the uninhibited sulphidogenic system.

The distribution of the VFA in the controls and under sulphate reduction inhibition with molybdate is depicted in Figures 4.11 and 4.12 respectively for the sulphidogenic and methanogenic systems.

a) b)

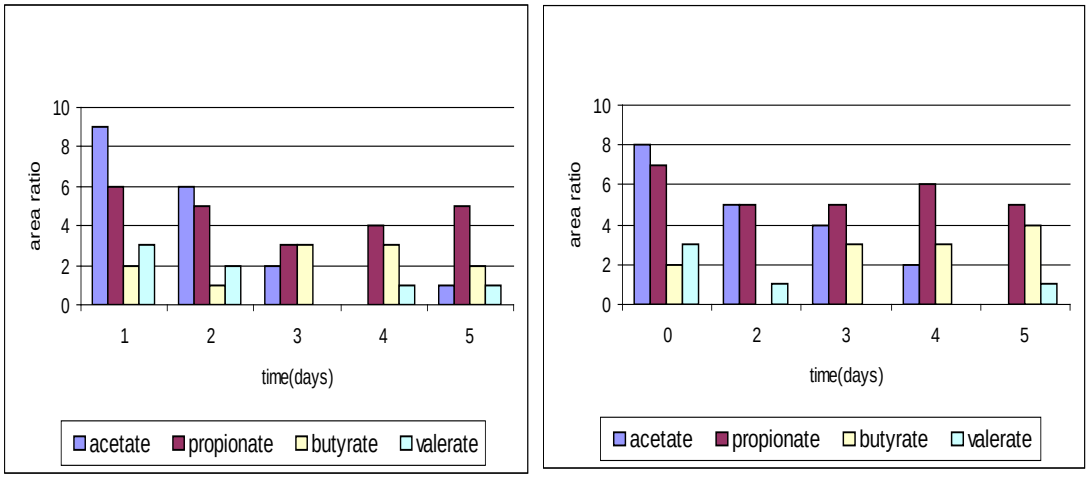


Figure 4.11 Change in the distribution of four volatile fatty acids in the methanogenic (a) and sulphidogenic (b) systems. (n=3, standard deviations were below 5%).

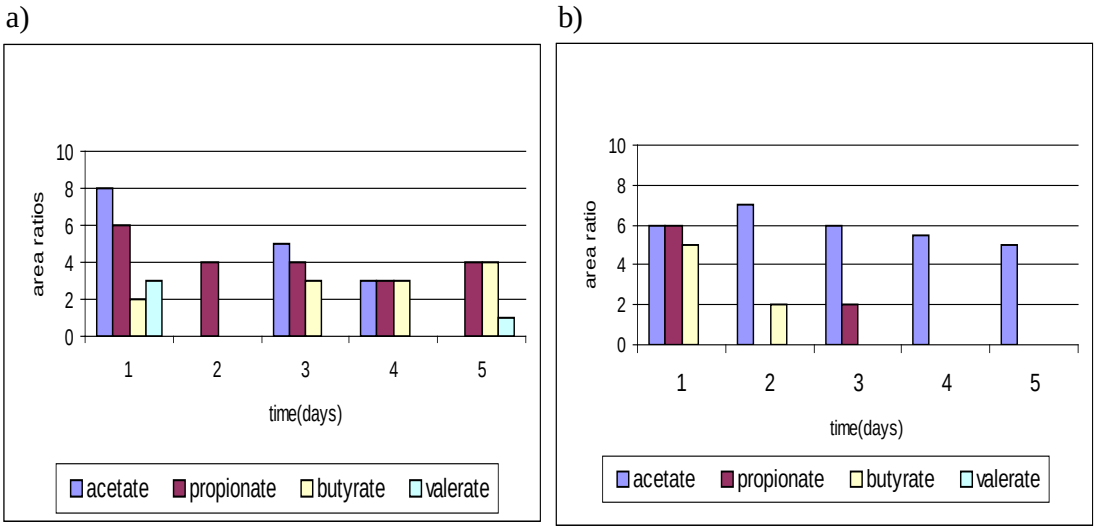


Figure 4.12 Change in the distribution of four volatile fatty acids in the methanogenic (a) and sulphidogenic (b) systems showing sulphate reduction inhibition with molybdate. (n=3, standard deviations were below 5%).

Figure 4.12 shows a similar VFA consumption pattern of VFA to that reported in Figure 4.8, where Figure 4.11 (a) shows that there was immediate utilization of acetate in the methanogenic system as it was totally consumed after day 2. This was followed by its reappearance on day 3, where after it was again consumed by day 5. Propionate decreased during the two days and its concentration then remained relatively constant until the end of the experiment. Butyrate also seemed to accumulate slightly while valerate was initially consumed but reappeared again at day 5. The sulphidogenic system (Figure 4.11b) on the other hand showed a utilization of propionate, butyrate and valerate while acetate accumulated. The addition of molybdate to the methanogenic system (Figure 4.12a) had very little effect on the general pattern of utilization but when added to the sulphidogenic system (Figure 4.12b), the pattern of utilization was similar to that of the methanogenic system as was expected.

The results of the study of utilization of VFA under methanogenic and sulphidogenic conditions were not conclusive, and could not provide definite proof that the enhanced hydrolysis of PSS under sulphate reducing conditions was linked to the ability of SRB to utilize a wider range of VFA. However, the calculated rates when sulphate reduction was inhibited with molybdate were, CODp removal ($61mg.L^{-1}.d^{-1}$), CODf utilization ($9mg.L^{-1}.d^{-1}$), and total VFA utilization ($30mg.L^{-1}.d^{-1}$) and pH (6.5). These rates and pH were significantly similar to the methanogenic control rates of CODp ($55mg.L^{-1}.d^{-1}$), CODf ($12mg.L^{-1}.d^{-1}$), total VFA ($33mg.L^{-1}.d^{-1}$) and pH

(6.6). These significant similarities served as preliminary conclusion that SRB play a significant role in the observed enhanced hydrolysis of PSS. This resulted in only limited accumulation of soluble products that could influence hydrolysis via a negative feedback system. Thus, more experiments would be necessary to conclusively prove the role of SRB and the effect of VFA on the enhanced hydrolysis of PSS.

4.4 Conclusion

The inhibition of sulphate reduction and reducing sugar uptake with molybdate and toluene respectively was successfully used to study the rates of uptake and distribution of VFA under methanogenic and sulphidogenic system respectively. This enabled more insight into the role played by sulphidogenic conditions on the hydrolysis of PSS to yield VFA, however the results of the study were not conclusive enough to explain the exact mechanism in place.

Form the results of the above study, the following conclusions were drawn:

- Molybdate is a successful inhibitor of sulphate reduction as this led to a decrease in sulphate reduction and pH;
- The enhanced hydrolysis of PSS under sulphidogenic conditions is dependent on the presence of SRB, and although it is suspected that this may be related to the ability of SRB to utilize a wider spectrum of VFA, thereby avoiding possible feedback inhibition, this was not proved conclusively.
- In the presence of high sulphate and neutral pH, the process of sulphidogenesis is favoured over methanogenesis, however, the inhibition of SRB with molybdate resulted in a pattern of VFA utilization similar to that of a methanogenic system.

Chapter 5

Conclusion: The Bio-Sulphidogenic Enhanced Hydrolysis of Primary Sewage Sludge

PSS has proved to be a readily available and cost-effective source of carbon driven biological processes including the use of SRB in the treatment of wastewaters. The data collected from this study was used to develop a descriptive model that serves to describe the process of PSS hydrolysis. The results obtained by Whittington-Jones (2000) in the operation of the RSBR were confirmed after studying the degradation of PSS under controlled flask conditions. The removal rate of CODp and therefore the solubilization of complex carbon to yield soluble products was enhanced under sulphate reducing conditions as compared to methanogenic conditions. The soluble products considered in this study were reducing sugars and VFA as the processes of acidogenesis and acetogenesis were of interest.

Over time there was a relative production and utilization of soluble products from PSS hydrolysis. However, from the rates calculated in this study, it was evident that under sulphate reducing conditions, the removal rates of these products were higher than under methanogenic conditions. The rates of reducing sugar production were calculated in the methanogenic and sulphidogenic systems. These rates were considered to be more accurate production rates at which reducing sugars are produced from the direct hydrolysis of PSS as their uptake was successfully inhibited using toluene. This rate of reducing sugar production was determined from the linear accumulation of reducing sugars and were found to be 167 mg. $L^{-1}.hr^{-1}$ under sulphidogenic conditions and 51 $mg.L^{-1}.hr^{-1}$ under very specific methanogenic conditions.

The utilization rates of total VFA were calculated for the two systems and it was noted that there is enhanced VFA utilization under sulphidogenic ($60mg.L^{-1}.d^{-1}$) as compared to the methanogenic ($33mg.L^{-1}.d^{-1}$) system. This enhanced utilization rate was attributed to the sulphidogenic system's ability to use a wider range of volatile fatty acids as compared to the methanogenic system. This ability then prevents the accumulation of these products as was evident in the sulphidogenic system. As proposed in the earlier study, the accumulation of these volatile fatty acids would trigger a negative feedback inhibition, which would lead to the decrease in pH, and affect the enzymes involved in the hydrolytic process. This would therefore

result in a lower particulate COD hydrolysis rate as observed in the methanogenic (57.mg.L^{-1}.d^{-1}) compared to the sulphidogenic (102mg.L^{-1}.d^{-1}) system. This was further confirmed by the distribution of VFA on the GC, where the methanogenic system was utilizing acetate as compared to propionate, butyrate and valerate until acetate was depleted. In the case of the sulphidogenic system, the converse of the methanogenic trend was noted where there was an accumulation of acetate and utilization of the other VFA. However, there was evidence that the uptake of reducing sugars is not necessary for the production of VFA as there were traces of VFA reappearance after depletion even though the uptake of reducing sugars was inhibited with toluene. The inhibition of sulphate reduction with molybdate led to the sulphidogenic system behaving more like a methanogenic system. This was supported by the inhibited sulphidogenic system's rate of particulate COD removal of 61mg.$L^{-1}d^{-1}$, which was not significantly different to the 57mg.L^{-1}.d^{-1} and 50mg.L^{-1}.d^{-1} obtained for the methanogenic control and experimental systems. The rate of total VFA utilization in the inhibited sulphidogenic and the methanogenic control and experimental systems were 30mg.L^{-1}.d^{-1}, 33mg.L^{-1}.d^{-1} and 35mg.L^{-1}.d^{-1} respectively for the specific conditions chosen. This data further served to confirm that the presence of SRB have a significant role to play in the enhanced rate of hydrolysis that was reported before.

The descriptive model developed in this study is centered on the ability of SRB to utilize a wider spectrum of these volatile fatty acids, which was observed in this study, thus no accumulation of VFA was observed in the sulphidogenic system. As a result, the occurrence of a negative feedback inhibition is avoided and the rate of hydrolysis is not affected. A diagrammatic representation of this model is shown on figure 5.1.

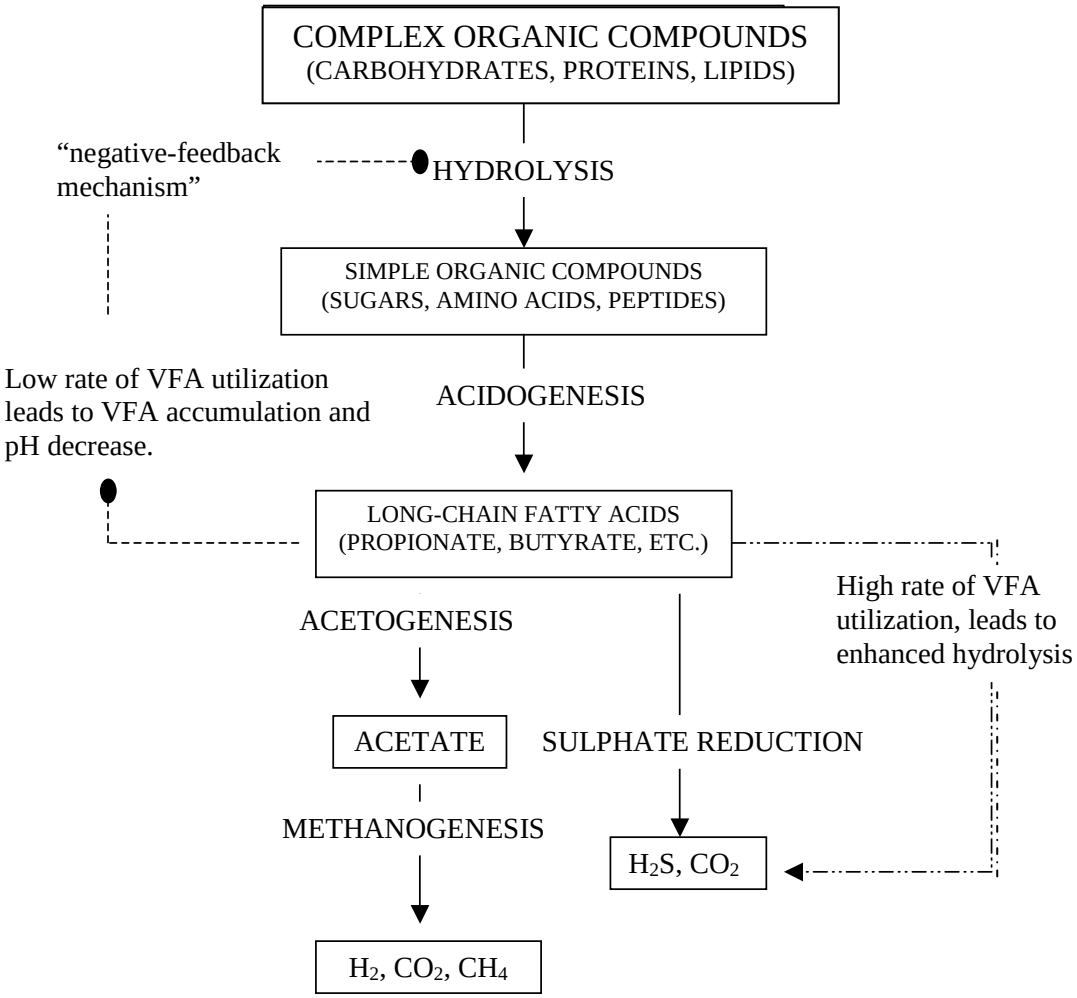


Figure 5.1 Flow diagram summarizing the enhanced hydrolysis under sulphidogenic system showing the possible "negative feedback inhibition

References

Aguilar A., Casa C. and Lema J.M. 1995. Degradation of volatile fatty acids by differently enriched methanogenic cultures: Kinetics and inhibition. *Wat. Res.,* **29** (2): 505-509.

APHA 1994. Standard Methods for examination of water and wastewater. 16th Edition, Washington.

Banat I.M, Lindstrom E.B., Nedwell D.B and Balba M.T. 1981. Evidence of two distinct functional groups of sulphate-reducing bacteria in salt marsh sediment. *Applied and Environmental Microbiology,* **42** (6): 985-992.

Banat I., Nedwell D. and Balba T.M. 1983. Stimulation of methanogenesis by slurries of salt marsh sediments after the addition of molybdate to inhibit sulphate reducing bacteria. *Journal of General Microbiology*, **117**: 89-92.

Banks D., Younger P.L., Amesen R., Iversen E.R and Banks S.E. 1997. Mine-water chemistry: the good the bad and the ugly. *Environmental Geology,* **32** (3): 157-171.

Banerjee A., Elefsiniotis P. and Tuhtar D. 1998. Effect of HRT and temperature on the acidogenesis of municipal primary sludge and industrial wastewater. *Wat. Sci. Tech.*, **38**: 417-423.

Banister S.S. & Pretorius W.A. 1998. Optimization of sludge acidogenic fermentation for biological nutrient removal. *Water SA*, **24**: 35-41.

Barber W.P. & Stuckey D.C. 1999. The use of the anaerobic baffled reactor (ABR) for wastewater treatment: a review. *Water Res.*, **33**: 1559-1578.

Basson M.S. & Van Rooyen J.A. 2001. Practical application of probabilistic approaches to the management of water resource systems. *Journal of Hydrology*, **241**: 53-61.

Bechard G., Rajan S. and Gould W.D. 1993. Characterization of a microbiological process for the treatment of acidic drainage. In: *Biohydrometallurgical Technologies.* Torma A.E., Apel M.L. and Brierley C.L. (eds). The Minerals, Metal and Materials Society, pp 277-286.

Benner R., Maccubin A.E. and Hodson R.E. 1984. Preparation, characterization and microbial degradation of specifically radiolabelled [^{14}C] lignocelluloses from marine and freshwater macrophytes. *Appl. Environ. Microbiol.,* **47**: 381-389.

Benner R., Weliky K. and Hedges J.I. 1990. Early diagenesis of mangrove leaves in a tropical estuary: molecular–level analyses of neutral sugars and lignin-derived phenols. *Geochim. Cosmochim. Acta,* **54**: 1991-2001.

Boon A.G. & Lister A.R. 1975. Formation of sulphide in rising main sewers and its prevention by injection of oxygen. *Progress in Water Technol*., **7** (2): 289-300.

Boshker H.T. & Cappenberg T.E. 1994. A sensitive method using 4-methylumbelliferyl-B-cellobiose as substrate to measure (1,4)-B-glucanase activity in sediments. *Appl. Environ. Microbiol.,* **60**: 3592-3596.

Boschker H.T., Bertilsson S.A., Dekkers E.M.J. and Cappenberg T.E. 1995. An inhibitor-based method to measure initial decomposition of naturally occurring polysaccharides in sediments. *Appl. Environ. Microbiol*., **61**: 2186-2192.

Boshoff G.A. 1999. Development of integrated biological processing for the biodesalination of sulphate- and metal-rich wastewaters.PhD Thesis. Rhodes University, Grahamstown, South Africa.

Braun M. and Stolp H. 1985. Degradation of methanol by a sulphate reducing bacterium. *Arch. Microbiol*., **142**: 77-80.

Brinch P.P., Rindel K. and Kalb K. 1994. Upgrading to nutrient removal by means of internal carbon from sludge hydrolysis. *Wat. Sci. Tech*., **29**: 31-40.

Bunce N.J., Chartrand C. and Keech P. 2001. Electrochemical treatment of aqueous acidic ferrous sulphate and copper sulphate as models of acid mine drainage. *Water Res.* **35**(18): 4410-4416.

Canfield D.E. 2001. Isotope fractionation by natural populations of sulphate reducing bacteria. *Geochemica et Cosmochimica Acta.* **65**(7): 1117-1124.

Canziani R., Pollice A. and Ragazzi M. 1996. Design considerations on primary sludge hydrolysis under psychrophilic conditions. *Environ. Technol.*, **17**: 747-754.

Christensen B., Laake M. and Torleiv L. 1996. Treatment of acid mine water by sulfate-reducing bacteria; results from a bench-scale experiment. *Water Res.*, **30**: 1617-1624.

Clancy P.B., Venkataraman N. and Lynd R. 1992. Biochemical inhibition of sulphate reduction in batch and continuous anaerobic digesters. *Wat. Sci. Tech*., **25** (7): 51-60.

Chang I.S., Shin P.Y. and Kim B.H. 2000. Biological treatment of acid mine drainage under sulphate reducing conditions with solid waste material as a substrate. *Water Res.*, **34**(4): 1269-1277.

Cole M.B., Arnold D.E. and Watten B.J. 2001. Physiological and behavioural responses of stonefly nymphs to enhanced limestone treatment of acid mine drainage. *Water Res.*, **35**(30): 625-632.

Coleberg P.J. & Young L.Y. 1985. Anaerobic degradation of soluble fractions of [^{14}C-lignin} lignocellulose. *Applied and Environmental Microbiology.***23**: 345-349.

Cypionka H. & Pfenning N. 1986. Growth yields of *Desafotomaculum orientis* with hydrogen in chemostat culture. *Arch. Microbiol*., **143**: 396-399.

Davison W., Reynolds C.S., Tipping E. and Needham R.F. 1989. Reclamation of acid waters using sewage sludge. *Environ. Pollut.*, **57**: 251- 274.

Department of Water Affairs, Management of the Water resources of South Africa, 1986.

Department of Water Affairs and Forestry 1987. A white paper on the national water policy for South Africa, pp.1-37.

Dicker H.J & Smith D.W. 1985. Metabolism of low molecular weight organic compounds by sulphate-reducing bacteria in a Delaware salt marsh. *Microb. Ecol*., **11**: 317-355.

Du Bois M., Gilles K.A., Hamilton J.K., Rebers P.A. and Smith F. 1956. Colorimetric method for determination of sugars and related substances. *Analyt. Chem*., **28**: 350-356.

Eastman J.A. & Ferguson J.F. 1981. Solubilization of particulate organic carbon during the acid phase of anaerobic digestion *Journal WPCF*, **53**: 352-366.

Edwards P.J, Bolton C.P, Ranson C.M. and Smith A.C. 1997. The River Pelenna treatment project. IMWA Symposium, Johannesburg, pp 17-32.

Elefsiniotis P. & Oldham W.K. 1994. Anaerobic acidogenesis of primary sludge: the role of solids retention time. *Biotech. Bioeng.*, **44**: 7-13.

Elliot P., Ragusa S. and Catcheside D. 1998. Growth of sulphate reducing bacteria under acidic conditions in an upflow anaerobic bioreactor as a treatment for acid mine drainage. *WaterRes.*, **32**: 3724-3730.

Espinosa A., Rosas L., Ilangovan K. and Noyola A. 1995. Effect of trace metals on the anaerobic degradation of volatile fatty acids in molasses stillage. *Wat. Sci. Tech*., **32** (12): 121-129.

Evangelou V.P. 2001. Pyrite micro encapsulation technologies: Principles and potential fields of application. *Ecological Engineering.* **17**: 165-178.

Foucher S., Battagalia B.F., Ignatiadis I. and Morin D. 2001. Treatment by sulphate reducing bacteria of Chessy of acid mine drainage and metals recovery. *Chemical Engeneering Science.* **56**: 1639-1645.

Gazea B., Adam K. and Kontopoulos. A. 1996. A review of passive systems for the treatment of acidic mine drainage. *Miner. Eng.*,**9**: 23-42.

Gibson G.R 1990. Physiology and ecology of sulphate reducing bacteria. *J. of Appl. Bacteriol*., **69**: 769-797.

Glombitza F. 2001. Treatment of acid lignite flooding water by means of microbial sulphate reduction. *Waste Management,* **21**: 197-203.

Ghosh S. 1991. Pilot-scale demonstration of two-phase anaerobic digestion of activated sludge. *Wat. Sci. Tech.* **23**: 1179-1188.

Grootvlei (Pty) Mines Ltd. 1997. Monitoring of the Blesbok Spruit: impacts of treated mine effluents from Grootvlei Mine (Company report).

Gujer W. & Zehder A.J.B 1983. Conversion process in anaerobic digestion. *Wat. Sci. Tech.*, **15**:127-167.

Hamilton Q.U.I, Lamb H.M, Hallet C. and Proctor J.A. 1997. Passive treatment systems for the remediation of acid mine drainage Wheal Jane, Cornwall, UK. IMWA Symposium, Johannesburg, pp 33-56.

Hammack R.W. & Edenborn H.M. 1992. The removal of nickel from mine waters using bacterial sulphate reduction. *Appl. Microbiol. Biotechnol*., **37**: 674-678.

Hansen T.A 1988. Physiology of sulphate reducing bacteria. *Micobiological SciencesI,* **3**: 81-84.
Hatziconstantinou G.J., Yannakopoulos P. and Andreadakis A. 1996. Primary sludge hydrolysis for biological nutrient removal. *Wat. Sci. Tech.,* **34**: 417-423.

Henrichs S.M. 1986. Early diagenesis of organic matter in marine sediments: Progress and perplexity. *Mar. Chem*., **39**: 119-149.

Henze M. & Mladenivski C. 1991. Hydrolysis of particulate substrate by activated sludge under

aerobic, anoxic and anaerobic conditions. *Water Res.*, **25**: 61-64.

Henze M.R. & Pieterse M.J. 1978. Acid mine drainage in the Republic of South Africa. *Prog. Water Technol.,* **9**: 981-1000.

Heukelekian H. & Balmat J.L. 1959. Chemical composition of the particulate fractions of domestic sewage. *Sew. Ind. Wastes,* **31**: 413-423.

Hunter J.V. & Heukelekian H. 1965. The composition of domestic sewage fractions. *Journal WPCF,* **37**: 1142-1163.

Jarvis A.P. & Younger P.L. 1999. Design, construction and performance of a full-scale compost wetland for mine spoil drainage treatment, Quaking Houses, UK. Unpublished report.

Johnson D.B. 1995. Acidophilic microbial communities: candidates for bioremediation of acidic mine effluents. *Int. Biodet. Biodeg*., **35**: 41-58.

Johnson D.B. 2000. Biological removal of sulphurous compounds from inorganic wastewaters. In: Lens P.N.L. and Hulsoff-Pol L. (eds). *Environmental technologies to treat sulfur pollution: Priciples in engineering.* IWA Publishing. London. Pp 175-193.

Kalin M..M., Cairns J. and McCready R. 1991. Ecological engineering methods for acid mine drainage treatment of coal waters. *Resour. Conserv. Recycl.*, **5**: 265-275.

Karhadkar P.P, Audic J.M, Faup G.M. and Khanna P. 1987. Sulphide and sulphate inhibition of methanogenesis. *Water Research,* **21**: 1061-1066.

Kim S.K., Matsui S., Pareek S., Shimizu Y. and Matsude t. 1997. Biodegradation of recalcitrant organic matter under sulphate reducing and methanogenic conditions in the landfill column reactors. *Wat. Sci. Tech.*, **36**: 91-98.

King G.M. 1986. Characterization of B-glucosidase activity in intertidal marine sediments. *Appl. Environ. Microbiol.,* **51**: 373-380.

King P.H, Norris L.R and Randall C.W. 1975. Sulphuric acid neutrilization and concurrent sulphate removal by precipitation with barium. Proceedings of the 30th Purdue Industrial Waste Conference. Ann. Science Publishers Inc., pp. 390-399.

Knapp J.S. & Howell J.A. 1978. Treatment of primary sewage sludge with enzymes. *Biotech. Bioeng.*, **20**: 1221-1234.

Lawrence A. W. and McCarthy P. L. 1969, Kinetics of methane fermentation in anaerobic treatment. *J. wat. Pollut. Control Fed.*, **41**: 1-17.

Lens P.N., De Poorter M.P., Cronenberg C.C. and Verstraete W.H. 1995. Sulphate reducing and methane producing bacteria in aerobic wastewater treatment systems. *Wat. Res.*, **29**: 871-880.

Lens P.N.L., Van Den Bosch M.C., Hulsoff-Pol L.W and Lettinga G. 1998. Effect of staging on volatile fatty acid degradation in a sulfodogenic granular sludge reactor. *Water Res.*, **32**; 1178-1192.

Lilley I.D., Wentzel M.C., Loewenthal R.E. and Marais G.V.R. 1990. Acid fermentation of primary sludge at 20^{o}C. Research Report, Civil Engineering Dept., University of Cape Town, W64, pp 87.

Maree J.P., Gerber A. and Strydom W.F. 1986. A biological process for sulphate removal from industrial effluents. *Water SA*, **12**: 139-144.

Maree J.P., Bosman D. and Jenkins G.R. 1989. Chemical removal of sulphate, calcium and heavy metals from mining and power station effluents. *Wat. Sew. Effl.*, 9 (3):10-26.

Maree J.P. & Hill E. 1989. Biological removal of sulphate from industrial effluents and concomitant production of sulphur. *Wat. Sci. Tech.*, **21**: 265-276.

Maree J.P, Hulse G., Pods D. and Schutte C.E. 1991. Pilot-scale biological sulphate removal utilizing producer gas as energy source. *Wat. Sci. Tech.*, **30** (12): 275-285.

Maree J.P., van Tonder G.J. and Millard P. 1996. Underground neutralization of mine water

with limestone. *Report to the Water Research Commission*, CSIR. WRC Report No. 609/1/96.

Marty D. 1981. Distribution of different anaerobic bacteria in Arabian Sea sediments. *Mar. Biol.*, **63**: 277-281.

Mays P.A. & Edwards G.S. 2001. Comparison of heavy metal accumulation in natural wetland and constructed wetlands receiving acid mine drainage. *Ecological Engineering,* **16**: 487-500.

McCartney D.M. & Oleszkiewicks J.A. 1993. Competition between methanogens and sulfate reducers: Effect of COD:sulfate ratio and acclimation. *Wat. Environ. Res.*, **65** (5): 656-660.

McCarty P. L. & Vath C. A. 1962, Volatile fatty acid digestion at high loading rates, *Int. J. air Wat. Poll.*, Pergamon Press, **6**: 65-73.

Mc Giness S., Sanger L.S and Atkinson K. 1997. The care and feeding of constructed wetlands. IMWA Symposium, Johannesburg, pp 123-131.

Middleton A.C & Laurence A.W. 1977. Kinetics of microbial sulphate reduction. *J. Wat. Pollut. Control. Fed*., **49**:1659-1670.

Molipane N.P. 1999. Biological sulphate reduction utilising hydrolysis of a complex carbon source. M.Sc. Thesis, Rhodes University, Grahamstown, South Africa.

National Water Act 36 of 1998.

Nielson H.P & Hvitved-Jacobsen T. 1989. Effect of sulphate and organic matter on the hydrogen sulphide formation in biofilms of filled sanitary sewers. *Water Pollution Control Federation Journal*, **60** (5): 627-634.

Novaes R.F.V. 1986. Microbiology of anaerobic digestion. *Wat. Sci.Tech.*, **18**: 1-14.

Novak J.T & Ramesh M.S. 1975. Stimulation in anaerobic degradation. *Water Research,* **9**: 963-967.

Odendaal P. 2001. Dynamics of the South African water scene over the last 21 years: A regional perspective. *Water Sewage and Effluent,* **21** (5): 24-31.

Ohron D., Cokgor E.U and Sozen S. 1999. Experimental basis for the hydrolysis of slowly biodegradable substrate in different wastewaters. *Wat. Sci. Tech.* **39**(1): 87-95.

Pareek S., Kim S.K., Matsui S. and Shimizu Y. 1998. Hydrolysis of (ligno) cellulosic materials under sulfidogenic and methanogenic conditions. *Wat. Sci. Tech.*, **38**: 193-200.

Parker W.J, Monteith H.D and Melcer H. 1994. Estimation of anaerobic biodegradation rates for toxic organic compounds in municipal digestion. *Wat. Res.* **28** (8): 1779-1789.

Peck, H.D. 1959. The ATP dependent reduction of sulphate with hydrogen in extracts of Desulfovibrio desulfuricans. *Proc. Natl. Acad. Sci*. **45**: 701-708.

Penaud V., Delgenes J.P., Torrijos M., Moletta R., Vanhoutte B. and Cans, P. 1997. Definition of optimal conditions for the hydrolysis and acidogenesis of pharmaceutical microbial biomass. *Process Biochem.*, **32**: 515-521.

Peppas A., Komnitsas K. and Halikia I. 2000. Use of organic covers for acid mine drainage control. *Minerals Engineering.* **33**(5): 563-574.

Postgate J.R 1984. The sulphate reducing bacteria. 2nd edition, Cambridge University Press, London, New York, pp. 107-120.

Pulles W., Howie D., Otto D. and Easton J. 1995. A manual on mine water treatment and management practices in South Africa. Water Research Commission, Report No. TT 80/96.

Qabiti A. J., Bories A. and Garcia J. L. 1990, Effects of sulfate on lactate and C_2-, C_3- volatile fatty acid anaerobic degradation by a mixed microbial culture. *Antonie van Leeuwenhoek*, **58**:

241-248.

Radchenko O.S. & Tisharev A.B. 1991. The role of sulphate reducing bacteria in anaerobic purification of wastewater. *Soviet J. of Chem. Technol*., **13** (2): 99-106.

Ramsingh L, Joubert H., Geldenhuys J. and Potgieter M. 1998. Determination of the effect of Lesotho Highlands Water on the Vaal dam. *Papers Volume 2, Benneial Conference and Exhibition of the Water Institute of Southern Africa,* 1998: 2B-3.

Ranade D.R., Dighe A.S., Bhirangi S.S., Panhalkar V.S. and Yeole T.Y. 1999. Evaluation of the use of sodium molybdate to inhibit sulphate reduction during digestion of distillery waste. *Bioresource Technology*, **68**: 287-291.

Robins N.S. & Younger P.L. 1997. Coal abandonment–mine water in surface and near-surface environment: some historical evidence from the UK. IMWA Symposium, Johannesburg, pp 253-261.

Robinson J.F.D. & Robb G.A. 1995. Methods for control and treatment of acid mine drainage. Coal International, July, pp. 152-156.

Rose P.D., Boshoff G.A., Van Hille R.P., Wallace L.C.M., Dunn K.M. and Duncan J.R. 1998. An integrated algal sulphate reducing high rate ponding process for the treatment of acid mine drainage wastewaters. *Biodegradation*, **9**: 247-257.

Rowley M.V, Warkentin D.D and Yan V.T. 1994. The biosulphide process: integrated biological chemical acid mine treatment: results from laboratory piloting. International and reclamation Mine drainage Conference, Pittsburg, P.A., pp. 205-213.

San Pedro D.C., Mino T. and Matsuo T. 1994. Evaluation of the rate of hydrolysis of slowly biodegradable COD (SBCOD) using starch as a substrate under anaerobic, anoxic and aerobic conditions. *Wat. Sci. Tech*., **30**: 191-199.

Sazowsky I.D., Foos A. and Miller C.M 2000. Lithic controls on the removal of iron and the remediation of acid mine drainage. *Water Res.*, **34**(10): 2742-2746.

Schoeman J.J. & Steyn A. 2001. Investigation into alternative water treatment technologies for the treatment of underground mine water discharged by the Grootvlei Propietary Mines Ltd into the Blesbokspruit in South Africa. *Desalination.* **133**(1): 13-30.

Scott R. 1995. Flooding of Central and East Rand gold mines: an investigation into controls over the inflow rate, water quality and the predicted impacts of flooded mines. *Water Research Commission, Report No. 486/1/95*.

Siegrist H., Renggli D. and Gujer W. 1993. Mathematical modelling of anaerobic digestion: A bioconversion mechanism and kinetic model.

Shimizu T., Kudo K. and Nasu Y. 1993. Anaerobic waste activated sludge digestion-a bioconversion mechanism and kinetic model. *Biotech. Bioeng*., **41**: 1082-1091.

Skalsky D.S. & Daigger G.T. 1995. Wastewater solids fermentation for volatile acid production and enhanced biological phosphorus removal. *Wat. Environ. Res.*, **67**: 230-237.

Smith R.L. & Klug M.J. 1981. Electron donors utilized by sulfate-reducing bacteria in Eutrophic lake sediments. *Applied Environmental Microbiology*, **42** (1): 116-121.

Stams A. M. J., Kremer D. R., Nicolay K., Weenk G. H. and Hansen T. A. 1984, pathway of propionate formation in *Desulfobulbus propionicus*. *Arch. Microbiol.*, **139**: 167-173.

Szwezyk U. & Pfenning N. 1987. Complete oxidation of catechol by the strictly anaerobic sulphate reducing *Desulfobacterium catecholicum sp*. Nov., *Arch. Microbiol*., **147**:163-168.

Torien D. F. & Hattingh W. H. J. 1969, Anaerobic digestion 1. The microbiology of anaerobic digestion. *Water Research* Pergamon Press, **3**: 385-416.

Tsukamoto T.K. & Miller G.C. 1999. Methanol as a carbon source for microbial treatment of acid mine drainage. *Water Res.,* 33**(6)**: 1365-1370.

Tuttle J.H., Dugan P.R. and Randles C.I. 1969. Microbial dissimilatory sulfur cycle in acid mine

water. *J. Bacteriol*., **97**: 594-602.

Ueki K., Kotaka K., Itoh K. and Ueki A. 1988. Potential availability of anaerobic treatment with digester slurry of animal waste for the reclamation of acid mine water containing sulphate and heavy metals. *J. Ferment. Technol*., **66**: 43-50.

Ueki K., Ueki A., Takahashi K. and Iwatsu M. 1992. The role of sufate reduction in methanogenic digestion of municipal sludge. *J. Gen. Appl. Microbiol.* **38**: 195-207.

Van Zyl H.C. 1996. Environmental systems in Amcoal. Mining Environmental management, March, pp. 18-21.

Vavillin V.A., Rytov S.V. and Lokshina L.Y. 1996. A description of hydrolysis kinetics in anaerobic degradation of particulate organic matter. *Bioresource Technol.*, **56**: 229-237.

WRC report No 230/1/90 1990. The Chemical removal of sulphates. *Report to the Water Research Commission by the Pollution Research Group, University of Natal.* Pretoria, South Africa.

Widdel F. & Bak F. 1986. Anaerobic degradation of phenol and phenol derivatives by the *Desulfobacterium phenolicum sp*. Nov. *Arch. Microbiol*., **146**:177-180.

Widdel F. 1988. Microbiology and Ecology of sulfate- and sulfur-reducing bacteria. In: *Biology of Anaerobic Microorganisms*. Zehnder, J.B. (ed.). John Wiley and Sons, New York, pp 469-584.

Whittington-Jones K. 2000. Sulphide-enhanced hydrolysis of PSS: implications for the bioremediation of sulphate-enriched wastewaters. PhD thesis, Rhodes University, Grahamstown, South Africa.

Wu W., Hickey R.F and Zeikus J.G. 1991. Characterization of metabolic performance of methanogenic granules treating brewery wastewater: role of sulphate reducing bacteria. *Appl. Environ. Microbiol.* **57**: 3483-3449.

Younger P.L. 1998a. Design, construction and initial operation of full-scale compost-based passive systems for treatment of coal mine drainage and spoil leachate in the U.K. IMWA Symposium, Johannesburg, pp 413-424.

Younger P.L., Curtis T.P., Jarvis A. and Pennell R. 1997. Effective passive treatment of aluminium-rich acidic colliery spoil drainage using a compost wetland at Quaking Houses, County Durham. *J. Chartered Inst. Water Environ. Mgt.*, **11**: 200-208.

Zehnder A.J.B. 1988. Biology of sulfur bacteria. In: Biology of anaerobic microorganisms. John Wiley & Sons. New York.

APPENDIX 1: PREPARATION OF ANALYTICAL REAGENTS

1.0 COD DETERMINATION

COD solution A and B

2.0 REDUCING SUGARS DETERMINATION

2.1 Cu^{2+} Reagent

Table A1.1. Analytical reagents used for the analysis of reducing sugars.

Reagent	**Quantity for 1L**
$CuSO_4.5H_2O$	4g
Na_2CO_3	24g
$C_4H_4KNaO_6.4H_2O$	16g
Na_2SO_4	180g
dH_2O	1000ml

2.2 Arsenomolybdate Reagent

Reagent 1

Table A1.2. **Analytical reagents used for the preparation of arsenomolybdate reagent 1**

Reagent	**Quantity for 1L**
$(NH_4)_6Mo_7O_{24}4H_2O$	50g
dH_2O	900ml
Conc H_2SO_4	42ml

Reagent 2

Table A1.3. **Analytical reagents used for the preparation of arsenomolybdate reagent 2**

Reagent	**Quantity for 1L**
$Na_2HasO_4.7H_2O$	6g
dH_2O	50ml

Mix reagents 1 and 2 and incubate at 37^oC for 24 hours and store in a brown bottle.

3.0 SULPHIDE DETERMINATION

3.1 Solution A

Table A1.4. Analytical reagents used to prepare solution A for sulphide determination

Reagent	**Quantity for 1L**
N,*N*-dimethyl-*p*-phenylenediamine dihydrochloride $(CH_3)_2N.C_6.H_4.NH_2.2HCl$	4g
dH_2O	1000ml

3.2 Solution B

Table A1.5. Analytical reagents used to prepare ferric chloride solution for sulphide determination

Reagent	**Quantity for 1L**
$FeCl_3.6H_2O$	16g
dH_2O	1000ml

3.3 Zinc Acetate solution

Table A1.6. Analytical reagents used for the preparation of zinc acetate

Reagent	Quantity for 1L
$(CH_3.COO)_2Zn.2H_2O$	10.44g
dH_2O	1000ml

3.4 Sulphide Stock

Table A1.7. Analytical reagents used for the preparation of sulphide stock solution

Reagent	Quantity for 1L
Na_2S	0.750g
dH_2O	1000ml

4.0 SULPHATE DETERMINATION

4.1 Stock Solution

Table A1.8. Analytical reagents used to prepare stock solution for sulphate determination

Reagent	Quantity for 2L
p-hydroxy-bromo-acetic acid	5.52g
HPLC grade CH_3OH	250ml
ddH_2O	1750ml

4.2 HPLC Mobile Phase: prepared fresh daily

Table A1.9. Analytical reagents used for the preparation of the HPLC mobile phase for sulphate determination

Reagent	Quantity for 1L
Stock solution	300ml
ddH_2O	700ml

Adjust pH to 4.8, filter with 0.45mm nylon filter and degas under pressure

5.0 VOLATILE FATTY ACID DETERMINATION

5.1 Steam Distillation

Table A1.10. Analytical reagents used in steam distillation of VFA

Reagent	**Quantity for 1L**
a. conc. H_2SO_4	500ml
dH2O	500ml
b. NaOH 0.1*N*	1.63g
Oxalic acid 0.1*N*	2.52g
c. Phenolphthalein indicator solution	10g
dH_2O	1000ml
d. CH_3COOH	1.9ml
dH_2O	1000ml

5.2 Liquid extraction

Dichloromethane was used as an extracting solvent.

APPENDIX II: PROTOCOLS FOR ASSAYS

1. COD DETERMINATION: SQ 118 METHOD

1.1 Principle: The solutions A and B are filled into a reaction cell containing the sample. After d9igestion with the thermoreactor TR 205, the solution is cooled and then analysed with photometer SQ 118 (Merck Test Kit).

1.2 Analysis

- Into a reaction tube add 3ml sample
- 0.3ml solution A
- 2.3ml solution B
- Mix the reaction
- Heat the reaction tube on the thermoreactor TR 205 at 148^{o}C for 2hrs
- Cool the reaction tube for 15 minutes
- Prepare a blank with water
- Read the COD concentration (mg.L^{-1}) on the photometer SQ 118.

2. REDUCING SUGARS DETERMINATION

2.1 Principle: A calorimetric reaction for the determination of reducing sugars was employed. When the sample is heated, an alkaline solution of copper tartrate and cuprous oxide is formed, which gives rise to molybdenum blue when it reacts with arsenomolybdate. Na_2SO4 is included to minimize the amount of atmospheric oxygen into the reaction mixture.

2.2 Analysis

- Into a clean test tube add 0.75ml of sample
- 1ml of the Cu^{2+} reagent

- Boil at 90^{o}C for 20 minutes in a water bath
- Cool the reaction for 10 minutes
- Add 1ml of the arsenomolybdate reagent
- Mix the reaction
- Stand the reaction for 5 minutes
- Prepare blank with water
- Read the absorbence at 420nm and convert to mg.L^{-1} glucose from the standard curve

3. SULPHIDE DETERMINATION

3.1 Principle: A calorimetric method was employed to determine the concentration of sulphide in the samples. H_2S in the presence of amine-dihydrochloride solution and ferric chloride gives water its reducing properties resulting in a negative redox potential.

3.2 Analysis

- In a clean test tube add 0.1ml ZnAc
- 1ml sample
- 1ml solution A
- 1ml solution B
- Mix the reaction
- Stand the reaction for 1hr
- Prepare blank with water
- Read the absorbence at 670nm and calculate the sulphide concentration in mg.L^{-1} form the standard curve

4.0 SULPHATE DETERMINATION

4.1 Principle: Samples were assayed using HPLC for sulphate presence. Yhe anionic column employed was used to separate present ions at different retention times. The retention time for sulphate was read using a known sulphate standard.

4.2 Analysis:

4.2.1. Sample preparation

- Collect sample and spin down at 3000rpm for 3 min
- Filter sample through a MAGNA, nylon, supported, 0.45 micron, 25mm filter
- Sample is ready for dilution
- Add 1ml of sample to 1ml of ZnAc
- Filter through MAGNA, nylon, supported, 0.45 micron, 25mm filter
- Wash the filter with 4ml of ddH_2O
- Pass through two isolute ^{TM} C18(EC) 1ml columns to remove the organics
- Wash the column with 4ml ddH_2O
- Make up to 10ml in a volumetric flask

4.2.2 Mobile phase preparation

- In a clean 1L volumetric flask, add 300ml of the stock solution
- Make up to 1L with ddH_2O
- Filter under pressure with a MAGNA, nylon, supported, 0.45 micron, 25mm filter
- Degas the mobile phase

5.0 VOLATILE FATTY ACID DETERMINATION

Two methods were employed for VFA determination, the steam distillation method and the GC method.

5.1 Principle:

5.1.2. Steam distillation: method was used to determine total VFA in the sample. Sulphuric acid is added to the sample to hydrolyse the carbon to simple sugars, this mixture is then boiled to distil VFA.

5.1.3. The GC method: a liquid extraction method is used to extract the clean sample into dichloromethane. The sample is then dried using sodium sulphate, before injecting into the GC. The operating parameters of the GC are optimised to initial temperature of 40^{o}C for 1min, rate 4^{o}/min and final temperature of 200^{o}C for 1min. Standard solutions were also employed in order to identify the different peaks.

5.2 Analysis:

5.2.1 Steam distillation method:

- In a clean 500ml round bottom flask
- Dilute sample in a 1:1 ratio with dH_2O (100ml sample + 100ml dH_2O)
- Add 5ml concentrated sulphuric acid/100ml sample
- Distil at 80^{o}C, discard the first 10ml
- Collect 130-150ml
- Add few drops phenolphthalein
- Titrate the distillate with standardised 0.04*N* NaOH
- Calculate concentration of VFA as acetic acid using the formula

5.2.2 GC

- Collect 1ml sample
- Spin down at 3000rpm for 3min
- Filter with MAGNA, nylon, supported, 0.45 micron, 25mm filter
- Add 0.5ml DCM
- Inject into GC at initial temperature 40^{o}C, initial time 1min, rate 4^{o}C/min and final temperature 200^{o}C

APPENDIX III: STANDARD CURVES

A.3.1 Carbohydrate Standard Curve

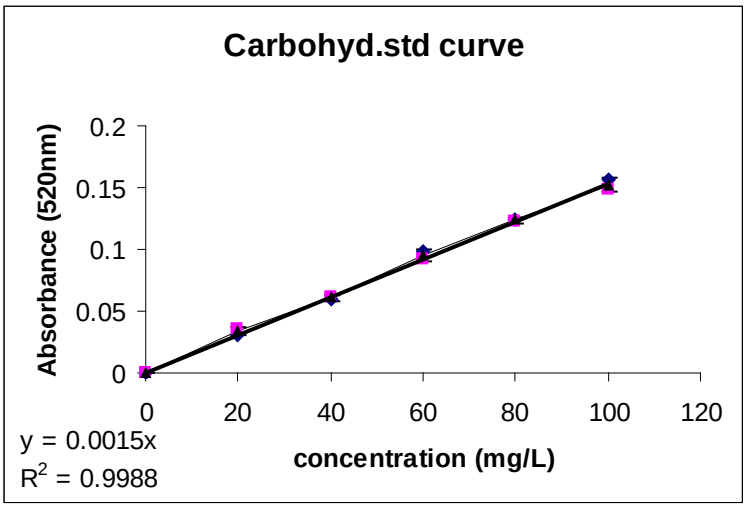


Figure A.3.1: Carbohydrate Standard Curve.

A.3.2 Sulphide Standard Curve

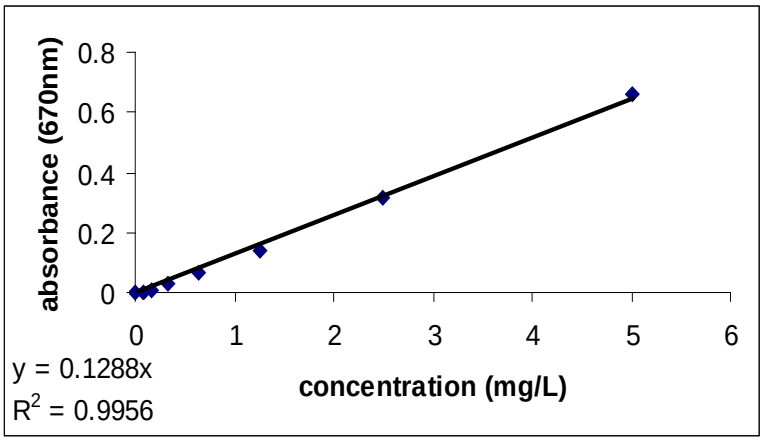


Figure A.3.2: Sulphide Standard Curve

A.3.3 Sulphate Standard Curve

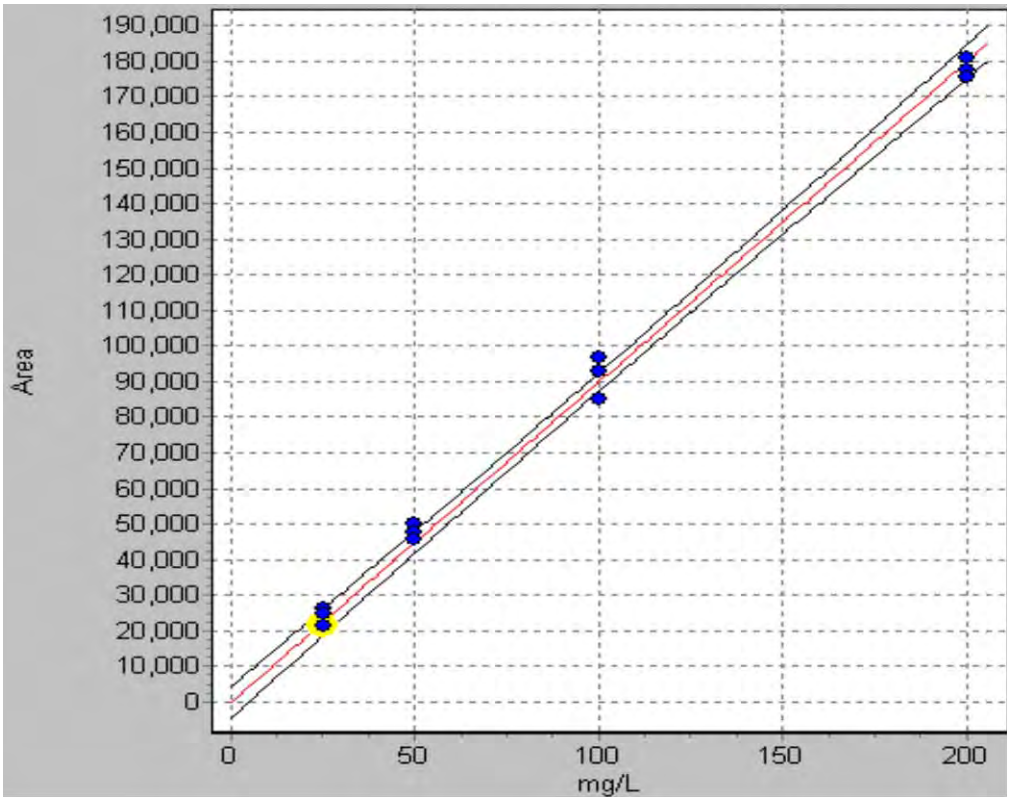


Figure A.3.3 Sulphate Standard Curve