SULPHIDE-ENHANCED HYDROLYSIS OF PRIMARY SEWAGE SLUDGE: IMPLICATIONS FOR THE BIOREMEDIATION OF SULPHATE-ENRICHED WASTEWATERS

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Abstract

The potential application of sulphate reducing bacteria for the bioremediation of acid mine drainage has already been recognised, and offers significant financial advantages over conventional chemical treatment approaches. Although the technology has been demonstrated successfully on both small- and large-scale, it's extensive implementation has been constrained by the provision of suitable and cost effective electron donor and carbon sources. Primary sewage sludge is readily available in large quantities, but the slow rate of solubilization and low yield of soluble products do not apparently favour its use for this application. A number of pre-treatment steps have been introduced in an attempt to improve the yield and rates under methanogenic conditions. However, although early work suggested that degradation of lignocellulose and proteins may be more rapid under sulphate reducing conditions, the fate of primary sewage sludge under these conditions has been ignored. It was proposed that by combining the hydrolysis of primary sewage sludge and biological sulphate reduction, in a settling sludge bed, both processes would be enhanced. The aim of this study was to test this hypothesis on laboratory- and pilot-scale, and attempt to elucidate the underlying mechanism involved.

The solubilization of primary sewage sludge was enhanced in the presence of sulphate reduction in continuous laboratory-scale reactors. Particulate matter accumulated in the bed of non-sulphidogenic systems, but not in sulphidogenic ones. This was attributed to increased solubilization and the smaller average floc size in the latter. Solubilization occurred within the settling sludge bed of the reactors, and offered a possible explanation for the better performance of the multiple- over single-stage reactor. A pilot-scale Falling Sludge Bed Reactor was constructed at Grootvlei Gold Mine, Springs, South Africa, and resulted in the solubilization of more than 70% of the influent primary sewage sludge. The system was also found to be highly resilient to severe perturbations, and returned rapidly to steady-state. Flask studies revealed that the hydrolysis of both proteins and complex carbohydrates was accelerated in the presence of biological sulphate reduction or sulphide. A study of the enzymology of sludge digestion revealed that sulphate reduction had little direct effect on the activity of the hydrolytic enzymes, but that reactor design was critical in the prevention of washout of these enzymes. Finally, a descriptive

model was developed to explain the enhanced hydrolysis of primary sewage sludge. The model incorporated the effect of sulphidogenesis on floc fracture and reflocculation, and likely implications for mass transfer limitations.

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List of Abbreviations

AMD = Acid Mine Drainage

ABR = Anaerobic Baffled Reactor

BNR = Biological Nutrient Removal

BT = Blend Tank

COD = Chemical Oxygen Demand ($mg.L^{-1}$)

COD_f = Filtered COD ($mg.L^{-1}$)

COD_p = Particulate COD ($mg.L^{-1}$)

COD_t = Total COD ($mg.L^{-1}$)

EPS = Extracellular Polymeric Substances

FSBR = Falling Sludge Bed Reactor

GDW = Grahamstown Disposal Works

HDS = High Density Sludge

HDPE = High Density Polyethylene

HRT = Hydraulic Retention Time

MS = Multiple-Stage

MWR = Mine Water Reservoir

PE = Polyethylene

PSS = Primary Sewage Sludge

SDS = Sodium Dodecyl Sulphate

SDS-PAGE = SDS-Polyacrylamide Gel Electrophoresis

SHT = Sludge Holding Tank

SRB = Sulphate Reducing Bacteria

SRT = Sludge Retention Time

SS = Single-Stage

STR = Stirred Tank Reactor

UASB = Upflow Anaerobic Sludge Blanket

VFA = Volatile Fatty Acids

VSS = Volatile Suspended Solids

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Chapter 1

General Introduction

1.1. Water usage and mining: a South African perspective

Acid mine drainage (AMD) is a worldwide environmental hazard associated with current and past mining activities, and poses a serious threat to the quality of valuable surface water. The acidic run-off from slimes dams is the main source of water pollution from gold and coal mining activities. Once the spent ore is brought to the surface, it is subject to the most acidic of all weathering reactions (equation 1, 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

This reaction, which may be enhanced by bacterial activity (Fortin *et al*., 1995; Johnson, 1995), is initially limited by pH values that are higher than optimal. However, once the pH has decreased, the reaction is only limited by oxygen supply. The AMD is characterised as having a low pH and high concentration of sulphate, iron and suspended solids. Henzen and Pieterse (1978) reported sulphate concentrations in polluted tributaries of the Vaal River of up to 729mg.L^{-1}, while concentrations in water just downstream from slimes dams ranged from 400-1160mg.L^{-1}. Wittmann and Förstner (1976) also analysed water samples from the Witwatersrand area and found sulphate concentrations in excess of 3000mg.L^{-1}. AMD often contains high concentrations of heavy metals, especially iron, copper, cobalt, nickel, zinc and manganese (Wittmann and Förstner, 1976, 1977). Many of these heavy metals are potentially toxic by virtue of their ability to bind to biologically active molecules, especially proteins. When these metal ions come into contact with hydrogen sulphide, produced by the bacterial reduction of sulphate, they form insoluble metal sulphides. These will then precipitate out of solution at high pH values (Widdel, 1988).

The gold-bearing reefs on the East Rand have a shallow basin-like structure, and have been actively mined for the last one hundred years. During this period, water flowed into the mines

from the surface and from dolomitic aquifers. In order to prevent the flooding of shafts by underground water, this brackish water (3500-4000mg.L^{-1} salt) is pumped to the surface (Wittmann and Förstner, 1976, 1977; Scott, 1995). The water is unsuitable for use by either agriculture or industry, and so is allowed to evaporate in large pans or is discharged into surface bodies (Scott, 1995). As the mines in the region became inter-linked, the removal of sub-surface water was allocated to a few of the deeper mines. A small proportion of the total volume was pumped by Grootvlei No. 3 and No. 4 shafts. Between 1988 and 1991, the mean volume of water being pumped from the East Rand mining basin was 65.4 ML.day^{-1} (Scott, 1995). By 1997, Grootvlei was pumping 110ML.d^{-1} (Grootvlei (Pty) Mines Ltd, 1997). Currently, heavy metals are removed by a High Density Sludge (HDS) process and the mean iron concentration in the water being discharged into the Blesbok Spruit is less than 1mg.L^{-1}. However, at times, the concentration exceeds 44mg.L^{-1}. The high salt content of the water is still problematic, with sulphate being the dominant contaminant of concern in the effluents from South African mining operations (Pulles *et al*., 1995). Failure to correct the situation will result in the loss of the wetland as well as a decline in the quality of farmland being irrigated with water from the Blesbok Spruit.

When mining ceases in the area, cessation of pumping will result in the filling of disused shafts, and will eventually lead to uncontrolled decanting of the mine drainage. Both alternatives require that mine drainage is adequately treated prior to discharge, long after all profitable mining operations have ceased. Thus, low cost, long-term alternatives to current treatment practices will have to be employed.

1.2. Current initiatives

Attempts to reduce the pollution caused by AMD are aimed at increasing the pH of the water and reducing the concentrations of metals and salts to acceptable levels. Ideally, the methods used should be relatively inexpensive, easy to carry out and produce as little solid waste as possible (Gazea *et al.*, 1996), and must be suitable for the remediation of large volumes of water. Current practises are reviewed below.

1.2.1. Physico-chemical treatment

Popular non-biological treatment methods include lime treatment (Thompson, 1980; Barnes and Romberg, 1986) and the Bethlehem process (Henzen and Pieterse, 1978). The former requires that unslaked, hydrated or dolomitic lime be added to the AMD. Each has its own advantages and disadvantages, for example, dolomitic lime (limestone, $CaCO_3$) reacts very slowly in water that has a high concentration of ferrous iron. Hydrated lime reacts faster, but produces a greater volume of sludge (Henzen and Pieterse, 1978). The Bethlehem process results in the formation of a denser sludge than regular treatment. Settled solids are aerated before lime is added, thus allowing all the ferrous iron to oxidise to the ferric state, and then precipitate. Neutralisation with $CaCO_3$ results in the partial removal of sulphate, as $CaSO_4$, as well as Fe^{3+} and aluminium. The chemical cost of limestone is approximately 29% of that of lime (Maree *et al*., 1992).

Although chemical neutralisation is effective, the major drawbacks are the costs associated with the purchase of reagents (lime), the transport and disposal of metal-laden sludge and plant construction (Gazea *et al.*, 1996). A more cost-effective means of lime treatment involves directing the flow of AMD over a bed of exposed limestone. This is, however, not effective if the AMD contains high concentrations of ferrous iron. The iron rapidly oxidises to form a ferric hydroxide precipitate that coats the limestone and reduces its ability to contribute alkalinity. A possible solution is to allow the AMD to come into contact with the limestone under anoxic conditions, thus preventing the oxidation of ferrous iron. Aluminium armouring may, however, remain a problem under anaerobic conditions (Johnson, pers. Comm., 1999)

Investigations into the use of membrane technology for the desalination of mine drainage were initiated by the South African Chamber of Mines in an effort to reduce corrosion and scaling in pipes, to reclaim water for re-use and to meet discharge requirements (SA Water Bulletin, 1987). Both electrodialysis and tubular reverse osmosis produced excellent results, and the water after treatment by the former was suitable for human consumption. However, steps had to be introduced to prolong the life and minimise fouling of the membranes. The SPARRO process (Slurry Precipitation Recycle Reverse Osmosis) was developed from the seeded RO concept by the Chamber of Mines (Chamber of Mines Research Organisation, 1988). The

advantages of this process are that the product is high quality gypsum that can be sold, and the recovery ratios are very high, reducing the quantity of brine requiring disposal. This process has been successfully tested on a pilot-scale at ERPM Hercules Shaft, but as with other membrane technologies, set-up and maintenance costs are high and fouling of the membranes reduces process efficiency (Chamber of Mines Research Organisation, 1988).

The production of ferrite from AMD has been explored as a possible chemical treatment that does not result in the formation of large quantities of secondary hazardous materials such as metal hydroxide sludge (Wang *et al*., 1996). Ferrite refers to magnetic oxides containing other materials in addition to iron. Spinel ferrite can exhibit ferrimagnetic properties, and may be used in the manufacture of recording media and magnetic markers. Research has shown that nearly all metals could be removed from simulated AMD as ferrites, but removal of smaller precipitates has proved difficult. Furthermore, the rate of the reaction at ambient temperatures may limit its applicability to the treatment of large volumes of AMD.

The common disadvantages of all presently available physico-chemical approaches to the treatment of AMD are the high costs associated with set-up, chemicals, maintenance, and disposal of hazardous metal-rich sludge. This is prohibitive when the treatment measures have to be continued long after the mines have ceased operation. Furthermore, although the neutralisation is effective in the removal of metals and increases the pH to acceptable levels, removal of sulphate is generally poor.

1.2.2. Biological treatment

Biological treatment systems have a number of advantages over traditional chemical treatment methods, predominantly financial ones. Costs associated with chemical reagents, labour and sludge removal are negligible. Instead, costs are usually measured in terms of land (Gazea *et al.*, 1996), making biological treatment particularly attractive in developing countries such as South Africa. Biological treatments include treatment by wetlands and treatment by bacterial consortiums, both *in situ* and in specially designed reactors.

Tuttle *et al*. (1969) noticed that when AMD passed through sawdust or naturally occurring

wetlands, the pH increased and the metal loading decreased. Since then, the treatment of AMD by passive technology, such as natural and constructed wetland systems, has received much attention (Johnson, 1995; Gazea *et al*., 1996; Younger *et al*., 1997; Banks *et al*., 1997; Younger, 1998a, 1998b). In the early stages of establishment, macrophytes and algae play a particularly important role in the remediation process and supply the surrounding water with oxygen, which is then available for oxidation of metal ions. The metals may then precipitate out of solution, usually as metal hydroxides. Alternatively, produced oxygen is used by aerobic bacteria to degrade organic waste material. Direct accumulation of metal ions may also play a role, and has been demonstrated in bryophytes (Engleman and McDiffett, 1996). It is now generally believed that pH increase and metal removal by a combination of ion exchange, metal uptake by plants and filtration of colloidal and suspended materials, is of less importance than that resulting from microbial-mediated transformations, particularly once the wetland becomes established (Johnson, 1995). Wetlands are highly complex ecosystems, and incorporate a range of alkali-generating microbial activities including sulphate and iron reduction, denitrification and methanogenesis (Kalin *et al*., 1991). The effectiveness of wetlands depends largely on the rate at which alkalinity can be produced by these microbial consortiums, and is dependent on the provision of suitable organic substrates for the alkali-generating microbes (Kalin *et al*., 1991; Johnson, 1995). This may be achieved by adding bulk materials periodically, and sources of carbon that have produced good results include peat, hay, straw, sawdust (Kalin *et al*., 1991), spent mushroom compost and combinations of horse or cow manure and straw (Younger *et al*., 1997). To date, no information regarding the fate of the compost and rates of hydrolysis under sulphate reducing conditions is available.

Although the wetland system has the potential to improve the quality of AMD, if pH values are very low and the metal concentrations very high, then a combination of natural lime treatment, together with aerobic and anaerobic wetlands has to be used to achieve satisfactory results. The system appears to work well for effluent with low metal concentrations, but may not be able to decrease initially high metal concentrations. Gazea *et al*. (1996) provided examples from Large Tracy Wetland, Montana. There, the concentration of iron was initially high (284mg.L^{-1}) and was only reduced to 271mg.L^{-1} after wetland treatment. The pH of the water actually decreased from 2.7 to 2.6 through the process. Thus, this system is less than perfect, particularly because

its operation is dependent on factors beyond the control of the user. Johnson (1995) suggested that a higher level of control may be achieved if microbial remediation of AMD is conducted in active biological systems i.e. specially designed bioreactors. These will also be more suited to handling large volumes, which if treated by wetlands will require a large surface area (Rose *et al*., 1998).

Sulphate reducing bacteria (SRB) are found in a wide range of anaerobic environments, particularly in the anoxic sediments of freshwater (Elsgaard *et al.*, 1994) and marine (Marty, 1981; Hines and Buck, 1982) systems. Recently, they have even been found in anoxic micro-environments in aerobic wastewater treatment systems (Lens *et al.*, 1995) and in highly acidic environments (Johnson, 1995; Elliot *et al*., 1998). However, these anaerobic organisms generally function best at pH values of 6-7 and at temperatures of around 30^{o}C (Widdel, 1988). While the upper limit of their tolerance to sulphate is reported to be approximately 2600mg.l^{-1} (Maree *et al*., 1986), their tolerance to sulphide is approximately an order of magnitude lower. The overall reaction carried out by SRB, such as *Desulfovibrio* sp. is as follows:

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

This generalised equation shows how the reduction of sulphate leads to the production of hydrogen sulphide and bicarbonate. The sulphide will react with dissolved heavy metals to produce insoluble metal sulphides, depending on the pH and the solubility of the particular metal sulphide. Bicarbonate is also important for the remediation of AMD as it will act to increase the pH of the solution.

The majority of studies involving this group have centred on their role in the corrosion of metal (Peng and Park, 1994), and concrete sewers. They have also been found to compete effectively with methanogens for electron donors, resulting in the contamination of methane biogas with toxic hydrogen sulphide. Thus, until recently, they have been considered a “pest group” and studies have been aimed at reducing their efficiency. The potential involvement of these microbes for the bioremediation of AMD and other sulphate- and metal-rich industrial effluents has also been realised (Tuttle *et al*., 1969; Maree and Strydom, 1985; Maree *et al*., 1986, 1987;

Maree and Hill, 1989; Widdel and Hansen, 1992). Examples of the use of sulphate reducing bioreactors on large-scale applications include treatment of contaminated groundwater at Budelco zinc refinery (Netherlands) by the THIOPAQ process (Scheeren *et al*., 1993), citric acid-production wastewater (Colleran *et al*., 1998) and tannery effluent (Rose *et al*., 1998).

As is the case with the construction of artificial wetlands, one of the most important obstacles to the implementation of biological treatment systems on a large-scale is the availability and cost of a suitable carbon source and electron donor. Under anaerobic conditions, SRB are able to oxidise a range of organic acids such as lactate, acetate and propionate as well as hydrogen. Sulphate is then used as an electron acceptor, and is reduced to sulphide (Widdel, 1988). Recent 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 (Holliger *et al*., 1988; Widdel, 1988; Drzyzga *et al*., 1993; Kuever *et al*., 1993; Pavlosthathis, 1994). They are, however, not able to hydrolyse large, complex organic molecules such as proteins and carbohydrates, and are thus reliant on a combination of hydrolytic and acidogenic bacteria to provide suitable electron donors. The mechanism by which complex molecules, such as proteins and carbohydrates, are hydrolysed to simpler molecules, which may be used by SRB, is discussed in detail in section 1.3. Inexpensive carbon sources that have been evaluated for their ability to sustain biological sulphate reduction include algal biomass and tannery effluent (Boshoff *et al*., 1996), cattle waste (Ueki *et al*., 1988), molasses (Maree and Hill, 1989), lactate and cheese whey (Oleszkiewicz and Hilton, 1986; Herrera *et al*., 1991), producer gas (Du Preez *et al*., 1992; Du Preez and Maree, 1994) and sewage sludge (Butlin *et al*., 1956; Burgess and Wood, 1961; Conradie and Grutz, 1973).

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. Béchard *et al.* (1993) ran synthetic AMD through channels containing straw and alfalfa, but found that unless sucrose was added to the system, sulphate reduction was very low and there was no significant increase in the pH of the water. When carbon was not limiting, i.e. when sucrose was added, the pH of the system increased from 3.5 to 6.9 and the initial iron concentration (200mg.L^{-1}) dropped by 75%. Christensen *et al*. (1996) found that if whey (mainly lactose, proteins, ash and

fat) was fed to batch systems (initial sulphate concentration of 900mg.L^{-1} and a pH of about 4) at fairly high concentrations (5.7% v/v), that the pH increased to 7.6. Surprisingly, the sulphate concentration also increased from 10mM to 60mM. The concentration of sulphide at the end of the experiment was between 40 and 150mg.L^{-1}. Both lactate and molasses resulted in more than 91% removal of zinc and a pH increase from 3.7 to 8.5 in another bench-scale system (Riekkola-Vanhanen and Mustikkamäki, 1997). Maree *et al*. (1987) found that when molasses was provided as an energy source for biological sulphate reduction, the concentration of sulphate in artificial AMD decreased from 2480mg.L^{-1} to 180mg.$L^{-1.}$ Thus, a number of relatively simple organic compounds may be used as electron donors by SRB and successfully increase the pH and decrease sulphate and metal concentrations in artificial mine water. These substrates are, however, too expensive to be used on full-scale remediation efforts, where large volumes of water are to be treated. Thus, researchers have looked towards high chemical oxygen demand (COD) effluents as potential substrates to drive sulphate reduction. Riekkola-Vanhanen and Mustikkamäki (1997) added press juice from silage (15g lactic acid.kg^{-1}) to a lake containing sulphate-rich AMD and reported that while the zinc concentration decreased , the concentration of iron remained constant. The pH of about 6.5 showed little change at any depth. It was thought that the poor result was due to a combination of the very low water temperatures (<5^{o}C) and the fact that iron remained in its dissolved ferrous form in the lake, and could not be removed from the column by forming an iron sulphide precipitate. A bench-scale trial was carried out using the same effluent as an energy source and it was found that iron removal was 100%, and that zinc removal was proportional to bacterial numbers. Perhaps the most successful attempt at *in situ* treatment was carried out in a shallow lake using sewage (Davison *et al*., 1989). After increasing the pH of the water by adding hydrated lime, it was expected that the sewage would drive sulphate reduction that, in turn, would neutralise any further acid that entering the lake. The pH of the system increased to 10 after the addition of the lime, but after two years had decreased to its former value of less than 3. The failure of this attempt to treat AMD using SRB was ascribed to the shallow nature of the lake. A combination of wind and wave action was able to scour the sewage from the lake bottom, and it was removed from the system.

These examples highlight a number of important points regarding the remediation of AMD by

SRB. A wide range of substrates may be used successfully in bench-scale experiments, but may fail to produce satisfactory results on scale-up. Although one of the advantages of the natural treatment system vs. active treatment is its self-regulating capacity and low maintenance requirements, it is clear that some control is required in order to achieve satisfactory results. Thus, a higher quality, yet relatively inexpensive, biological treatment may best be achieved through the use of carefully designed reactors. A prerequisite for the bioremediation of large volumes of AMD using SRB, is the availability of a large quantity of inexpensive electron donor material. Primary sewage sludge (PSS) meets the above requirements but must be converted to a soluble form before it can be used by populations of SRB.

1.3. Primary sewage sludge as an electron donor for sulphate reduction

Pipyn and Verstraete (1979) set four questions that should be answered when evaluating the potential of a substrate for methane production. If slightly modified, these will also apply to substrates being considered as a potential energy source for sulphate reduction, and are:

1. To what extent is the material convertible to a substrate that can be used by SRB?
2. What is the maximum rate at which the process can proceed?
3. What is the best suited process in terms of pre-treatment, digestion and handling of the residue?
4. What are the overall economics in terms of waste treatment on the one hand, and capital expenditure on the other?

Current options for the disposal of PSS are costly and are unlikely to be sustainable over the long term. As worldwide production of PSS increases, novel and cost-effective methods for the disposal of PSS must be investigated. Thus, from an economic standpoint, the bioremediation of sulphate-rich mine waters using PSS as an electron donor is an attractive option. It is of particular importance to developing countries, where the use of relatively expensive electron donors, such as ethanol, for bioremediation of any sulphate-rich waters is impractical. Not only is PSS readily available, but by using it, two hazardous effluents will be treated simultaneously. The only major cost that may be incurred is the piping of PSS from a collection point to the site where it is to be used. 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 and Daigger, 1995; Canziani *et al*., 1996; Hatziconstantinou *et al.*, 1996; Andreasen *et al.*, 1997; Banister and Pretorius, 1998). The success has, however, been limited despite the apparently large carbon source available. As yet, the optimization of PSS hydrolysis for the production of large quantities of soluble products has not been used for biological sulphate reduction.

The majority of known SRB grow optimally on relatively simple molecules such as short-chain volatile fatty acids (VFA) (Widdel, 1988). Thus, a large proportion of the energy of PSS will remain trapped in complex molecules until freed by hydrolytic and acidogenic bacteria. The degradation of sewage sludge in anaerobic systems is slow. The value calculated for $t^{1/2}_{vs}$ (the half time of volatile solids) was 56 days (Pipyn and Verstraete, 1979). This was significantly higher than the values calculated for other high COD wastes such as piggery slurry (24 days), yeast production plant effluent (10 days) and industrial secondary sludge (16 days). The slow digestion was thought to be the result of the relatively high concentration (0.2-0.5g.L^{-1}) of mineral oil in the PSS. The concentration of cellulose, which is fairly resistant to degradation (Woodward, 1987), may have comprised a significant proportion of the dry matter. Ghosh (1991) suggested that a significant proportion of the readily degradable COD is trapped by recalcitrant cell components such as the cell walls. Thus, cell lysis and/or hydrolysis would be required before the cell components could be utilised by the acidogenic bacteria.

The process of hydrolysis involves the cleavage of complex organic molecules (proteins, lipids and carbohydrates), to smaller compounds such as sugars, amino acids and peptides. Products of this reaction are then cleaved further during acidogenesis to long-chain fatty acids and VFA (Figure 1.1). Hydrolysis, the initial cleavage step, is considered to be rate-limiting (Nyns *et al.*, 1979; Eastman and Ferguson, 1981; Ghosh, 1991; San Pedro *et al.*, 1994; Eliosov and Argaman, 1995; El-Fadel *et al.*, 1996; Vavilin *et al.*, 1996; Penaud *et al.*, 1997). The rate at which hydrolysis proceeds is best described by first order kinetics (Eastman and Ferguson, 1981; Shimizu *et al*., 1993) and may be strongly influenced by environmental and operational parameters. These include pH, temperature, microbial biomass, type and concentration of particulate substrate, particle size and product concentration (Eastman and Ferguson, 1981).

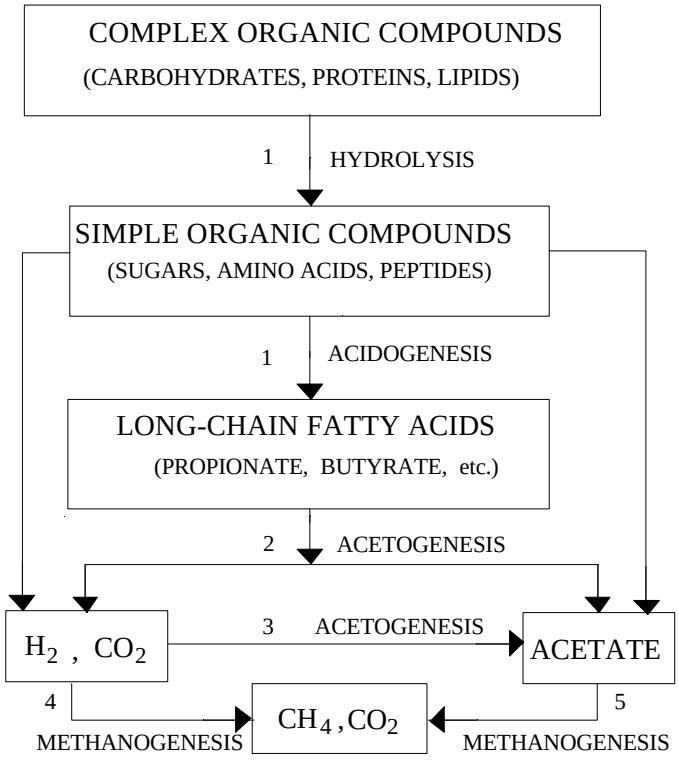


Figure 1.1. Metabolic steps and microbial groups involved in anaerobic digestion: 1) fermentative bacteria; 2) H_2-producing acetogenic bacteria; 3) H_2-consuming acetogenic or homoacetogenic bacteria; 4) CO_2-reducing methanogenic bacteria; 5) acetoclastic methanogenic bacteria (Novaes, 1986).

Table 1.1 summarises rates and yields of soluble products for the anaerobic hydrolysis of PSS, as well as other organic molecules of varying complexity. Reported values for the rate of hydrolysis and the yields from primary sludge are limited, and comparison of published figures is complicated by the fact that the criteria by which the rates were calculated are often different. Calculations have been based on production of ammonia (Henze and Mladenovski, 1991), soluble products such as COD and VFA (Eastman and Ferguson, 1981; Lilley *et al.*, 1990; Brinch *et al.*, 1994, Canziani *et al.*, 1996; Hatziconstantinou *et al.*, 1996; Andreasen *et al.*, 1997; Banister and 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. As hydrolysis is the rate-limiting step, the process of acidogenesis and internalisation are relatively rapid (San Pedro *et al.*, 1994).

Thus, rates calculated by this method probably describe the hydrolysis/acidogenesis step rather than hydrolysis alone. Futhermore, due to the rapid uptake of soluble products, especially VFAs, the rates and yields of the combined process are probably underestimates. Rates calculated from the removal of particulate VSS (Shimizu *et al.*, 1993) or specific complex molecules (San Pedro *et al.*, 1994) are probably more accurate. The value of 0.12 hr^{-1} obtained by Eastman and Ferguson (1981) is considerably higher than that of 0.16day^{-1} (Lilley *et al.*, 1990), although both rates were calculated from the production of soluble products. The difference may partially be a result of the different temperatures at which the two values were obtained (Table 1.1). Alternatively, such differences may have been due to differences in sludge composition. Shimizu *et al*. (1993) showed that different molecular components of PSS are degraded at significantly different rates. Lipids and cellulose were hydrolysed relatively slowly, at 0.76 and 0.52day^{-1} respectively, while proteins and carbohydrates were both degraded at approximately 1.2day^{-1}. Less complex organics, such as starch (San Pedro *et al.*, 1994) and the organics released from sonicated activated sludge (Shimizu *et al.*, 1993) exhibited even higher rates of hydrolysis, with values of 3.28 and 1.2day^{-1} respectively. Thus, as the rate of hydrolysis describes the overall degradation, the relative concentrations of slowly and more rapidly degradable compounds will influence the rate. Although the rate of hydrolysis will affect residence time and therefore, reactor size, percentage yield (expressed either as soluble products produced per unit of complex substrate, or percentage of the complex substrate solubilized) will determine the feasibility of downstream processes. 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 probably affected by environmental and operational parameters, expected yields are low, with the average being less than 20%. Some of the parameters affecting both yield and the rate of hydrolysis/acidogenesis are reviewed below.

1.4. Reactor Design

The choice of reactor design is critical to the success of the process as a whole, and must allow for the optimization of the liquefaction of PSS, as well as the sulphate reduction step. The feasibility of using PSS as a source of electron donors for biological sulphate reduction has been successfully demonstrated in a 1m^3 stirred tank reactor (Molipane, 1999). The performance of

Table 1.1. Summary of hydrolysis data obtained from reported studies.

Feed	k (day^{-1})	Yield (%)	Temp(^oC)	Sludge recycle	Scale	pH	*HRT (hr)	**SRT (day)	Reference
Primary sludge	-	15 - 35	150	none	full	<4	-	-	Karlsson & Göransson, 1993
Primary sludge	-	5	16-20	none	lab	6.7	15d	-	Canziani *et al*., 1996
Primary sludge	-	Poor	16	yes	pilot	-	1.5	10	Canziani *et al.*, 1996
Primary sludge	-	10 - 15	25	none	Lab/ pilot	-	3d	10	Brinch *et al.*, 1994
Primary sludge	0.16	17	20	none	Lab (batch)	-	-	-	Lilley *et al.*, 1990
Primary sludge	-	9	18 - 28	none	Lab (batch)	-	6	-	Banister and Pretorius, 1998
Primary sludge	-	4.8 – 22.4 mean = 10	<20	none	Lab	-	6	-	Hatziconstantinou *et al.*, 1996
Primary sludge	-	5	18	None	Lab	-	2	2	Hatziconstantinou *et al.*, 1996
Primary sludge	-	35	24	10%	Pilot	-	2	4 –5	Hatziconstantinou *et al.*, 1996
Primary sludge	$0.12hr^{-1}$	27	35	none	lab	5.1	1.5	-	Eastman and Ferguson, 1981
Primary sludge	-	9 - 16	20	none	full	-	-	-	Andreasen *et al.*, 1997
Activated sludge	-	2.5	8 - 17	none	full	-	-	-	Andreasen *et al.*, 1997
Sludge mix	0.25	-	35	none	lab	-	10d	-	Siegrist *et al.*, 1993
Raw sewage	0.22	-	20	none	lab	-	5d	-	Balmat, 1957
Waste Activated Sludge	0.16	65	37	yes	lab	7	-	-	Shimuzu *et al.*, 1993
Released organics	1.2	90	37	yes	lab	7	10d	-	Shimuzu *et al.*, 1993
Starch	3.28	-	20	none	Lab (batch)	7	-	-	San Pedro *et al.*, 1994

HRT = Hydraulic Retention Time; **SRT = Sludge Retention Time

the system in terms of removal of both sulphate (85%) and COD (65%) was highly satisfactory. Although the stirred tank reactor is widely used for the digestion of PSS (Ghosh *et al*., 1975; Nyns *et al*., 1979; Toerien and Maree, 1987; Elefsiniotis and Oldham, 1994) it is not ideally suited to combined sludge digestion and sulphate reduction on the scale required for the bioremediation of high-flow AMD. Biomass retention in these reactors is poor (Kosaric and Blaszczyk, 1990), and considering the relatively poor adhesion of SRB (Isa *et al*., 1986; Widdel, 1988), loss of this population is expected to be high. In fact, the ability of methanogenic bacteria to out-compete SRB for electron donors, even in the presence of sulphate, has been attributed to the superior ability of the former to form dense flocs and thus resist washout from bioreactors (Isa *et al*., 1986). Due to the enormous volumes of AMD to be treated, the demand for electron donors is extremely high and the feasibility of the process depends on maximising the flow of electrons to the SRB i.e. methanogens must not be allowed to dominate.

SRB have been retained successfully within a range of modern high-rate anaerobic digesters such as the Upflow Anaerobic Sludge Blanket (UASB) (Sam-Soon *et al*., 1991; Lens *et al*., 1998), Granular Sludge Bed Reactor (Omil *et al*., 1996), Anaerobic Filter bioreactor (Lens *et al*., 1995), Upflow Packed Bed Reactor (Colleran *et al*., 1998), Multi-Stage Reversing-Flow Bioreactor (Takahashi and Kyosai, 1988) and the Anaerobic Baffled Reactor (Barber and Stuckey, 1999). Although retention of biomass, including SRB, within these reactors is high, the majority of the reactors are unsuitable for the enhanced hydrolysis of PSS. The influent to these systems should ideally contain a low concentration of suspended solids (Kosaric and Blaszczyk, 1990; Lettinga and Hulshoff-Pol, 1991). More importantly, retention of SRB is dependent on the ability of the bacteria to adhere to inert packing material or dense bacterial flocs. Based on the proposed hypothesis, this ability is likely to be impeded, and because of the upward flow of water within the reactors, the susceptibility of bacteria to washout would be high. Brinch *et al.* (1994) proposed that a HRT of 2 to 3 days was required in order to optimise the hydrolysis of PSS in terms of soluble COD production. HRT is limited by reactor volume and the size of the effluent stream to be treated, but considerably longer HRTs have been shown to improve process performance (Elefsiniotis and Oldham, 1994; Banister and Pretorius, 1998). It has been found that maximum soluble products are produced from PSS at SRT of 8 to 10 days, depending on temperature (Canziani *et al.*, 1996; Hatziconstantinou *et al.*, 1996; Banister and

Pretorius, 1998). Other studies have shown that under meso- or psychrophilic conditions, a SRT of between 10 and 20 days is required to obtain maximum solubilization of complex substrates, and maximum product formation (Elefsiniotis and Oldham, 1994; Shimizu *et al.*, 1993). The rate of COD solubilization was independent of SRT. Thus, for the solubilization of complex organic substrates, it is preferable to operate the hydrolysis process with a SRT far longer than the HRT. This may be achieved through the recirculation of reactor solids, which alone resulted in a 13% improvement of COD removal in an anaerobic system treating the waste from an enzyme production plant (Pipyn and Verstraete, 1979). Longer sludge retention times are thought to selectively benefit the acidogenic population within the reactors (Elefsiniotis and Oldham, 1994), and results in the accumulation of bacteria and their associated enzymes (Hatziconstantinou *et al.*, 1996).

Implementation of sludge recirculation will introduce a certain amount of mixing into the digester contents. The effect of this parameter on the anaerobic process, and particularly on the hydrolysis step, is poorly understood. Contradictory theories have been formulated regarding the mechanism and effect of mixing on the production of soluble products. Unfortunately, no studies have incorporated both sludge recirculation and mixing. Perot *et al.* (1988) proposed that the mixing benefited soluble product production by maintaining organics in suspension, thus increasing the contact between biomass and substrate. Experiments showed that VFA production was indeed enhanced by mixing. As the percentage solubilization of volatile solids did not increase, it was suspected that the apparent improvement in product formation was not due to enhancement of the hydrolysis step, but rather to inhibition of the methanogens. Banister and Pretorius (1998) believed that mixing increased the contact between bacteria and inhibitory compounds, which would result in decreased process performance. Slow, intermittent stirring was found to be optimal, and lead to an increase in VFA yield. Initial conversion of COD to VFA was, however, low. A combination of sludge recirculation and mixing might allow for the enhanced process performance, in terms of both yield and conversion rate, as a result of facilitating biomass and enzyme accumulation, continual elutrition of potentially inhibitory products, and increased contact between enzyme and substrate.

Although the volume of sulphate-rich water being pumped to the surface by many of the mines in South Africa is large and typically ranges from 36 to 64 $ML.day^{-1}$ (Scott, 1995), at present, Grootvlei Mine pumps volumes of up to $130ML.d^{-1}$ (Grootvlei Mine, 1997). This will place further constraints on the configuration of the reactors. The costs of constructing many of the high-rate reactors of the size required to accept such large effluent volumes would be prohibitory, and would reduce the advantage of biological bioremediation over the present chemical processes. In South Africa, where large expanses of land are available, the use of relatively simple "trench reactors" would be appropriate. They must, however, be designed in such a way so as to meet the requirements discussed above.

1.5. Research Hypothesis

The greatest challenge that needs to be overcome before PSS can be used effectively as an electron donor to drive a range of biological processes is to increase both the rate of solubilization and the yield of soluble products.

Jain *et al*. (1992) presented a mathematical model that accurately described the anaerobic digestion of cow dung, a complex substrate. The two factors that had the greatest impact on the rate-limiting hydrolytic step were the concentration of the hydrolytic enzymes and the contact between these enzymes and their substrates. Particle size has also been shown to have a profound impact on the rate of anaerobic digestion of complex substrates (Balmat, 1957; Levine *et al*., 1985; Szikriszt *et al*., 1988; Choi *et al*., 1997; Madhukara *et al*., 1997; Müller *et al*., 1998). Vavilin *et al*. (1996) modelled the degradation of particulate substrates and proposed that the hydrolysis rate constant was a function of the ratio between the characteristic sizes of the hydrolytic bacteria and the substrate particles. Based on the above findings, any increase in the enzyme concentration in a digester, or a reduction in mass transfer limitations or particle size will result in an increase in the rate of hydrolysis of complex particulate substrates. The terms hydrolysis and solubilization are used inter-changeably throughout the literature. To avoid confusion in this study, hydrolysis refers to the chemical cleavage of molecules, while solubilization refers to the change of solids from the settleable to the suspened or soluble (i.e. $<0.45\mu m$) state.

To date, no information is available regarding the composition and formation of flocs in non-pellet forming anaerobic treatment systems. For the purpose of this study, it was assumed that the composition of the flocs in PSS is representative of the composition of the sludge, and contains roughly equal proportions of carbohydrates, lipids and proteins (Heukelekian and Balmat, 1959; Hunter and Heukelekian, 1965; Levine *et al*., 1985). It was also assumed that those factors known to be responsible for flocculation in non-bulking activated sludge systems, i.e. non-covalent bonds between bacteria, extracellular polymeric substances (EPS), and metal ions (Eriksson and Alm, 1991; Bruus *et al*., 1992; Urbain *et al*., 1993), are also essential for maintaining the integrity of the anaerobic flocs. For the purposes of this study, EPS is defined as being any extracellular organic molecules, including proteins and carbohydrates. Large undigested macromolecules present in the PSS would also be incorporated into the flocs.

Hydrolytic enzymes in biological treatment systems are thought to be bound to bacterial cell walls and the floc matrix, with minimal enzyme activity in the bulk solution (Frølund *et al*., 1995; Confer and Logan; 1998; Goel *et al*., 1998). Thus, flocculation is essential for the retention of both biomass and enzymes within the reactor system. However, it is proposed that flocculation results in severe mass transfer limitations, and will have a negative impact upon the rate of hydrolysis of complex macromolecules. Li and Ganczarczyk (1990) proposed that substrates and products diffusing to and from cells must overcome the diffusional resistance created by EPS. Indeed, any macromolecules within the floc could offer significant resistance to the passive movement of soluble molecules to and from the floc. If mass transfer to and from the floc is reduced, accumulation of product within the floc will result in product inhibition of enzyme production, and a severe reduction or cessation of hydrolysis. For this reason, it would be advantageous for cyclical floc fracture followed by reflocculation. Upon floc fracture, any products trapped within the flocs would be released, and upon reflocculation, hydrolytic enzymes would be concentrated around freshly incorporated substrate. As the mean size of flocs is dependent on the relative rates of flocculation and floc fracture, and increase in the rate of floc fracture would result in a smaller mean floc size. Thus, the area-to-volume ratio of the floc would be increased and would benefit hydrolysis of the floc components, assuming that the majority of the hydrolysis occurs on the floc surface.

Traditionally, anaerobic stabilisation of PSS is performed under methanogenic conditions, and rarely results in more than 30% solubilization (Table 1.1). Pipes (1961) suggested that stabilization under sulphate reducing conditions might offer significant advantages for disposal of PSS. Indeed, more recent studies have shown that the rate and extent of the solubilization of ligno-cellulose, an abundant compound in primary sludge (Heukelekian and Balmat, 1959; Hunter and Heukelekian, 1965; Elefsiniotis and Oldham, 1994), was enhanced in the presence of sulphur compounds (Khan and Trottier, 1978; Kim *et al*., 1997; Pareek *et al*., 1998). Hydrolysis of large protein molecules may also be enhanced as sulphide is a strong reducing agent and is capable of reducing disulphide linkages that are essential for maintaining the three-dimensional conformation of large proteins. Although the concentration of lipids in PSS can be as high as that of the carbohydrate and protein components (Levine *et al*., 1985), they are not degraded during the rate-limiting hydrolytic phase of anaerobic digestion (Eastman and Ferguson, 1981). Instead, they are degraded by β-oxidation in the acidogenic phase, and as such will not be considered during this study.

The main factors limiting the rate of hydrolysis of PSS appear to be the following:

- Mass transfer limitation due to floc size and structure
- Reduced contact between hydrolytic enzymes and their substrates
- Poor retention of biomass and enzymes due to low immobilisation efficiency
- Inefficient separation of soluble products and undigested material

Based on the above, it is proposed that the solubilization of PSS will be enhanced under sulphate reducing conditions because of a decrease in both particle and floc size, as a result of enhanced hydrolysis of macromolecular proteins and carbohydrates. Furthermore, incorporation of a novel settling sludge bed will offer significant advantages in terms of cyclical flocculation and floc fracture, separation of the soluble products from undigested material and in the retention of small flocs, bacterial biomass and associated hydrolytic enzymes.

1.6. Research Objectives

In order to use PSS as a carbon source and electron donor for biological sulphate reduction on the scale required for the remediation of large volumes of AMD in South Africa, the hydrolysis step of this complex substrate must be understood and optimised. Current knowledge is poor and a number of questions need to be answered:

a) Can PSS be used as an efficient carbon source and electron donor for biological sulphate reduction?
b) Will the product of the reaction i.e. sulphide, enhance the hydrolysis of the substrate?
c) If so, what is the underlying mechanism involved in the sulphide-enhanced hydrolysis of PSS?
d) Does a settling sludge bed offer any advantage over conventional reactor systems?
e) Does the process work on scale-up?

Chapter 2

The Effect of Reactor Design and Sulphate Reduction on Solubilization of Primary Sewage Sludge: Results from a Laboratory-Scale Reactor

2.1. Introduction

The limited success using PSS as a carbon source for a range of biological processes, such as BNR and sulphate reduction, despite the large carbon source apparently available may be ascribed to the inefficient hydrolysis of the complex substrate, and emphasised the need for control of the hydrolysis process. Operational parameters such as mixing (Perot *et al*., 1988; Banister and Pretorius, 1998), temperature (Gujer and Zehnder, 1983; Perot *et al*., 1988; El-Fadel *et al*., 1996; Van Lier *et al*., 1997; Banerjee *et al*., 1998) and SRT (Skalsky and Daigger, 1995) have all been shown to influence the rates and degree of hydrolysis of complex substrates. Thus, for optimization of the combined solubilization/sulphate reduction process, it would be advantageous to conduct both reactions within a well-controlled reactor system.

A reactor incorporating a settling sludge bed is thought to offer significant advantages over conventional upflow-type high-rate digesters. It is proposed that such a system will result in increased solubilization of PSS, while being relatively inexpensive to construct and operate. The aims of this study were thus to evaluate the performance of a novel laboratory-scale settling sludge bed reactor in terms of the hydrolysis of PSS, and to examine the effects of sulphate reduction and reactor configuration on the solubilization process. The effect of sulphate reduction on floc formation was also investigated.

2.2. Methods

2.2.1. Reactor design and operation

The reactor was composed of one or more interconnected units (Figure 2.1a) with the feed of PSS and sulphate-rich water entering the first unit near the surface (Figure 2.1b). The particulate organic matter in the PSS settles into the base of the unit, and is then recirculated and re-enters the reactor adjacent to the influent stream, allowing a period of

reaction. A portion of the influent water is drawn into the bed, while the remainder containing solubilized material flows horizontally. Liquefaction of the PSS and sulphate reduction occurs in the bed of the reactor. Although the retention of biomass is reliant on flocculation, the predominantly downward flow of liquid is thought to offer significant advantages, in terms of biomass retention, over the upward flow in the UASB-type reactors. The recirculation of sludge may be handled in single- or multiple-stage operations.

Two single-stage and two three-stage reactors were used, so as to allow for comparison of both reactor configurations under sulphidogenic (+ sulphate) and non-sulphidogenic (- sulphate) conditions. All reactor vessels were constructed from 2L inverted plastic (PET) bottles, with a neck angle of 60^{o}. The overflow of the reactors was positioned to allow for a working volume of 1.7L. Multiple-stage (MS) reactors (Figure 2.1a) were constructed from three single-stage (SS) reactors inter-connected by silicone rubber tubing (5mm i.d.). Each of the three successive components was positioned on a marginally lower level to allow passive overflow from one stage of the reactor to the next. The headspaces of each stage were interconnected by silicone rubber tubing so as to allow periodic purging of the headspace with nitrogen gas. The sludge that settled in the base of each reactor unit was continuously recycled to the top of the same reactor unit at a rate of 20% reactor volume/hour, using a variable speed peristaltic pump (Watson-Marlow 504S). Turbulence created by the inflow of recycled sludge resulted in the formation of a suspended sludge bed within the reactor. The height of the bed could be regulated by altering the recycle rate, and was initially maintained at approximately 50mm below the overflow in the single-stage reactors and stage 1 of the MS systems. The positions of the sample ports in the sides of the reactor (Figure 2.1b) allowed samples to be drawn from the center of the sludge bed. The overflow of each reactor (effluent) was sampled via taps in the interconnecting tubes.

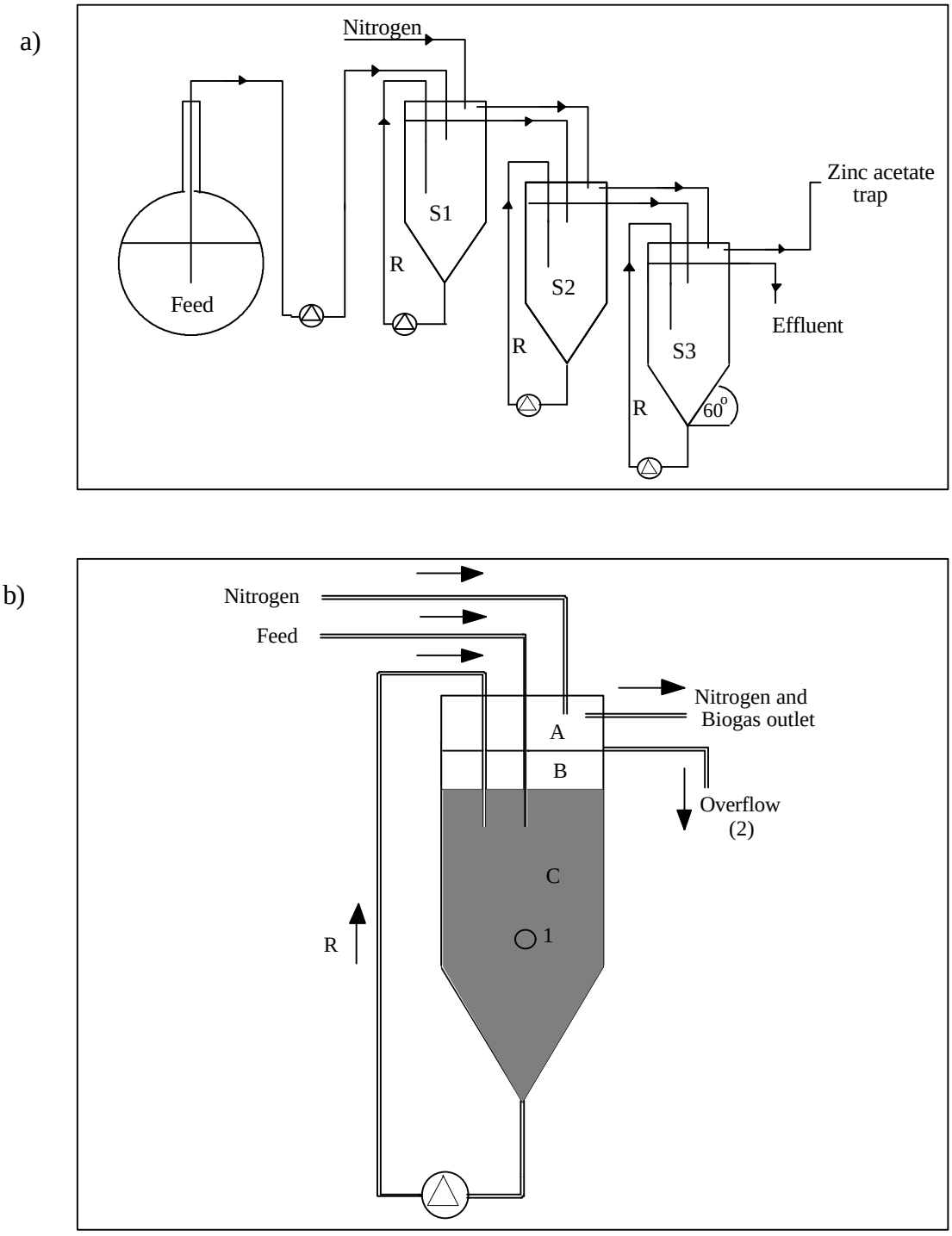


Figure 2.1. The laboratory-scale reactor. a) Multiple-stage reactor system (S = stage); b) detail of a single stage (A = headspace, B = Top zone, C = Bed) showing the location of sample ports (1 = Bed, 2 = Effluent). R = recycle. The position of the gas inlets and outlets is also shown.

All four reactor systems were operated at a HRT of 2 days, at room temperature (22-25^{o}C). Fresh PSS was collected every two days from Grahamstown Disposal Works (GDW). After collection, the PSS was passed through a sieve (2mm mesh size) and stored at 4^{o}C until required. Fresh feed was made up every 48-hours to minimise build-up of large bacterial populations in the feed reservoirs. Sieved PSS was diluted with tap water to obtain a feed with a COD of 2000 mg.L^{-1}. Na_2SO_4 was added to the feed of the sulphidogenic system to obtain a final sulphate concentration in the feed of 2000mg.L^{-1}. The ratio of COD: sulphate in the feed was thus 1:1. The glass feed reservoir was placed on a magnetic stirrer, and the contents stirred continuously, at low speed, to prevent settling of particulate organic matter. The neck of the feed reservoir was closed to limit oxygen transfer into the feed. The reactors were allowed to stabilize for 14 days, after which time it was assumed that steady state had been reached. Samples were drawn and analyzed at regular intervals for a period of 38 days after which time the experiment was terminated.

2.2.2. Analytical procedures

2.2.2.1. Chemical Analysis

Merck Spectroquant® test kits were used to determine concentrations of sulphate (Merck # 14791) and COD (Merck # 14541). Filtered COD (COD_f) was determined by passing samples through a Glass Microfibre Filters (type GF/A; Whatman Ltd. # 1820 025), and calculating the COD of the filtrate. This method was modified from Lilley *et al*. (1990) and represented the soluble COD fraction. Prior to determination of COD, all samples were acidified with 32% HCl to pH<2 and shaken for 10 minutes, to remove any sulphide. Although the accuracy of COD quantification may have been improved if sludge samples were macerated prior to dilution and analysis, it was felt that this might have resulted in an artificial underestimation of the particulate COD fraction (COD_p). Instead, determination of total COD (COD_t) was carried out in triplicate and the mean value calculated, in an attempt to obtain accurate quantification of the COD_t of the highly particulate sludge samples. COD_p was calculated as the difference between the COD_t and COD_f of a sample. The percentage sulphate removed was calculated as the difference between the concentration of sulphate in the feed and that in the effluent of a

particular reactor or reactor system. pH was measured with an electronic pH meter (Cyberscan 2000, Wirsam Scientific).

2.2.2.2. Floc size

Sludge samples were drawn from various points within the reactor systems, and diluted 10X with a 10% (v/v) formalin solution. Formalin acts as a fixing agent and has been used successfully to preserve the three-dimensional structure of activated sludge flocs (Li and Ganczarczyk, 1990). Each sample was gently mixed and a drop placed onto a glass slide using an automatic pipette with an enlarged tip aperture. A glass coverslip was then placed over the sample, ensuring that minimal pressure was applied. Although the presence of a coverslip may have resulted in a flattening and expansion of the flocs, it was envisaged that this effect was minimized by the fixation step. The flocs were then viewed under a light microscope, photographed and the area of the flocs (mm^2) calculated using image analysis software (Sigma Scan Image™). Between 50 and 100 flocs per sample were measured. Statistical software (Statgraphics 7.0) was used to compare the mean floc size of the various samples.

2.3. Results

2.3.1. Solubilization of particulate organic matter

Due to periodic dilution of municipal PSS after rains, and inherent difficulties associated with obtaining an accurate measure of COD in a particulate sludge sample, influent COD to the bench-scale systems showed some fluctuation (Figures 2.2a, 2.3a) over the experimental period. The concentration of COD_f in the feed to all four systems was consistently low (Figures 2.2a, 2.3a) and rarely increased to above $500mg.L^{-1}$ at any stage of the reactor systems, except in the beds (stage 1) of the MS systems. The mean concentrations of COD_f in the beds (stage 1) were $711mg.L^{-1}$ and $950mg.L^{-1}$, for the + sulphate and – sulphate MS systems, respectively (Table 2.1). In comparison, the COD_f in the beds of the SS systems were only $274mg.L^{-1}$ (+ sulphate) and $231mg.L^{-1}$ (- sulphate) (Table 2.1).

Sulphate reduction appeared to enhance the solubilization of particulate organics in the multiple-stage reactor, but not to the same degree in the single-stage system. COD_p increased steadily in bed 1 of the – sulphate MS system, from 6862mg.L^{-1} on day 0, to 24027mg.L^{-1} on day 39 (Figure 2.3b). In the equivalent stage of the + sulphate system,

Table 2.1. Mean COD concentrations (mg.L^{-1}) for bench-scale reactors over the 38 day experimental period. SS = single-stage reactors; MS = multiple-stage reactors. Standard deviations are indicated in brackets. Percentage changes are reported in Table 2.2.

	COD_t		COD_p		COD_f	
Sample	+ sulphate	- sulphate	+ sulphate	- sulphate	+ sulphate	- sulphate
Feed (SS)	1545 (548)	1808 (681)	1343 (525)	1511 (712)	205 (106)	296 (227)
Bed (SS)	5886 (3988)	4779 (2956)	4752 (3824)	4557 (2992)	274 (120)	231 (112)
Effluent (SS)	1271 (975)	1157 (1339)	1035 (1016)	964 (1375)	236 (120)	202 (152)
Feed (MS)	1674 (336)	1754 (452)	1479 (372)	1532 (423)	195 (98)	222 (116)
S1 Bed (MS)	10576 (5777)	20595 (7826)	9864 (5425)	19644 (7087)	711 (497)	950 (901)
S1 Effluent (MS)	2250 (1686)	834 (386)	1929 (1613)	535 (324)	321 (162)	299 (153)
S2 Effluent (MS)	1434 (1265)	415 (211)	1205 (1240)	250 (211)	229 (111)	161 (65)
S3 Effluent (MS)	1235 (956)	257 (141)	1044 (923)	128 (148)	190 (118)	129 (31)

periods of particulate accumulation were followed by periods of rapid removal of particulate COD (Figure 2.3b). The concentration of COD_p reached a maximum on day 4 (18171mg.L^{-1}) but then decreased to 6025mg.L^{-1} (day 13). COD_p began to accumulate again in the bed around day 30. The data suggests that there was, on average, comparatively little accumulation of COD_p within the beds of the SS systems (Figure 2.2b). This is misleading as the bed of the – sulphate SS system was very compact, with the top of the bed being situated below the lowest sample port. Although the actual concentration of CODp in the bed of the – sulphate SS system was not determined, it was possibly far higher than indicated.

Percentage COD removal data were transformed (arcsin$\sqrt{}$proportion) prior to statistical analysis, in an attempt to reduce the variability. Although the mean removal of COD_t

was higher in the – sulphate systems (Table 2.2), the mean removals for the four reactor systems were not significantly different (ANOVA, df = 3, 44; P>0.05). The periodic increase in COD in the effluent of the systems resulted in high standard deviations. These would have affected the statistical analysis, but should not detract from the significance of the results.

In the MS systems, 84% of the COD was removed in the – sulphate system compared with 21% removal in the + sulphate system. Removal in the SS systems was 67% and 42% for the – sulphate and + sulphate systems, respectively. During those periods when the concentration of COD_p in the bed of stage 1 (MS + sulphate) showed a rapid decrease (Fig. 2.3b), the COD_t of the effluent of stage 1 increased (Figure 2.4a). There was thus a net loss of COD_t from stage 1 of the MS + sulphate system, as indicated by the mean change of 39% for that stage (Table 2.2).

Table 2.2. Comparison of mean percentage removal of sulphate and COD from bench-scale reactors. SS = single-stage reactors, MS = multiple-stage reactors. Standard deviations are indicated in brackets.

Sulphate	Sample	% COD change	% sulphate removal
+	Stage 1 MS	+39 (101)	34 (8)
+	Stage 2 MS	-8 (84)	50 (19)
+	Stage 3 MS	-21 (61)	59 (9)
-	Stage 1 MS	-50 (21)	-
-	Stage 2 MS	-75 (12)	-
-	Stage 3 MS	-84 (8)	-
+	SS	-42 (39)	21 (9)
-	SS	-67 (21)	-

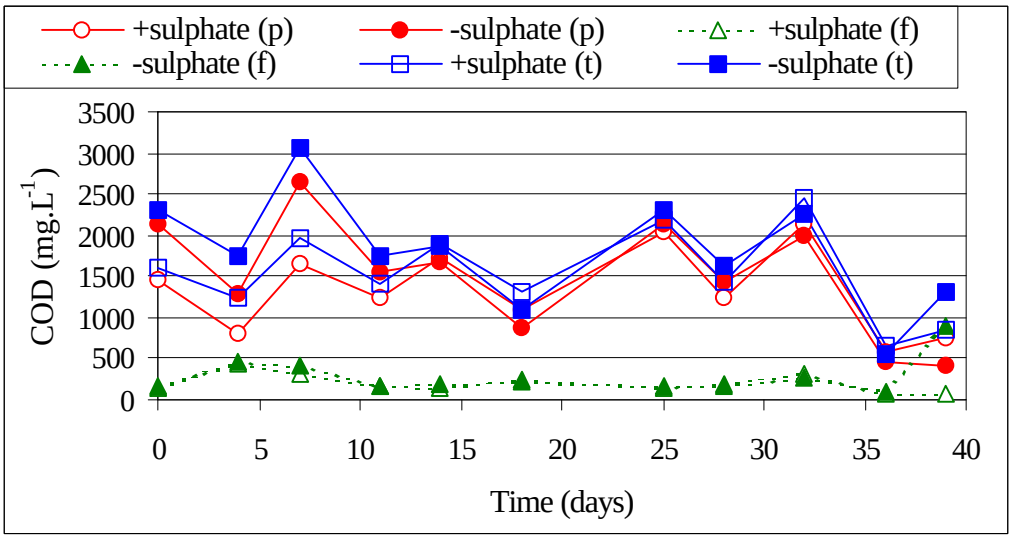
The COD concentration of the effluents of both the SS systems were relatively constant for the first 30 days of the experimental period (Figure 2.2c). The peak in COD concentration in the effluent of the + sulphate SS system on day 25 occurred at the same time as a large decrease in the COD of the sludge bed of that system (Figure 2.2b). On

day 32, the COD concentration in the beds of both SS systems showed a significant decrease (Figure 2.2b) and, again, this coincided with a significant increase in the COD_t of the effluents of both systems (Figure 2.2c).

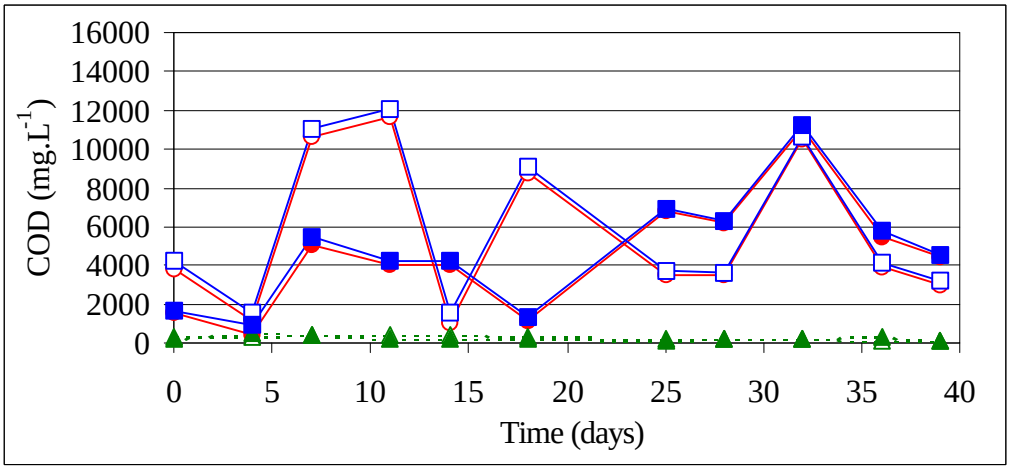
While the COD_t in the effluent of stage 1 of the – sulphate MS system remained relatively low for the duration of the experimental period (mean concentration = 834mg.L^{-1}, Table 2.1), that of the + sulphate system showed large fluctuations (Figure 2.4a). The COD_t in the effluent of stage 1 (+sulphate) ranged between 924mg.L^{-1} (day 10) and 5775mg.L^{-1} (day 13). The mean value was 2250mg.L^{-1} (Table 2.1). The effluent COD_t concentrations of stages 2 and 3 of the + sulphate system showed similar fluctuations. The small peaks in effluent COD_t of stage 1 on days 7 and 22 occurred at the same time as larger peaks in effluent of stages 2 (Figure 2.4b) and 3 (Figure 2.4c). However, the peak on day 28 was only seen in the effluents of stages 1 and 3.

These results showed that COD accumulated in the – sulphate systems and that removal was higher in the + sulphate systems due to increased solubilization. Furthermore, the solubilization events occurred in the beds of the reactors, and emphasised the need for recirculation. It is proposed that the higher solubilization in the multiple-stage reactors was linked to higher total bed volume and, consequently, the extended period that particulate matter remained trapped within the bed.

a)



b)



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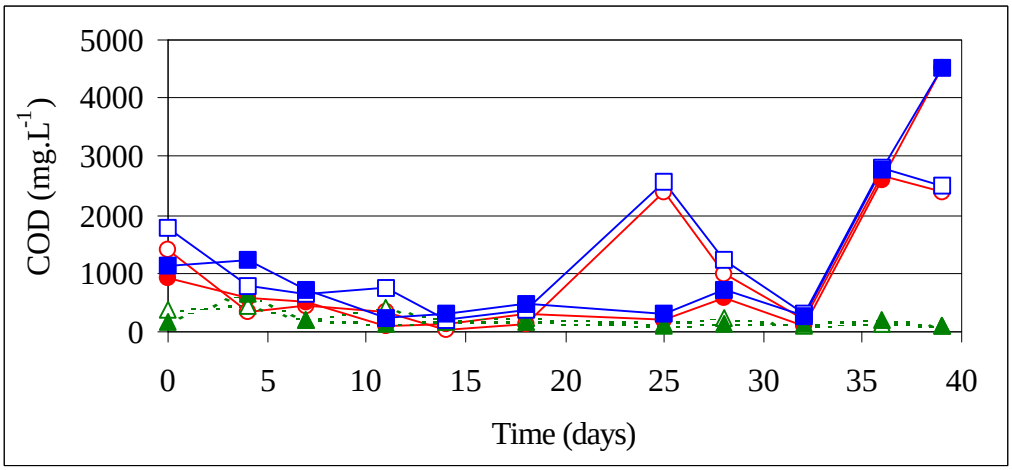


Figure 2.2. COD concentration in the single-stage reactors. a) Feed, b) Bed, c) Effluent. t = COD_t; p = COD_p; f = COD_f

a)

b)

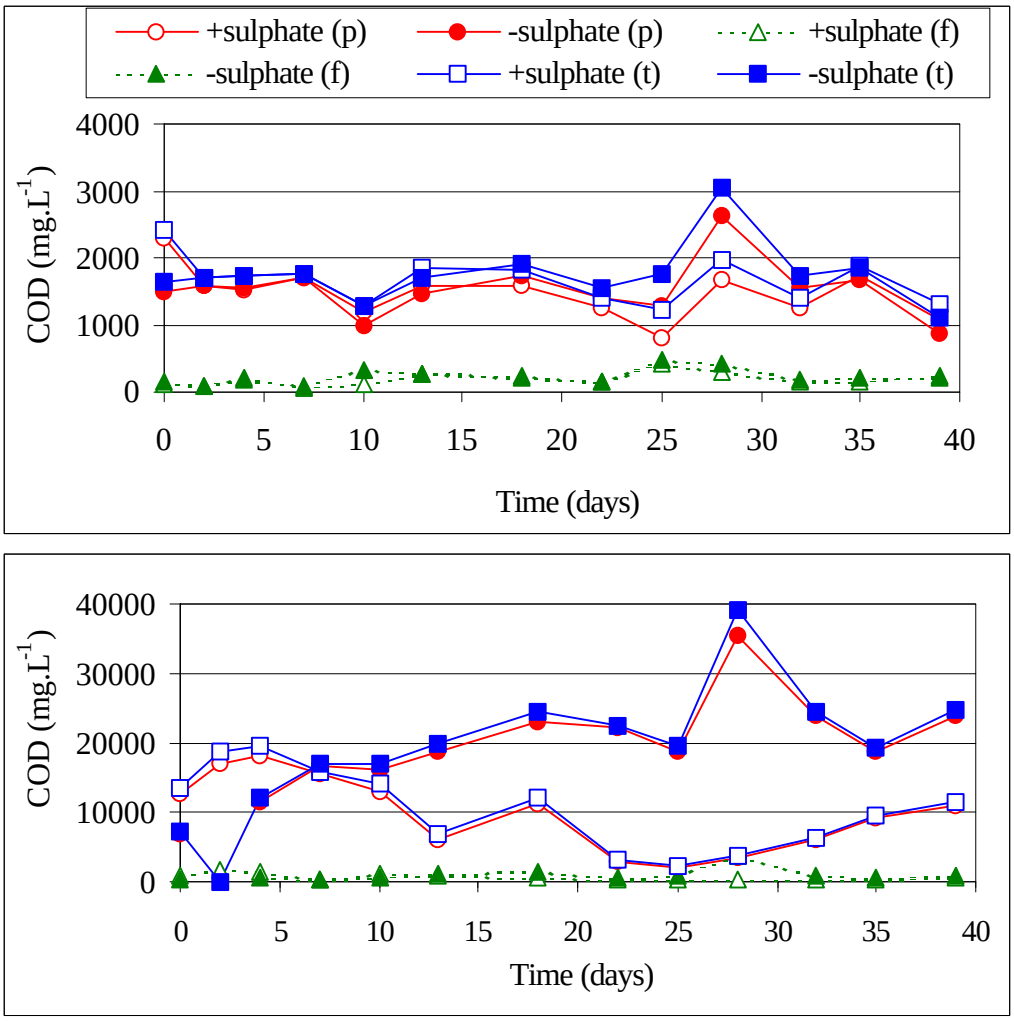
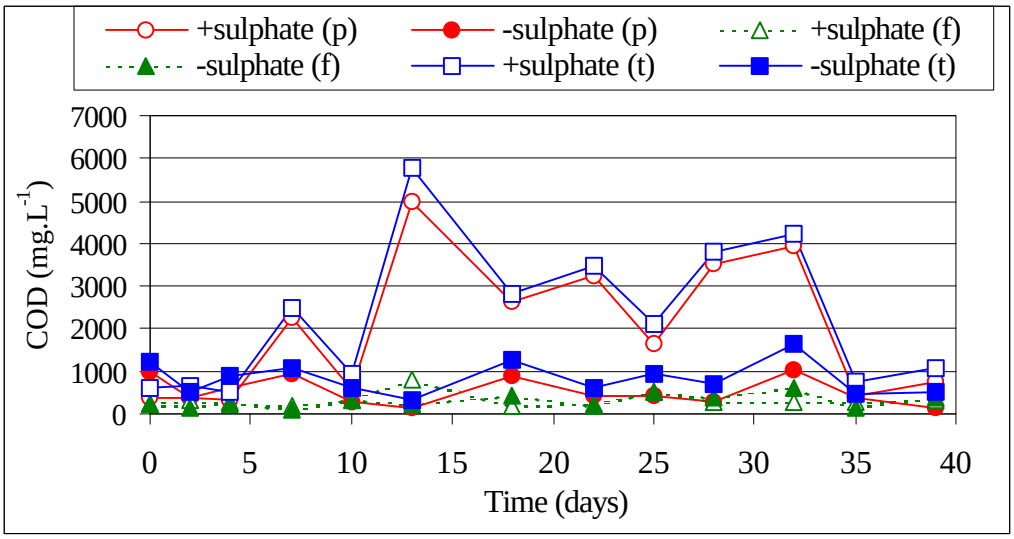


Figure 2.3. COD concentrations in stage 1 of the multiple-stage reactors. a) Feed, b) Bed. t = COD_t; p = COD_p; f = COD_f

a)



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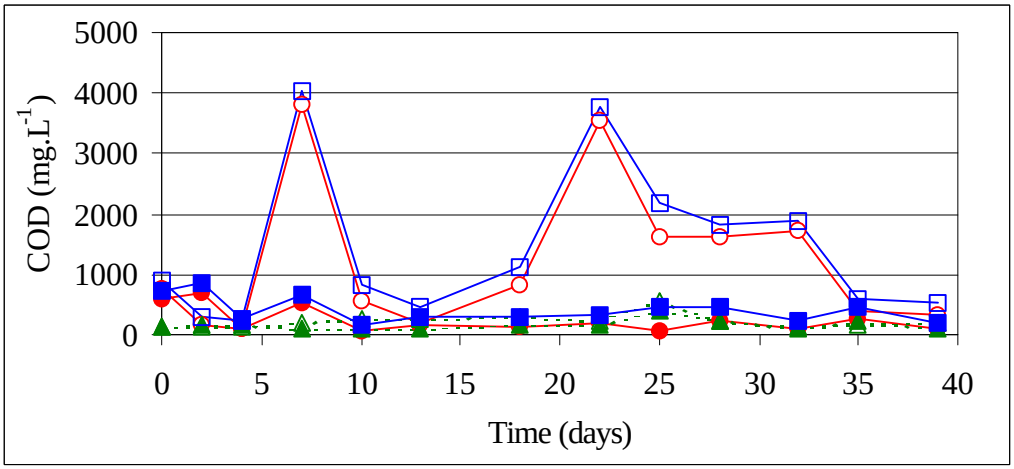


c)

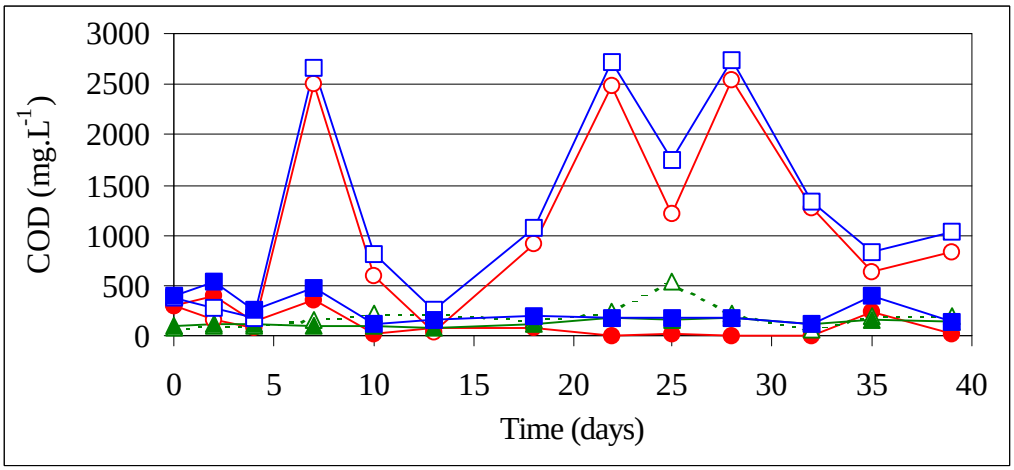


Figure 2.4. COD concentrations in the effluents of the multiple-stage reactor. a) Stage 1, b) Stage 2, c) Stage 3. t = COD_t; p = COD_p; f = COD_f

2.3.2. Sulphate reduction

Overall, the mean percentage sulphate removal in the multiple-stage system (59%) exceeded that of the single-stage system (21%) (Table 2.2). The concentration of sulphate in the influent of the + sulphate systems showed some fluctuation (Figure 2.5), and could be attributed to fluctuations in the level of sulphate reduction occurring within the feed reservoir. This was presumably related to the concentration of soluble COD and although it was a problem with the experimental design, it did not detract from the value of the data obtained.

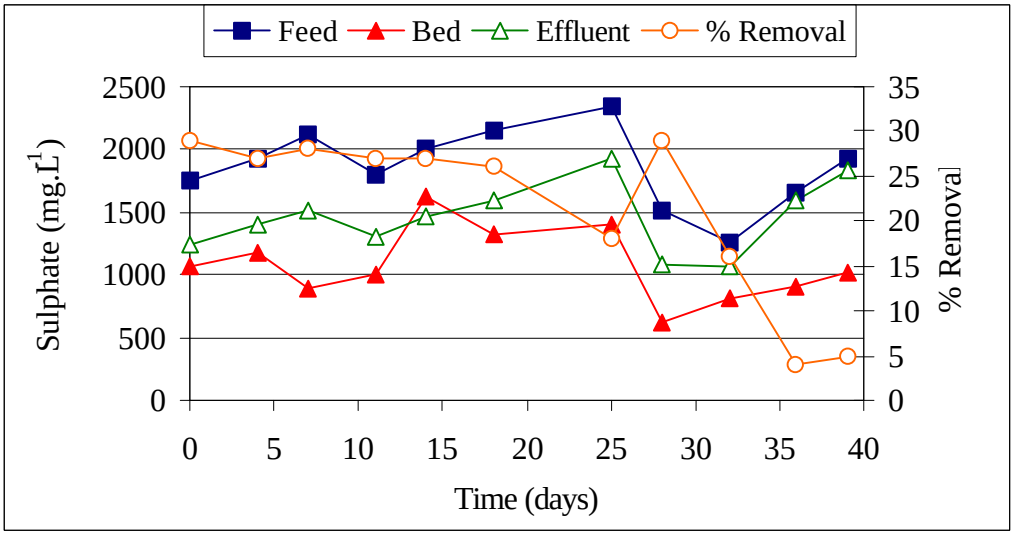


Figure 2.5. The concentration and percentage sulphate removal in the + sulphate single-stage reactor.

These fluctuations in the feed sulphate, eg. on day 32, resulted in simultaneous fluctuations in the sulphate levels in both the bed and effluent of the SS system. The total percentage removal of sulphate dropped sharply after day 27, and was probably related to the washout of particulate COD from the system at that time (Figure 2.2). The mean concentration of sulphate in the bed of the SS reactor (1076mg.L^{-1}) was lower than in the effluent (1537mg.L^{-1}) (Table 2.3), and indicated that sulphate removal was higher in the bed than in the aqueous phase. Sulphate removal was highest in the first stage of the multiple-stage systems, although further removal took place in stages 2 and 3 (Tables 2.2, 2.3), and may be related to the availability of COD_f. Although the concentration of sulphate in the feed of the MS reactor was relatively constant, there was a large amount

of variation in the sulphate concentration of the bed (stage 1) (Figure 2.6a). The percentage sulphate removal in the bed of stage 1 varied between 20 and 80%, and the concentration of sulphate between 400mg.L^{-1} and 1550mg.L^{-1} (Figure 2.6a).

Table 2.3. Mean sulphate concentrations and pH in bench-scale reactors. SS = single-stage reactors; MS = multiple-stage reactors. Standard deviations are indicated in brackets.

	Sulphate (mg.L^{-1})		pH	
Sample	+ SO_4	-SO_4	+SO_4	-SO_4
Feed (SS)	1858 (306)	<10	7.0 (0.2)	7.0 (0.2)
Bed (SS)	1076 (282)	<10	7.4 (0.3)	6.8 (0.3)
Effluent (SS)	1455 (274)	<10	7.4 (0.3)	6.8 (0.3)
Feed (MS)	2123 (185)	<10	6.9 (0.3)	6.9 (0.2)
Stage 1 Bed (MS)	844 (374)	<10	7.2 (0.4)	6.6 (0.3)
Stage 1 effluent (MS)	1378 (208)	<10	7.4 (0.3)	6.9 (0.2)
Stage 2 effluent (MS)	1039 (397)	<10	7.6 (0.3)	7.1 (0.2)
Stage 3 effluent (MS)	851 (199)	<10	7.5 (0.3)	7.0 (0.2)

The percentage sulphate removal in the three stages varied with time, although all showed similar trends (Figure 2.6). The decrease in percentage sulphate removal on days 7 and 25 coincided with washout of CODp from the reactors (Figure 2.4).

2.3.3. pH

The mean pH values for the +sulphate systems exceeded those of the – sulphate systems, at all stages (Table 2.3), although the differences were not substantial. In both the SS and MS + sulphate systems, the pH within the reactors exceeded that of the feed, and was an indication of active sulphate reduction (see reaction 2, chapter 1). In the – sulphate systems, the pH within the reactors was slightly lower than that of the feed. The effluent of stage 3 of the MS + sulphate reactor was higher than that from the SS + sulphate reactor, as a result of more sulphate reduction in the MS system.

a)

b)

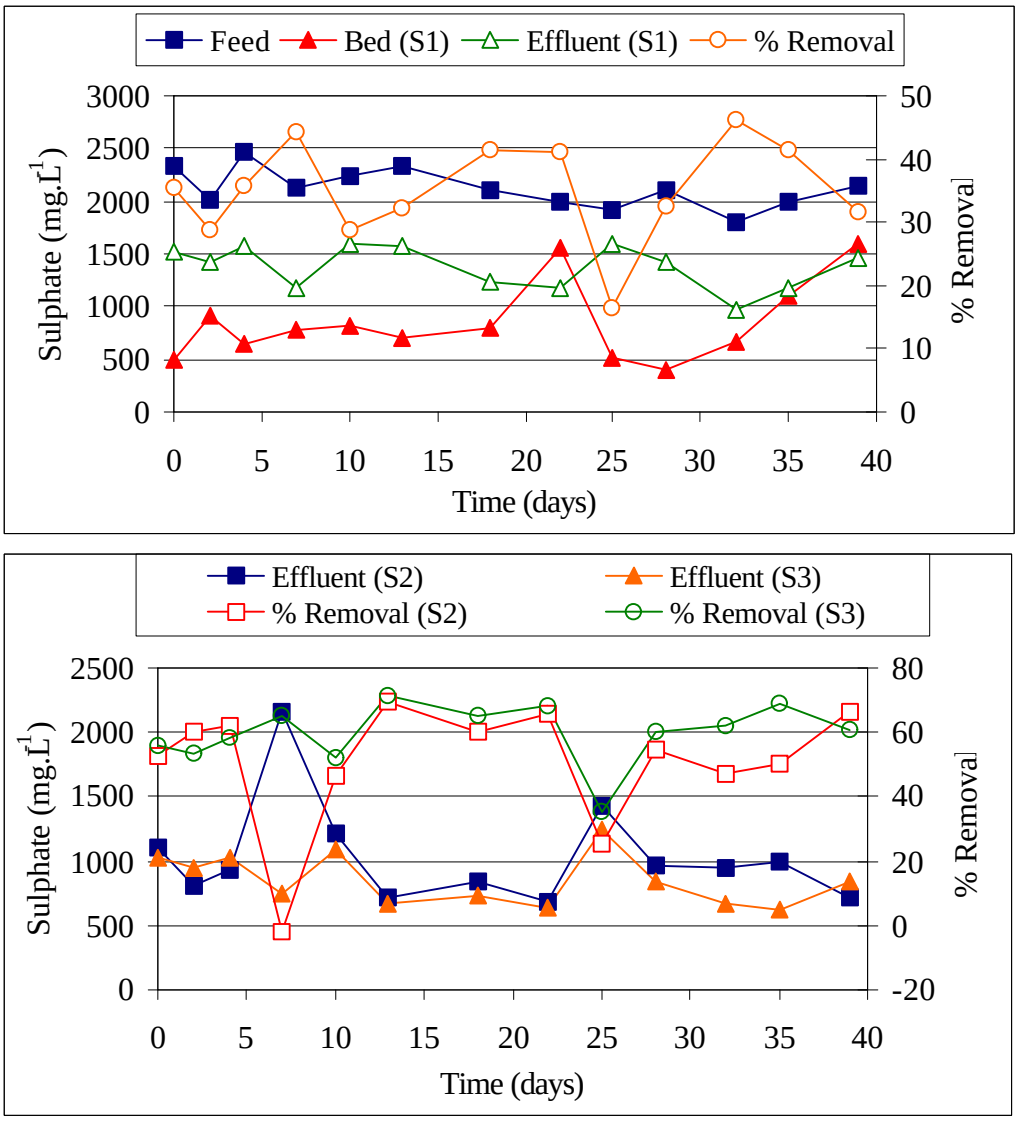


Figure 2.6. The concentration and percentage removal of sulphate in the + sulphate multiple-stage reactor. a) Feed, Bed and Effluent of Stage 1, b) Effluents of Stage 2 (S2) and Stage 3 (S3).

2.3.4. Floc size

Fixation of the raw PSS samples appeared to stabilize the floc matrix, and no obvious flattening of the flocs by the cover slip was observed. Microscopic examination of the flocs revealed the presence of dense particulate matter embedded within fine EPS (Figure 2.7).

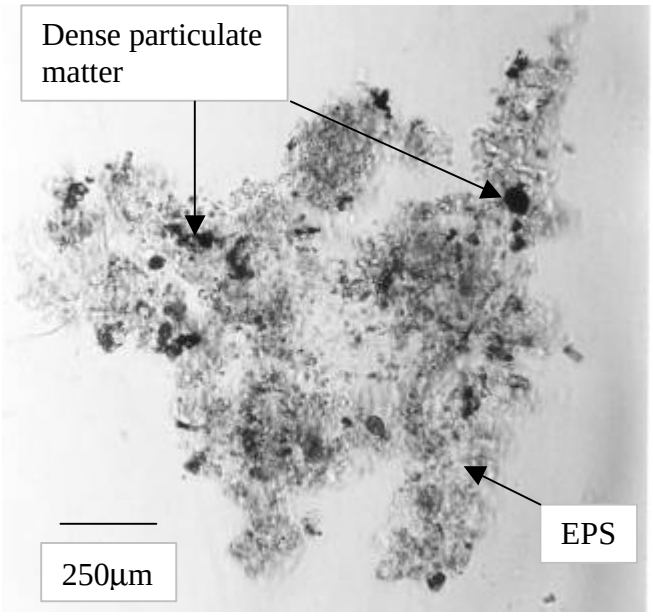
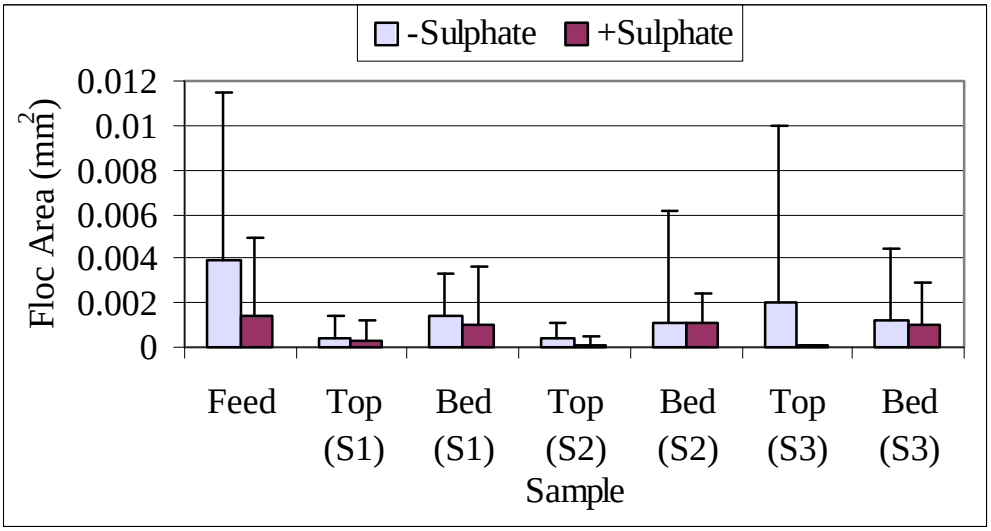


Figure 2.7. Floc morphology as seen under the light microscope (40x magnification). The dense particulate matter embedded within EPS is clearly visible.

The floc-area data sets from most samples contained outliers, and resulted in large standard deviations (Figure 2.8). To reduce some of the variation, all data were log-transformed prior to statistical analysis. As the data were not normally distributed, means were analysed using a Kruskal-Wallis analysis for non-parametric data. The mean areas of the flocs from 18 samples were found to be significantly different ($P<0.001$; $df = 17$). As expected, the flocs in the upper zone of the reactors were smaller than those in the corresponding beds (Figure 2.8). This, alone, would have accounted for the significant finding. Thus, in order to determine whether sulphate reduction had any effect on floc size, it was necessary to analyse the data obtained from the reactor feed and beds separately from that of the upper zones. Similarly to previous observations, the differences observed in Figure 2.8 were significant ($P<0.001$; $df = 9$). Unfortunately, the Kruskal-Wallis test is unable to provide details regarding the exact position of the differences. From Figure 2.8, it appeared as if the flocs in the feed of the + sulphate system were significantly smaller than those in the feed of the – sulphate system. This was confirmed using a T-test

($P < 0.01$; df = 132). Thus, although the flocs in the beds of both the single- and multiple-stage were smaller in the + sulphate systems, this may have been a direct result of a significant difference in floc size within the feed of the two systems. Furthermore, the size of the flocs in the three stages of the multiple stage systems appeared to remain constant. The relatively high mean value and standard deviation obtained for the top of stage 3 (- sulphate) was the result of 9 relatively large flocs in the sample. The presence of these could not be explained.

a)



b)

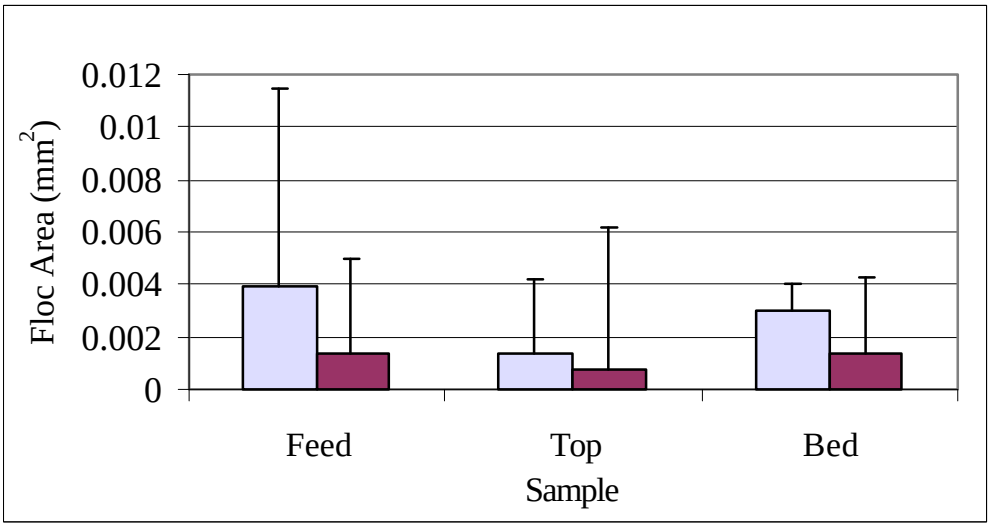


Figure 2.8. Mean area of flocs within the (a) Multiple- and (b) Single-Stage reactors, with and without sulphate.

2.4. Discussion

Anaerobic solubilization of PSS was enhanced significantly in the presence of biological sulphate reduction. Particulate organic matter tended to accumulate in the non-sulphidogenic reactor, and resulted in the formation of a dense sludge bed. Any sudden increases in the COD of the influent resulted in similar increases in the COD concentration of the bed. In contrast, the sludge bed in the sulphidogenic reactors was slurry-like, and accumulation of particulate organic matter within the bed was gradual. Furthermore, it did not reflect the periodic increases in the influent COD. These differences were attributed to a higher rate of hydrolysis of complex substrates, and higher rates of floc fracture within the sulphidogenic environment.

The mean percentage removal of COD in the outflow from the non-sulphidogenic reactor was significantly higher than that in the sulphidogenic system, and was the result of accumulation of undigested organic matter rather than higher rates of PSS solubilization. The retention of fine particulate organic matter within a digester, as well as the integrity of the sludge bed, is reliant on the ability of the organic matter to flocculate. As the flocs grow in size, their density and settleability increases, while susceptibility to washout decreases. The significant difference in the mean size of flocs in the effluents and beds of the laboratory-scale reactors was evidence for the importance of the floc fracturing process in the performance of the sulphidogenic system. While a proportion of the calculated COD loss was the result of solubilisation of particulate matter, a significant fraction was the direct result of a sudden loss of integrity of the sludge bed followed by washout of fine particulate matter. These periods of rapid reduction in the COD concentration of the sludge beds coincided with an elevation in the concentrations of particulate organic matter in the effluents of the various stages of the sulphidogenic systems. A study of floc size revealed that flocs within the feed and beds of the sulphidogenic system (+ sulphate) were significantly smaller than in the – sulphate system. As floc size is dependent on the relative rates of flocculation and floc fracture, it is proposed that the process of biological sulphate reduction has two possible effects. It either reduces floc binding and reflocculation or is actively involved in the fracturing of the floc structure. The cyclical nature of the solubilization in the sulphidogenic systems

implied that the effect of sulphate reduction on flocculation was not continuous and immediate, but that it required a period of incubation.

Under these conditions, the multiple-stage reactor system would offer a significant advantage to the overall performance of the digestion process in terms of percentage removal of undigested but biodegradable matter. Small flocs of undigested organic matter that were washed from the first and second stages would have passed into the next stage of the reactor. There they may then have undergone flocculation and would have been retained within the reactor, where hydrolysis of organic particulate matter would have occurred. This process of floc fracture and reflocculation within the successive stages of the multiple-stage reactor would have extended the time that undegraded material was exposed to the solubilization process, and thus increased the degree of degradation.

The current study supported previous findings that a complex carbon source could be used as a source of electron donor to drive biological sulphate reduction (Rose *et al*., 1998). The mean total percentage sulphate reduced in the laboratory-scale reactors, calculated as the difference between the influent and effluent sulphate concentrations, was relatively low. The mean percentage sulphate reduced in stage 1 of the MS reactor was slightly less than that reduced in the single-stage system. However, some sulphate was also reduced in stages 2 and 3 of the MS system, and total percentage sulphate reduced in the MS system exceeded that of the SS system by 19%. The quantity of sulphate reduced in the successive stages of the MS system decreased, and was thought to reflect a limitation of available soluble electron donors. Although unused soluble organic matter was present in the effluent of stages 2 and 3, this may have been in a form not suitable to the local population of SRB. SRB are known to prefer certain volatile fatty acid over others, and utilization of acetate is thought to be particularly poor (Omil *et al*., 1996; Lens *et al*., 1998).

In terms of the hypothesis proposed in Chapter 1, it has been shown that the mean size of flocs is reduced in a sulphidogenic environment, possibly because of an effect of

biological sulphate reduction on floc fracture or reflocculation. Although the lack of accumulation of organic matter within the sulphidogenic reactors is not evidence for a high rate of solubilization, the rate of solubilization is expected to have been higher than in the non-sulphidogenic reactor as a result of reduced floc size. The improved performance of the multiple-stage reactor may have been related to the higher sedimentation capacity. However, although performance of the multiple-stage reactor system was better than that of the single-stage system, the current reactor design did not prevent loss of particulate organic matter and possibly, associated hydrolytic enzymes. A reduction of this loss by appropriate reactor design could further enhance the rate of hydrolysis of PSS. Further improvements to the design of the process were required to lower the amount of sulphate removal taking place in the feed reservoir, and might include reducing the residence time at this stage. It is now proposed that sulphate reduction and the solubilization process are mutually beneficial and both reactions should thus be confined to a single reactor in order to achieve optimum results.

2.5. Conclusions

- This was the first indication that the solubilization of PSS is enhanced under sulphate reducing conditions.
- Mean floc size was significantly smaller in the sulphidogenic than in the non-sulphidogenic system.
- The 3-stage reactor system out-performed the single-stage system in terms of percentage sulphate removed.
- Improved reactor design was required to minimise the loss of sludge and biomass.
- The combination of a linear flow settler design above a sulphidogenic settling sludge bed appears to enhance the solubilization of PSS.
- The inclusion of recycle of hydrolysed material promotes the separation of soluble product, which passes forward for polishing, and the unreacted material which settles and is subjected to further hydrolysis.
- It is postulated that three distinct stages of the process include floc fracturing, hydrolysis and then sulphate reduction. The process of sulphate reduction, in turn, enhances the other two steps.

Chapter 3

Solubilization of Primary Sewage Sludge in a Pilot-Scale Falling Sludge Bed Reactor

3.1. Introduction

Conventionally, scale-up of novel reactor designs proceeds in 10-fold increments. However, for a number of reasons it was decided to proceed directly from the lab-scale reactor to a 23m^3 pilot-scale system. A 10L perspex settling sludge bed reactor was operated for a period of 1 week, but recycle of dense settled sludge on that scale resulted in severe channelling and was thus unsatisfactory for evaluation of the settling sludge bed concept. Due to this problem and the relatively low cost of constructing the 23m^3 pilot-scale system, it was decided to proceed directly with the evaluation of the process at the larger scale.

The site chosen for the pilot plant was Grootvlei Gold Mine on the Far East Rand Mining Basin (FERMB) where a Department of Water Affairs and Forestry-sponsored AMD treatment evaluation exercise was being undertaken. It is estimated that the mine discharges 130 $ML.d^{-1}$ highly saline water into the nearby Blesbok Spruit, an ecologically sensitive Ramsar site (Van Wyk and Munnik, 1998). Prior to discharge, the concentration of iron in the water is reduced to the accepted discharge levels by lime addition in a High Density Sludge (HDS) Plant (Grootvlei Mines, 1997).

The results obtained from the laboratory-scale settling sludge bed reactors indicated that retention of small sludge flocs was poor, and resulted in periodic washout of particulate organic matter from the reactor. Furthermore, results also indicated that the 3-stage reactor system offered significant advantages, in terms of both PSS stabilisation and sulphate removal, over the single-stage system. The introduction of an upper region of linear flow and a lower region consisting of a sulphidogenic settling sludge bed, as well as the inclusion of recycle of settled sludge, appeared to facilitate separation of soluble product from the undigested material, while encouraging flocculation of undigested substrate, bacteria and enzymes.

In order to minimise cost of construction, while at the same time maximising performance, the pilot-scale reactor was constructed as a modified trench-style settler. A single elongated reactor that would allow for a longer period of settling replaced the 3-stage system. Internally, the lower third of the trench was divided longitudinally into three valleys, while the upper region of the reactor was undivided (Figure 3.1). It was proposed that this configuration would allow for maximum settling of small flocs and controlled recycle of the settled sludge. Simpler horizontal-flow trench-type anaerobic digesters have proved highly successful in the digestion of hog manure in Taiwan (Hong, 1986).

The hydrolysis unit or Falling Sludge Bed Reactor (FSBR), as it is now known, formed the first part of a multiple-unit process train (Figure 3.1) for the bioremediation of AMD. The process train, known as the "Rhodes Process", consisted of three principal stages namely sludge solubilization, sulphate removal, and polishing. Supplementary sulphate removal was carried out in an Anaerobic Baffled Reactor (ABR) and was followed by polishing in two high-rate algal ponding systems. Provision was made for the removal of heavy metals from the AMD by sulphide precipitation prior to it being combined with PSS in a blend tank (BT). However, as Grootvlei Mine was operating a full-scale HDS plant for metal removal, this step was unnecessary. Due to the complexity of each of the stages, the present study was limited to the performance of the FSBR. Research into the sulphate reducing, metal removal and polishing stages is ongoing. The aim of the present study was to monitor the performance of the FSBR in terms of the solubilization of PSS in a sulphate-rich environment, and to characterise the spatial variation in the COD within the reactor.

3.2. Methods

3.2.1. Configuration and operation

The pilot plant for the bioremediation of sulphate-rich mine drainage was erected at Number 3 shaft, Grootvlei Gold Mine, Springs, South Africa. The hydrolysis reactor was constructed from a modified metal shipping container (6.0 x 2.5 x 2.5m) by the Grahamstown Engineering Company. After modification, the hydraulic volume of the

reactor was 23m^3. The layout and dimensions of the entire pilot plant are given in Figure 3.1.

The container was divided into three contiguous sections of equal size, with the lower meter of each section forming a valley (Figure 3.1). These valleys were constructed by welding 10mm mild steel plates into the base of the container at 45^o angles. The joins between the plates and the reactor sides were not watertight. This allowed water to fill the dead-spaces under the plates to prevent buckling of the plates when the reactor was full. A pipe (i.d. = 150mm) containing slots was placed into the base of each valley. These pipes, which spanned the width of the reactor and passed through the wall on both sides, allowed for the recycle of settled sludge. The HRT of the reactor was maintained at 2 days, and the mean organic loading rate was 1.57kg COD.m^{-3}.d^{-1}. Sludge was drawn sequentially from each of the valleys for pre-determined time periods (valley 1 = 60s, valley 2 = 30s, valley 3 = 10s) and then pumped to the reactor inlet pipe where it mixed with influent feed. Influent passed into the reactor via an 80mm steel pipe, with 40mm sockets placed just below water level, and axially orientated toward the effluent draw-off crown wier. Effluent flowed over the crown weir, placed 2.2m above the reactor base, before it flowed, gravitationally, into the baffled reactor. Three 2cm i.d. pipes were inserted into each valley through the roof of the reactor (Figure 3.1). They allowed samples to be drawn from depths of 0.2, 0.75 and 1.7m below the surface of the reactor. Two inspection manholes with bolted steel covers were installed into both ends of the reactor roof.

Screened PSS was delivered to the pilot plant twice weekly from the Springs ANCOR Water Care Works operated by the East Rand Water Care Company (ERWAT), and stored in 5m^3 High Density Polyethylene (HDPE) holding tanks. The PSS was then pumped into a 2.7m^3 Sludge Holding Tank (SHT). Based on daily analysis of the sewage and mine water (AMD), a predetermined volume of PSS was mixed, in the BT, with iron-free mine water from the HDS process, via a mine water reservoir (MWR, to maintain a feed, from the BT to the FSBR, with a COD: SO_4 ratio of approximately 2:1.

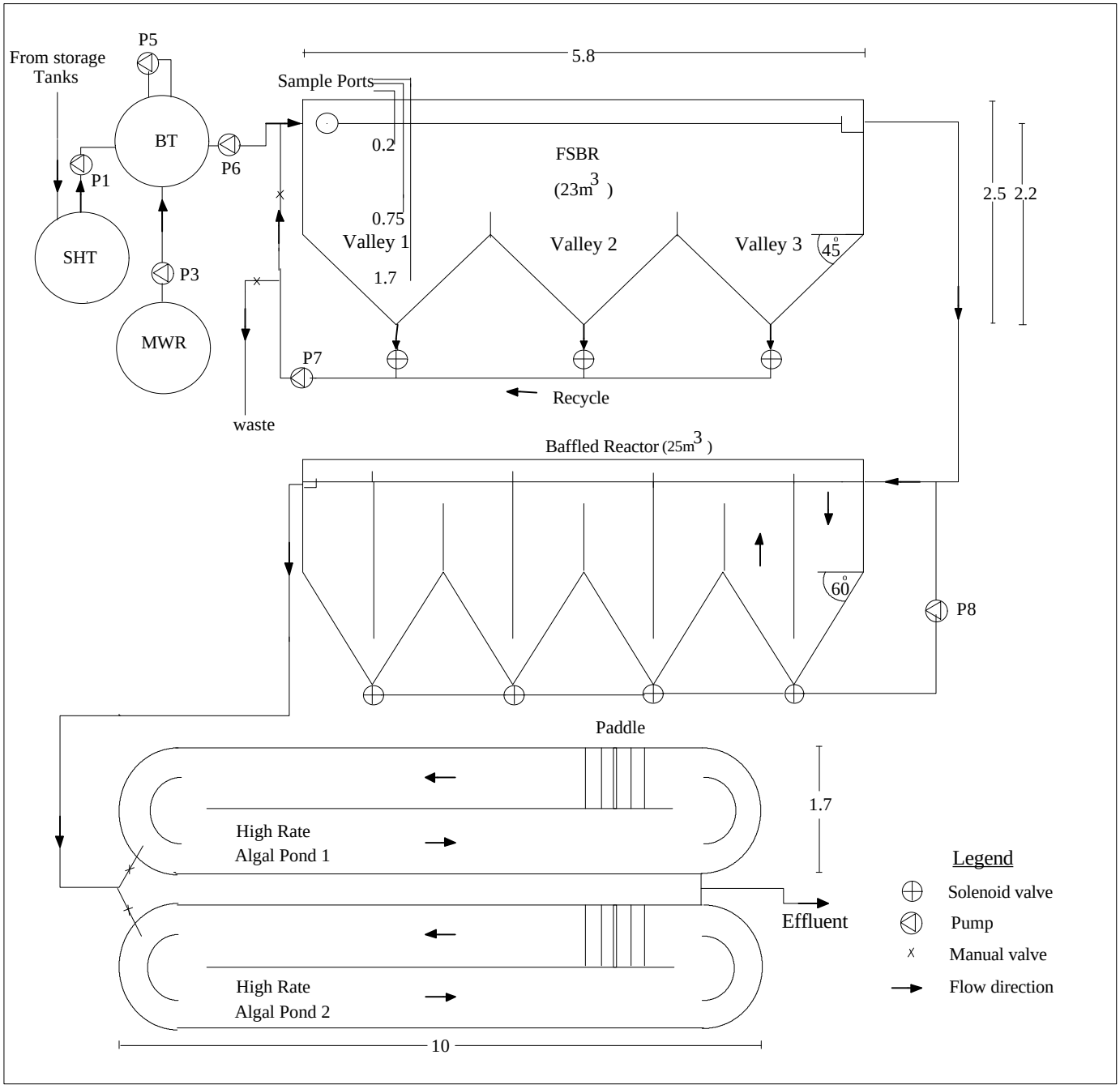


Figure 3.1. The complete "Rhodes Process" for the bioremediation of sulphate-rich AMD. All measurements are given in metres. Pump specifications are given in Appendix 1. BT = Blend Tank; MWR = Mine Water Reservoir; SHT = Sludge Holding Tank; FSBR = Falling Sludge Bed Reactor.

To maintain an operational temperature of above 20^{o}C during winter, a counter-current heat exchange system was installed. The heat exchanger consisted of a series of 25mm

galvanised pipes, placed into the reactor, through which warm untreated underground mine water was allowed to flow. The mine water had a temperature of between 24 - 27^{o}C.

3.2.2. Analytical procedures

The methods used to analyse concentrations of COD and sulphate, as well as pH are described in Chapter 2. Suspended solids and settleable solids were calculated as described in Standard Methods (APHA, 1989). Sludge profiles were studied by inserting a 2.5m transparent perspex tube through roof of the reactor. Once the tube had reached the base of the reactor, the upper end was stoppered and the tube removed. The depth of the dense and expanded regions of the sludge bed was recorded for each of the three valleys. Loose sludge was characterised as consisting of distinct flocs, while the compact sludge was tar-like, and of a uniform consistency.

3.2.3. Sampling regime

The concentrations of COD_t and sulphate in the influent and effluent of the hydrolysis unit were determined daily for 120 days. Sludge profiles were monitored on a regular basis. A complete sample profile of the reactor was carried out on three occasions, during September, November and December 1998. At these times, samples were drawn from the blend tank and effluent of the hydrolysis unit, as well as from three depths within each of the three valleys of the reactor. Samples (500mL) were collected and analysed for COD_t, COD_f, COD_p, sulphate, pH and suspended solids.

3.3. Results

3.3.1. Overall performance

Mean removal of COD and removal of sulphate, for the three audit periods, were 57.9 (± 6)% and 31.4 (± 14)%, respectively (Table 3.1). Both parameters exhibited some variation within the sample periods. The mean pH of the effluent, pH 7.0, was slightly higher than that of the influent value of pH 6.4. Values for COD_p and COD_f were not calculated during the November audit, and, consequently, the mean values for these parameters were only calculated from two sample periods. A high percentage of influent

COD_t (85.5%) was in the form of particulate organic matter, with a small percentage (14.5%) being in the soluble form. The average volume of settleable solids in the PSS during September was 990mL. This dropped to 90mL in the BT due to dilution, and was between 1 and 3mL in the effluent of the FSBR. This indicated that the rate of solubilization of PSS within the FSBR was high.

Table 3.1. Summary of the FSBR performance during the three audit periods. All values in $mg.L^{-1}$ unless otherwise stated. Standard deviations are indicated in brackets.

	Sample Times			
Parameter	September	November	December	Mean
COD_t in	3003	3207	3553	3254 (278)
COD_t out	1439	1362	1274	1358 (82)
COD_p in	2568	-	2779	2670 (154)
COD_p out	841	-	986	913 (102)
COD_f in	442	-	774	608 (234)
COD_f out	598	-	288	443 (219)
SO_4 in	1656	1673	1771	1700 (62)
SO_4 out	1407	956	1128	1163 (227)
COD_t removed	1564	1845	2279	1896 (360)
% COD_t removed	51.2	57.5	64.1	57.9 (6)
SO_4 removed	249	717	643	536 (251)
% SO_4 removed	15.0	42.8	36.3	31.4 (14)
pH in	6.5	6.6	6.0	6.4 (0.3)
pH out	6.6	7.5	7.1	7.0 (0.4)

A detailed record of COD removal and sulphate removal during September is shown in Table 3.2. The COD: SO_4 ratio of the feed was maintained at 2:1 for the duration of the pilot plant study, except for a period of six days in September. During this period, the ratio was reduced to 1.5:1 by decreasing the COD concentration. This change had little influence on the overall performance of the reactor (Table 3.2). However, within 24 hours after the change was introduced, there was an increase in gas production, and solids began to rise to the surface of the reactor. The composition of this gas was not determined.

Based on the data given in Tables 3.1 and 3.2, and a stoichiometric requirement of 2g COD required to reduce 1g sulphate (Isa *et al*., 1986; Lens *et al*., 1995), it is possible to calculate the minimum percentage of particulate COD hydrolysed. It must also be assumed that only soluble COD can be utilized by SRB, and that COD_p is unavailable to this group. Taking the mean data from the three audit periods (Table 3.1), 536mg.L^{-1} SO_4 was reduced, which would require 1072mg.L^{-1} of soluble COD (COD_f).

$$\text{Total CODf}_{\text{required}} = (\text{CODf}_{\text{SO4 removal}} - \text{CODf}_{\text{in}}) + \text{CODf}_{\text{out}}$$

$$= (1072\text{-}608) + 443$$

$$\therefore \text{CODf}_{\text{required}} = 907\text{mg.L}^{-1}$$

The concentration of COD_p remaining in the reactor was 1757mg.L^{-1}.
Thus, a minimum of 51.6% of the particulate COD that remained in the reactor had to have been hydrolysed to produce enough soluble COD to have supported the amount of sulphate reduction that took place. The minimum percentage of COD_p hydrolysed on the day of the December audit was only 44%. Using similar calculations, it was possible to estimate what fraction of the COD_t retained in the reactor was used for sulphate reduction and, consequently, what percentage was likely to have been removed by settling. Values, based purely on COD_t of the influent and effluents and sulphate removed, are given in Table 3.2. Mass balance figures based on a 2gCOD/g sulphate removed showed shortfalls or surplus of COD. These values ranged from shortfalls of 52% to a surplus of 78.8%. On average for the month of September, there was a mean surplus of 36.8% (± 36). Thus, a certain proportion of the COD was being removed by the FSBR, but was not utilised for sulphate reduction. This may reflect that portion of the PSS which is highly recalcitrant.

During the course of the operation, the system was exposed to a number of perturbations that demonstrated the robustness of the process under shock load conditions. These incidents had an immediate and profound effect on destabilising the FSBR, affecting both COD (Figure 3.2a) and sulphate concentrations (Figure 3.3a). However, once the source of the disturbance had been removed, the systems quickly returned to steady-state operation.

Table 3.2. Summarised performance of the FSBR during September 1998. All values in $mg.L^{-1}$ unless otherwise indicated.

Date	$COD:SO_4$ (feed)	COD_t removed (influent – effluent)	Sulphate removed	Theoretical COD used in sulphate reduction	% surplus (+) or shortfall (-) in COD requirement
2	2:1	917	304	608	+33
3	2:1	1505	763	1526	-1.4
4	2:1	1838	194	388	+78.8
5	1.5:1	400	258	516	-29.0
9	1.5:1	596	453	906	-52.0
10	1.5:1	1078	215	430	+60.1
11	1.5:1	1118	180	360	+67.7
12	2:1	602	248	496	+17.6
13	2:1	1611	465	930	+42.2
16	2:1	1494	704	1408	+5.7
17	2:1	1851	798	1596	+13.7
18	2:1	1845	717	1434	+22.3
19	2:1	1954	720	1440	+26.3

The first incident took place during the start-up phase of the programme, and was due to accidental injection of NaOH into the mine water feed line from the HDS plant. This resulted in unstable operating conditions, characterised by an increase in pH of the feed to 11 on days 37-55 (Figure 3.2c). The combined sulphate removal in the BT and hydrolysis reactor exceeded 60% during this period (Figure 3.3b). This may be explained by enhanced sulphate removal under alkaline conditions. The increased pH had little effect on COD_t concentrations (Figure 3.2a). Within five days of stopping inflow of the NaOH into the system, pH returned to between 7.5 and 8 (Figure 3.2c), and influent sulphate to normal operating concentrations of around 1674 $mg.L^{-1}$ (Figure 3.3a).

The second incident took place during days 78 to 85 (Figures 3.2 and 3.3). This challenge to the system was the result of fresh water leaking into the mine water feed line through a faulty valve. Sulphate levels in the feed fell to 1000 $mg.L^{-1}$ (Figure 3.3a). COD removal decreased dramatically during this period (Figure 3.2b), although the COD concentration of the feed remained constant (Figure 3.2a). The percentage sulphate removal showed a significant improvement (Figure 3.3b), although this was most likely to have been an

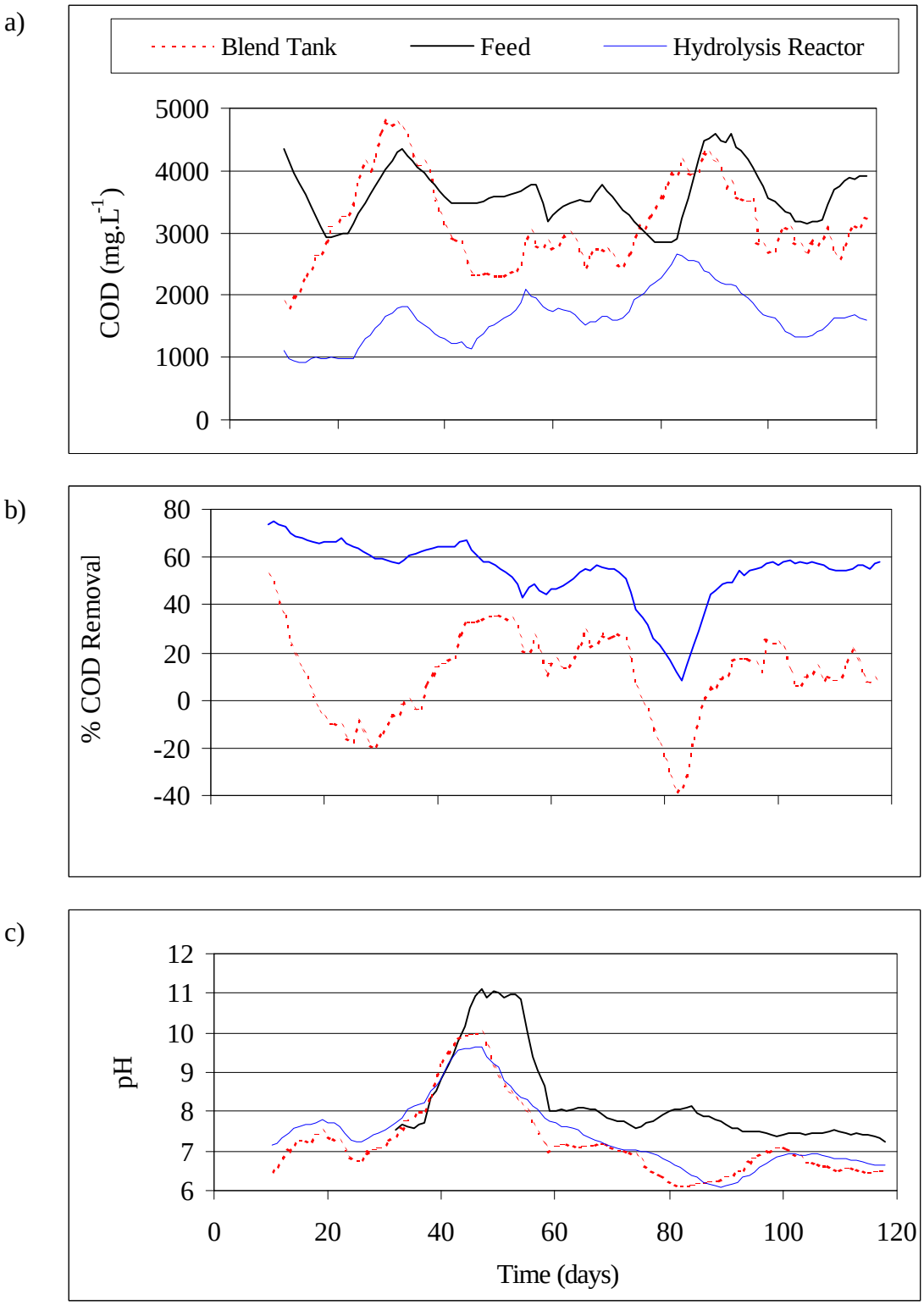


Figure 3.2. Performance of the pilot-scale Falling Sludge Bed Reactor. a) COD, b) percentage COD removed, c) pH. Trends for all data were derived using the moving average.

a)

b)

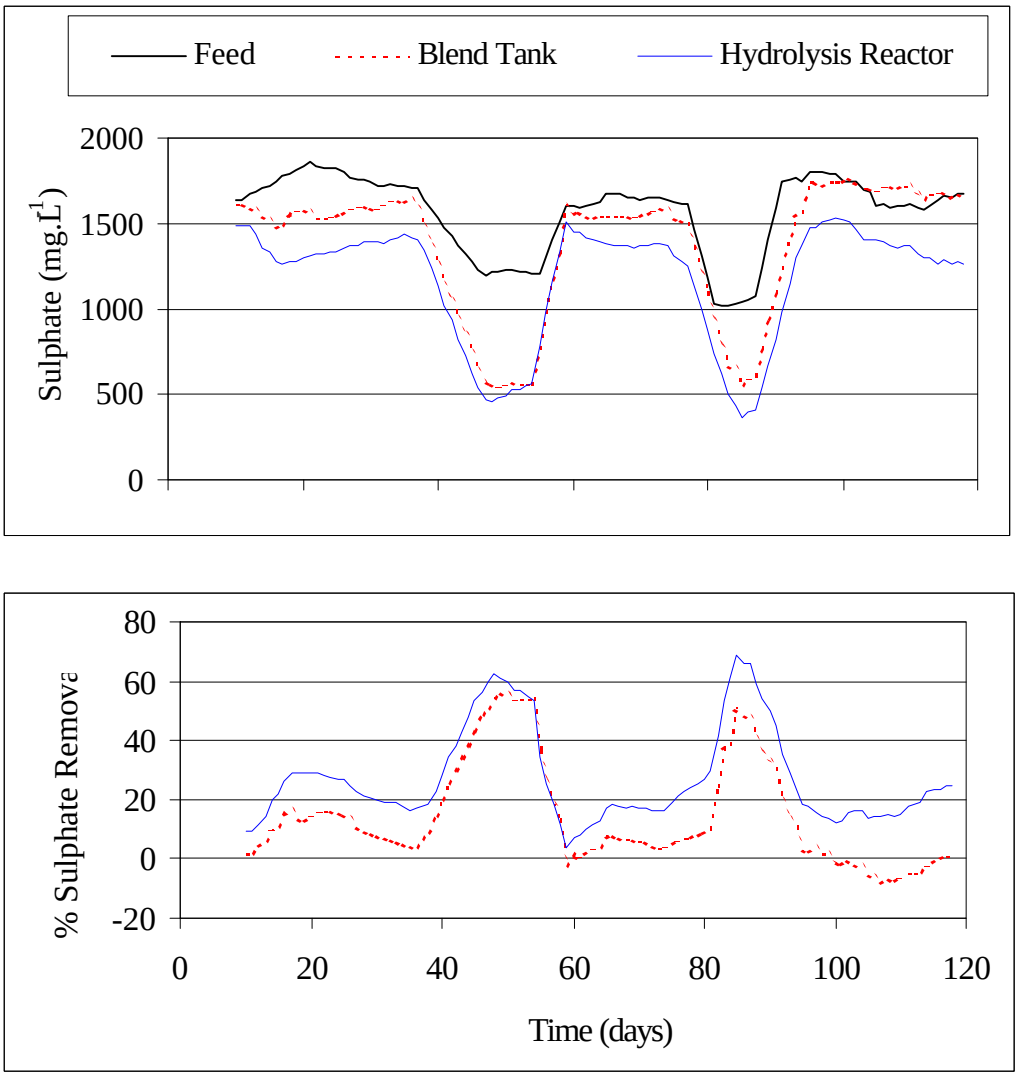


Figure 3.3. Performance of the pilot-scale Falling Sludge Bed Reactor. a) Sulphate removal, b) percentage sulphate removed.

artefact as a result of dilution. The pH of the hydrolysis unit also showed a slight drop during this period (Figure 3.2c).

3.3.2. Profile analysis

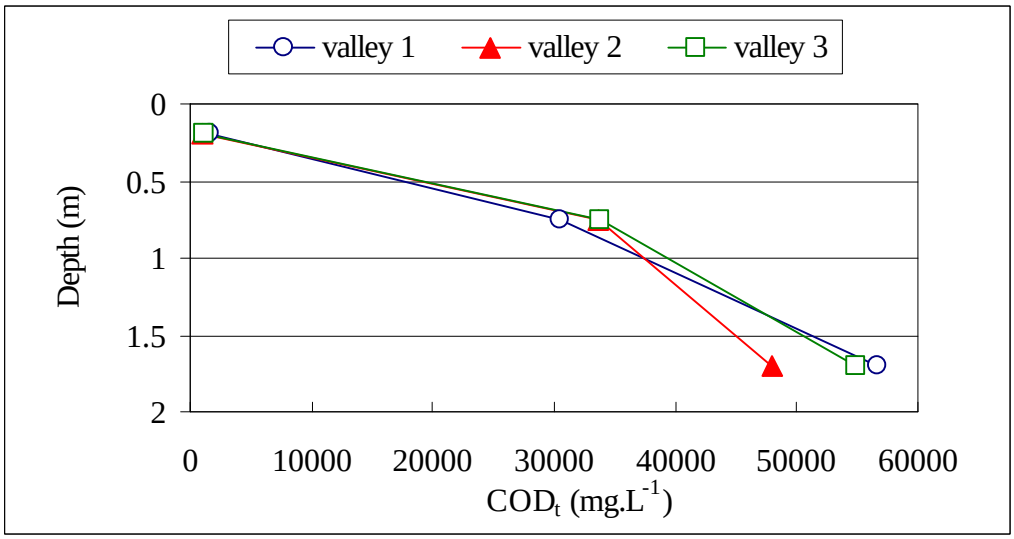
The mean concentrations of all COD fractions showed similar profiles in all three valleys (Figure 3.4). The COD concentration just below the surface of the reactor was low and increased significantly with depth. The difference between the upper and middle samples was far larger than that between the middle and lowest sample, with mean COD_t in the

beds between 50 000 and 60 000mg.L^{-1} (Figure 3.4a). These values showed little variation over the entire experimental period. The mean concentration of COD_f in the middle of valley 3 was significantly higher than in the other two valleys, at the same depth, due to a high concentration of COD_f at that position during the September audit. The COD_f decreased to a relatively lower concentration in the bed of valley 3(Figure 3.4c). The COD_f concentration in the bed of valley 1 was almost twice that of the other two valleys.

The mean sulphate concentration at the various depths exhibited a profile opposite to that of COD, with a dramatic decrease in sulphate concentration with depth (Figure 3.5a). The mean concentration near the surface of valley 1 was slightly lower than valley 2 and 3 (Figure 3.5a). The concentration of sulphate decreased from between 1000 and 1200mg.L^{-1} on the surface, to approximately 300mg.L^{-1} in the beds. This profile may be linked to the availability of hydrolysis products towards the base of the reactor. The presence of 200 – 400mg.L^{-1} sulphate in the base of the FSBR might have been as a result of inhibition of sulphate reduction by high sulphide concentrations. The accumulation of COD_f supports this idea and, as expected, the pH in the lower region of the FSBR was lower than at the surface. The increase in pH from the middle to bottom of the first two valleys was probably due to constant removal of soluble products from the lower region during the recycling of sludge. Sludge was drawn less frequently from the third valley and may have allowed for the accumulation of product, and a drop in pH, in this region. There was very little difference in the mean pH profiles of the valleys at different depths, with the largest difference being 0.2 pH points (Figure 3.5b).

Suspended solids were measured during the September audit. The profiles were similar to those of COD_t and COD_p, showing an increase in suspended solids concentration as a function of depth (Figure 3.5c). There was little difference in the top and middle samples from all valleys, but the concentration of suspended solids in the bed of valley 1 was almost double that in the beds of valleys 2 and 3.

a)



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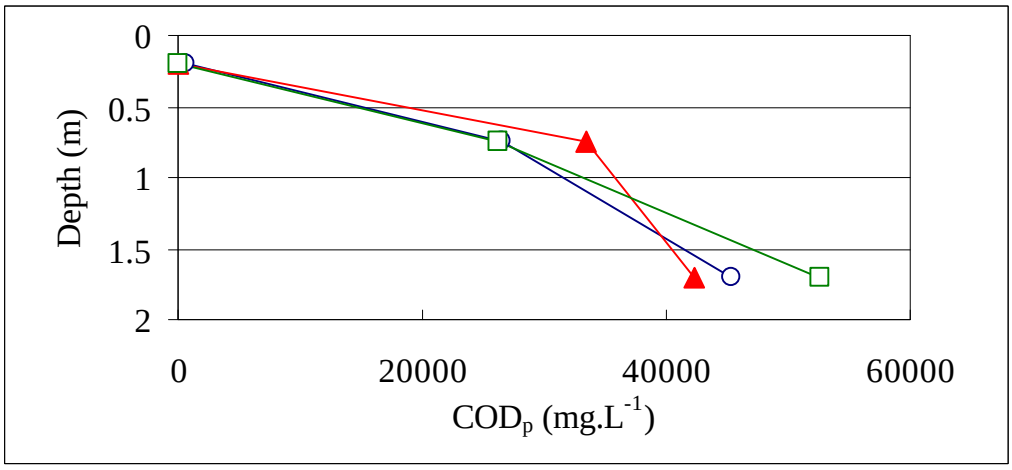

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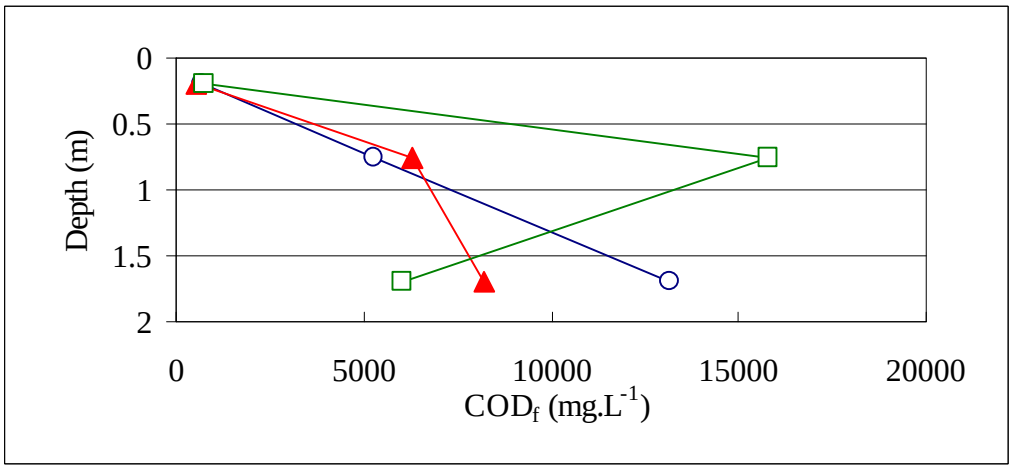
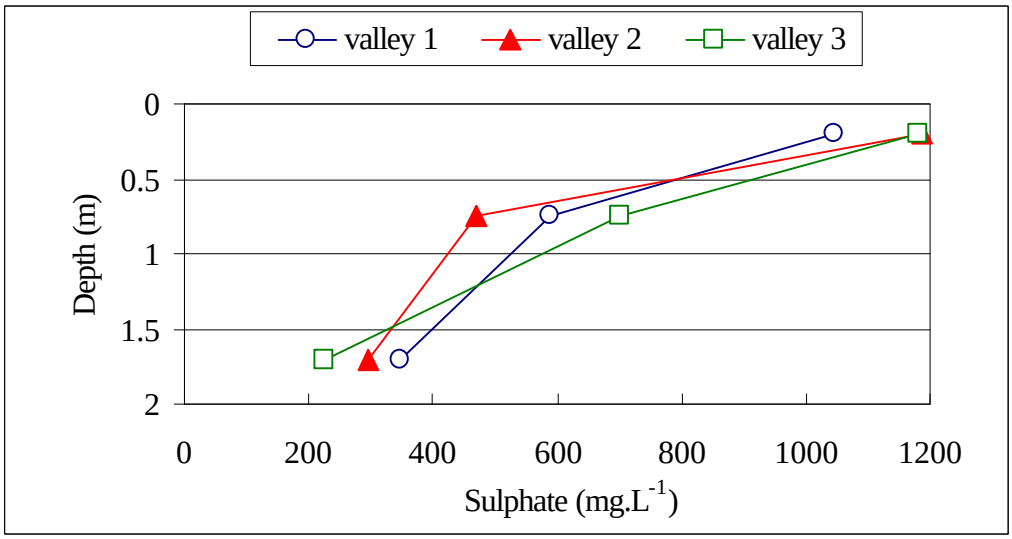


Figure 3.4. Mean COD concentrations at three depths in the FSBR for the three audit periods. Standard deviations were omitted for clarity.

a)



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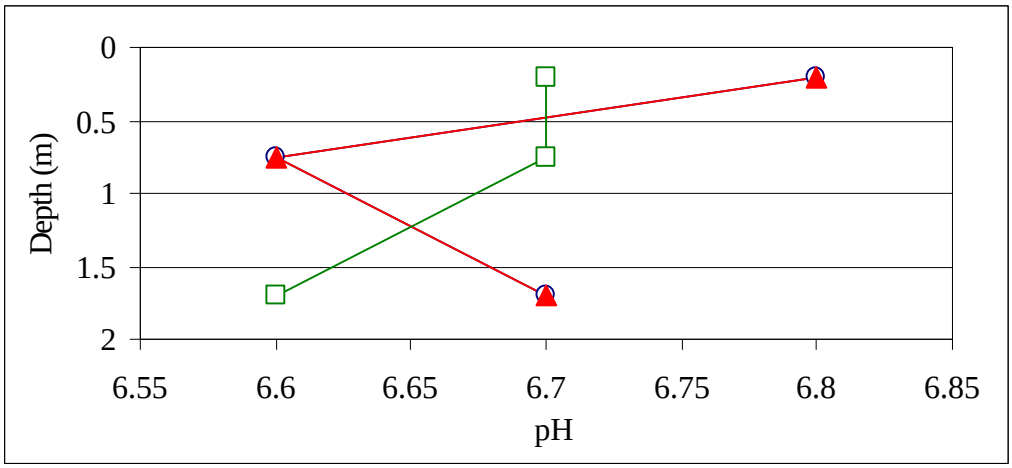

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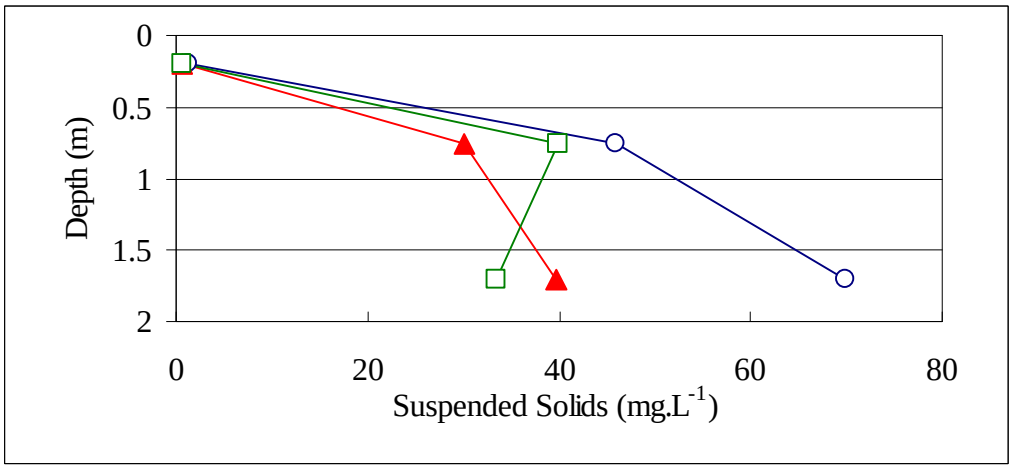


Figure 3.5. Mean sulphate concentrations, pH and suspended solids concentrations at three depths in the pilot-scale FSBR for three audit periods (n = 1 for suspended solids). Standard deviations were omitted for clarity.

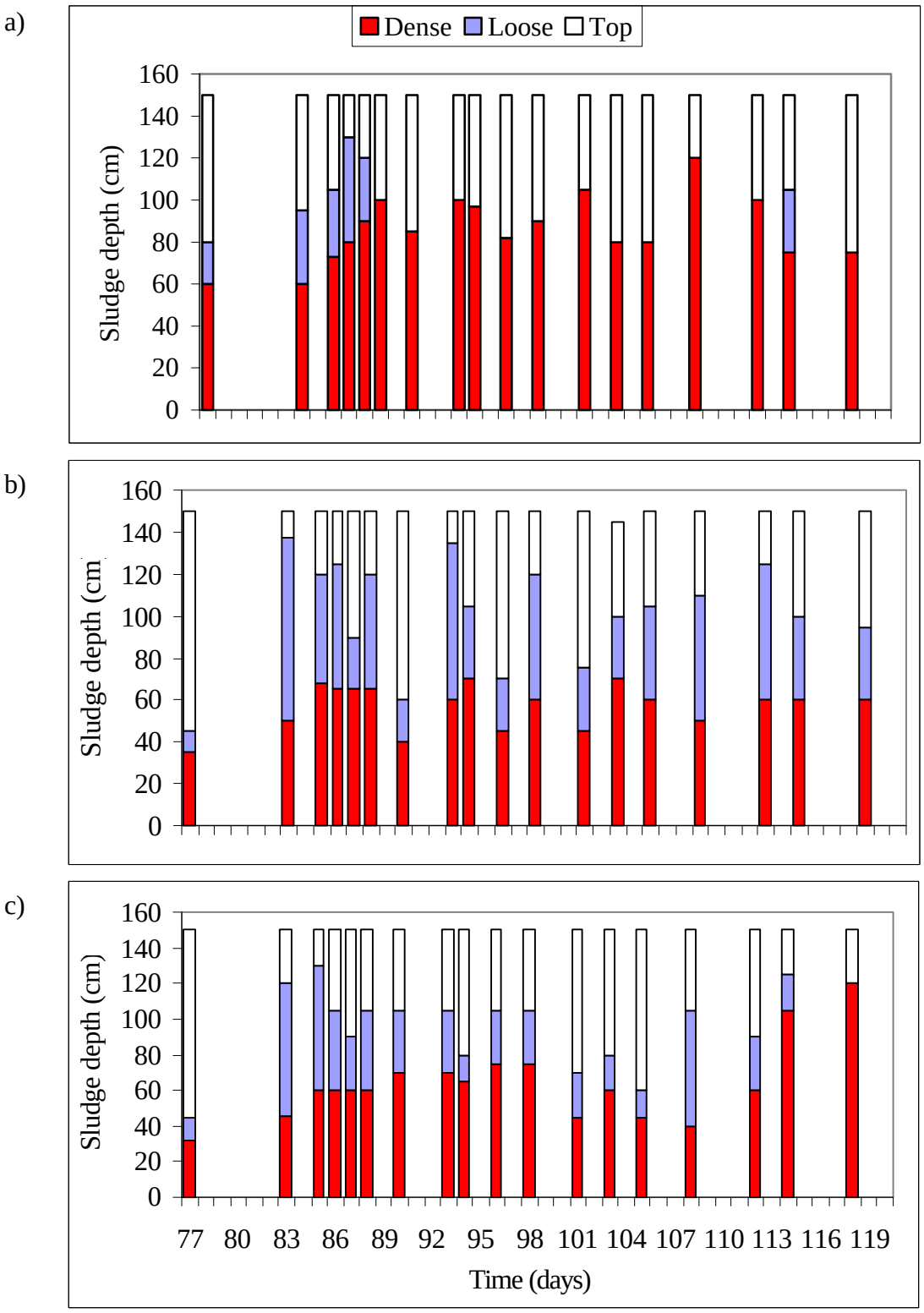


Figure 3.6. Analysis of sludge in three valleys within the pilot-scale FSBR after steady state had been reached. a) valley 1, b) valley 2, c) valley 3. The depth of the loose and dense sludge is indicated.

Accumulation of sludge within the FSBR was minimal, although some accumulation did occur within valley 3 after day 113 (Figure 3.6). The profiles also show that the entire sludge bed in valley 1 was dense, while the beds in valleys 2 and 3 consisted of a dense bed under a loose bed. This result showed the improved performance of the linear settler function.

3.4. Discussion

The degree of solubilization of PSS in the pilot-scale FSBR was significantly higher than any previous solubilization reports (Table 1.1) . At the same time, the reactor proved to be highly resilient, and was able to withstand unplanned perturbation events without prolonged loss of performance. The performance of the pilot-scale reactor during the perturbation events provided further evidence for a possible link between biological sulphate reduction and enhanced solubilization of PSS. The decrease in the sulphate concentration of the feed, as a result of the freshwater leak, resulted in a significant increase in the COD:SO_4 ratio of the feed. This would have, theoretically, favoured methanogenesis (Li *et al*., 1996), and may have resulted in a reduction in the activity of the SRB population. The decrease in COD removal during that period may have been as a result of lower alkalinity as result of reduced biological sulphate reduction. However, when the pH of the reactor was artificially elevated by the injection of NaOH, the increase in percentage sulphate removal had little effect on COD removal. The increase in pH was, by itself, not sufficient to increase the rate of COD solubilization. The rapid re-establishment of equilibrium within the FSBR following these perturbations was further evidence to support the use of this type of reactor configuration.

Although not yet fully optimised, approximately 70% of the particulate COD in primary sewage is effectively solubilized in the FSBR, in the presence of an active SRB population. This exceeds published maximum yields of around 35% (Karlsson and Göransson, 1993; Hatziconstantinou *et al*., 1996). The majority of sulphate reduction is expected to occur within the second stage of the reactor system (Figure 3.1) and consequently, the low percentage of sulphate removed within the first stage of the "Rhodes Process" i.e. the FSBR, is of less concern.

The calculated values of the minimum percentage COD_p that was solubilized were likely to be underestimates. It is thought that the quantity of sulphate reduced, and consequently, the quantity of COD_f utilised, was actually higher than represented by the data. Re-oxidation of the sulphide gas on the surface layer of the reactor, may have resulted in a cycle of sulphate reduction and sulphide oxidation within the reactor. Thus, an estimation of the quantity of sulphate reduced made by comparing influent and effluent sulphate concentrations, was probably inaccurate. The presence of elemental sulphur on the surface of the reactor contents is evidence of this oxidation. An increase in the concentration of sulphate on the surface of the reactor from the inlet to the outlet is further evidence of sulphide oxidation within the hydrolysis unit (Figure 3.5a). When the headspace of reactor was purged with nitrogen, the concentration of sulphate on the surface decreased along the length of the reactor (results not shown). The sulphate concentration in the effluent before and after purging was, however, similar. Any utilisation of COD_f by methanogenic bacteria would also have resulted in an underestimation of the quantity of COD_p solubilized. Under the conditions within the hydrolysis unit, i.e. potentially high concentrations of sulphide and a low $COD_f : SO_4$ ratio, methanogenesis would have been severely retarded. Methanogens are only expected to out-compete SRB at $COD:SO_4$ concentrations of above 2:1 (Li *et al*., 1996).

With a mean $COD:SO_4$ ratio of 0.5 near the surface of the reactor, SRB would be expected to dominate, if they could resist being washed out. However, in the sludge bed, where the sulphate concentration is lower and the concentration of COD_f is high, the $COD:SO_4$ ratios are 15 and 30 for the middle and bed, respectively. Under these conditions, in the absence of inhibitory concentrations of sulphide, methanogens would dominate (Li *et al*., 1996). The free sulphide in the sludge bed never exceeded $100mg.L^{-1}$ (data not shown), and would thus probably not severely inhibit methanogenesis. Characteristic signs of methanogenesis, such as gas bubbles and lifting of sludge by gas, were only seen over the short period when the ratio of $COD:SO_4$ in the feed was lowered to 1.5:1. At this stage, no explanation is offered for this phenomenon, and it appears that methanogenesis is low.

Profile data showed that the highest proportion of sulphate removal occurred in the upper to middle region of the sludge bed, with a smaller proportion taking place in the base of the reactor (Figure 3.5a). The pH profile was expected to reflect the activity of the SRB at the different depths, with the highest pH in the middle zone. The reverse was found, with the pH in the bed being lower than that in the upper zones (Figure 3.5a). This was probably a result of the accumulation of volatile fatty acids in the lower zones. In the bed, only a small quantity of the soluble hydrolysis products would have been consumed by SRB, because of the low sulphate concentration. Thus, the recycle of the volatile fatty acids from the bed, to a region of high sulphate and with an active population of SRB, is critical for the maintenance of a pH close to neutral. Alternatively, the high concentration of soluble COD in the base of the reactor could be used to support nutrient removal, with the particulate fraction of the recycle being returned to the hydrolysis unit.

The pilot-scale FSBR has been operated at an organic loading rate of 1.57kg $COD.m^{-3}.d^{-1}$ for 10 months. Although accidental, the injection of both freshwater and NaOH into the reactor system demonstrated the stability of the system, and its ability to recover rapidly after major upsets. The system effectively solubilized complex particulate COD, i.e. PSS, and offered a high yield of soluble COD_f. These soluble products were used successfully as a source of electron donors for the biological reduction of sulphate.

The biodegradability of the COD in the effluent of the FSBR was determined in order to assess whether a second hydrolysis unit was required. 250mL (n = 2) of the effluent from the pilot-scale FSBR was digested anaerobically at ambient temperature for three days. The COD of the samples were determined before and after digestion. The results of the degradation experiment showed that 27.6% of the total COD in the effluent of the FSBR was biodegradable within 3 days, although the largest consumption was of the soluble fraction. Solubilization of the particulate fraction was only 13% (Table 3.3). Thus, a certain amount of soluble product would only have been released in the second stage of the process, and would have been consumed by the SRB resident there. The experiment also indicated that approximately 45% of the soluble COD in the effluent was not utilised

by SRB, although the concentration of sulphate within the experimental flasks exceeded 500mg.L^{-1}.

Table 3.3. Mean percentage decrease in the COD of FSBR effluent after digesting anaerobically for 3 days (n = 2).

	Mean % removal	SD
COD_t	27.6	8
COD_f	54.5	0.1
COD_p	13.6	13

Thus, both readily and more slowly degradable carbon was available for sulphate reduction after solubilization. However, a second stage was required in order to lengthen the effective period of digestion, thereby optimising the yield of soluble product available for biological processes such as sulphate reduction or nutrient removal. Even higher yields and sulphate reduction may be achieved if the HRT within the second stage is increased.

Thus, the FSBR has been shown to be a relatively robust alternative to conventional PSS disposal methods, while simultaneously producing soluble COD to drive downstream biological processes.

3.5. Conclusions

- An indirect but reliable estimate showed that the minimum percentage solubilization of 71% had to have been achieved within the FSBR. This value is almost double any previously published yields. The volume of settleable solids of the PSS was reduced by 99.8% in the blend tank and FSBR.
- The mean percentage sulphate removal in the FSBR was only 31%, but was higher when re-oxidation of sulphide was prevented.
- The biological process within the FSBR was highly resilient, and recovered quickly after perturbation events.

- The COD in the effluent of the FSBR could be degraded further if the period of digestion was extended, indicating that a two-stage process was required.
- The study raised questions about the mechanism of enhanced hydrolysis of PSS under sulphate reducing conditions, including the effect on floc solubilization, enzymology and the hydrolysis of complex organic molecules.

Chapter 4

The Effects of Sulphate Reduction and Sulphide on the Particulate Solubilization and Lignocellulose Hydrolysis Processes

4.1 Introduction

The rate at which the biological degradation of complex organic substrates proceeds is limited by the rate of the hydrolytic step (Eastman and Ferguson, 1981; Pavlostathis and Giraldo-Gomez, 1991; San Pedro *et al*., 1994; Vavilin *et al*., 1996; Confer and Logan, 1997a, 1997b, 1998), and is best described by first-order kinetics (Gujer and Zehnder, 1983). Balmat (1957) and Eastman and Ferguson (1981) proposed that the overall hydrolysis rate constant for sewage solids represented a number of separate biochemical reactions, each having their own rate constant. Furthermore, it was shown that one of the factors responsible for variations in rate constants of biological oxidation of sewage solids, was the particle size of the substrates. The hydrolysis rate constant for particulate organics greater than 1μm was 200-300 percent lower than the rate for particulates <1μm (Balmat, 1957). Levine *et al*. (1985) obtained similar results when studying the size distribution of contaminants in wastewater. After digesting PSS anaerobically for 10 days, the concentration of particulate organics < 1.2μm decreased by 85%, while that of the particulates > 100μm, or settleable solids (Balmat, 1957), was reduced by only 9.3%.

Carbohydrates and lignin make up a significant, but variable proportion of sewage. Estimates have ranged from about 25% of the settleable solids fraction of raw sewage (Heukelekian and Balmat, 1959; Hunter and Heukelekian, 1965) to as high as 42% of the total solids in PSS (Elefsiniotis and Oldham, 1994). Despite the variable nature of PSS, it usually contains a considerable amount of lignocellulose. Reported concentrations suggest that up to 60% of the total carbohydrates in PSS may be in the form of cellulose (Heukelekian and Balmat, 1959; Hunter and Heukelekian, 1965), with lignin contributing up to 6% of the settleable solids fraction of sewage (Heukelekian and Balmat, 1959). Common sources of this complex organic matter may include paper, rags and vegetable matter (Knapp and Howell, 1978).

Cellulose is a homopolymer composed of D-glucose units linked by β (1-4)-glucosidic bonds (Bisaria and Ghose, 1981). While the amorphous region of cellulose is easily degraded, the crystalline region is structurally resistant to enzymatic attack (Gharpuray *et al*., 1983). Cellulose never occurs naturally in its pure form, and is always closely associated with a variety of other polysaccharides, in particular lignin and hemicellulose (Janes, 1969; Bisaria and Ghose, 1981). Hemicellulose is a polysaccharide usually consisting of a β -1,4 -linked D-xylose backbone with arabinofuranose and methylglucuronic acid side groups. Enzymes responsible for the hydrolysis of this compound include endoxylanase and β - xylosidase. Non-xylose components are removed by a range of enzymes including α-1,3-L-arabinofuranosidase and the debranching α-(1,2)-D-glucuronidase (Teunissen and Op den Camp, 1993). Lignin is not a carbohydrate, but a polymer of phenylpropane units linked together by ether and carbon-carbon bonds, and is highly resistant to biological breakdown. During the commercial Alkaline Pulping process, also referred to as the Kraft process or sulphate pulping, lignin is solubilised in the presence of alkalinity (Clayton, 1969). The β-aryl ether bonds are cleaved to yield quinone methide intermediates. The rate of delignification is accelerated in the presence of sulphide, and it is thought that the mechanism of the delignification reaction is slightly different to that in the presence of alkalinity alone. In the presence of sulphide, the quinone methide intermediates are converted to episulphides and guaicol compounds. The formation of episulphides is thought to facilitate the pulping process by preventing re-polymerisation of the phenylpropane subunits.

The abundance of lignocellulosic wastes and their potential as a cheap energy source has lead to continued research into improved utilization of this compound. As the susceptibility of cellulose to biological hydrolysis is dependent on its accessibility to cellulase enzymes, it is expected that the presence of associated polysaccharides will reduce the rate of cellulose hydrolysis. Gharpuray *et al*. (1983) reported that removal of lignin with peracetic acid resulted in a dramatic increase in the rate of hydrolysis of wheat straw, and the hydrolysis of bagasse was improved when a xylanase pretreatment

step was introduced (Bisaria and Ghose, 1981). Removal of hemicellulose was thought to have increased the accessibility of cellulose to exo- and endo-glucanases. Enzymatic pre-treatment using fungal cellulases has been investigated (Cinq-Mars and Howell, 1977), but has not been employed on a large scale.

Khan and Trottier (1978) demonstrated that degradation of cellulose decreased by 80% after removal of all sulphur compounds from the growth medium, and that the ability to degrade cellulose was restored upon the addition of a range of sulphur compounds. Recent attempts at improving the degradation of solid waste in landfills, revealed that solubilization of lignocellulosic wastes was greater under sulphate reducing conditions than during the conventional methanogenic digestion. 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 may be enhanced by the presence of relatively high sulphate concentrations. They showed that addition of 1000 $mg.L^{-1}$ sulphate improved both the rate and percentage solubilization of paper in lab-scale landfills. By following degradation of cellulose at $37^{o}C$ in 100mL batch reactors, hydrolysis rates under methanogenic and sulphate reducing conditions were calculated as 0.048 and 0.069 day^{-1}, respectively. Within 10 days, 48% of cellulose in the methanogenic reactor, and 75% under sulphate-rich conditions, had been degraded. Pareek *et al*. (1998) reported similar enhancement of cellulose digestion in the presence of active sulphate reduction. They found that the percentage and rate of paper mineralization, under sulphidogenic conditions, was approximately twice that under methanogenic conditions. Furthermore, a relatively high lignin content was associated with a lower percentage mineralization, although the relationship was not linear. The effect of continuous production of alkalinity, during sulphate reduction, on the hydrolysis of lignocellulose was not considered, although the low natural buffering capacity during digestion of predominantly carbohydrate waste can lead to digester failure (Banks and Humphreys, 1998).

The aim of the current study was to determine whether the solubilization of the particulate fraction of PSS was increased in the presence of biological sulphate reduction and, if so, if the effect could be linked to improved hydrolysis of lignocellulose.

4.2. Methods

4.2.1. Effect of sulphate reduction on particulate organic matter

The design of the experiment used to investigate the effect of elevated sulphide concentrations and pH, on the degradation of COD_p, is shown in Table 4.1. The experiment was conducted, in triplicate, in 1000mL flasks, and the contents stirred using magnetic beads. The pH of the flasks was adjusted, after sulphide addition, with 32% HCl or 50g.L^{-1}NaOH. Sulphide was added to experimental flasks in the form of a concentrated solution (1g.L^{-1}) of $Na_2S.9H_2O$. An equal volume of distilled water was added to the control flasks. The PSS was collected from GDW approximately 1 hour before use, and passed through a sieve (2mm mesh size) to remove any large particulates. The COD concentration was adjusted to 2000mg.L^{-1} with distilled water. The bacterial inoculum was obtained from a 10L fed-batch stirred tank reactor (STR) that had been operating under sulphate reducing conditions for approximately 1 year. PSS was used as the sole carbon source for the system (2000mg.L^{-1} COD), and the COD:SO_4 ratio was maintained at approximately 1:1. In an attempt to minimise the quantity of sulphide added to the control flasks, nitrogen gas was bubbled through the inoculum for 15 minutes prior to inoculation. At the start of the experiment, and after each sampling event, the headspace of each flask was flushed with nitrogen before sealing.

Based on data from the preliminary experiments, it was decided that any change in the solubilization rate of particulate COD would be evident within the first 48 hours of incubation. Thus, samples were analysed at the start of the experiment (t=0) and after termination of the experiment, at 48 hours. All flasks were incubated at 25^{o}C (± 3) in the dark. Sulphide was removed from all samples prior to determination of COD concentrations, as described in Chapter 2. Sulphide concentrations were determined using NN'-Diethyl-*p*-phenylenediamine (Rees *et al*., 1971). The sulphide standard curve is shown in Appendix 2. Alkalinity (mg.L^{-1} $CaCO_3$) was determined by titrating samples

to pH 3.7 with O.1M HCl (APHA, 1989). All other analytical methods used are described in Chapter 2. All percentage data were transformed (arcsin√proportion) prior to statistical analysis.

Table 4.1. Summary of the experimental design used to determine the effect of elevated sulphide concentrations on the hydrolysis of particulate COD.

Experiment	Sulphide ($mg.L^{-1}$)	COD ($mg.L^{-1}$)	Inoculum (mL)
-sulphide (pH7)	0	2000	100
+sulphide (pH7)	100	2000	100
-sulphide (pH10)	0	2000	100
+sulphide (pH10)	100	2000	100

4.2.2. Effect of sulphate reduction on hydrolysis of complex polysaccharides

Solubilization of the carbohydrate fraction of PSS under methanogenic conditions was compared to solubilization under conditions of elevated sulphide and sulphate. All experiments were conducted in sealed 500mL volumetric flasks, with a working volume of 400mL. Triplicate experimental and control flasks were inoculated with sieved (2mm mesh size) sludge (10% v/v) from the methanogenic anaerobic digester at GDW. Fresh PSS was used as the sole carbon source, and was prepared as described above, prior to incubation. The composition of the control (methanogenic) and experimental flasks is detailed in Table 4.2. Sulphide and sulphate were added as concentrated solutions of $Na_2S.9H_2O$ and Na_2SO_4, respectively. Before sealing the flasks, the pH of the flasks was adjusted to pH 7 and the headspace of each was flushed with nitrogen gas. All flasks were incubated on a shaker (100 rpm) at room temperature ($25^oC \pm 3$) in the dark.

The hydrolysis of insoluble complex carbohydrates, was followed by monitoring the changes in the concentration of soluble carbohydrates. The carbohydrate standard curve (Appendix 3) was prepared using glucose, as this is released during the hydrolysis of cellulose. Microbial uptake of the soluble carbohydrates was inhibited by the addition of toluene, without affecting the extracellular hydrolysis of the polysaccharides (Boschker *et*

al., 1995). Flasks were incubated for 7 days, at which time 3% (v/v) toluene was added to each flask (t=0). The headspace of the flasks was flushed with nitrogen, the flasks re-sealed, and incubated under the same conditions for a further 6 hours (t=6). Samples of 50mL were collected at t=0 and t=6.

Table 4.2. Composition of the control and experimental flasks used to assess the impact of sulphate and sulphide on the solubilization of complex carbohydrates.

Treatment	Sulphide ($mg.L^{-1}$)	Sulphate ($mg.L^{-1}$)	Final Volume (mL)
Control	0	0	400
Elevated sulphide	100	0	400
Elevated sulphate	0	2000	400

These were centrifuged at low speed (Universal 16A, 3000 rpm) for 10 minutes, and the supernatant passed through a GF/A glass microfibre filter (Whatman). The concentration of carbohydrate in the filtrate (i.e. soluble carbohydrate) was determined by the phenol-sulphuric acid method (Dubois *et al*., 1956). Preliminary studies indicated that this method was sensitive to sulphide. Thus, all samples were acidified to <pH 2 with concentrated H_2SO_4 and sparged with nitrogen for 15 minutes, prior to carbohydrate determination. Preliminary investigations showed that this was sufficient to remove all free sulphide from samples. Again, all data were transformed (arcsin√proportion) prior to statistical analysis.

4.2.3. The effect of sulphate reduction and sulphide on delignification

The effect of sulphate reduction and sulphide on the rate of delignification was examined in flask experiments. Details of the experimental setup are shown in Table 4.3. All contained 500mL of sieved PSS (prepared as above) and were inoculated with 200mL of sludge from the municipal anaerobic digester at GDW. The final concentration of sulphide in the + sulphide treatment was elevated to $500mg.L^{-1}$ with a concentrated sulphide solution ($Na_2S.9H_2O$). The initial sulphate concentration in the two + sulphate treatments was $2000mg.L^{-1}$, also added as a concentrated stock solution. The final liquid volume of each flask was made to 2L with tap water. A pellet of known SRB was added

to the + sulphate + SRB treatment while the only SRB present in the + sulphate – SRB treatment were those present in the inoculum or PSS. The pellet of SRB was prepared by isolating bacteria from the pilot-scale FSBR (Chapter 3) on modified Postgate medium B (Hiligsmann *et al*., 1998). A single colony was then allowed to multiply in liquid Postgate medium. After 3 days, 100mL of the medium was centrifuged (Universal 16A, 3000 rpm) and the bacterial pellet placed into the experimental flask.

Before sealing the flasks, the pH of each was adjusted to pH 7 as described previously, and the headspace of each flask was flushed with nitrogen gas. The flasks were then incubated on a shaker (100 rpm) at room temperature ($25^{o}C \pm 3$) for the duration of the experiment.

Table 4.3. Composition of control and experimental flasks used to assess the impact of sulphate reduction and sulphide on the hydrolysis of lignin in PSS. All volumes in mL.

Treatment	Sludge inoculum	Sulphide Stock	Sulphate Stock	Water	PSS
Control	200	0	0	1300	500
+ sulphide	200	500	0	800	500
+ sulphate – SRB	200	0	500	800	500
+ sulphate + SRB	200	0	500	800	500

Samples (100mL) were collected from each flask at 0 (30 minutes after initial setup), 24-, 48- and 72-hours, and the pH of each flask adjusted to the desired value before resealing. 10mL of each sample was filtered through a GF/A filter (Whatman), the filtrate acidified and then sparged with nitrogen gas for 10 minutes to remove all residual sulphide. A control, containing sulphide but no phenol, was treated in the same way to ensure that the method removed all sulphide that may have interfered with the colorimetric assay. The concentration of total phenolic material in each sample was determined using the method described by Box (1983). The standard curve was constructed using laboratory grade phenol (Merck) and is shown in Appendix 4. Each assay was conducted in triplicate.

4.3. Results

4.3.1. Effect of sulphate reduction on particulate organic matter

The mean percentage decrease in CODp in the four treatments were significantly different (ANOVA, df = 3,8; $P<0.05$). The highest solubilization of particulate COD was achieved at pH 10 and at elevated sulphide concentrations (Table 4.4). However, the percentage increase in percentage solubilization, when comparing the control and experiment at each of the pH values, was greater at pH 7. At a neutral pH, addition of sulphide resulted in a mean difference of 375% while at pH 10, the increase was only 53%. The addition of sulphide combined with an elevation of the pH resulted in more than a 500% increase in the percentage removal of COD_p, relative to the control (i.e. – sulphide at pH7).

For one of the repeats, the particulate concentration was calculated at both t=24 and t=48. Results showed that in both the experiments and controls, solubilization of particulates did not only occur in the first 24 hours of incubation (Figure 4.1). Under conditions of elevated sulphide concentrations, at both pH 7 and pH 10, the percentage of particulates solubilized within the first and second 24-hour periods were approximately equal. Thus, the effect of the elevated sulphide concentration in this system was cumulative over time, rather than instantaneous. Purging of the inoculum sludge with nitrogen prior to innoculation failed to remove all dissolved sulphide. Thus, the sulphide concentrations in the controls were higher than $20 mg.L^{-1}$ at the start of the experiment (Table 4.4).

Table 4.4. The effect of elevated concentrations of sulphide, and pH, on the hydrolysis of particulate COD in batch studies. Standard deviations are indicated in brackets.

Treatment	Mean % decrease in CODp	Sulphide t=0 ($mg.L^{-1}$)	Sulphide t=48 ($mg.L^{-1}$)	% change in alkalinity
-sulphide (pH7)	3.1 (5)	23.8 (8)	21 (8)	11.9
+sulphide (pH7)	10.4 (9)	77 (10)	68 (20)	20.0
-sulphide (pH10)	16.8 (2)	32 (13)	27 (12)	9.1
+sulphide (pH10)	24.0 (4)	108 (20)	91 (30)	9.2

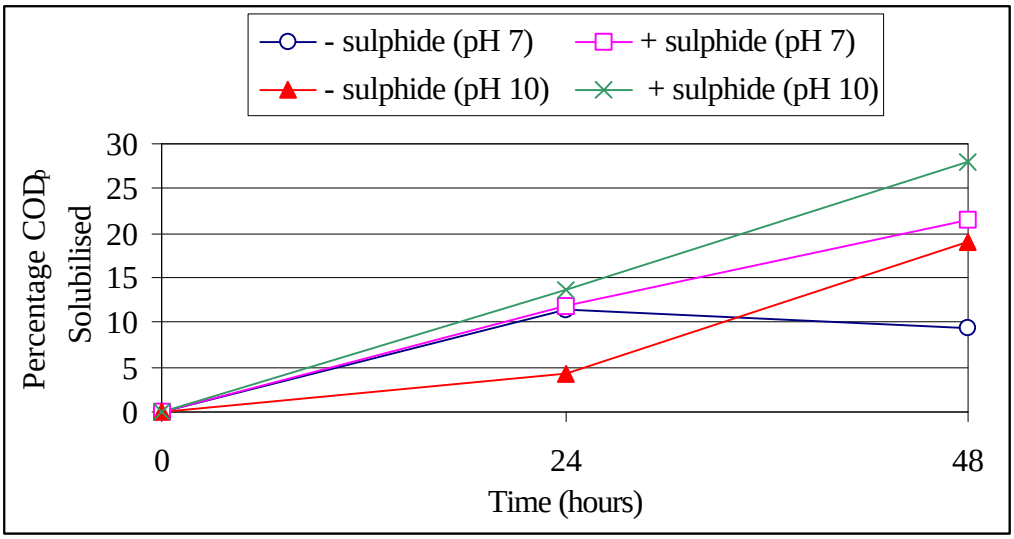


Figure 4.1. Percentage solubilization of PSS particulates after 24- and 48-hours.

4.3.2. Effect of sulphate reduction on hydrolysis of complex polysaccharides

The addition of sulphide, either chemically or biogenically through sulphate reduction, resulted in significantly enhanced hydrolysis of complex carbohydrates (ANOVA, df = 2,6; $P<0.01$). The concentration of soluble carbohydrate in the methanogenic systems decreased by a mean of 1.5% in the 6-hour period after addition of toluene. In the same time period, the concentration of soluble carbohydrate in the sulphate reducing and sulphide-addition systems increased by 39.3% and 25.4% respectively (Table 4.5). The reason for the higher efficiency of the sulphate reducing system is not understood but may be related to the continuous production of alkalinity or removal of the small phenolic end-products of lignin solubilization.

The mean concentration of glucose in the +sulphide treatment at t=0 was higher than that in the control and +sulphate treatment. The increase in the concentration of sulphide within the sulphate-rich systems, from 0.3mg.L^{-1} at the start of the experiment to 25mg.L^{-1} at t=0 (7 days later), indicated that sulphate reduction had occurred. A sulphide concentration of as low as 25mg.L^{-1} in the sulphate reducing system was enough to enhance the solubilization of the complex carbohydrates within the PSS.

Table 4.5. The effect of sulphate and sulphide addition on the hydrolysis of complex carbohydrates. Standard deviations are indicated in brackets. n = 3. t = 0 hours indicates the time of toluene addition.

Treatment	Sulphide ($mg.L^{-1}$)		Soluble Carbohydrate ($mg.L^{-1}$)		% Increase in Soluble Carbohydrate
	Start	t=0	t=0	t=6	
Control	0.8	0 (0)	33 (1)	32 (8)	-1.5
+ sulphate	0.3	25 (2)	28 (0.3)	39 (17)	39.3
+sulphide	68.5	31 (9)	41 (5)	52 (15)	25.4

The pH of all three treatments had decreased by the end of the digestion period (7 days) indicating that hydrolysis and acidogenesis had occurred (Table 4.6). The pH of the + sulphide treatment showed the largest decrease, and dropped from pH 7 at the start of the experiment to pH 5.8 on day 7. The pH of the control and sulphate reducing treatments on day 7 were pH 6.1 and pH 6.4, respectively.

Table 4.6. The effect of sulphide and sulphate addition on the pH of the digester contents, after digesting anaerobically for 7 days. Standard deviations are indicated in brackets.

Treatment	pH (start)	pH (t=0)
Control	7	6.1 (0.3)
+ Sulphate	7	6.4 (0.1)
+ Sulphide	7	5.8 (0.1)

4.3.3. The effect of sulphate reduction and sulphide on delignification

Sulphate reduction and high concentrations of sulphide had a significant impact on the concentration of soluble phenolic compounds in the 2L batch reactors, and provided indirect evidence that solubilization of lignin was enhanced under these conditions (Figure 4.2). The total concentration of phenolic material in the + sulphate – SRB and + sulphate + SRB treatments increased by 107% and 86% respectively ($12.7 mg.L^{-1}$ to 26.3 and $23.7 mg.L^{-1}$), over the experimental period. The increase in the + sulphide treatment

was 27% if calculated as the difference between the calculated concentration at t = 0 (63.5mg.L^{-1}) and t = 3 (81.2mg.L^{-1}). However, if one considers that the initial concentration of soluble phenolic material immediately before introduction of sulphide (although not determined) was probably around 11mg.L^{-1} (Figure 4.2), then the increase was approximately 638%.

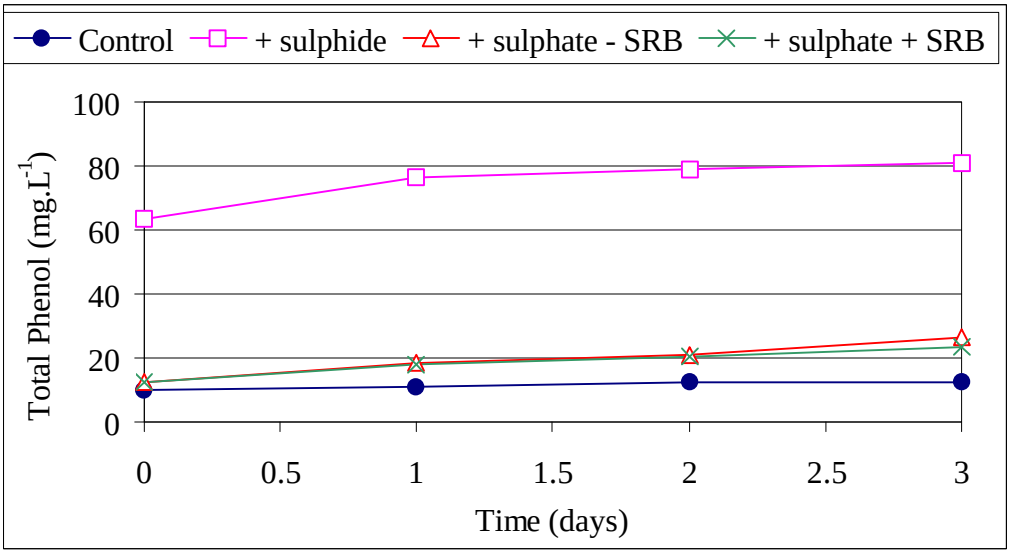


Figure 4.2. The effect of sulphide and sulphate reduction on the concentration of total soluble phenolic material in PSS when digested anaerobically (n=1).

Table 4.7. Change in sulphide concentrations in batch experiments used to determine the effect of sulphide and sulphate reduction on the concentration of soluble phenolic compounds in PSS. All concentrations in mg.L^{-1}.

	Time (days)			
Treatment	0	1	2	3
Control	1.6	5.5	3	7.1
+ sulphide	447	446	468	451
+ sulphate – SRB	1.0	13	29	45
+ sulphate + SRB	0	13	30	45

The addition of SRB, added to ensure a strong population of these bacteria, had no noticeable effect on the concentration of soluble phenolic material or sulphide (Table 4.7) over the experimental period. The rate of sulphate reduction, determined from the

increase in the concentration of sulphide in both of the + sulphate flasks, was similar. The increase in soluble lignin was closely correlated to the sulphide concentration in the treatments.

4.4. Discussion

An increase in pH and sulphide concentration had a positive effect on the solubilization of COD_p, under anaerobic conditions. Both variables might have influenced the solubilization step individually, but as maximum reduction of particulate organics was achieved at pH 10, and at an elevated sulphide concentration, it is suspected that a synergistic effect could be involved. Biochemical hydrolysis of PSS is an enzymatic process, requiring a suite of enzymes capable of hydrolysing a wide range of organics (Boczar *et al*., 1992; Nybroe *et al*., 1992; Frølund *et al*., 1995; Goel *et al*., 1998). Each enzyme in the suite will have its own pH optimum, and the hydrolysis process will proceed at a maximum rate when the pH of the system is close to the optimum pH of the majority, or most important, of the enzymes. Results of the present study suggested that, for the anaerobic digestion of PSS, this value is closer to pH 10 than to neutral.

As predicted, hydrolysis of the complex carbohydrates in PSS was accelerated by the addition of either sulphide or sulphate to the digester at pH 7. As the pH in the pilot-scale reactor never exceeded pH 7 under normal operating conditions, all experiments into the effect of sulphide on the hydrolysis of carbohydrates were only conducted at pH 7. Gharpuray *et al*. (1983) investigated the effect and mode of action of a variety of pre-treatments on the rate of hydrolysis of lignocellulose. They concluded that the factors involved, in order of importance, were the specific surface area, the lignin content and the crystallinity of the substrate. An increase in the specific surface area or a decrease in the lignin content or crystallinity was found to result in a higher rate of hydrolysis. Fan *et al*. (1980) also reported a highly linear relationship between the crystallinity index and the rate of hydrolysis, but that the specific surface area was of less importance. There is no evidence to suggest that either form of sulphur compound (sulphate or sulphide) is able to influence either the surface area or crystallinity of cellulose. The increased hydrolysis of carbohydrates and production of soluble phenolic compounds both strongly support the

hypothesis that a significant proportion of the enhanced hydrolysis of particulate matter in PSS occurs as a result of enhanced hydrolysis of the carbohydrate fraction. Furthermore, the enhanced hydrolysis of the carbohydrate fraction seems to be linked to the increased solubilization of lignin, after which the access of hydrolytic enzymes to these substrates is improved.

The first step in the manufacture of paper is the separation of cellulose from the lignin with which it is associated. During Alkaline Pulping, lignin is hydrolysed by a combination of alkalinity and sulphide (Clayton, 1969), both of which are products of sulphate reduction. The alkali results in a swelling of the wood chips and is also responsible for the degradation of lignin and long polysaccharide chains. The smaller lignin molecules produced during the alkaline hydrolysis process are soluble, and can be utilised by bacteria (Zeikus *et al*., 1982; Schink *et al*., 1992). The commercial pulping process is performed at a temperature of 170^{o}C and at a pH of 13.5 – 14.

While no evidence has been reported indicating a similarity of mechanisms between sulphidogenic hydrolysis of lignocellulose and the Alkaline Pulping process, a number of factors emerging from this study indicate some similarities. Although the reaction might proceed under the current experimental conditions, temperature and pH affect the rate of hydrolysis of lignin and the reaction is expected to be slow. If sulphide is responsible for the observed improvement in hydrolysis, then it is expected that the rate of hydrolysis would increase with an increase in alkalinity. This would explain the higher rate of carbohydrate hydrolysis under sulphate reducing conditions i.e. in the + sulphate treatment. Although alkalinity was not measured directly, the amount of alkalinity produced in each of the treatments could be estimated crudely from the change in pH over the 7-day period. The elevated sulphide concentration resulted in increased hydrolysis of cellulose, and should have resulted in a subsequent increase in the concentration of volatile fatty acids. As very little alkalinity is produced during the anaerobic digestion of carbohydrates, there would only have been a limited quantity of natural buffer to limit the drop in pH. Although the degradation of lignin and cellulose in the sulphate reducing system was possibly greater than in the + sulphide treatment, the

continual production of alkalinity, during the process of sulphate reduction, would explain the less significant decline in pH.

The ability of SRB to utilize aromatic compounds as a carbon source has been reported by a number of authors (Holliger *et al*., 1988; Widdel, 1988; Schnell and Schink, 1991; Schnell and Schink, 1992; Schink *et al*., 1992; Gorny and Schink, 1994; Pavlosthathis, 1994; Reichenbecher and Schink, 1997) and cannot be excluded as a possible mechanism to partially explain the enhanced hydrolysis of lignocellulose. The compounds used are similar in structure to the phenypropane sub-units of lignin, and included benzoate, phenol, indoles and catechols. Widdel (1988) cited evidence that benzoate-degrading species, such *as Desulfococcus niacini* and *Desulfobacterium vacuolatum* were able to completely oxidise phenylpropionate to benzoate. Strain SAX, like the majority of SRB able to utilise aromatic compounds, was isolated from marine environments and was found to degrade benzoate, 4-hydroxybenzoate and phenol to CO_2 (Drzyzga *et al*., 1993). Kuever *et al*. (1993) isolated a sulphate reducer, D*esulfotomaculum* strain Groll, from a freshwater ditch. The strain was found to completely degrade a wide range of aromatics, including catechol and *m*- and *p*-cresol, by O-demethylation, and it was speculated that this strain might be able to participate in the bioconversion of lignin monomers. There is indirect evidence that SRB, capable of degrading aromatic compounds, are present in municipal digesters. Fang and Zhou (1997) used municipal digester sludge to inoculate a lab-scale UASB reactor. After acclimation on sucrose, benzoate and *m*- and *o*-cresol were added to the feed. Eventually, these aromatic compounds were the sole carbon source. The percentage removals achieved were 99.5% for benzoate, and 11% and 8.3% for *m*- and *o*-cresol, respectively. Furthermore, during batch studies, degradation of benzoate was 125% faster in the presence of 1250mg.L^{-1} sulphate, than in a sulphate-free medium. Thus, based on current knowledge, it is possible to suggest that certain species of SRB might be able to degrade lignin, and in this way enhance the hydrolysis of lignocellulosic wastes. This would explain the enhanced hydrolysis when sulphate was added to landfills (Kim *et al*., 1997; Pareek *et al*., 1998), and in the present flask studies. This mechanism alone is insufficient to explain the results of this study, as it could not have been responsible for the enhanced hydrolysis observed in the + sulphide treatments.

The quantity of sulphide oxidised to sulphate was probably minimal as the headspace of all the flasks were flushed with oxygen-free nitrogen before incubation, and no elemental sulphur was seen in the flasks on day 7. The ability of SRB to utilize small phenolic compounds as a carbon source may have contributed to the relatively low concentration of soluble phenolic compounds in the + sulphate treatments when compared to the + sulphide treatment. However, the observed difference was more likely to have been due to the greater quantity of lignin being solubilised in the presence of higher concentrations of sulphide.

The enhanced hydrolysis of the carbohydrate fraction of PSS contributes significantly to the observed enhancement of particulate organic matter in PSS under sulphate reducing conditions. The underlying mechanism could in some ways be similar to that used in the commercial Alkaline Pulping process, and involves the solubilization of lignin in the presence of sulphide and alkalinity, although occurring at much reduced rates at standard temperatures and pressures. This, in turn, increases the possibility of contact between hydrolytic enzymes and substrates such as cellulose and hemicellulose. The direct hydrolysis of lignin by SRB may contribute to the removal of lignin.

4.5. Conclusions

- Solubilization of COD_p was enhanced significantly in the presence of sulphide at both pH 7 and pH 10.
- The rate of hydrolysis of complex carbohydrates is enhanced in the presence of sulphide. The rate of hydrolysis is higher in the presence of biological sulphate reduction than when sulphide was added chemically, possibly due to the biological production of alkalinity.
- The rate of delignification is enhanced in the presence of sulphide or biological sulphate reduction and it is suggested that the mechanism could resemble that of the Alkaline Pulping process.
- Successful delignification is known to increase the exposure of cellulose and hemi-cellulose to attack and degradation by hydrolytic enzymes.

Chapter 5
Hydrolysis of Protein in Conditions of Elevated Sulphide Concentrations

5.1. Introduction

Estimates of the protein content of municipal PSS have varied from 17.2 to 29% (Heukelekian and Balmat, 1959; Eastman and Ferguson, 1981; Levine *et al*., 1985; Pavlostathis and Giraldo-Gomez, 1991). Although a significant proportion of PSS is in the form of insoluble protein, very little is known about the hydrolysis of this class of organic compound under anaerobic conditions. Factors such as solubility, tertiary protein structure and type of end-groups are considered to influence the rate of protein hydrolysis (McInerney, 1988). As with all enzymatic processes, hydrolysis of protein is affected by pH, although there is no consensus with regards to an optimum pH range for proteolytic activity. Hanaki *et al*. (1987) monitored the degradation of protein in whey at 37^{o}C, and reported maximum hydrolysis of protein at pH 7. Penaud *et al*. (1997) found that the percentage solubilization of protein in pharmaceutical wastewater increased from 5% at pH 5 to 82% at pH 9. Contrary to this, the maximum degradation of protein in PSS was found to be pH 4.5, with lower solubilization at increased pH (Elefsiniotis and Oldham, 1994). The maximum percentage solubilization of PSS appears to be affected by both temperature and retention time. Eastman and Ferguson (1981) reported 54% solubilization of protein at 35^{o} C at a retention time of 2.75 days. When PSS was digested at 18 – 22^{o} C, the percentage solubilization of protein was only 38.7% at a solids retention time of 5 days, and increased to 54% after 20 days (Elefsiniotis and Oldham, 1994). Ghosh (1987) found maximum reduction of crude protein, in a mixture of PSS and waste activated sludge, of 23% after 3 days and only 27% after 15 days. This was evidence that a certain fraction of the protein contained in sludge is not easily biodegradeable.

To date, no studies have investigated the fate of proteins in PSS under sulphate reducing conditions. Pipes (1961) proposed that one possible advantage of digesting municipal waste under sulphate reducing conditions was the degradation of recalcitrant proteins in

the presence of high sulphide concentrations. Although polypeptides themselves can not be used as electron donors by SRB (Widdel, 1988), the sulphide produced during biological sulphate reduction is a strong reducing agent, and is capable of cleaving the disulphide linkages that are essential for maintaining the conformity of large proteins. If the hydrolysis of sludge proteins is accelerated in the presence of sulphate reduction, this might contribute to the enhanced solubilization of COD_p observed upon the addition of sulphide (HS^-).

The aim of this study was to investigate the fate of the protein component of PSS when digested anaerobically in the presence of elevated sulphide concentrations or active sulphate reduction. When studying the effect of sulphide on the hydrolysis of the carbohydrate fraction of PSS, it was possible to monitor the rate of hydrolysis through the accumulation of hydrolysis end-products i.e. glucose. A similar technique could not be found for the study of protein hydrolysis, as the common methods for estimation of protein concentrations detects large proteins as well as smaller polypeptides. Recently, Ehlers and Cloete (1999) compared the microbial communities in activated sludge systems by subjecting extracted bacterial proteins to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). It was thought that a similar approach could be used to follow the hydrolysis of sludge proteins, by following the disappearance of large proteins and the appearance of lower molecular weight products.

The effect of sulphide addition on the hydrolysis of sludge proteins was studied at two pH values, and the rate of hydrolysis compared using SDS-PAGE.

5.2. Methods

5.2.1. Analytical methods

Protein concentrations were determined using the Folin-Lowry method (Lowry *et al*., 1951). The standard curve was prepared with Bovine Serum Albumin (Böehringer Mannheim), and is shown in Appendix 5. Preliminary evaluation of the technique revealed that relatively low concentrations of sulphide (greater than $10mg.L^{-1}$) in sludge samples interfered with the analysis, and resulted in falsely elevated estimates of protein

concentrations. However, sulphide concentrations in the crude protein extracts (after solvent precipitation) used for electrophoresis were less than 2mg.L^{-1} and did not interfere with protein determination.

The methods used in the determination of sulphate and sulphide concentrations, pH and alkalinity are given in previous chapters.

5.2.2. General Operating Conditions for SDS-PAGE

All gels were run in a Hoefer gel system (SE 600 series) with a LKB 2197 power supply. To promote even running of all samples, the running buffer was kept cold by placing the lower buffer chamber on ice. Cold water was also circulated through the submerged heat exchanger in the lower buffer chamber. The settings used remained constant for all runs and were as follows: 200V, 35A and 100W. Under these conditions, each run took 11.5 hours. After completion of a run, the gels were stained with Coomassie Brilliant Blue R250. The protocol for staining and de-staining is given in Appendix 6.3. Gels were then photographed and analyzed using Kodak Digital Science 1D image analysis software. Due to the appearance of breakdown products in the High Molecular Weight Marker, the density of the marker bands could not be used directly to determine the quantity of protein in each of the experimental bands. To overcome this problem, the actual quantity of protein in each of the marker bands was determined by comparing the density of the marker bands with the density of a band of known protein mass. The standard band contained 5µg of BSA (Böehringer Mannheim).

In order to test the hypothesis, it was essential that no reducing agents other than sulphide were introduced into the system. Thus, non-reducing gradient SDS-PAGE (Laemmli, 1970) was used throughout this study. During trial studies, a 7.5 - 22% gradient was used. No high MW bands were detected, and the separation of low molecular weight bands was poor. It was envisaged that by changing the gradient to 5 – 30%, larger proteins might penetrate into the gel matrix, and that separation of the low MW bands would improve. The composition of the separating and stacking gels is given in Appendix 6.4. All chemicals used in the preparation and running of SDS-PAGE gels

were electrophoresis grade. The protein concentration of each sample was determined prior to loading samples onto the gels. The volume of each sample loaded was then adjusted to ensure that all wells contained the same quantity of protein.

5.2.3. Optimization of technique for visualization of sludge protein

One of the aims of the optimization step was to use minimal pretreatment of PSS samples prior to running SDS-PAGE. Thus, the first samples run on the system were raw PSS. Fresh PSS was collected from GDW and sieved (2mm mesh-size) to remove any large organic matter. 1ml of raw PSS was then diluted 1:1 with fresh sample buffer and run on a 7.5-22% gradient gel. The gradient was increased to 5-30% in an attempt to increase the number of bands formed.

The poor results obtained when raw PSS was loaded onto the gel were ascribed to a high concentration of impurities in the sample and adsorption of proteins to sludge flocs by electrostatic forces. Samples were centrifuged (Universal 16A, 3000 rpm for 10min) and the supernatant filtered through a GF/A glass microfibre filter (Whatman) to remove some of the impurities that may have reduced the visibility of the protein bands within the gel. The lack of banding suggested that the proteins might have been strongly attached to the floc matrix, probably by electrostatic forces. Thus, a significant proportion of the total protein was probably removed during the filtration step. In an attempt to release the proteins from the floc matrix prior to filtration, and thereby increase the concentration of protein in the filtrate, all samples were treated with SDS (sodium dodecyl sulphate). 1ml of 10% SDS (solution D, Appendix 6.1) was added to 9mL of PSS. After mixing thoroughly, the samples were allowed to stand at room temperature for 1 hour. The samples were then centrifuged (Universal 16A, 3000 rpm for 10 minutes) and filtered. The protein concentration of SDS-treated samples (filtrate and supernatant) was compared to that of untreated samples i.e. raw PSS. The effect of adding maceration of the PSS as an additional pretreatment was also investigated. Unsieved PSS was circulated through a large sludge grinder (Mini-Monster Model 20 000 in-line grinder with 5 and 11 tooth double edge 4130 alloy steel cutters, 0.75kW, 525V, JWC Environmental, Irvine, California, U.S.A.) but not sieved. Although no

organic material was removed from the raw PSS sample, the size of some of the larger components was reduced. The pretreatment steps examined are recorded in Table 5.1.

Although bands had appeared after the introduction of the pretreatment steps, they were barely detectable. The possibility of increasing the intensity of the bands by concentrating the protein was investigated. The effectiveness of solvent extraction and ammonium sulphate precipitation (Cantor, 1982) was compared. Details of both procedures are given in Appendix 7. Samples treated by a combination of centrifugation, filtration and protein extraction steps were compared using SDS-PAGE.

Table 5.1. Combinations used to determine the effect of pretreatment of sludge samples prior to PAGE.

Lane	Treatment
1	Macerated (raw sludge – SDS)
2	Macerated + SDS (centrifuged + filtered)
3	Macerated + SDS (centrifuged only)
4	High Molecular Weight Marker
5	Sieved (raw sludge - SDS)
6	Sieved + SDS (centrifuged + filtered)
7	Sieved + SDS (centrifuged only)

5.2.4. Effect of sulphide, sulphate reduction and pH on protein hydrolysis

The experiment was conducted in 250mL flasks. All flasks contained 175mL of fresh macerated PSS, and were inoculated with 25mL of sludge from the anaerobic digester at GDW. The experimental design used to test for the effect of sulphide and sulphate reduction on the hydrolysis of protein is shown in Table 5.2. Two flasks were stored at 4^{o}C for 7 days, and were used as undigested controls. Preliminary studies (van Jaarsveld, pers. comm., 1999) showed that no protease activity occurred in PSS at 4^{o}C. In order to test if sulphide cleaved sludge proteins in the absence of biological activity, control flasks were incubated with and without sulphide. Although the concentration of free sulphide within the pilot-scale reactor rarely exceeded 150mg.L^{-1}, the sulphide concentration in

Table 5.2. Design of experiment to examine the effect of sulphide and sulphate reduction on the hydrolysis of sludge protein at pH 7.

Flask	Treatment	Incubation temperature	Sulphide ($mg.L^{-1}$)	Sulphate ($mg.L^{-1}$)	MoO_4 (mM)
1	Control – sulphide	4°C	0	0	0
2	Control + sulphide	4°C	500	0	0
3	Digested – sulphide	25°C	0	0	0
4	Digested + sulphide	25°C	100	0	0
5	Digested + sulphide	25°C	500	0	0
6	Digested + sulphate reduction	25°C	0	2000	0
7	Digested – sulphate reduction	25°C	0	2000	30

the control flask was elevated to $500mg.L^{-1}$. This was to ensure enough sulphide was present to react with the protein. The remaining flasks were incubated on a shaker at 25°C for 7 days. One flask was maintained under methanogenic conditions, as a secondary control. $100mg.L^{-1}$ and $500mg.L^{-1}$ sulphide was added to two of the flasks (as $Na_2S.9H_2O$) to test for the effect of elevated sulphide concentrations, in the presence of normal biological activity, on the hydrolysis of sludge proteins. The effect of sulphate reduction and sulphate alone was also tested. Two treatments contained $2000mg.L^{-1}$ sulphate (added as Na_2SO_4). 30mM molybdate was added to one of the flasks to inhibit sulphate reduction (Lens *et al*., 1995). The pH of all flasks was adjusted to 7 with 32% HCl or $50g.L^{-1}$ NaOH. The volume of liquid within all flasks was equalised with distilled water (final volume = 250mL), so as to ensure approximately equal concentrations of protein in all treatments. The original PSS was not diluted to a COD of $2000mg.L^{-1}$ so as to maximize the recovery of protein for SDS-PAGE. The headspaces of all flasks were flushed with oxygen-free nitrogen gas and the flasks sealed. After 7 days, the proteins were extracted and concentrated using the acetone method (Appendix 7.1), and then analyzed by SDS-PAGE (5-30% gradient).

The second phase of the experiment was designed to provide information on the rates of protein hydrolysis, with and without sulphide, under neutral and alkaline conditions.

These experiments were also conducted in 4 x 250mL flasks. All flasks contained 200mL fresh macerated PSS (see above), and were inoculated with 25mL of sludge from the municipal anaerobic digester. The sulphide concentration in two of the flasks was elevated to 100mg.L^{-1} by adding sodium sulphide solution. The pH of one of the control (- sulphide) and + sulphide flasks was adjusted to 7, and that of remaining flasks to pH 10. The headspace of the flasks was flushed with oxygen-free nitrogen gas, and the flasks sealed with rubber bungs. They were then incubated on a shaker at 25^{o}C. Samples of 50mL were removed from each flask before incubation (t=0) and on days 1, 3 and 5. The proteins were extracted and concentrated, as described above, immediately after sampling. The re-suspended pellets were stored under nitrogen at 4^{o}C until analyzed using SDS-PAGE.

5.3. Results

5.3.1. Optimization of SDS-PAGE for visualization of sludge proteins

The bands observed on gels loaded with raw PSS were indistinct. A light band was observed in the upper region of the gels, and corresponded to proteins with a molecular weight of approximately 112kDa. Diffuse bands corresponding to proteins in the region of 25kDa and 15kDa were also observed. The estimation of the size of the proteins was not accurate, as lanes did not run parallel.

The low intensity of the bands was indicative of only small quantities of protein migrating into the gel. Some of the proteins were probably bound to the sludge matrix, and thus were removed during the filtration step. Incubation of samples in 1% SDS resulted in release of protein from the sludge matrix into the soluble fraction (Table 5.3), and should have resulted in more protein moving into the gel. This should have resulted in formation of bands of higher intensity. Recovery of protein in the filtrate increased after addition of SDS. The concentration of protein in the untreated filtrate was 17% of that in the unfiltered sludge sample (total sludge protein), while that in the SDS-treated filtrate was 62% (Table 5.3).

Table 5.3. Mean (n=3) concentration of protein in raw and SDS-treated samples. Standard deviations are indicated in brackets.

Sample	Mean Protein ($mg.L^{-1}$)	% of total protein
Untreated raw PSS	1.76 (0.09)	100
Filtrate – SDS	0. 3 (0.05)	17
Filtrate + SDS	1.09 (0.08)	62

The concentration of protein was $4465mg.L^{-1}$ and $4932mg.L^{-1}$ in the macerated and sieved sludge samples, respectively. Although the samples were taken on the same day, they were taken from different points at the disposal works. This would have accounted for the difference in the protein concentration of the two samples. When the samples were analyzed by SDS-PAGE, bands corresponding to proteins of approximately 45kDa were visible, but were too indistinct for comparative studies. The bands in the lanes loaded with macerated sludge were slightly darker than those loaded with the sieved samples, and centrifuged samples (+SDS) produced darker bands than samples that had been filtered after centrifugation. The filtration step was thus disadvantageous.

Table 5.4. The effect of the extraction step on the concentration of protein in samples. Values indicate the mean protein concentrations (n=3) of treated samples after protein extraction and concentration. Standard deviations are indicated in brackets.

Sample	Protein ($mg.L^{-1}$)
+ SDS (control)	3532 (733)
- SDS (control)	1266 (352)
+ SDS (acetone extraction)	4643 (101)
- SDS (acetone extraction)	3332 (305)
+ SDS (ammonium sulphate extraction)	4221 (443)
- SDS (ammonium sulphate extraction)	3310 (1172)

Both extraction procedures resulted in an increase in the protein concentration in the samples after re-suspending the pellets in buffer (Table 5.4), with higher concentrations of protein in the SDS-treated samples. When comparing the efficiency of the two extraction methods after SDS treatment, the acetone extraction resulted in a 31.4%

increase in the protein concentration over the untreated samples. Extraction of protein by the ammonium sulphate method resulted only in a 19.5% increase. Inclusion of the extraction step lead to a significant improvement in the number of bands that could be seen after SDS-PAGE (Figure 5.1). The majority of the bands corresponded to proteins in the 29 – 55kDa size-range. Bands formed by the SDS-treated samples were more visible than those formed by untreated samples.

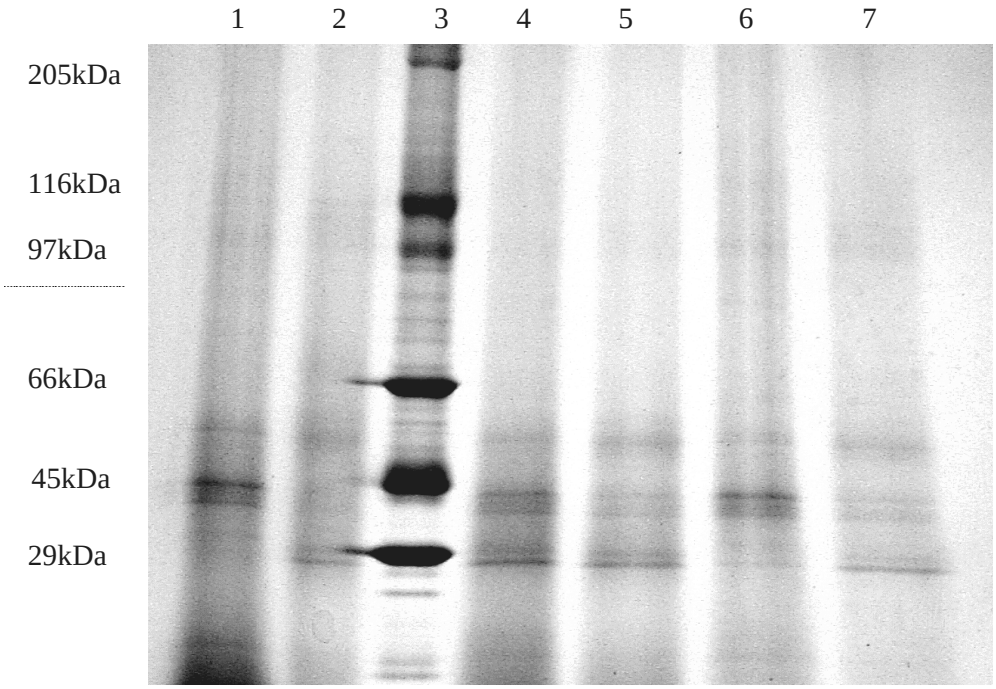


Figure 5.1. A comparison of sludge proteins after extraction and concentration by acetone or ammonium sulphate. Lanes: 1= Control + SDS; 2 = Control - SDS; 3 = Marker; 4 = Acetone extracted + SDS; 5 = Acetone extracted – SDS; 6 = Ammonium sulphate extracted + SDS; 7 = Ammonium sulphate extracted – SDS.

5.3.2. Effect of sulphide, sulphate reduction and pH on protein hydrolysis

The effect of different concentrations of sulphide and biological sulphate reduction on the hydrolysis of sludge proteins was examined using SDS-PAGE. Again, the bands of interest corresponded to proteins in the 45 to 26kDa size-range (Figure 5.2). The banding pattern was consistently repeated and appeared to be representative of the local sludge proteins, with approximately 4 major breakdown products. In order to aid interpretation of the results, the intensity of the bands was calculated against the BSA standard, and is

illustrated in Figure 5.3. Four distinct bands were present in the sulphide-free control (lane 2), at 41, 37, 29 and 26kDa. The two larger protein bands (41 and 37kDa) were absent from the control that contained 500mg.L^{-1} sulphide (lane 3). The quantity of protein in the remaining two bands was only slightly less than in the respective bands in the sulphide-free control (Figure 5.3a).

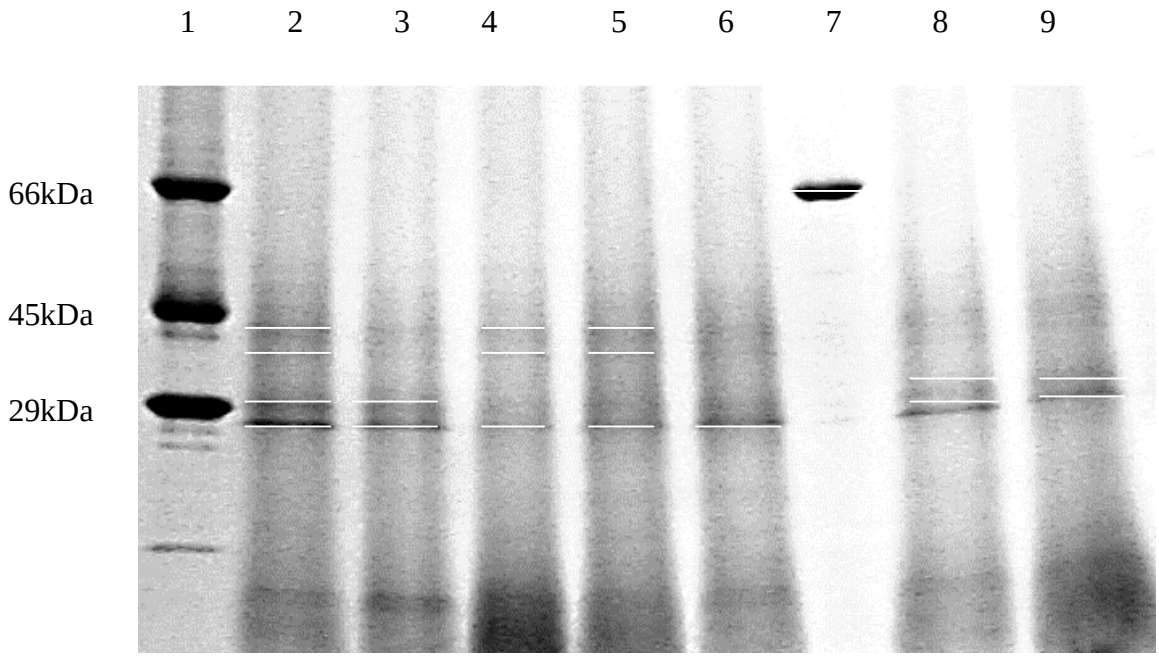


Figure 5.2. The effect of sulphide and sulphate reduction on the hydrolysis of proteins in primary sludge. The molecular weights of the marker proteins (lane 1) are indicated. Lanes: 2 = sulphide-free control, 3 = control + 500mg.L^{-1} sulphide, 4 = digested – sulphide, 5 = digested + 100mg.L^{-1} sulphide, 6 = 500mg.L^{-1}sulphide, 7 = BSA, 8 = digested + sulphate + sulphate reduction, 9 = digested + sulphate – sulphate reduction. Lines indicate protein bands detected by Kodak 1D with the sensitivity level set at –2.

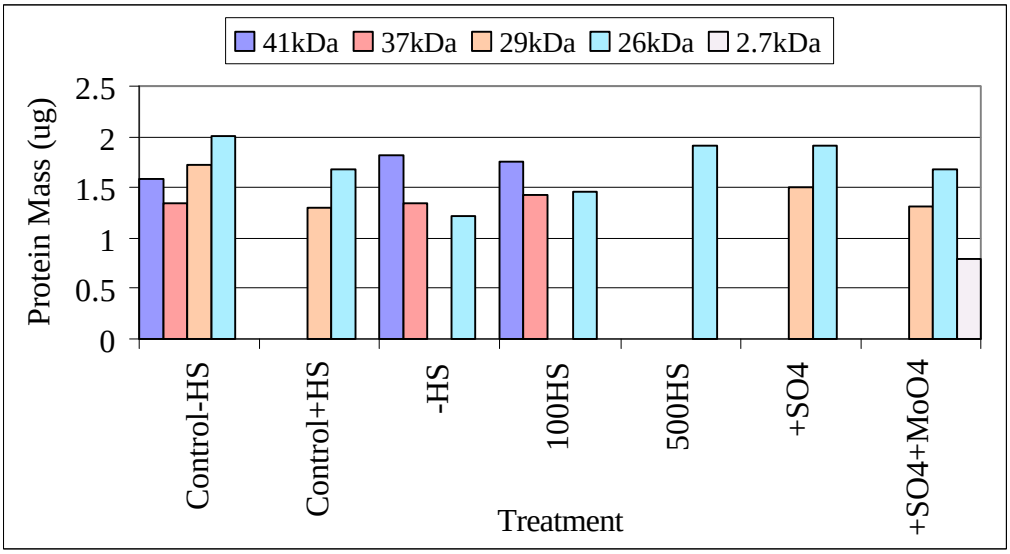
When the PSS was digested in the presence of 500mg.L^{-1} sulphide (lane 6), only one protein band was seen at 26kDa. The quantity of protein in that band was slightly greater than in the undigested sulphide-rich sample (lane 3), but lower than in the sulphide-free control (lane 2) (Figure 5.3a). The quantity of 41kDa protein in the – sulphide treatment increased slightly, while the quantity of 26kDa protein decreased, relative to the sulphide-

free control. The addition of 100mg.L^{-1} sulphide before digestion appeared to make no difference to the hydrolysis of sludge protein. The position and intensity of the banding patterns in lanes 4 and 5 are almost identical after 7 days incubation. After 7 days, the concentration of sulphide in the sulphate-rich sample (lane 8) had increased from 1.5 to 68.4mg.L^{-1}, and indicated that active sulphate reduction had occurred.

The total quantity of protein in each of the distinct bands was calculated, and the results shown in Figure 5.3b. The quantity of protein in the sulphide-free control was higher than in any of the other treatments. The total protein in the + sulphide control was approximately 50% of that in the – sulphide control (Figure 5.3b). This could be explained either by sulphide preventing the migration of protein onto the gel or that the presence of sulphide resulted in cleavage of proteins to small polypeptides that moved off the gel. The appearance of the small 2.7kDa band when sulphate reduction was inhibited by the addition of molybdate, suggests that the latter was more likely. The lowest quantity of protein was found in those samples that contained 500mg.L^{-1} sulphide, with the quantity of protein in the digested samples being lower than in the control. The addition of 100mg.L^{-1} sulphide to the digested samples resulted in a decrease of nearly 30% in the total quantity of protein. The quantity of protein was also not affected by the presence or absence of active sulphate reduction, possibly because the concentration of sulphide in these treatments remained low.

The results of SDS-PAGE showing the degradation of sludge proteins, in the presence and absence of sulphide, over a period of 5 days are shown in Figure 5.4. The quantity of protein in each of the bands is shown in Figures 5.5 and 5.6. The addition of sulphide appeared to have a negligible effect on the banding pattern at pH 10, but some differences were detected between the two treatments at pH 7. In all four treatments, bands corresponding to larger proteins of approximately 60 to 70kDa appeared after day 3. The largest protein band, of 71.4kDa, appeared in the – sulphide (pH 7) treatment on day 5 (Figure 5.4a, 5.5a). A band of 58kDa was present in that treatment throughout the experiment, but only appeared in the remaining treatments on days 3 (+ sulphide, pH 7) and 5 (pH 10).

a)



b)

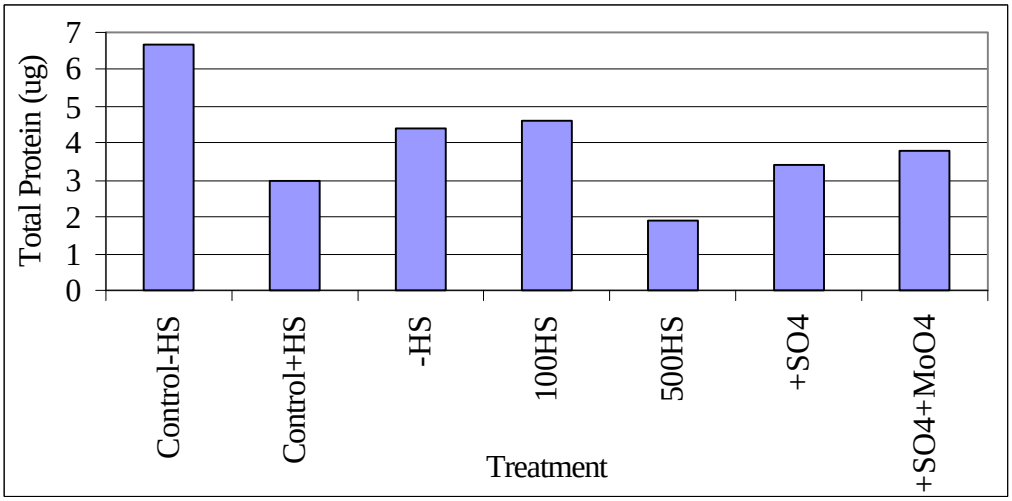


Figure 5.3. The effect of sulphide and sulphate reduction on the hydrolysis of sludge proteins. a) the quantity of protein in each of the bands after SDS-PAGE; b) the sum of protein in all bands. –HS = no added sulphide; +HS = elevated sulphide; +MoO4 indicates addition of molybdate.

There was an increase in the number of bands in the 26 to 47kDa region towards the end of the incubation, in all treatments (Figure 5.4, 5.5 and 5.6). This increase in the number of bands occurred on day 3 in the + sulphide (pH 7) treatment, but only on day 4 or 5 in the remaining treatments. Sulphide appeared to have a limited impact on digestion of the sludge protein at pH 10 (Figure 5.6). The quantity of protein in the 32kDa band was 1.5µg on day 0 in the – sulphide treatment, and remained relatively constant until day 5.

In the + sulphide treatment, the initial quantity of protein in this band was only 0.66μg on day 0. It increased to a maximum of 1.8μg on days 1 and 3, and then decreased to 1μg by day 5. Similar fluctuations were seen in the 44kDa bands in both treatments. However, while the quantity of protein in this band reached a maximum of 1.19μg on day 1 in the – sulphide treatment, the maximum quantity in this band in the + sulphide treatment was 2.29μg on day 3. The quantity of protein in this band decreased again by day 5, in both treatments.

Although the banding patterns in the two treatments at pH 7 were similar, the quantity of protein in the various bands varied between treatments (Figure 5.5). In the + sulphide treatment, the quantity of protein in the 26kDa bands increased steadily from 1.23μg on day 0 to 2.48μg on day 5. In the – sulphide treatment, the quantity of protein in the same band decreased from 2.05μg on day 0 to 1.42μg on day 1, and was absent for the remainder of the experiment. The quantity of protein in the 45kDa band in the + sulphide treatment decreased slightly between days 0 and 5. This band was absent from the – sulphide treatment on day 0, but increased to 2.35μg on day 1, after which time it remained constant. If the bands seen after SDS-PAGE were hydrolysis products of larger sludge proteins, it was expected that the total quantity of protein in these bands would have increased over the 5-day digestion period. The quantity of protein in each of the bands for a particular sample (see Figure 5.5 and 5.6) were summed (Figure 5.7). The total protein increased from days 0 to 5 in all samples, although the increase was greater in the + sulphide samples at both pH values.

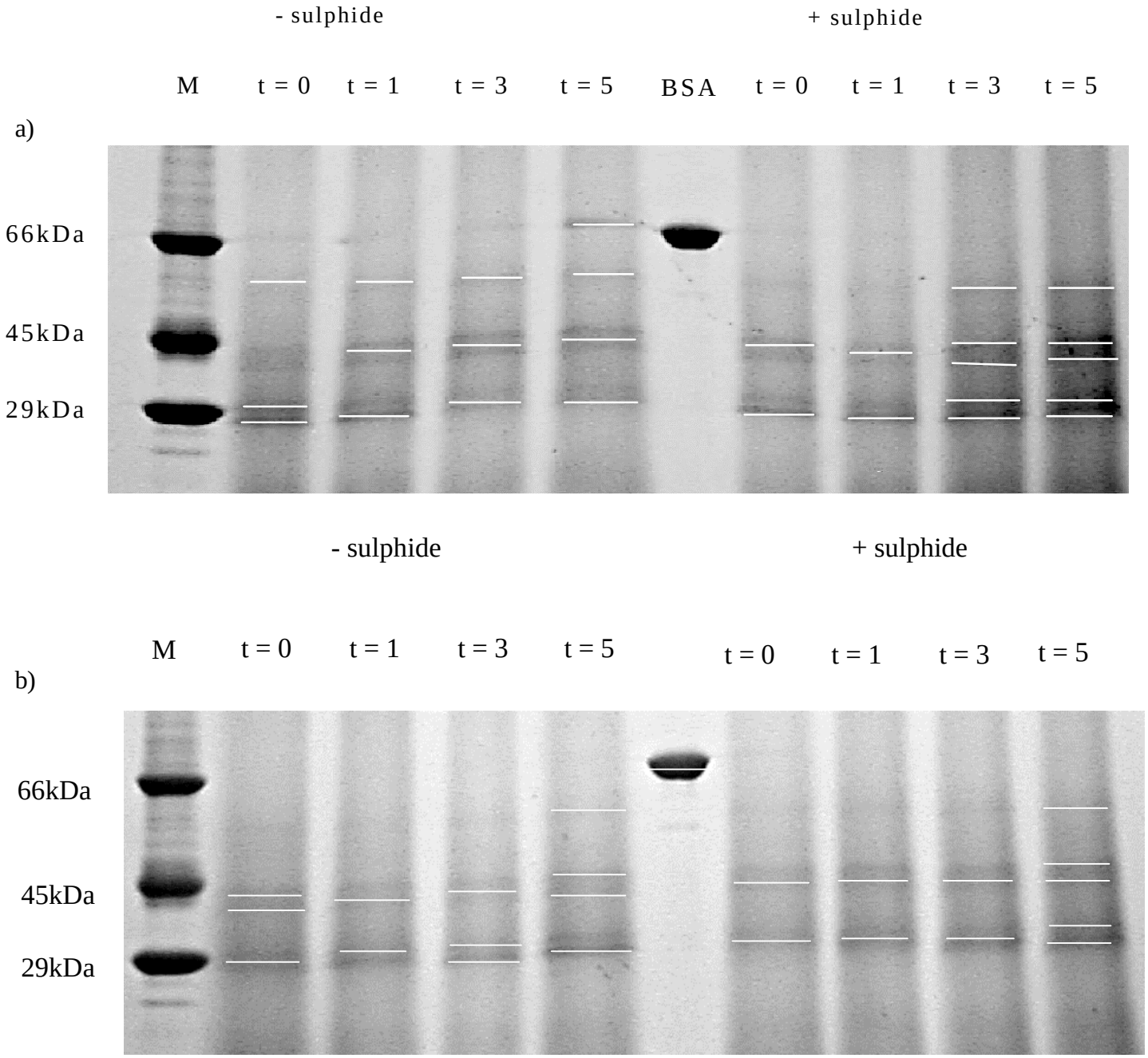
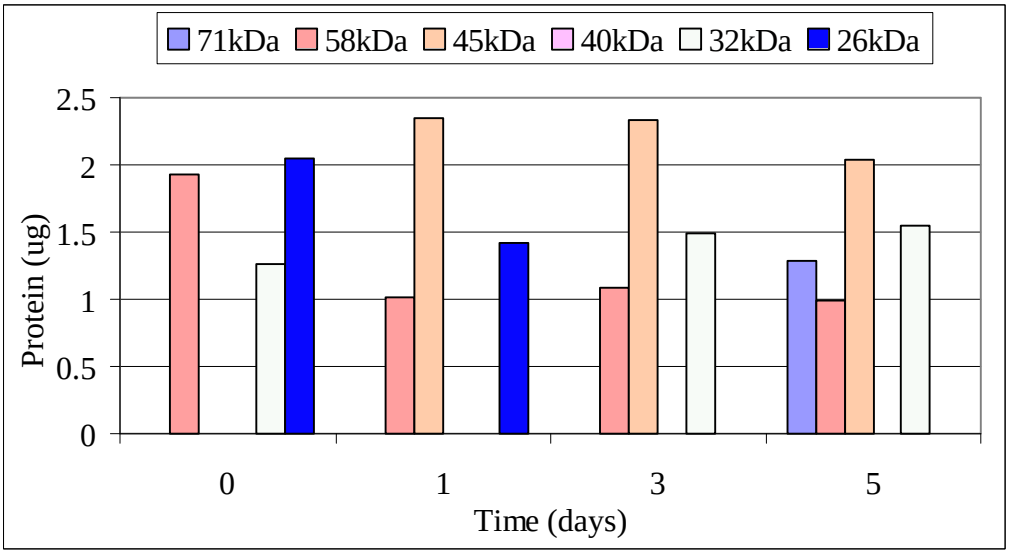


Figure 5.4. Hydrolysis of sludge proteins over a 5-day period in the presence and absence of sulphide, at (a) pH 7 and (b) pH 10. Bands indicated were detected by Kodak 1D image analysis with sensitivity set at –2. M = Molecular Weight

a)



b)

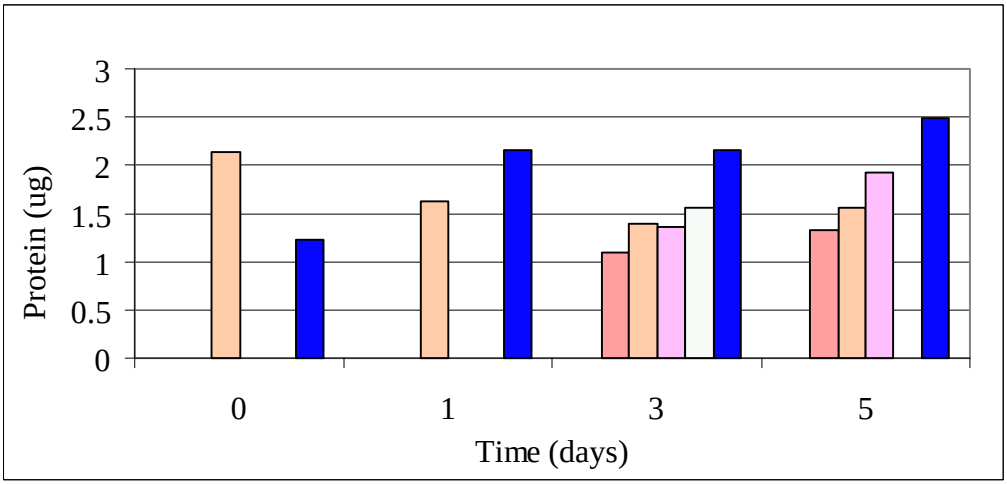
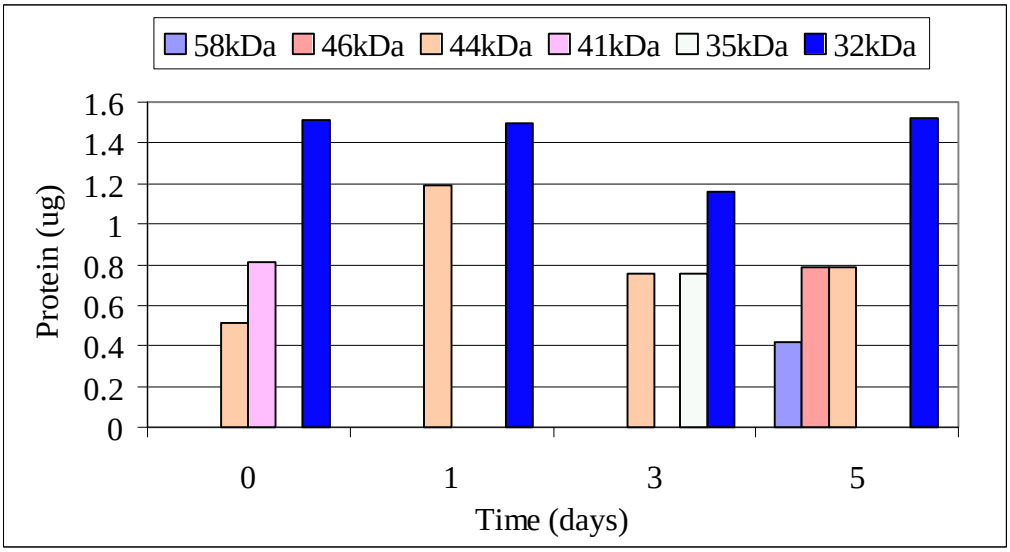


Figure 5.5. Quantity of protein in the bands seen on SDS-PAGE after running primary sewage sludge that had been digested at pH 7. (a) – sulphide; (b) + sulphide.

a)



b)

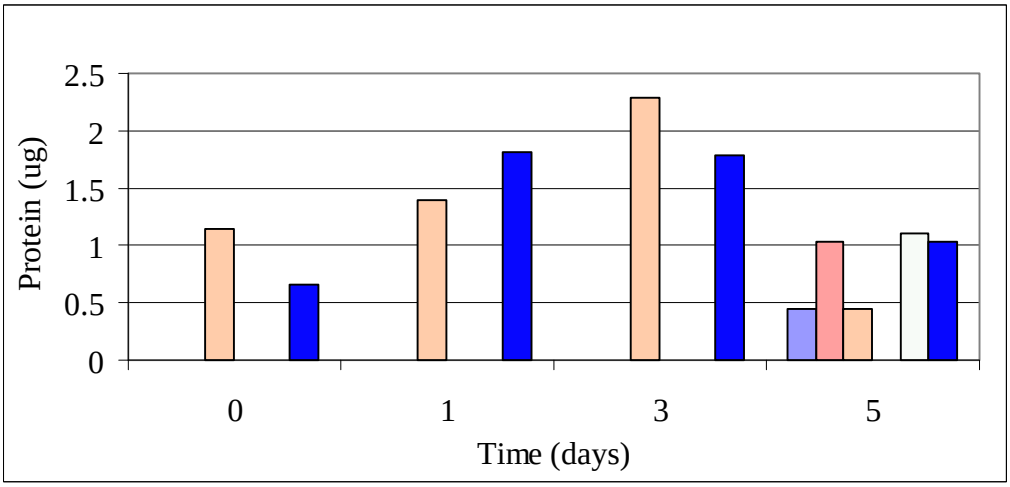
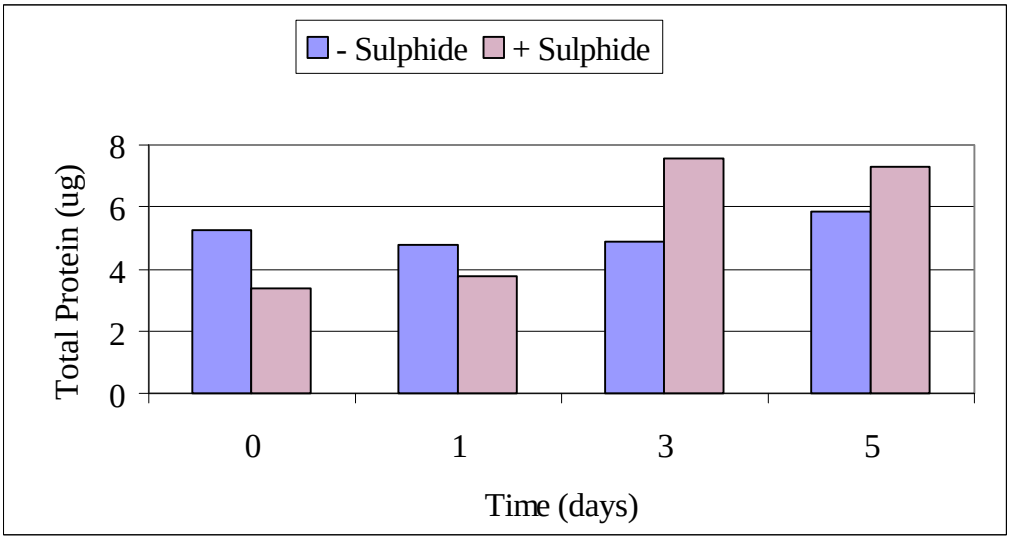


Figure 5.6. Quantity of protein in the bands seen on SDS-PAGE after running primary sewage sludge that had been digested at pH 10. (a) – sulphide; (b) + sulphide.

a)



b)

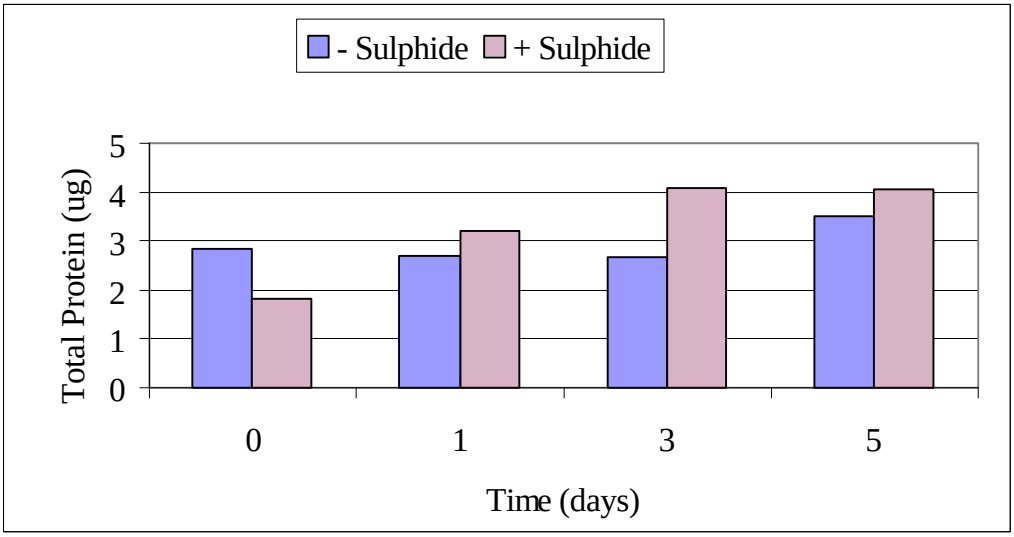


Figure 5.7. The effect of sulphide and pH on the total quantity of protein in all the bands on days 0 to 5. a) pH 7 ; b) pH 10.

5.4. Discussion

Hydrolysis of the protein component of PSS could be monitored using SDS-PAGE, but pretreatment steps were required. The two main problems associated with untreated PSS samples were the adsorption of protein to sludge flocs, and the low quantity of protein that migrated into the gel matrix. When 60μg of the high molecular weight standard was loaded, bands were clearly visible. However, even if 200μg of sludge protein was loaded, the bands were barely visible. Both of these problems were overcome by first releasing a significant quantity of protein from the sludge flocs in the presence of 1% SDS, and then concentrating the protein by applying the acetone extraction procedure. As both the extraction and concentration steps resulted in visible banding, the acetone method was used in later experiments because of the relatively short time required for the procedure.

Studies into the composition and physico-chemical interactions within activated sludge flocs have shown that proteins may be a significant component in the formation of activated sludge flocs. Furthermore, the integrity of the flocs is maintained by electrostatic forces (Bruus *et al*., 1992; Urbain *et al*., 1993). Nothing is known about the role of electrostatic interactions in the formation of flocs in anaerobic treatment systems. The release of protein upon addition of SDS to PSS implied that at least a portion of the protein is adsorbed to the flocs in this manner.

The banding patterns of proteins in the PSS were consistent, although samples were not all collected at the same time. This would appear to imply that the products of protein hydrolysis are limited and are of similar molecular weights. Alternatively, the majority of hydrolysis products were very small and migrated off the gel, while a limited number of products formed visible bands. The lack of larger hydrolysis products might also be explained by a variation in the rate of protein hydrolysis depending on protein size. McInerney (1988) listed the tertiary structure of proteins as one of the factors that could influence their rate of hydrolysis. It is reasonable that large polypeptide chains, with complex three-dimensional structures, are relatively resistant to enzymatic hydrolysis. Their large size and complex shape would prevent migration into the gel. However, once

hydrolysis of the protein had proceeded beyond a certain stage, although the proteins would still be too large to be seen on the gel, further hydrolysis would be rapid, with the formation of small peptide chains. These proteins would then migrate into the gel, and would be visible as bands corresponding to proteins in the small size-range. Disappearance of these bands, representing proteins in the 20 – 50kDa range, upon digestion with sulphide, indicates that it is these smaller proteins that are cleaved by sulphide. Further evidence for the rapid hydrolysis of smaller proteins in the presence of sulphide is the appearance of a 2.7kDa band when sulphate reduction was inhibited.

The fact that some of the bands were present even in the undigested controls suggested that at least some of the digestion of sludge proteins occurred prior to collection i.e. within the sewer. Alternatively, the proteins in the bands were not only hydrolysis products, but also included bacterial hydrolytic enzymes that have been shown to accumulate within the matrix of sludge flocs (Frølund *et al*., 1995; Goel *et al*., 1998). The consistent bands present even in the controls, may have been produced by these enzymes that were released from the sludge flocs upon treatment with SDS. An unknown proportion of the protein contained in PSS was almost certainly too large to penetrate the gel matrix. After each run, wells still contained organic material, although the quantity was reduced if the sludge had been macerated before extraction of the protein. The appearance of bands corresponding to proteins of slightly higher molecular weights, on days 3 to 5 of the time series study, proved that at least some of the proteins on the gels must have been hydrolysis products. This is supported further by the increase in the total quantity of small proteins within all treatments over the 5-day digestion period.

The hydrolysis of the proteins was enhanced in the presence of sulphide, with a reduction in the total quantity of protein at higher sulphide concentrations. The number of visible bands was reduced when sludge was digested in the presence of 500mg.L^{-1} sulphide. The total concentration of protein was reduced by approximately 30% upon addition of 100mg.L^{-1} sulphide, and the banding pattern was similar to that of the sulphide-free control. Although the concentration of sulphide measured in the pilot-scale FSBR was

generally below 150mg.L^{-1}, this was likely to have been an underestimate. Based on the quantity of sulphate reduced, and the pH within the reactor, it is likely that loss of sulphide to the headspace had occurred, and that the actual concentration of sulphide within the FSBR was far higher than the calculated concentration. Only two concentrations of sulphide were used in the study, and the threshold for the enhanced hydrolysis of sludge proteins by sulphide may be lower than 500mg.L^{-1}. Sulphide concentrations up to 300mg.L^{-1} have no negative effect on the biological hydrolysis step (Rudolfs and Amberg, 1952).

The role of SRB in the enhanced hydrolysis of sludge protein is probably limited to the production of sufficiently high concentrations of sulphide. The concentration of sulphide in the sulphidogenic treatment was less than 100mg.L^{-1}, and should have been insufficient to result in enhanced protein hydrolysis. Considering that the presence of SRB themselves was inconsequential, and that the sulphide concentration was below the estimated threshold, it was surprising that the banding pattern of the sulphidogenic system was more similar to that of the control with 500mg.L^{-1} sulphide than the sulphide-free control. No explanation can be determined for this observation. The alkalinities of the sulphidogenic treatment and sulphide-rich control were identical (1866 mg.L^{-1}) and higher than that of the sulphide-free control (1416mg.L^{-1}). The higher alkalinity was, however, not the reason for the reduced banding. The banding pattern of the non–sulphidogenic sulphate-rich treatment was similar to that of the sulphidogenic treatment and sulphide-rich control, although the alkalinity was significantly higher (3866 mg.L^{-1} $CaCO_3$).

The study of the rates of hydrolysis of proteins over 5 days showed that the hydrolysis products of sludge protein appeared in the treatments after 3 days. Hanaki *et al*. (1987) reported that anaerobic digestion of protein in whey required a retention time of at least 12.9 days, but that digestion of individual proteins could be achieved after 5 days. The percentage degradation of protein was not calculated in the present study, but significant degradation would probably have required more than 3 days digestion. The presence of 100mg.L^{-1} sulphide did affect the degradation of protein over the 5-day period, at both

pHs. The accumulation of small protein fragments was greater in the presence of sulphide, and it is proposed that this was the result of more rapid cleavage of sludge proteins under these conditions. Due to time constraints, the fate of those proteins too large to move onto the gel, and of those small polypeptides that may have passed off the gel, was not studied. This information is vital to understanding the mechanism behind sulphide-enhanced hydrolysis of sludge proteins, and is part of an ongoing investigation.

5.5. Conclusions

- The effect of SDS indicated that proteins were held onto the sludge flocs by electrostatic forces.
- The rate of degradation of intermediate size proteins was high, even in the absence of sulphide, and offered an explanation for the absence of bands on the gels corresponding to proteins of this size.
- A sulphide concentration of 100mg.L^{-1} resulted in an increase in the rate of hydrolysis of sludge proteins, relative to that in the sulphide-free control. The rate was increased further at higher sulphide concentrations.
- The enhanced hydrolysis of sludge proteins in the presence of sulphide is thought to make a significant contribution to the enhanced degradation of particulate organic matter that was observed.

Chapter 6

The Effect of Sulphide, pH and Reactor Design on Enzymatic Activity in Primary Sewage Sludge

6.1. Introduction

A significant proportion of the total organic matter in PSS is in the form of complex macromolecules (Heukelekian and Balmat, 1959; Levine *et al*., 1985). During the process of anaerobic digestion, these macromolecules are hydrolysed by a variety of extracellular bacterial enzymes, to produce smaller constituent molecules that can be internalised by the bacterial community (Eastman and Ferguson, 1981; Novaes, 1986; Pavlostathis and Giraldo-Gomez, 1991; Jain *et al*., 1992; Confer and Logan, 1997a, 1997b, 1998). The study of the relative activity of these extracellular hydrolytic enzymes has proven to be a useful tool for understanding the biology and role of operational changes in biological treatment systems. However, to date these studies have been confined to the activated sludge process (Boczar *et al*., 1992; Nybroe *et al*., 1992; Frølund *et al*., 1995; Goel *et al*., 1998). No information is currently available regarding the use of enzyme profiles for the study of anaerobic treatment systems.

The rate of enzymatic reactions is first order with respect to enzyme concentration, and thus loss of enzymes from a reactor will have a negative impact on the rate of hydrolysis of complex organics. Jain *et al*. (1992) successfully modeled the anaerobic digestion of cow dung and concluded that the rate of hydrolysis was related to the mass transfer of enzymes, i.e. contact between the enzymes and substrates. Their results also indicated that the rate of hydrolysis was dependent on the concentration of the enzymes when substrate was in excess. Vavilin *et al*. (1996) proposed a two-phase model for the digestion of complex wastes, where colonization of particulate substrate was followed by degradation of the surface at a constant depth per unit time. This two-phase model showed a relatively good fit to experimental data obtained during the digestion of complex wastes such as swine waste, PSS and cattle manure. An important implication of this model is that degradation of particulate matter by free enzymes, i.e. those not attached to the surface of bacterial cells, is insignificant.

In Chapter 2, the size of sludge flocs in sulphidogenic lab-scale reactors was shown to be smaller than in the non-sulphidogenic systems. As a result, the sludge bed within the sulphidogenic system was less compacted, and the small flocs more susceptible to washout from the reactor. No information concerning the location of hydrolytic enzymes in anaerobic systems is available, but results from studies on activated sludge plants have shown that the bulk of hydrolytic enzymes are associated with the bacterial cells or sludge flocs (Boczar *et al*., 1992; Frølund *et al*., 1995; Confer and Logan, 1998; Goel *et al*., 1998). Assuming that hydrolytic enzymes in anaerobic treatment systems are also closely associated with sludge flocs, a loss of small flocs would result in a loss of enzymes. Consequently, the degradation of complex organic macromolecules would be reduced in the sulphidogenic system. The potential loss of enzymes from sulphidogenic systems, and the role of reactor design and operation in limiting this loss needs to be addressed.

The negative effect of sulphide, even in relatively high concentrations, on the hydrolysis step of anaerobic digestion is thought to be minimal (Rudolfs and Amberg, 1952; Maillacheruvu *et al*., 1993). The impact of sulphide was, however, influenced by reactor design and complexity of the influent. No studies have investigated the possible effect of sulphide on the activity or production of the hydrolytic enzymes themselves. Enzyme "fingerprints" have been used to compare microbial activity in activated sludge systems. Nybroe *et al*. (1992) compiled "fingerprints" using 4 enzymes. There was no correlation between the enzyme "fingerprints" and operational conditions, but they were closely related to the influent composition. These fingerprints can provide useful information regarding any positive or negative effects of sulphide on enzyme activity within anaerobic digesters. The close correlation between the enzyme "fingerprints" and substrate type suggests that the fingerprint will show variation among different regions of a reactor, as complex substrates are degraded. By looking for changes in the fingerprint in various stages or regions of a complex reactor, such as the FSBR, it is possible to determine whether different microbial populations reside in the different regions. This would provide useful information concerning the spatial microbial succession within the reactor.

The aims of this study were to investigate the effect of sulphide addition on the washout and activity of a range of hydrolytic enzymes in laboratory and the pilot-scale anaerobic digesters. An attempt was made to explain the enhanced hydrolysis of PSS within the FSBR in terms of reactor configuration and enzyme activity. Results obtained so far indicated that enhanced hydrolysis of cellulose upon the removal of protective lignin is a key element of the enhanced hydrolysis of PSS under sulphate reducing conditions. β-glucosidase is responsible for the hydrolysis of cellulose and, as such , was expected to show higher activity in the presence of active sulphate reduction.

The study of enzyme activity was undertaken using the API ZYM enzyme test kits (bioMérieux). The kit provides for rapid, semi-quantitative colorimetric tests for a wide range of hydrolytic enzymes, including lipases, esterases, proteases, aminopeptidases, phosphatases and glucosyl hydrolases. It has been used successfully to study the enzymology of activated sludge systems (Boczar *et al*., 1992), as well as to identify anaerobic bacteria (Tharagonnet *et al*., 1977). The results of this analytical system are however, semi-quantitative, and therefore caution must be exercised when comparing the relative activity of a particular enzyme either in different reactor systems or at different points within a single reactor.

6.2. Methods

6.2.1. Analytical methods and the API ZYM system

API ZYM test kits were obtained from bioMérieux (# 2 520 0), and used according to the manufacturer's instructions. All of the enzymes included in the kit are listed in Table 6.1. Milli-Q® water was used for dilutions and maintenance of humidity. 65µL of sample was placed into each cupule, and the strips incubated at 35°C for 4 hours. After incubation and addition of the two reagents (ZYM A and ZYM B), strips were held under a bright light for 5 minutes to remove any residual yellow colouration. This yellow colour is due to the presence of excess Fast Blue that did not react.

Table 6.1. Enzymes tested for by the API ZYM test kit. The number of each enzyme is used in place of enzyme names in all subsequent figures.

Number	Enzyme	Number	Enzyme
1	Control	11	Acid Phosphatase
2	Alkaline Phosphatase	12	Naphthol-AS-BI-phosphohydrolase
3	Esterase (C 4)	13	α-galactosidase
4	Esterase lipase (C 8)	14	β-galactosidase
5	Lipase (C 14)	15	β-glucuronidase
6	Leucine arylamidase	16	α-glucosidase
7	Valine arylamidase	17	β-glucosidase
8	Cysteine arylamidase	18	N-acetyl-β-glucosaminidase
9	Trypsin	19	α-mannosidase
10	Chymotrypsin	20	α-fucosidase

The estimation of enzyme activity was based on comparison of colour on the API test strips with colours on a reference chart. The assays are based on the enzymes within the samples reacting with known substrates within the cupules. The intensity of the colour produced is proportional to the quantity of substrate hydrolysed and that, in turn, is proportional to the quantity of enzyme in the sample. All activities are indicated as nmol of substrate hydrolysed. Due to the dark colouration and high viscosity of some of the sludge samples, accurate comparison of colour was impossible. These samples were diluted appropriately with sterile distilled water prior to analysis of enzyme activity. The dilution factor was taken into account when calculating the quantity of substrate converted.

The methods used to determine pH and sulphide concentrations are given in previous chapters.

6.2.2. Lab-scale reactors

The design and operation of the single- and multiple-stage lab-scale reactors is described in detail in Chapter 2. 50mL samples were collected from the feed and from various points in the four reactor systems, after the reactors had been running for 38 days at steady-state. Three samples were collected from the single-stage systems: feed, bed and

effluent. Samples were collected from the feed of the multiple-stage system, as well as from the bed and overflow from each of the three stages. The activity of the hydrolytic enzymes in each sample was determined using the API ZYM kits. As the samples were not lysed prior to the assays, the activity of intracellular enzymes would have been detected only to the extent to which spontaneous cell lysis had taken place. It was already known that the presence of sulphide interferes with a number of colorimetric assays. For this reason, any possible effects of sulphide on the API ZYM system had to be tested, and if necessary, taken into account when calculating the enzyme activity of samples that contained sulphide. The cupules on a single API ZYM test strip were inoculated with a sterile 100mg.L^{-1} sulphide solution (65µL sulphide solution per cupule). The strip was then incubated and analysed in the same way as experimental tests.

6.2.3. Pilot-scale reactor

Samples were collected during September 1998, after the pilot-plant had been running at steady state for 3.5 months. Samples (1L) were collected from the feed (blend tank) as well as from the top, middle and bottom of each of the three valleys in the hydrolysis unit. Samples were stored in airtight plastic bottles, and analysis of the enzyme activity was initiated within 30 minutes of collection. After inoculation of the API ZYM strips, they were incubated at room temperature (25^{o}C) for 4 hours.

6.2.4. The effect of pH and sulphide on enzyme activity in PSS

The design of the experiment used to investigate the effect of elevated sulphide concentrations and pH on the enzyme activity of PSS is shown in Table 6.2. The experiment was conducted in 1000ml flasks, and the contents stirred using magnetic beads. The pH of the flasks was adjusted, after sulphide addition, with 32% HCl or 50g.L^{-1} NaOH. Sulphide was added to experimental flasks in the form of a concentrated solution (1g.L^{-1}) of $Na_2S.9H_2O$. An equal volume of distilled water was added to the control flasks. The PSS was collected from GDW approximately 1 hour before use, and passed through a sieve (2mm mesh size) to remove any large particulates. The COD

concentration was adjusted to 2000mg.L^{-1} with distilled water. The bacterial seed was obtained from the methanogenic anaerobic digester at GDW.

Table 6.2. Summary of the experimental design used to determine the effect of elevated sulphide concentrations on the enzyme activity of PSS.

Experiment	Sulphide (mg.L^{-1})	COD (mg.L^{-1})	Seed (mL)
-sulphide (pH7)	0	2000	100
+sulphide (pH7)	100	2000	100
-sulphide (pH10)	0	2000	100
+sulphide (pH10)	100	2000	100

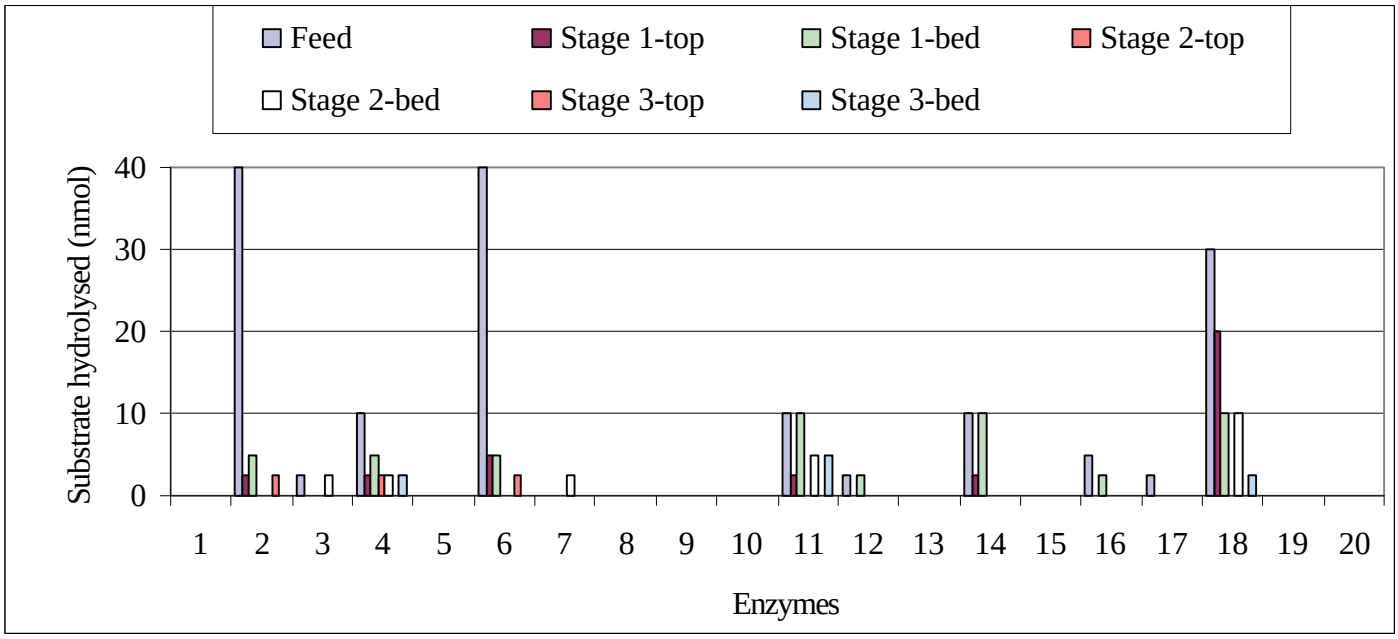
In an attempt to minimise the quantity of sulphide added to the control flasks, nitrogen gas was bubbled through the seed for 15 minutes prior to inoculation. At the start of the experiment, and after each sampling event, the headspace of each flask was flushed with nitrogen before resealing. All flasks were incubated at 25^{o}C (± 3) in the dark. Samples were collected after 36 hours, and the enzyme activity analysed using API ZYM kits.

6.3. Results

6.3.1. Lab-scale reactors

Approximately half of the enzymes tested showed activity in the reactors, with a larger number of enzymes being active in the + sulphate systems than the - sulphate equivalents (Figures 6.1 and 6.2). Both β-glucosidase and valine arylamidase showed low activity in the + sulphate multiple-stage system but no activity in the – sulphate system (Figure 6.1). Activity of β-glucosidase was, however, only detected in the feed of the + sulphate multiple-stage system, but not in the feed of either of the single-stage systems. Likewise, low activity of α-glucosidase was detected in the bed of the single-stage + sulphate system, but not in the –sulphate system (Figure 6.2). Esterase C4 (3), esterase lipase C8 (4) and the two phosphatase enzymes (2, 11) showed activity in all four reactor systems.

a)



b)

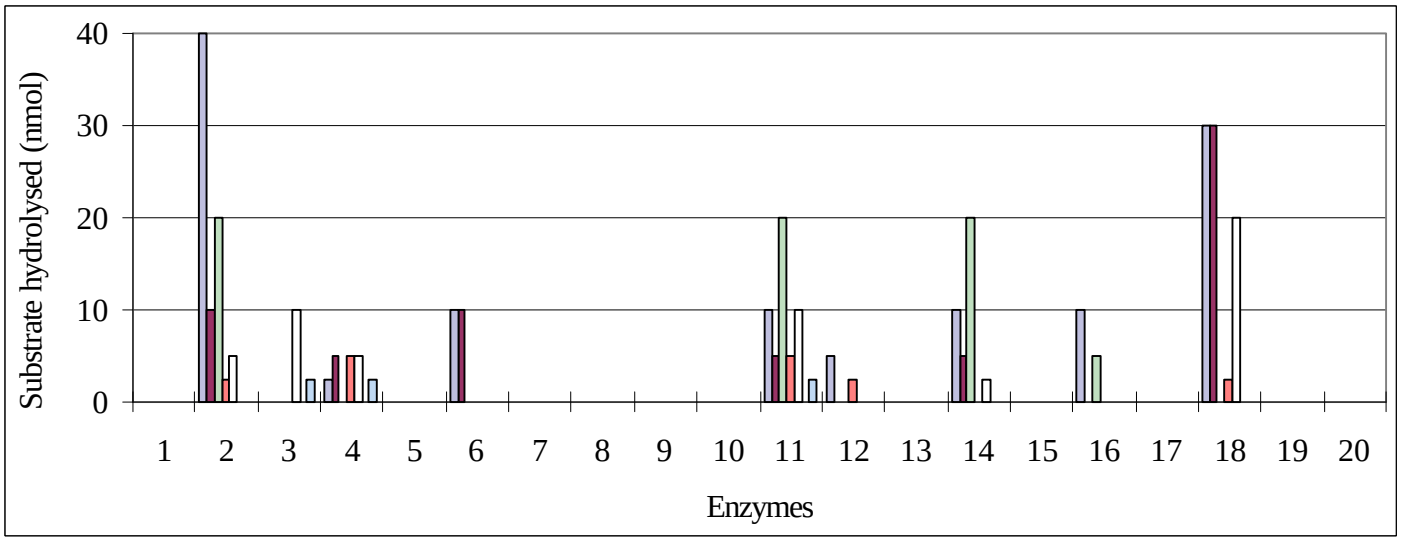
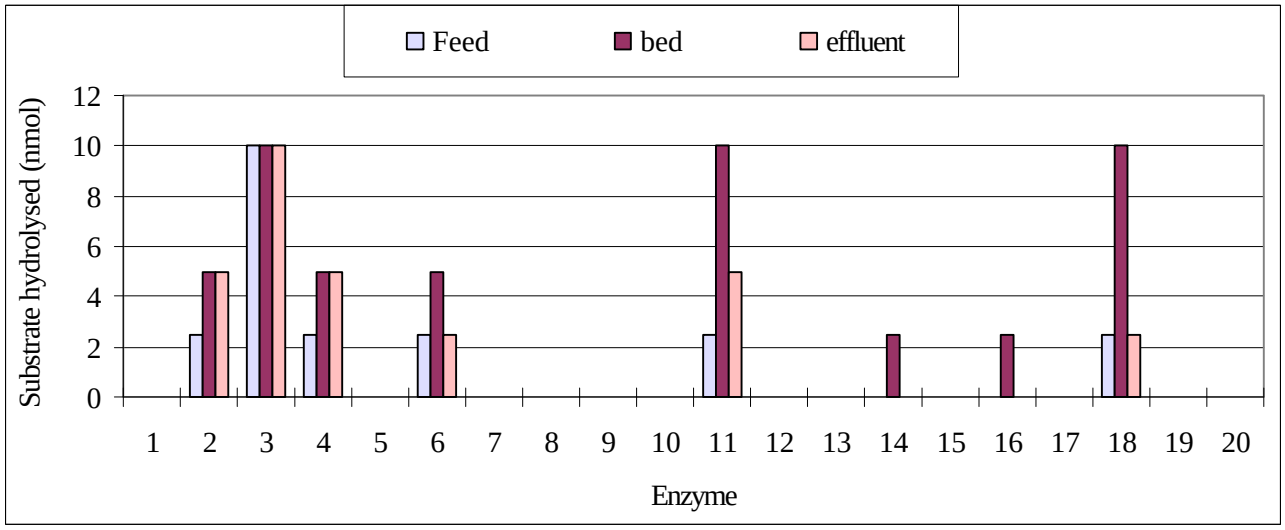


Figure 6.1. Results of the API-ZYM analysis showing the activity of 19 enzymes in the multiple-stage lab-scale reactors. a) + sulphate system; b) – sulphate system. The key to the numbers is given in Table 6.1.

a)



b)

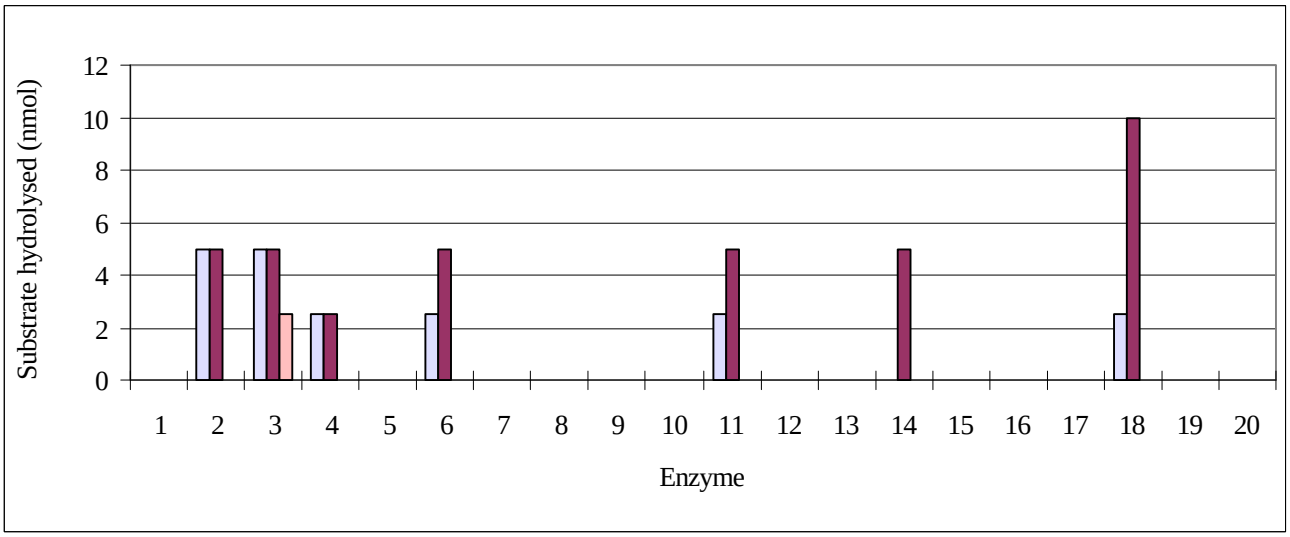


Figure 6.2. Results of the API-ZYM analysis showing the activity of 19 enzymes in the single-stage lab-scale reactors. a) + sulphate system; b) – sulphate system. The key to the numbers is given in Table 6.1.

Enzyme activity was detected in the effluent of the + sulphate single-stage system for 6 of the enzymes. In contrast, only slight esterase C4 (3) activity was detected in the effluent of the – sulphate single-stage reactor (Figure 6.2). The enzyme activity in the effluents of the multiple-stage systems was low, although a range of enzymes exhibited activity in the effluents of both the + sulphate and the – sulphate systems (Figure 6.1).

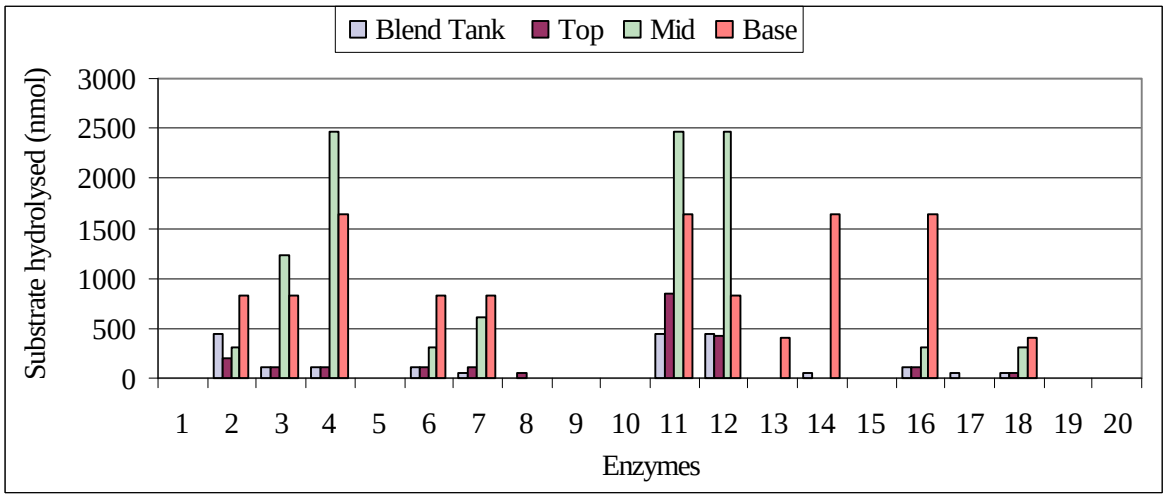
The single-stage systems acted as enzyme accumulators, with higher activity of most enzymes in the bed than in the influent (Figure 6.2). The activity of esterase C4 (3), in both systems, and alkaline phosphatase (2) in the – sulphate system was equivalent in the influent and bed. The activity of enzymes in the + sulphate multiple-stage reactor declined through the system, with lower activity in the bed of stage 1, than in the feed (Figure 6.1a). This was particularly true for phosphatase alkaline (2), leucine arylamidase (6) and N-acetyl-β-glucosaminidase (18) that exhibited very high activity in the feed. The activity of phosphatase acid (11) and β-galactosidase (14) was equivalent in the feed and bed (stage 1) of the + sulphate system, and higher in the bed than the feed of the – sulphate system (Figure 6.1).

6.3.2. Pilot-scale reactor

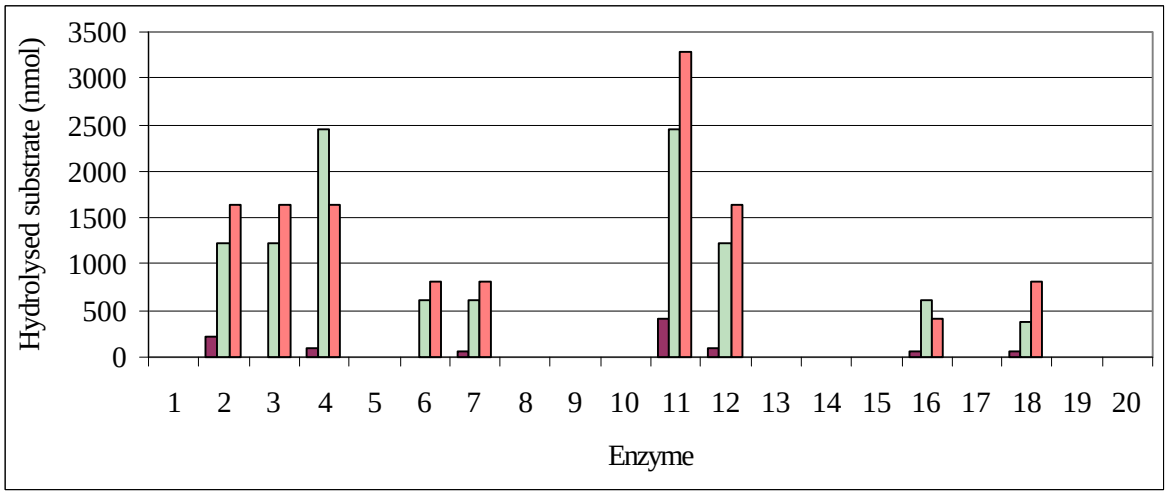
The enzyme activity within the pilot-scale hydrolysis unit was high when compared to the activity within the lab-scale reactors (Figure 6.3). However, any comparisons between the lab- and pilot-scale systems must be made with caution, as the specific activity of the enzymes was not calculated. A number of the enzymes showed no activity, and included both proteases (9, 10), some of the glucosyl hydrolases (15, 19, 20) and lipase C14 (5). Cysteine arylamidase (8) activity was not detected in any of the samples except for the top of valley 1, where its activity was very low (Figure 6.3a). The activity of β-galactosidase (14) and α-galactosidase (13) was only detected in the blend tank and in some of the samples taken from valley 1 (Figure 6.3).

The activity of enzymes in the samples taken from the BT and the top of the valleys was always low, and increased significantly towards the base of the reactor. The activity of most of the enzymes tested was higher in the base than in the middle region, although the activity of some of the enzymes did not conform to this pattern. In valley 1, esterase lipase C4 (3), esterase lipase C8 (4), phosphatase acid (11) and naphthol-AS-BI-phosphohydrolase (12) all exhibited higher activity in the middle region than in the base (Figure 6.3a). This pattern of activity was also seen for esterase lipase C8 (4) and α-glucosidase (16) in valley 2 (Figure 6.3b).

a)



b)



c)

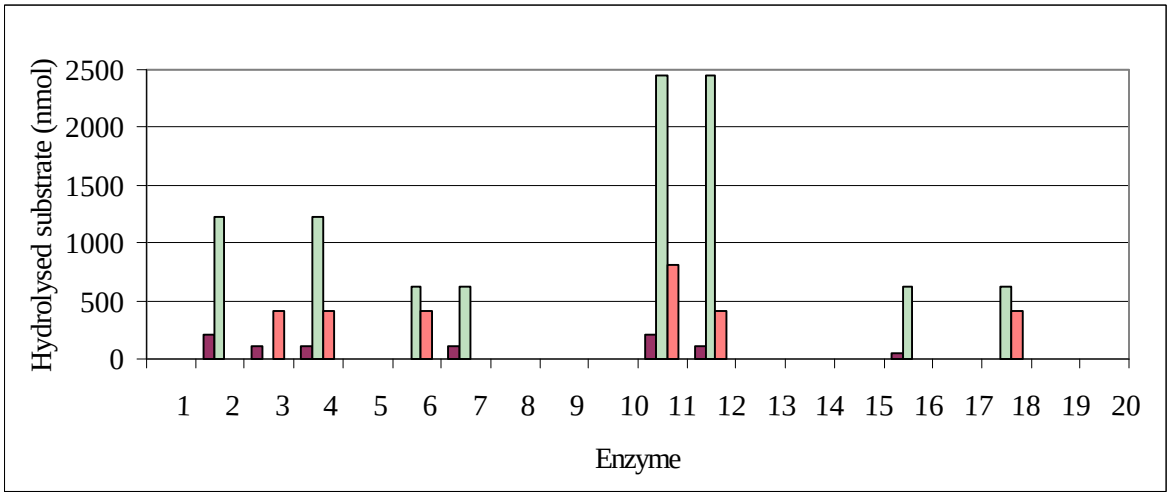


Figure 6.3. Enzyme activity in the pilot-scale hydrolysis unit in a) BT and valley 1, b) valley 2 and c) valley 3. The list of enzymes is given in Table 6.1.

In valley 3, the activity of each of the enzymes was lower in the base of the reactor than in the middle (Figure 6.3c). Some enzymes, such as phosphatase alkaline (2), valine arylamidase (7) and α-glucosidase (16) showed activity in the top and middle regions of the reactor, but no activity in the base. In order to obtain a more accurate assessment of the regions of high and low within the pilot-scale reactor, the total enzyme activity (as nmol substrate converted) was calculated for each of the samples (Table 6.3).

Table 6.3. Total enzyme activity (as nmol substrate converted) of samples from 9 regions in the pilot-scale reactor. The total activity of the feed was 1980nmol.

Depth (m)	Valley 1	Valley 2	Valley 3
0.2	2100	997	892
0.75	10459	10822	9840
1.7	11480	12710	2870

The total activity in the top of all valleys, and in the base of valley 3 were very low in comparison to the other 5 areas.

6.3.3. Effect of pH and sulphide on enzyme activity in PSS

Both protease enzymes, trypsin (9) and chymotrypsin (10), and a number of the glucosyl-hydrolases (13, 15, 17, 19, 20) showed no activity in the PSS at either of the pH values, even in the absence of sulphide (Figure 6.4). Lipase C14 (5), β-galactosidase (14), α-glucosidase (16) and N-acetyl-β-glucosaminidase (18) showed very low activity at pH 7, and no activity at pH 10. There was no evidence of enhanced enzyme activity in the presence of sulphide, but a number of enzymes showed inhibition of varying intensity. The data was not converted to specific activity, and any quantitative comparisons between the flasks must be treated with caution. Lipase C14 (5) only showed low activity at pH 7 in the absence of sulphide, and no activity in the presence of sulphide. Sulphide resulted in significant inhibition of leucine arylamidase (6) at pH 7, but not at the higher pH. Both phosphatase enzymes (2, 11) showed higher activity at pH 7 than

pH 10, and also showed greater inhibition by sulphide at the lower pH. Sulphide had a strong inhibitory effect on the activity of the esterases (3, 4) at both pH values.

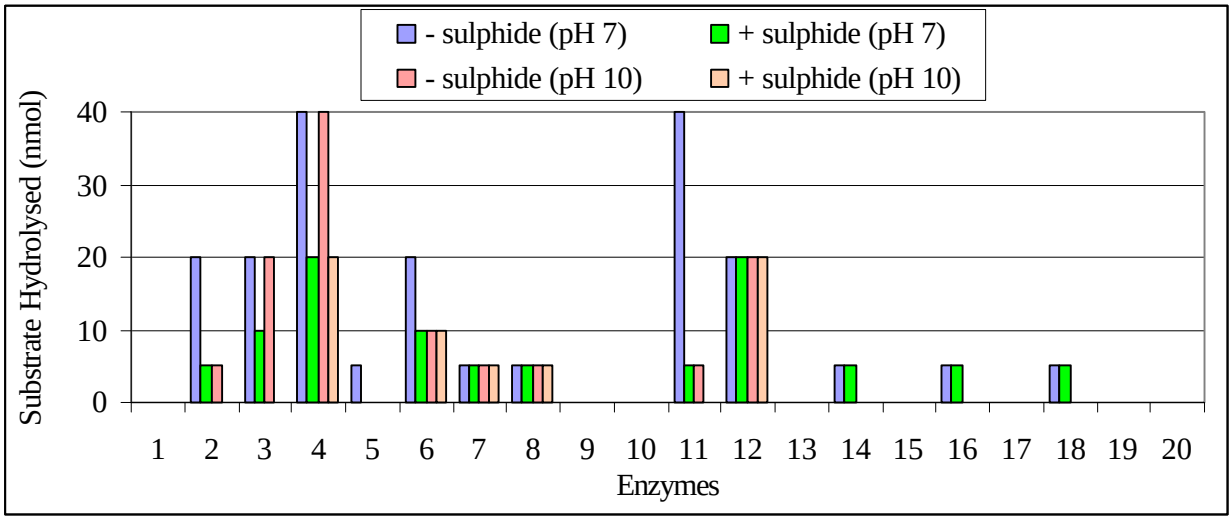


Figure 6.4. Enzyme activity in PSS at pH 7 and pH 10, in the presence and absence of sulphide. The list of enzymes is given in Table 6.1.

6.4. Discussion

The results of this study into the effect of pH and sulphide on the activity of hydrolytic enzymes make an important contribution to understanding the process of enhanced hydrolysis of PSS under sulphidogenic conditions. A reduction or total absence in the activity of certain enzymes at pH 10, when compared to the activity at pH 7, supported previously published findings. The rate of solubilization of complex organic wastes, particularly carbohydrates, under anaerobic conditions is highest at pH 6 –7 (Eastman and Ferguson, 1981; Perot *et al*., 1988; Elefsiniotis and Oldham, 1994; Penaud *et al*., 1997). The pH at which optimum solubilization of complex organic material is attained, is thought to reflect the optimum operating pH of the most important hydrolytic enzymes. Penaud *et al*. (1997) found that hydrolysis of protein in pharmaceutical waste increased with pH, at least until pH 9.

It is widely accepted that while anaerobic digestion is adversely affected by the presence of sulphide, this is limited to the gasification step. The initial hydrolytic step is not considered to be affected by sulphide in concentrations up to 300mg.L^{-1} (Rudolfs and

Amberg, 1952; Aulenbach and Heukelekian, 1955; Lawrence *et al*., 1966; Maillacheruvu *et al*., 1993). In light of the present findings, while this might describe the effect of sulphide on the overall performance of the hydrolytic process, it appears to be an over-simplification when considering enzymological effects. Comparisons between the activity of enzymes in the various reactor systems and flasks must be interpreted with caution as the API ZYM assays were only semi-quantitative, and specific activities were not calculated. Nevertheless, the enzyme profiles of the lab-scale reactors indicated that two enzymes, valine arylamidase and β-glucosidase, were only active in the sulphidogenic reactors. There was no evidence of inhibition of hydrolytic enzymes by sulphide in these reactors. The results obtained from the flask studies appeared contradictory. None of the enzymes exhibited activity in the + sulphate systems but not in the – sulphate systems, as was seen in the lab-scale reactors. Furthermore, lipase C14 (5) was totally inhibited in the presence of sulphide at pH 7, and the phosphatases (2, 11) and esterase lipases (3, 4, 5) also appeared to be inhibited. Based on these results alone, it was not possible to determine whether the apparent inhibition was at the enzyme or bacterial level i.e. whether sulphide affected the action of the enzyme itself or the ability of the bacteria to produce the enzyme in the same quantity as under non-inhibitory conditions.

The lack of inhibition of the same enzymes in the continuous systems implies that the actual function of the enzyme is not adversely affect by sulphide. It is proposed that in the short-term flask experiments, hydrolytic bacteria acclimated to methanogenic conditions are inhibited by 100mg.L^{-1} sulphide. However, in the continuous lab-scale systems, either these same hydrolytic bacteria adapted to elevated sulphide concentrations or were replaced by sulphide-tolerant species. The production of extracellular hydrolytic enzymes is closely linked to the available substrates (Nybroe *et al*., 1992) i.e. an increase in the concentration of a particular substrate will stimulate bacteria to produce enzymes specific to that substrate. It has already been shown that hydrolysis of carbohydrates and proteins is enhanced in the presence of sulphide. If this results in the production of additional substrates, compared to non-sulphidogenic systems, it is reasonable to expect that additional enzymes will be produced. In this way,

it is possible to explain the larger number of enzymes exhibiting activity in the sulphidogenic lab-scale systems, although sulphide was apparently also responsible for a decline in enzyme activity in the short-term flask studies. A number of enzymes showed activity in the feed of the multiple-stage reactors, but not in the feed of the single-stage reactors. This was most likely due to the different times at which the samples were collected. All samples for the enzyme activity studies were collected after the reactors had been at steady-state for 38 days, and as the single-stage reactors were started 7 days later than the multiple-stage systems, the samples were also collected 7 days later. Because of this delay, the enzyme activity of the feeds to the single and multiple-stage reactors may have differed slightly in composition and baseline enzyme activity. However, as the feed sludge was collected from exactly the same source and contained very little industrial effluent, the differences were expected to be insignificant. More importantly, the feed of the multiple-stage reactors had been in the feed reservoir for almost 48 hours before being analysed for enzyme activity, while that of the single-stage system had only been in the reservoir for less than 3 hours. This difference in the age of the feed would have allowed for any effect of sulphide on enzyme production, whether direct or indirect, to have been initiated in the feed of the multiple-stage system but not in the feed of the single-stage system. Bearing this in mind, any direct comparisons between the enzyme activity of the multiple- and single-stage systems must be made with caution. The different ages of the feeds could explain the presence of β-glucosidase in the feed of the multiple-stage system but not the single-stage system. In the former, sufficient time may have elapsed for the lignin to be dissolved and the cellulose exposed. This would then have resulted in induction of β-glucosidase production. The feed of the single-stage system was relatively fresh and sufficient removal of lignin may not yet have taken place for the exposure of the underlying cellulose and the subsequent induction of β-glucosidase production. The absence of β-glucosidase activity in the flask studies could also not be explained, unless it was below the level of detection of the assay.

α-glucosidase activity was detected in the beds of the first stages of both multiple-stage reactors and in the bed of the + sulphate single-stage system. As this enzyme is responsible for the hydrolysis of starch, a common biological compound, its absence

from the bed of the – sulphate single-stage reactor was surprising. Results from the pilot-scale FSBR indicated that the activity of this enzyme was related to the concentration of the substrate. The activity of α-glucosidase decreased from valley 1 to valley 3 in the pilot-scale reactor. Because starch is rapidly biodegradable (San Pedro *et al*., 1994) it is thought that the concentration of starch decreased rapidly along the length of the reactor, and was responsible for the reduced α-glucosidase activity.

Since the initial velocity of an enzymatic reaction is first order with respect to enzyme concentration, the performance of the hydrolysis reactor would benefit from the accumulation of hydrolytic enzymes. Conversely, loss of enzymes from the reactor would have a negative impact on the rate of hydrolysis. The enzyme profiles of the single-stage lab-scale reactors showed that the loss of enzymes from the sulphidogenic reactor was higher than that from the – sulphate reactor, and can be linked to enhanced hydrolysis under sulphidogenic conditions. Under conditions that result in rapid hydrolysis of particulate organic matter, the resultant reduction in floc size leads to increased loss of flocs from the reactor. If the bulk of hydrolytic enzymes is closely associated with the sludge flocs, as is the case in activated sludge systems (Boczar *et al*., 1992; Frølund *et al*., 1995; Goel *et al*., 1998), then loss of enzymes would accompany floc loss. Although the hydrolysis of organic matter is high in the + sulphate system, so is the potential for washout of enzymes. The high accumulation of hydrolytic enzymes within the pilot plant can be attributed to good retention of undigested organic matter with which enzymes are most likely to be associated. The enzyme profiles from the two multiple-stages reactor systems were similar. The activity of most of the enzymes in the beds of stage 1 was equal to or lower than that of the influent, and indicated that no accumulation of enzymes had occurred. The retention time in each of the stages was 1/3 of that in the single-stage reactors, and it is thought that this facilitated washout of sludge and enzymes from the reactors. This would also account for the low enzyme activity in stages 2 and 3. Enzyme activity in the effluent of the pilot-scale FSBR was low in comparison to that in the beds of valleys 2 and 3. This indicated that retention or production of enzymes was high in the pilot plant.

6.5. Conclusions

- Sulphide may have a negative effect on the activity of hydrolytic enzymes in the short term, but did not adversely affect activity in continuous experiments.
- Washout of hydrolytic enzymes from sulphidogenic systems was higher than from the non-methanogenic systems. Reactor design and retention times are therefore considered critical if washout of bio-catalyst and hydrolytic enzymes from sulphidogenic hydrolysis reactors is to be minimised.
- The absence of β-glucosidase activity in the flask studies and in the + sulphate single-stage reactor system did not support the current hypothesis. However, the API ZYM kits used in this study are only semi-quantitative and further studies of the activity of hydrolytic enzymes under sulphate reducing conditions should employ more traditional enzymological techniques.

Chapter 7

Conclusion: The role of Sulphide and Reactor Design in the Enhanced Solubilization of Primary Sewage Sludge

The empirical data from this study was used to develop a model describing the process of enhanced solubilization of PSS under sulphate reducing conditions. The rate and extent of hydrolysis of complex substrates, such as PSS, is dependent on the enzyme concentration and contact between enzymes and the substrate (Jain *et al.*, 1992), as well as colonisation of the surface of particulates by hydrolytic bacteria (Vavilin *et al.*, 1996). It is proposed that the products of biological sulphate reduction both directly and indirectly facilitate contact between enzyme and substrate, while simultaneously increasing the surface area of particulate substrates available to colonisation by hydrolytic bacteria.

The effect of sulphate reduction on the hydrolysis of both the protein and carbohydrate fractions of PSS was examined. The soluble proteins in digested and undigested PSS samples were small size, probably as a result of digestion within the sewer. Sulphide was shown to enhance the hydrolysis of proteins in PSS, and was thought to have been the major contributing factor to the process of enhanced solubilization of the PSS. Based on the profiles obtained by SDS-PAGE, it is proposed that initial hydrolysis of sludge proteins was slow, and that sulphide was responsible for the cleavage of smaller polypeptides in the 20 – 70 kDa size-range. The concentration of sulphide required for enhanced hydrolysis of proteins may have been less than the 500mg.L^{-1} measured in this study and it is possible that concentrations were sufficiently high in localised areas of high sulphate reduction, within the FSBR. A concentration of less than 100mg.L^{-1} sulphide was shown to enhance the solubilization of lignocellulose. Experimental results indicated that the Alkaline Pulping process (Clayton, 1969) could provide a useful model for understanding the underlying mechanism. Lignin is solubilised in the presence of alkalinity and is prevented from re-polymerising by HS^- ions. The increase in soluble

phenolic compounds upon digestion of PSS in the presence of biological sulphate reduction or sulphide, indicated that this reaction was probably occurring in this system. Cellulose is closely associated with lignin in plant tissues, and is protected from contact with cellulase enzymes by a lignin shield. After solubilisation of lignin by alkalinity and sulphide ions, the underlying cellulose is exposed and may be hydrolysed enzymatically. As lignocellulose is known to contribute significantly to the total mass of PSS (Heukelekian and Balmat, 1959; Hunter and Heukelekian, 1965; Elefsiniotis and Oldham, 1994), enhanced solubilization of this fraction will improve the overall mineralisation of PSS significantly. Furthermore, this mechanism explains the improved rates and degree of solubilization of lignocellulosic wastes in landfills when operated under sulphate reducing conditions (Kim *et al.*, 1997; Pareek *et al.*, 1998). Various authors have shown that certain species of sulphate reducing bacteria can utilise phenolic compounds as their sole carbon source (Holliger *et al.*, 1988; Widdel, 1988; Schink *et al.*, 1992; Drzyzga *et al.*, 1993; Kuever *et al.*, 1993; Pavlostathis, 1994). Thus, the possibility that SRB may directly degrade lignin cannot be excluded and requires further investigation.

The absence of any enhanced β-glucosidase activity under sulphate reducing conditions was unexpected. Upon exposure of the substrate, cellulose, induction of enzyme production was expected. The observed accumulation of glucose observed reported in Chapter 4 could have been due to hydrolysis of starch, particularly as α-glucosidase was detected in the flask, laboratory and pilot-scale studies. Starch is, however, rapidly degradable (San Pedro *et al.*, 1994) and it is unlikely that high concentrations were present in the flasks at the time when the accumulation of glucose was recorded. Thus, the soluble carbohydrates must have been derived from the hydrolysis of cellulose. The API ZYM kits used in this study are qualitative but only semi-quantitative, and may not have been suitable for the detection of cellulase in anaerobic systems. Further studies into the effect of sulphate reduction and sulphide on the enzymology of PSS digestion are required, and should employ more rigorous enzymological techniques. Based on the results of the semi-quantitative enzyme kits, sulphide did not appear to inhibit or enhance enzyme activity. The reduction in enzyme activity seen in the flask studies was

explained in terms of inhibition of enzyme production by hydrolytic bacteria, rather than inhibition of the enzymes themselves. The inhibition was removed if a period of acclimation was introduced i.e. in the continuous reactor system. The enzymological study did reveal that the design and operation of the pilot-scale FSBR facilitated the accumulation of enzymes within the system, and is thought to have been partially responsible for the enhanced hydrolysis that was observed.

The average floc size within a digester is dependent on the relative rates of flocculation and floc fracture. Constant floc growth is offset by enzymatic (Nielsen *et al.*, 1996) and shear-induced (Spicer and Pratsinis, 1996) deflocculation. The smaller average floc size in the sulphidogenic lab-scale reactors indicated that deflocculation was accelerated under those conditions. The mechanism of enhanced floc fracture in the FSBR is poorly understood, but is likely to involve enhanced solubilisation of carbohydrates and proteins, in the presence of biological sulphate reduction. Little is known about the composition and formation of flocs in non-granulating anaerobic digesters. If it is assumed that the composition and structure of these flocs is similar to those in non-bulking activated sludge systems, then the composition is likely to reflect that of the feed to the digester. As such, a significant proportion of the flocs will be proteins and carbohydrates, held together by non-covalent bonds (Forster, 1982; Eriksson and Alm, 1991; Bruus *et al.*, 1992; Urbain *et al.*, 1993). It has already been shown that the solubilization of lignin and proteins are enhanced under sulphate reducing conditions. The increased rate of floc fracture is most likely due to a loss of integrity of the floc due to enhanced hydrolysis of important structural components such as lignin, cellulose and proteins. Cinq-Mars and Howell (1977) provided further evidence for the importance of cellulose in maintaining the integrity of flocs in PSS. When they treated PSS with fungal cellulase, the gel-like characteristic of the raw sludge changed to that of a slurry of fine particles in less than 2 hours.

A reduction in floc size may offer further advantages to the solubilization of organic wastes. Particulate organic matter of varying size would also be incorporated into the flocs, and would be enveloped in non-particulate organic molecules, or EPS. Li and

Ganczarczyk (1990) suggested that the significant quantity of EPS in activated sludge flocs provided diffusional resistance to the movement of substrates and products to and from the flocs. Likewise, EPS and other macromolecular components of the flocs in anaerobic digesters may offer steric resistance to the passage of bacteria and hydrolytic enzymes. Although bacteria would also be incorporated into the growing flocs, the majority of degradation of the floc components is likely to be from the outer surface where the concentration of bacteria will be high. As such, the macromolecules within the floc will be protected from enzymatic degradation. As the flocs disintegrate, macromolecules that were previously protected from enzyme attack are exposed, and may be degraded by hydrolytic enzymes. By increasing the frequency of floc cleavage, it is possible to facilitate hydrolysis by increasing the contact between enzyme and substrate. Furthermore, deflocculation will allow hydrolytic bacteria and their associated enzymes to penetrate into the floc matrix, where hydrolysis of previously unexposed macromolecules will take place. The rate at which freshly exposed particulate organics are degraded is related to the size of the particles, with small particles degraded more rapidly than larger ones (Torrijos *et al.*, 1993; Wentzel *et al.*, 1995; Vavilin *et al.*, 1996). Figures 7.1, 7.2 and 7.3 summarise the proposed mechanism for the accelerated hydrolysis of PSS under sulphate reducing conditions. Figure 7.1 provides a summary of the sequence of the most important events involved in the process in the order in which they are thought to occur, while the indirect and direct effects of the various components of the reaction on each other are shown in Figure 7.2. Finally, the location, within the first valley of the FSBR, of each of the steps involved in the process of enhanced solubilization are outlined in Figure 7.3.

The disadvantage of smaller flocs is their increased susceptibility to washout from reactors. As hydrolytic bacteria and hydrolytic enzymes are closely associated with the floc matrix (Boczar *et al.*, 1992; Frølund *et al.*, 1995; Confer and Logan, 1998; Goel *et al.*, 1998) they will also be removed from the reactor. The study of the enzymology of the lab-scale reactors revealed that the loss of hydrolytic enzymes from the sulphidogenic reactors did exceed that from the sulphate-free systems. Accumulation of enzymes within

the pilot-scale FSBR emphasised the importance of reactor design in the retention of biomass and hydrolytic enzymes.

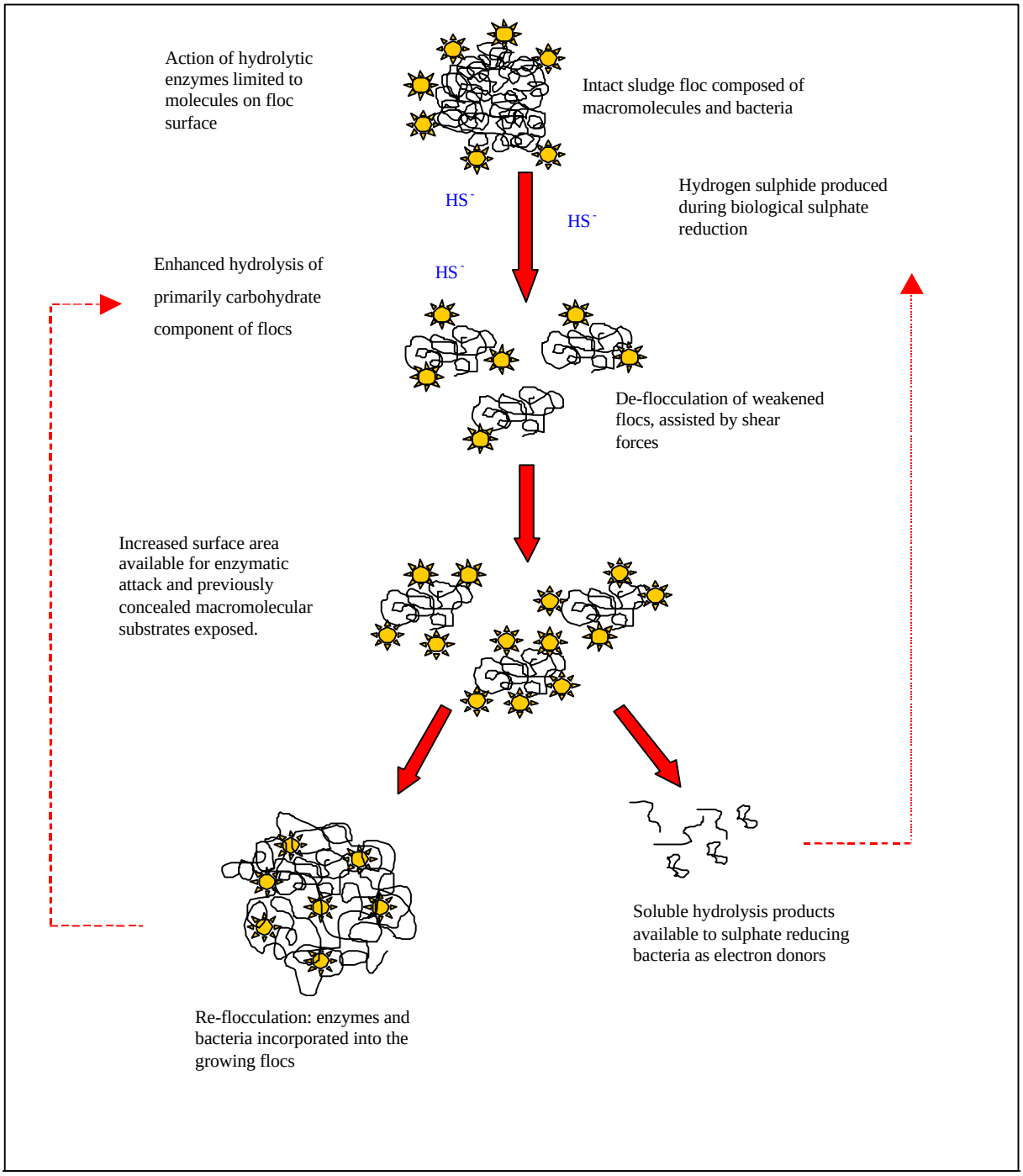


Figure 7.1. Flow diagram summarising the factors involved in the process of enhanced hydrolysis of PSS under sulphate reducing conditions.

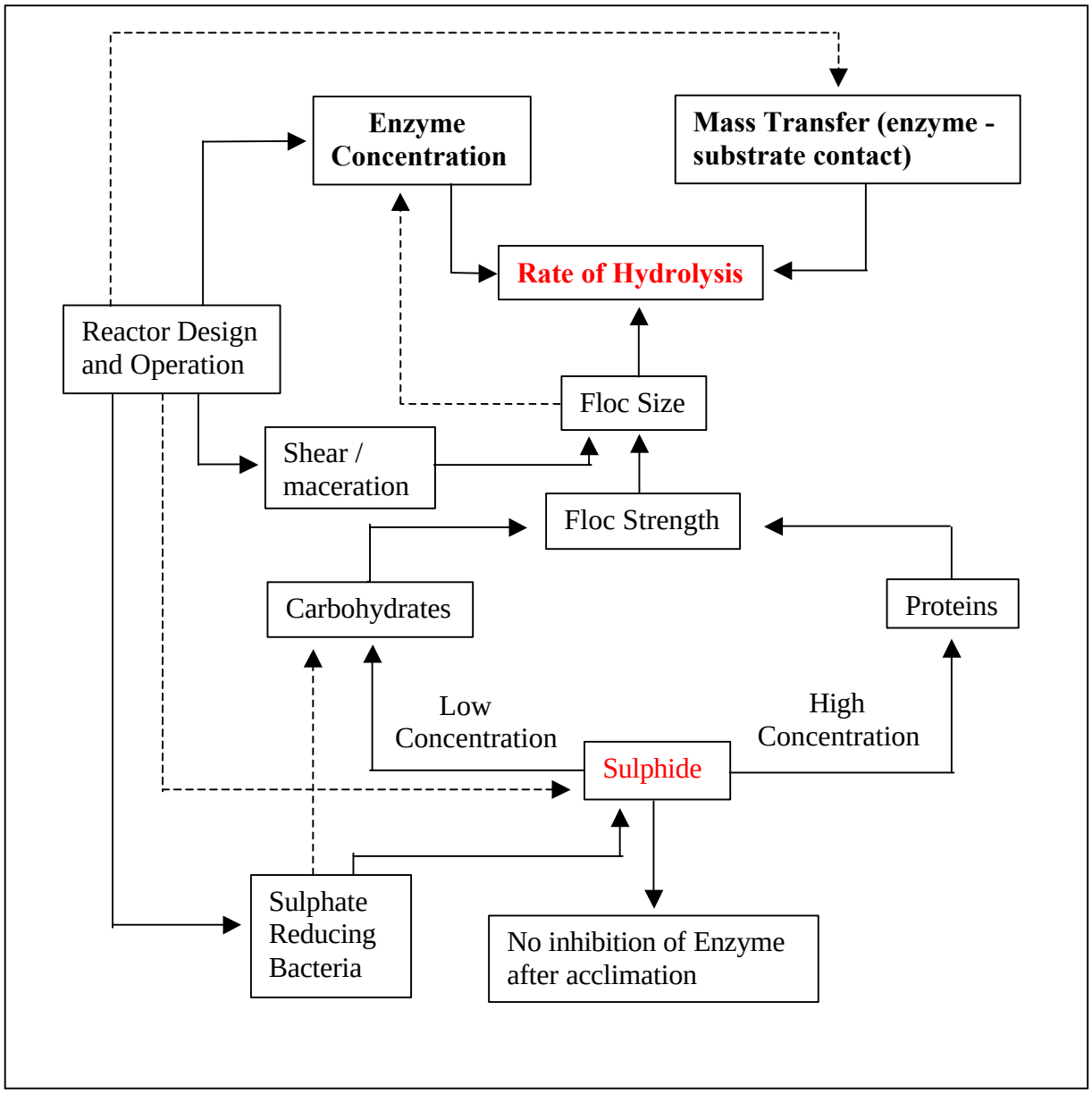


Figure 7.2. Flow diagram indicating the interrelationships of factors on the process of enhanced hydrolysis of PSS under sulphidogenic conditions. Dotted lines indicate possibly lower level impacts.

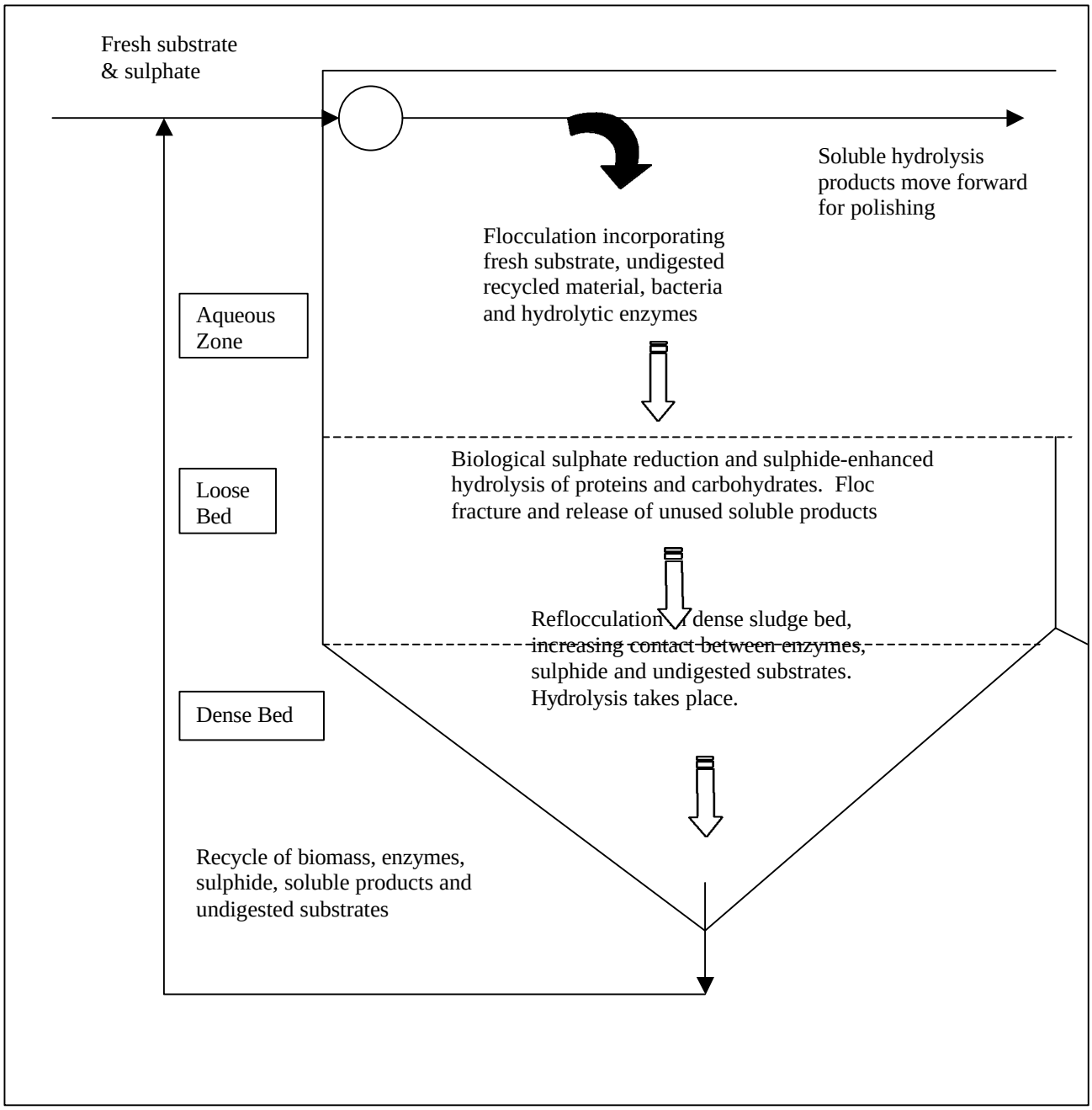


Figure 7.3. Diagramatic representation of the proposed spatial distribution within the first valley of the FSBR, of the stages involved in enhanced hydrolysis of PSS under sulphate reducing conditions.

The design of the FSBR and incorporation of sludge recycle are thought to be critical components in the success of the enhanced hydrolysis process. Based on the significant decrease in total enzyme activity in the base of valley 3, it is proposed that non-degradable or recalcitrant organic matter accumulated in this region. This has important implications for the operation of the hydrolysis unit. Although accumulation of solids within the FSBR was slow, periodic wastage of sludge from the reactor will be necessary. The results of the present study indicated that wastage of sludge from valley 3 would maximise removal of non-biodegradable organic matter while minimising loss of viable hydrolytic bacteria. However, accumulation of rapidly settling non-biodegradable solids in valley 1 is also likely to be high, and will have to be removed. Optimization of the desludging process will form part of the ongoing study.

The results also suggested that while recirculation of sludge from the base of valley 1 and valley 2 to the inlet is critical, recycle of sludge from the base of valley 3 to the inlet will likely be of less value. Recirculation from the base of the first two valleys will facilitate contact between undigested substrate in the feed and hydrolytic enzymes, in a sulphide-rich environment. Volatile fatty acids will also be introduced to the middle of the reactor where a significant proportion of active sulphate reduction occurred. Mixing will also serve to reduce floc size by shear forces. After the flocs re-enter the reactor, they will grow and eventually settle into the base of the reactor. It is proposed that during this phase of floc growth that enzymes and fresh substrates are brought into close proximity and that this will facilitate the hydrolysis process.

The “Rhodes Process” offers a relatively inexpensive yet effective alternative to conventional chemical processes used in the remediation of large volumes of sulphate-enriched wastewaters. It is especially attractive for the remediation of AMD where, in many cases, remediation must continue although the profitable mining operations have long since ceased. The results of this study have also shown that the solubilization of a complex and hazardous waste, PSS, is enhanced when digested in the presence of biogenic or chemical sulphide. Thus, it may also offer an alternative to costly sludge

disposal practices such as inceneration. Lastly, the indications that sulphide and / or biological sulphate reduction can assist in the cleavage of slowly biodegradable organic compounds, such as lignin, may find application in the remediation of related phenol-like compounds found in many wastewaters.

In summary, the results of this investigation indicate that the process relies on a reduction of floc stability in the presence of sulphide as a result of an increased rate of hydrolysis of lignin, carbohydrates and proteins. Deflocculation and mixing are essential, and results in exposure of previously inaccessible macromolecules to cleavage by hydrolytic enzymes. The configuration and operation of the reactor appears to be critical to the optimum performance of the process due to mass transfer events occurring in the falling sludge bed i.e. 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.

References

Andreasen, K., Peterson, G., Thomsen, H. & Strube. 1997. Reduction of nutrient emission by sludge hydrolysis. *Wat. Sci. Tech.*, *35*: 79-85.

American Public Health Association (APHA). 1989. *Standard Methods for the Examination of Water and Wastewater*, 17th Edition. 1989. American Public Health Association, Washington D.C.

Aulenbach, D.B. & Heukelekian, H. 1955. Transformation and effects of reduced sulphur compounds in sludge digestion. *Sew. Ind. Wastes*, 27: 1147-1159.

Balmat, J.L. 1957. Biochemical oxidation of various particulate fractions of sewage. *Sew. Ind. Wastes*, *29*: 757-761.

Banerjee, A., Elefsiniotis, P. & 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.

Banks, C.J. & Humphreys, P.N. 1998. The anaerobic treatment of a ligno-cellulosic substrate offering little natural pH buffering capacity. *Wat. Sci. Tech*., *38*: 29-35.

Banks, D., Younger, P.L., Arnesen, R.T., Iversen, E.R. & Banks, S.B. 1997. Mine-water chemistry: the good, the bad and the ugly. *Environ. Geol.*, *32*: 157-174.

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.

Barnes, H.L. & Romberg, S.B. 1986. Chemical aspects of acid mine drainage. *J. Water Pollut. Control. Fed*., *40*: 371-384.

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

Bisaria, V.S. & Ghose, T.K. 1981. Biodegradation of cellulosic materials: substrates, microorganisms, enzymes and products. *Enzyme. Microb. Technol*., *3*: 90-104.

Boczar, B.A., Begley, W.M. & Larson, R.J. 1992. Characterization of enzyme activity in activated sludge using rapid analysis for specific hydrolases. *Water Environ. Res*., *64*: 792-797.

Boschker, H.T., Bertilsson, S.A., Dekkers, E.M.J. & 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., Duncan, J, and Rose, P.D. 1996. An algal-bacterial intergrated ponding system for the treatment of mine drainage waters. *J. Appl. Phycology*, *8*: 442.

Box, J.D. 1983. Investigation of the Folin-Ciocalteau phenol reagent for the determination of polyphenolic substances in natural waters. *Water Res*., *17*: 511-525.

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

Bruus, J.H., Nielsen, P.H. & Keiding, K. 1992. On the stability of activated sludge flocs with implications to dewatering. *Water Res*., *26*: 1597-1604.

Burgess, S.G. & Wood, L.B. 1961. Pilot plant studies in production of sulphur from sulphate-enriched sewage sludge. *J. Sci. Food Agric*., *12*: 326-341.

Butlin, K.R., Selwyn, S.C. & Wakerley, D.S. 1956. Sulphide production from sulphate-enriched sewage sludges. *J. Appl. Bacteriol*., *19*: 3-15.

Cantor, C.R. 1982. *Protein Purification: principles and practice*. Springer-Verlag, New York, pp 47-59.

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

Chamber of Mines Research Organisation. 1988. New desalination programme on stream. R&D News CM, October.

Choi, H.B., Hwang, K.Y. & Shin, E.B. 1997. Effects of anaerobic digestion of sewage sludge pretreatment. *Wat. Sci. Tech*., *35*: 207-211.

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

Cinq-Mars, G.V. & Howell, J. 1977. Enzymatic treatment of primary municipal sludge with *Trichoderma viridae* cellulase. *Biotech. Bioeng.*, *19*: 377-385.

Clayton, D.W. 1969. The chemistry of alkaline pulping. In: *Pulp and Paper Manufacture, 2nd edition, volume 1: The Pulping of Wood*. R.G. MacDonald & J.N. Franklin (eds.). McGraw-Hill Book Company, New York, pp 347-438.

Colleran, E., Pender, S., Philpott, U., O'Flaherty, V. & Leahy, B. 1998. Full-scale and laboratory-scale anaerobic treatment of citric acid production wastewater. *Biodegradation, 9*: 233-246.

Confer, D.R. & Logan, B.E. 1997a. Molecular weight distribution of hydrolysis products during biodegradation of model macromolecules in suspended and biofilm cultures I. Bovine serum albumin. *Water Res., 31*: 2127-2136.

Confer, D.R. & Logan, B.E. 1997b. Molecular weight distribution of hydrolysis products during biodegradation of model macromolecules in suspended and biofilm cultures II. Dextran and dextrin. *Water Res., 31*: 2137-2145.

Confer, D.R. & Logan, B.E. 1998. A conceptual model describing macromolecule degradation by suspended cultures and biofilms. *Wat. Sci. Tech., 37*: 231-234.

Conradie, P.J.A. & Grutz, P.W.E. 1973. The treatment of acid mine waste in a mixture with sewage sludge in an anerobic digester. Report to the Chamber of Mines (File No. w6/534/3). National Institute for Water Research, Pretoria.

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

Drzyzga, O., Küver, J. & Blotevogel, K. 1993. Complete oxidation of benzoate and 4–hydroxybenzoate by a new sulfate reducing bacterium resembling *Desulfoarculus*. *Arch. Microbiol., 159*: 109-113.

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

Du Preez, L.A., Odendaal, J.P., Maree, J.P. & Ponsonby, M. 1992. Biological removal of sulphate from industrial effluents using producer gas as an energy source. *Environ. Technol*., *13*: 875-882.

Du Preez, L.A. & Maree, J.P. 1994. Pilot-scale biological sulphate and nitrate removal utilizing producer gas as an energy source. *Wat. Sci. Tech*., *30*: 275-285.

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

Ehlers, M.M. & Cloete, T.E. 1999. Protein profiles of phosphorus- and nitrate-removing activated sludge systems. *Water SA*, *25*: 351-356.

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

El-Fadel, M., Findikakis, A.N. & Leckie, J.O. 1996. Temperature effects in modelling solid waste biodegradation. *Environ. Technol.*, *17*: 915-935.

Eliosov, B. & Argaman, Y. 1995. Hydrolysis of particulate organics in activated sludge systems. *Water Res*., *29*: 155-163.

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

Elsgaard, L., Prieur, D., Mukwaya, G.M. & Jorgensen, B.B. 1994. Thermophilic sulfate reduction in hydrothermal sediment of lake Tanganyika, East Africa. *Appl. Environ. Microbiol., 60*: 1473-1480.

Engleman, C.J. & McDiffett, W.F. 1996. Accumulation of aluminium and iron by bryophytes in streams affected by acid-mine drainage. *Environ. Pollut*., *94*: 67-74.

Eriksson, L. & Alm, B. 1991. Study of flocculation mechanisms by observing effects of a complexing agent on activated sludge properties. *Wat. Sci. Tech*., *24*: 21-28.

Fan, L.T., Lee, Y-H. & Beardmore, D.H. 1980. Mechanism of the enzymatic hydrolysis of cellulose: Effects of major structural features of cellulose on enzymatic hydrolysis. *Biotech. Bioeng*., *22*: 177-199.

Fang, H.H.P. & Zhou, G. 1997. Anaerobic degradation of benzoate and cresol isomers in sulfate-rich wastewater. *Wat. Sci. Tech*., *36*: 7-14.

Forster, C.F. 1982. Sludge surfaces and their relation to the rheology of sewage sludge suspensions. *J. Chem. Tech. Biotechnol*., *32*: 799-807.

Fortin, D., Davis, B., Southam, G. & Beveridge, T.J. 1995. Biogeochemical phenomena induced by bacteria within sulfidic mine tailings. *J. Ind. Micro*., *14*: 178-185.

Frølund, B., Griebe, T. & Nielsen, P.H. 1995. Enzymatic activity in the activated-sludge floc matrix. *Appl. Microbiol. Biotechnol*., *43*: 755-761.

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

Gharpuray, M.M., Lee, Y. & Fan, L.T. 1983. Structural modification of lignocellulosics by pretreatment to enhance enzymatic hydrolysis. *Biotech. Bioeng*., *25*: 157-172.

Ghosh, S. 1987. Improved sludge gasification by two-phase anaerobic digestion. J. *Environ. Eng., 113*: 1265-1284.

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

Ghosh, S., Conrad, J.R. & Klass, D. 1975. Anaerobic acidogenesis of wastewater sludge. *Journal WPCF, 47*: 30-45.

Goel, R., Mino, T., Satoh, H. & Matsuo, T. 1998. Enzyme activities under anaerobic and aerobic conditions in activated sludge sequencing batch reactor. *Wat. Res*., *32*: 2081-2088.

Gorny, N. & Schink, B. 1994. Complete anaerobic oxidation of hydroquinone by *Desulfococcus* sp. Strain Hy5: indications of hydroquinone carboxylation to gentisate. *Arch. Micrbiol*., *162*: 131-135.

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

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

Hanaki, K., Matsuo, T., Nagase, M. & Tabata, Y. 1987. Evaluation of effectiveness of two-phase anaerobic digestion process degrading complex substrate. *Wat.Sci. Tech., 19*: 311-322.

Hatziconstantinou, G.J., Yannakopoulos, P. & Andreadakis, A. 1996. Primary sludge hydrolysis for biological nutrient removal. *Wat. Sci. Tech.*, 34: 417-423.

Henze, M. & Mladenovski, C. 1991. Hydrolysis of particulate substrate by activated sludge under aerobic, anoxic and anaerobic conditions. *Water Res*., *25*: 61-64.

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

Herrera, L.J., Hernàndez, P, Ruíz & Gantenbein, S. 1991. *Desulfovibrio desulfuricans* growth kinetics. *Environ. Toxicol. Water Qual*., *6*: 225-238.

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

Hiligsmann, S., Jacques, P. & Thonart, P. 1998. Isolation of highly performant sulfate reducers from sulfate-rich environments. *Biodegradation*, *9*: 285-292.

Hines, M.E. & Buck, J.D. 1982. Distribution of methanogenic and sulfate-reducing bacteria in near-shore marine sediments. *Appl. Environ. Microbiol., 43*: 447-453.

Holliger, C., Stams, A.J.M. & Zehnder, A.J.B. 1988. Anaerobic degradation of recalcitrant compounds. In: *Anaerobic Digestion*. E.R. Hall and P.N. Hobson (eds.). Pergamon Press, Oxford, UK, pp 211-224.

Hong, C.M. 1986. Horizontal-type anaerobic digester. The second International Conference on Environment and Housing for Livestock. Pig Research Institute, Taiwan.

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

Isa, Z., Grusenmeyer, S. & Verstraete, W. 1986. Sulphate reduction relative to methane production in high-rate anaerobic digestion: microbiological aspects. *Appl. Environ. Microbiol.*, *51*: 580-587.

Jain, S., Lala, A.K., Bhatia, S.K. and Kudchadker, A.P. 1992. Modelling of hydrolysis controlled anaerobic digestion. *J. Chem. Tech. Biotechnol*., *53*: 337–344.

Janes, R.L. 1969. The chemistry of wood fibres. In: *Pulp and Paper Manufacture, 2nd edition, volume 1: The Pulping of Wood*. R.G. MacDonald and J.N. Franklin (eds.). McGraw-Hill Book Company, New York, pp 33-72.

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

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

Karlsson, I. & Göransson, J. 1993. Thermic sludge treatment. *Wat. Sci. Tech*., *27*: 449-456.

Khan, A.W. & Trottier, T.M. 1978. Effect of sulfur-containing compounds on anaerobic degradation of cellulose to methane by mixed cultures obtained from sewage sludge. *Appl. Environ. Microbiol*., *35*: 1027-1034.

Kim, S.K., Matsui, S., Pareek, S., Shimizu, Y. & 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.

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

Kosaric, N. & Blaszczyk, R. 1990. Microbial aggregates in anaerobic wastewater treatment. *Adv. Biochem. Eng. Biotechnol.*, *42*: 27-62.

Kuever, J., Kulmer, J., Jannsen, S., Fischer, U. & Blotevogel, K. 1993. Isolation and characterization of a new spore-forming sulfate-reducing bacterium growing by complete oxidation of catechol. *Arch. Microbiol*., *159*: 282-288.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*: 680-685.

Lawrence, A.W., McCarty, P.L. & Guerin, F.J.A. 1966. The effects of sulphides on anaerobic treatment. *Air Wat. Pollut. Int. J., 10*: 207-221.

Lens, P.N., De Poorter, M.P., Cronenberg, C.C. & 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., Hulshoff-Pol, L.W. and Lettinga, G. 1998. Effect of staging on volatile fatty acid degradation in a sulfidogenic granular sludge reactor. *Water Res*., *32*: 1178-1192.

Lettinga, G. and Hulshoff-Pol, L.W. 1991. UASB-process design for various types of wastewaters. *Wat. Sci. Tech*., *24*: 87-107.

Levine, A.D., Tchobanoglous, G. & Asana, T. 1985. Characterization of the size distribution of contaminants in wastewater: treatment and reuse implications. *Journal WPCF*, *57*: 805-816.

Li, D. & Ganczarczyk, J.J. 1990. Structure of activated sludge flocs. *Biotech. Bioeng*., *35*: 57-65.

Li, Y., Lam, S. and Fang, H.P. 1996. Interactions between methanogenic, sulfate-reducing and syntrophic acetogenic bacteria in the anaerobic digestion of benzoate.

Water Res., *30*: 1555-1562.

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

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem*., *193*: 265-275.

Madhukara, K., Srilatha, H.R., Srinath, K., Bharathi, K. & Nand, K. 1997. Production of methane from green pea shells in floating dome digesters. *Process Biochem.*, *32*: 509-513

Maillacheruvu, K.Y., Parkin, G.F., Peng, C.Y., Kuo, W., Oonge, Z.I. & Lebduschka, V. 1993. Sulfide toxicity in anaerobic systems fed sulphate and various organics. *Water Environ. Res.*, *65*: 100-109.

Maree, J.P., Du Plessis, P. & van Der Walt, C.J. 1992. Treatment of acid effluents with limestone instead of lime. *Wat. Sci. Tech*., *26*: 345-355.

Maree, J.P., Gerber, A. & Hill, E. 1987. An integrated process for biological treatment of SO_4-containing industrial effluents. *Journal WPCF, 59*: 1069-1074.

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

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. & Strydom, W.F. 1985. Biological sulphate removal in an upflow packed bed reactor. *Water SA*, *19*: 1101-1106

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

McInerney, M.J. 1988. Anaerobic hydrolysis and fermentation of fats and proteins. In: *Biology of Anaerobic Miroorganisms*. A.J. Zehnder (ed.). John Wiley and Sons, Inc., New York, pp 373-415.

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

Müller, J., Lehne, G., Schwedes, J., Battenberg, S., Näveke, R., Kopp, J., Dichtl, N., Scheminski, A., Krull, R. & Hempel, D.C. 1998. Disintegration of sewage sludges and influence on anaerobic digestion. *Wat. Sci. Tech*., *38*: 425-433.

Nielsen, P.H., Frølund, B. & Keiding, K. 1996. Changes in the composition of extracellular polymeric substances in activated sludge during anaerobic storage. *Appl. Microbiol. Biotechnol*., *44*: 823-830.

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

Nybroe, O., Jørgensen, P.E. & Henze, M. 1992. Enzyme activities in wastewater and activated sludge. *Water Res*., *26*: 579-584.

Nyns, E.J., Naveau, H.P., Chome, R. & Bertrand, Y. 1979. Digesters - a world-wide review. In: *Proceedings of the first International symposium on anaerobic digestion, University College, Cardiff, Wales*. D.A. Stafford, B.I. Wheatley and D.E. Hughes (eds.). Applied Science Publishers Ltd., London, pp 37-53.

Oleszkiewicz, J.A. & Hilton, B.L. 1986. Anaerobic treatment of high sulphate wastes. *Can. J. Civ. Eng., 13*: 423-428.

Omil, F., Lens, P., Hulshoff-Pol, L. and Lettinga, G. 1996. Effect of upward velocity and sulphide concentration on volatile fatty acid degradation in a sulphidogenic granular sludge reactor. *Process Biochem*., *31*: 699-710.

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

Pavlostathis, S.G. 1994. Anaerobic processes. *Water Environ. Res.*, *66*: 342-356.

Pavlostathis, S.G. & Giraldo-Gomez, E. 1991. Kinetics of anaerobic treatment. *Wat. Sci. Tech*., *24*: 35-59.

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

Peng, C. & Park, J.K. 1994. Electrochemical mechanisms of corrosion influenced by sulfate-reducing bacteria in aquatic systems. *Water Res., 28*: 1681-1692.

Perot, C., Sergent, M., Richard, P., Phan Tan Luu, R. & Millot, N. 1988. The effects of pH, temperature and agitation speed on sludge anaerobic hydrolysis- acidification. *Environ Technol Lett.*, *9*: 741-752.

Pipes, W.O. 1961. Sludge digestion by sulphate reducing bacteria. In: *Proceedings of the 15th Industrial Waste Conference*, pp 308-319.

Pipyn, P & Verstraete, W. 1979. Waste classification for the digestibility in anaerobic systems. In: *Proceedings of the first International symposium on anaerobic digestion, University College, Cardiff, Wales.* D.A. Stafford, B.I. Wheatley and D.E. Hughes (eds.). Applied Science Publishers Ltd., London, pp 151-166.

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

Rees, T.D., Gyllenspetz, A.B. & Docherty, A.C. 1971. Determination of trace amounts of sulphide in condensed steam with N'N-diethyl-p-phenylenediamine. *Analyst*, *96*: 201-208.

Reichenbecher, W. & Schink, B. 1997. *Desulfovibrio inopinatus*, sp. Nov., a new sulfate-reducing bacterium that degrades hydroxyhydroquinone (1,2,4-trihydroxybenzene). *Arch. Micrbiol*., *168*: 338-344.

Riekkola-Vanhanen, M. & Mustikkamäki, U.P. 1997. *In situ* treatment of acid mine drainage by sulphate reducing bacteria in an open pit mine. In: *Proceedings of Biomine International Conference*, *Sydney, Australia*. E-ROM3.1-E-ROM3.8.

Rose, P.D., Boshoff, G.A., Van Hille, R.P., Wallace, L.C.M., Dunn, K.M. & 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.

Rudolfs, W. & Amberg, H.R. 1952. White water treatment. II. Effect of sulfides on digestion. *Sew. Ind. Wastes*, *24*: 1278-1287.

Sam-Soon, P.A.L.N.S., Loewenthal, R.E., Wentzel, M.C. and Marais, G.vR. 1991. Effect of sulphate on pelletisation in the UASB system with glucose as substrate. *Water SA*, *17*: 47-56.

San Pedro, D.C., Mino, T. & 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.

SA Water Bulletin. 1987. Mine water desalination demonstrated. June, pp 14-15.

Scheeren, P.J.H., Kock, R.O. & Buisman, C.J.N. 1993. Geohydrological contaminant system and microbial water treatment plan for metal contaminated groundwater at Budelco. International Symposium World Zinc, pp 373-384.

Schink, B., Brune, A., & Schnell, S. 1992. Anaerobic degradation of aromatic compounds. In: *Microbial Degradation of Natural Products*. G. Winkelmann (ed.). VCH Publishers, Weinheim, pp 219-242.

Schnell, S. & Schink, B. 1991. Anaerobic aniline degradation via reductive deamination of 4-aminobenzoyl-CoA in *Desulfobacterium anilini*. *Arch. Microbiol*., *155*: 183-190.

Schnell, S. & Schink, B. 1992. Anaerobic degradation of 3-aminobenzoate by a newly isolated sulfate reducer and a methanogenic enrichment culture. *Arch. Microbiol*., *158*: 328-334.

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*.

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

Siegrist, H., Renggli, D. & Gujer, W. 1993. Mathematical modelling of anaerobic mesophilic sewage treatment. *Wat. Sci. Tech*., *27*: 25-36.

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

Spicer, P.T. & Pratsinis, S.E. 1996. Shear-induced flocculation: the evolution of floc structure and the shape of the size-distribution at steady state. *Water Res*., *30*: 1049-1056.

Szikriszt, G., Frastell, B., Norrman, J. & Bergstrom, R. 1988. Pilot- scale anaerobic digestion of municipal solid waste after novel pretreatment. In: *Anaerobic Digestion*. E.R. Hall and P.W. Hobson (eds.). Pergamon Press, Oxford, U.K, pp 375-382.

Takahashi, M. & Kyosai, S. 1988. Development of multi-stage reversing-flow bioreactor (MRB) for wastewater treatment. *Wat. Sci. Tech.*, *20*: 361-367.

Teunissen, M.J. & Op den Camp, H.J.M. 1993. Anaerobic fungi and their cellulolytic and xylanolytic enzymes. *Antonie van Leeuwenhoek*, *63*: 63-76.

Tharagonnet, D., Sission, P.R., Roxby, C.M., Ingham, H.R. & Selkon, J.B. 1977. The API ZYM system in the identification of gram-negative anaerobes. *J. Clin. Path*., *30*: 505-509.

Thompson, J.G. 1980. Acid mine waters in South Africa and their amelioration. *Water SA*, *6*: 130-134.

Toerien, D.F. & Maree, J.P. 1987. Reflections on anaerobic process biotechnology and its impact on water utilization in South Africa. *Water SA*, *13*: 137-144.

Torrijos, M., Cerro, R-M., Capdeville, B., Zeghal, S., Payraudeau, M. & Lesouef, A. 1993. Sequencing batch reactor: a tool for wastewater characterization for the IAWPRC model. In: *Proceedings of the 1st Int. Spec. Conf. On Microorganisms in Activated Sludge and Biofilm Processes*, Paris, pp 81-90.

Tuttle, J.H., Dugan, P.R. & 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.

Urbain, V., Block, J.L. & Manem, J. 1993. Bioflocculation in activated sludge: an analytical approach. *Wat. Res*., *27*: 829-838.

Van Lier, J.B., Rebac, S. & Lettinga, G. 1997. High-rate anaerobic wastewater treatment under psychrophilic and thermophilic conditions. *Wat. Sci. Tech*., *35*: 199-206.

Van Wyk, J.J. & Munnik, R. 1998. Dewatering of the East Rand Mining Basin: a critical evaluation of the Government's approach towards solving the associated environmental problems. *Report 3C-1, Department of Water Affairs and Forestry, South Africa.*

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

Wang, W., Xu, Z. & Finch, J. 1996. Fundamental study of an ambient temperature ferrite process in the treatment of acid mine drainage. *Environmental Sci. Technol.*, *30*: 2604-2608.

Wentzel, M.C., Mbewe, A. & Ekama, G.A. 1995. Batch test for measurement of readily biodegradable COD and active organism concentrations in municipal waste waters. *Water SA*, *21*: 117-124.

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.

Widdel, F. & Hansen, T.A. 1992. Dissimilatory sulphate or sulphur reducing bacteria. In: *The Prokaryotes, Volume 1*, A. Ballows, H.G. Truper, M. Dworkin, W. Harder and K. Schleifer (eds.). Springer-Verlag, Berlin, pp 583-624.

Wittman, G.T.W & Förstner, U. 1976. Heavy metal enrichment in mine drainage: II. The Witwatersrand goldfields. *South Afr. J. Sci., 72*: 365-370.

Wittman, G.T.W & Förstner, U. 1977. Heavy metal enrichment in mine drainage: V. The Orange Free State goldfields. *South Afr. J. Sci., 73*: 374-378.

Woodward, J. 1987. Utilization of cellulose as fermentation substrate: problems and potential. In: *Carbon substrates in biotechnology*. J.D Stowell, A.J. Beardsmore, C.W. Keevil and J.R. Woodward (eds.). IRL Press, Oxford, pp 45-65.

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. 1998b. The creation of floodplain wetlands to passively treat polluted minewaters. In: *Hydrology in a Changing Environment*, Volume 1. Proceedings of the British Hydrological Society Internation Conference, Exeter. H. Wheater and C. Kirby (eds.). John Wiley and Sons, Chichester, pp 495-502.

Younger, P.L., Curtis, T.P., Jarvis, A. & 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.

Zeikus, J.G., Wellstein, A.L. & Kirk, T.K. 1982. Molecular basis for the biodegradative recalcitrance of lignin in anaerobic environments. *FEMS Microbiol. Lett., 15*: 193-197.

Appendices

Appendix 1. Pump specifications for pilot-scale Falling Sludge Bed Reactor

Pump Number (Figure 3.1)	Description	Flow Rate
P1	variable speed sludge pump	0-200L.h^{-1}
P3	variable speed centrifugal pump	variable
P5	high speed shear pump	10m^3.h^{-1}
P6	variable speed open impeller pump	0.3-8m^3.h^{-1}
P7	variable speed sludge pump	max. = 27m^3.h^{-1}

Appendix 2. Sulphide standard curve

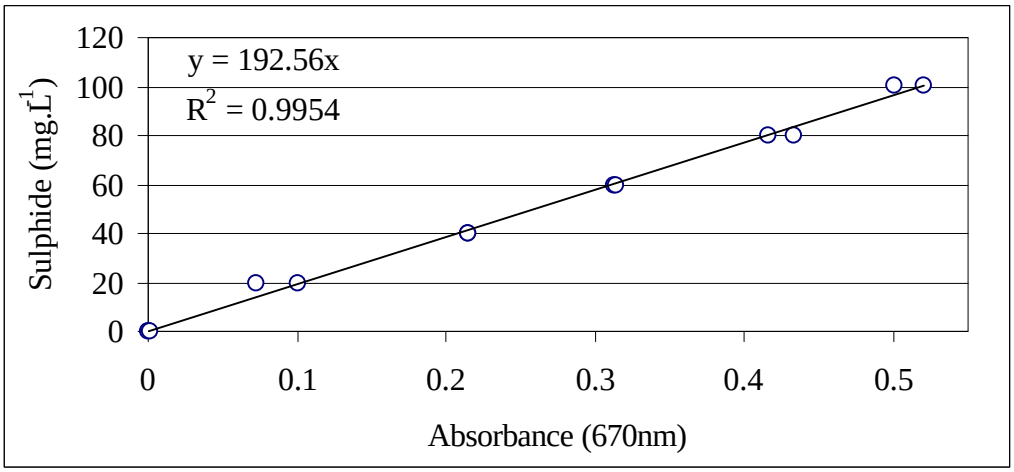


Figure A2.1. Sulphide standard curve.

Appendix 3. Soluble carbohydrate standard curve

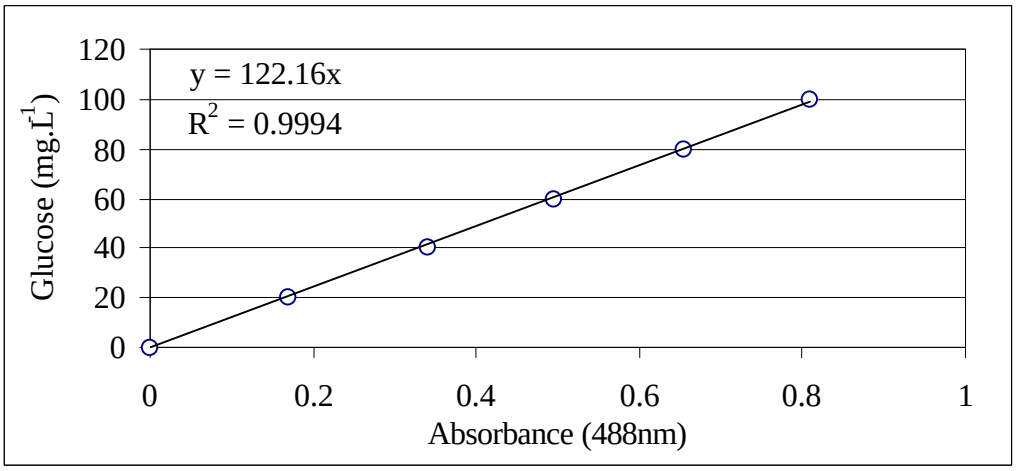


Figure A3.1. Soluble carbohydrate standard curve prepared with glucose.

Appendix 4. Total phenol standard curve.

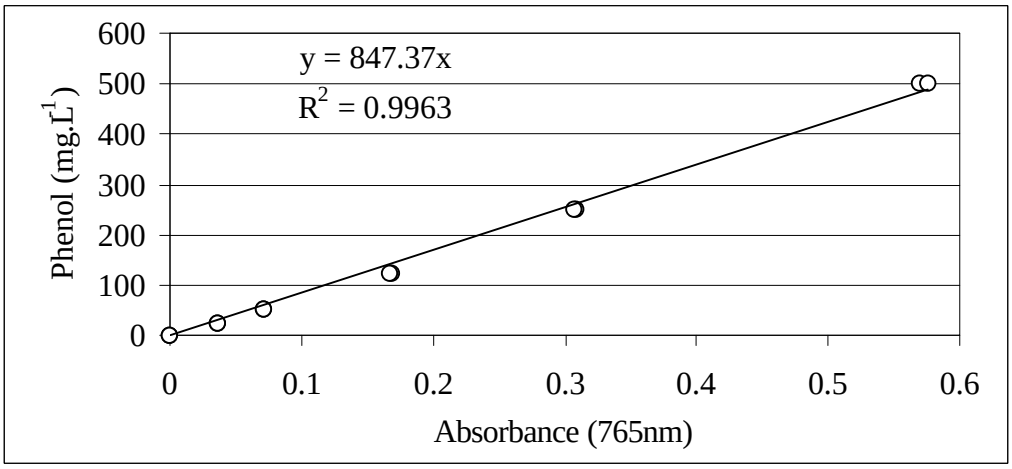


Figure A4.1. Total phenol standard curve

Appendix 5. Protein Standard Curve

Table A5.1. Volumes used in the preparation of the protein standard curve. The protein stock solution was made by dissolving 20mg BSA in 200mL distilled water.

Protein Conc. ($mg.L^{-1}$)	BSA stock solution (mL)	Distilled water (mL)
100	10	0
80	8	2
60	6	4
40	4	6
20	2	8
0	0	10

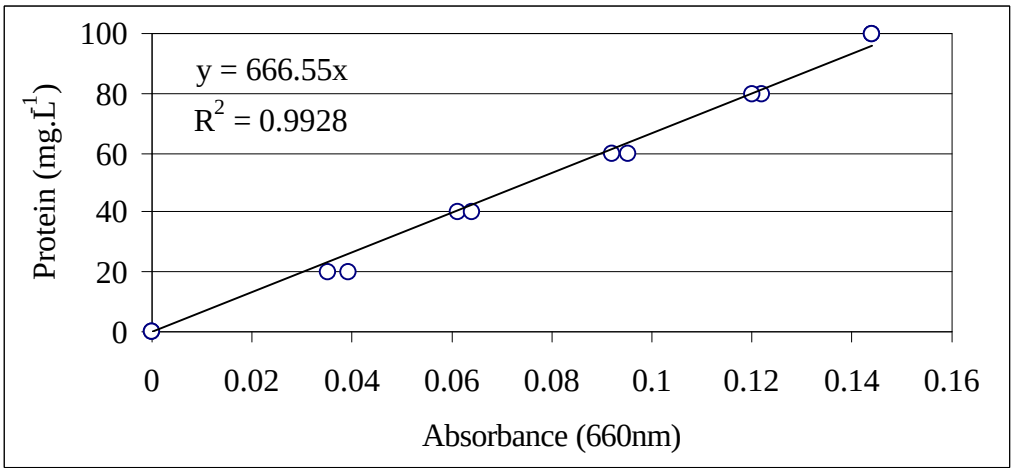


Figure A5.1. Protein standard curve prepared with Bovine Serum Albumin.

Appendix 6. Preparation of non-reducing gradient SDS-PAGE

A6.1. Stock solutions

Solution A: Acrylamide / bisacrylamide stock

Dissolve 29.2g acrylamide and 0.8g N'N-bis-methylene-acrylamide in distilled water and make volume to 100mL. To make a 45% stock solution, dissolve 43.8g acrylamide and 1.2g N'N-bis-methylene-acrylamide.Store in the dark at 4^{o}C. Stable for a maximum of 1 month.

Solution B: 1.5 M Tris-HCl, pH 8.8

Dissolve 18.5g Tris in 80mL distilled water. Adjust the pH to 8.8 with 1M HCl and make the volume to 100mL. Store at 4^{o}C for weeks.

Solution C: 0.5 M Tris-HCl buffer, pH 6.8

Dissolve 6g Tris in 80mL distilled water. Adjust the pH to 6.8 with 1 M HCl and make the volume up to 100mL. Store at 4^{o}C. Stable for weeks.

Solution D: 10% (w/v) SDS

Dissolve 10g SDS in distilled water with gentle stirring. Make the volume up to 100mL with distilled water and store at room temperature. Stable for months.

Solution E: Sample buffer (non-reducing)

Table A6.1. Volumes used to prepare non-reducing sample buffer.

Reagent	Volume (mL)
Distilled water	4.4
Solution C	1.0
Glycerol	0.8
Solution D	1.6
0.2% (w/v) bromophenol blue	0.2

Prepare sample buffer immediately before use. Dilute samples 1:1 with sample buffer and heat for 5 minutes at 95^{o}C.

Solution F: Electrode (running) buffer

Dissolve 30g Tris, 144g glycine and 10g SDS in 2.0L distilled water. Adjust the pH to 8.3. Before use, dilute 1L of the stock solution with 4L distilled water.

Ammonium persulphate solution

Dissolve 0.1g $(NH_4)_2S_2O_8$ in 1mL distilled water. Make fresh immediately before use.

A6.2. Preparation of Molecular Weight Marker

60µg High Molecular Weight Marker Mix (Sigma) was dissolved in 100µL sample buffer. The mix was heated at 95^{o}C prior to loading.

Table A6.2. Composition of the High Molecular Weight Standard Protein Mix (Sigma).

Protein	Molecular Weight (kDa)
Myosin	205000
β-Galactosidase	116000
Phosphorylase-b	97400
Bovine Serum Albumin	66000
Egg Albumin	45000
Carbonic Anhydrase	29000

A6.3. Staining and destaining of gels

A6.3.1. Staining:

0.1% w/v Coomassie Brialliant Blue R250 was made by mixing 50mL methanol with 70mL glacial acetic acid. 1g Coomassie Brilliant Blue R250 was dissolved in the mix, and the volume made up to 1L with distilled water. The stain was filtered (Whatman # 1) prior to use. Each gel was stained in 500mL stain for 2 hours.

A6.3.2. Destaining:

A mixture of 10% methanol and 5% glacial acetic acid was found to be an effective destain. Gels were placed in the destain mix and agitated over night. The gels were removed from the destain and washed twice in distilled water.

A6.4. Preparation of gels

Table A6.3. Preparation of separating gels for SDS-PAGE. A 30% acrylamide stock solution was used. All volumes in mL.

Reagent	5% gel	7.5% gel	20% gel
Distilled water	8.55	7.275	1.0175
Solution B	3.75	3.75	3.75
Solution D	0.15	0.15	0.15
Solution A	2.5	3.75	10.0
Ammonium Persulphate	0.075	0.075	0.075
TEMED (Sigma)	0.0075	0.0075	0.0075

Table A6.4. Preparation of 5 % and 30% separating gels using a 45% acrylamide stock solution. All volumes in mL.

Reagent	5% gel	30% gel
Distilled water	9.417	1.0175
Solution B	3.75	3.75
Solution D	0.15	0.15
Solution A	1.6	10.0
Ammonium Persulphate	0.075	0.075
TEMED (Sigma)	0.0075	0.0075

Table A6.5. Preparation of 4 % stacking gels for SDS-PAGE. All volumes in mL.

Reagent	Using 30% acrylamide stock solution	Using 45% acrylamide stock solution
Distilled water	6.1	6.55
Solution C	2.5	2.5
Solution D	0.1	0.1
Solution A	1.3	0.85
Ammonium Persulphate	0.05	0.05
TEMED (Sigma)	0.025	0.025

Appendix 7. Precipitation of sludge proteins

A7.1. Solvent extraction

- Dilute 3mL raw sludge sample with an equal volume of cold (4^oC) acetone
- Incubate at 4^oC for 15 min.
- Centrifuge at 20000 rpm for 15min (Beckman L8-80M ultracentrifuge, SW41 rotor).
- Pour off the supernatant and re-suspend the pellet in 1mL cold buffer (0.5M Tris-HCl, pH 6.8).
- Place samples in vacuum desiccator for 15min to remove excess solvent.

A7.2. Ammonium sulphate precipitation

- Add 1.548g $(NH_4)_2SO_4$ to 3mL sludge sample (80% salt solution).
- Allow to stand for 30min on ice.
- Centrifuge at 20000 rpm for 15min (Beckman L8-80M ultracentrifuge, SW41 rotor).
- Re-suspend the pellet in 1mL cold buffer (0.5M Tris-HCl, pH 6.8).
- Dialyze in 0.5M Tris-HCl buffer (pH 6.8) for 24 hours.