# Addressing the Challenges Facing Biological Sulphate Reduction as a Strategy for AMD Treatment:

## *Analysis of the reactor stage: raw materials products and process kinetics*

Report to the
**Water Research Commission**

by

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# EXECUTIVE SUMMARY


## BACKGROUND

The contamination of surface and groundwater by acid mine drainage (AMD) and acid rock drainage (ARD) and the consequences for the environment, agriculture and human health are serious concerns in the regions of South Africa impacted by mining activities. Acid drainage is generated via the oxidation of sulphide minerals, typically pyrite, when exposed to oxygen and water. The process is usually catalysed by iron and sulphur oxidising microorganisms. In South Africa, mine water can be divided into two broad categories. The first, AMD, originates from the rebound of groundwater through abandoned mine workings, once dewatering has ceased and is characterised by large volumes of heavily impacted water. This is occurring in the underground basins caused by gold mining in the Witwatersrand and has been the subject of much media attention. The authorities have devised an emergency plan to address the situation to avoid uncontrolled discharges. The plan involves active dewatering, followed by conventional oxidation, neutralisation and metal precipitation, with the potential of reverse osmosis to recover potable quality water. The volume and composition of the AMD precludes the application of biological treatment options in most cases. The second type, referred to as acid rock drainage (ARD) in this report, originates from diffuse sources, such as waste rock dumps, tailings impoundments, coal discard heaps and unworked pits. These sites are more numerous, are likely to affect a greater area and can persist for decades. However, they have received relatively little attention from the media or the authorities. Acid rock drainage from diffuse sources as well as end-of-pipe sources are more amenable to biological treatment.

The biological treatment of ARD is centred on the activity of sulphate-reducing bacteria (SRB), which are able to reduce sulphate to sulphide, coupled to the oxidation of an electron donor, typically an organic carbon molecule. A number of commercial processes, based on biological sulphate reduction, have been developed, but their widespread application has been constrained by three factors. These are the cost of the electron donor, the relatively slow growth of sulphate reducers and the associated kinetic constraints, and the management of the sulphide product. The research presented in this report addressed the first two constraints by assessing the potential of whole and partially digested microalgae as the electron donor and investigating novel reactor configurations aimed at biomass retention and recycling. The handling of the sulphide product was previously addressed in van Hille and Mooruth (2013). Together, these studies demonstrate potential for development of a feasible integrated process for operation in a passive, semi-passive or active configuration, allowing treatment of these ARD sources with concomitant sulphur recovery.

## AIMS

The aims of the project were as follows:

1. Critically evaluate existing SRB-based technologies (active and passive).
2. Evaluate microalgae as a carbon source and electron donor for SRB systems, both in terms of digestibility and the ability to cultivate sufficient quantities. Evaluate the potential to cultivate algae using treated AMD as the basis for the growth medium.
3. Evaluate the effect of decoupling the hydrolysis and acidogenesis steps from the sulphate reduction.
4. Review reactor options for the retention of biomass and the creation of specific reaction zones.
5. Investigate the use of cross-flow microfiltration as a model biomass retention strategy for the sulphate reduction reactor.
6. Investigate novel reactor configuration to achieve biomass retention that has the potential for application in active and passive systems.

## METHODOLOGY

A set of baseline data was generated for suspended culture, using continuously stirred tank reactors (CSTRs) and a defined growth medium with lactate as the electron donor and carbon source. Three feed sulphate concentrations (1.0, 2.5 and 5.0 g/ℓ) were tested, at a constant chemical oxygen demand (COD) to sulphate ratio (0.7). The reactors were operated at hydraulic retention times (HRTs) from 5 days to 12 hours and steady state data were used to determine volumetric sulphate reduction rates (VSRR).

Three experimental reactor configurations were evaluated: a standard reactor without active agitation, a standard reactor coupled to a cross-flow microfiltration unit for biomass recycling, and a novel linear flow channel reactor (LFCR) with carbon fibres for biomass retention. Experiments were confined to a feed sulphate concentration of 1 g/ℓ and HRTs from 4 days to 12 hours.

The potential of whole cell and anaerobically digested microalgae as electron donors was evaluated in standard CSTRs. *Spirulina*, a cyanobacterium and *Scenedesmus*, a green alga were assessed as candidate substrates for anaerobic digestion, both as whole cells and mechanically pre-treated slurries. The resulting digestate was characterised in terms of COD and volatile fatty acid (VFA) profile. Anaerobically digested *Spirulina* was used as the electron donor in sulphate reduction studies. The experiments were conducted in standard CSTRs at feed sulphate concentrations of 2.5 and 5 g/ℓ. A COD to sulphate ratio of 0.7 was maintained.

The potential for growing algae on treated ARD (raw and nutrient supplemented) was evaluated in small-scale growth studies. A number of known species as well as uncharacterised environmental isolates were tested. Selected isolates were grown in airlift photobioreactors to compare growth on treated ARD against defined growth media.

**RESULTS AND DISCUSSION**

The baseline study data were consistent with previous studies conducted under similar conditions. At a feed sulphate concentration of 1 g/ℓ, efficient (>85%) sulphate reduction was observed at HRTs from 5 days to 1 day, with a linear increase in the VSRR and complete utilisation of the lactate substrate. The sulphate reduction efficiency fell significantly at a 12 hour HRT, to around 50%, although lactate conversion remained complete. The VSRR increased to 41 mg/ℓ.h, but deviated from the linear trend. The results suggest washout of part of the sulphate reducing community and a shift toward a lactate fermenting community.

The data generated at the feed sulphate concentrations of 2.5 and 5 g/ℓ showed lower sulphate reduction efficiencies (50-60%) at the longer HRTs (4-5 days), becoming even less efficient at the shorter HRTs. Lactate conversion was complete in the reactor receiving 2.5 g/ℓ sulphate across the HRTs and ranged from 90% to 60% in the 5 g/ℓ reactor, indicating that the majority of the lactate was consumed by fermenters, rather than sulphate reducers. The shift in community structure was consistent with previous studies.

Sulphate reduction in the non-agitated control reactor was similar to that in the CSTR, indicating that constant agitation was not necessary. A separate mixing study confirmed that while mixing was slow in the non-agitated reactor, complete mixing was achieved in less than the HRT for all dilution rates tested. Biomass accumulation, as suspended flocs, was observed.

The inclusion of the cross-flow microfiltration unit to ensure biomass recycle significantly improved the sulphate reduction efficiency of the system at low HRTs. The permeate from the membrane was free of cells, indicating 100% efficiency in terms of biomass recycling as expected using filtration through an exclusion membrane. The pH of the permeate was typically 0.5 pH units higher than the bulk fluid; its light yellow colour suggested the presence of polysulphides. These catalysed the abiotic oxidation of a portion of the sulphide to elemental sulphur in the permeate drainage tube. The deposition of elemental sulphur resulted in periodic blocking of the permeate line, resulting in overflow from the reactor and the loss of some biomass. The problem became more significant at lower HRTs, with the proportion of permeate relative to overflow falling from 76% at a HRT of 1.5 days to 44% at a HRT of 12 hours. The problem was most likely caused by the permeability of the silicone tubing and the autocatalytic effect of polysulphide. It could be addressed by selecting materials that excluded oxygen more efficiently. Notwithstanding the loss of some of the accumulated biomass, the VSRR at a 12 hour HRT was approximately 65 mg/ℓ.h, 50% higher than in the CSTR under similar conditions, clearly demonstrating the benefit of biomass recycling and the associated increase in the biomass concentration.

The carbon microfibres used as the site of biofilm formation for cell retention in the LFCR proved to be an excellent support material. They were rapidly colonised, demonstrated by a substantial amount of attached biomass being removed with them at the end of the experiment. The benefit of the biomass retention was apparent at HRTs below 24 hours. The VSRR at a 12 hour HRT was approximately 47.5 mg/ℓ.h, almost 20% higher than in the baseline study. The microbial community was particularly robust, surviving extremely alkaline (pH 13) and acidic (pH 2.5) conditions for several hours following an event where over a third of the reactor volume was replaced with 0.5 M sodium hydroxide. It is unlikely that a purely planktonic community would have been able to recover under similar circumstances. The lack of turbulent mixing in the LFCR

limited the partial oxidation of the sulphide, resulting in a higher aqueous concentration. This increased the selective pressure on non-sulphate reducing species and prevented the proliferation of lactate fermenters at low HRTs. It was not possible to exclude oxygen completely from the headspace and the reactor developed a floating sulphur biofilm at the air-water interface, resulting in a significant decrease in the effluent sulphide concentration relative to that in the bulk liquid owing to the characteristic flow patterns.

Biological sulphate reduction was achieved using whole *Spirulina* biomass as the electron donor and carbon source, although the performance was inconsistent. More predictable performance was achieved when the hydrolysis and acidogenesis reactions were decoupled from the sulphate reduction. *Spirulina* proved to be a good substrate for anaerobic digestion with relatively rapid liberation of volatile fatty acids, predominantly acetate and butyrate. Mechanical pre-treatment did not significantly improve the concentration of VFAs leaving the anaerobic digestor. Digestion of whole cell *Scenedesmus* by anaerobic digestion was less efficient than Spirulina, probably due to the recalcitrance of the cellulosic cell wall. Mechanical pre-treatment was required to facilitate more rapid formation of VFAs.

The use of effluent from the anaerobic digestion of *Spirulina* as an electron donor and carbon for sulphate reduction was successfully demonstrated at feed sulphate concentrations of 2.5 and 5 g/ℓ. The digestate was blended with the sulphate feed to ensure the COD:sulphate ratio was maintained at 0.7. A high degree of sulphate reduction was obtained at hydraulic HRTs of 5, 4 and 3 days.

In order to operate a sulphate reducing system cost effectively on raw or partially digested microalgae, the biomass should be cultivated on site, preferably using wastewater or treated ARD as the basis of the medium. Effluent from the LFCR showed a consistently low sulphide concentration, which was reduced to zero following a brief period of aeration. While *Spirulina* could not grow on the aerated channel reactor effluent (ACRE), a number of other known species and uncharacterised environmental isolates could, although at growth rates lower than in defined media.

**GENERAL**

The primary aims of the research; to evaluate the potential of microalgae as an electron donor, and to demonstrate the benefits of recycling or retaining the sulphate reducing biomass have been met. Decoupling the hydrolysis and acidogenesis stage from the sulphate reduction was necessary to ensure stable and efficient sulphate reduction.

**CONCLUSIONS**

The most important conclusions that can be drawn from the research are:

- While the CSTR with suspended culture is a useful research tool to provide excellent kinetic data, operating conditions are constrained by the maximum specific growth rate of the community members and washout occurs when the dilution rate exceeds this. This may be overcome by retaining or recycling biomass.
- Biomass retention, by attachment to carbon microfibres, or recycling, using the cross-flow microfiltration system, resulted in significantly higher volumetric sulphate reduction rates at low (12 hour) HRT. The maximum values, 47.5 and 65 mg/ℓ.h respectively, were 20% and 50% higher than that achieved in a CSTR with no biomass retention.
- The LFCR required no mixing or external energy input and is suitable for incorporation into a passive or semi-passive treatment system. The development of a floating sulphur biofilm at the air-water interface suggests the reactor could be optimised to integrate sulphate reduction and partial sulphide oxidation into a single unit.
- The effluent from the anaerobic digestion of microalgae contained sufficient residual COD, primarily in the form of acetate and butyrate, to sustain efficient sulphate reduction. Blending the digestate and simulated AMD to maintain the desired COD to sulphate ratio of 0.7 resulted in sulphate reduction efficiencies similar to those achieved using defined media. In addition, the absence of fermentable fatty acids, such as lactate, in the digestate eliminated the competition for substrate observed when lactate was used as the electron donor.

- Decoupling the algal hydrolysis and acidogenesis stage from the sulphate reduction allowed a degree of control over the composition of the feed to the sulphate reducing reactor. In these systems an increased efficiency in sulphate reduction was observed.
- Mechanical pre-treatment to rupture the algal cells enhanced the hydrolysis and acidogenesis during anaerobic digestion of biomass with cellulosic cell walls, but was not necessary in the case of *Spirulina*.
- It was possible to cultivate certain algal species on simulated AMD that had been through the biological treatment process. The productivities were lower than on defined media, but could be improved by blending the effluent with fresh water or nutrient supplementation.

**RECOMMENDATIONS**

Analysis of the data generated during this research suggested a number of recommendations for future work. These include the following:

- Investigation of the effect of biomass retention and recycling at higher feed sulphate concentrations.
- Evaluation of the potential of integrating sulphate reduction and sulphide oxidation into a single reactor unit.
- Determination of the effect of temperature fluctuations on sulphate reduction efficiency, particularly in the LFCR.
- Optimisation of the membrane reactor unit to eliminate oxygen effectively and prevent the formation of elemental sulphur.
- Evaluation of a wider range of substrates for anaerobic digestion, including agricultural residues that could be abundant in the region of a future treatment facility.
- Completion of the techno-economic evaluation of the LFCR and membrane systems.

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# ACRONYMS and ABBREVIATIONS

| | |
|---|---|
| ACRE | Aerated channel reactor effluent |
| AD | Anaerobic digestion |
| ALD | Anoxic limestone drain |
| AMD | Acid mine drainage |
| ARD | Acid rock drainage |
| BSR | Bacterial sulphate reduction |
| COD | Chemical oxygen demand |
| CSTR | Continuously stirred tank reactor |
| HLPC | High performance liquid chromatography |
| HRT | Hydraulic retention time |
| LFCR | Linear flow channel reactor |
| MBR | Membrane bioreactor |
| MCRT | Mean cell retention time |
| OLR | Organic loading rate |
| PSS | Primary sewage sludge |
| RSBR | Recycling sludge bed reactor |
| SEM | Scanning electron microscopy |
| SRB | Sulphate reducing bacteria |
| STR | Stirred tank reactor |
| UASB | Upflow anaerobic sludge bed |
| VFA | Volatile fatty acid |
| VSLR | Volumetric sulphate loading rate |
| VSRR | Volumetric sulphate reduction rate |

# CHAPTER 1: BACKGROUND

## 1.1 INTRODUCTION

Acidic waters from mines and mining related operations continue to be a significant problem within the industrial sector, not only in South Africa but other parts of the world. As the global population and the demand for commodities continue to expand, the rapid increase in industrial activity is resulting in a greater generation of wastewaters. These wastewaters currently pose a threat to the surrounding ecosystems and habitats. In South Africa, mine water can be divided into two broad categories; that originating from groundwater rebound through abandoned mine workings, referred to as acid mine drainage or AMD in this report, and that derived from more diffuse sources, referred to as acid rock drainage (ARD). The first occurs once dewatering has ceased and is characterised by large volumes of heavily impacted water. The volume and composition of the AMD precludes the application of biological treatment options in most cases. The second type originates from diffuse sources, such as waste rock dumps, tailings impoundments, coal discard heaps and unworked pits. These sites are more numerous, are likely to affect a greater area and can persist for decades, but have received relatively little attention from the media or the authorities. Acid rock drainage from diffuse sources is more amenable to biological treatment using active, passive or semi-passive processes.

Acid mine drainage and acid rock drainage are generally rich in sulphates, sulphides and dissolved metals, although circumneutral discharges that are less saline may also occur.

## 1.2 GENERATION OF ACID ROCK DRAINAGE

Acid rock drainage is essentially caused by the exposure of sulphidic minerals to both oxygen and water as a consequence of mining and processing of metal ores and coal (Johnson and Hallberg, 2005). The sulphide minerals may be exposed as tailings or waste rock, ore stockpiles or in operating and abandoned mine workings. Acid rock drainage can be generated abiotically, through chemical weathering, but the presence of iron and sulphur oxidising microorganisms can increase the kinetics of the process up to a thousand-fold. The reactions involved are detailed below (Equations 1-4) (Akcil and Koldas, 2006).

$$FeS_2 + \frac{7}{2}\,O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \qquad \text{Equation 1}$$

$$Fe^{2+} + \frac{1}{4}O_2 + H^+ \rightarrow Fe^{3+} + \frac{1}{2}\,H_2O \qquad \text{Equation 2}$$

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+ \qquad \text{Equation 3}$$

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{3+} + 2SO_4^{2-} + 16H^+ \qquad \text{Equation 4}$$

Pyrite ($FeS_2$) is the most abundant sulphide mineral and is the primary mineral responsible for ARD generation. The process is initiated due to weathering and oxidation (Equation 1) at a neutral pH. The first reaction is abiotic. The reaction described by Equation 2 may be abiotic, but occurs slowly under acidic conditions in the absence of catalytic microorganisms. The generation of ARD is significantly enhanced when the second reaction is catalysed by aerobic iron-oxidising bacteria such as *Acidithiobacillus ferrooxidans, Leptospirillum ferroxidans* and *Leptospirillum ferriphilum* (Zagury *et al*., 2007; Johnson and Hallberg, 2003).

These particular bacteria are characterised as being acidophilic, aerobic chemoautotrophic species which are most active between pH 1.0 and pH 3.5. The iron-oxidisers are capable of increasing the rate of $Fe^{2+}$ oxidation (Equation 2) by several orders of magnitude (Gazea *et al*., 1996). Ferric iron has limited solubility and if the pH is higher than pH 2.3-3.5, it precipitates as oxyhydroxide, releasing $H^+$ and therefore lowers the pH as per Equation 3 (Zagury *et al*., 2007). The oxyhydroxide precipitate gives water a red-orange colour, which is a common characteristic of ARD discharge.

In addition to the oxidative reactions, the ferric ions may react with more pyrite as per Equation 4, producing more ferrous iron to drive Equation 2. In the presence of sufficient dissolved oxygen, a continuous cycle is

maintained (Johnson and Hallberg, 2003). The process becomes self-sustaining as the pH continues to decrease, as more ferric iron will remain in solution to chemically attack the pyrite.

A second group of microorganisms, capable of oxidising reduced sulphur species, are typically found in these environments and contribute to ARD formation. This group, which includes *Acidithiobacillus thiooxidans* and *At. caldus*, utilises reduced sulphur species as the electron donor to produce sulphate and protons. The proton acidity contributes to the low pH which typically characterises AMD. A consequence of the low pH is the dissolution of acid-labile minerals, leading to the further release of heavy metals and ions contributing to salinity.

The closure of deep-level mines poses a particularly serious threat in terms of uncontrolled AMD discharges. These workings typically intersect the water table, requiring active dewatering during operation (Adams *et al.*, 2000). Upon cessation of mining activities the dewatering is typically stopped, allowing groundwater rebound to occur (Scott, 1995; Younger, 1997). During this process previously dewatered voids gradually fill with water until a surface overflow point is encountered. Rebound not only results in a repositioning of the water table and surface discharges, but can also have a profound effect on the water quality. During dewatering water passes through the workings along discrete flow-paths which are well washed and as such any soluble minerals are flushed from them. When the workings are left to flood all the void spaces come into contact with water. Regions that have been previously unsaturated are likely to be encrusted with "acid generating salts" (iron hydroxyl-sulphates formed by partial oxidation of pyrite under unsaturated conditions), that rapidly dissolve liberating mineral and proton acidity as well as sulphate (Younger, 1997). This is termed vestigial acidity and results in a highly polluted "first flush" scenario, where active treatment will typically be required. The rate of depletion of vestigial acidity is primarily controlled by hydraulic factors and a number of models have been proposed to predict this (Younger, 2000). In contrast, juvenile acidity arises from the continued oxidation of sulphide minerals as a consequence of seasonal fluctuations in the water table or percolation through waste rock and tailings impoundments. Theoretically, juvenile acidity can persist until all the exposed sulphides have been depleted, which may take tens to hundreds of years. These effluents are less heavily polluted and are more amenable to passive treatment.

## 1.3 TREATMENT TECHNOLOGIES

A variety of technologies have been developed for the treatment of AMD and ARD. The established methods are based on oxidation, neutralisation, precipitation and sedimentation. The oxidation converts iron and aluminium to their less soluble oxidised form, which makes subsequent precipitation more efficient.

The most appropriate treatment is dependent upon the volume of the effluent, concentration type of contaminants and the pH of the water (Gazea *et al.*, 1996). Acid drainage treatment technologies can be divided into two broad categories, active and passive treatment systems.

### 1.3.1 Active treatment technologies

Active treatment typically involves the installation of agitated reactors or similar units, which require constant energy input. Furthermore, the addition of alkaline chemicals and reagents to treat the acidic effluent can become costly, given that the drainage may persist for several decades, or longer, at decommissioned mine sites (Gazea *et al.*, 1996). Many of the active treatment technologies depend on the addition of lime or limestone, which are non-renewable resources. Lime addition to sulphate rich effluents typically results in substantial gypsum precipitation, which needs to be managed. The long-term sustainability of many active treatment technologies is therefore questionable, both from an economic and environmental perspective. There is a diverse range of active treatment technologies, such as chemical precipitation, ion-exchange, membrane technology and biological sulphate reduction.

#### *1.3.1.1 Chemical treatment*

The most commonly used chemical treatment method is the addition of an alkaline material to raise the pH, in conjunction with aeration, to accelerate the rate of chemical oxidation of ferrous iron. The most common reagents are lime ($Ca(OH)_2$), slaked lime, calcium carbonate ($CaCO_3$), sodium carbonate ($Na_2CO_3$) or sodium hydroxide (NaOH). However, each compound varies in cost and effectiveness and therefore the

preferred agents are generally lime or calcium oxide, due to economic concerns (Johnson and Hallberg, 2005). Liming as a treatment process is effective in the removal of sulphate to the saturation level of gypsum ($CaSO_4.2H_2O$) and neutralisation of acidity (Equations 5 and 6) as well as the precipitation of dissolved metals as metal hydroxides. However, the resulting sludge (gypsum and metal hydroxides) is voluminous and unstable at a low pH, leading to resolubilisation of the metal hydroxides (reverse of Equation 7) (Lorax International, 2003). The major disadvantage of these treatment processes are the production and disposal of the sludge and the high cost of chemicals (Lorax International, 2003; Johnson and Hallberg, 2005).

$$Ca(OH)_2 + H_2SO_4 \rightarrow CaSO_4.2H_2O(s)(5)$$ Equation 5

$$CaCO_3 + H_2O + H_2SO_4 \rightarrow CaSO_4.2H_2O + CO_2$$ Equation 6

$$Ca(OH)_2 + H_2SO_4 + Me \rightarrow 2H^+ + CaSO_4 + Me(OH)_2$$ Equation 7

The high density sludge (HDS) process represents a technological advancement over conventional chemical technologies. It makes use of iron oxide seeds, which are added to the neutralisation reactor. The seeds promote secondary nucleation which results in the deposition of new precipitate on the existing particles. This enhances the precipitation process and leads to the formation of a denser, more granular sludge which significantly enhances the solid-liquid separation efficiency (Loewenthal *et al*., 2001; Hove *et al*., 2009). The HDS process has been selected as the initial step for the management of AMD from the dewatering of the Witwatersrand basins.

#### *1.3.1.2 Adsorption and ion-exchange*

Adsorption refers to the binding of charged species in solution to reactive groups, with opposite charge, on a solid support. The technology was widely investigated for the polishing of residual heavy metals from partially treated waste streams, with much of the focus on using biologically derived adsorbents. Despite the body of research this technology has found very limited application.

Ion-exchange refers to the replacement of a particular ion or group of ions in solution with more benign or desirable counter-ions that balance the surface charge of the solid exchanger. The resins are derived from both natural (zeolite) and synthetic (synthetic polymers) sources. They may be manufactured to contain single or multiple functional groups, depending on the application (Chiarle *et al*., 2000).

The GYP-CIX process is an example of an ion exchange technology developed specifically to treat AMD, whereby the cations ($Ca^{2+}$) are removed from the water via several fluidised contacting stages with a strong acid cation resin. Thereafter the anions ($SO_4^{2-}$) are removed via a weak base anion resin, resulting in a treated water product with a neutral pH with very little dissolved sulphates, metals and other substances. Thereafter the resin is regenerated and recycled with the only by-product being the gypsum sludge (Lorax International, 2003; Feng *et al*., 2000). The process was evaluated in a 2001 study by Schoeman and Steyn as one of three potential options for the treatment of AMD at the Grootvlei mine. In addition, it was one of the 13 processes evaluated during stage 1 selection for the Emalahleni Water Reclamation plant, but was not considered for further evaluation (Günther and Mey, 2008). More recently Earth Metallurgical Solutions has proposed an ion exchange based process for the treatment of AMD in Gauteng. Richard Doyle, the CEO, was quoted in Mining Weekly (19 November 2010) saying that the company had recently completed trials which indicated that AMD and associated reverse osmosis (RO) brines could be treated to produce potable water and high value products such as explosive components and thermal salts. The article implied that value recovery from AMD made process commercially viable.

#### *1.3.1.3 Membrane technology*

The two commercial technologies which utilise membranes in mine water treatment are reverse osmosis and electrodialysis. Electrodialysis involves an electric potential being utilised to move dissolved ions through a selectively permeable membrane. Similarly, reverse osmosis involves the forceful movement of water through a semi-permeable membrane (which excludes all but pure water) via high-pressure pumps. Reverse osmosis is a very flexible technology in that it can treat numerous types of wastewater. However, the membrane may be severely affected by fouling, depending on the quality of the feed water (Lorax International, 2003). Reverse osmosis has emerged as the technology of choice for second stage treatment of AMD in South Africa, following an initial neutralisation and precipitation step, and has been employed at

the Emalahleni Water Reclamation Plant for several years, achieving very high water recoveries (Günther and Naidu, 2008). Despite the high water recoveries, a substantial amount of hypersaline brine is produced which needs to be managed. This, together with the lime and energy requirements means the process remains costly. Eutectic freeze crystallisation is being considered as an option to assist with brine management (Nathoo *et al*., 2009).

#### *1.3.1.4 Biological sulphate reduction (BSR)*

Biological sulphate reduction (BSR) has the potential to be a more economical alternative to the costly physical and chemical processes described above. This technology is essentially dependent on the ability of anaerobic sulphate reducing bacteria (SRB) to utilise sulphate as their terminal electron acceptor. Most SRB are heterotrophic and require an organic carbon source (volatile fatty acid or short chain alcohol) as the electron donor. A small number of autotrophic species exist that are able to utilise hydrogen as the electron donor and fix carbon dioxide. Sulphate reduction may be assimilatory, where the sulphide is incorporated in sulphur-containing amino acids, or dissimilatory, where the sulphide is released to the external medium. The latter process forms the basis of AMD remediation processes as it is not directly linked to biomass growth. A generalised reaction for dissimilatory sulphate reduction is shown below (Zagury *et al*., 2007; Oyekola, 2008).

$$2CH_2O + SO_4^{2-} \rightarrow 2HCO_3^- + H_2S \qquad \text{Equation 8}$$

Whilst the sulphate is reduced to sulphide there is the simultaneous generation of alkalinity, predominantly as bicarbonate ($HCO_3^-$). From an ARD treatment perspective the alkalinity acts to neutralise the acidity while the sulphide is available for the precipitation of metals as metal sulphides (Johnson and Hallberg, 2005). Metal sulphides are particularly insoluble, even at relatively low pH values and produce more compact precipitates than hydroxide equivalents. Theoretically, sulphide precipitation is a highly effective method to reduce heavy metal concentrations to insignificant levels and thermodynamic data suggest that individual metal sulphides can be sequentially precipitated by adjusting the pH (Hammack *et al*., 1993). However, the extremely high supersaturation induced by the low solubility promotes primary nucleation, resulting in the precipitation of a large number of very small (< 0.2 µm) particles, complicating downstream separation (Mokone *et al.,* 2010).

Sulphide is a toxic, corrosive and malodorous compound which needs to be removed from the treated effluent prior to ultimate discharge. While metal sulphide precipitation can potentially remove a portion of the sulphide, the fact that most ARD is derived from pyrite ($FeS_2$) means that if the sulphate reduction process is more than 50% efficient there will always be residual sulphide. In reality the residual sulphide is normally significantly higher as the majority of the iron is removed prior to the sulphate reduction step. One attractive option for the management of residual sulphide is the partial oxidation to elemental sulphur, which can be recovered as a value adding product. This has been achieved in an active process (Janssen *et al*., 1995) and more recently in a passive system (van Hille and Mooruth, 2011; Molwantwa and Rose, 2013).

### 1.3.2 Passive treatment technologies

Natural processes typically ameliorate ARD pollution. As the contaminated water flows through the receiving systems the toxicity is remediated naturally as a result of chemical and biological reactions and dilution with uncontaminated waters. These phenomena formed the basis for the development of passive treatment technologies, which depend on naturally occurring chemical and biological reactions. Ideally these systems require no further addition of chemicals and little or no operational and maintenance inputs. Passive systems depend on processes that are kinetically slower than those involved in active systems and thus require longer hydraulic retention times (HRTs) and larger areas to achieve similar results (Hedin *et al*., 1994). As a consequence the application of passive systems tends to be limited to low volume, relatively benign wastewaters, typical of the juvenile acidity phase of AMD and to ARD from diffuse sources or at end-of-pipe from certain processes.

The interest in passive systems was sparked by research in the late 1980s which indicated that natural *Sphagnum* wetlands improved the quality of mine drainage without incurring any obvious ecological damage (Wieder and Lang, 1992). A number of experimental wetlands were constructed to mimic the *Sphagnum* moss wetlands. However, *Sphagnum* moss was not readily available, proved difficult to transplant and had the tendency to accumulate heavy metals to toxic levels within a few months (Spratt and Wieder, 1988).

Despite the initial setbacks research continued and a design evolved that proved tolerant to years of exposure to contaminated mine drainage and was effective at lowering the concentration of dissolved metals. The systems typically consisted of a series of small wetlands (< 1 ha), vegetated with cattails (*Typha latifolia*) (Wieder, 1989).

During later development the importance of anaerobic processes in metal removal was recognised. It was found that in such situations a complex ecosystem was not required and treatment cells could operate effectively without plants. Recent evidence (White *et al*., 2011) suggests that plant derived organics actually reduce the efficiency of wetlands, primarily due to the complexation of metals by phenolic compounds.

Pre-treatment systems were also developed, where the acidic waters were contacted with limestone in an anoxic environment prior to entering the settling pond or wetland system (Gazea *et al*., 1996).

#### *1.3.2.1 Anoxic limestone drains (ALDs)*

Anoxic limestone drains are an alternative method of adding alkalinity to ARD and developed as a popular "pre-treatment" stage in passive systems (Younger, 1995). An ALD is essentially buried under several metres of clay and a plastic liner is placed as an additional gas barrier between the limestone and the clay soil. Hence the ALD is almost entirely sealed from atmospheric oxygen and the accumulation of $CO_2$ promoted. The ARD water is then directed downwards via a well to the limestone whilst minimising the amount of exposure to the atmosphere (Gazea *et al*., 1996).

It is imperative that the ferrous iron remain in its reduced form. If oxidised to ferric the iron precipitates as ferric hydroxide forming a layer on the surface of the limestone. This is known as "armouring" and significantly reduces the effectiveness of the limestone and its ability to dissolve. Furthermore the accumulation of carbon dioxide is encouraged as it accelerates the rate of dissolution of limestone and hence the rate of increase in concentration of alkalinity.

Therefore, in order for an ALD to be an effective treatment method the acidic water should have a low ferric iron ($Fe^{3+}$ < 2 mg/ℓ) and aluminium concentration ($Al^{3+}$ < 2 mg/ℓ) and a low dissolved oxygen concentration (DO < 1 mg/ℓ) in order to prevent armouring (Gazea *et al*., 1996; Johnson and Hallberg, 2005).

#### *1.3.2.2 Wetlands*

The use of wetlands as passive treatment method for ARD is a relatively low-cost alternative. However, it does require a large area of land and is a slow biological process. Wetland systems can be broadly divided into two categories, aerobic and anaerobic wetlands.

Aerobic wetlands have been used effectively to treat n*et al*kaline waters. These typically contain sufficient alkalinity to buffer the acidity produced by metal hydrolysis. The aerobic systems rely primarily on oxidation reactions and the metals precipitates as hydroxides, oxyhydroxides and oxides. Aerobic wetland cells are designed to retard the flow of water sufficiently for metal oxidation, hydrolysis, precipitation and settling of the precipitate to occur. The hydrolysis reactions release protons, which retard the oxidation rate if sufficient buffering is not available. In such cases crushed limestone may be added to maintain the pH between 5.5 and 6.5, which enhances the precipitation of oxidised iron, aluminium and manganese, the primary constituents of n*et al*kaline mine waters (Younger, 1995; Gazea *et al*., 1996).

The design of aerobic constructed wetlands is similar to natural wetlands. They consist of basins and channels with a relatively impermeable base to reduce seepage. The efficiency of aerobic wetlands is dependent on dissolved oxygen concentration, so the systems are designed to include features that enhance aeration, such as steps or waterfalls. Each aeration step provides sufficient oxygen to reduce the iron concentration by approximately 50 mg/ℓ (Hedin *et al.,* 1994). Aerobic wetlands are designed to be shallow (10-50 cm) to further enhance aeration, but may include deeper (1-2 m) regions for sludge accumulation. The length to width ratio is typically 10 or greater to provide sufficient HRT.

Anaerobic or compost wetlands are mainly used in the treatment of acidic waters, whereby alkalinity is generated through bacterial activity and limestone dissolution. To encourage sulphate reduction a rich organic substrate (electron donor), such as peat, wood chips or cow manure is provided, typically in layers 30-45 cm thick (Gazea *et al*., 1996). A compost loading in the region of 250-300 kg/m$^2$ is normally used.

## 1.4 APPLICATION OF BIOLOGICAL TECHNOLOGIES TO TREAT ACID MINEWATER IN SOUTH AFRICA

### 1.4.1 Rhodes BioSURE process

Development of the Rhodes BioSURE process began at Rhodes University in the early 1990s, with observations of enhanced degradation of complex organic wastes in sulphate reducing tannery ponds (Rose, 1992; Boshoff *et al.,* 1996; Rose *et al.,* 1996; Dunn, 1998). These observations prompted the development of an integrated bioprocess for the treatment of ARD that relied on algal primary production to provide the electron donor for sulphate reduction, known as the Integrated Algal Sulphate Reducing Ponding Process for Acid Metal Wastewater Treatment, or ASPAM process (Rose *et al*., 1999). However, when the extent of AMD pollution on the East Rand became apparent it was clear that a more abundant electron donor would need to be sourced and the focus shifted to primary sewage sludge (PSS).

Encouraging results were obtained with PSS, which prompted the development of the recycling sludge bed reactor (RSBR) and a detailed study on the mechanisms of PSS degradation, specifically the enzymatic pathways involved. The fundamental research underpinning the understanding is reviewed by Rose (2013).

The RSBR concept was developed and scaled up through 2 ℓ, 10 ℓ, 3 $m^3$ to 23 $m^3$ reactor configurations. In these studies a multi-compartment baffle reactor was investigated in a second stage unit operation. In the second reactor the soluble and suspended COD, derived from the hydrolysis and fracturing of the PSS flocs in the RSBR, provided a readily available electron donor/carbon source for the separate optimisation of the sulphate reduction reaction. The sludge bed in the upflow chambers of the baffle reactor, provided for the immobilization of generally poorly adhering sulphate reducing bacteria and also the entrapment of particulate organics. Complete anaerobic digestion was reported under these conditions, with sulphate as the principal terminal electron acceptor. Control of the COD:sulphate ratio around 2:1 was found to be necessary to prevent a shift to methanogenic conditions, where the evolution of gas could cause the disruption of the up-flow sludge bed and washout of both the sludge bed and SRB (Corbett, 2001).

These findings were incorporated in the scale-up design of a pilot plant designed to treat 40 $m^3$/day of AMD. The pilot plant was constructed at the Grootvlei Gold Mine, near Springs on the Eastern Witwatersrand. At the time Grootvlei was the last remaining operating mine and was responsible for dewatering the entire Eastern Basin. Between 70-100 Mℓ/day of AMD was pumped from the mine and treated in a high density sludge process. The neutralised, metal-free effluent from the HDS process still contained a significant sulphate load and was used as the feed to the pilot operation (Corbett, 2001). The sulphide generated from the sulphate reduction was reacted with iron hydroxide sludge, converting it to an iron sulphide. The stability of the amorphous iron sulphide upon exposure to air was a concern and sludge management remained a challenge.

The encouraging performance of the pilot plant led to the development of a demonstration scale (1.6 Mℓ/day) plant using an upflow RSBR. Ultimately, in 2005, a full scale plant at the ERWAT Ancor sewage treatment works, where the PSS was readily available. The full scale plant received 10 Mℓ/day of post-HDS effluent from Grootvlei and 2 Mℓ/day of iron hydroxide sludge (Rose, 2013) using eight upflow sludge banket reactors with external sludge recycle. The process was designed to remove sulphate to levels below 250 mg/ℓ, equating to the removal of more than 12 t/day of sulphate. Sewage sludge was utilised at a rate of 0.85 mg biodegradable COD/mg sulphate reduced.

The process was operated successfully for a number of years, but has been decommissioned. This was due to changes at the utility, rather than failure of the technology. In discussion with Professor Rose, he indicated the biological sulphate reduction could have a future for the remediation of acid minewater, but would require the co-operation of utility companies, which have the infrastructure, technology and expertise required to manage large volumes of wastewaters on a daily basis and at as low a cost as possible. With these factors in place, Professor Rose considered it possible that treatment of acid minewater could be feasible over the long time-frames that need to be contemplated. He cautioned that decisive input from Government would most likely be required to facilitate the co-operation across various interest sectors necessary to realise it.

### 1.4.2 Paques biological sulphate removal technology

The Paques Thiopaq process was implemented at the Landau Colliery in Witbank to treat acid minewater at the Navigation site. The plant was commissioned as one of the options to be evaluated by Anglo Coal and Ingwe Collieries (BHP Billiton) for the Emalahleni Mine Water Reclamation plant and was managed by IST Technik. The design specifications were for the treatment of 20 Mℓ per day, with a water recovery of greater than 95%. The raw water had a pH of 3.12 and the following major components: sulphate (2500 mg/ℓ), calcium (536 mg/ℓ), iron (81 mg/ℓ), aluminium (16 mg/ℓ) and manganese (23 mg/ℓ). Targets for product water quality were sulphate (<200 mg/ℓ), calcium (<30 mg/ℓ) and heavy metals at less than 0.15 mg/ℓ. A demonstration scale plant, capable of treating 3 Mℓ/day, was commissioned and operated for several years. The performance of the plant was encouraging, with effluent sulphate concentrations typically below 500 mg/ℓ. The operators did encounter challenges, particularly with scaling of the heat exchangers. In addition, the licensing fees and the cost of the electron donor (ethanol) counted against the technology. Despite relatively stable performance the system was not selected for implementation (Günther and Mey, 2006), receiving low scores on “operability issues”, “risk management”, “reliable achievement of project targets” and “safety, health and environment”.

### 1.4.3 Integrated Managed Passive (IMPI) process and passive bio-neutralisation

The disadvantages of active treatment systems and enhanced sustainability of more passive systems has been highlighted in the preceding sections. In South Africa, the research into passive treatment options has focussed heavily on the development of passive anaerobic sulphate reduction technologies (Pulles *et al*., 2003). The research has been led by Pulles Howard and de Lange (PHD) and Golder Associates Africa (GAA), with contributions from a number of academic institutions, particularly Rhodes University, the University of Cape Town and the University of Pretoria and has addressed the problem at both a fundamental science and more applied pilot plant level (Pulles and Heath, 2009).

The programme addressed two primary challenges, the low sulphate reduction efficiencies reported for systems developed abroad and significant inhibition of existing systems at pH levels below pH 4.5.

The development of the IMPI process began in 1995, with the aim of developing a system that could achieve high rates of sulphate reduction over a sustained period, utilising lignocellulosic material as the source of electron donor. The hydrolysis of lignocellulose was identified as the rate limiting step. The long-term reactor studies that were undertaken as part of the programme allowed the characterisation of five distinct phases of sulphate reduction in passive systems (Coetser *et al*., 2005; Molwantwa *et al*., 2010). These were a lag phase (90-150 days), where the microbial community became established and adapted to the environment, a high performance phase (< 8 months), during which high rates of sulphate reduction were maintained, followed by a crash phase and finally a sustained phase, where relatively low rates of sulphate reduction were observed. Eventually, sulphate reduction ceased once all the hydrolysable lignocellulose had been consumed. Pulles and Heath (2009) suggested that many published studies were not conducted for long enough to reach the crash phase, resulting in optimistic projections of longer-term performance.

The initial target of the research was to develop a system capable of sustained sulphate reduction of over 600 mM/$m^3$.d, considered the threshold for economic viability. Based on the understanding that hydrolysis of lignocellulose was rate limiting, research was conducted to investigate the potential of pre-treatment, using white rot fungi to break down the lignin matrix. The technical feasibility of the system was demonstrated, but unfavourable economics led to the termination of the research.

The second approach, pursued in collaboration with Rhodes University, focussed on understanding the mechanisms of lignocellulose hydrolysis. The research suggested that the degradation of lignocellulose was enhanced under sulphidogenic conditions (Roman, 2006) and that the provision of some readily usable organic carbon (molasses) could significantly reduce the effect of the crash phase and enhance the level of sulphate reduction in phase 4 (sustained phase). These insights led to the development of the patented degrading packed bed reactor (DPBR), which formed the basis of the IMPI process (Figure 1). The DPBR has an optimised packing configuration, with layers of different carbon sources designed to ensure efficient performance. The upper part of the reactor is responsible for removing dissolved oxygen and generating sulphide and alkalinity, which the lower part of the reactor is the site of accelerated lignocelluose degradation and volatile fatty acid production. To ensure efficient operation the influent is typically supplemented with molasses (0.05-0.1%) as a readily utilisable carbon source (Coetser *et al*., 2005).

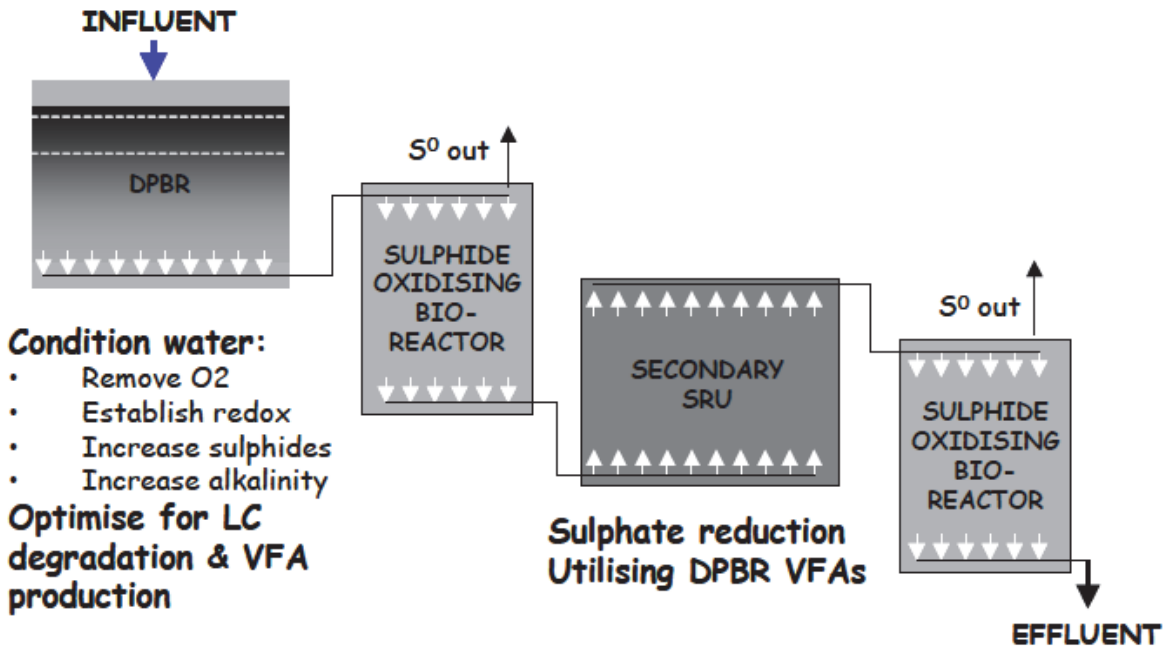



*Figure 1: Schematic representation of the IMPI process (Pulles and Heath, 2009)*

The overall configuration typically consisted of two sulphate reducing units, the DPBR and a secondary sulphate reduction unit, and two biological sulphide oxidising units which are operated under conditions that promote the partial oxidation of sulphide to elemental sulphur, rather than the complete oxidation back to sulphate. The sulphide oxidation units were not supplied with organic carbon.

A long term (4 years) study conducted at Vryheid Coronation Colliery (VCC) showed stable performance, with sulphate reduction rates significantly higher than the 600 mM/m$^3$.d target. Performance improved over the summer months, confirming the impact of temperature on sulphate reduction rate.

A full scale system, designed to treat 200 m$^3$ of minewater, was constructed at the Middelburg mine in Mpumalanga. The system contained a novel sulphide oxidation reactor, the linear flow channel reactor (LFCR) which made use of a floating sulphur biofilm to achieve partial oxidation of sulphide. The system was plagued by a number of construction and operational issues, as well as challenges with the LFCR and performance did not meet expectations. A detailed study into the LFCR was conducted at the University of Cape Town, leading to further optimisation in design and operating parameters (van Hille and Mooruth, 2013).

The passive bio-neutralisation programme was launched in 2003, with the aim of treating acid minewaters (pH <3). The patented DPBR formed the basis for the treatment system. A detailed microbial ecology study identified three distinct microbial communities and it was speculated that the success of the system depended on the interaction and tight special orientation of these communities. The first community was responsible for removing residual oxygen from the system and establishing the necessary redox environment (-250 to -350 mV). The second community, characterised by *Clostridium* species enabled the efficient degradation of lignocellulose and the generation of substrate, small charge-neutral carbon compounds, for the sulphate reducing bacteria than made up the third community. Alkalinity, in the form of bicarbonate, is generated following the oxidation of the organic electron donor, coupled to the sulphate reduction reaction. Laboratory scale tests, treating simulated minewater with an average pH of 3, have shown consistent performance over a six year period, with effluent pH values ranging between pH 5.5 and pH 7.

## 1.5 CONSTRAINTS TO MORE WIDESPREAD APPLICATION

A number of acid minewater treatment technologies based on biological sulphate reduction have been tested at pilot, demonstration and commercial scale in South Africa, but the application of the technologies has not been widespread. This can be attributed to a mixture of political and technical reasons.

The rebound of groundwater through the Witwatersrand basins and the potential impacts of uncontrolled discharge has commanded the attention of the media and the authorities in recent years. The anticipated volume and composition of the AMD largely precludes the application of biological treatment options, so the spotlight has been focussed on chemical treatment processes. The perception of biological treatment options has been influenced by public statements from a number of high profile individuals who are sceptical of their feasibility. In an interview with Edmund Furter of sheqafrica.com (26/11/2010) Professor Terence McCarthy, an advisor to the Inter-ministerial Committee on AMD, was quoted as saying "I do not like bioremediation in principle, I recommend standard technology, with acidity treatment by adding lime, and flocculation, leaving a smaller waste stream", when asked about the Rhodes BioSURE and other biotechnological processes. Similarly, personal communication with representatives of a number of engineering and environmental consultancies at events such as the WISA AMD forum has revealed an inherent scepticism of biological processes, with several people citing the absence such systems in South Africa as justification.

From a technical perspective we have identified three challenges that would need to be overcome to make systems based on biological sulphate reduction more attractive. These are the provision of a low cost, readily usable electron donor, the enhancement of reaction rates and improved management of the resulting sulphide. The demonstration of a well-designed pilot plant, that addresses the challenges listed above, is essential to convince Government and industry of the potential of biological treatment.

## 1.6 PROJECT AIMS

The aims addressed in the project were as follows:

1. Critically evaluate existing SRB-based technologies (active and passive).
2. Evaluate microalgae as a carbon source and electron donor for SRB systems, both in terms of digestibility and the ability to cultivate sufficient quantities. Evaluate the potential to cultivate algae using treated AMD as the basis for the growth medium.
3. Evaluate the effect of decoupling the hydrolysis and acidogenesis steps from the sulphate reduction.
4. Review reactor options for the retention of biomass and the creation of specific reaction zones.
5. Investigate the use of cross-flow microfiltration as a model biomass retention strategy for the sulphate reduction reactor.
6. Investigate novel reactor configuration to achieve biomass retention that has the potential for application in active and passive systems.

The initial project proposal included an aim related to the techno-economic evaluation of the novel reactor configurations, but this aim was removed following discussion with the reference group. The project team felt that the novel configurations needed to be tested under a wider range of conditions before a meaningful evaluation could be undertaken.

# CHAPTER 2: MATERIALS AND METHODS

## 2.1 INTRODUCTION

This chapter presents an overview of the algal and microbial cultures used in this research, a description of the individual reactor units and the detail of the routine analyses performed. The specific experimental programme relating to each of the sets of experiments is described in detail in the relevant chapters.

## 2.2 ALGAL AND MICROBIAL CULTURES

### 2.2.1 *Scenedesmus* sp.

Stock cultures of the microalga *Scenedesmus* (isolated from ponds at Cape Carotene, Upington) were maintained at ambient laboratory temperature (25°C ± 2ºC) in 500 mℓ flasks, sparged (2 ℓ/min) with air and illuminated with three fluorescent bulbs (120 µmol photon/$m^2$.s at the surface) from one side of the flask. All *Scenedesmus* cultures were grown on *Botryococcus* media comprising of: nutrients (0.75 g/ℓ $NaNO_3$, 0.075 g/ℓ $K_2HPO_4$, 0.175 g/ℓ $KH_2PO_4$, 0.025 g/ℓ $CaCl_2.2H_2O$ and 0.025 g/ℓ NaCl) and a metal solution (0.75 g/ℓ $Na_2EDTA$, 0.017 g/ℓ $FeCl_3.6H_2O$, 0.041 g/ℓ $MgCl_2.4H_2O$, 0.005 g/ℓ $ZnCl_2$, 0.002 g/ℓ $CoCl_2.6H_2O$, and 0.004 g/ℓ $Na_2MoO_4.2H_2O$) at a concentration of 6 mℓ/ℓ. Unless otherwise stated, all regents were analytical grade, sourced from Merck. The cultures were further inoculated into airlift photobioreactors (3.2 ℓ) and a raceway pond (50 ℓ) for generation of the required biomass for subsequent experiments.

### 2.2.2 *Spirulina* sp.

Stock cultures of the cyanobacterium *Spirulina* (isolated from disused ponds at Western Tanning, Wellington) were maintained at ambient laboratory temperature (25°C ± 2ºC) in 2 ℓ flasks, continuously mixed with a magnetic stirrer and illuminated with three fluorescent bulbs (120 µmol photon/$m^2$.s at the surface) from one side of the flask. All *Spirulina* cultures were grown on Zarrouk's media comprising of: nutrients (18 g/ℓ $NaHCO_3$, 2.5 g/ℓ $NaNO_3$, 0.5 g/ℓ $K_2HPO_4$, 1 g/ℓ $K_2SO_4$, 0.04 g/ℓ $CaCl_2.2H_2O$, 1 g/ℓ NaCl, 0.2 g/ℓ $MgSO_4.7H_2O$, 0.01 g/ℓ $FeSO_4.7H_2O$ and 0.08 g/ℓ EDTA), metal solution A5 (2.86 g/ℓ $H_3BO_3$, 1.81 g/ℓ $MnCl_2.4H_2O$, 0.22 g/ℓ $ZnSO_4.7H_2O$, 0.08 g/ℓ $CuSO_4.5H_2O$ and 0.0124 g/ℓ $Na_2MoO_4$) at a concentration of 1 mℓ/ℓ and metal solution B6 (56.6 mg/ℓ $K_2CrO_7$, 47.8 mg/ℓ $NiSO_4.7H_2O$ and 4.2 mg/ℓ $CoSO_4.7H_2O$) at a concentration of 1 mℓ/ℓ. The cultures were further inoculated into airlift photobioreactors (3.2 ℓ) and a raceway pond (50 ℓ) for generation of the required biomass for subsequent experiments.

### 2.2.3 *Chlamydomonas debaryana*

Stock cultures of *Chlamydomonas debaryana* (isolated from a wastewater treatment facility in Potchefstroom) were maintained in small (100 mℓ) bottles on 3N BBM growth medium, under constant aeration. Continuous illumination was provided by two fluorescent bulbs providing 50-80 µmol/$m^2$.s photosynthetically active radiation (PAR). The growth medium consisted of: nutrients (0.75 g/ℓ $NaNO_3$, 0.075 g/ℓ $MgSO_4.7H_2O$, 0.005 g/ℓ NaCl, 0.075 g/ℓ $K_2HPO_4.3H_2O$, 0.175 g/ℓ $KH_2PO_4$ and 0.025 g/ℓ $CaCl_2.2H_2O$), trace elements (0.75 g/ℓ $Na_2EDTA$, 97 mg/ℓ $FeCl_3.6H_2O$, 41 mg/ℓ $MnCl_2.4H_2O$, 5 mg/ℓ $ZnCl_2.6H_2O$, 2 mg/ℓ $CoCl_2.6H_2O$ and 4 mg/ℓ $Na_2MO_4.2H_2O$) at a concentration of 6 mℓ/ℓ and 1 mℓ/ℓ of vitamin B1 stock (1.2 g/ℓ) and vitamin B12 stock (1 g/ℓ).

### 2.2.4 *Parachlorella hussii*

*Parachlorella hussii* was isolated from a laboratory scale reactor treating thiocyanate containing effluent in the CeBER laboratories at UCT. The stock culture was maintained in small (100 mℓ) bottles on 3N BBM growth medium, under constant aeration. Continuous illumination was provided by two fluorescent bulbs providing 50-80 µmol/$m^2$.s PAR.

### 2.2.5 Anaerobic digestion inocula

Two different inocula in were used to provide the required anaerobic microbes in all anaerobic digesters. The first inoculum was obtained from an anaerobic digester treating brewery effluent (South African Breweries (SAB), Newlands, Cape Town). The second component of the inoculum was obtained from a 1 ℓ stock reactor that had been adapted to *Spirulina*. The stock reactor was inoculated with 20% (vol/vol) activated sewage sludge. The stock digester was operated in fed batch mode with intermittent loading of *Spirulina*. Gas production and composition were monitored to ensure that the digester was operating efficiently. The digester was harvested when necessary and the volume gradually increased back to the operating volume of 1 ℓ by addition of *Spirulina* slurries. The method of inoculation utilised in this study ensured a relatively consistent initial community of microorganisms in each digester.

### 2.2.6 Sulphate reducing bacteria (SRB) inoculum

The SRB mixed microbial community was obtained from the Department of Microbiology, Biochemistry and Biotechnology at Rhodes University, originally from the anaerobic compartment of a facultative pond at the Grahamstown sewage treatment works, and has been maintained at UCT since 1999. The stock culture has been maintained on modified Postgate B medium consisting of: 0.5 g/ℓ $KH_2PO_4$, 1 g/ℓ $NH_4Cl$, 2 g/ℓ $MgSO_4.7H_2O$, 1 g/ℓ $Na_2SO_4$, 1 g/ℓ yeast extract, 6 mℓ/ℓ 60% sodium lactate solution (Sigma), 0.3 g/ℓ sodium citrate. Previously, the stock culture has been used to generate cultures adapted to ethanol and acetate (Moosa *et al*., 2002; Erasmus 2005).

## 2.3 REACTOR UNITS

### 2.3.1 Photobioreactors

A portion of the algal biomass used for batch digestion studies was generated in 3.2 ℓ glass and stainless steel, internal loop airlift reactors described fully by Langley *et al*. (2012) and shown in Figure 2. The photobioreactors consisted of a 600 mm high, 100 mm outside diameter (OD) column and a 50 mm OD draught tube. Air, normal or enriched with $CO_2$ was sparged into the column through a 0.22 µm stainless steel HPLC inlet filter, located at the base of the draught tube. Air and $CO_2$ flow rates were controlled with a Brooks 5850S Thermal Mass Flow Controller. The two gas streams passed through an inline mixer and were fed to each column at a flow rate of 2 ℓ/min. Light was supplied continuously by three Osram 18 watt cool white fluorescent bulbs at a distance of 3 cm from the column surface, providing 300 µmol/$m^2$.s PAR.

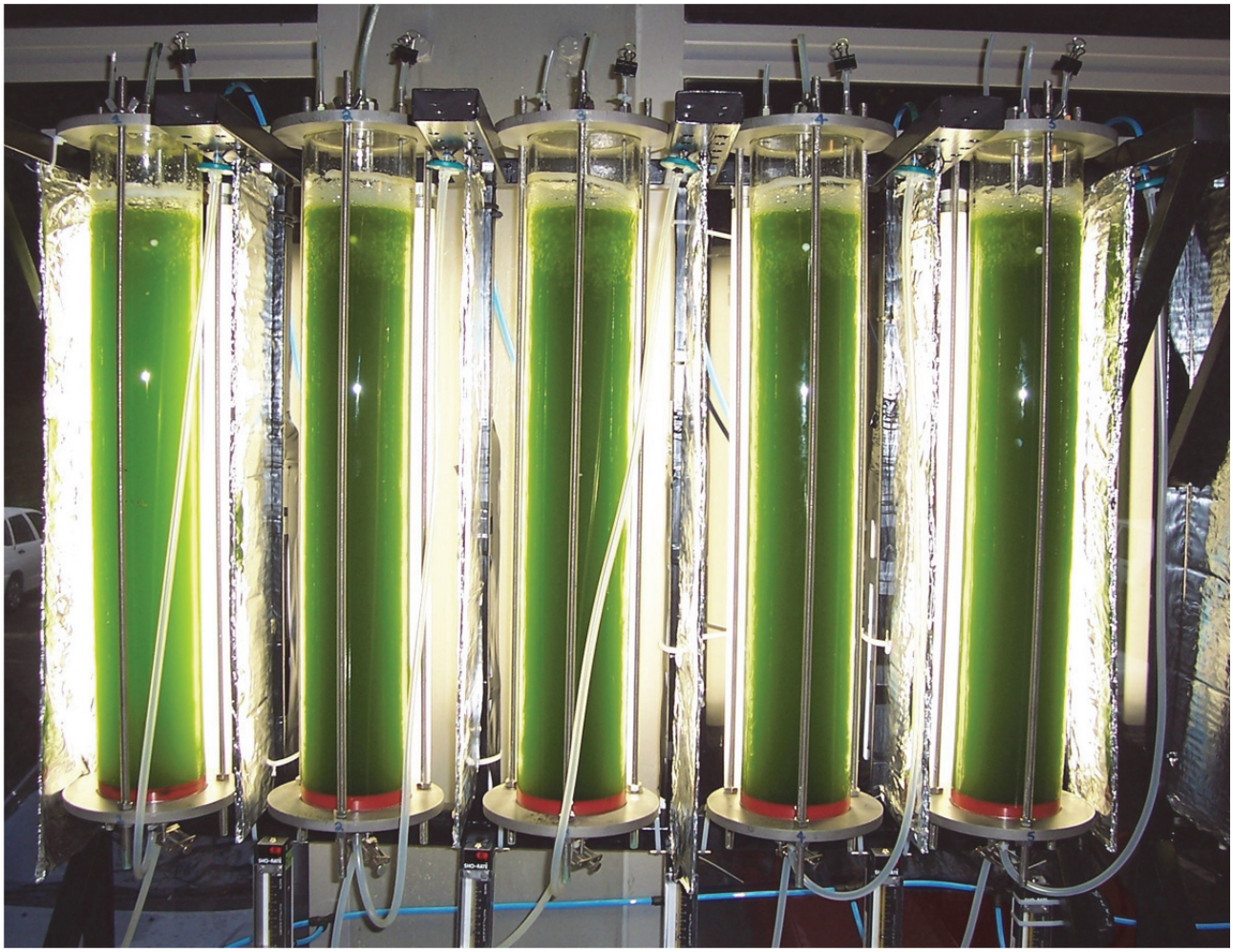


*Figure 2: Photograph showing a bank of five airlift photobioreactors used to perform the algal growth experiments*

### 2.3.2 Raceway pond

A 50 ℓ Perspex raceway pond with a paddle wheel was used for larger-scale algal cultivation. Where aeration was required, it was introduced through a 6.35 mm stainless steel tube, with 1 mm holes drilled every 20 cm, positioned on the reactor floor. Circulation of the medium was achieved using a four bladed paddle wheel. The speed could be varied depending on the requirements for the particular algal species. The liquid depth was 10 cm and the total surface area was 0.51 $m^2$. Six 58 watt and two 36 watt fluorescent bulbs provided continuous light to the cultures. A photograph of the raceway pond is shown in Figure 3.

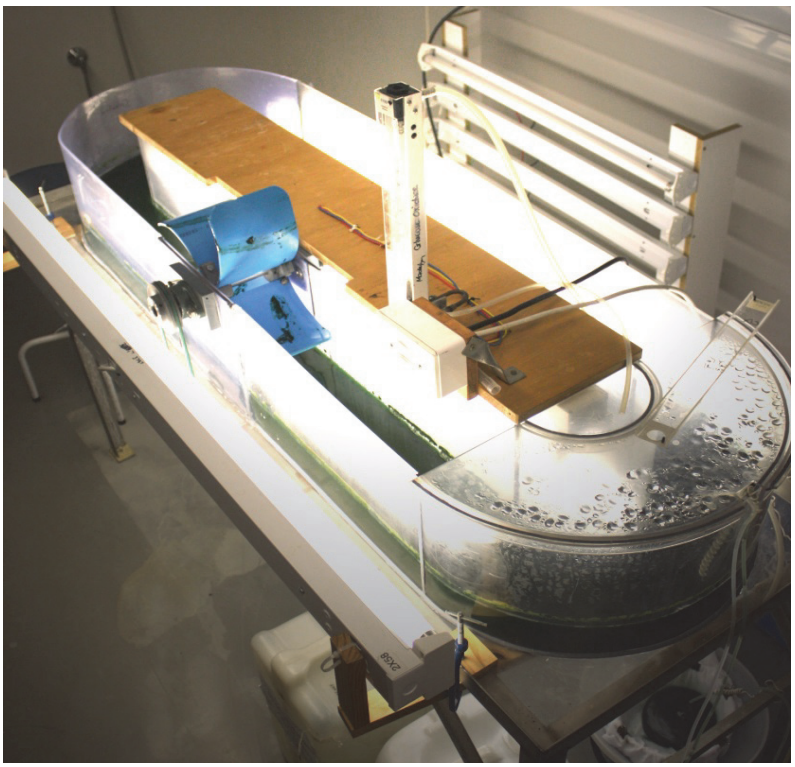


*Figure 3: Photograph showing the raceway used for larger-scale algal cultivation*

### 2.3.3 Anaerobic bench-top batch reactors

The digestion experiments were carried out in continuously mixed bench-top batch reactors. The reactors were constructed from 1 ℓ Duran Schott bottles and were operated with minimal headspace. The lid of the reactor was modified to include three ports. The first port was used for biogas collection, the second for liquid sampling and third for biogas sampling to determine composition. The units were placed in a controlled environment room at 37±2ºC, and were continuously mixed on an orbital shaker (140 rpm). Biogas was collected using water displacement vessels filled with saturated sodium chloride (NaCl) solution to minimise the dissolution of $CO_2$. To prevent any backflow of NaCl into the reactors, 1 ℓ trap bottles were inserted on all biogas lines. These trap bottles were fitted with a secondary biogas sampling point. The volume of biogas produced was corrected for normal temperature and pressure (STP). The digester pH could be controlled by injecting 5 M sodium hydroxide (NaOH).

### 2.3.4 Fed-batch anaerobic digester

The semi-continuous anaerobic reactor, shown in Figure 4, consisted of: a 10 mm thick PVC tube (640 × 100 (ID) mm) fitted with an overflow (510 mm from base) and continuous feed port (5 mm from base). The positioning of the overflow port allowed for a 4 ℓ liquid volume and 1 ℓ headspace volume. The PVC tube was machined and glued into a solid PVC base (120 × 120 × 12 mm). The inner lining of the tube was fitted with four (640 × 1 × 10 mm wide) Perspex strips, which acted as baffles to improve mixing. The top of the tube was flanged such that a lid could be screwed on with four butterfly nuts and bolts. The lid was sealed with a 105 mm (OD) silicone O-ring, which was placed in a groove that had been machined into the base of the lid. The lid was fitted with four ports. The first port had a stainless steel tube (510 × 8 mm) running through to a ball valve (10 bar specified) for substrate feeding. The second port was fitted with a 12.7 mm check valve (3 bar cracking pressure) to serve as the safety relief valve. The third port was fitted with a 3.18 mm pressure gauge (4 bar rating) for headspace pressure readings. The fourth port was fitted with a ball valve (10 bar specified) for gas collection and sampling. Continuous mixing was achieved using a magnetic stirrer.

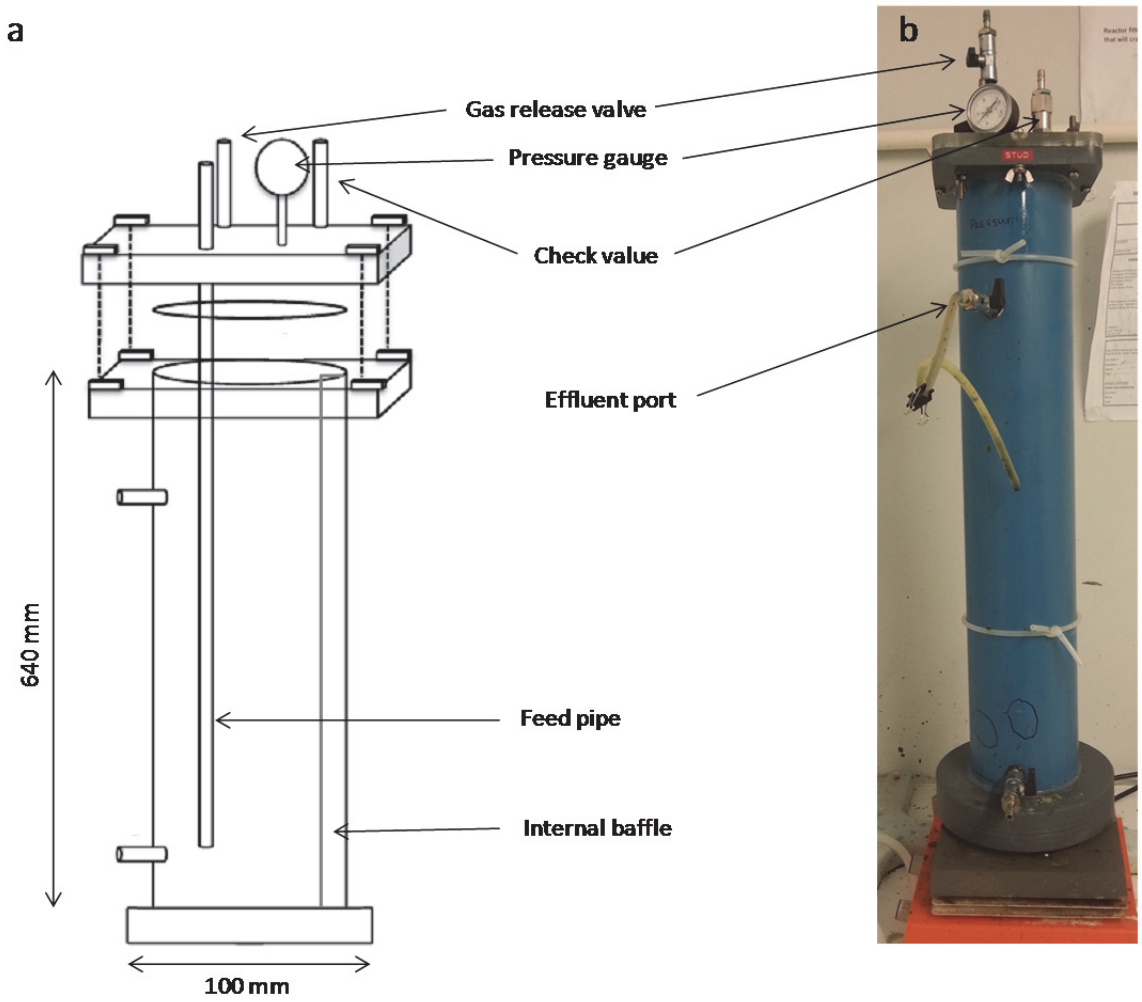



*Figure 4: Schematic diagram (a) and photograph (b) of the reactor used for the fed-batch anaerobic digestion of algae*

### 2.3.5 Continuously stirred biological sulphate reduction reactor

Continuous experiments were performed in glass reactors with a working volume of 1 ℓ. These were refined from the reactor setup reported by Moosa *et al*. (2002) and Oyekola *et al*. (2009) for kinetic studies of biological sulphate reduction using suspended culture and are shown in Figure 5. The reactor height was 200 mm, with a liquid volume height of 118 mm, and diameter 104 mm. Agitation was provided by an overhead stirrer powering a four-bladed marine impeller (58 mm diameter) at 300 rpm. The reactor was fitted with four vertical baffles (10 mm width) to prevent vortex formation. The reactor lid was specially designed and constructed to reduce the possibility of air ingress. The impeller shaft passed through a column containing three rubber lip seals, which effectively sealed the unit. In addition, the lid was fitted with a feed port, a sampling port and a gas venting port. Offgas was passed through a series of sulphide stripping units (1 M NaOH) for health and safety reasons and to allow the quantification of any sulphide lost as $H_2S$ gas. Temperature was controlled at 30°C by pumping heated water through the external jacket or placing the reactors in a temperature-controlled water bath. Feed solution was continuously pumped into the reactor using a variable speed peristaltic pump and, in the case of reactors fed on algae; the additional slurry was injected in through a port in the reactor lid at designated time intervals.

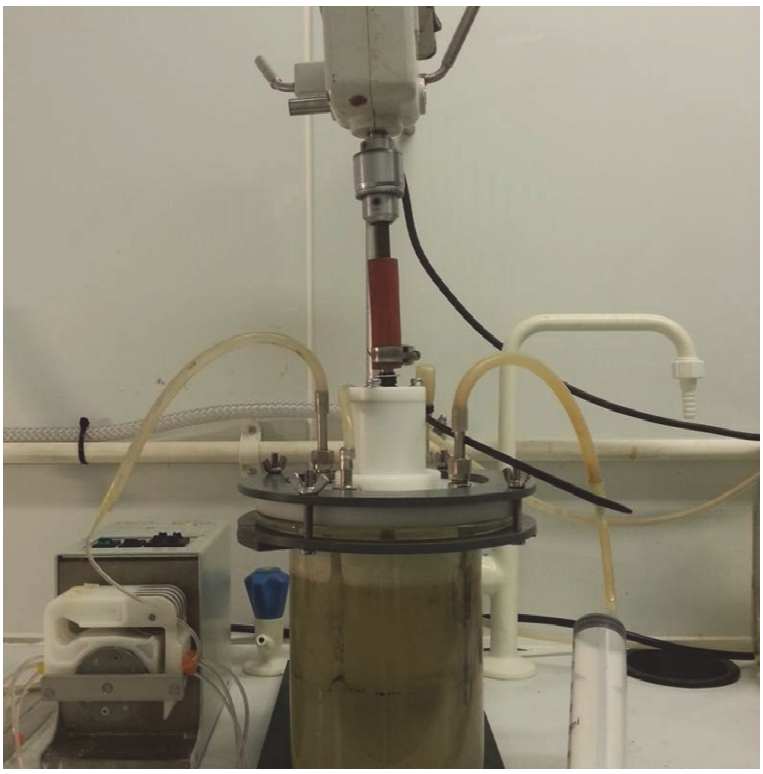


*Figure 5: Photograph of the 1 ℓ CSTR used to generate baseline data for biological sulphate reduction, using suspended culture under reactor conditions designed for biokinetic studies*

### 2.3.6 Sulphate reduction reactor with microfiltration unit

The reactor configuration for the cross-flow microfiltration unit is shown in Figure 6, with a photograph of the unit in Figure 7. The system consists of the standard 1 ℓ glass reactor coupled to a microfiltration unit. The contents of the reactor are pumped through the membrane unit at a rate of 1.7 ℓ/min, meaning the entire volume passes through the membrane every 35 seconds. As a result, the reactor can be considered well mixed and additional agitation by the impeller was not required. This was confirmed through mixing studies.

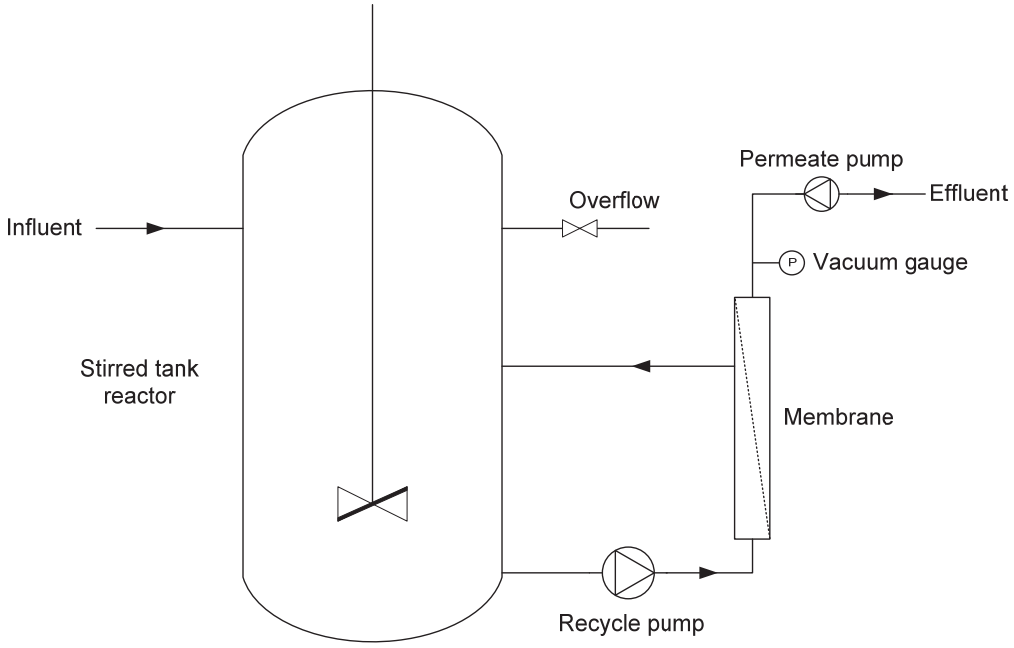



*Figure 6: Schematic of sulphate reduction bioreactor coupled to membrane filter for biomass retention*

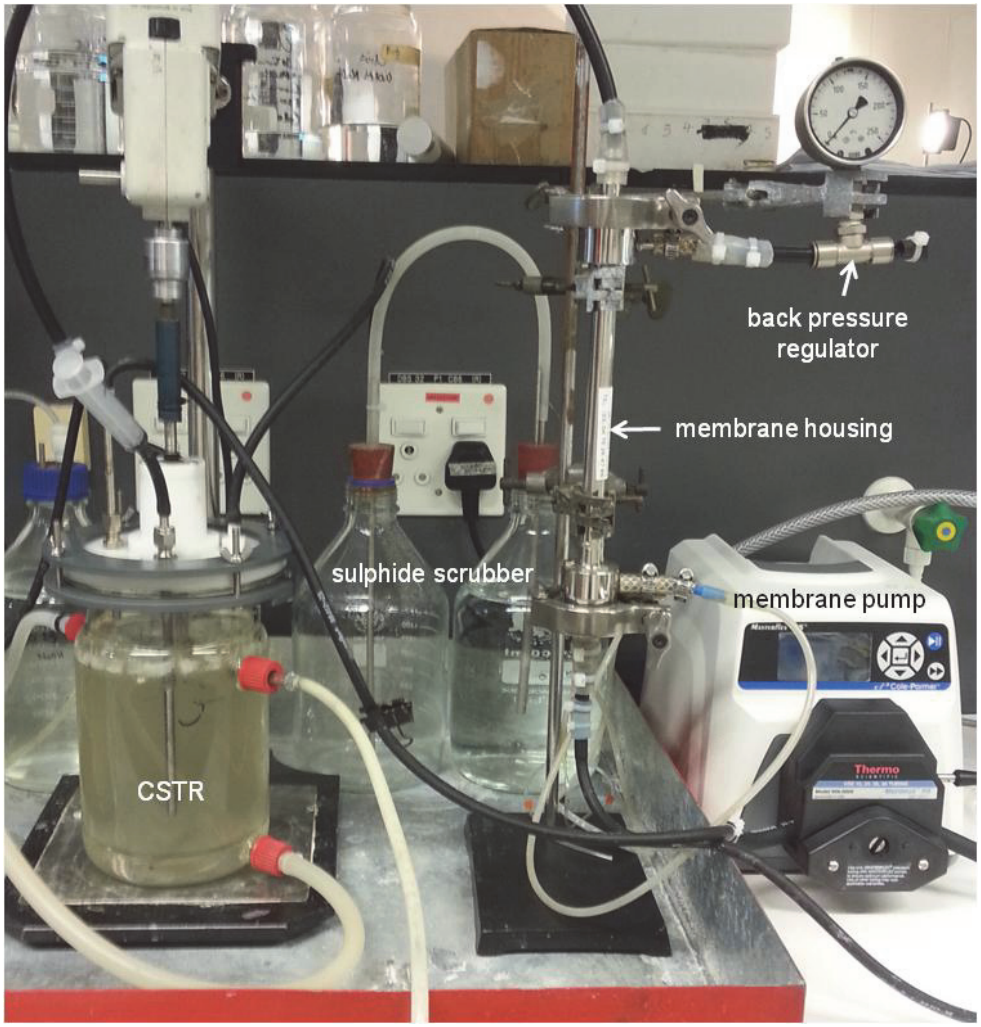



*Figure 7: Photograph of the cross-flow microfiltration unit connected to the sulphate reduction reactor.*

### 2.3.7 Biological sulphate reduction channel reactor with carbon microfibres

The channel reactor provided a flow-through reactor with medium stratification in place of homogeneity. It was constructed from Perspex (11 mm thickness). The front wall of the reactor was fitted with three sets of sample ports, located 60 mm, 120 mm and 180 mm from the inlet. At each distance, there were three ports, 25 mm, 55 mm and 90 mm from the base of the reactor. Each port was fitted with a GC septum and samples were withdrawn with a hypodermic needle. The reactor was fitted with three feed ports (25 mm, 60 mm and 95 mm from the base) in the left wall and three effluent ports (15 mm, 50 mm and 85 mm from the base) in the right wall. When the top outlet port was utilised the liquid height in the reactor was 85 mm, giving a working volume of 2.125 ℓ. The reactor was fitted with a lid and an airtight silicon seal. A port fitted 10 mm below the lid in the left and right hand walls allowed the headspace to be flushed. A strip (38 mm wide) of carbon microfibres (AMT Composites, Cape Town) was attached to the bottom of the lid so that the fibres were submerged in the liquid. The strip had a bundle of microfibres (180 mm long) attached at 7 mm intervals on each side (Figure 8).


*Figure 8: Photograph of carbon microfibres showing macrostructure (A) and detail of a single bundle (B) illustrating the large surface area for microbial attachment*

The reactor was operated continuously by pumping feed in from the uppermost feed port and collecting effluent from the uppermost effluent port (Figure 9).

Subsequent to the collection of the first dataset, using the configuration described above, the reactor was modified. A heating coil was included (fitted to the lowest of the three ports in the left and right walls), facilitating temperature control outside of the constant environment room. In addition, the configuration of the carbon fibres was optimised by attaching them to a submerged strip, rather than fixing them to the roof of the reactor (Figure 10). This allowed the lid to be removed without disturbing the fibres, so samples could be removed for scanning electron microscopy (SEM) and other analyses.

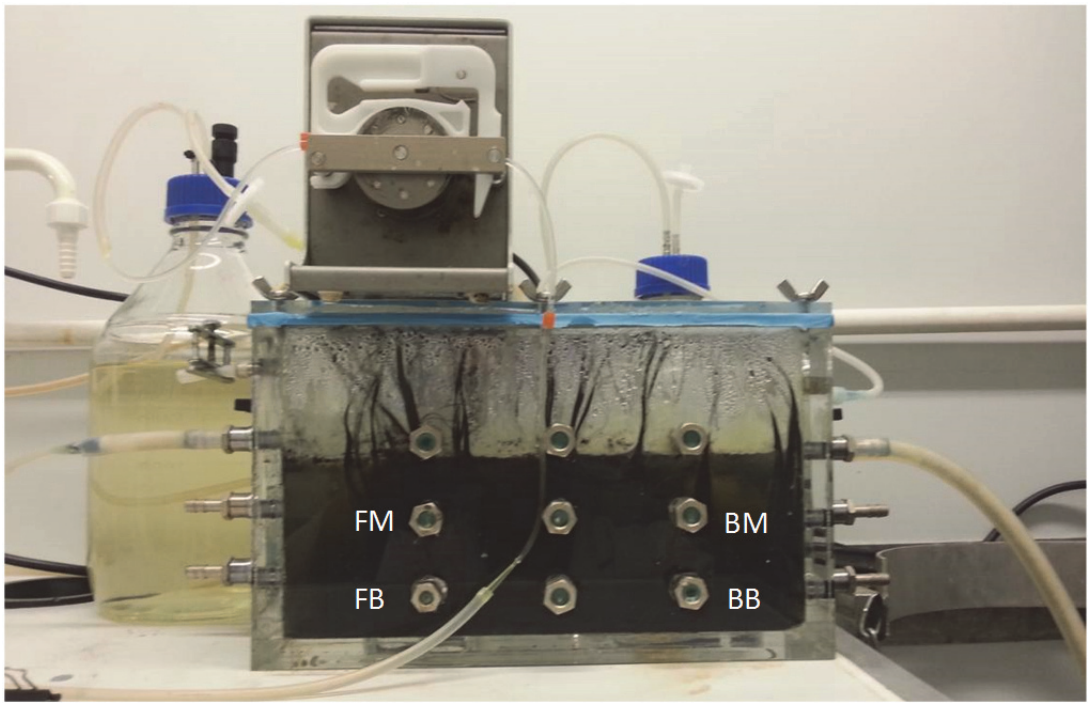



*Figure 9: Photograph showing the channel reactor configuration. The sample ports from which samples were drawn are highlighted*

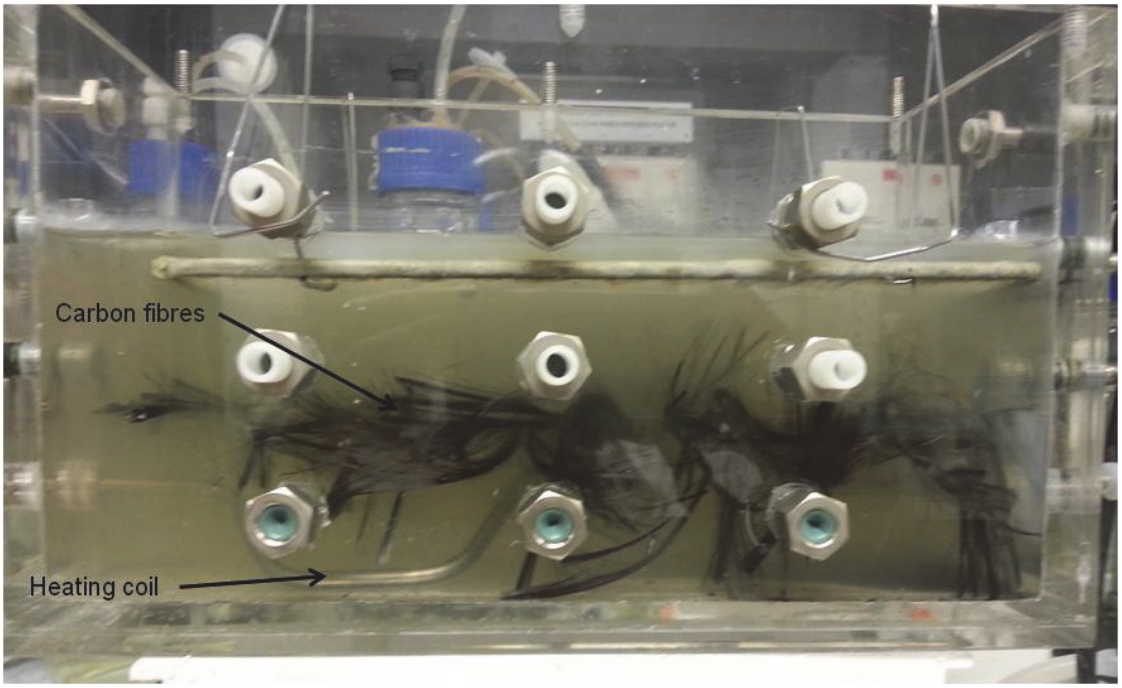



*Figure 10: Photograph of the modified channel reactor illustrating the heating coil and optimised arrangement of the carbon fibres*

## 2.4 BEAD MILL

Batch phase bead milling was used as the mechanical pre-treatment to rupture the algal cells. The "bead milling" was achieved by agitation in a 1 ℓ stirred tank reactor, at 900 rpm, using a 20 mm diameter Rushton turbine; mixing was enhanced by four 10 mm wall baffles. Based on the research of Scholtz *et al.* (1997), the mill was loaded with glass beads (1 mm diameter) at 35% vol/vol for the smaller *Scenedesmus* and with larger beads (4 mm diameter) at the same loading for the filamentous *Spirulina* cells. The mill ran for a time period that allowed for complete disruption of the algal cells.

## 2.5 ANALYTICAL METHODS

### 2.5.1 Biomass concentration using optical density

Algal biomass concentration was determined daily by measuring optical density at 750 nm with a Helios spectrophotometer and converting these to dry mass concentration using a calibration curve. The sample was first diluted to ensure that the absorbance measured did not exceed 1. The final concentration was adjusted using this dilution factor. Bacterial cultures were similarly measured at a wavelength of 660 nm.

### 2.5.2 Biomass concentration using dry weight

Algal biomass dry weight was measured by filtration of a 5 mℓ sample through a pre-weighed 0.22 µm glass fibre filter paper, which was then dried at 80°C overnight before being re-weighed.

### 2.5.3 pH

All pH testing was done on a Cyberscan 2500 micro pH meter. The meter was calibrated daily using standard (pH of 4.0 and 7.0) buffer solutions.

### 2.5.4 Sulphide

Aqueous sulphide was quantified using the colorimetric DMPD method (APHA, 2005). The principle of the method is reaction of aqueous sulphide with N,N-dimethyl-p-phenylenediamine (DMPD), catalysed by ferric ions, to produce methylene blue. An appropriate volume of sample (10-4800 µℓ) is added to 200 µℓ of 1% zinc acetate. The volume is made up to 5 mℓ with deoxygenated water, after which 500 µℓ of 0.4% N,N-dimethyl-p-phenylene diamine (in 6 M HCl) and 500 µℓ of 1.6% ferric chloride (in 6 M HCl) are added. The sample is mixed well and left to react for a minimum of 5 minutes after which the absorbance is read at 670 nm and the concentration determined relative to a standard curve. The assay has a maximum detection limit of just over 1 mg/ℓ so significant dilution is required. This is typically achieved by using a small volume (20-50 µℓ) of sample.

### 2.5.5 Ammonium ions

Ammonium ion concentration was determined by HPLC using a Waters Breeze 2.0 system equipped with a Hamilton PRP-X200 Polymeric cation exchange column (4.1 × 150 mm) and a conductivity detector. The system was run isocratically using a 4 mM nitric acid in 30% methanol mobile phase. The HPLC was run at ambient laboratory temperature (25±2°C) with a mobile phase flow rate of 1 mℓ/min. The pressure in the column did not exceed 1600 psi. Sample injection volumes of 100 µℓ were used. To quantify the ion concentration, standard solutions (50, 100, 500 and 1000 mg/ℓ $NH_4^+$) were prepared using $NH_4Cl$.

### 2.5.6 Sulphate, nitrate and phosphate ions

Aqueous sulphate, nitrate and phosphate ion concentrations were measured by HPLC using a Waters Breeze 2.0 system equipped with a Waters IC-Pak A HR (Anion High resolution) column and a conductivity detector. The system was run isocratically using a sodium borate-gluconate mobile phase at a flow rate of 1 mℓ/min.

The HPLC was run at ambient laboratory temperature with a mobile phase flow rate of 1 mℓ/min. The pressure in the column did not exceed 2000 psi. Sample injection volumes of 100 µℓ were used. To quantify the ion concentrations standard solutions (20, 40, 60, 80 and 100 mg/ℓ $SO_4^{2-}$, $Cl^-$, $NO_3^-$ and $PO_4^{3-}$) were prepared using sodium sulphate ($Na_2SO_4$), sodium chloride (NaCl), sodium nitrate ($NaNO_3$) and potassium dihydrogen phosphate ($KH_2PO_4$) respectively.

### 2.5.7 Soluble, solid and total COD

All COD measurements were carried out using the Merck reagent test protocol for high (1500-10000 mg/ℓ) concentrations. The method is based on the oxidation of the sample with a hot sulphuric acid solution containing potassium dichromate, with silver sulphate as the catalyst. The chloride is masked with mercury sulphate. The concentration of unconsumed yellow $Cr_2O_7^{2-}$ ions or green $Cr_3^+$ ions is then determined photometrically and used to quantify oxygen demand. The reactions were performed in glass COD tubes, to which 2.2 mℓ of COD reagent A and 1.8 mℓ of COD reagent B were added. The sample (1 mℓ) was added to the reagents in the tube and the contents mixed using a vortex mixer. A blank was prepared using 1 mℓ of deionised water instead of the sample. The tubes were heated at 150°C for 120 minutes in a heating block, then allowed to cool to room temperature. The absorbance was measured using a spectrophotometer at 610 nm. To quantify the COD concentrations, standard solutions (0, 2500, 5000, 7500 and 10000 mg/ℓ COD) were prepared using potassium hydrogen phthalate.

### 2.5.8 Volatile fatty acids (VFAs)

A full volatile fatty acids (VFAs) analysis was conducted to quantify the concentration of lactic, acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids present in all digesters over the duration of digestion. The concentration of each VFA was determined using HPLC on a Waters Breeze 2 HPLC system equipped with a Bio-Rad Organics Acids ROA column and a UV (210 nm wavelength) detector. The system was run isocratically using a mobile phase of 0.01 M $H_2SO_4$ at a flow rate of 0.6 mℓ/min. The pressure in the

column did not exceed 2000 psi. Sample injection volumes of 100 µℓ were used. To quantify the VFA concentrations, standard solutions (100, 200, 300, 400 and 500 mg/ℓ for each acid) were prepared.

### 2.5.9 Volatile solids

Volatile solids (VS) were quantified using the standard method (APHA , 2005). The sample (1 mℓ) was filtered through a pre-weighed 25 mm diameter glass fibre acetate (GFA) filter. The filter was placed in a furnace at 105°C overnight to determine the moisture content. The filter was heated further at 550°C for two hours. The amount of ash left behind was quantified by re-weighing the filter. From this the total mass of volatile solids was calculated and a relative % VS determined. All VS testing was done in triplicate to quantify the error associated with the method.

# CHAPTER 3: BASELINE SULPHATE REDUCTION STUDIES

## 3.1 INTRODUCTION

One of the primary aims of this research was to assess the impact of biomass retention and recycling on sulphate reduction performance. Historically, CSTRs with suspended biomass have been used in studies to determine sulphate reduction kinetics and characterise the microbial community with respect to maximum growth rates. This configuration was selected to perform the baseline studies, against which the novel reactor systems could be compared.

## 3.2 EXPERIMENTAL PROGRAMME

### 3.2.1 Continuously stirred tank reactors (CSTRs)

The baseline data were generated using a similar set of experimental reactors and operating conditions to those used by Moosa (2001) and Oyekola (2008), reported in the literature through Moosa *et al*. (2002, 2005, 2006) and Oyekola *et al*. (2009, 2010). A series of standard CSTRs were operated at 30°C, using the modified Postgate B medium as the feed source. Reactors were operated at feed sulphate concentrations of 1, 2.5 and 5 g/ℓ. The lactate concentration in the feed was adjusted to maintain a COD to sulphate ratio of 0.7 across all the reactors. The reactors were started at a HRT of 5 days and sampled at least once per HRT. Once steady state had been established and monitored for at least three HRTs, the feed rate was increased, reducing the HRT to 4 days. The process was repeated a number of times, collecting data at 3, 2, 1.5, 1 and 0.5 days. For the reactors with feed sulphate concentrations of 2.5 and 5 g/ℓ, the experiment was terminated at a HRT of 1 day.

The reactors were sampled by removing 10 mℓ of solution and immediately using 20 µℓ to perform a sulphide assay. The pH and redox potential were measured using the remaining sample. A subsample (2 mℓ) was transferred to a 2 mℓ Eppendorf tube, to which 40 µℓ of zinc chloride solution was added. The tubes were mixed on a vortex mixer, then centrifuged at 14000 × *g* for 7 minutes. The supernatant was used to prepare samples for analysis of anions and VFAs by HPLC.

### 3.2.2 Assessment of abiotic sulphide oxidation

Data obtained using the CSTRs showed that the measured sulphide concentrations were significantly lower than expected, based on the extent of sulphate reduction. The methodology has been validated and there was no loss of sulphide during the course of sampling and analysis. There was, however, visual evidence of elemental sulphur at the interface between the liquid and the headspace in the reactor, with precipitation clearly visible as a layer on the surface of the liquid. Despite this nominally being a closed system, it was not easy to exclude oxygen totally. This is due largely to the porosity of the tubing used, for which there no viable alternative was found.

Therefore, a supplementary study was performed, aimed at determining the rate of abiotic sulphide oxidation in the reactor, to assess whether the loss of sulphide observed could be accounted for by this, or whether it was likely that biological sulphide oxidation was responsible for the majority of the sulphide loss.

The reactor was filled with water and supplemented with lactate (200 mg/ℓ), bicarbonate (500 mg/ℓ) and sulphide (250 mg/ℓ) using stock solutions to achieve the desired final concentrations. The pH was adjusted to pH 7.5 using sulphuric acid, to best approximate the conditions within an active reactor. The reactor was operated in batch mode, with stirring at 200 rpm. The reactor was sampled, initially at one hour intervals, then less frequently, by removing 5 mℓ from the bulk fluid and testing pH, redox potential and dissolved sulphide. Changes in appearance of the bulk liquid were noted.

## 3.3 RESULTS AND DISCUSSION

### 3.3.1 Biological sulphate reducing reactors

The data presented in this section represent a summary of steady state data at the different retention times across the three reactors. Steady state was assumed when the change in key parameters, particularly residual sulphate concentration, was less than 10% for three successive HRTs following a change in system conditions.

The steady state profiles for sulphate consumption and measured sulphide, at a feed sulphate concentration of 1.0 g/ℓ are shown in Figure 11. The theoretical sulphide concentration, based on the molar concentration of sulphate reduced, is also shown. The sulphate reduction is relatively consistent across the range of HRTs from 5 days down to 1 day, with between 870 and 920 mg/ℓ of the 1000 mg/ℓ feed being consumed. Based on molar stoichiometry, the theoretical sulphide concentration was just below 300 mg/ℓ. However, the actual measured sulphide concentration was significantly lower, suggesting either a loss of sulphide to the surroundings or further reaction of the sulphide.

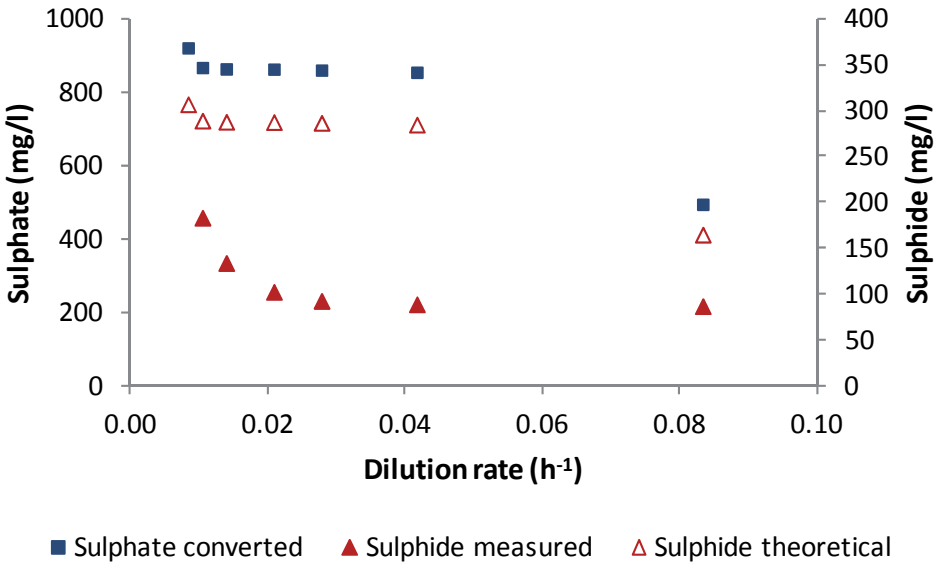



*Figure 11: Sulphate converted and measured and theoretical sulphide concentrations for the CSTR receiving 1 g/ℓ $SO_4^{2-}$*

The reactor unit was sealed and any offgas passed through a sodium hydroxide sulphide scrubber, where gaseous hydrogen sulphide ($H_2S$) would be converted to aqueous bisulphide ($HS^-$). Analysis of the bisulphide concentration indicated no significant loss of $H_2S$ from the reactor. This is consistent with the steady state pH (7.4 ± 0.15). Under these conditions the majority of the aqueous sulphide would exist as the $HS^-$. The result suggests partial oxidation or precipitation of a portion of the sulphide, although no metal sulphide precipitate could be observed. Visual observation of the reactor showed an elemental sulphur deposit at the air/water interface. This deposition was clearly visible when the reactors were taken down at the end of the experiment (Figure 12).

*Figure 12: Photograph showing the deposition of elemental sulphur on the walls of the reactor near the air/water interface as a result of partial oxidation of sulphide*

There was a significant decrease in the amount of sulphate reduced at a HRT of 12 hours (dilution rate of 0.083/h), with the conversion efficiency falling to around 50%. Despite the reduction in the sulphate conversion, the conversion of lactate remained at 99% (Figure 13). Based on the amount of sulphate reduced, the expected residual lactate concentration at this HRT should have been over 1000 mg/ℓ. However, a residual concentration of below 30 mg/ℓ was detected. This indicates that the sulphate conversion was not limited by lactate concentration. This observation also suggests that while sulphate reducers oxidised lactate at a rate near their $\mu_{max}$ another group of microorganisms characterised by higher $\mu_{max}$ and $K_s$ values for lactate utilisation were able to proliferate due to increased lactate loading at the high volumetric loading rate. Consequently, there was no accumulation of lactate. The decline in sulphate conversion was most likely a consequence of wash out of a portion of the sulphate reducing community when the reactor was operated at a dilution rate greater than their $\mu_{max}$. These data are similar to those obtained by Moosa *et al*. (2002, 2005), Oyekola *et al.* (2009; 2010; 2012) and Baksaran and Nemati (2006). The study showed that the decrease in sulphate reduction efficiency coincided with a decrease in acetate formation and increase in propionate formation (Oyekola *et al*., 2009). Propionate production is an indication of lactate fermentation (Heimann *et al*., 2005). In addition Oyekola *et al.* (2012) performed a qualitative assessment of microbial community structure, which confirmed that the diversity of sulphate reducers decreased with increasing dilution rate.

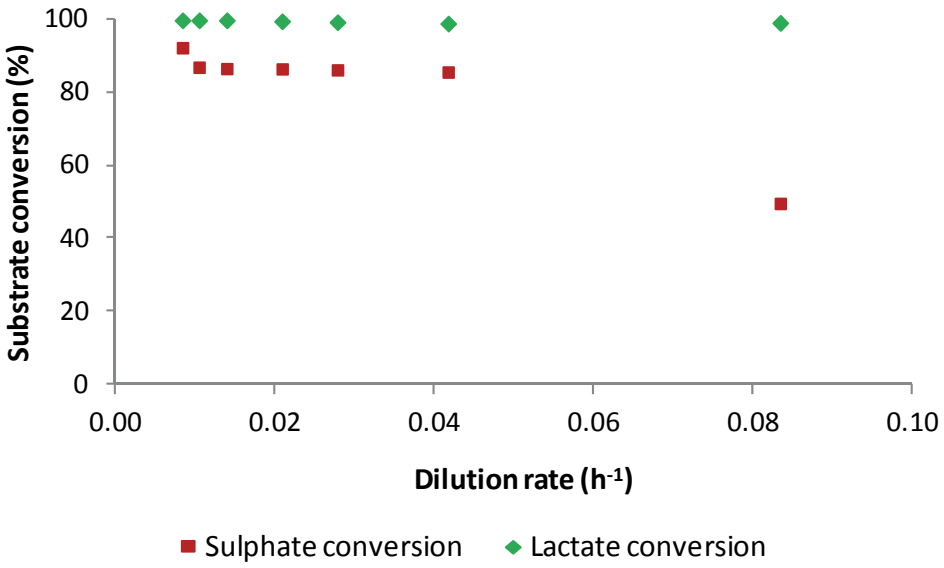



*Figure 13: Proportion of sulphate and lactate converted as a function of dilution rate (HRT) for the CSTR receiving 1 g/ℓ $SO_4^{2-}$*

The data from the reactor operated at a feed sulphate concentration of 2.5 g/ℓ showed a very different trend, with the sulphate reduction efficiency falling consistently with the decrease in HRT (Figure 14). The maximum sulphate conversion achieved was only 54%, at a HRT of 5 days. Across the range of HRTs studied, the lactate conversion was maintained at 100%, despite the significant decline in the sulphate conversion, as the volumetric loading rate was increased (Figure 15). These observations suggest the presence of lactate fermenting non-sulphate reducing bacteria that became predominant as the dilution rate was increased. The proliferation of these organisms reduces the amount of lactate available for oxidation by the sulphate reducing bacteria. The increase in propionate concentration, confirming lactate fermentation, has been confirmed under similar operating conditions (Oyekola *et al*., 2009).

The measured sulphide concentrations were again significantly lower than that expected on the basis of sulphate reduced. The aqueous sulphide concentrations measured were even lower than those measured in the reactor fed 1 g/ℓ sulphate, despite the reduction of more sulphate at the longer HRTs (4 and 5 days).

The low sulphide concentration most likely contributed to the predominance of lactate fermenters. Oyekola (2008) showed a similar trend in sulphate and lactate utilization rates at feed sulphate concentrations of 2.5 and 5 g/ℓ. However, at a feed sulphate concentration of 10 g/ℓ, lactate oxidation by SRB accounted for the majority of lactate utilization. This was attributed to the high aqueous sulphide concentration (300-600 mg/ℓ), which was postulated to inhibit the lactate fermenters selectively.

A similar observation was reported by Laanbroek *et al*. (1983), who cultured a lactate oxidiser (*Desulfovibrio* spp.) and a lactate fermenting microbe (*Veillonella* spp.) under the same conditions. The $\mu_{max}$ value of the *Veillonella* was reduced by 50% as the sulphide concentration increased from 17 to 165 mg/ℓ, while that of the lactate oxidiser remained unchanged.

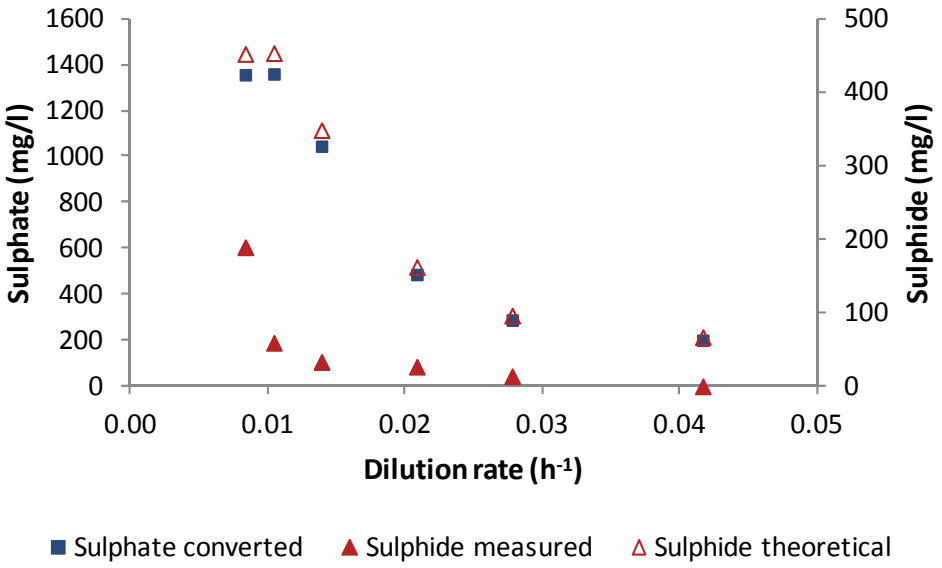



*Figure 14: Sulphate converted and measured and theoretical sulphide concentrations for the CSTR receiving 2.5 g/ℓ $SO_4^{2-}$*

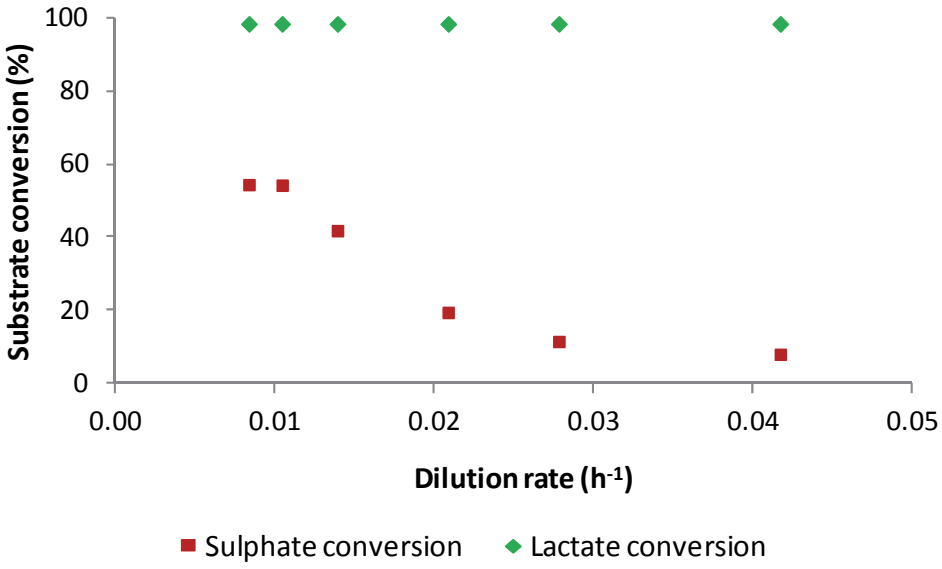



*Figure 15: Proportion of sulphate and lactate converted as a function of dilution rate (related to HRT) for the CSTR receiving 2.5 g/ℓ $SO_4^{2-}$*

The data generated at a feed sulphate concentration of 5 g/ℓ showed a similar trend to that of the 2.5 g/ℓ reactor, with respect to the relationship between HRT and sulphate reduction efficiency, which could again be attributed to a shift in dominance between lactate oxidisers and lactate fermenters. However, at the longer HRTs (5 and 4 days; dilution rates 0.008 and 0.010 $h^{-1}$), the aqueous sulphide concentration remained above 200 mg/ℓ (Figure 16), which provided some competitive advantage to the sulphate reducers. The measured sulphide concentration was again significantly lower than the theoretical value based on sulphate reduction.

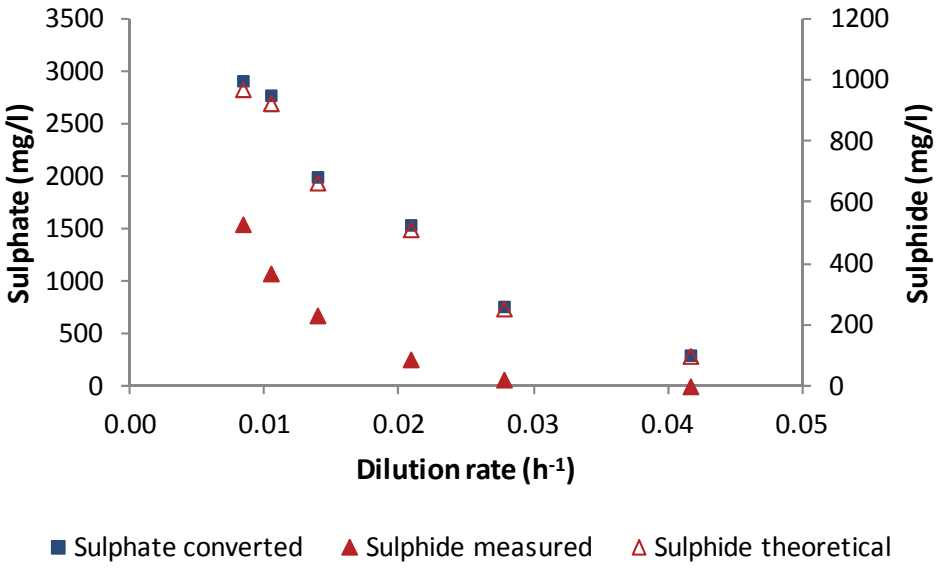



*Figure 16: Sulphate converted and measured and theoretical sulphide concentrations for the CSTR receiving 5 g/ℓ $SO_4^{2-}$*

A significant difference between the 2.5 and 5 g/ℓ reactors was observed in the extent of lactate utilisation, which was essentially complete across the range of HRTs in the 2.5 g/ℓ reactor, but incomplete for all HRTs in the 5 g/ℓ reactor (Figure 17). The implication is that the lactate loading was higher than the capacity of the microbial community to use it. The fact that the lactate utilisation decreased more substantially between the 4 and 2 day HRTs (0.010 and 0.021 $h^{-1}$) than between 2 and 1 day ($0.042^{-1}$) is consistent with the lactate fermenters having a higher $\mu_{max}$ when not inhibited by sulphide.

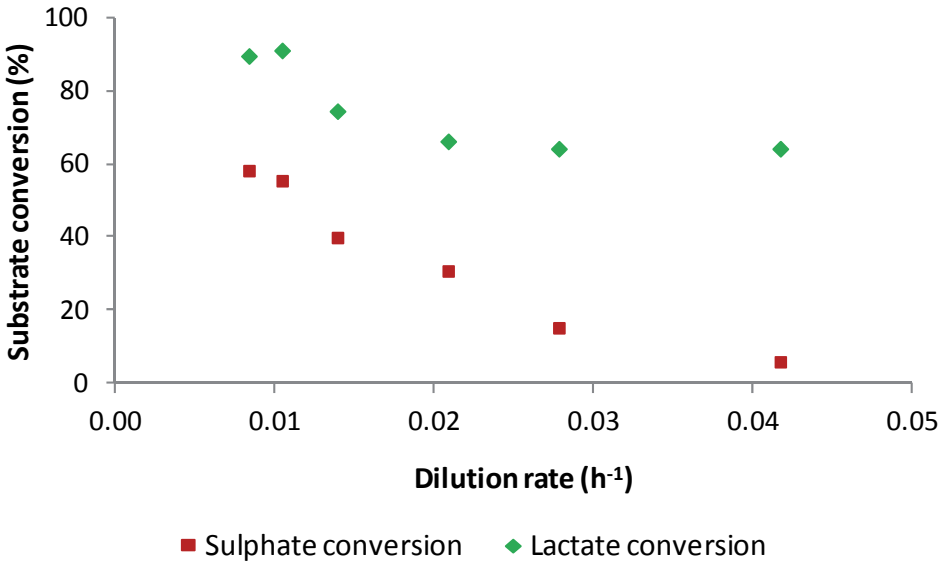



*Figure 17: Proportion of sulphate and lactate converted as a function of dilution rate (HRT) for the CSTR receiving 5 g/ℓ $SO_4^{2-}$*

The kinetic data can be summarised by considering the volumetric sulphate reduction rates for the three reactors (Figure 18). At a feed sulphate concentration of 1 g/ℓ, where there is less competition between sulphate reduction and lactate fermentation, there is an almost linear increase in VSSR at HRTs between 5 and 1 day. The data points lie close to the theoretical maximum, confirming a high level of efficiency. However, at a HRT below 1 day the VSSR deviated significantly from the theoretical value, indicating a loss of efficiency that can be attributed to washout of part of the sulphate reducing community. The reduced conversion for the reactors fed with 2.5 and 5 g/l sulphate resulted from competition between the lactate

fermenters and lactate oxidisers for the lactate available. However, the lactate fermenters also showed inhibition in the presence of increasing sulphide concentration in solution.

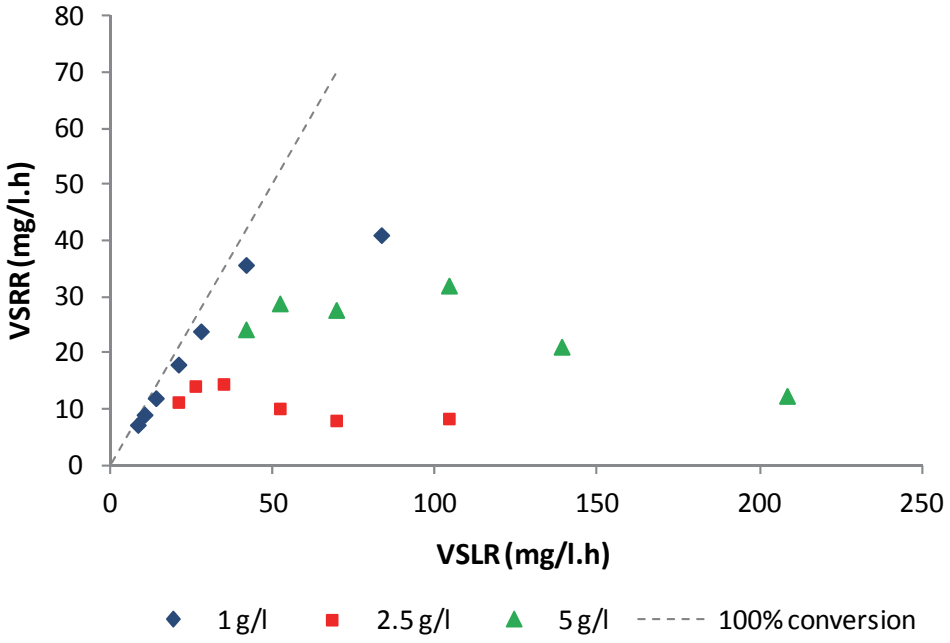



*Figure 18: Relationship between volumetric sulphate loading rate (VSLR) and volumetric sulphate reduction rate (VSRR) for the reactors fed 1, 2.5 and 5 g/ℓ sulphate. The dashed line represents the theoretical relationship for 100% sulphate conversion.*

### 3.3.2 Abiotic oxidation of sulphide

The experiment performed using the abiotic STR showed that the rate of abiotic sulphide oxidation was relatively slow, requiring 4 days for the measured sulphide concentration to decrease to zero (Figure 19). The appearance of the fluid in the abiotic reactor changed over the duration of the experiment, with the presence of polysulphides, confirmed by the characteristic yellow-green colour at certain stages. This was accompanied by a fairly large change in the pH and redox potential measurements (Figure 20). This degree of fluctuation was not observed in the biological CSTRs.

Polysulphides can be formed as a result of chemical sulphide oxidation. Polysulphide species have been observed as intermediates under conditions where elemental sulphur is formed through chemical oxidation (at pH 7). Chen and Morris (1972) found that polysulphide ion oxidation occurred more rapidly than sulphide oxidation and therefore polysulphide ions were found to act as a catalyst for sulphide oxidation. It was confirmed that the polysulphide oxidation followed the reaction stoichiometry in the pH range 7-9. Elemental sulphur was only observed if the average sulphur content (x) exceeded 2 (Equation 9).

$$2S_x^{2-} + O_2 + H_2O \rightleftharpoons S_2O_3^{2-} + 2S^{2-} + 2S_{x-2}^{2-} + 2H^+ \qquad \text{Equation 9}$$

Polysulphide solutions are generally yellow-green in colour, whereas thiosulphate solutions are colourless. Therefore, discolouration of the solution and formation of a precipitate ($S^0$) would indicate that the reaction given in Equation 10 was taking place (Bruser *et al*., 2000). This auto-oxidation is known to occur when the solution is exposed to the atmosphere.

$$S_x^{2-} + \frac{3}{2} O_2 \rightarrow S_2O_3^{2-} + (x-2)S^0 \qquad \text{Equation 10}$$

Under mildly alkaline conditions and in equilibrium with excess inorganic sulphur, the average polysulphide chain length (x) varies from 4.39-5.5 (van den Bosch, 2008). Kamyshny *et al*. (2004) showed that the specific chain length is not affected by pH or total polysulphide concentration.

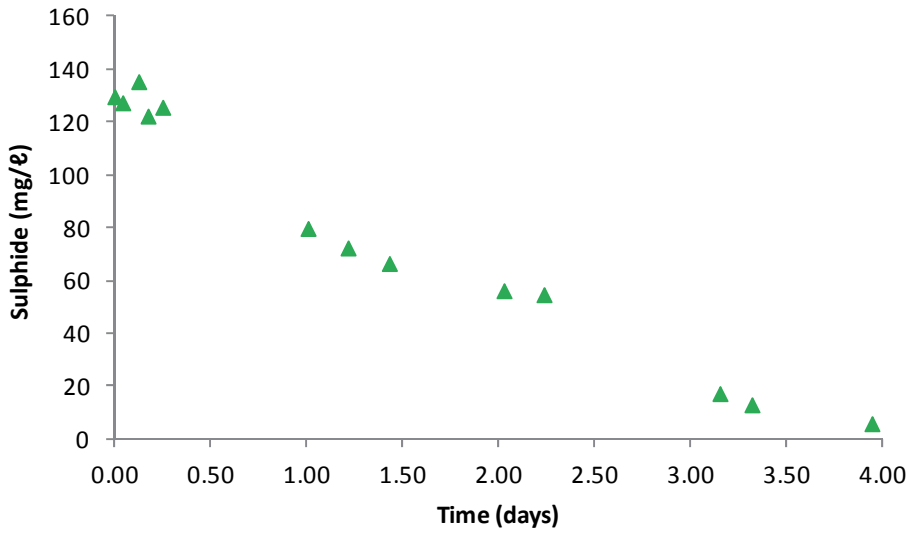



*Figure 19: Rate of sulphide oxidation in the abiotic STR*

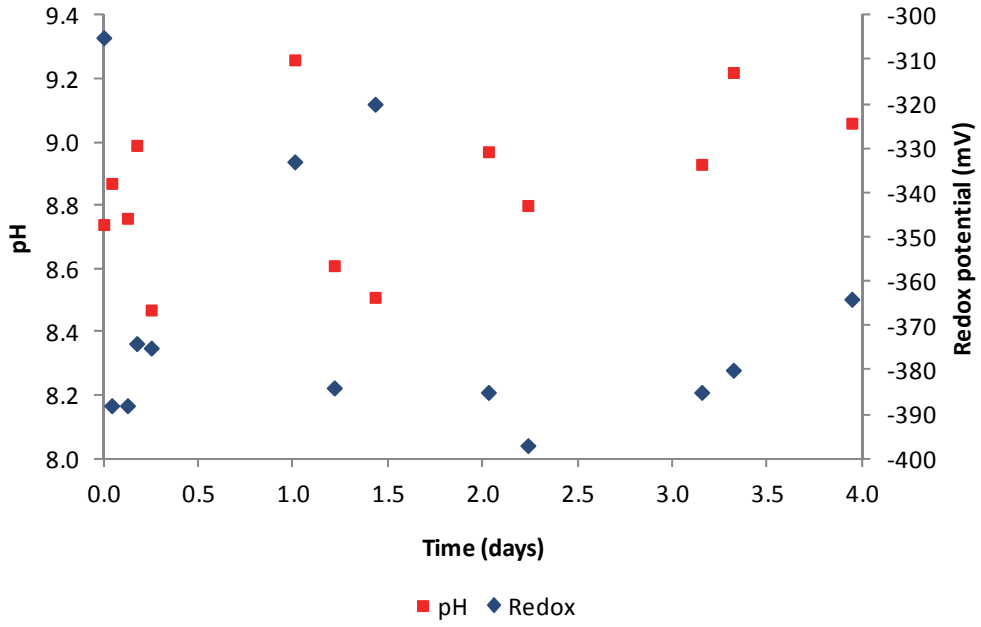



*Figure 20: Redox potential and pH data from the abiotic sulphide oxidation study*

The pH of the solution in the reactor plays a critical role in the overall distribution of sulphide species. At pH < 9.17 the majority of the aqueous sulphide is present as $HS^-$. If the pH is increased above pH 9.17 the majority of the sulphide occurs as polysulphides ($S_x^{2-}$). Therefore, an equilibrium exists in the region of pH = 9.17, as described by the following expression (van den Bosch, 2008):

$$K_x = \frac{[S_x^{2-}][H^+]}{[HS^-]} \times \frac{\gamma_{S_x^{2-}} \gamma_{H^+}}{\gamma_{HS^-}} \qquad \text{Equation 11}$$

van den Bosch and co-workers (2008) discovered that over a lower pH range (pH 8.5-9) about 18-40% of the total sulphide was present as polysulphides, while at the higher pH range (pH 9.6-10.1) the polysulphide content increased to between 73% and 89%. The observations made during this experiment were consistent with the proposed chemistry, with the colour associated with polysulphides most evident around 24 hours, when the pH in the reactor exceeded pH 9.2.

The relatively slow rate of abiotic sulphide oxidation means that the discrepancy between the measured sulphide concentrations and that expected on the basis of sulphate reduced cannot be due to abiotic oxidation. This implies the presence of sulphide oxidising microorganisms in the reactors. The observation of elemental sulphur at the air-liquid interface suggests that a significant amount of the unaccounted for sulphide was partially oxidised to elemental sulphur.

## 3.4 CONCLUSIONS

The baseline studies of biological sulphate reduction in CSTRs, using lactate as the carbon source and electron donor, generated data very similar to those reported by Oyekola *et al.* (2009, 2010 and 2012), who performed experiments under similar conditions, using the same inoculum source.

Greater than 85% conversion of the sulphate was achieved at a sulphate feed concentration of 1 g/ℓ and HRT of 1 day or more (approaching theoretical conversions). The results confirmed a substantial decrease in the efficiency of sulphate reduction at HRTs below 1 day, which was most likely the result of washout of a component of the sulphate reducing community in a suspended cell culture with no biomass retention. While a steady state was achieved in the reactor receiving feed at 1 g/ℓ sulphate at a 12 h HRT, the reduction efficiency was only around 50%.

In addition, the results obtained at the higher feed sulphate concentrations (2.5 and 5 g/ℓ) confirmed the competition for the lactate substrate between sulphate reducers and lactate fermenters. The latter utilise lactate more rapidly at high lactate concentrations and have a faster growth rate than the SRB, but have a lower affinity for lactate at low concentrations and are more susceptible to inhibition by sulphide.

In all cases there was a significant difference in the measured sulphide concentration in the reactor and the expected concentration, based on reaction stoichiometry and the amount of sulphate reduced. This could not be accounted for by the loss of gaseous hydrogen sulphide from the reactor, or the abiotic oxidation of sulphide. The most likely explanation was the partial oxidation of sulphide to elemental sulphur by sulphide oxidising microorganisms.

# CHAPTER 4: NOVEL BIOREACTOR CONFIGURATIONS FOR BIOMASS RETENTION

## 4.1 INTRODUCTION

Biomass retention or recycling is one of the most important aspects of modern anaerobic technology (Vallero *et al*., 2005). Uncoupling of the hydraulic retention time (HRT) and mean cell retention time (MCRT) by self-aggregation (e.g. granular sludges) or biofilm formation is essential for the successful operation of conventional high rate anaerobic bioreactors (Young and McCarty, 1989; Lettinga *et al*., 1980). However, conventional anaerobic reactors for sulphate reduction systems have presented a challenge with respect to biomass retention as the microorganisms typically do not attach well to solid supports and the granules or biofilms and are washed out from granular sludge or biofilm systems. The unsuccessful immobilisation of specific strains has been reported in fluidised bed (Nagpal *et al*., 2000), upflow anaerobic granular sludge bed (UASB) (Omil *et al*., 1997) and hybrid (UASB + packed bed) (O'Flaherty and Colleran, 1999) reactor systems.

A complete retention of all microorganisms in the bioreactor, including newly added bacterial species with a specific metabolic capacity, can be achieved in anaerobic membrane bioreactors (Vallero *et al*., 2005). In addition, membrane bioreactors (MBRs) are not dependent on granulation or biofilm formation, so that MBRs can also be operated with cell suspensions or flocs with poor settling characteristics. Thus, inoculation of the MBRs with a pure culture or a combination of known bacterial species can be performed without any risk of their washout (Vallero *et al*., 2005). This is of particular interest for biological systems that depend on the retention of a large population of slow growing microorganisms that perform a specific metabolism, even at a very low HRT.

Carbon microfibres have become readily available and represent a relatively cheap biomass support option. When suspended in an aqueous environment they present a very large surface area for biomass attachment, without taking up a significant volume in the reactor. A recent study by Matsumoto *et al*. (2012) showed that carbon microfibres provided an excellent support material for wastewater treatment biofilms. They compared the attachment of four pure cultures to a range of materials, including carbon fibres and a range of synthetic fibres. All species attached preferentially to the carbon microfibres and this was attributed to the removal of the energy barrier to attachment, explained using the Derjaguin-Landau-Verwey-Overbeek theory.

## 4.2 EXPERIMENTAL PROGRAMME

The HRT of the continuous suspended cell culture is limited by the maximum specific growth rate of the culture. At HRTs providing dilution rates that exceed this, biomass washout occurs. This is consistent with the washout of slower growing sulphate reducing species observed at HRTs below 1 day during the generation of the baseline data. In order to increase the efficiency of the system, it is necessary to de-couple hydraulic and mean cell retention time (MCRT) by biomass retention or recycle, thereby increasing the "catalyst" concentration and effective rate of conversion under those conditions. To assess the benefit of biomass retention, the standard CSTR was operated without agitation, to facilitate floc formation. In addition, two novel reactor configurations were assessed: the coupling of the CSTR to a membrane filtration unit for biomass recycle and the use of a channel reactor fitted with carbon micro-fibres to provide surface area for colonisation and associated biomass retention.

### 4.2.1 Non-agitated control reactor

The control reactor was set up initially in an identical fashion to the reactor used to collect the baseline data at a feed sulphate concentration of 1 g/ℓ (Sections 2.3.5 and 3.2.1). However, on day 12 the agitation was discontinued, with the exception of a 15 second burst prior to sampling. The intention was to observe whether floc formation or colonisation of the reactor internals by biofilm occurred in the absence of continuous agitation and whether this affected the overall performance. The mixing time study showed that

when the impeller was switched on, the reactor became homogeneous in approximately 6.3 seconds, so the 15 second agitation pulse prior to sampling was to ensure that a representative sample was obtained. From day 98, a sample was withdrawn prior to the burst of mixing and again afterward to compare the results.

A 10 mℓ sample was withdrawn from the reactor and a fraction (20 µℓ) immediately used to perform a sulphide assay. A second fraction (2 mℓ) was transferred to a 2 mℓ Eppendorf tube, to which 40 µℓ of $ZnCl_2$ (100 g/ℓ stock) was added. The tube was mixed on a vortex mixer and then centrifuged at 14000 × *g* for 7 minutes. The supernatant was filtered through a 0.22 µm membrane filter and stored for subsequent HPLC analysis (anions and VFAs). The remaining sample was used to measure the pH and redox potential. Effluent from the reactor was collected in a sealed bottle and the volume quantified to confirm the HRT. The silicone tubing used in the head of the peristaltic pump does become distorted over time, so the pump setting needed to be adjusted periodically to maintain the desired feed rate. The HRT in the reactor was calculated based on the effluent volume and averaged to determine the mean HRT for each portion of the experiment. The mean flow rates and number of HRTs under each set of conditions are summarised in Table 1.

*Table 1: Summary of the operating conditions for the non-agitated control reactor and the reactor connected to the microfiltration unit. The total volume represents the effluent volume collected during operation at each particular HRT. 'HRTs' represents the total number of HRTs under each set of conditions*

| Desired RT (days) | Mean flow rate (mℓ/min) | Mean HRT (days) | Total volume (ℓ) | HRTs |
|---|---|---|---|---|
| 4 | 0.170 | 4.10 | 11.96 | 11.96 |
| 3 | 0.219 | 3.17 | 7.61 | 7.61 |
| 2 | 0.339 | 2.05 | 19.24 | 19.24 |
| 1.5 | 0.466 | 1.49 | 9.49 | 9.49 |
| 1 | 0.661 | 1.05 | 17.69 | 17.69 |
| 0.75 | 0.900 | 0.77 | 10.29 | 10.29 |
| 0.5 | 1.347 | 0.52 | 7.90 | 7.90 |

#### *4.2.1.1 Mixing time study*

The mixing time studies were conducted in a standard 1 ℓ CSTR, set up identically to the control reactor. The macromixing time was determined on the basis of colour change, using a phenolphthalein indicator. The reactors were filled with 1 ℓ of 0.001 M sodium hydroxide solution, to which 2 mℓ of phenolphthalein was added. A 2 m ℓ pulse of 1M hydrochloric acid was subsequently added via the feed inlet pipe. The acid was added in excess to ensure the neutralization reaction was diffusion limited. The acid and base concentrations were chosen to ensure the density difference was less than two percent. Following the acid pulse addition, the peristaltic pump was connected to pump water into the reactor, via the feed inlet pipe, and remove liquid from the reactor, via the effluent outlet pipe. The flow rate of the peristaltic pump was chosen to match a 0.5, 1, 1.5, 2, 3 and 4 day HRTs. The mixing regime within the reactor was visually observed by the colour change of the phenolphthalein from pink to clear, with the mixing time defined as the time at which the last of the colour was seen to disappear. In addition, the pH (Eutech CyberScan pH 1100 meter connected to a data logger) was measured, at 1 second intervals. Once the pH reached a steady state, the mixing was considered complete.

### 4.2.2 Continuous reactor with attached microfiltration unit

The purpose of coupling the microfiltration unit to the continuous reactor was to separate biomass from suspension and recycle it back to the reactor, while discharging a cell-free permeate. This was chosen to build up a very high cell density. It was hypothesised that the high cell density would support efficient sulphate reduction at HRTs below 1 day, owing to the de-coupling of the MCRT and HRT.

The reactor coupled to the membrane filtration unit was commissioned at the same time as the control reactor and feed was provided using the same multichannel peristaltic pump. Therefore, the feed rates, overall hydraulic retention times and number of hydraulic retention times under each set of conditions was identical to the non-agitated control (Table 1). However, the pore size of the membrane available at the start of the experiment (500 kDa) was too small and the peristaltic pump was unable to generate sufficient

transmembrane pressure. The membrane was replaced with a ceramic membrane with a pore size of 0.2 µm and a more powerful pump was acquired. As a result, the reactor was operated without biomass recycling for the first 54 days. During this period the reactor was stirred at 300 rpm.

Once the functional membrane unit was attached, permeate was pumped out of the membrane unit at the same rate as feed was pumped into the reactor. Under normal operating conditions no effluent was collected from the overflow port of the reactor. However, if the membrane became fouled, reducing the transmembrane flux, or the permeate pipe became blocked, the volume in the reactor would accumulate and discharge through the overflow port. When this occurred, the membrane needed to be de-fouled.

De-fouling of the membrane was achieved by reversing the flow, such that liquid was pumped into the space outside the membrane, rather than through the lumen, generating a back-pressure. The movement of fluid from outside to inside should dislodge biofilm from the inner wall. Approximately once per month the membrane was disconnected and flushed with 0.1 NaOH for 60 minutes to destroy biofilm growth.

The reactor was sampled as described for the control reactor, while permeate was sampled by collecting the outflow for 5-10 minutes. Reactor and permeate samples were analysed for pH, redox potential, anions and VFAs.

### 4.2.3 Linear flow channel reactor with carbon microfibres

#### *4.2.3.1 Prior to upset period*

The channel reactor was initially operated at a 5.5 day HRT (0.27 mℓ/min) to allow for colonisation of the microfibres. This was achieved successfully over a period of 20 days. From day 20, the feed rate was increased to achieve a HRT of 4 days. Samples (2 mℓ) were removed daily from the middle (FM) and lower (FB) sample ports in the first and third (BM and BB) rows (Figure 9). The pH and sulphide concentration were measured immediately, after which the remainder of the sample was treated with 40 µℓ of zinc chloride (100 g/ℓ) and centrifuged at 14000 × *g* for 7 minutes to remove sulphide as zinc sulphide. The supernatant was filtered through a 0.22 µm nylon membrane filter and retained for HPLC analysis (VFAs and anions). Effluent from the reactor was collected in a sealed bottle over varying time intervals and the volume quantified to confirm the HRT. A portion of the collected effluent was treated for HPLC analysis, while the rest was used to measure pH and redox potential.

On day 34 approximately 800 mℓ of 0.5 M NaOH was siphoned into the reactor from the sulphide trap. This resulted in a significant deviation from the steady state conditions. In an attempt to re-establish the culture and its desired operating conditions, the feed flow rate was increased to 1.4 mℓ/min (1 day HRT) and 5 mℓ of 5 M sulphuric acid was injected through each of the four sample ports.

#### *4.2.3.2 After the upset period*

The culture recovered fully after the NaOH event and reached steady state at a four day HRT on day 47, after which the HRT was reduced by increasing the feed rate. The operating conditions of the channel reactor are summarised in Table 2. The liquid in the effluent bottle was collected at regular intervals and the volume used to calculate the flow rate. The flow rate did change with time as the tubing in the pump head was compressed, necessitating regular changing of the pump settings to maintain the desired flow rate and less frequent changing of the tubing. Therefore, the data presented in Table 2 represent mean values across the relevant time intervals.

*Table 2: Summary of the operating conditions of the channel reactor after recovery from the upset event. The total volume represents the effluent volume collected during operation at each particular retention time. HRTs represents the total number of hydraulic retention times under each set of conditions*

| Desired HRT (days) | Mean flow rate (mℓ/min) | Mean HRT (days) | Total volume (ℓ) | HRTs |
|---|---|---|---|---|
| 4 | 0.361 | 4.09 | 5.317 | 2.47 |
| 3 | 0.465 | 3.18 | 12.120 | 5.64 |
| 2.5 | 0.594 | 2.49 | 9.400 | 4.37 |
| 2 | 0.713 | 2.07 | 11.375 | 5.29 |
| 1.5 | 0.950 | 1.55 | 14.605 | 6.79 |
| 1 | 1.412 | 1.05 | 16.505 | 7.68 |
| 0.75 | 1.869 | 0.79 | 28.660 | 13.33 |
| 0.5 | 2.535 | 0.58 | 38.850 | 18.07 |
| 1.3 | 1.135 | 1.30 | 8.560 | 4.03 |

## 4.3 RESULTS AND DISCUSSION

### 4.3.1 Non-agitated control reactor

The reactor took approximately 30 days to establish steady state conditions. During this period the pH in the reactor decreased from pH 7.5-7.6 to approximately pH 7 (Figure 21). At the same time the redox potential decreased to a steady state level in the region of -400 mV, which is typical of efficient sulphate reduction.

The pH and redox potential values remained relatively stable as the hydraulic retention time was decreased from 4 days to 1 day, with steady state conditions maintained for several HRTs at each level. The differences in pH and redox potential of the samples taken before and after the burst of mixing were minor, suggesting the reactor was relatively well mixed, even in the absence of active agitation. On day 135, after 7.5 HRTs of steady state operation at the 1 day HRT, the reactor inexplicably failed. The appearance changed dramatically, with the reactor contents becoming black, rather than the typical brownish-grey. This was accompanied by a noticeable drop in pH and significant increase in the redox potential, from -400 mV to as high as -350 mV on day 141. The effect on sulphate reduction efficiency was most significant (Figure 22).

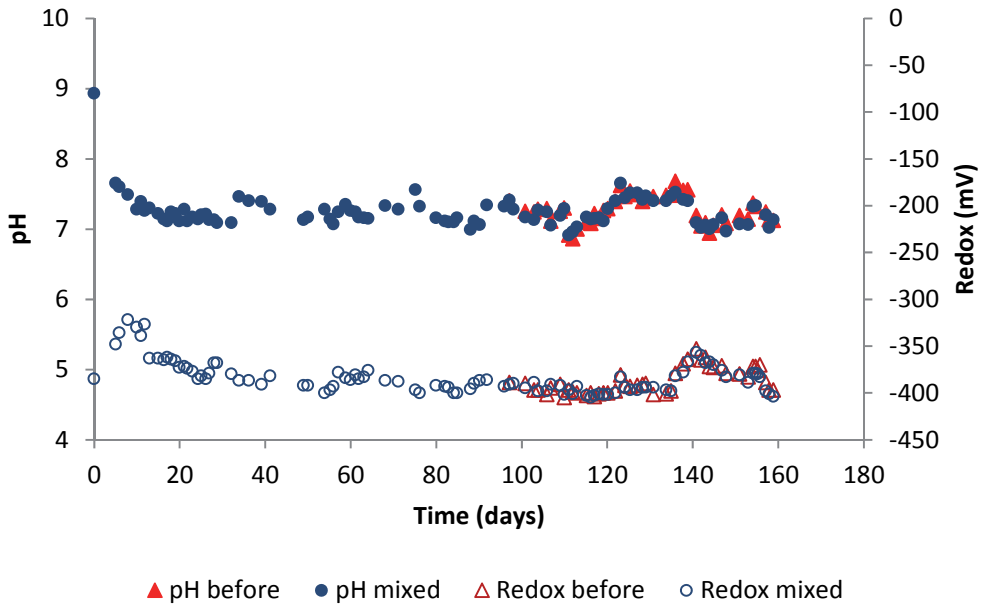



*Figure 21: Redox potential and pH data from the non-agitated control reactor with a feed sulphate concentration of 1 g/ℓ. "Before" refers to sampling without mixing the reactor. "Mixed" refers to the sample taken following the 15 s agitation pulse*

The residual sulphate concentration in the reactor decreased from around 800 mg/ℓ to the steady state concentration of between 140 and 150 mg/ℓ during the first 30 days. At steady state, the sulphate reduction efficiency was between 85 and 90%, similar to that in the baseline study for 1 g/ℓ sulphate in the feed. A spike in residual sulphate concentration was observed when the HRT was decreased as the culture adapted to the increased loading rate. The adaptation period became shorter as the experiment progressed and the reactor became increasingly colonised with attached biofilm and suspended flocs.

The difference in residual sulphate between samples taken before and after active mixing was typically low, although more substantial in a number of cases. Where the differences were significant the value prior to mixing was generally higher. The sample was drawn from near the bottom of the reactor, at a similar level to where fresh feed was introduced. Given the mixing regime within the reactor, this is where inhomogeneity is expected to be greatest.

The response to the upset event that occurred on day 135 was rapid and dramatic, with sulphate reduction effectively ceasing and the measured sulphate concentration increasing to levels similar to that of the feed by day 147. The system recovered some sulphate reduction capacity after day 147, confirmed by the continued presence of sulphide in the reactor (Figure 23), but the system did not return to steady state.

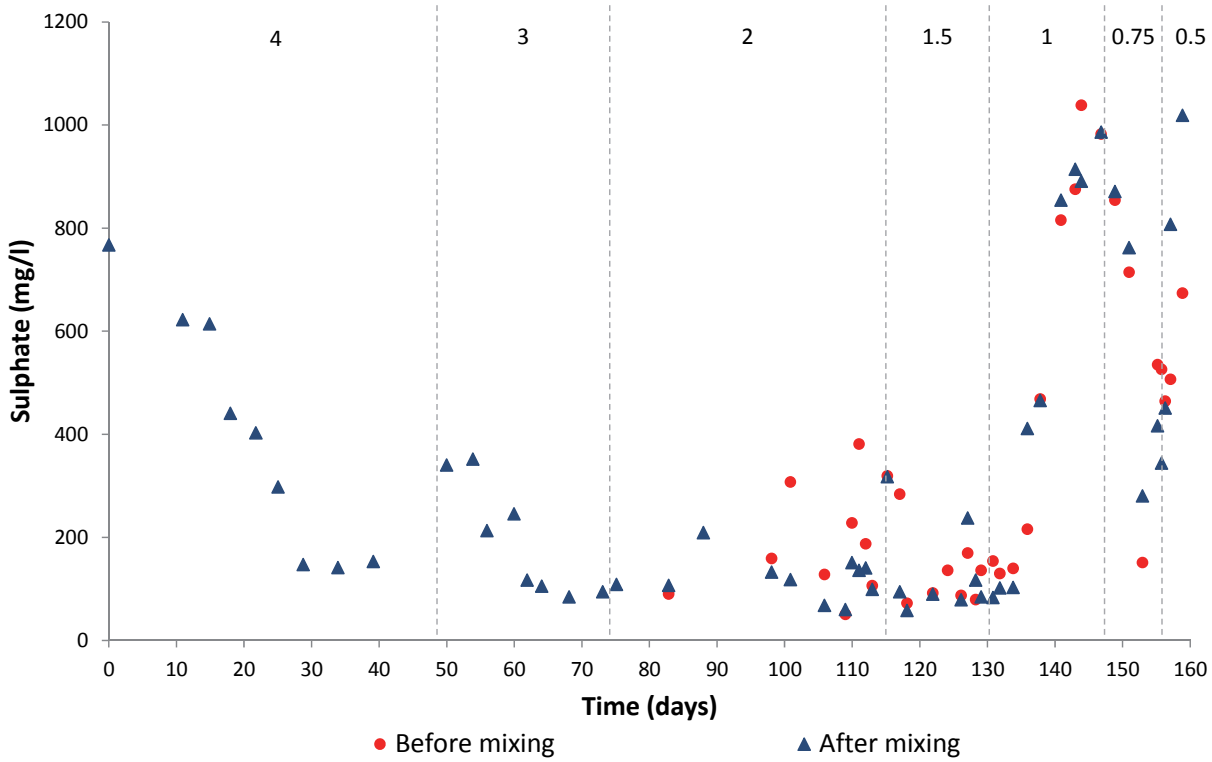



*Figure 22: Residual sulphate data from the non-agitated control reactor operated at a feed sulphate concentration of 1 g/ℓ sulphate. The change in HRT is indicated by the dashed lines with the nominal mean HRT during the period recorded by the floating numeral at the top.*

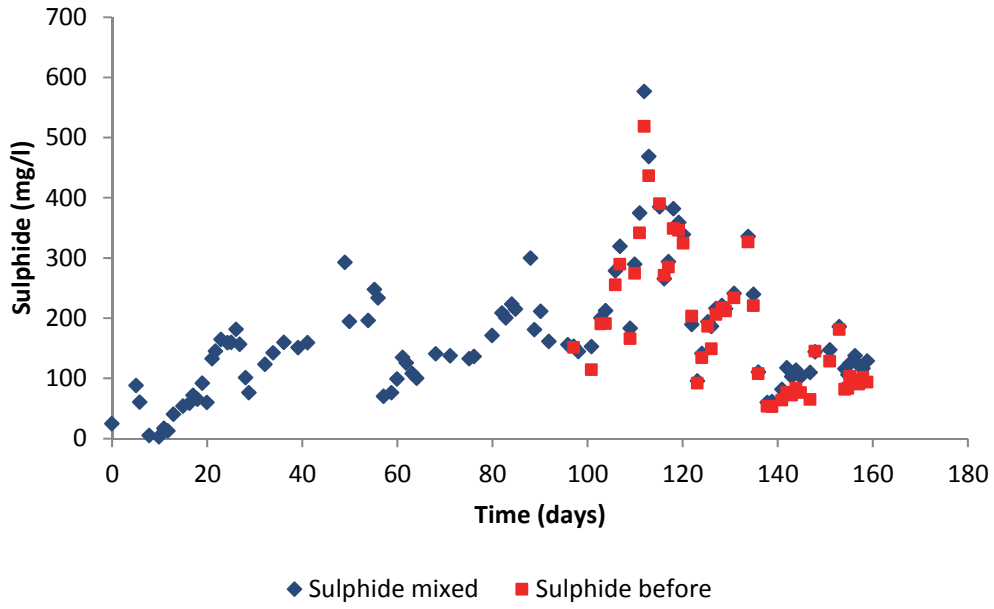



*Figure 23: Sulphide concentration in solution drawn from the non-agitated control reactor operated with a feed containing 1 g/ℓ sulphate. "Before" refers to the sample taken prior to agitation. "Mixed" refers to the sample taken following the 15 s agitation pulse*

The sulphide concentration measured in the reactor was most susceptible to external influences, given the nature of sulphide chemistry and the difficulty in excluding all oxygen from the system. Significant fluctuations in the sulphide concentration were observed, even under steady state conditions with respect to sulphate reduction, due to the nature of sulphide chemistry. Therefore, sulphide concentration should not be used to assess whether a reactor has reached steady state. On day 115 the overflow pipe from the reactor became blocked, resulting in the accumulation of liquid in the reactor and the elimination of the headspace. This resulted in a minor improvement in sulphate reduction, but a substantial increase in measured sulphide,

as partial oxidation of the sulphide was prevented. Similarly, on day 123 a problem with feed pumping resulted in air being pumped into the reactor for several hours. Again, this had very little impact on sulphate reduction, but a significant impact on the sulphide concentration.

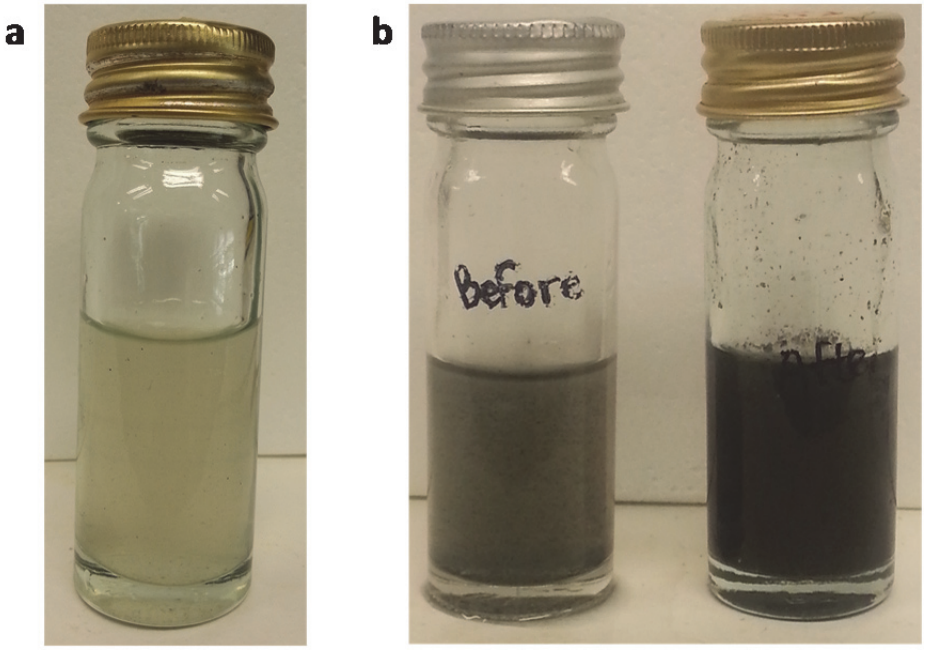



*Figure 24: Photograph showing the difference in appearance of the sample from the non-agitated control reactor (a) prior to the upset condition and (b) after. The two samples in (b) depict the appearance before (left) and after (right) agitation*

The cause of the reactor failure is not clear and seems unlikely to have been an effect of sulphate loading or dilution rate, given the fact that the system had been in steady state under those conditions for a number of HRTs. The change in appearance was dramatic (Figure 24). In most sulphate reducing systems a black colour is associated with the formation of metal sulphides, typically iron, but there was no iron in the feed to the reactor. It is possible that a shift in microbial community structure may have been the cause. Samples for DNA extraction were taken prior to and after the change. However, the capacity to do the community analysis was not available at the time and the stored DNA will be analysed at a later stage.

#### *4.3.1.1 Mixing time study*

The mixing time study was performed to assess whether an unstirred reactor could be considered well mixed within a specific HRT. The phenolphthalein indicator method was useful as it provided a visual assessment of the mixing regime. In the absence of active agitation the contents of the reactor partitioned into distinct zones based on the small density difference between the acid and the hydroxide in the bulk fluid (Figure 25). The clear zone, indicating neutralisation of the hydroxide moved upwards as water was pumped in through the feed pipe, indicating that mixing by convection was more rapid than mixing by diffusion. A similar situation was reported for the LCFR (Mooruth, 2013).

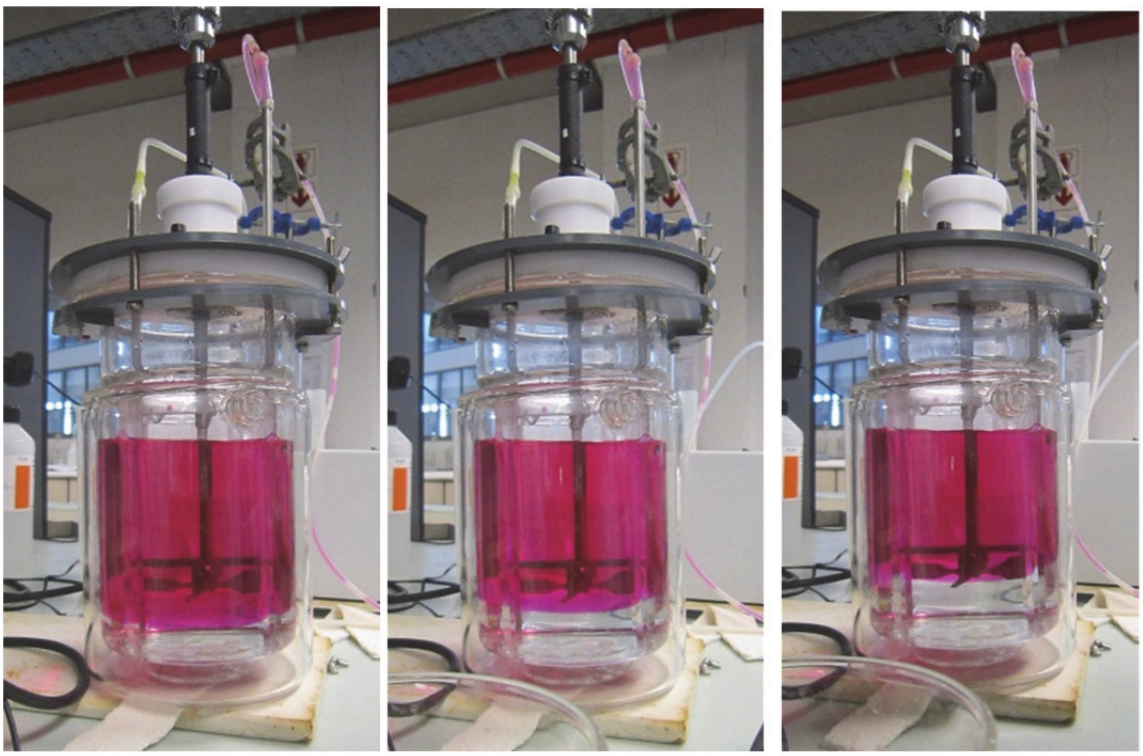



*Figure 25: Mixing profile in the non-agitated reactor, showing progression with time. Hydraulic retention time in the reactor was 2 days*

In tests where no fresh feed was pumped into the reactor and mixing was entirely dependent on the diffusion of the small volume of acid through the reactor, the average time to achieve complete neutralisation (i.e. disappearance of all colour) was 23.2 ± 0.2 hours. When the impeller was turned on at 300 rpm complete mixing was achieved in 6.7 ± 0.2 seconds.

The mixing time data for the different feed rates are summarised in Figure 26. In all cases, the mixing time was lower than the HRT, indicating that diffusion did play a minor role. However, there was no clear trend between HRT and mixing time, with shorter mixing times recorded for the higher HRTs. This trend was unexpected, but when viewed in the context of the temperature the trend becomes clearer. The experiments were performed at ambient temperature on the laboratory bench. The temperature in the laboratory is typically controlled at 25 ± 1°C, but an extended period during which the air conditioning was not consistent resulted in the fluctuations observed. The temperature in the sulphate reducing reactors was controlled at 30°C so the mixing time was likely lower than 12 hours. A more systematic study on the effect of temperature on mixing time in non-agitated reactors should be performed.

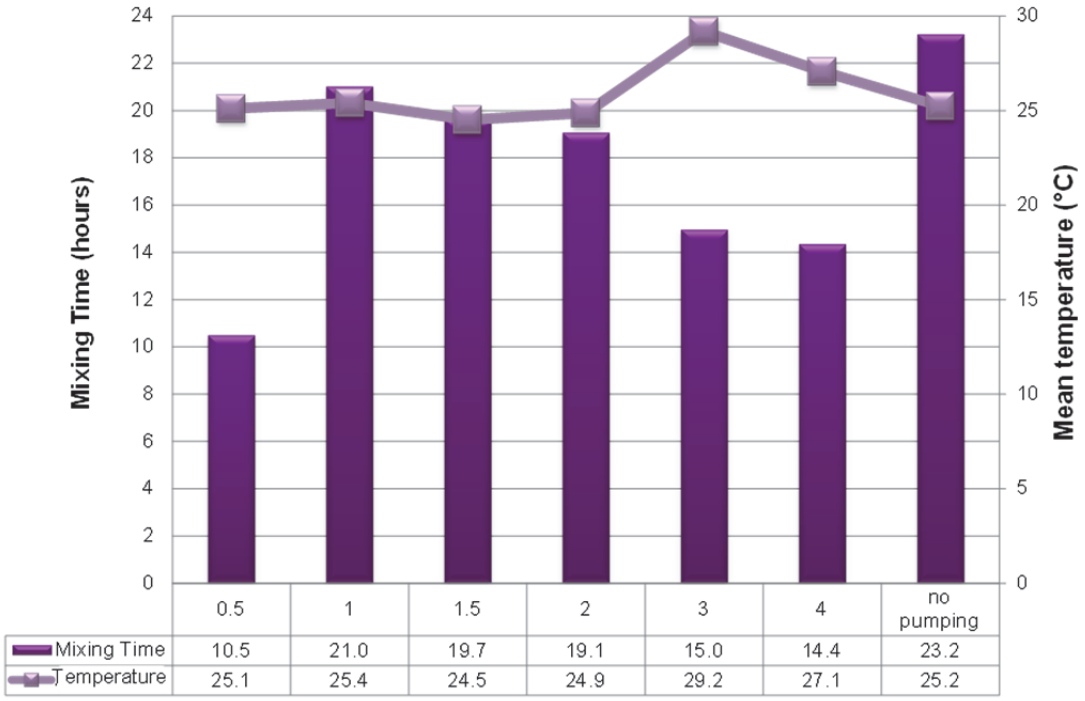



*Figure 26: Summary of data from the mixing time study showing the relationship between HRT, temperature and mixing time*

### 4.3.2 Continuous reactor with microfiltration unit

The redox potential in the reactor decreased from an initial value of around -350 mV to a steady state between -390 and -400 mV during the first 30 days of operation (Figure 27), as the sulphate reduction efficiency increased prior to attaining steady state at a four day HRT (day 30). During the same time the pH increased from around pH 7 to pH 7.4-7.5. The pH profile differed from that in the control reactor, which showed a gradual decrease to around pH 7 over the same period. The reason for this was not immediately apparent, but could be related to the establishment of a slightly different microbial community owing to the removal of selective pressure associated with a continuous flow-through system.

The redox potential of the permeate was typically similar to that of the bulk reactor fluid, except during periods where permeate flow decreased due to fouling or blockage of the tube draining the membrane unit. Under these circumstances oxidation of the permeate was near complete, resulting in a less negative redox potential.

The pH of the permeate from the microfiltration membrane was consistently higher than the pH measured in the reactor, typically by 0.5 pH units.

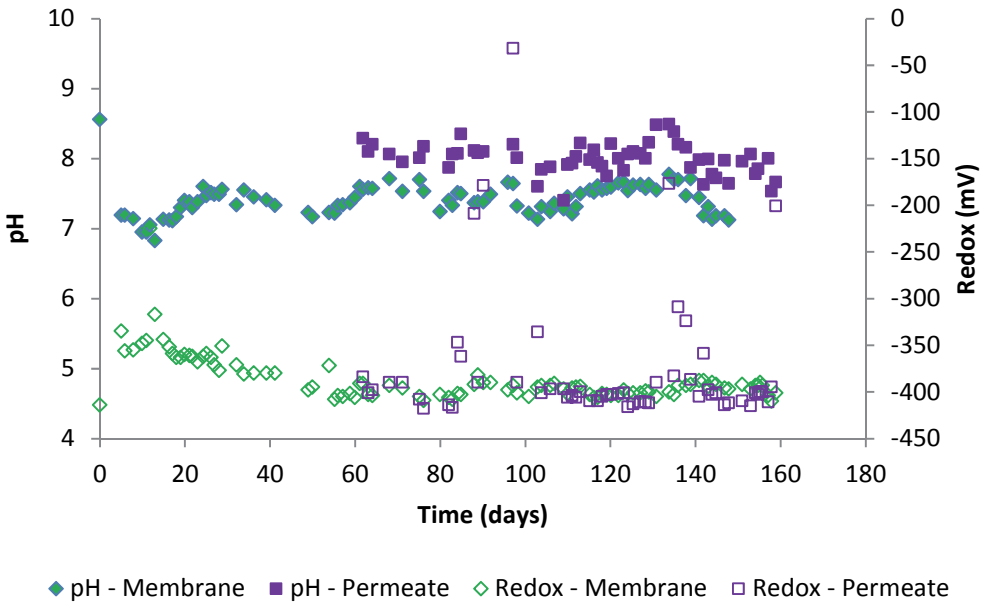



*Figure 27: Redox potential and pH data from the continuous reactor with microfiltration unit*

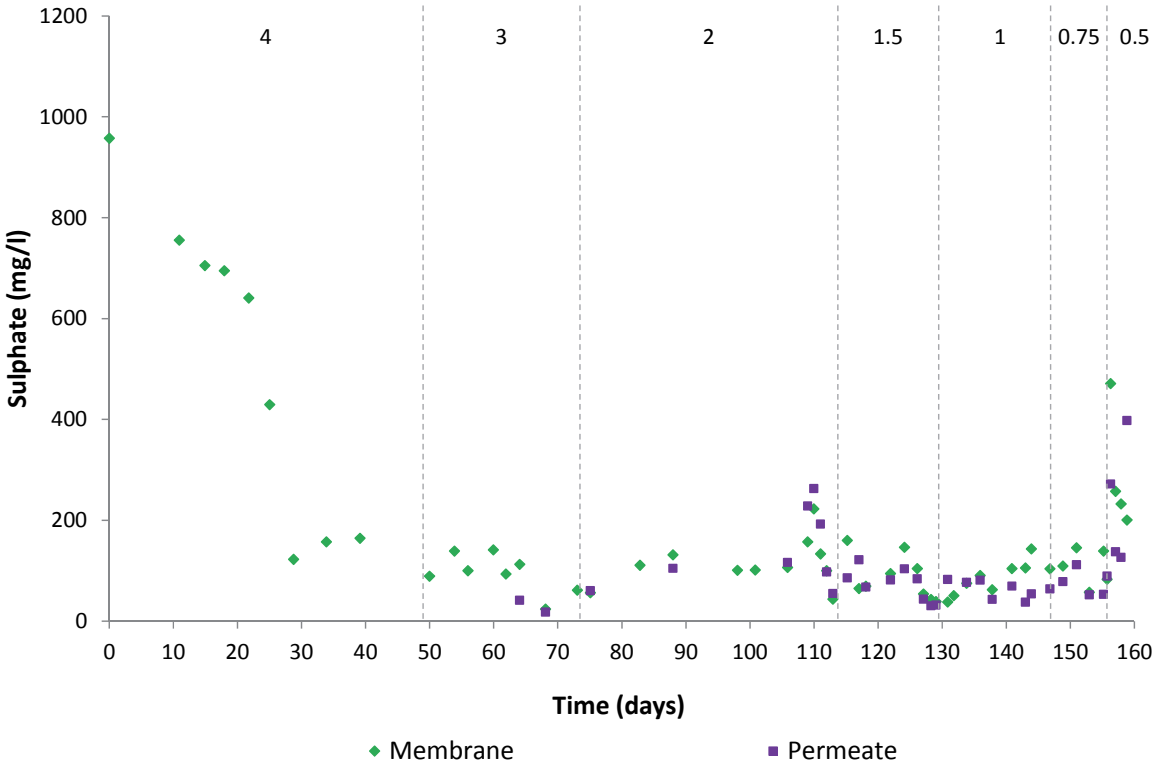



*Figure 28: Residual sulphate data from the continuous reactor with microfiltration unit, treating 1 g/ℓ sulphate. The change in HRT is indicated by the dashed lines with the nominal mean HRT during each period represented by the floating numeral at the top. 'Membrane' refers to the sample from the bulk reactor fluid.*

The residual sulphate data (Figure 28) gave an indication of the efficiency of the system across the range of HRTs. The system took around 30 days to reach steady state at the initial 4 day HRT. The residual sulphate at steady state was approximately 140 mg/ℓ, representing a removal efficiency of over 85%. This was consistent with the baseline data under similar operating conditions. The residual sulphate was relatively unchanged down to a HRT of 0.75 days and increased slightly at 0.5 days. The sulphate concentration measured in the permeate was similar or slightly lower in most cases, indicating that while partial oxidation of sulphide to sulphur occurred in the drainage tube, complete oxidation to sulphate did not.

The low residual sulphate, even at a HRT of 0.5 days, was indicative of very efficient performance. The recycling of the majority of the biomass, particularly under conditions where slower growing species would be washed out, resulted in a very high biomass concentration that could sustain efficient sulphate reduction at a high volumetric loading rate. The average VSRR measured across the 0.5 day HRT was 64.18 mg/ℓ.h, over 50% higher than that achieved during the baseline study. A comparison of the volatile sulphate reduction rates across the different reactor configurations is shown in Figure 44, where the results are discussed in more detail.

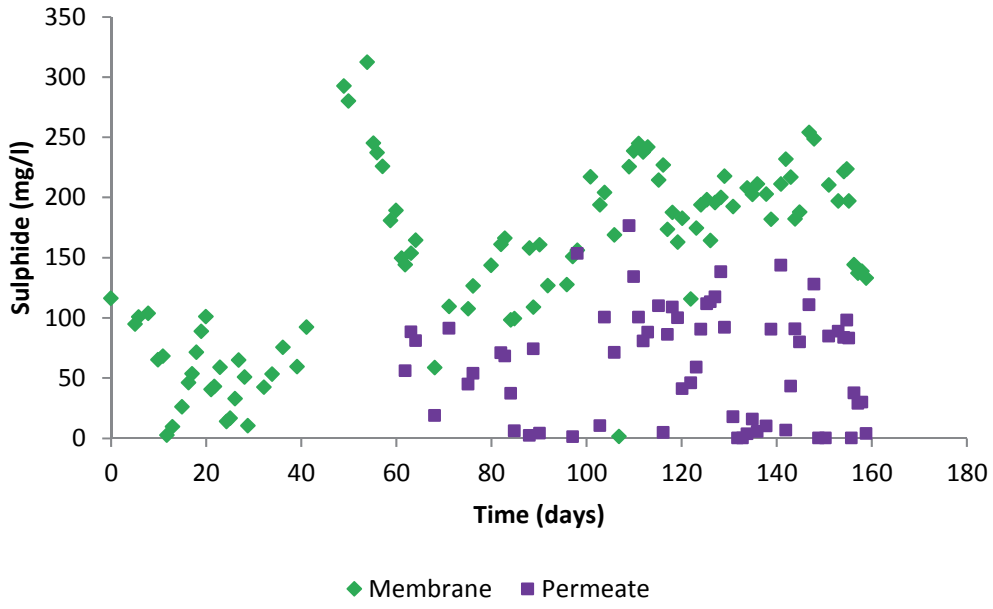



*Figure 29: Sulphide data from the continuous reactor with microfiltration unit treating 1 g/ℓ sulphate. 'Membrane' refers to the sample from the bulk reactor fluid*

The sulphide concentration in the reactor was typical of a CSTR during the first 40 days of operation (Figure 29) with the measured values significantly lower than the expected. On day 50 the overflow line became blocked by an accumulation of biofilm and elemental sulphur, resulting in the accumulation of liquid in the reactor to the point where the entire headspace was filled. This resulted in a substantial increase in the sulphide concentration, due to the longer effective HRT and more importantly the complete exclusion of oxygen from the reactor. The sulphide concentration approached the expected value, based on the extent of sulphate reduction. Following the perturbation the sulphide concentration increased gradually as the volumetric sulphate loading increased.

The sulphide concentration of the permeate was consistently lower than that in the bulk fluid, fluctuating around 100 mg/ℓ when the system was operating efficiently. The lower sulphide concentration, coupled with the increased pH suggested the partial oxidation of some of the sulphide to elemental sulphur. The silicone tubing used to drain the permeate is permeable to oxygen. Under oxygen limiting conditions the partial oxidation of sulphide is favoured, according to the reaction below (Kuhn *et al.*, 1983)

$$2HS^- + O_2 \rightarrow \tfrac{1}{4}S_8 + 2OH^- \qquad \text{Equation 12}$$

The generation of hydroxide ions accounts for the increase in pH. As the pH increased, a portion of the remaining sulphide could be converted to polysulphides. The permeate was typically pale yellow (Figure 30), consistent with the presence of some polysulphide. Where permeate flux was reduced, more complete oxidation of sulphide occurred, resulting in the zero values recorded on a number of occasions (Figure 29).

*Figure 30: Photograph showing the difference between the sample from the membrane reactor (left) and the membrane permeate (right) collected during operation at a HRT of 1 day*

The sample from the reactor clearly contained suspended biomass, which became more apparent as the experiment progressed. The appearance of attached biofilm on the reactor walls and the presence of elemental sulphur in the bulk fluid prevented accurate quantification by either dry mass or optical density. The permeate sample was consistently cell free, indicating that the cells were recycled back to the reactor with the retentate. The absence of cells in the permeate was confirmed by light microscopy.

#### *4.3.2.1 Challenges associated with the membrane unit*

While the addition of the membrane unit resulted in the highest volumetric sulphate reduction rate across the reactor configurations tested, the system was not without challenges. The most significant was associated with the ingress of a limited amount of oxygen, leading to the partial oxidation of sulphide to elemental sulphur.

Typically, in a membrane system such as this, fouling occurs on the inner wall of the membrane due to biofilm attachment, resulting in a decrease in permeate flux across the membrane and an increase in backpressure. This can be alleviated by back-flushing the membrane. In the current experiment some fouling of the membrane did occur, but a more common problem was the deposition of particulate sulphur in the drainage tube and the tubing in the head of the peristaltic pump (Figure 31). This accumulated to the point where flow was restricted or ceased altogether, resulting in effluent draining from the reactor overflow port, rather than exiting as permeate. To avoid this, the pipes needed to be removed and flushed of sulphur regularly, although when the blockages occurred at night reduction in permeate flow was inevitable. The problem was more pronounced at the shorter HRTs. At the 1.5 day HRT 76% of the total effluent was made up of permeate and 24% as overflow from the reactor. The proportion exiting as permeate fell to 67%, 69% and 44% at the 1 day, 0.75 day and 0.5 day HRTs respectively.

Upon completion of the experiment the membrane was removed from the housing for closer inspection. While there was some biofilm growth on the base plate (Figure 32) this would not affect performance. The lumen of the membrane was relatively free of biofilm. However, there was significant deposition of elemental sulphur on the outer surface of the membrane (Figure 33), which could not easily be removed. This was most likely responsible for the deteriorating performance toward the end of the experiment. Restoration of the membrane required soaking in chloroform, followed by the physical scouring of the deposit off the membrane surface.

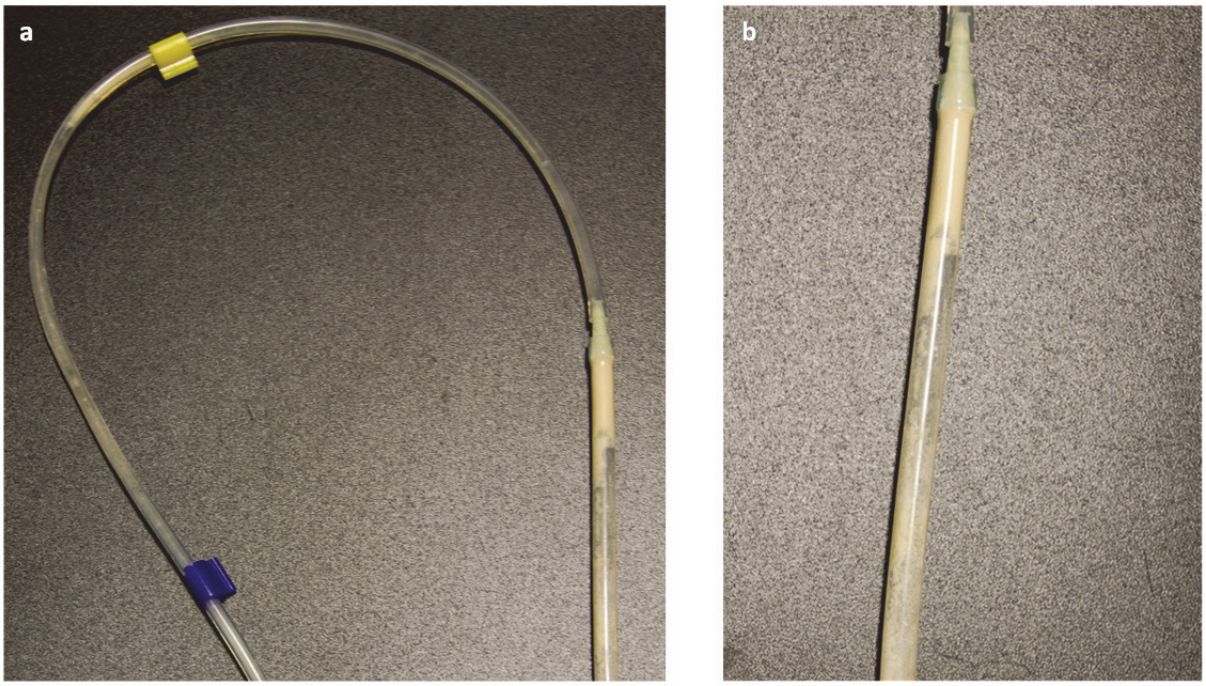



*Figure 31: Photographs showing (a) the deposition of elemental sulphur in the peristaltic pump and effluent tubing and (b) a close-up of the effluent tubing*

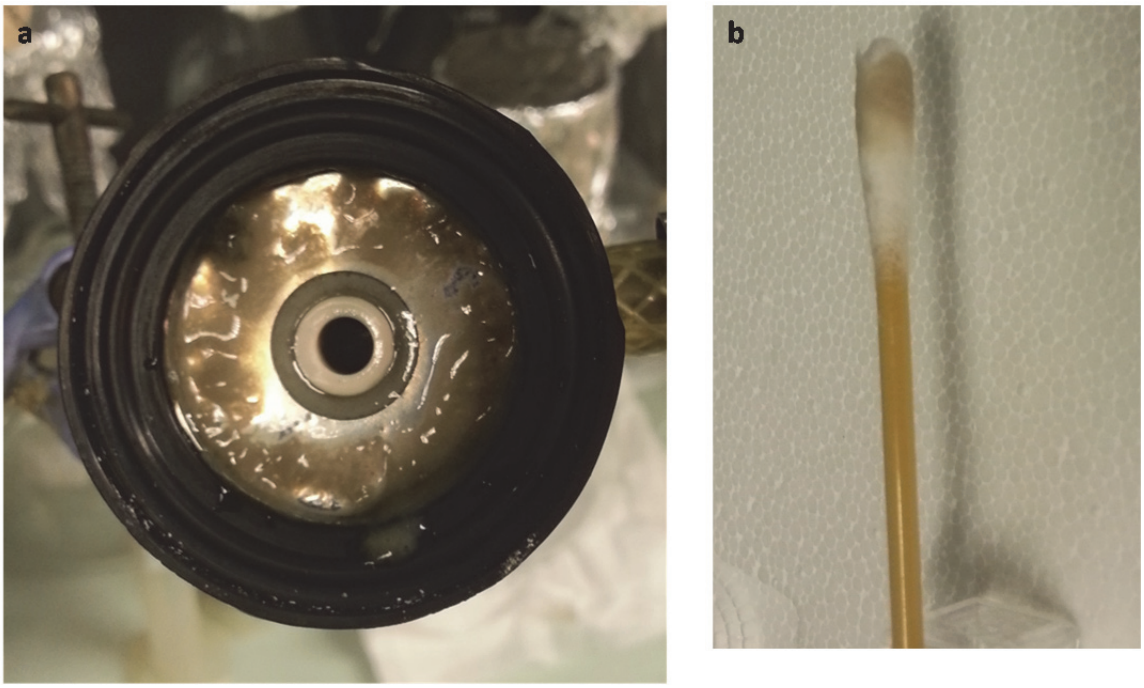



*Figure 32: Photographs of (a) the base plate support the membrane showing biofilm growth on the base-plate and (b) biofilm scraped from the lumen of the membrane*

*Figure 33: Photograph showing elemental sulphur deposition on the outer surface of the membrane. The sulphur layer is progressively thicker toward the right hand side of the image*

### 4.3.3 Linear flow channel reactor with carbon microfibres

The LFCR was operated for over 150 days, from an initial HRT of 5.5 days to a minimum HRT of 0.5 days. The progress of the experiment was interrupted by a major upset event between day 33 and 34, when negative pressure in the reactor headspace resulted in approximately 800 ml of 0.5 M NaOH being siphoned into the reactor from the sulphide scrubber. The event provided an opportunity to evaluate the resilience of the retained biomass. The data prior to the event are presented and discussed separately to those "after".

#### *4.3.3.1 Performance prior to upset conditions*

During the initial period of operation, while the microbial community became established, only the two sample points, toward the end of the reactor (BM and BB), were sampled (see Figure 9). The reactor was initially dosed with chemical sulphide to a concentration of approximately 50 mg/ℓ to ensure the redox potential was sufficiently low to promote sulphate reduction. There was little change in pH (Figure 34) or sulphide concentration (Figure 35) during the first seven days, indicating only limited sulphate reduction activity. A pump failure on day 9 resulted in the reactors operating in batch mode for almost 24 hours. This resulted in a significant increase in the pH within the reactor. This was most likely a combination of more complete utilisation of the VFAs in the feed and the oxidation of some of the sulphide under oxygen limiting conditions. The reactor was designed to exclude oxygen, but the silicone tubing is gas permeable, so some oxygen ingress was likely to occur. The presence of visible colloidal sulphur particles in suspension supported this explanation.

Once the pump had been replaced the pH gradually decreased and reached a relatively steady state around pH 7.2, a slight increase from the feed pH of 6.7. The sulphide concentration increased significantly from day 15, indicating increased biological activity. This corresponded to the appearance of distinct biofilm formation on the submerged carbon fibres.

The channel reactor was a scaled-down version of that used for sulphide oxidation by a floating sulphur biofilm, which formed the basis of an associated project (van Hille *et al*., 2012). The hydrodynamics within the larger reactor (2.5 m length) were extensively studied and the results showed that flow was laminar, even at a relatively low HRT (1 day), and dominated by convective flow, with little diffusion. Furthermore, as a consequence of the flow pattern, vertical stratification was evident and this was a factor of the relative density difference between the incoming fluid and the bulk volume in the reactor. The stratification observed in this reactor was similar.

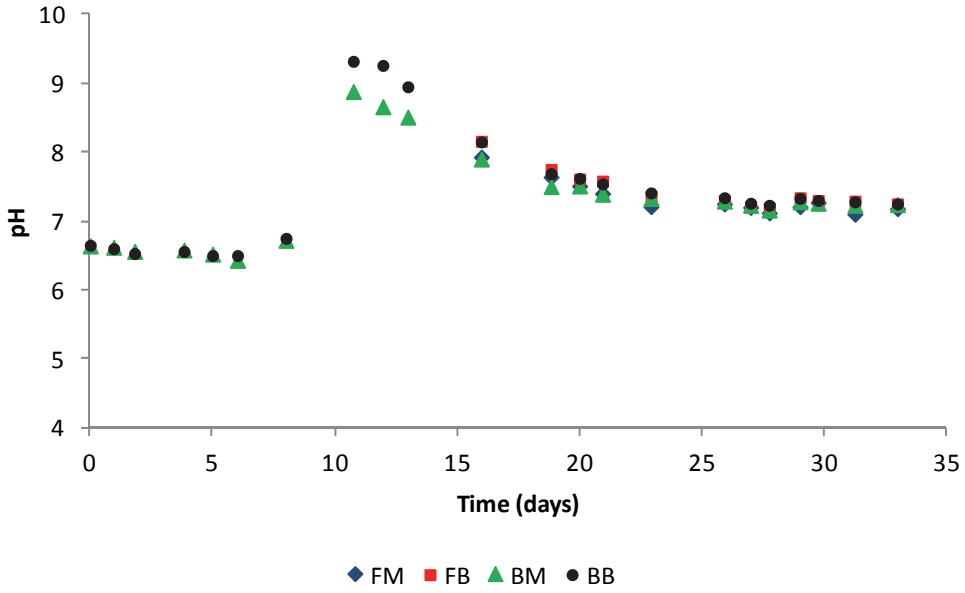



*Figure 34: Data for pH taken from different sampling points within the carbon fibre channel reactor. FM and FB represent the middle and bottom ports in the set nearest the inlet and BM and BB the middle and bottom for the set of ports nearest the outlet (see Figure 9).*

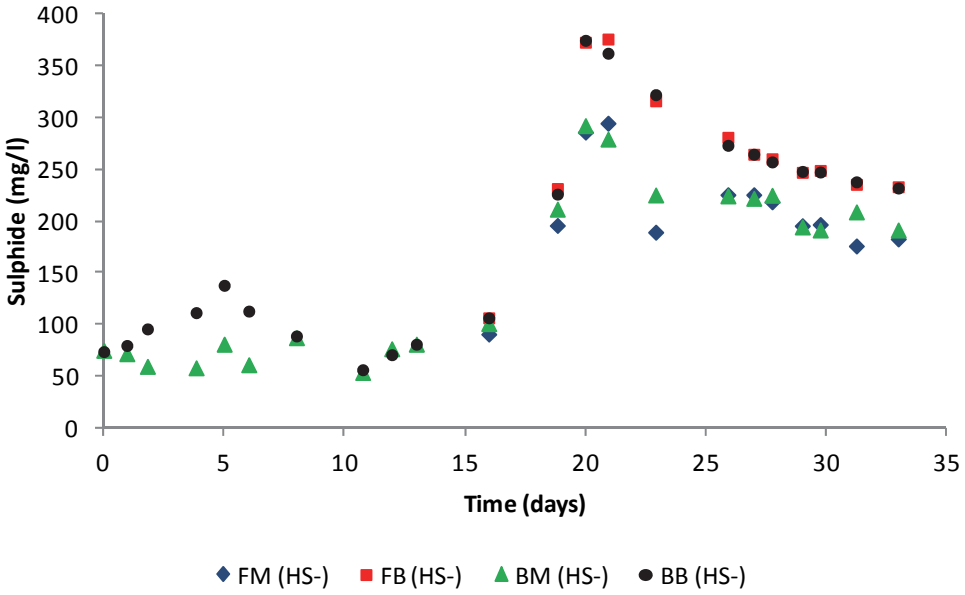



*Figure 35: Sulphide concentration data from different sampling points within the carbon fibre channel reactor. FM and FB represent the middle and bottom ports in the set nearest the inlet and BM and BB the middle and bottom for the set of ports nearest the outlet (see Figure 9).*

At a 5 day HRT, the sulphide concentration stabilised at around 250 mg/ℓ in the lower half and 200 mg/ℓ in the upper half. These values were similar to the steady state concentration observed in the CSTRs.

The residual sulphate data showed a similar vertical trend, with concentrations of 125 and 12 mg/ℓ measured in the FM and FB samples on day 20. The sulphate concentrations were reduced further towards the back end of the reactor, with 76.5 and 6.3 mg/ℓ recorded for the BM and BB samples respectively.

The composition of the reactor effluent differed significantly from that within the reactor. Throughout the first 34 days the sulphide concentration did not exceed 70 mg/ℓ and the pH was typically 0.5 to 0.75 units higher. This could be accounted for by sulphide oxidation in the effluent pipe (silicone), most likely accelerated by sulphide oxidising microorganisms. The coating of elemental sulphur that developed on the inside of the

effluent pipe was clearly visible. The effluent sulphate concentration (181 mg/ℓ) was also significantly higher than that within the reactor, indicating more complete oxidation of some of the sulphide.

#### *4.3.3.2 Performance post upset conditions*

Between day 33 and day 34 approximately 800 mℓ of 0.5 M NaOH was siphoned from the sulphide gas trap into the reactor due to negative pressure within the reactor. This may have been caused by the dissolution of headspace gas. This caused the pH to increase by 4-5 units, representing an increase in $OH^-$ ions of several orders of magnitude (Figure 36). The appearance of the liquid in the reactor changed from murky to clear. The feed rate to the reactor was increased to 1.47 mℓ/min (equivalent to 1 day HRT) to flush out the high pH solution and sulphuric acid was injected via the four sampling ports. The consequence of the acid injection was to generate very acidic conditions in the bottom half of the reactor, due to the density of the sulphuric acid, while the upper half remained alkaline. As a result of the increased flow rate, the pH in the upper and lower portions rapidly converged and by day 38 the pH throughout the reactor ranged between pH 6.7 and pH 7.1, similar to the pre-upset values.

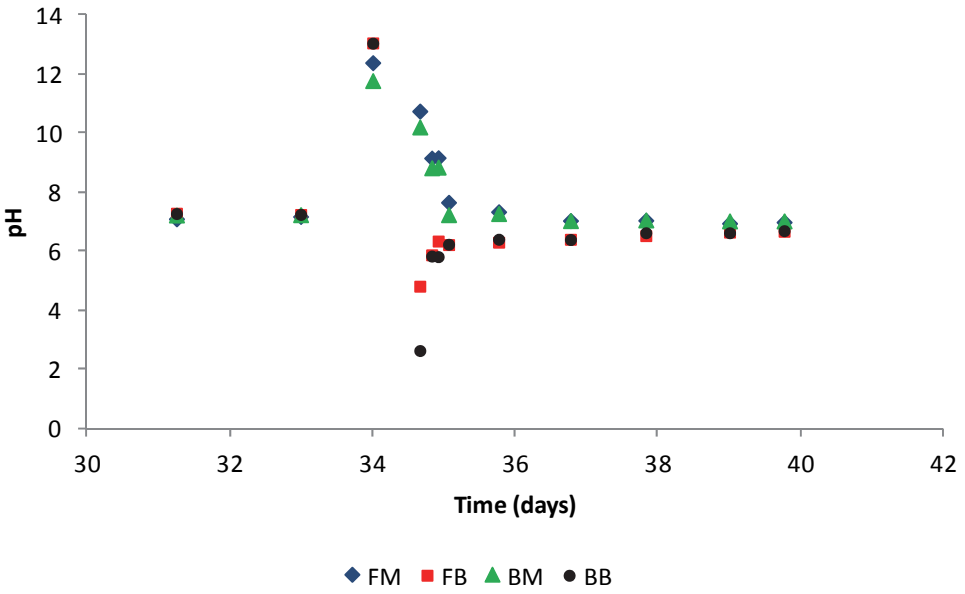



*Figure 36: Data for pH taken from different sampling points within the carbon fibre channel reactor FM and FB represent the middle and bottom ports in the set nearest the inlet and BM and BB the middle and bottom for the set of ports nearest the outlet (see Figure 9).*

The rapid increase in pH had a negative effect on the sulphate reduction performance, resulting in an almost 50% reduction in the sulphide concentration (Figure 37). However, the microbial community remained viable, even in the lower half of the reactor where the pH fluctuated between pH 13 and pH 2.6 within a 24 hour period. Despite the increased feed rate between day 35 and 36, the sulphide concentration remained relatively stable and continued to increase gradually. This illustrated the enhanced robustness of the attached microbial community. It is unlikely that a planktonic culture would have survived the pH fluctuations so well, particularly when coupled with a six-fold increase in dilution rate.

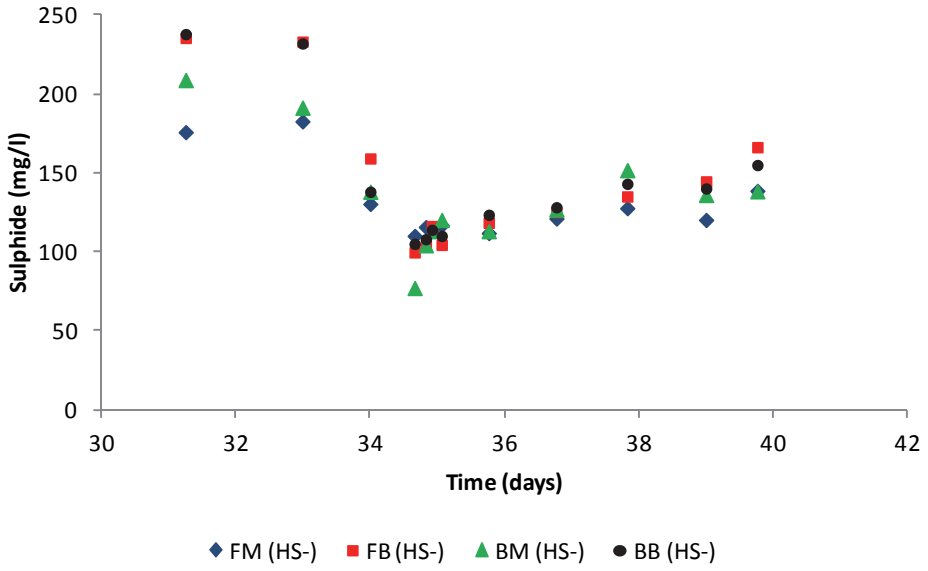



*Figure 37: Sulphide concentration data from different sampling points within the carbon fibre channel reactor. FM and FB represent the middle and bottom ports in the set nearest the inlet and BM and BB the middle and bottom for the set of ports nearest the outlet (see Figure 9).*

Steady state, with respect to sulphate reduction, was restored by day 40 and on day 45 the HRT was reduced to 4 days. The sulphide data (Figure 38) show that a relatively high sulphide concentration was maintained in the reactor, even at a 0.5 day HRT. At the longer HRT there was some inhomogeneity in the reactor, a function of the hydrodynamics within the reactor, with higher concentrations in the lower half of the reactor. While the sulphide levels did decrease to an extent as the HRT decreased, a relatively steady state was observed at each HRT and the decreased performance with decreasing HRT below the critical maximum specific growth rate of the cells, observed in the CSTR, did not occur owing to a de-coupling of the hydraulic and biomass dilution rates. Biofilm growth on the carbon fibres was clearly visible, demonstrating biomass retention.

The pH data for the same period are shown in Figure 39 and show a trend similar to the sulphide. The pH of the feed was between pH 6.8 and pH 7.0, so the decrease in pH at the shortest HRT is probably due to some acetic acid formation.

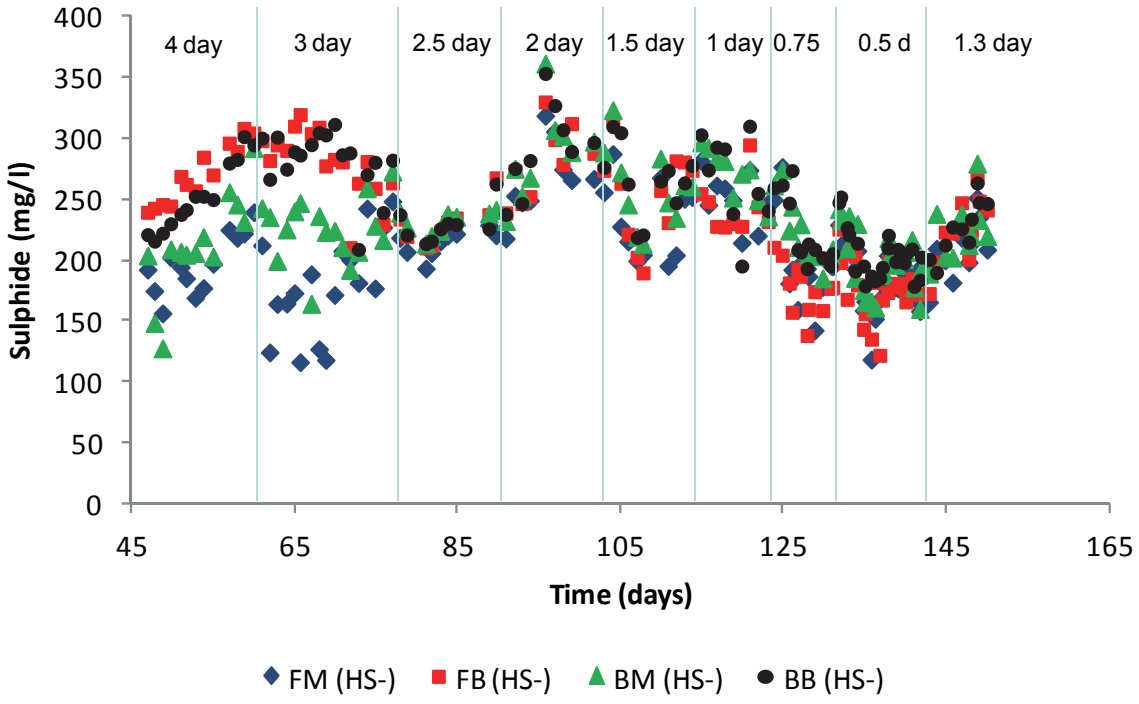



*Figure 38: Aqueous sulphide concentration as a factor of HRT, measured at four points in the reactor. FM and FB represent the middle and bottom ports in the set nearest the inlet and BM and BB the middle and bottom for the set of ports nearest the outlet (see Figure 9).*

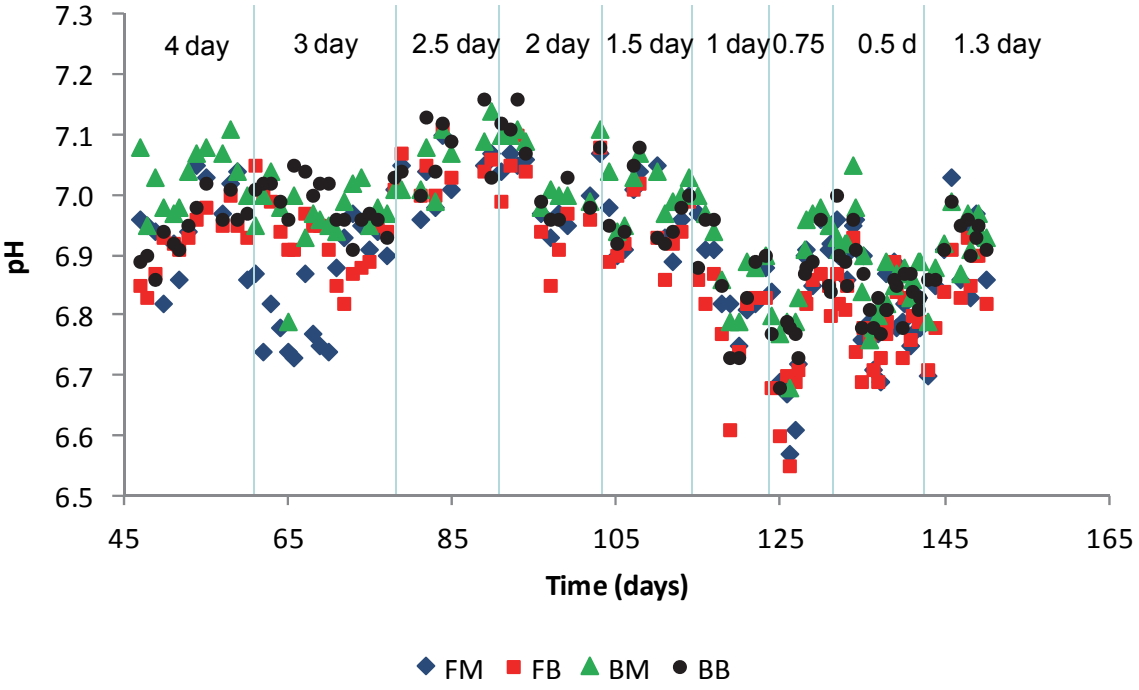



*Figure 39: pH as a factor of HRT, measured at four points in the reactor. FM and FB represent the middle and bottom ports in the set nearest the inlet and BM and BB the middle and bottom for the set of ports nearest the outlet (see Figure 9).*

Due to the lack of turbulent mixing in the reactor, a degree of sulphide oxidation occurred at the air-liquid interface, resulting in the formation of an elemental sulphur layer at the surface, due to partial sulphide oxidation, most likely facilitated by sulphide oxidising microbes. As a consequence, the sulphide concentration of the effluent was lower than that of the bulk fluid, while the pH was also significantly higher (Figure 40).

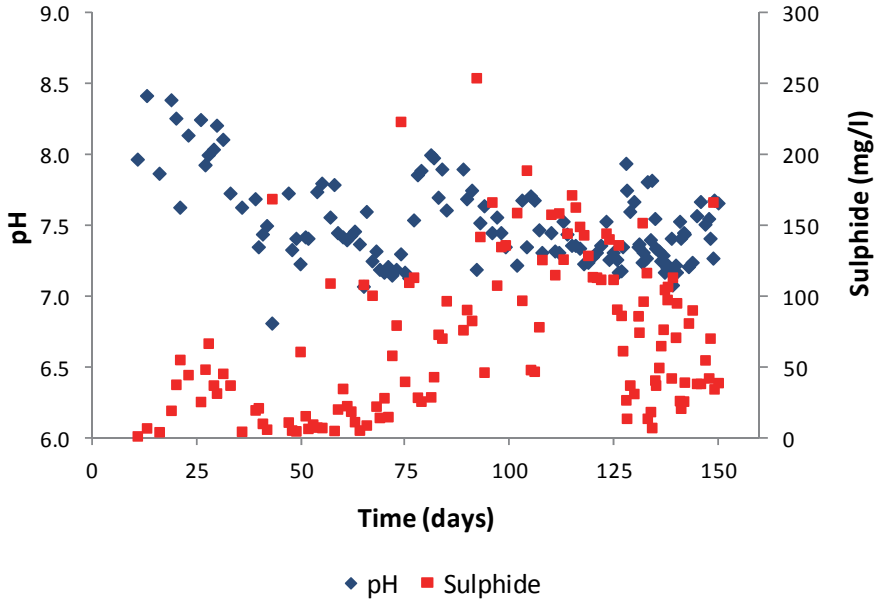



*Figure 40: pH and sulphide measured in the effluent bottle*

A comparison of the sulphide concentration in the effluent bottle and the closest sampling port is shown in Figure 41. Again, at the shortest HRT the extent of sulphide oxidation at the surface was lowest.

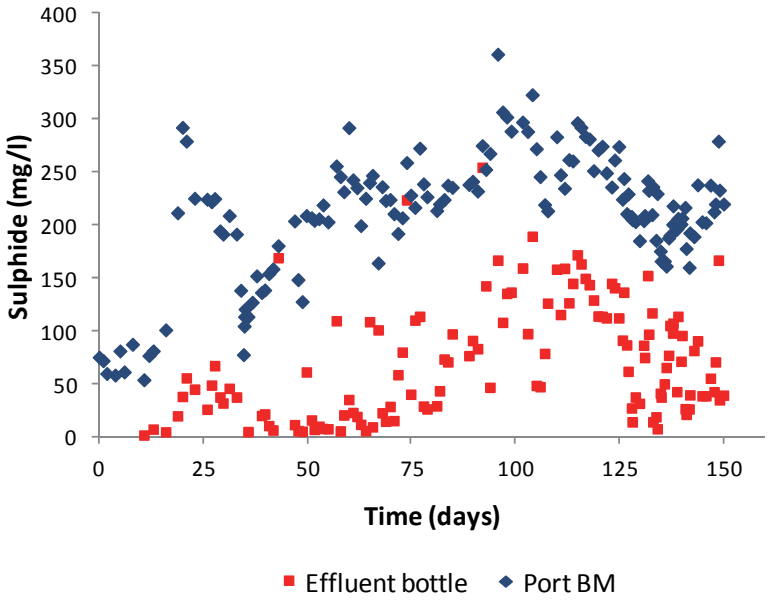



*Figure 41: Comparison of the aqueous sulphide measured at port BM and in the effluent bottle*

#### *4.3.3.3 Evidence of biomass retention*

Upon completion of the study the channel reactor was dismantled. The original configuration had the carbon fibres attached to lid of the reactor, so when the lid was removed the fibre bundles were lifted out of the bulk liquid. This provided the first real evidence of the extensive biomass retention on the carbon fibres (Figure 42). Sections of the biofilm were removed and preserved for future analysis by electron microscopy and molecular techniques.

A thick layer of elemental sulphur had formed at the air-liquid interface and this was disrupted by the removal of the fibres. Sections of the sulphur layer remained attached to the biofilm (Figure 43). The sulphur film was similar in structure to that observed during the operation of a dedicated sulphide oxidation reactor (Mooruth, 2013) and suggested the possibility of combining the sulphate reduction and sulphide oxidation processes within a single reactor, rather than as two separate reactors.


*Figure 42: Photograph showing the extent of colonisation of the carbon fibres in the channel reactor*

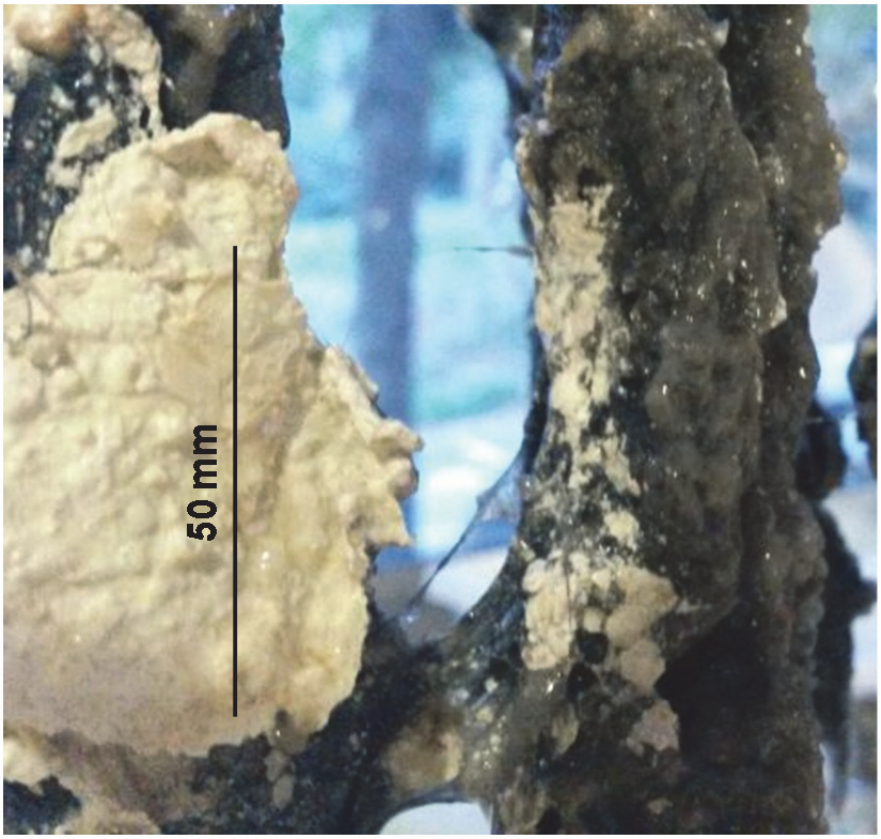



*Figure 43: Close up photograph of the biofilm attached to the carbon fibres showing elemental sulphur deposits*

### 4.3.4 Comparison of performance across reactor configurations

The performance in each of the different reactor configurations can be compared by considering the volumetric sulphate reduction rate relative to the volumetric sulphate loading rate (Figure 44). At a feed

sulphate concentration of 1 g/ℓ, the performance was similar at HRTs from 5 days down to 1 day, with a sulphate reduction efficiency of between 85-95%. Significant divergence in performance was observed at lower HRTs, where washout of a portion of the sulphate reducing community occurred in the stirred tank reactors as the dilution rate exceeded the maximum specific growth rate. The two systems that were characterised by either recycling of the biomass (BSR reactor coupled to membrane filtration unit) or efficient retention of the biomass (carbon microfibre channel reactor) maintained higher VSRRs, owing to the requisite decoupling of the MCRT and HRT. At the HRT at which washout is first observed in the CSTR (0.5 day), for the channel reactor the VSRR was approximately 20% higher than the baseline study, while for the membrane system it was over 50% higher, despite the challenges associated with elemental sulphur formation.

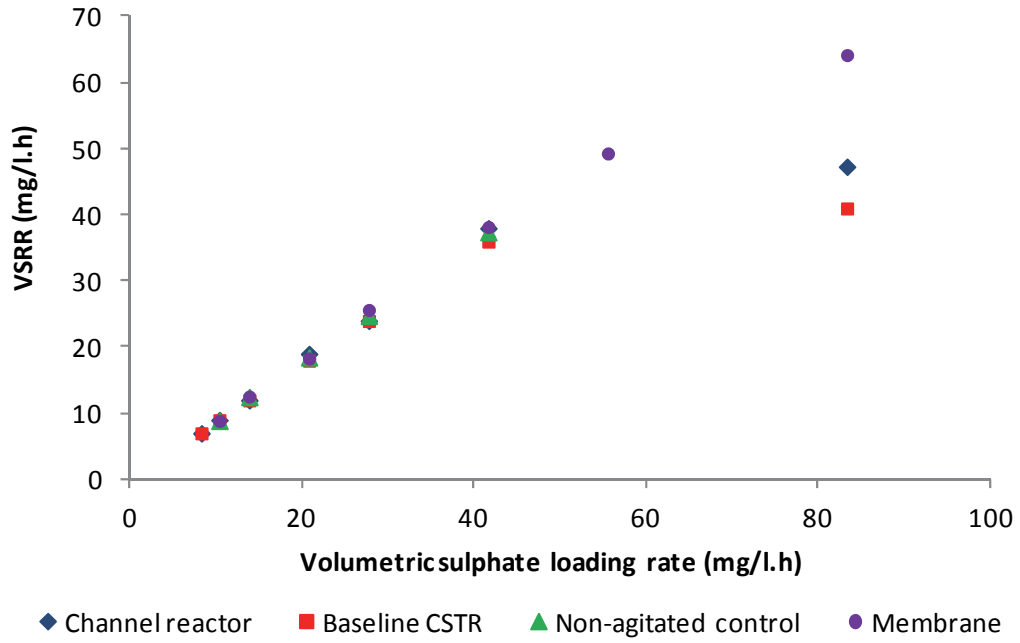



*Figure 44: Comparison of the VSRRs across the four reactor configurations at a feed sulphate concentration of 1 g/ℓ*

## 4.4 CONCLUSIONS

Decoupling of mean cell and hydraulic retention times was achieved successfully in both the LFCR, where biofilm formation on the carbon microfibres was very efficient and ensured biomass retention, and the reactor fitted with the microfiltration membrane, where the membrane permeate was consistently cell free, demonstrating biomass recycle. The maintenance of the biomass within the reactor resulted in significantly improved performance at low HRTs, with the volumetric sulphate reduction rate in the channel and membrane coupled reactors being 20% and 50% higher respectively than the baseline CSTR data at a HRT of 0.5 days.

In both systems, the complete elimination of oxygen was not possible, resulting in the partial oxidation of a portion of the sulphide formed to elemental sulphur. In the channel reactor this occurred primarily in a floating biofilm, similar to that observed in the dedicated sulphide oxidation reactor, with some sulphide oxidation also occurring in the effluent pipe. This suggests that sulphide oxidation and sulphur recovery could be coupled with sulphate reduction in this configuration.

The sulphur formation presented more of a problem in the membrane system as particulate sulphur blocked the permeate drainage line and peristaltic pump tubing, restricting permeate flow, as well as forming a layer on the outer surface of the membrane, reducing transmembrane flux. As a consequence, a significant amount of the accumulated biomass was lost as overflow from the reactor. Despite this, the membrane coupled system resulted in the most efficient sulphate reduction.

# CHAPTER 5: EVALUATION OF MICROALGAE AS AN ELECTRON DONOR

## 5.1 INTRODUCTION

The high cost of traditional electron donors for sulphate reduction, such as ethanol, methanol, carbohydrates and VFAs as well as associated transport costs (Gopal, 2005) has constrained the application of technologies based on sulphate reduction for the treatment of acidic minewaters. The use of complex, lignocellulosic materials has been investigated, but slow and inconsistent degradation have limited the efficiency of subsequent sulphate reduction.

The drive toward renewable energy sources has focused significant attention on biomass as a source of renewable energy. Microalgae, in particular, have been extensively studied as a source of biomass for energy generation, due to their productivity, lipid content and $CO_2$ fixation ability (Cheng *et al*., 2006; De Schamphelaire and Verstraete, 2009; Chisti, 2007, Harrison *et al.,* 2013). They have been shown to be more photosynthetically efficient than higher plants and can be grown in a simple salts medium on a large scale (Illman *et al*., 2000). Algal biomass can be used directly, converted into ethanol, methanol, hydrogen, methane, or lipids extracted to produce fuel oils (Illman *et al*., 2000). Based on the literature relating to the anaerobic digestion of algae, their potential as an electron donor and carbon source for sulphate reduction will be investigated in this study.

### 5.1.1 Anaerobic digestion of algae

Anaerobic digestion incorporates a series of processes in which microorganisms break down biodegradable material in the absence of oxygen (McKendry, 2002). Anaerobic digestion technology is widely used to harness energy from renewable sources. This process produces methane (65-70%) and carbon dioxide (30-35%) rich biogas suitable for energy production, as an alternative to fossil fuels (Gunaseelan, 1997; McKendry, 2002). The biogas produced from anaerobic digestion can be utilised directly or indirectly to derive energy in a number of processes. Examples of these processes are: use in a combined heat and power unit (CHP) where the gas is combusted to produce heat and electricity; directly compressed or liquefied to produce a transport fuel (De Schamphelaire and Verstraete, 2009) or purified and used in the production of more traditional transport fuels such as petroleum or diesel (De Schamphelaire and Verstraete, 2009). The anaerobic digestion literature focuses primarily on biogas composition and yield per unit of substrate, with little consideration given to the composition of the digestate. Some studies report a residual COD value, but not a more detailed analysis.

### 5.1.2 Organic loading rate

The organic loading rate (OLR) determines the amount of volatile solids loaded into the digester. If the OLR is too high, the acidogenic bacteria proliferate, causing a decrease in pH and a low biogas production rate. Various OLRs have been reported in literature based on the amount of volatile solids (VS) per litre of material fed. The OLR is selected according to the type of substrate (e.g. low for high nitrogen organics) and reactor configuration (Speece, 1983). Typical ranges reported are 1.4-3.5 g VS/ℓ.day (Golueke *et al.,* 1957; Chen, 1987; Chandra *et al.*, 2006; Antonopoulou and Lyberatos, 2009). From the perspective of generating a substrate for biological sulphate reduction it may be beneficial to operate a digester at an OLR higher than the optimum for biogas production in order to produce a digestate with a high VFA load.

## 5.2 SELECTION OF ALGAL SPECIES

*Spirulina* sp. (cyanobacteria) and *Scenedesmus* sp. (microalgae) were chosen for investigation as part of this study. Both species are indigenous and have been isolated as part of associated research projects. A number of other algal species were tested for their ability to grow on aerated channel reactor effluent, to

assess their potential as part of a larger, integrated process. Some of these species have been identified on the basis of their 18S ribosomal RNA gene sequence, while others have not yet been identified.

### 5.2.1 Spirulina

*Spirulina* is a microscopic, multicellular blue-green alga (cyanobacterium), normally occurring as a spiral filament, although the spiral may uncoil under certain conditions. *Spirulina* has been found in both marine and alkaline fresh water environments. *Spirulina* is the common name for two species of cyanobacteria, *Arthrospira platensis*, and *Arthrospira maxima*. While they are routinely referred to as algae, because of their aquatic environment and ability to photosynthesise, cyanobacteria are not related to any of the various eukaryotic algae (Ciferri, 1983). *Arthrospira* are free-floating filamentous cyanobacteria characterised by cylindrical, multicellular trichomes in an open left-hand helix (Venkataraman, 1997). They occur naturally in tropical and subtropical lakes with high pH (above 9.5) and high concentrations of carbonate and bicarbonate (above 10 g/ℓ). These species were once classified in the genus *Spirulina*. There is now agreement that they are in fact *Arthrospira*; nevertheless, the older term *Spirulina* remains in use for historical reasons (Ciferri, 1983). Most cultivated *Spirulina* is produced in open-channel raceway ponds, with paddle-wheels used to agitate the water (Venkataraman, 1997).

### 5.2.2 Scenedesmus

*Scenedesmus* sp. is a small, non-motile colonial green alga consisting of cells aligned in a flat plate. The cells are usually cylindrical but may be more lunate, ovoid, or fusiform. Each cell contains a single parietal, plate-like chloroplast with a single pyrenoid. The cell walls may be covered in bumps or reticulations that are best viewed with scanning electron microscopy (SEM). *Scenedesmus* is commonly found in the plankton of freshwater rivers, ponds, and lakes. Growth of the microalgae typically takes place in closed photobioreactors at pH 7 and through direct gas liquid mass transfer of carbon dioxide (Hartig *et al*., 1988).

The macro-composition of a given substrate can help to determine the digestion potential in terms of methane yields (Sialve *et al*., 2009). It also enables the C:N:P nutrient ratio available to the anaerobic microbes to be determined. The composition of nutrients within algal cells is dependent on the environment in which it has grown (Sialve *et al*., 2009).

The average total energy content of algae is around 25.1 J/g (Chen and Oswald, 1998). During anaerobic digestion, an optimised mesophilic system recovers around 60% of this energy (Golueke *et al*., 1957). The remaining 40% is typically resistant to release during digestion because of substrate properties such as degradation resistant biopolymers contained within the cell walls (Blokker *et al*., 1998). A pre-treatment of lignocellulosic material is essential for the removal of lignin and hemicelluloses, which can enhance hydrolysis of cellulose and thus improve yields (Sun and Cheng, 2002; Wyman *et al*., 2005). The cell walls of algal biomass protect the cell from the physicochemical and biological environment, including enzymes produced by the anaerobic microbe, reducing the cells' biodegradability and so creating a strong resistance to hydrolysis. Golueke *et al*. (1957) and Sanchez and Travieso (1993) reported the presence of whole cells in their digesters after long periods of time, confirming the resistance to degradation. Physical factors that influence the hydrolysis of cellulose have been identified. These include the porosity (accessible surface area) of materials, cellulose fibre crystallinity, and the lignin and hemicellulose content (Himmel and Overend, 1994). Therefore the removal of lignin and hemicelluloses, reduction of cellulose crystallinity and increase of porosity during pre-treatment is expected to have positive effects on the hydrolysis efficiency of enzymes such as cellulases (Sun and Cheng, 2002). Algal biomass is typically characterised by a relatively high (> 10%) hemicellulose content. Ververis *et al*. (2007) investigated cellulose, hemicellulose, lignin and ash contents for various organic materials. The results emphasised the high content of hemicellulose (average 16.3%).

Hydrolysis of cellulose may occur in anaerobic digestion, dependent on the microbial community present, but is difficult and may be rate limiting if present in high concentrations (Yen and Brune, 2007). This makes investigation into cell disruption relevant. A number of technologies exist for the disruption of cell walls for the release of proteins, lipids, nucleic acids and carbohydrates (Doucha and Livansky, 2008; Harrison 2011). Of all the available technologies, the following have been investigated for algal biomass pre-treatment: physical (ultrasonification, Samson and LeDuy, 1983), thermal (hot water hydrolysis, Chen and Oswald,

1998; Samson and LeDuy, 1983) and thermo-chemical (heating with addition of NaOH, Chen and Oswald, 1998; Samson and LeDuy, 1983) pre-treatments.

## 5.3 EXPERIMENTAL PROGRAMME

### 5.3.1 Biological sulphate reduction using whole *Spirulina* biomass as carbon source

The 1 ℓ CSTRs were operated at a HRT of four days. The digester maintained on whole-cell *Spirulina* was inoculated with a mixture of the stock culture (10% v/v) and overflow from the *Spirulina* AD (10% v/v). It was continuously fed with a basal salt solution (modified Postgate B medium without lactate, citrate and yeast extract). Algal slurry (approximately 24 g wet mass in 50 mℓ water) was injected into the reactor on day one of each four day cycle. A parallel digester was operated on modified Postgate B medium.

#### *5.3.1.1 Sampling protocol*

A sample (10 mℓ) was drawn from the reactor at regular intervals (5 per week). A portion of the sample was used immediately to determine sulphide concentration. A fraction (1.9 mℓ) of the sample was placed in a 2 mℓ Eppendorf tube and 40 µℓ of zinc chloride solution added to react with the sulphide. The tube was centrifuged at 6500 × *g* to remove the zinc sulphide, then filtered through a 0.22 µm membrane filter and stored for HPLC analysis. The remainder of the sample was used to measure pH and redox potential.

### 5.3.2 Batch anaerobic digestion studies

#### *5.3.2.1 Algal cell disruption*

Bead milling was used to rupture both *Scenedesmus* spp. and *Spirulina* spp. cells physically. The method involved loading 1 ℓ of concentrated algae slurry into the bead mill and operating continuously for a period of time that would allow for complete disruption. The algal cells were disrupted at concentrations of 40 g/ℓ and 20 g/ℓ. Two 2 mℓ samples were taken every 30 min for *Spirulina* spp. disruption and hourly for *Scenedesmus* spp. disruption. The samples were spun down at 12000 rpm (9660 x *g*) for 10 minutes. The supernatant was decanted and utilised for optical density, soluble COD, pH and VFA analyses. The solid pellet remaining was diluted to 2 mℓ using deionised $H_2O$ and used for solid COD analysis. Photographs taken, using a diluted 20 µℓ volume, through a 100× objective lens were used to obtain confirmation of cell disruption and for determination of complete disruption. The mill ran for a time period that allowed for complete disruption of the algal cells.

#### *5.3.2.2 Whole cell slurry preparation*

To obtain the desired slurry concentration for addition to the anaerobic digesters the following process was followed. A sufficient mass (wet weight) of algal cells were harvested. The dry mass of a fraction of the w*et al*gae was determined to allow for the calculation of the moisture content. Once the moisture content of the w*et al*gae had been determined, sufficient wet mass was diluted with deionised $H_2O$ to obtain the desired dry weight slurry concentration. The accuracy of the process was calculated by performing a dry mass determination of a fraction of the slurry. This procedure was applied to both *Scenedesmus* and *Spirulina* algal slurries.

#### *5.3.2.3 Ruptured cell slurry preparation*

For the digesters loaded with ruptured biomass the same initial procedure was followed. This produced an unruptured algal slurry with the correct biomass concentration. After this had been done the slurry was loaded into the bead mill and operation initiated. Once complete disruption had occurred the slurry was separated from the beads using sedimentation and cloth filtration (100 µm pore size).

### 5.3.3 Batch digestion studies

The following experimental protocol was followed for all batch digestion experiments. The digesters were loaded with 20 g dry weight (DW) of the appropriate substrate. The substrate was either in the form of wet biomass or a slurry of ruptured cells with an initial loading of 20 g DW. Tap water was added to a total volume of 800 mℓ. The reactor was inoculated with 100 mℓ of the *Spirulina* stock inoculum and 100 mℓ of the SAB inoculum. The reactor was made airtight using a custom cast silicone seal, which fitted inside the screw cap. The sampling and gas collection port were connected and the reactor prepared for anaerobic operation by sparging with nitrogen for 5 min. All ports, with the exclusion of the gas exhaust port that was positioned into the gas collection system, were sealed. The reactors were loaded onto an orbital shaker rotating at 140 rpm in a temperature controlled room at 37°C.

#### *5.3.3.1 Sampling protocol*

The sampling protocol for all batch digesters involved daily gas phase sampling where a 100 µℓ Hamilton syringe was used to sample directly from the digester headspace. Every second day 2 mℓ of liquid sample was removed from the digesters via the liquid phase sampling port. The sample was separated into two 2 mℓ Eppendorf tubes (1 mℓ of sample in each). One of the tubes was used for pH analysis and immediately stored at -20°C for future sampling, if necessary. The second sample was spun down at 12 000 rpm for 10 minutes. The supernatant was decanted from the solid pellet. A 400 µℓ sample of the supernatant was further diluted to 2 mℓ, filtered through a 0.22 µm Millipore filter and used for all HPLC analysis. A further 400 µℓ sample of the supernatant was diluted to 1400 mℓ and used in the COD analysis. The solid pellet remaining was diluted to 2 mℓ and used for COD analysis. Note: all dilutions were conducted using deionised $H_2O$.

### 5.3.4 Continuous digestion studies

The 1 ℓ CSTRs were operated at a HRT of four days. The digester maintained on whole-cell *Spirulina* was inoculated with a mixture of the stock culture (10% v/v) and overflow from the *Spirulina* AD (10% v/v). It was fed continuously with a basal salt solution (modified Postgate B medium without lactate, citrate and yeast extract). Algal slurry (approximately 24 g wet mass in 50 mℓ water) was injected into the reactor on day one of each four day cycle. A parallel digester was operated on modified Postgate B medium.

#### *5.3.4.1 Sampling protocol*

A sample (10 mℓ) was drawn from the reactor at regular intervals (5 per week). A portion of the sample was immediately used to determine sulphide concentration. A fraction (1.9 mℓ) of the sample was placed in a 2 mℓ Eppendorf tube and 100 µℓ of zinc acetate solution added to react with the sulphide. The tube was centrifuged at 6500 × *g* to remove the zinc sulphide, then filtered through a 0.22 µm membrane filter and stored for HPLC analysis. The remainder of the sample was used to measure pH and redox potential.

### 5.3.5 Biological sulphate reduction using partially digested *Spirulina* biomass

Two continuous stirred tank reactors were set up and fed effluent from the anaerobic digester as the carbon source and electron donor, in order assess the proof of concept. No additional nutrients were provided. Constraints on the number of reactors available and the amount of effluent that could be generated from the digester meant that a limited number of reactors could be maintained. The decision was taken to test the 2.5 and 5 g/ℓ sulphate concentrations, as the data generated at these conditions using lactate as the feed identified challenges. Effluent from the digester was collected until 2 ℓ was available. The effluent was filtered through a fine mesh (50 µm) cloth, then centrifuged at 5 000 × *g* for 10 minutes to remove particulate material that could block the feed pipes. The COD of the supernatant was determined. The centrifuged effluent and sulphate stock solution were placed in separate feed bottles and the pumping rates calibrated to achieve the desired overall HRT in the reactor and maintain a COD:$SO_4^{2-}$ ratio of 0.7. The concentration of the sulphate stock solution was adjusted appropriately. The calculation and calibration was performed each time fresh digester effluent was added, although the COD of the effluent was relatively consistent so only small changes were required.

Both sets of reactors were initially operated at a HRT of 5 days (dilution rate 0.0083/h). The reactors were sampled at least twice per HRT and the pH, redox potential and sulphide concentration determined immediately. A fraction (2 mℓ) of the sample was treated with 40 µℓ of zinc chloride (100 g/ℓ) and centrifuged at 14000 × *g* for 7 minutes to remove sulphide as zinc sulphide. The supernatant was filtered through a 0.45 µm nylon membrane filter and retained for HPLC analysis (VFAs and sulphate). A COD analysis was performed on selected samples, with a minimum of one analysis per HRT.

The data were used to determine when steady state was achieved. Once steady state had been maintained for a further three HRTs, the feed rate was increased to achieve the next HRT.

### 5.3.6 Algal growth studies

The promising results achieved using partially digested algae to sustain biological sulphate reduction prompted an investigation into the potential of cultivating algae on effluent from the sulphate reduction unit, with the view to developing an integrated system. The configuration of the channel reactor resulted in partial oxidation of a significant proportion of the sulphide at the surface of the reactor, resulting in a much lower sulphide concentration in the effluent. It was therefore chosen for further study. The initial tests were performed using *Spirulina* as it was the preferred substrate for anaerobic digestion. The study was extended to a broader range of known species and environmental isolates, as recommended by the Reference Group.

#### *5.3.6.1 Spirulina cultivation on reactor effluent*

The potential of using the reactor effluent as the basis for an algal cultivation medium was investigated using *Spirulina*. Experiments were performed in 500 mℓ Erlenmeyer flasks with a liquid volume of 200 mℓ. Duplicate flasks were set, containing 200 mℓ of centrifuged reactor effluent, or mixtures of reactor effluent and Zarrouk's medium (75:25, 50:50 and 25:75). Control flasks contained 200 mℓ of Zarrouk's medium. The flasks were inoculated with a *Spirulina* slurry to an initial algal concentration of 0.15 g/ℓ. The flasks were agitated using a magnetic stirrer and maintained in a constant environment room at 37°C, under constant illumination. A 5 mℓ sample was removed daily and the pH, redox potential and optical density (750 nm) determined. The biomass concentration was calculated using a standard curve of dry biomass concentration as a function of optical density.

#### *5.3.6.2 Aeration of channel reactor effluent*

Many passive treatment systems contain an oxidation cascade, either to promote iron oxidation at the front end of the system or prior to polishing at the back end. The channel effluent contained some residual sulphide and still had a redox potential of around -340 mV, so it was decided to pre-oxidise the effluent prior to algal cultivation. The collected effluent, in 5 ℓ batches, was aerated using an air-stone at an air flow rate of 2 ℓ/min. A sample was taken every 30 min and the pH, redox potential and residual sulphide determined. Once the redox potential had increased to close to 0 mV the effluent was used to test algal growth. A sample of the aerated channel reactor effluent (ACRE) was filtered for anion analysis to determine phosphate and nitrate content.

#### *5.3.6.3 Assessment of cultivation potential*

Algal growth studies were performed in glass bottles with a working volume of 100 mℓ, connected to an aeration manifold. Air was bubbled through the cultures at approximately 0.2 ℓ/min. Three cultures were tested, *Spirulina, Scenedesmus* and mixed culture from a wastewater treatment plant. A 10% inoculation was used (i.e. 10 mℓ in 90 mℓ aerated channel effluent). An initial sample (4 mℓ) was removed and the optical density determined at 750 nm, against a blank of the aerated effluent. Samples were taken on a daily basis to measure pH and optical density. Following the initial set, a number of further tests were performed using micro and macroalgae isolated from natural environments.

#### *5.3.6.4 Growth rate studies in airlift photobioreactors*

The growth rate of selected algal species, which showed positive growth on the ACRE, was determined in the airlift photobioreactors. The standard photobioreactor configuration was used, with an aeration rate of 2 ℓ/min. The air was not supplemented with addition $CO_2$. Three reactors were operated in parallel. The first acted as a positive control, with 3N BBM as the growth medium. The second contained ACRE as the growth medium, while the third contained ACRE supplemented with nitrogen and phosphate to the same concentrations as the 3N BBM. The reactors were inoculated with seed culture to an initial $OD_{750}$ of 0.1, corrected for the background absorbance of the ACRE. Reactors were sampled daily to determine the pH and cell density.

## 5.4 RESULTS AND DISCUSSION

### 5.4.1 Continuous sulphate reduction using whole cell *Spirulina*

The ability of the sulphate reducing culture to consistently reduce sulphate and generate sulphide using whole-cell *Spirulina* as the sole carbon source and electron donor was demonstrated (Figure 45). The measured sulphide concentration in the first week after inoculation was high, most likely due to the metabolism of residual volatile fatty acids carried over with the inocula. Following that the sulphide concentration fell significantly, to below 50 mg/ℓ, before starting to increase by the second week. The sulphide concentration appeared to stabilise within a range of 70 to 100 mg/ℓ, but the degree of variation was high and steady state was not achieved. This was in contrast to the lactate-fed reactors, where steady state was achieved and maintained at a four day HRT. The pH of the system remained relatively constant within the optimal range for sulphate reduction.

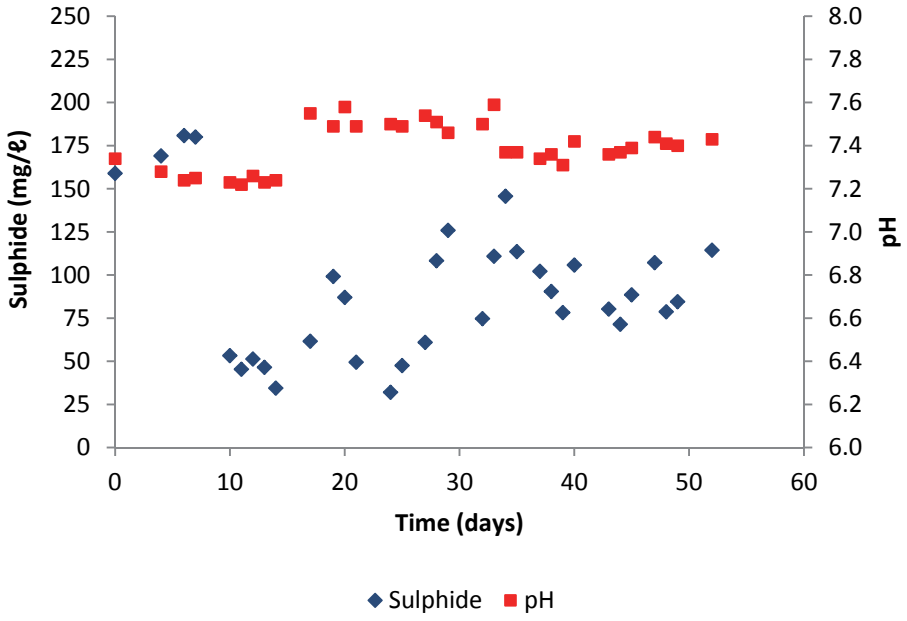



*Figure 45: Sulphide and pH data for sulphidogenic bioreactor fed on whole-cell Spirulina*

While the experiment showed that sulphate reduction could be achieved using whole cell *Spirulina* as the electron donor and carbon source, the inability to achieve steady state meant that kinetic parameters could not be determined accurately. Based on the residual sulphate concentration data, the sulphate reduction rate was 40-50% of that achieved using lactate as the electron donor and carbon source.

The relative instability of the algae-fed reactor and resultant cyclic behaviour may indicate a sensitivity of microbes involved in algal digestion to sulphide concentration and prompted a more detailed study on the degradation of algae under anaerobic conditions to evaluate the rate of VFA generation and assess the potential of algae as a suitable carbon source in either a one-stage or two-stage process.

### 5.4.2 Anaerobic digestion of algal biomass

Most SRB utilise VFAs as the electron donor and carbon source for sulphate reduction. Where a complex organic carbon source is utilised, the material must first undergo a series of hydrolytic and acidogenic degradation reactions. These are typically catalysed by non-sulphate reducing bacteria and the reactions are similar to the initial phases of anaerobic digestion for biogas generation. Therefore, in order to evaluate the generation of VFAs from algae a series of batch anaerobic digestion reactors were operated. Two very different algal species were selected, *Spirulina* for its ability to grow in saline media and its potential to be used in an integrated process with carbon sequestration and *Scenedesmus* for its high productivity and apparent competitive advantage under typical outdoor cultivation conditions (neutral pH, ambient temperature, low salinity).

#### *5.4.2.1 Digestion of whole cells*

The first round of experiments was performed on whole algal cells. Batch digesters were operated in triplicate and the data presented represent the mean values. The progress of digestion was monitored through solid and soluble COD concentrations (Figure 46). The COD of the solid biomass represented oxygen demand by complex organics comprising the cell wall and intracellular content of the algae. The soluble COD represented the demand for oxygen from organic compounds in the aqueous phase of the digesters. For the *Spirulina* digesters a rapid decrease in solid COD was accompanied by an increase in soluble COD (days 0 to 4). This is most likely accounted for by osmotic shock. The Zarrouk's medium has a high dissolved salts concentration (EC > 20 mS/cm), to which the cells are adapted. Placing the cells in a low salt solution would result in the rapid ingress of water causing cells to rupture. In addition, *Spirulina* has a soft cell wall made of complex sugars and proteins, unlike the cellulosic walls of most other algae, hence is easily disrupted and more prone to rapid digestion (Kozenko and Henson, 1996). This accounts for the consistent decrease in solid COD up to day 30. The soluble COD decreased from day 4 as biogas production became pronounced and stabilised at the same time point, 30 days. At this point both the residual solid and soluble components were resistant to further degradation.

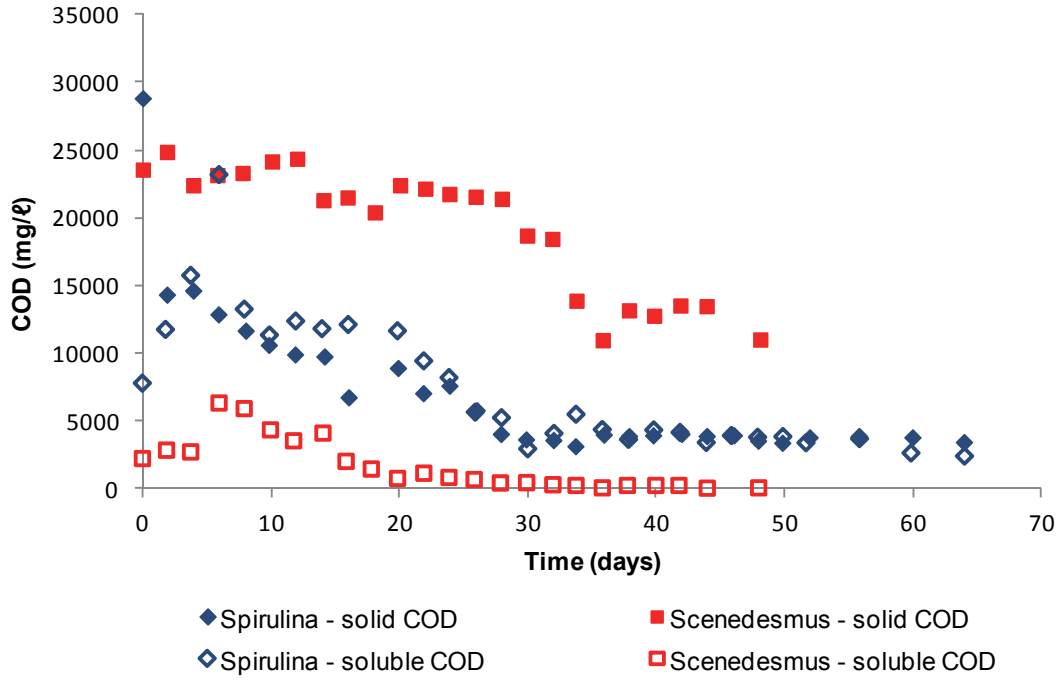



*Figure 46: Solid and soluble COD data for the batch digestion of whole-cell Spirulina and Scenedesmus*

The solid COD in the *Scenedesmus* spp. digesters only decreased significantly at a later stage (day 26) indicating lower biodegradability. The cell walls of *Scenedesmus* are composed of highly resistant, non-hydrolysable aliphatic biopolymers. The biopolymers are composed of long-chain even-carbon-numbered unsaturated o-hydroxy fatty acid monomers. The polyether nature of these algaenans makes them highly resistant against degradation (Blokker *et al.*, 1998). The cell walls also are composed of large amounts of

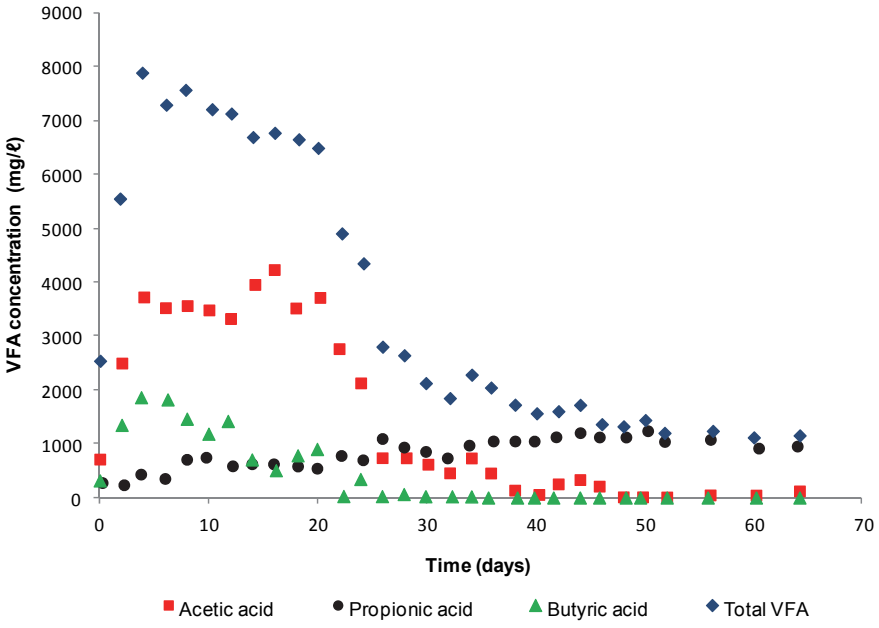
hemicellulose, which is characterised by slow hydrolysis (Yen and Brune, 2007). The soluble COD concentration of the *Scenedesmus* digesters decreased to the detection limit of the assay, indicating complete oxidation of the soluble organic fraction. However, a significant proportion of the solid material was not biodegradable and the overall liberation of soluble organics suitable for conversion to VFAs was significantly lower than for *Spirulina*, suggesting that whole-cell *Scenedesmus* would not be an effective carbon source for bacterial sulphate reduction, particularly in active systems, in the absence of cell breakage.

Biomass production, due to microbial growth, also contributed to an increase in solid COD. However, in AD systems the contribution rarely exceeds 0.5 g/ℓ and so the impact on the COD is low (Lyberatos and Skiadas, 1999).

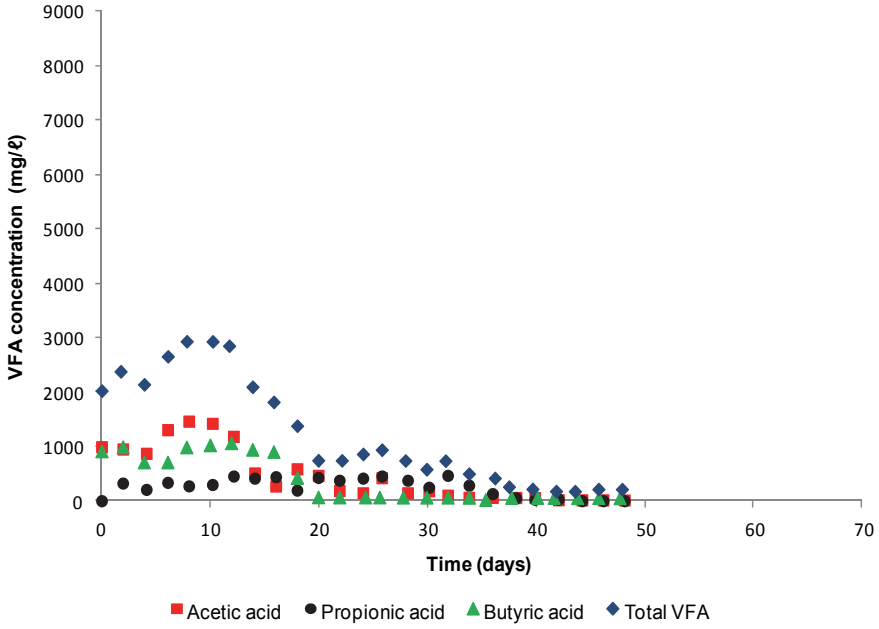
Volatile fatty acids, particularly acetate, are the most important substrate for sulphate reducing bacteria. Acetate is produced via the microbial conversion of longer chain fatty acids (butyrate and propionate). Therefore, monitoring these key volatile fatty acids informs the final feasibility analysis. Trends in VFA metabolism have been used as process indicators in many laboratory, pilot and industrial scale digesters (Ahring *et al.*, 1995; Boltes *et al*., 2008; Buyukkamaci and Filibeli, 2004; Hickey and Switzenbaum, 1991). The real time analysis of VFAs allows digester stability to be monitored and, if necessary, actions taken to control the system. For batch anaerobic digestion, the acidogenic (including acetogenisis) and methanogenic stages should be clearly identifiable from changes in the key VFAs within the digester (Boltes *et al*., 2008). This is important when considering AD as a precursor to sulphate reduction as the HRT in the AD should be sufficiently short to promote acidogenesis over methanogenesis.

For both *Spirulina* (Figure 47 (a)) and *Scenedesmus* (Figure 47 (b)), large amounts of VFAs were produced from day 0 to 4. The most significant VFAs were acetic, butyric and propionic acids. A consequence of the high VFA production was a decrease in pH. The *Spirulina* culture decreased from pH 6.5 to 6.1 and the *Scenedesmus* culture from pH 6.6 to 6.2. The additional alkalinity in the *Spirulina* substrate, most likely a carry-over from the growth medium, buffered the system and despite the high VFA production the pH did not drop excessively. The accumulation of these acids indicated that the system was in the acido- and aceto-genisis phases of AD. The acids formed at a higher rate than the acetic acid consumption by the methanogenic microbes. After the initial high rate of production of these specific acids, the total VFA concentration stabilised. The anaerobic microbial consortia can also be sensitive to the hydrogen partial pressures ($pH_2$) (Lyberatos and Skiadas, 1999). Whilst acetate production is independent of hydrogen partial pressure, the degradation of carbohydrates to butyrate and propionate cannot proceed at high hydrogen partial pressures (Lyberatos and Skiadas, 1999). A high $pH_2$ will thus lead to inhibition of butyrate and propionate metabolism, leading to their accumulation.

The difference in the AD of the two un-ruptured algal species was clearly visible. Volatile fatty acid generation was greater when the reactors were loaded with *Spirulina* (8000 mg/ℓ Total VFAs) compared to that loaded with *Scenedesmus* (2800 mg/ℓ Total VFAs). The ratio at which the key acids (acetate:butyrate:propionate) were produced also varied: 8:4:1 for the *Spirulina* system and 3:2:1 for the *Scenedesmus* system at maximum total VFA concentrations. The higher concentration of VFAs produced is related directly to the availability of easily fermentable organics and simply degradable biomass within the digesters (Angelidaki *et al*., 1999). In their general model for AD, Moletta *et al*. (1986) proposed that an organic substrate is comprised of more recalcitrant organics (non-soluble proteins, lipids etc.) and easily fermentable organics (glucose equivalent), which are fermented by acidogenic bacteria to produce organic acids (acetate equivalent). The high acid production relates directly to the high rate of fermentation. *Spirulina* rupture readily due to osmotic shock, releasing a larger amount of organic compounds for hydrolysis and fermentation. *Scenedesmus* has a strong cell wall made up of complex biopolymers resistant to hydrolysis. In addition, the growth media has a lower ionic strength (roughly 2 mS/cm) and so the cells did not experience osmotic shock when loaded into the digester. Hence fewer readily available organics were present in the aqueous phase of the slurry for conversion to VFAs during the initial stages of digestion. Degradation of the cellulosic cell wall was required to liberate organics and the enzymatic degradation of cellulose is slow (Samson and LeDuy, 1983) explaining the lower amount of VFAs is the *Scenedesmus* reactors.



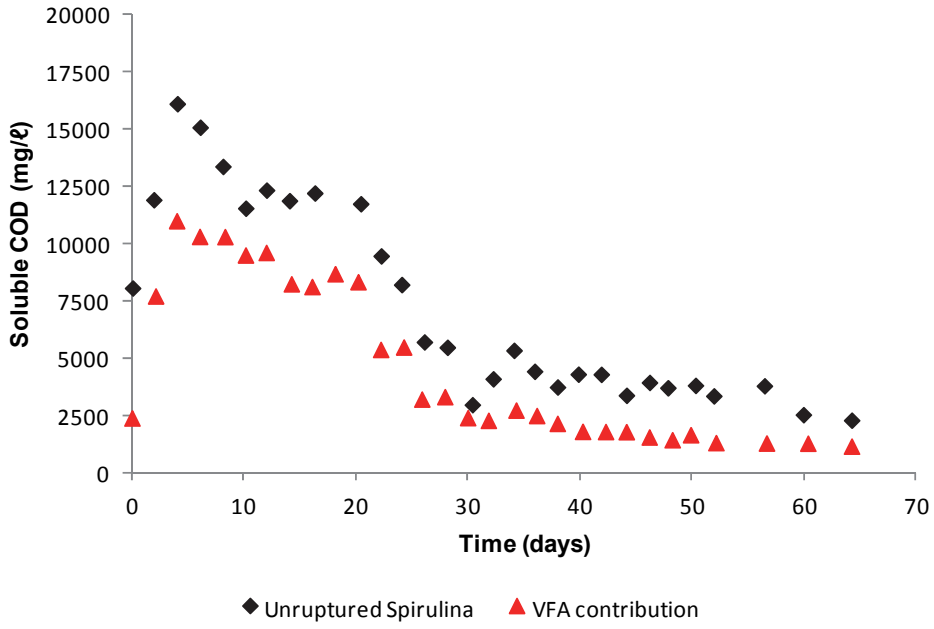

(a) *Spirulina* substrate loaded digesters



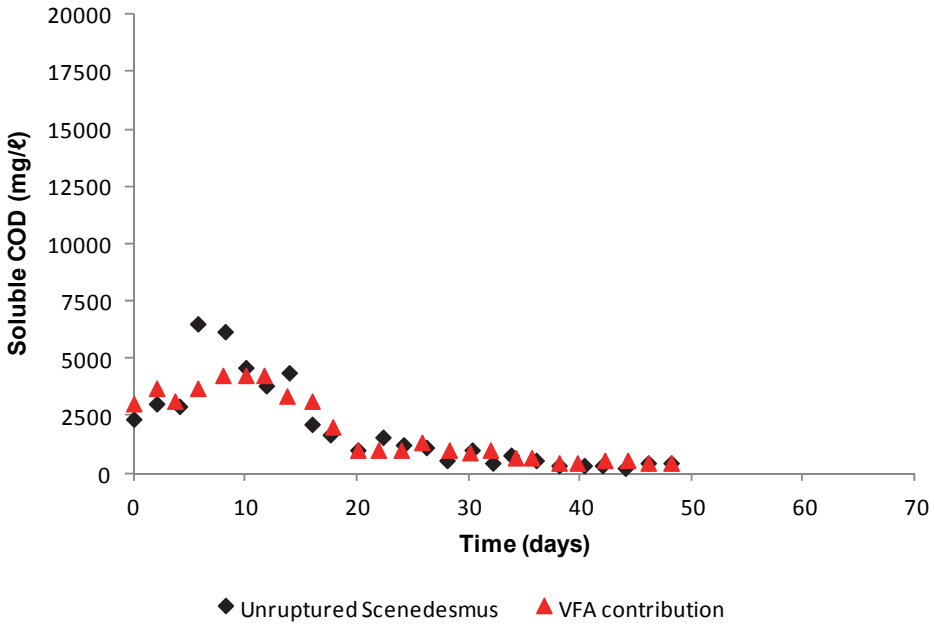

(b) *Scenedesmus* substrate loaded digesters

*Figure 47: Volatile fatty acid profiles as a function of time in batch digesters fed whole algal cells*

The digestion of unruptured algal cells showed that *Spirulina* was more amenable to digestion, liberating more VFAs, primarily due to the ease with which the cells broke open and the non-cellulosic cell wall. Unruptured *Scenedesmus* did not appear a viable option to support sulphate reduction, even in a two-stage process.




(a) *Spirulina* whole cell loaded digesters




(b) *Scenedesmus* whole cell loaded digesters

*Figure 48: Proportion of soluble COD accounted for by VFAs for the digestion of Spirulina and Scenedesmus*

The proportion of soluble COD accounted for by VFAs (Figure 48) was high for the digestion of both algal species. In the case of *Spirulina* there was a greater divergence, almost certainly accounted for by the high concentration of pigments liberated when the cells ruptured, particularly the water soluble phycocyanin.

Biogas production was high during the first four days in both cases, but the gas primarily comprised carbon dioxide, liberated during the acidogenic reactions. Significant methane production was only observed after day 12 in the *Scenedesmus* reactors and day 16 in the *Spirulina* reactors. Therefore, the HRT in the anaerobic digestion phase of a two-stage system should be below 12 days.

#### *5.4.2.2 Digestion of ruptured cells*

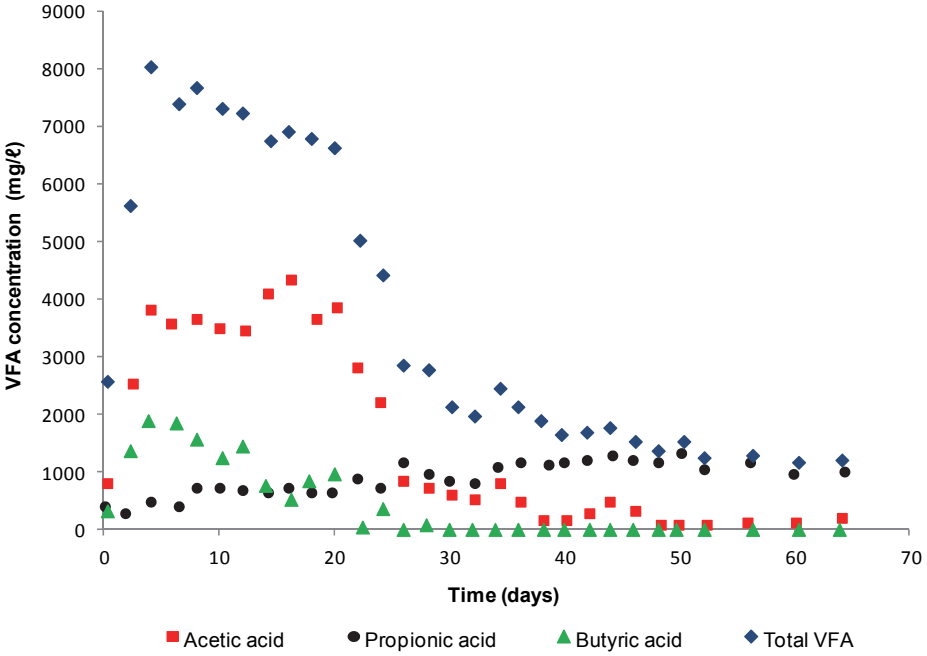
Bead milling was selected as the physical pre-treatment method to disrupt both *Spirulina* and *Scenedesmus* cells prior to digestion. The current project did not focus on optimisation of the disruption method, but rather to ensure complete digestion, such that the impact on AD efficiency could be determined.

To evaluate the extent of disruption, samples were taken every half an hour and investigated by light microscopy. At the chosen operating conditions (40 g DW/ℓ, impeller speed 900 rpm, 35% loading with 4 mm diameter) complete disruption was achieved after 3.5 hours of milling, with approximately 90% disruption achieved after two hours in the stirred tank mill. In a previous study by Cisneros and Rito-Palomares (2004), batch bead milling of *Spirulina maxima* cells was conducted to release c-phycocyanin. The study reported that extent of disruption decreased with increasing initial wet weight concentration when operating for a constant time period. With the specific operating conditions; impeller speed 800-1100 rpm, 38% 5mm glass bead loading, initial wet weight concentration of 12 g/ℓ; an 85% disruption was achieved after 30 minutes of milling in a conventional bead mill (much increased P/V).

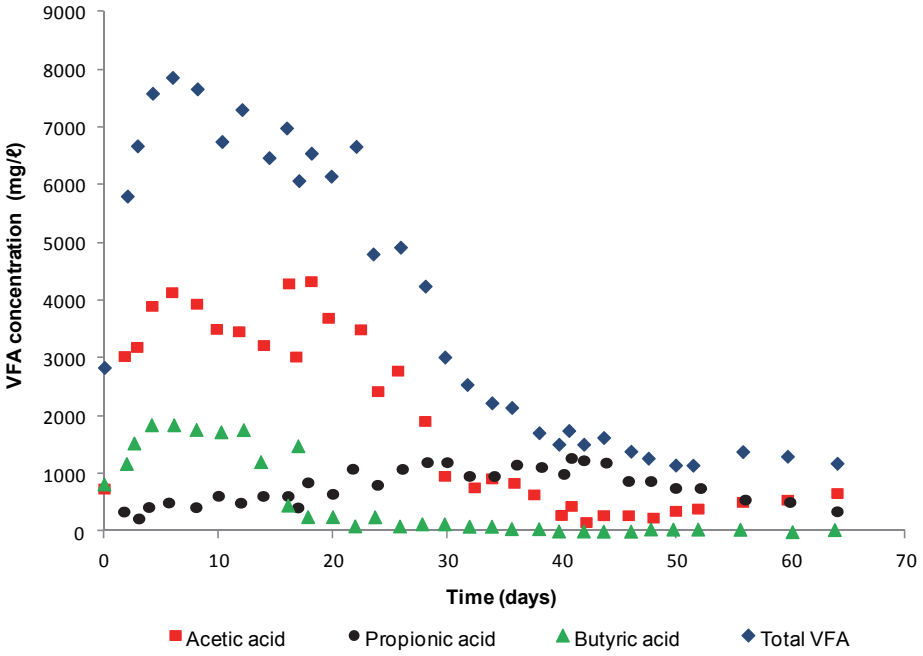
In the case of *Spirulina* a significant increase in soluble COD (18250 mg/ℓ to 41250 mg/ℓ) was observed over the period of milling. This was accompanied by a significant decrease in solid COD (46400 mg/ℓ to 24400 mg/ℓ). The increase in soluble COD was largely accounted for by propionic, iso-butyric and lactic acids, suggesting that longer chain fatty acids are more prevalent than short chain fatty acids (e.g. acetate) in the cytoplasm.

In the case of *Scenedesmus* a longer milling period was required to achieve complete disruption. This was expected due to the smaller size and strong, cellulosic cell wall (Sialve, 2009; Harrison,2011). Cell disruption, characterised by cells that had released their intracellular contents and appeared clear under the microscope, was observed during the first hour of milling, but a significant number of intact and partially disrupted cells were still clearly visible. This was also the case after two and three hours, but to a lesser extent. Complete disruption was observed after four hours of milling. The soluble COD of the slurry increased significantly (5700 to 10300 mg/ℓ) whilst that of the solid matter decreased to a lesser extent (43300 to 39000 mg/ℓ). The smaller proportional change can be accounted for by the smaller size of the *Scenedesmus* cells, resulting in a larger surface area to volume ratio and a greater proportion of the total COD being accounted for by solid matter, even after complete disruption. In the case of *Scenedesmus* the increased soluble fraction was primarily accounted for by iso-valeric, propionic and lactic acids.

The mechanical disruption of the cells had a limited impact on the subsequent liberation of VFAs during batch anaerobic digestion in the case of *Spirulina* (Figure 49). This result was not unexpected, given the ease with which *Spirulina* cells ruptured as a result of osmotic shock. The data confirm that mechanical pre-treatment would not be necessary during a two-stage AD-BSR process using *Spirulina*.




(a) *Spirulina* whole cell loaded digesters

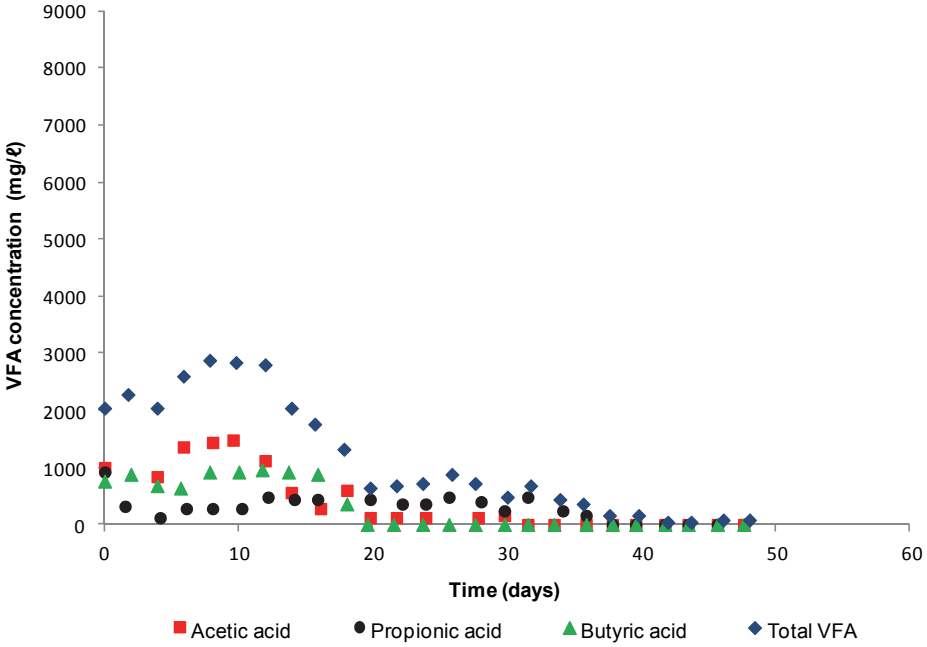



(b) *Spirulina* ruptured cell loaded digesters

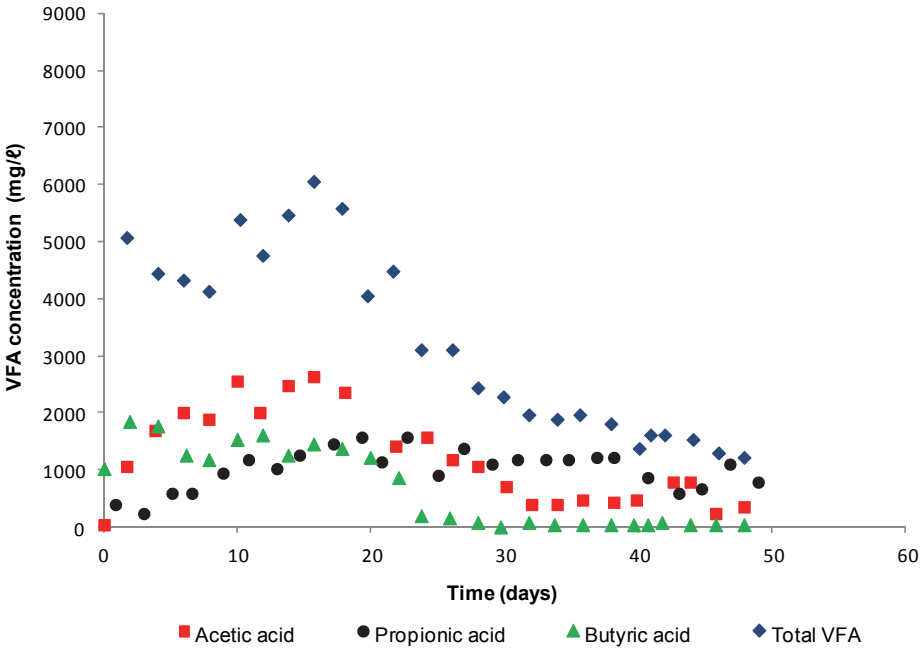
*Figure 49: Comparison of VFA liberation during batch AD of whole-cell and ruptured Spirulina*

The situation was very different for *Scenedesmus* (Figure 50), where loading the digesters with mechanically pre-treated cells significantly increased the liberation of VFAs. The highest total VFA concentration increased from approximately 3000 mg/ℓ (day 10) to 6000 mg/ℓ (day 18) as a consequence. The relative proportions of acetic, butyric and propionic acids remained similar, suggesting that the increase was most likely due to the liberation of intracellular components, rather than the accelerated digestion of the cellulosic cell wall.

The data presented in Figure 50 suggest that the utilisation of more conventional microalgal feedstocks has potential if mechanical pre-treatment or other accelerated hydrolysis step is included.




(a) *Scenedesmus* whole cell loaded digesters




(b) *Scenedesmus* ruptured cell loaded digesters

*Figure 50: Comparison of VFA liberation during batch AD of whole-cell and ruptured Scenedesmus*

### 5.4.3 Sulphate reduction using partially digested *Spirulina* biomass

The dataset for the reactors fed on digestate from the anaerobic digester was limited to the five, four and three day HRTs due to a shortage of algae for digestion and limited capacity in the digester, so sufficient digestate could not be generated to sustain the reactors at low HRTs.

Due to the fact that data are only available for three steady states, the graphs for this section show time course data, rather than steady state data.

*5.4.3.1 Feed sulphate concentration 2.5 g/ℓ*

The data presented in Figure 51 show a period of unsteady state operation for the first 23 days, during which the residual sulphate concentration decreased from >400 mg/ℓ to <100 mg/ℓ, at which point steady state was achieved. The sulphate conversion under steady state conditions was between 96% and 97%, which was significantly higher than the corresponding reactor receiving lactate as the electron donor (54-55%).

The retention time was decreased from five days to four days on day 34. This led to a short term increase in residual sulphate concentration, with the reactor appearing to approach the new steady state by day 53. At this point the feed to the reactor became partially blocked and the lower volumetric loading created the impression of very efficient performance. Once the problem was detected and rectified the residual sulphate concentration increased again and the new steady state was achieved from day 80, with the residual sulphate concentration remaining stable between 50 mg/ℓ and 60 mg/ℓ. Once again, the sulphate conversion was significantly higher than for the lactate-fed reactor.

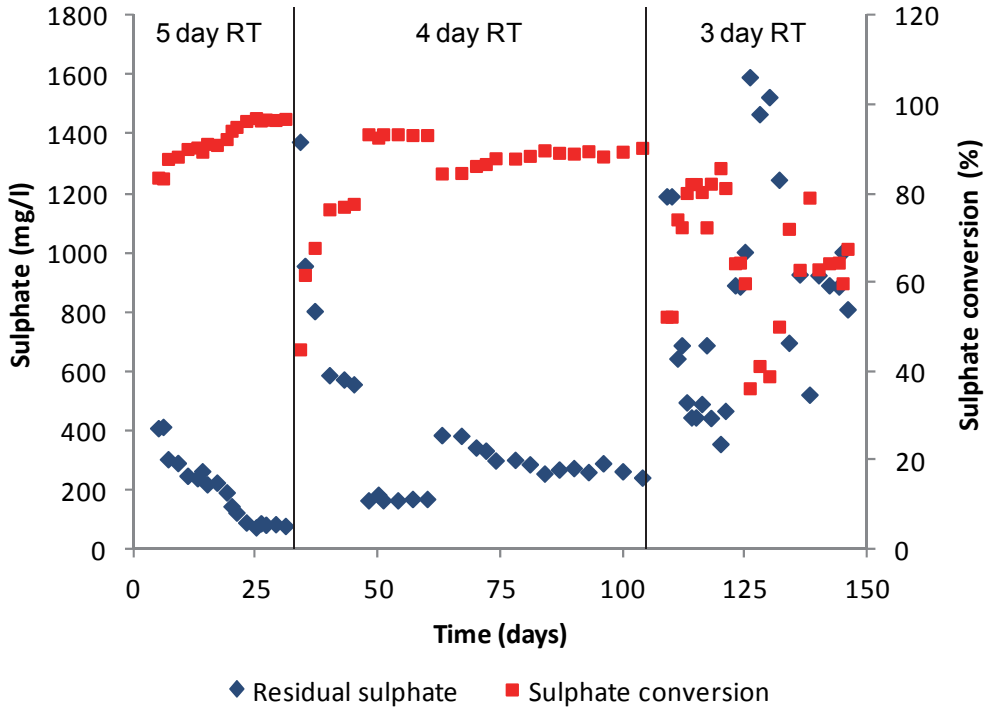



*Figure 51: Residual sulphate and sulphate conversion data for the CSTR receiving feed with a sulphate concentration of 2.5 g/ℓ and digestate as the electron donor, as a function of hydraulic residence time (RT)*

There are two factors that contribute to the very efficient sulphate reduction. The first is the composition of the feed, which contains primarily acetate, propionate and iso-valerate. Acetate is a two-carbon organic acid and cannot be fermented further, while propionate and iso-valerate are not easily fermented. Therefore, there is not competition for the electron donor, as occurred in the lactate-fed reactors. Secondly, a significant amount of the sulphide that would be produced from the efficient sulphate reduction is not retained in solution (Figure 52). If all the sulphide was retained in solution the concentration would exceed the levels that have been reported as inhibitory to sulphate reducing species. The sulphide is most likely partially oxidised to elemental sulphur, which was observed on the surface of the reactor and as a suspended colloid in the effluent, polysulphides or thiosulphate.

During December 2013, for a two week period, the reactor was poorly maintained, with only intermittent feeding. As a consequence, the feed was exhausted and air was pumped into the reactor. This resulted in the complete elimination of aqueous sulphide (Figure 52) and slight increase in redox potential. Upon resumption of correct maintenance the sulphide concentration did recover to the previous levels, but the sulphate reduction performance became inconsistent. The performance approached steady state again from about day 140.

Samples were taken and DNA extracted following the upset period and analysis of these will indicate if the loss of steady state was related to a change in community structure, possibly influenced by the lack of aqueous sulphide.

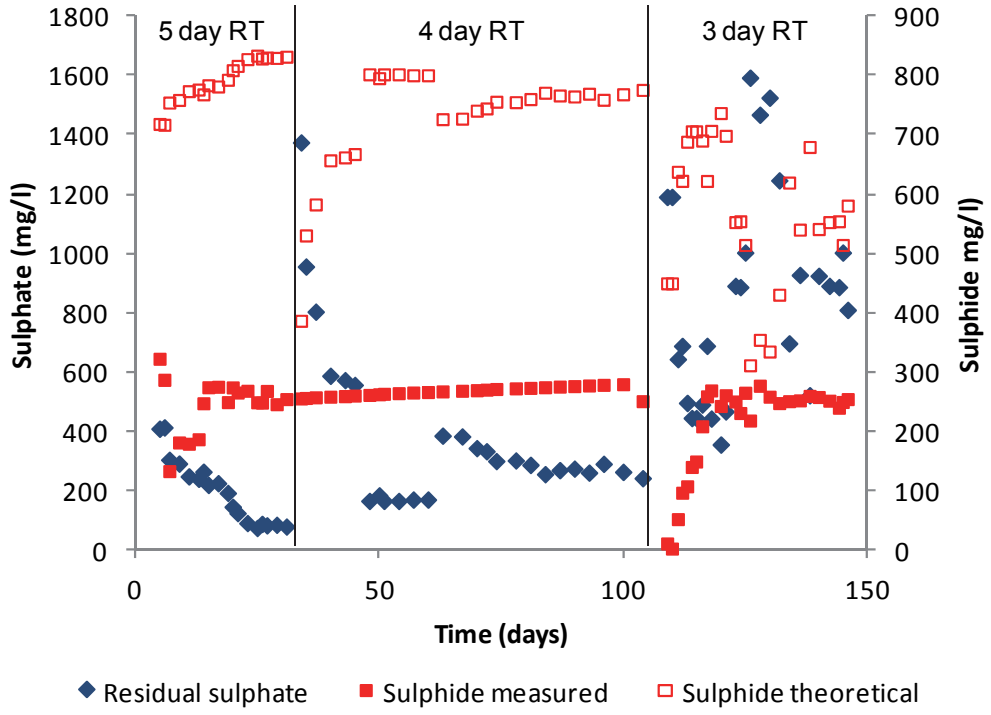



*Figure 52: Residual sulphate, measured and theoretical sulphide values for the CSTR receiving feed with a sulphate concentration of 2.5 g/ℓ and Spirulina digestate as the electron donor, as a function of hydraulic residence time (RT)*

Analysis of the VFA profile in the effluent from the reactor showed almost complete conversion of the acetate, propionate and iso-valerate in the feed. Acetate was the only VFA detected in the effluent, but the concentration did not exceed 10 mg/ℓ. The complete conversion of the VFAs is consistent with the extent of sulphate reduction achieved, given that the COD:sulphate ratio in the feed was 0.7.

### *5.4.3.2 Feed sulphate concentration 5 g/ℓ*

The data for the reactor receiving feed with sulphate at a concentration of 5 g/ℓ show very similar trends to the 2.5 g/ℓ case. There was an initial period of unsteady state, with steady state being achieved after 23 days at a five day HRT (Figure 53). The residual sulphate concentration at steady state was around 150 mg/ℓ, which represented a very high sulphate reduction efficiency. The residual sulphate increased briefly to over 1400 mg/ℓ following the decrease in HRT to three days, but fell rapidly thereafter. Steady state at the four day HRT was achieved shortly after the tubing had been replaced (day 82). The residual sulphate concentration at steady state was significantly higher (550-600 mg/ℓ) than at the five day HRT, resulting in a decrease in reduction efficiency to around 85%. This is still significantly higher than the 55% achieved in the lactate-fed reactor.

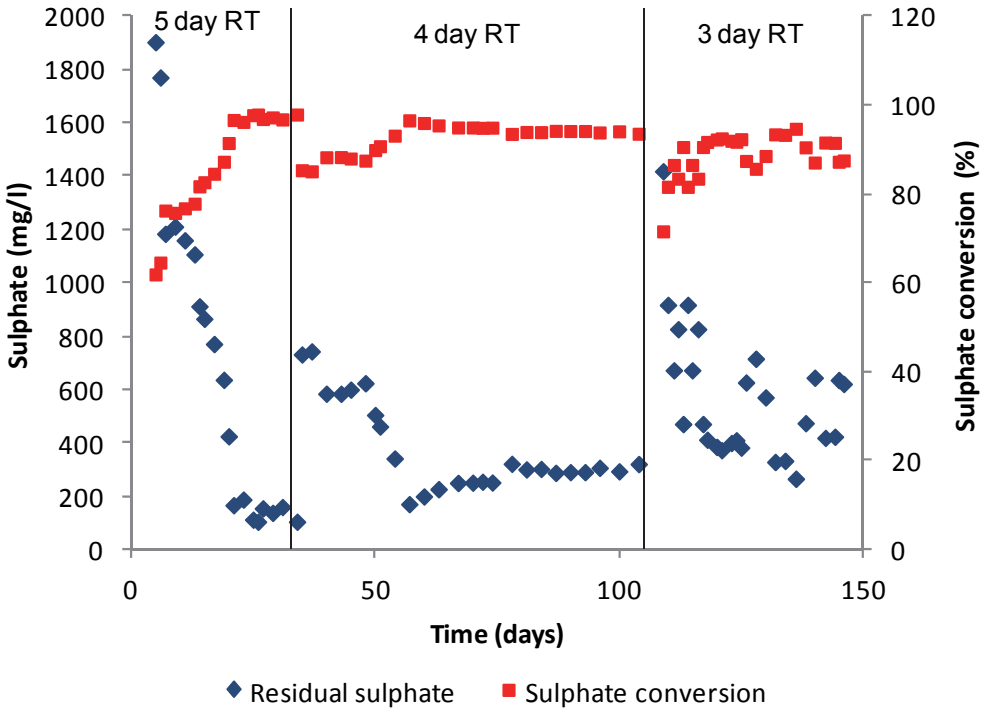



*Figure 53: Residual sulphate and sulphate conversion data for the CSTR receiving feed with a sulphate concentration of 5 g/ℓ and Spirulina digestate as the electron donor, as a function of hydraulic residence time (RT)*

The 5 g/ℓ reactor was subjected to the same period of neglect as the 2.5 g/ℓ reactor. While the consistency of the residual sulphate data was negatively affected, the overall sulphate reduction efficiency remained high. The higher concentrations of sulphate and electron donor in the feed would result in a higher biomass density, which may have resulted in the greater resilience observed.

The measured sulphide concentration was again very low, relative to the theoretical value based on the amount of sulphate reduced, which is a clear indication that some oxygen entered the reactor. The efficient mixing achieved by the marine impeller ensured continuous transport of sulphide to the reaction zone, so a significant portion of the sulphide generated was consumed. However, the amount of oxygen entering the system was relatively small so the reaction stoichiometry favoured partial oxidation.

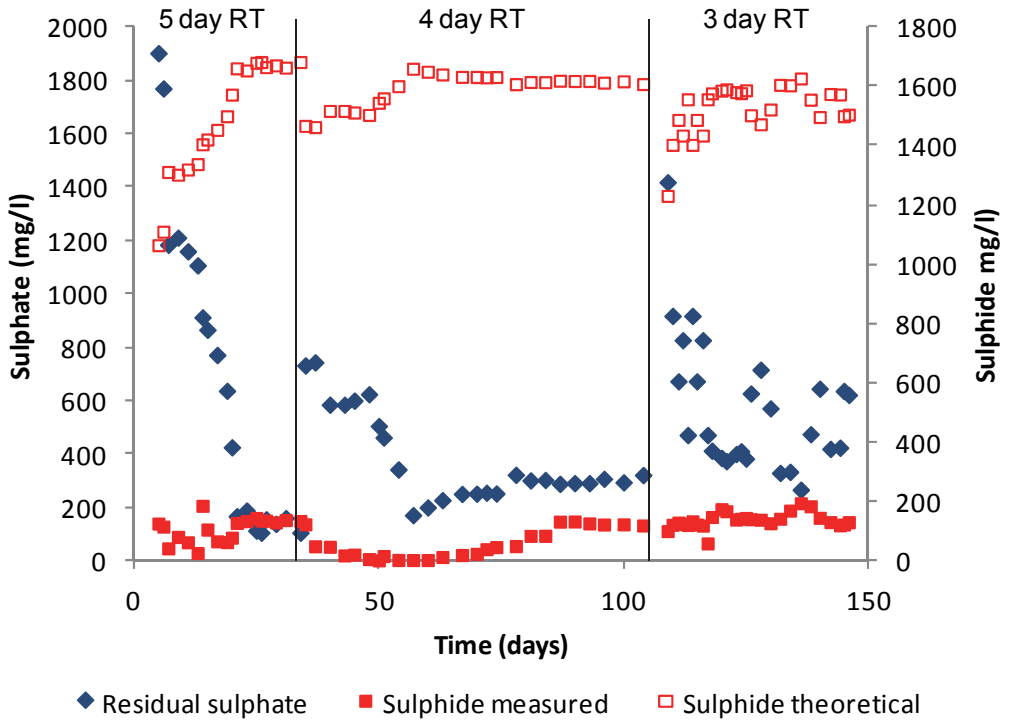



*Figure 54: Residual sulphate, measured and theoretical sulphide values for the CSTR receiving feed with a sulphate concentration of 2.5 g/ℓ and digestate as the electron donor, as a function of hydraulic residence time (HRT)*

### *5.4.3.3 Volumetric sulphate reduction rates*

The VSRR is the most relevant measure of reactor efficiency and the data for the digestate reactors are summarised in Figure 55. During steady state at the five day HRT the VSRR achieved in the 5 g/ℓ reactor (40.4 mg/ℓ.h) was double that achieved in the 2.5 g/ℓ reactor, reflecting the similar percentage conversions. By contrast, the VSRRs achieved in the corresponding lactate-fed reactors were 11.3 mg/ℓ.h and 24.3 mg/ℓ.h for the 2.5 g/ℓ and 5 g/ℓ reactors respectively. Following the reduction of the HRT to four days, the VSRR for the 2.5 g/ℓ reactor increased to 25.5 mg/ℓ.h which is consistent with the linear increase observed in the 1 g/ℓ lactate-fed reactor. The VSRR for the 5 g/ℓ reactor also increased by a similar proportion, to 49.2 mg/ℓ.h. The data for the 2.5 g/ℓ reactor at the three day HRT were inconsistent, so a steady state value could not be derived. The performance of the 5 g/ℓ reactor was more consistent, with a mean VSRR of 62.4 mg/ℓ.h, which was substantially higher than the 27.7 mg/ℓ.h obtained in the corresponding lactate-fed reactor.

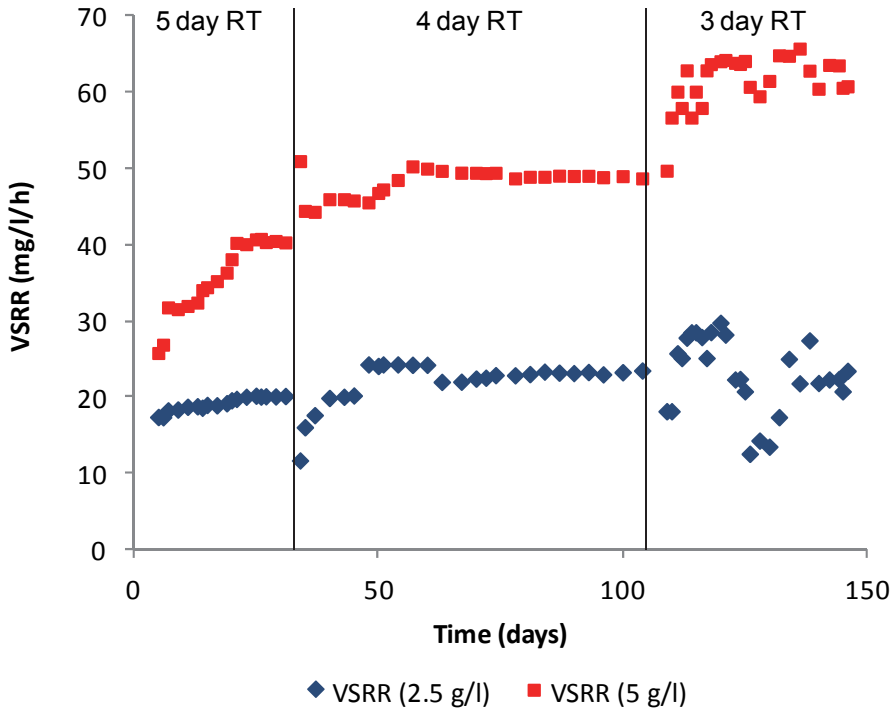



*Figure 55: Volumetric sulphate reduction rates achieved in the two CSTRs at five, four and three day hydraulic residence times (HRT)*

### 5.4.4 Algal growth studies

#### *5.4.4.1 Spirulina cultivation on channel reactor effluent*

The attempts to grow *Spirulina* directly on channel reactor effluent were not successful. The data are presented in Figure 56 and Figure 57. The algae grew well on the Zarrouk's medium, but in all cases the presence of SRB effluent, even at low proportions, resulted either the rapid death of the algal cells or a significant reduction in algal growth. *Spirulina* may be susceptible to sulphide, although it has been grown successfully in an integrated system treating simulated AMD, where tannery effluent provided the electron donor for the sulphate reduction component (Rose *et al*., 1998). In that system, the sulphide concentration in the aerobic cap of the facultative pond, which fed the algal pond, averaged approximately 75 mg/ℓ.

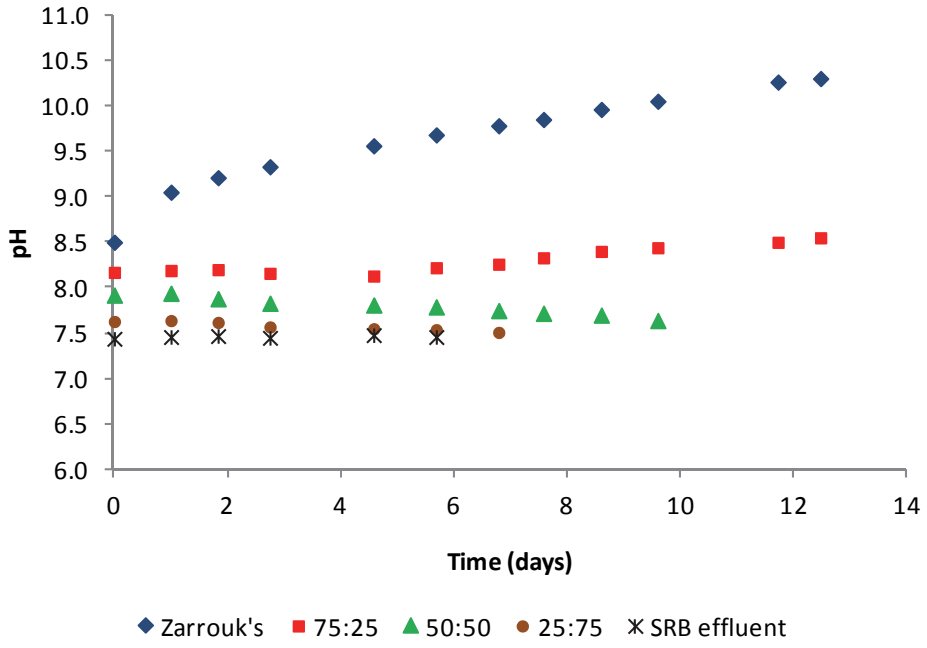



*Figure 56: pH data for batch Spirulina cultivation experiments using a combination of Zarrouk's medium and SRB reactor effluent. Where ratios are presented, the Zarrouk's fraction is displayed first*

Some growth of *Spirulina* was observed in the flask containing the 75:25 mix of Zarrouk's medium and effluent following a long adaptation period and initial decrease in biomass concentration. The observed growth rate was significantly lower than that observed in the control flasks.

Previous work by van Hille (2001) showed that it was possible to cultivate the cyanobacterium *Oscillatoria* sp. on effluent from the sulphate reducing unit of an integrated biological system for the treatment of acidic minewater. *Oscillatoria* is filamentous, like *Spirulina*, but grows preferentially attached to a solid support. In the integrated system, this was provided as a gravel filled channel. The work of van Hille (2001) was performed in an integrated system, where *Oscillatoria* was cultivated in effluent from the SRB reactor that was first aerated to strip off gaseous sulphide, in order to precipitate copper selectively. While this did not eliminate aqueous sulphide, it did reduce the concentration and raise the redox potential of the liquid. In addition, the flow rate was relatively low, compared to a batch flask where the algae were exposed to the full dose of sulphide in a single event. This served as motivation for the aeration of the channel reactor effluent in subsequent studies.

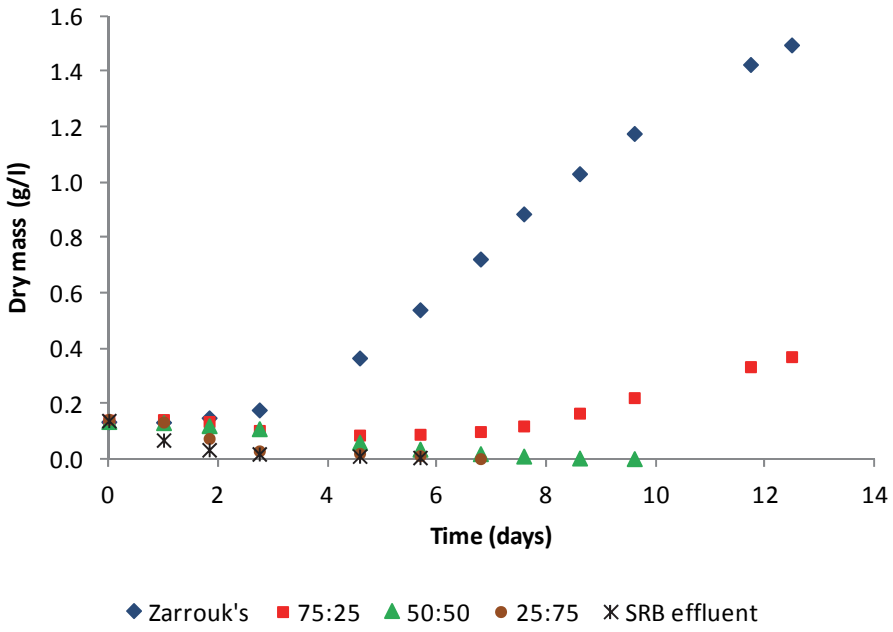



*Figure 57: Biomass concentration data (g dry mass per ℓ) for batch Spirulina cultivation experiments using a combination of Zarrouk's medium and SRB reactor effluent. Where ratios are presented, the Zarrouk's fraction is displayed first*

### *5.4.4.2 Aeration of the channel reactor effluent*

The effluent from the channel reactor, prior to aeration, had a sulphide concentration of 26.4 mg/ℓ, a pH of 7.61 and a redox potential of -340 mV. All the residual sulphide was either stripped out or oxidised within the first 30 minutes of aeration. The rapid increase in pH (Figure 58) is consistent with stripping as gaseous hydrogen sulphide, as a proton must be consumed to shift the equilibrium from $HS^-$ to $H_2S$. The pH and redox potential changed more slowly after the first 60 minutes.

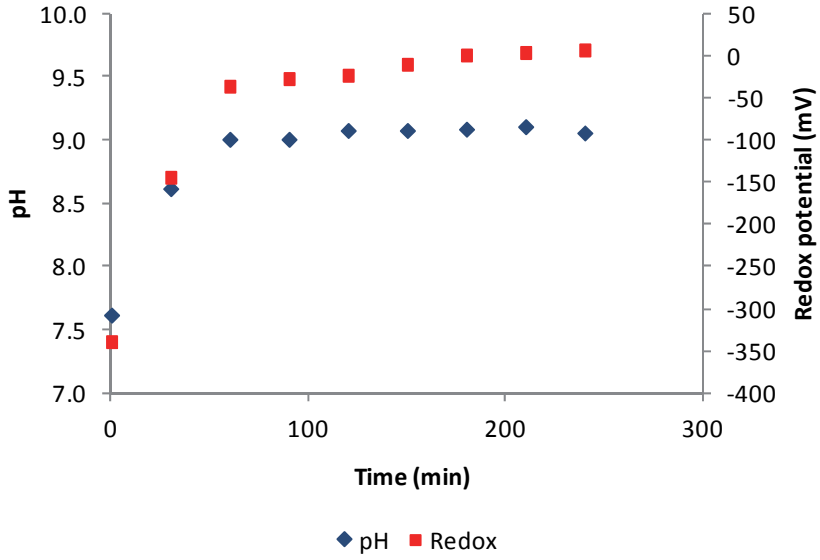



*Figure 58: Redox potential and pH of the LFCR effluent during aeration*

### *5.4.4.3 Assessment of cultivation potential*

The appearance of the bottles immediately after inoculation is shown in Figure 59. Initially, all three algal cultures showed a significant decrease in viability in the 48 hours after inoculation. Visually, the cultures appeared to lose colour and this was accompanied by a significant decrease in the measured OD. No further measurements were taken after the first 48 hours. However, after about 8-10 days the green colour

returned to the *Scenedesmus* and wastewater algae bottles. Observation of the cell morphology using light microscopy showed cells consistent with *Scenedesmus* and *Chlamydomonas* (Figure 60). The wastewater isolate was subsequently identified as *Chlamydomonas debaryanan*. In addition, growth was detected in the bottle inoculated with *Spirulina*, although the morphology was not consistent with *Spirulina*. The bottles were subcultured, using freshly aerated reactor effluent and this time no initial reduction in growth was observed. The *Chlamydomonas* sp. was selected for growth rate studies in the airlift reactors.

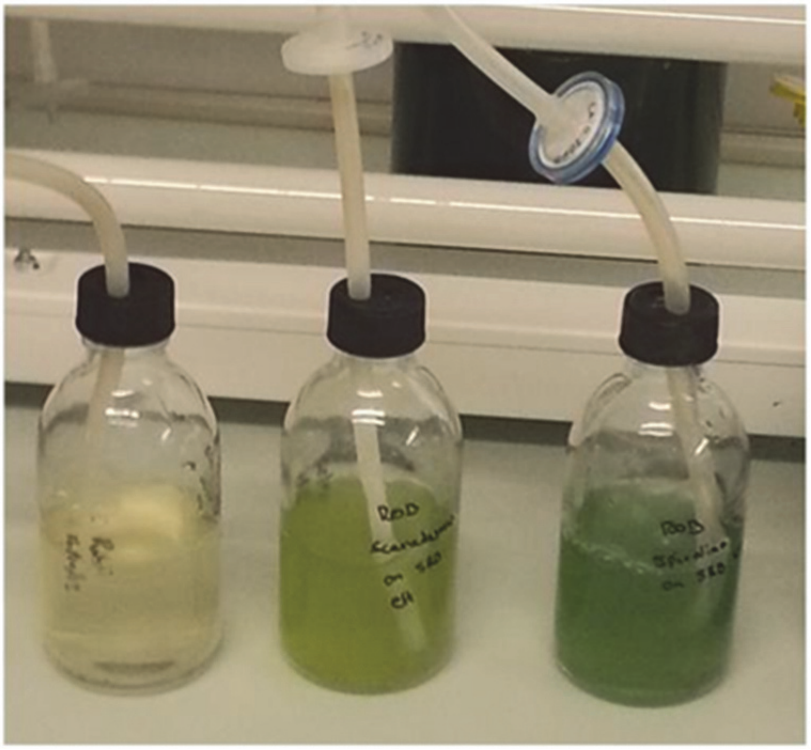


*Figure 59: Photograph of small bottles used to assess algal growth on aerated SRB channel effluent. Photograph taken immediately after inoculation. The bottle on the left was inoculated with the wastewater isolate, the centre with Scenedusmus and the right with Spirulina*

A second round of tests focussed on environmental isolates and included a number of macroalgal species. A number of these showed positive growth on the ACRE (Figure 61) and will be considered for further testing. Several macroalgal species have the ability to grow attached to solid supports. This creates the potential for easier harvesting, as the supports can be lifted out of the growth medium, harvested of algae and replaced. The concentration of unicellular microalgae from dilute suspensions is one of the major challenges standing in the way of their exploitation for commodity products at a commercial scale, although some species such as *Scenedesmus* have been shown to flocculate and sediment at the end of cultivation (unpublished data).

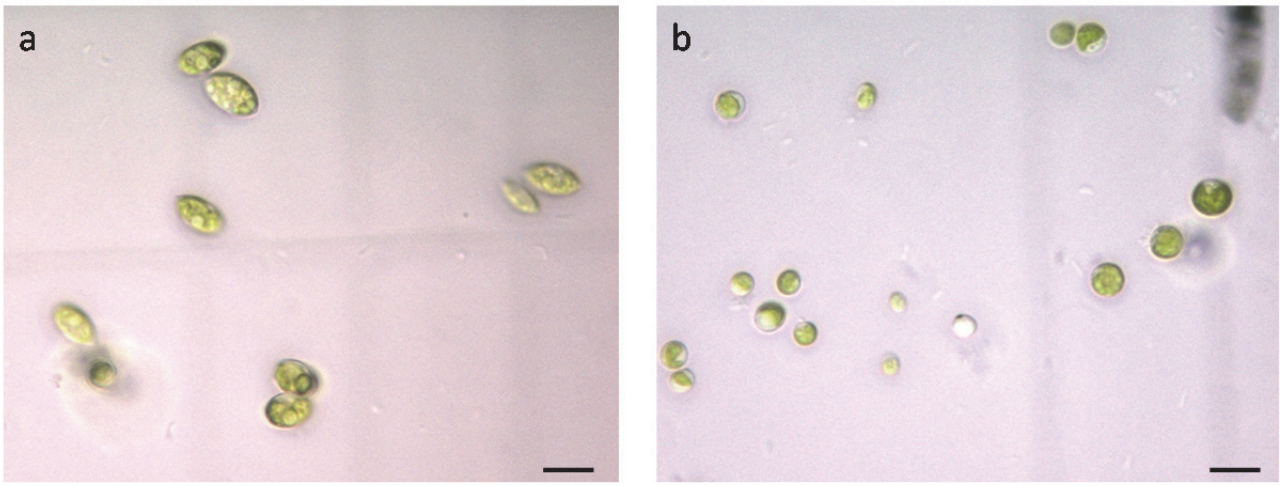



*Figure 60: Light microscope image of (a) Scenedesmus sp. and (b) Chlamydomonas debaryana grown on aerated channel reactor effluent. Images taken using a 100× objective lens. Scale bar represents 5 µm*

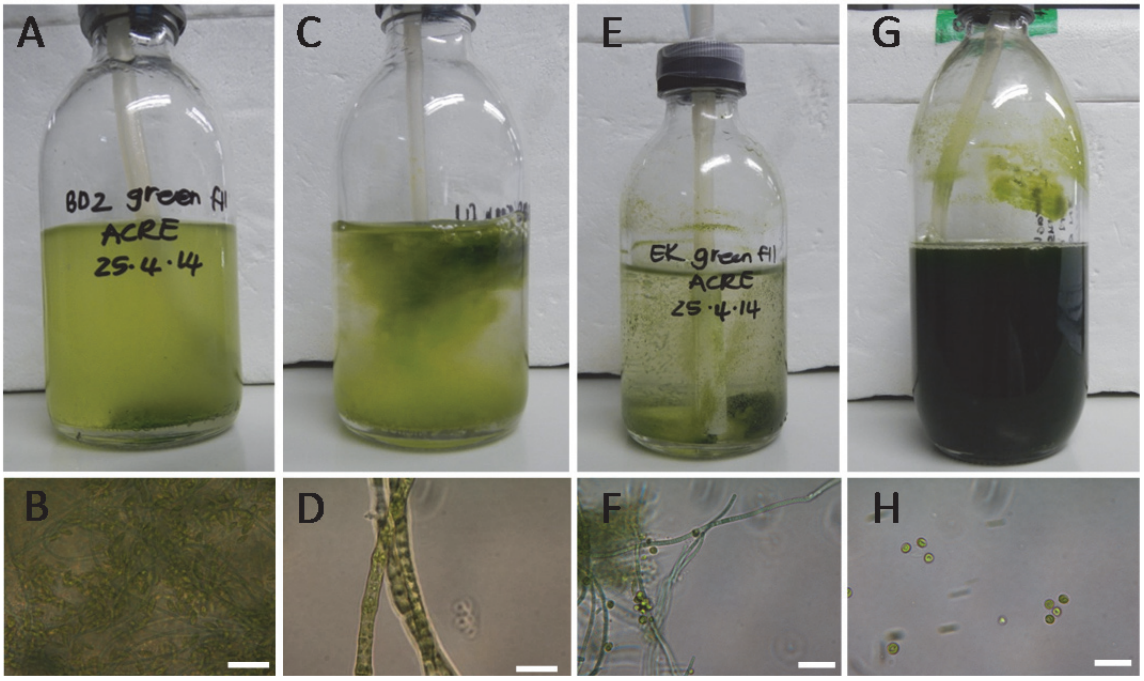



*Figure 61: Growth of filamentous algae (A-F) and a microalgal species, Parachlorella hussii (G and H) in ACRE waste. Filamentous algae BD2 (A and B) and BD3 (C and D) were isolated from Simondium, Western Cape, while EK (E and F) was isolated from the Elsieskraal in Pinelands. Microscope pictures (B, D, F and H) were taken using a 100 × objective lens. Scale bars depicted by white lines indicate 1 µm*

#### *5.4.4.4 Growth rate studies in airlift photobioreactors*

The isolated *Chlamydomonas* sp. was selected for growth rate experiments, both on defined 3N BBM and on supplemented and non-supplemented effluent ACRE. The results are summarised in Figure 62 Growth on the defined medium was faster and achieved a higher final cell density. There was no apparent difference in the growth rates between supplemented and non-supplemented effluent, indicating that the reduced growth rate was not necessarily a function of nitrogen and/or phosphorous limitation. The initial pH of the effluent reactors was around pH 9.4, while the BBM medium has a pH around 7.5. However, during growth the pH in the BBM-fed reactor increased to around pH 9.5, while the effluent reactors did not change significantly, suggesting that pH may not have been the cause.

Additional growth experiments, where the pH of the BBM is artificially elevated to pH 9.4 at the start of the experiment, were performed, but the result did not differ significantly from those shown in Figure 62.

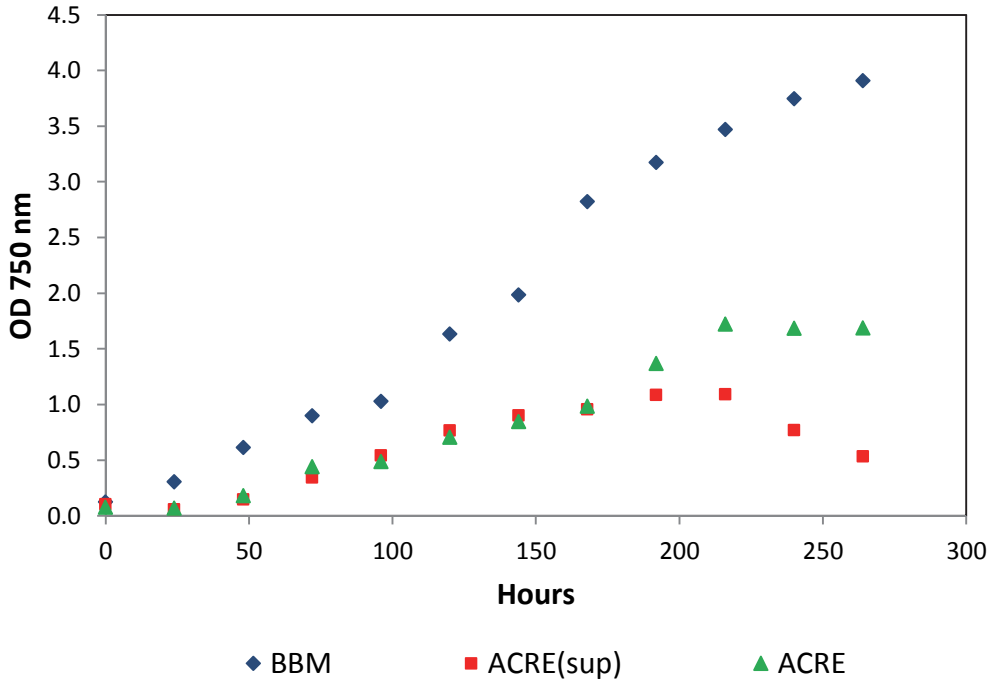



*Figure 62: Growth data for Chlamydomonas debaryana in 3.2 ℓ airlift reactors. BBM represents the defined growth medium, ACRE is aerated effluent from a sulphate reduction/sulphide oxidation system and ACRE (sup) is the effluent supplemented with nitrate and phosphate to the levels of the BBM*

A second study, using similar growth conditions was performed using *Parachlorella hussii.* This species was isolated from a continuous reactor treating cyanide- and thiocyanate-containing wastewater, suggesting it may be robust and capable of growth in effluent from other reactors. The isolate grew well on the 3N BBM medium. While growth on ACRE was observed in the small bottles, in batch mode, no growth was observed in the airlift reactor on ACRE. The algae did grow on ACRE supplemented with nitrogen and phosphorous, but at a lower growth rate. In addition, after four days the algae began to aggregate and stick to the walls of the reactor. At this point accurate OD measurements could no longer be obtained and the experiment was terminated.

## 5.5 CONCLUSIONS

The work presented in this chapter revealed that it is possible to sustain biological sulphate reduction using whole cell algae at the carbon source, although the performance was inconsistent. The *Spirulina* used in the experiment has a number of characteristics that make it more amenable than typical microalgae. Decoupling the VFA generation and sulphate reduction stages was undertaken to enable a greater chance of consistent performance.

Microalgae have been studied extensively as a substrate for anaerobic digestion. The AD research typically focuses on the biogas yield and does not characterise the digestate. Our research has shown that the digestion of microalgae liberates the VFAs necessary to sustain sulphate reduction and if the digester is operated with a higher than optimal organic loading rate the effluent will contain VFA concentrations equivalent to several g/ℓ of COD. A second option, in situations where the generation of biogas does not represent an associated benefit, would be to add some sulphate to the anaerobic digester feed. Methanogenic archaea are inhibited by lower sulphide concentrations than the hydrolytic and acidogenic bacteria so their inhibition would result in a greater proportion of the VFAs, particularly acetate, being available as substrate for the downstream processes.

*Spirulina* proved a very good substrate for AD and did not require mechanical pre-treatment to ensure efficient liberation of VFAs. *Scenedesmus* has a more robust, cellulosic cell wall, and mechanical disruption of the cells was required in order for similar levels of VFAs to be liberated during digestion.

The effluent from the AD of *Spirulina* proved to be an excellent substrate for sulphate reduction, at least at HRTs of 3-5 days. High sulphate reduction efficiencies were observed at feed sulphate concentrations of

2.5 and 5 g/ℓ. In addition the VFA profile in the effluent was not susceptible to further fermentation, so the problem of competition that affected the lactate-fed reactors under similar operating conditions was not experienced.

If partially digested algae are to be used as a substrate for sulphate reduction in an ARD treatment system, it is important that the algae are grown on site to avoid transport costs, ideally using a waste material as feedstock. Experiments performed using aerated effluent from the channel reactor as the basis of the algal growth medium showed positive growth for a number of species, although a period of adaptation was necessary in some cases. The channel reactor effluent was deficient in nitrogen and phosphate, so some supplementation or blending with a more nutrient rich medium would be advantageous.

# CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

## 6.1 CONCLUSIONS

The intention of this project was to address two of the major challenges that have prevented the more widespread application of technologies based on bacterial sulphate reduction in the field of AMD treatment, kinetics of biological sulphate reduction and the cost of the electron donor. The former was addressed by investigating novel reactor configurations aimed at improving sulphate reduction at low HRTs by biomass retention or recycling. The latter was addressed by investigating the use of microalgae, both as whole cells and partially digested biomass, as the carbon source and electron donor.

Baseline studies were conducted in CSTRs, using a defined medium with lactate as the carbon source and electron donor. The results were consistent with previous studies, showing efficient sulphate reduction in the system receiving a feed sulphate concentration of 1 g/ℓ at HRTs as low as one day, below which the efficiency fell off sharply, despite the VSRR increasing slightly. The data suggested washout of at least part of the sulphate reducing community and justified the subsequent investigation of biomass retention and recycling strategies.

The efficiency of the system was compromised at higher feed sulphate concentrations (2.5 and 5.0 g/ℓ) due to competition for the lactate substrate between lactate oxidising sulphate reducers and lactate fermenting species. This was consistent with previous studies and confirmed that the lactate fermenters had a higher growth rate, but lower affinity for lactate. In addition, the fermenters were more susceptible to inhibition by aqueous sulphide.

Three experimental systems were investigated, using lactate medium at a feed sulphate concentration of 1 g/ℓ. The conventional reactor, operated without agitation, demonstrated similar performance to the CSTR at HRTs as short as one day. At this point the reactor failed, possibly due to a change in microbial community structure, although this needs to be confirmed. Prior to reactor failure, distinct flocs were visible within the reactor and were retained under normal operating conditions.

The LFCR yielded very encouraging results. Colonisation of the carbon fibres was rapid and extensive, with a substantial amount of attached biomass removed from the reactor at the end of the study. As a consequence, the system was able to sustain relatively efficient sulphate reduction at a HRT of 0.5 days. The VSRR was 20% higher than in the CSTR operated at a similar VSLR. The channel reactor required no agitation or external energy input, so would be compatible with a semi-passive or passive system. The lack of turbulent mixing reduced sulphide losses, resulting in significantly higher aqueous sulphide concentrations. This would create a more selective environment, favouring sulphate reducers over lactate fermenters.

The integration of the crossflow microfiltration unit with the CSTR resulted in the greatest efficiency, measured as VSRR. Despite the problems encountered with sulphur formation and blocking of the permeate drainage line, the VSRR at a 0.5 day HRT was 50% higher than in the corresponding CSTR. The sulphur formation was most likely a consequence of using silicone tubing, which permitted significant oxygen penetration. This could be reduced in a scaled-up system, where non-porous materials of construction would be used. While the deposition of sulphur on the outer surface of the membrane reduced the transmembrane flux, it did not result in membrane damage. The inner surface did not foul and overall performance was restored after the sulphur coating was removed.

The research showed that it was possible to sustain a sulphate reducing community using whole algal biomass as the carbon source and electron donor, but achieving steady state was a challenge. By contrast, the use of partially digested algal biomass yielded very encouraging results. The majority of the residual COD in the digestate was in the form of VFAs, particularly acetate. These could be used directly by the SRB and efficient sulphate reduction was observed. An additional benefit of using digestate was that the dominant VFAs were not fermentable, so the SRBs were not subject to competition for the substrate.

The ability to generate the feedstock for an integrated system on site is important to reduce transport costs. In the case of algae, it would be ideal if wastewater or treated effluent could be used. Positive growth of a number of species was demonstrated on aerated channel reactor effluent, although the productivities were lower than on defined medium, possibly due to the very low nitrogen and phosphorous content of the

effluent. Blending of effluents or some nutrient supplementation may be required to achieve good productivity. The positive results achieved with a number of filamentous cyanobacteria and macroalgae were particularly encouraging as these may be grown on solid supports, making cost-effective harvesting more viable.

In conclusion, the two novel reactor configurations tested in this study have demonstrated significant improvements in sulphate reduction efficiency as a result of good biomass retention. The channel reactor could be applied in passive or semi-passive system, while the incorporation of the membrane unit would be appropriate for an active system. The potential of microalgae as a carbon source and electron donor was demonstrated and results were most encouraging when the hydrolysis, acidogenesis and acetogenesis steps were decoupled from the sulphate reduction. Cultivation of algae on treated ARD was demonstrated, although further optimisation is required.

The positive outcome of the laboratory scale research showed that there is definite potential to incorporate the advances into existing treatment systems and the development of novel, integrated processes for ARD treatment. Further research is essential, particularly to assess the impact of parameters, such as seasonal climate effects, that will be important to application in the field. A number of recommendations are suggested in the subsequent section. In addition, a number of the findings could have broader implications for other biotechnological processes. The success of the carbon microfibres as a surface for microbial attachment was remarkable. Many biotech processes benefit from biomass retention and carbon microfibres have the advantage of providing a very large surface area for attachment without sacrificing reactor volume. In addition, recent research has indicated that they could be used as electrodes in microbial fuel cells.

It is important that the momentum generated during this research is continued, particularly toward the goal of demonstrating the viability of a system based on biological sulphate reduction and sulphide oxidation at pilot scale. The channel reactor configuration lends itself to application in a modular fashion, reducing some of the challenges associated with scale-up. The research reported by van Hille and Mooruth (2013) outlined the necessary steps required to optimise the linear flow channel reactor for sulphide oxidation and sulphur recovery, to address the obstacles encountered during the previous attempt to demonstrate the technology in the field.

The project team has been in discussion with representatives from coal mining companies and there is a desire to pilot a novel integrated system, developed using the knowledge generated during this project, as early as 2015. In addition, further funding from the Water Research Commission has been secured to continue with the fundamental research that will underpin the development of the pilot plant. An associated WRC-funded project will continue the investigation of microbial community dynamics in sulphate reducing and sulphide oxidising systems, using the channel reactor configuration as the basis for the experimental work, so the data generated will have direct relevance. The outcome of this work has stimulated interest and support from the mining companies, which will make crossing the bridge between fundamental research and real world application significantly easier.

## 6.2 RECOMMENDATIONS

The outcomes of the research described in this report show that there is potential to address the challenges that currently prevent the broader application of technologies based on bacterial sulphate reduction. The laboratory-scale research has demonstrated “proof of concept” under relatively well controlled conditions, so it is necessary to assess the impact of parameters, such as seasonal variation in ambient temperature, that would influence performance in the field. Furthermore, there is substantial scope for optimisation of the reactor design and configuration of the individual units. Specific recommendations for further research are listed below.

The LFCR yielded encouraging results, from which a number of logical recommendations for continuing research can be made. These include:

- An investigation of the effect of reactor depth on sulphate reduction performance is suggested. A deeper channel would increase the volume of the reactor without increasing the required surface area, which would be beneficial for scale-up.
- An investigation of the ability of the system to treat solutions with higher feed sulphate concentrations, including the potential for sulphide inhibition, is recommended. The lack of turbulent

mixing resulted in improved retention of aqueous sulphide, but this may become inhibitory at higher feed sulphate concentrations.

- The deposition of elemental sulphur in a biofilm at the air-liquid interface suggests the possibility of integrating the sulphate reduction and sulphide oxidation reactors into a single unit. Previously, the LFCR has been optimised as a stand-alone sulphide oxidation unit, with the sulphate reduction taking place in a separate, upstream unit. Combining the two processes would significantly decrease the footprint and construction cost of an integrated process. The potential of harvesting elemental sulphur as a value adding product should be investigated.
- The effect of changes in temperature on sulphate reduction performance, particularly at temperatures below 20°C, should be determined. The current research was conducted at 30°C, but in the field the system would be exposed to large fluctuations in temperature. Sulphate reduction is typically less efficient at low temperature.
- The effect of scaling up of the reactor should be investigated. A number of 25 ℓ LFCRs were used previously to study sulphide oxidation (van Hille and Mooruth, 2013) and these could easily be modified to serve as sulphate reduction reactors. The system lends itself to modular scale-up, so a ten-fold increase in reactor size would provide valuable information.

The microfiltration membrane was very effective at retaining biomass, which resulted in significantly improved performance at low HRTs. From an operating perspective the formation of elemental sulphur presented the greatest challenge. Therefore, recommendations for future work include:

- Replacing as much of the silicone tubing with stainless steel piping or non-permeable plastic to exclude oxygen more effectively.

The channel and membrane reactor systems resulted in high biomass retention. In both cases it was difficult to quantify the biomass, so it would be useful to develop techniques that would facilitate biomass quantification. In addition, a study of the microbial ecology of the community resulting from biomass retention would be useful to determine whether the community remains similar to that in the CSTR or whether particular members of the community are enriched in the retained biomass systems. This will provide further value in combination with the use of Metagenomics tools to provide insight into the potential functionality of the community and the impact of dynamics of the microbial ecology on this.

Both the channel reactor and integrated membrane reactor showed significantly improved performance in volumetric sulphate removal rates, relative to the control, at low HRTs, thereby demonstrating proof of concept at a laboratory scale. A techno-economic evaluation of the potential for application at a larger scale should now be performed.

The utilisation of digestate from an anaerobic digester as the electron donor and carbon source for sulphate reduction has the potential to overcome the challenge of finding a cost effective substrate in sufficient quantity and in proximity to the site of application that can still sustain high rates of sulphate reduction. The results obtained using partially digested *Spirulina* were very encouraging, but *Spirulina* may not be a viable substrate at large scale as its mass cultivation is relatively expensive and it represents a potentially valuable commodity in itself. Therefore, it is recommended that the anaerobic digestion of a range of potential substrates be investigated to determine their susceptibility to digestion, biogas yield and the composition of the resulting digestate. The substrates tested should include agricultural residues from areas impacted by AMD and algal species, particularly macroalgae that could be cultivated on site, using available water resources of treated effluent.

The concept for a novel, integrated system that includes a linear channel reactor with carbon microfibres, capable of simultaneous sulphate reduction and sulphide oxidation has been developed. A successful application has been made to the WRC to support fundamental research into the concept and there has been interest from a mining company to test the concept at pilot scale at one of their sites.

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